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Regulatory mechanism of lncRNA NORAD on proliferation and invasion of ovarian cancer cells through miR-199a-3p

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Abstract. – OBJECTIVE: To explore the regulatory mechanism of lncRNA NORAD on proliferation and invasion of ovarian cancer cells through miR-199a-3p.

PATIENTS AND METHODS: Eighty-six ovarian cancer tissues and 86 tissues adjacent to cancer, human ovarian cancer cell lines SKOV3, HO-8910, A2780, OVCAR-3, and human normal ovarian epithelial cell line IOSE80 were collected. MiR-199a-3p-mimics, miR-199a-3p-inhibitor, miR-NC, si-NORAD, Sh-NORAD, and NC were transfected into HO-8910 and A2780 cells, the expression levels of lncRNA NORAD and miR-199a-3p in ovarian cancer tissues and cells were detected by qRT-PCR, and the expression levels of N-cadherin, E-cadherin, and vimentin in cells were detected by WB. Cell Counting Kit-8 (CCK-8), transwell, and cell scratch tests were used to detect proliferation, invasion, and migration of cells, and the relationship between lncRNA NORAD and miR-199a-3p was confirmed by the Dual-Luciferase reporter assay.

RESULTS: LncRNA NORAD was highly expressed and miR-199a-3p was lowly expressed in ovarian cancer, and the expression levels of LNCRNARAD and miR-199a-3p were negatively correlated. Cell experiments showed that inhibiting the expression of lncRNA NORAD or up-regulating the expression of miR-199a-3p could inhibit the proliferation, invasion, migration, and EMT of ovarian cancer cells, while up-regulating the expression of lncRNA NORAD or inhibiting the expression of miR-199a-3p could promote their proliferation, invasion, migration, and EMT. Dual-Luciferase reporter assay confirmed that there was a regulatory relationship between lncRNA NORAD and miR-199a-3p.

CONCLUSIONS: LncRNA NORAD was highly expressed in ovarian cancer tissues, while silencing lncRNA NORAD expression could inhibit the proliferation, invasion, migration, and EMT of ovarian cancer cells by regulating miR-199a-3p, which might be a new target for the diagnosis and treatment of ovarian cancer.

Key Words:
LncRNA NORAD, MiR-199a-3p, Ovarian cancer cells, Proliferation, Invasion, Regulatory mechanism.

Introduction

Ovarian cancer, as one of the common malignant tumors in women, is also one of the main causes leading to their death. In recent years, its morbidity and mortality are still rising continuously, posing a great threat to women's life and health[1,2]. Although some progress has been made in the treatment and diagnosis of ovarian cancer with the continuous development of medical technology in recent years, due to its not evident early symptoms and the characteristics of rapid disease progression, patients tend to ignore their early symptoms, which are usually already advanced at the time of treatment. This is also the reason why the 5-year survival rate of ovarian cancer patients is less than 40%[3,4]. Therefore, it is of great significance for clinical treatment and diagnosis to find the molecular mechanism related to the pathogenesis of ovarian cancer.

LncRNA is a non-coding RNA with a length of more than 200 nt and it has been found to play a very important role in the occurrence and development of tumors in recent years[5]. LncRNA NORAD is a lncRNA activated by DNA damage, and it is also a highly conserved and expressed LncRNA. It has been proved that there are expression disorders in many tumors[6]. Researchers[7] have found that lncRNA NORAD has important value in differential diagnosis of benign and malignant colorectal cancer lesions. Other studies[8] have discovered that lncRNA NORAD can promote the development of thyroid cancer by targeting miR-202-5p. However, the mechanism of

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its action in ovarian cancer has not been studied yet. MiRNA, as a non-coding single-stranded RNA, can regulate gene expression by binding to 3'-UTR of target gene[9]. MiR-199a-3p is a miRNA with expression imbalance in various tumors and can promote invasion and migration of gastric cancer cells[10] by targeting ETNK1. In the past, miR-199a-3p was proved to play the role of tumor suppressor gene in ovarian cancer[11], but its upstream regulatory mechanism has not been studied and discussed. We predicted that lncRNA DANCR and miR-199a-3p had a binding site through online database and in this study, we explored the regulatory mechanism of lncRNA NORAD on ovarian cancer cells to provide a new target direction for its treatment and diagnosis.

