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MiR-218 suppresses metastasis and invasion of endometrial cancer via negatively regulating ADD2

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Abstract. – OBJECTIVE: Endometrial cancer (EC) is one of the three most common gynecological cancers. Due to the lack of effective treatment for EC patients in an advanced stage, the mortality rate of EC is increasing rapidly. Hence, it is essential to seek for novel molecular therapeutic targets and biomarkers for EC. The aim of this study was to explore the role of miR-218 in the occurrence and development of EC and to investigate the possible underlying mechanism.

PATIENTS AND METHODS: The expression level of miR-218 in EC tissues and cell lines was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Wound healing assay and Matrigel assay were performed to determine the migration and invasion abilities of EC cells. Meanwhile, the potential targets of miR-218 were predicted by bioinformatics analysis and confirmed by Luciferase reporter gene assay. In addition, the protein expression level of Adducin 2 (ADD2) was assessed by Western blotting analysis.

RESULTS: QRT-PCR results revealed that miR-218 was significantly downregulated in EC tissues and cell lines. Wound healing assay and Matrigel assay demonstrated that miR-218 suppressed the migration and invasion abilities of EC cells. Online prediction databases predicted that ADD2 was a direct target of miR-218, which was verified by Luciferase reporter gene assay. Rescue experiments further validated that miR-218 could serve as a carcinoma suppressor by negatively regulating ADD2 expression in EC.

CONCLUSIONS: In the present study, we elucidated that miR-218 served as a tumor suppressor in EC by negatively regulating ADD2. This might bring a novel insight into new molecular therapeutic targets and biomarkers for EC.

Key Words:
Endometrial cancer, MiR-218, Migration, Invasion, ADD2.

Introduction

Endometrial cancer (EC) is one of the most common gynecological cancers worldwide[1]. The incidence rate of EC has greatly increased in the past few years[2]. In developed countries, EC remains the fourth most common malignant cancer in women[1]. Although considerable researches have been conducted, EC is still the leading cause of gynecological related mortality worldwide[3]. Due to the lack of effective treatments for EC patients in advanced or recurrent stages, the prognosis of these patients is still low[4]. Hence, it is essential to seek novel treatment strategies and biomarkers for EC.

MicroRNAs (miRNAs) are a type of endogenous, non-coding small RNA molecules with 19-24 nucleotides in length. They participate in post-transcriptional gene expression regulation through binding to the 3'-untranslated regions (3'-UTR) of target messenger RNAs (mRNAs). This may eventually result in the degradation of mRNAs or inhibition of transcription[5]. Accumulating evidence has proved that miRNAs play an essential role in physiological processes, including cell differentiation, proliferation, metastasis, invasion, apoptosis and angiogenesis[6-8]. Dysregulated expression of miRNAs can lead to various types of cancers, such as EC[9-11]. Recent studies have shown that miR-218 is abnormally expressed multiple tumors. Meanwhile, its dysregulation can affect various biological processes in the development of malignancy[12-14]. However, the exact physiological function of miR-218 in EC progression remains unclear. Therefore, the aim of this study was to investigate the role of miR-218 in EC.

Adducin 2 (ADD2) was firstly extracted and identified from red blood cell cytoskeleton[15]. ADD2 belongs to the adducin family, which

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is a class membrane skeleton proteins including ADD1, ADD2 and ADD3[16]. Currently, it is believed that adducin plays a vital role in tumorigenesis and metastasis through regulating oncogenic signal transduction pathways[17,18]. Consistently, the alternation of ADD2 can lead to various diseases along with malignant tumors[16]. However, the molecular mechanism underlying the diverse range of disorders in numerous diseases is still unknown. Hence, in the current study, we hope to introduce novel insights of ADD2 in EC.

In the present study, we found that miR-218 expression was significantly down-regulated in EC tissues and cell lines. Functional experiments revealed that miR-218 inhibited the migration and invasion abilities *in vitro*. Besides, ADD2 served as a direct downstream target of miR-218. To sum up, we considered that miR-218 suppressed the migration and invasion of EC by regulating ADD2.

Patients and Methods

Clinical Samples

This study was approved by the Ethics Committee of Luoyang Central Hospital Affiliated to Zhengzhou University. Informed consent was obtained from each subject before the study. From 2016 to 2017, 25 pairs of EC tissues and adjacent normal tissues were collected from EC patients who underwent treatment in Luoyang Central Hospital Affiliated to Zhengzhou University. The expression levels of miR-218 and ADD2 were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). All collected tissues samples were preserved in liquid nitrogen for subsequent experiments.