Patients and Methods

General Data

Eight-six patients who underwent ovarian cancer resection in our hospital from March 2016 to 2018 were collected, and 86 ovarian cancer tissues and 86 tissues adjacent to cancer were taken for detection during surgery with their consent. Inclusion criteria were as follows: patients diagnosed as ovarian cancer by pathological diagnosis; patients diagnosed with ovarian cancer for the first time. Exclusion criteria were as follows: patients who had received radiotherapy and chemotherapy; patients with other malignant tumors, severe liver, and kidney dysfunction, or serious infectious diseases; patients who refused to provide experimental specimens. All patients and their families agreed to participate in the study and signed an informed consent. The study has been approved by our Ethics Committee.

Experimental Materials and Reagents

Human ovarian cancer cell lines SKOV3, HO-8910, A2780, OVCAR-3, and human normal ovarian epithelial cell line IOSE80 were purchased from ATCC's agent BeNa Culture Collection Co., Ltd., qRT-PCR and reverse transcription kit (TransGen Biotech, Beijing, China), CCK-8 kit (Promega, Madison, WI, USA), transwell kit (Shanghai Fanke Biotechnology Co., Ltd., FK-lk019), phosphate-buffered saline (PBS), fetal calf serum (FBS; Gibco, Rockville, MD, USA), TRIzol reagent (Beijing Biolab Technology Co., Ltd.), Dual-Luciferase Assay Kit (Beijing Biolab Technology Co., Ltd.), Radio Immunoprecipitation Assay (RIPA), bicinchoninic acid (BCA) protein kit (Thermo Fisher Scientific, Waltham, MA, USA). N-cadherin, E-cadherin, vimentin, and β-Actin antibodies (Cell Signaling Technology, CST; Danvers, MA, USA), goat anti-rabbit IgG secondary antibody (Wuhan Boster Biological Technology Co., Ltd.), enhanced chemiluminescence (ECL) developer (Thermo Fisher Scientific, Waltham, MA, USA) and PCR instrument (Applied Biosystems; Foster City, CA, USA, 7500). All primers were designed and synthesized by Shanghai Sangon Bioengineering Technology Service Co., Ltd.

Cell Culture and Transfection

Human ovarian cancer cell lines SKOV3, HO-8910, A2780, OVCAR-3, and human normal ovarian epithelial cell line IOSE80 were placed in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% PBS, 2 ml penicillin, and streptomycin, and cultured in an environment of 37°C and 5% CO_2. When the adherent growth and fusion of cells reached 85%, 25% pancreatin was added for digestion. After digestion was completed, the cells were placed in the medium for continuous culture and passage. Then, the expression levels of lncRNA NORAD and miR-199a-3p in each cell line were detected. Next, HO-8910 and A2780 were selected for transfection, and miR-199a-3p-inhibitor (suppression sequence), miR-199a-3p-mimics (over-expression sequence), miR-NC negative control (miR-NC), targeted inhibition of NORAD RNA (si-NORAD), targeted over-expression of NORAD RNA (sh-NORAD), negative control RNA (NC) were transfected with Lipofectamine™ 2000 kit, and the operation steps were strictly carried out in accordance with the kit instructions.

Real-Time Quantitative PCR

First, total RNA in tissues and cells was extracted with TRIzol reagent. Then, 5 μg of total RNA was taken respectively for reverse transcription of cDNA according to the instructions of the kit, and 1 μL of synthesized cDNA was taken for amplification after transcription. The amplification system was as follows: 0.4 μL of upstream and downstream primers of 1 μL of cDNA, 10 μL of 2X TransScript® Tip Green qPCR SuperMix, Passive Reference Dye (50X) 0.4 μL, Nuclease-free Water supplemented to 20 μL. MiR-199a-3p used U6 as the internal reference, NORAD used β-Actin as the internal reference, and $2^{-\Delta CT}$ was used to analyze the data. Primer sequences were shown in Table I.

Cell Proliferation Test

The proliferation ability of HO-8910 and A2780 cells was evaluated by the CCK-8 kit. Cells 48 h after transfection were collected, diluted to 3×104 cell/ml, seeded into 96-well plates, inoculated with 100 μl of cells per well, and cultured under 37°C, 5% CO_2 environment. A total of 10 μl CCK-8 solution was added to each well at 0 h, 24 h, 48 h, and 72 h after the cells adhered to the wall. Once the reagent was added, the cells were continuously cultured in an incubator at 37°C and 5% CO_2 for 2 h. Then, the OD value was measured at 450 nm using an enzyme reader to detect the cell proliferation and draw the growth curve. The experiment was repeated 3 times.