Cell Culture

Four EC cell lines (ECC-1, Ishikawa, KLE, AN3CA) and one endometrial fibroblast cell line (T-HESC) were cultured in Dulbecco's modified Eagle' medium (DMEM) (Gibco, Rockville, MD, USA) supplemented by 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin and maintained in a 37°C, 5% CO_2 incubator.

Cell Transfection

Oligonucleotides for overexpressing or down-regulating miR-218 (mimics or inhibitor) and their negative controls were constructed by GenePharma (Shanghai, China). The plasmid pcDNA-3.1 (GenePharma, Shanghai, China) was used to up-regulate the expression of ADD2, while the empty pcDNA-3.1 plasmid was used as a negative control. SiRNA ADD2 (GenePharma, Shanghai, China) was transfected to cells to downregulate its expression. Transfection efficiency was determined by qRT-PCR.

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

EC tissue specimens or EC cell lines were involved to derive total RNA *via* using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse Transcription Kit (TaKaRa, Dalian, China) was taken part into synthesized cDNA subsequently. The complementary Deoxyribose Nucleic Acid (cDNA) was synthesized according to the instructions of the PrimeScriptTM RT MasterMix kit (Invitrogen, Carlsbad, CA, USA). QRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s and 72°C for 90 s, for a total of 40 cycles. The relative expression level of the target gene was expressed by $2^{-\Delta\Delta Ct}$. Primer sequences used in this study were as follows: miR-218, F: 5'-GCGGCTTTGTGCTTGATCTAA-3', R: 5'-GTGCAGGGTCCGAGGT-3'; ADD2, F: 5'- GTGCTTTGTGGAGGGTACTTCAT-3', R: 5'- TTGGACATCACAAATAGGACAATACTT-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Wound Healing Assay

To elucidate the effect of miR-218 on cell migration, wound healing assay was performed. Cells were first seeded into 6-well plates and transfected with miR-218 mimics or inhibitor. After 48 h, the culture medium was replaced with serum-free medium DMEM, and all linear scratches were made by pipette tips. The scratches were photographed at 0 h and 24 h to monitor the migration process, respectively. This experiment was repeated three times.

Transwell Assay

Transwell assay was then performed to detect the invasion ability of EC cells. Transwell chambers and 24-well plates were obtained from Corning (Corning, NY, USA). A total of 1×10^5 cells were suspended with 100 μL serum-free medium and transferred to the Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) coated upper chamber.

After 36 h, the invasive cells were captured by an inverted microscope (Olympus, Tokyo, Japan), and the number of cells was counted. 5 fields were randomly selected for each sample.

Luciferase Reporter Gene Assay

Bioinformatics predicted that ADD2 had potential binding sites with miR-218 in the 3'-UTR region. Hence, we used Luciferase reporter gene assay for validation. The pGL3 promoter vector containing wild ADD2 3'-UTR sequence or mutant ADD2 3'-UTR sequence was constructed (Genscript, Nanjing, China). Cell transfection was conducted according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Luciferase activity was detected by the Luciferase assay kit (Promega, Madison, WI, USA). This experiment was repeated three times.

Western Blotting

Total protein of cells was extracted by the radio-immunoprecipitation assay (RIPA) buffer (Thermo, Waltham, MA, USA) containing Phenylmethanesulfonyl fluoride (PMSF). Subsequently, total proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Roche, Basel, Switzerland). After blocking with 5% skimmed milk, the membranes were incubated with rabbit anti-ADD2 (1:1000, CST, Danvers, MA, USA) at 4°C overnight. After washing with Tris-Buffered Saline and Tween 20 (TBST) 3 times, the membranes were incubated with the corresponding secondary antibody at room temperature for 1 h. Rabbit anti-GAPDH (1:5000, CST, Danvers, MA, USA) was taken as a loading control. Relative protein expression level was evaluated by Image J software.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 Software (IBM, Armonk, NY, USA) was used for all statistical analysis. All experiments in this study were repeated at least three times. Experimental data were expressed as mean ± standard deviation (SD). Pearson correlation analysis was performed for correlation analysis. Student's unpaired *t*-test was used to compare the differences between the two groups. $p<0.05$ was considered statistically significant.