Cell Migration and Invasion Test

The ability of cell migration and invasion was evaluated by scratch-healing test and transwell test. For wound healing determination, 200 μl sterile loading gun head was used to divide the cells into a cell-free area, PBS was used to wash the divided cells, and a new culture medium was added for culture. At 0 h (W0) and 24 h (W24) after cell division, the cell migration ability was evaluated by microscope for scratches at three different positions. Transwell assay: first 200 μL DMEM culture solution containing 1×105 cells was added to the upper chamber, and 500 mL DMEM containing 20% FBS was added to the lower chamber. The substrate and cells that did not pass through the membrane surface in the upper chamber were wiped off after 48 h of culture at 37°C, washed with PBS for 3 times, fixed with paraformaldehyde for 10 min, washed with double distilled water for 3 times, stained with 0.1% crystal violet for 10 min after it was dried, and cell invasion was observed with a microscope.

Western Blot Test

The total protein was extracted from collected cultured cells by RIPA lysis, and its concentration was detected by the BCA method and adjusted to 4 μg/μL. The protein was separated by 12% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) electrophoresis and then transferred to polyvinylidene difluoride (PVDF) membrane after ionization. The PVDF membrane was blocked with 5% skimmed milk powder for 2 h, and finally sealed overnight at 4°C after adding N-cadherin (1:500), E-cadherin (1: 500), Vimentin (1: 500), and β-Actin (1: 1000). The membrane was washed to remove primary antibody, the horseradish peroxidase (HRP)-labeled goat anti-rabbit secondary antibody (1: 1000) was added, and the protein was incubated at 37°C for 1 h. After that, the membrane was rinsed 3 times with PBS, for 5 min each time. Afterwards, the protein bands on the membrane were developed in a dark room using the enhanced chemiluminescence reagent (ECL), and the excess liquid on the membrane was absorbed with a filter paper. The luminescent protein bands were scanned, and the gray value was analyzed using Quantity One software. The relative expression level of each protein = the gray value of the target protein band / the gray value of the β-Actin protein band.

Double-Luciferase Assay

According to the bioinformatics website STARBASE v2.0 (http://starbase.sysu.edu.cn/index.php), the potential binding site between NORAD and miR-199a-3p was predicted, relevant primers were designed, and the sequence combining NORAD with miR-199a-3p was cloned. Then, the fragment was inserted into Luciferase reporter gene plasmid pmirGLO, so as to construct wild-type NORAD reporter gene plasmid (NORAD-wt) and mutant NORAD reporter gene plasmid (NORAD-mut). NORAD-wt, NORAD-mut plasmid, and miR-199a-3p mimic were co-transfected into HO-8910 cells, cultured for 24 h, the cells were collected, Luciferase activity was detected by Luciferase detection kit, and the results were statistically analyzed.

Table I. Primer sequence for qRT-PCR assay.

	Forward primer	Reverse primer
NORAD	5'-TGATAGGATACATCTTGGACATGGA-3'	5'-AACCTAATGAACAAGTCCTGACATACA-3'
miR-199a-3p	5'-GCCGAACAGTAGTCTGCACAT-3'	5'-TATGGTTTTGACGACTGTGTGAT-3'
U6	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTTGCGT-3'
GAPDH	5'-GGAGCGAGATCCCTCCAAAAT-3'	5'-GGCTGTTGTCATACTTCTCATGG-3'

Statistical Analysis

SPSS 20.0 software package (IBM, Armonk, WI, USA) was used to carry out statistical analysis on the collected data, and GraphPad 7 software package (San Diego, CA, USA) was used to draw the required pictures; independent-samples t-test was used for inter-group comparison, one-way analysis of variance (ANOVA) was used for multi-group comparison, and LSD-t-test was used for post-hoc pairwise comparison; repeated measures analysis of variance was used for multi-time point expression, and Bonferroni and Pearson test were used for back testing to analyze the correlation between NORAD and miR-199a-3p in tissue; $p < 0.05$ was a significant statistical difference.

Results

Over-Expression of NORAD in Tissues and Cells of Ovarian Cancer

QPCR results showed that compared with tissues adjacent to cancer, NORAD was highly expressed in tissues and cells of ovarian cancer ($p<0.05$), while miR-199a-3p was lowly expressed ($p<0.05$); the correlation analysis revealed that the expression levels of NORAD and miR-199a-3p were negatively correlated (r= -0.856, $p<0.05$; Figure 1).

Effect of NORAD on Proliferation, Invasion, and Migration of Ovarian Cancer Cells

After HO-8910 and A2780 cells were transfected with Si-NORAD, Sh-NORAD and NC, it was found that the expression of NORAD in HO-8910 and A2780 transfected Si-NORAD cells was significantly lower than that in miR-NC transfected cells, while the expression of NORAD in HO-8910 and A2780 transfected Sh-NORAD cells was significantly higher than that in miR-NC transfected cells. By examining the biological functions of cells in the two groups, it was found that the proliferation, invasion, and migration ability of transfected Si-NORAD cells were significantly lower than that of miR-NC, while the proliferation, invasion, and migration ability of transfected Sh-NORAD cells were significantly higher than that of miR-NC. After transfection of Si-NORAD, the expression levels of N-cadherin and vimentin in transfected miR-NC cells reduced significantly and the expression of E-cadherin increased significantly. After transfection of Sh-NORAD, the expression levels of N-cadherin and vimentin in transfected miR-NC cells increased significantly and the expression of E-cadherin reduced significantly (Figure 2).

Effect of MiR-199a-3p on Proliferation, Invasion, and Migration of Ovarian Cancer Cells

After HO-8910 and A2780 cells were transfected with miR-199a-3p-mimics, miR-199a-3p-inhibitor and miR-NC, it was found that the expression of miR-199a-3p-mimics transfected with HO-8910 and A2780 cells significantly up-regulated compared with that of miR-NC transfected cells. However, the expression of miR-199a-3p-inhibitor transfected by HO-8910 and A2780 was significantly lower than that of miR-NC transfected cells. After examining the biological functions of cells in the two groups, it was found that the proliferation, invasion, and migration ability of transfected miR-199a-3p-mimics cells significantly reduced compared with that of miR-NC, while the proliferation, invasion, and migration ability of transfected miR-199a-3p-inhibitor cells significantly increased compared with that of miR-NC. After transfecting miR-199a-3p-mimics, the expression levels of N-cadherin and vimentin in transfected miR-NC cells significantly reduced and the expression of E-cadherin significantly increased, while after transfecting miR-199a-3p-inhibitor, the expression levels of N-cadherin and vimentin in transfected miR-NC cells significantly increased and the expression of E-cadherin significantly decreased (Figure 3).

Regulatory Relationship Between NORAD and MiR-199a-3p

Bioinformatics prediction tools found that NORAD might have direct effect on miR-199a-3p, and their binding sequences had similar binding positions. To verify whether NORAD could combine with miR-199a-3p 3'UTR, NORAD and miR-199a-3p were co-transfected into HO-8910 cells. Dual-Luciferase reporter gene results showed that the Luciferase activity of miR-199a-3p significantly reduced after NORAD and miR-199a-3p were co-transfected. The results demonstrated that NORAD could specifically bind to the 3'UTR of miR-214 and regulate its expression activity and level. In addition, we compared the expression of endogenous miR199a-3p between si-NC and si-NORAD transfected HO-8910, A2780 cells, and found that miR-199a-3p was up-regulated in reverse in HO-8910 and A2780 cells with NORAD inhibition (Figure 4).

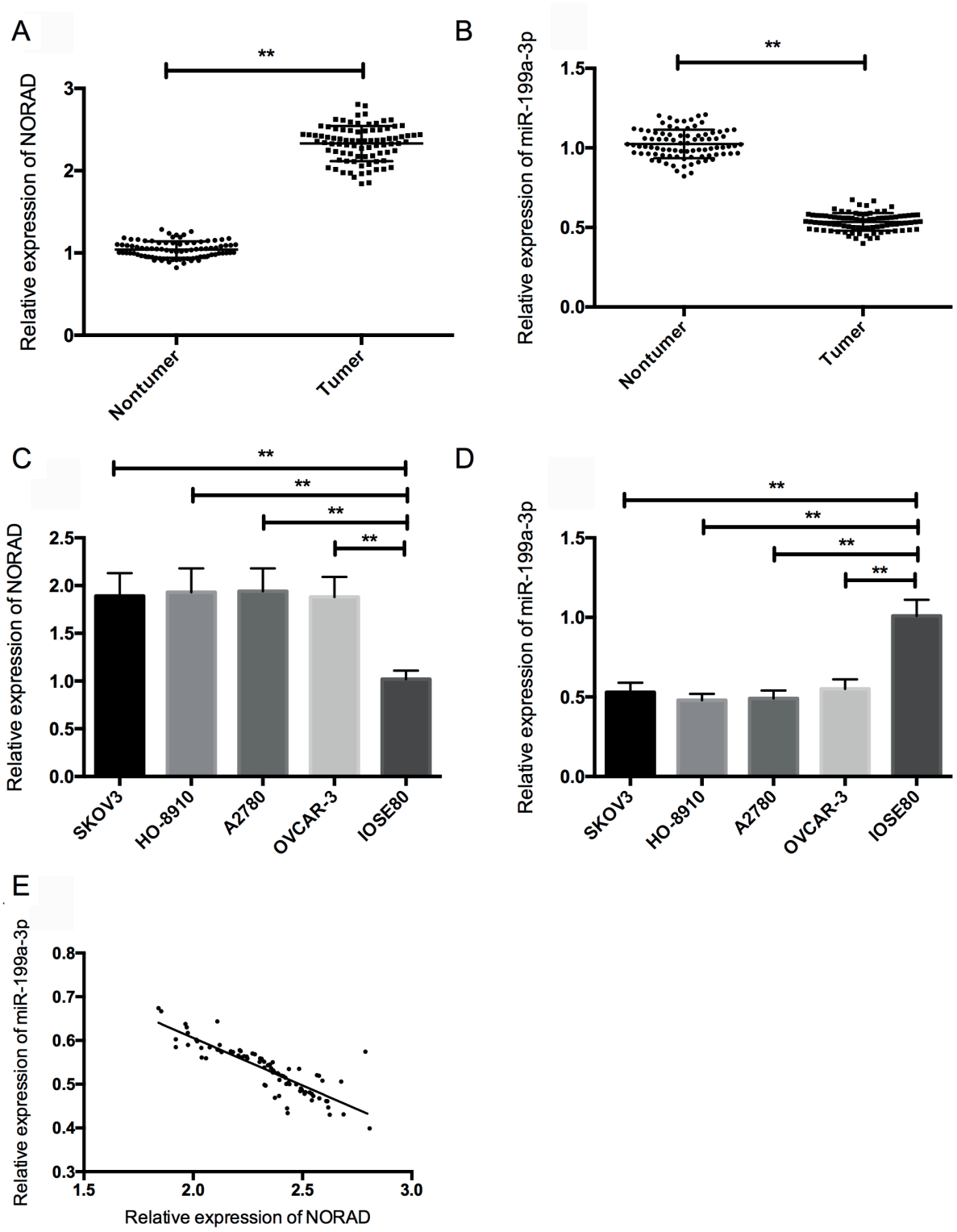

Figure 1. Over-expression of NORAD in tissues and cells of ovarian cancer. **A,** Expression of NORAD in ovarian cancer tissues. **B,** Expression of miR-199a-3p in ovarian cancer tissues. **C,** Expression of NORAD in ovarian cancer cells. **D,** Expression of miR-199a-3p in ovarian cancer cells. **E,** NORAD was negatively correlated with miR-199a-3p. ** indicates $p < 0.05$.

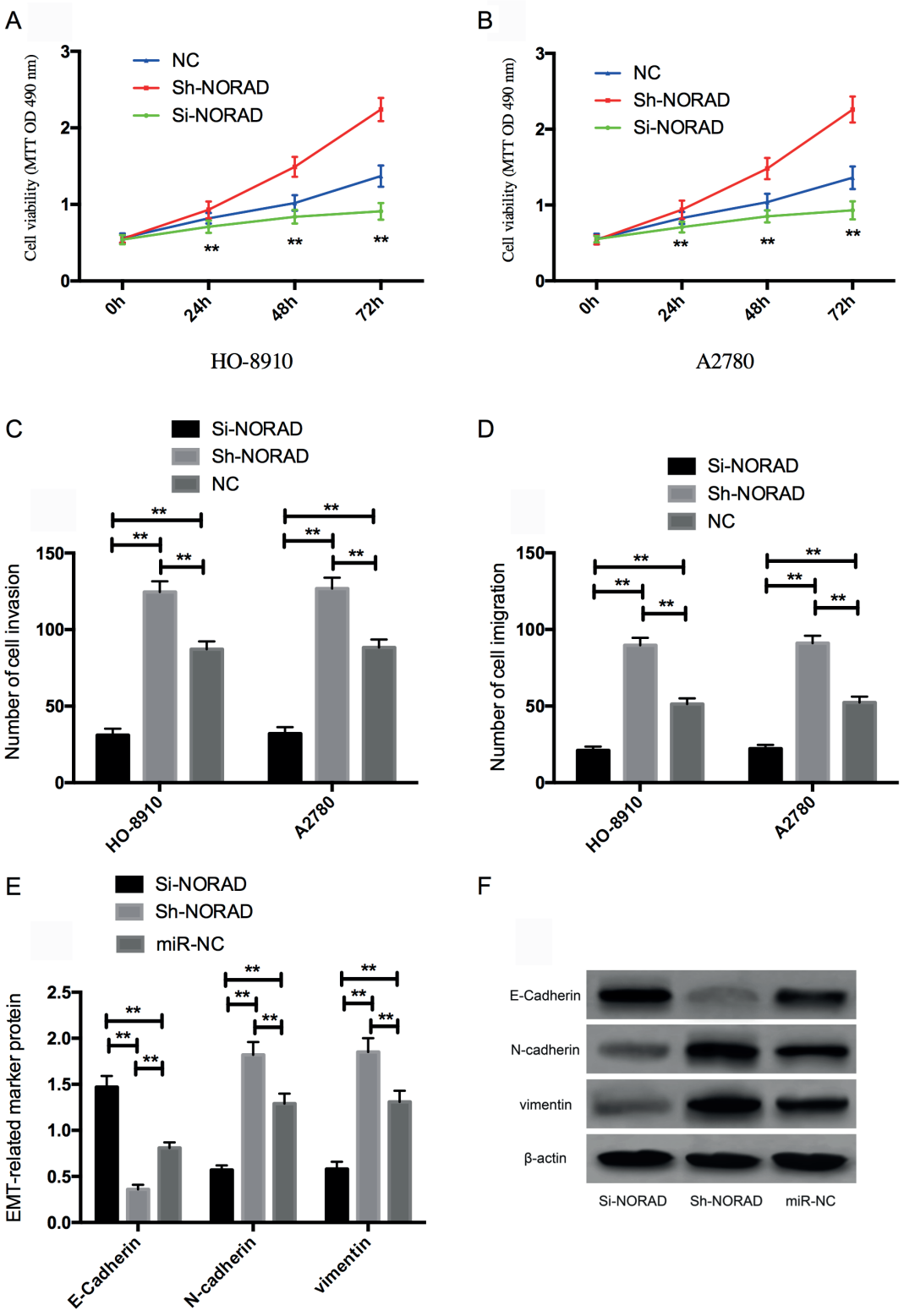

Figure 2. Effect of NORAD on proliferation, invasion and migration of ovarian cancer cells. **A-B,** Effect of NORAD on proliferation of ovarian cancer cells. **C,** Effect of NORAD on invasion of ovarian cancer cells. **D,** Effect of NORAD on migration of ovarian cancer cells. **E,** Effect of NORAD on EMT-related proteins in ovarian cancer cells. **F,** Western blot. ** indicates $p < 0.05$.

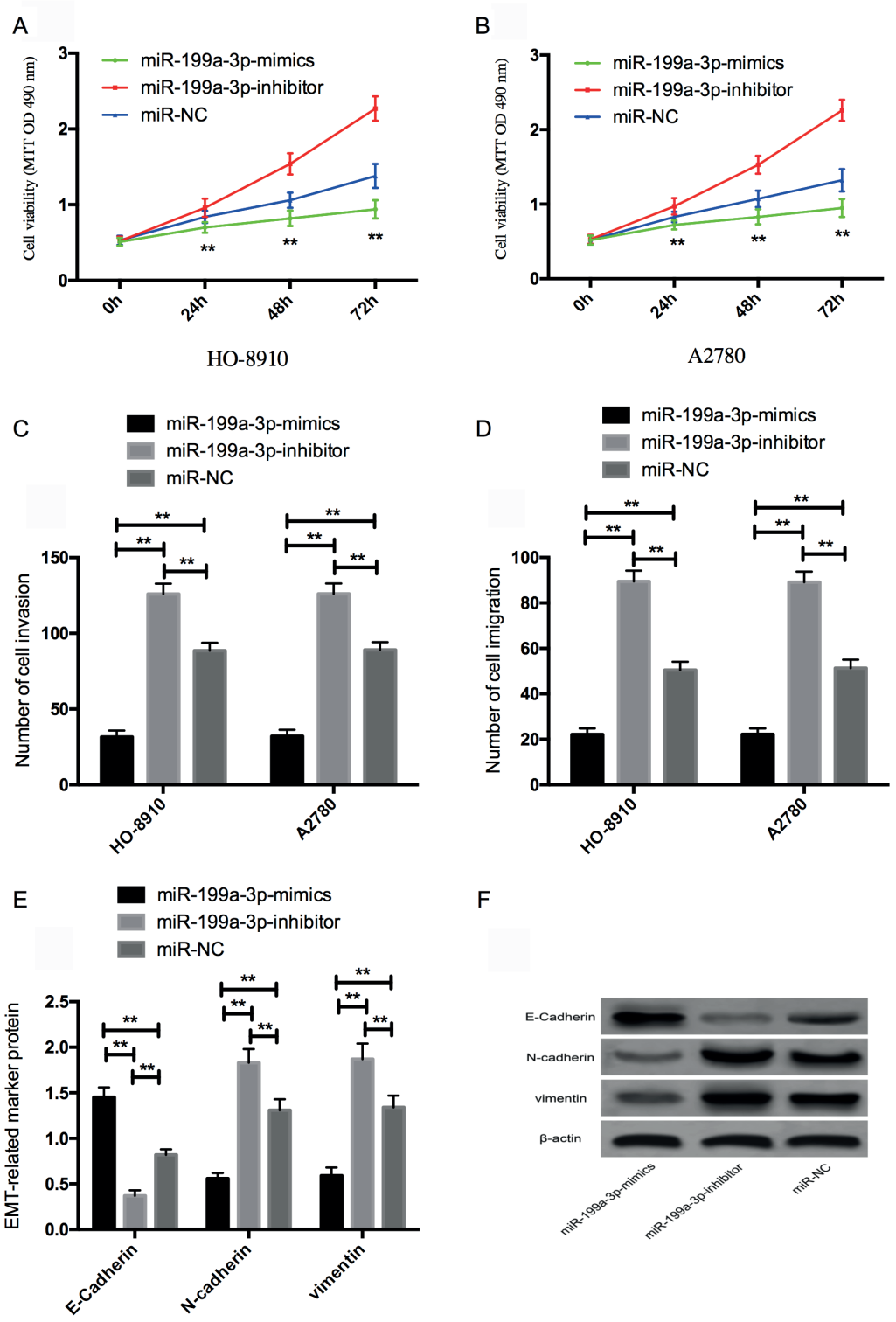

Figure 3. Effect of miR-199a-3p on proliferation, invasion and migration of ovarian cancer cells. **A-B,** Effect of miR-199a-3p on proliferation of ovarian cancer cells. **C,** Effect of miR-199a-3p on invasion of ovarian cancer cells. **D,** Effect of miR-199a-3p on migration of ovarian cancer cells. **E,** Effect of miR-199a-3p on EMT-related proteins in ovarian cancer cells. **F,** Western blot. ** indicates $p < 0.05$.

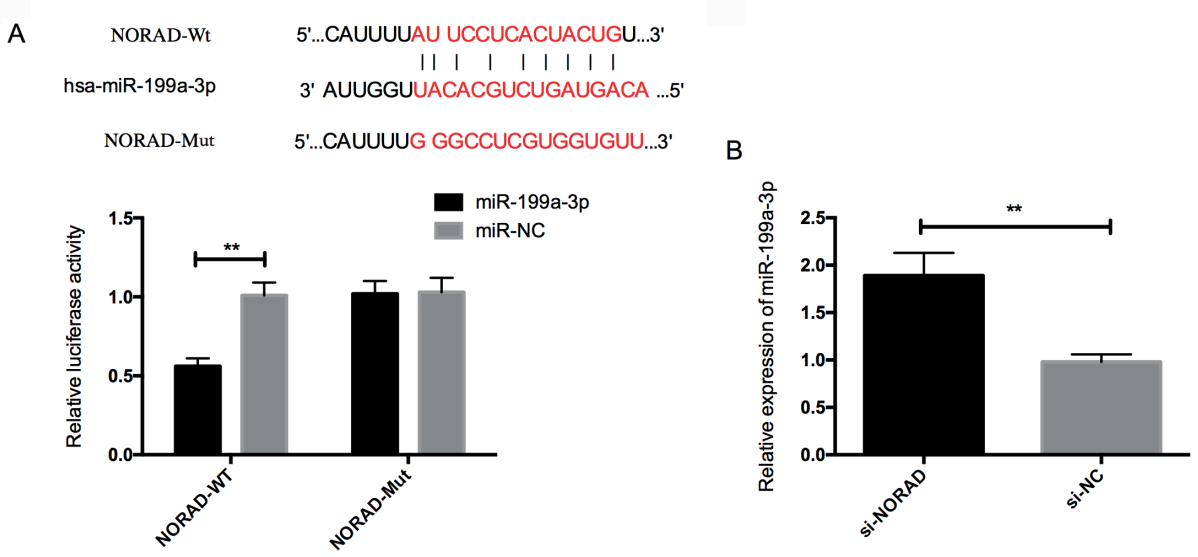

Figure 4. Regulatory relationship between NORAD and miR-199a-3p. **A,** Dual-Luciferase reporter enzyme and binding site. **B,** NORAD's influence on miR-199a-3p expression ** indicates $p < 0.05$.

Discussion

Ovarian cancer, as the most common gynecologic tumor, has a high mortality because most patients are already advanced at the time of diagnosis[12]. In recent years, despite the continuous development of medical technology, the treatment plan against surgery-assisted radiotherapy and chemotherapy for advanced ovarian cancer patients is still ineffective, and their five-year survival rate is still low[13]. Moreover, current diagnostic methods such as imaging or serum tumor markers are less sensitive to early ovarian cancer. Therefore, finding a new diagnostic and therapeutic target is very significant to ovarian cancer patients[14]. In recent years, increasing studies have found that epigenetic regulation of LncRNA plays a vital role in the occurrence and development of tumors and may be an important molecular target in diagnosis and treatment of clinical tumors[15].

In our research, we found that inhibiting the expression of LncRNA NORAD could inhibit the proliferation and invasion of ovarian cancer cells by regulating miR-199a-3p. Some reports[16] have identified that LncRNA NORAD can promote cell proliferation and glycolysis of non-small cell lung cancer by playing a sponge role on miR-136-5p. Yu et al[17] have revealed that silencing LncRNA NORAD can inhibit the proliferation and invasion of gastric cancer cells by regulating the RhoA/ROCK1 pathway. All these have indicated that LncRNA NORAD is a tumor suppressor gene in most tumors. We first detected the expression of LncRNA NORAD in tissues and cells of ovarian cancer, and discovered that LncRNA NORAD upregulated in its tissues and cells. Then, we respectively up-regulated and down-regulated NORAD in HO-8910 and A2780 cells of ovarian cancer, and the results showed that when NORAD down-regulated, proliferation, invasion, and migration of cells were significantly inhibited. Detection of EMT-related proteins also showed that EMT was significantly inhibited. When NORAD was upregulated, it was found that the proliferation, invasion, and migration of cells significantly enhanced, and EMT was also promoted. This suggested that NORAD played an oncogenic role in ovarian cancer and could effectively inhibit the proliferation and invasion of ovarian cancer cells when inhibiting its expression. Although NORAD was proved to promote EMT of ovarian cancer cells for the first time, it had been found in previous studies[8] that NORAD could promote EMT of thyroid carcinoma patients through interaction with miR-202-5p, which could also confirm our conclusion. However, the mechanism of NORAD in ovarian cancer is still unclear.

To further analyze the mechanism of NORAD in ovarian cancer, we predicted that lncRNA DANCR and miR-199a-3p had a binding site

through online database. In addition, some studies[18] have found that NORAD can regulate the proliferation and migration of osteosarcoma cells by competitive binding of miR-199a-3p in human osteosarcoma, but the regulatory mechanism of competitive binding in ovarian cancer has not been discussed. MiR-199a-3p had been verified to have expression disorders in various cancers, including ovarian cancer in the past[19,20]. In our study, we found that the expression of miR-199a-3p in tissues and cells of ovarian cancer significantly down-regulated, which was consistent with the results of previous studies[21]. Subsequently, we observed the effect of miR-199a-3p on the biological function of ovarian cancer cells by up-regulating and down-regulating their expression of miR-199a-3p. The results indicated that when the expression of miR-199a-3p was upregulated, the proliferation, invasion, migration, and EMT of ovarian cancer cells were significantly inhibited, but when the expression of miR-199a-3p was further down-regulated, their proliferation, invasion, migration, and EMT were further enhanced, which showed that miR-199a-3p played a part of tumor suppressor gene in ovarian cancer. Deng et al[22] indicated that miR-199a-3p played a part of tumor suppressor gene in ovarian cancer and inhibited invasion and metastasis of ovarian cancer tumor cells by regulating the expression of DDR1, which was also consistent with our conclusion. Then, to further prove the relationship between NORAD and miR-199a-3p, we carried out Dual-Luciferase reporter enzyme detection. The results showed that the Luciferase activity of miR-199a-3p significantly reduced after NORAD and miR-199a-3p were co-transfected, and miR-199a-3p was found to be up-regulated in reverse in HO-8910 and A2780 cells with NORAD inhibition, which further indicated the regulatory relationship between NORAD and miR-199a-3p.

Conclusions

LncRNA NORAD is highly expressed in ovarian cancer tissues. Silencing the expression of LncRNA NORAD can inhibit the proliferation, invasion, migration, and EMT of ovarian cancer cells by regulating miR-199a-3p, which may be a new target for its diagnosis and treatment. Nevertheless, there are still some deficiencies in this study. For example, we did not further analyze the molecular mechanism of miR-199a-3p in ovarian cancer, as well as the clinical value of LncRNA NORAD in patients. In the subsequent experiments, we will further enlarge the sample and carry out more in-depth basic experiments.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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