Results

MiR-218 was Downregulated in EC Tissues and Cell Lines

QRT-PCR was performed to detect the expression of miR-218 in EC tissues and cell lines. As shown in Figure 1A, we found that miR-218 expression in EC tissues was significantly lower than that of adjacent normal tissues. Consistently, the expression level of miR-218 in EC cell lines was remarkably lower than the endometrial fibroblast cell line T-HESC (Figure 1B). All the results demonstrated that miR-218 was negatively correlated with the progression of EC.

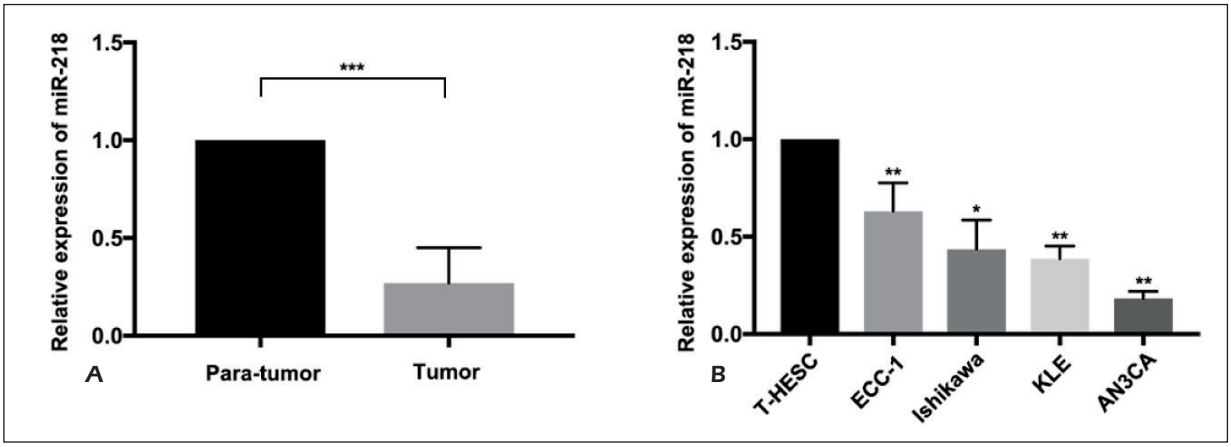

Figure 1. The expression level of miR-218 in EC tissues and cell lines. **A**, Relative expression level of miR-218 in 25 paired EC tissues and adjacent normal tissues was detected by qRT-PCR. **A**, QRT-PCR was used to detect the expression level of miR-218 in EC cell lines. *$p<0.05$, **$p<0.01$, ***$p<0.001$, compared with the control group. Data were expressed as mean ± SD.

MiR-218 Suppressed the Migration and Invasion of EC Cells

Subsequently, we transfected miR-218 mimics AN3CA cells to up-regulation its expression and transfected miR-218 inhibitor in ECC-1 cells to down-regulate its expression, respectively. QRT-PCR was performed to determine the transfection efficiency. As shown in Figure 2A, miR-218 expression was significantly upregulated in the mimics group. Reversely, compared with the inhibitor-NC group, the expression level of miR-218 was remarkably downregulated in the inhibitor group. Wound healing assay was to investigate the migration ability of EC cells. The results showed that the wound healing ability of the mimics group was significantly impaired when compared with the NC group. However, compared with the inhibitor-NC group, the wound healing ability of the inhibitor group was improved (Figure 2B). Subsequently, we performed the Matrigel assay to examine the effect of miR-218 on cell invasion. As shown in Figure 2C, compared with the control group respectively, miR-218 over-expression significantly decreased the number of invasive cells. However, miR-218 down-relation significantly increased the number of invasive cells. The above results all proved that miR-218 inhibited cell migration and invasion in EC.

ADD2 was a Downstream Target of miR-218

We then searched public available databases to predict the potential downstream targets of miR-218, including RNA22, TargetScan, miRWalk, and MiRanda. Results suggested that ADD2 was as a potential target of miR-218. Luciferase reporter gene assay was performed to verify our hypothesis. We found that the Luciferase activity of mimics group was significantly lower than that of the NC group when co-transfected with ADD2 WT 3'-UTR. In contrast, no significant difference was found in the Luciferase activity after co-transfection of mutant 3'-UTR (Figure 3). The above results revealed that miR-218 was a direct target of miR-218.

Furthermore, we used qRT-PCR to quantify the relative expression level of ADD2 in EC tissues. Results manifested that the expression of ADD2 in EC tissues was significantly higher than that of adjacent tumor tissues (Figure 4A). Pearson correlation analysis demonstrated that miR-218 expression was negatively correlated with ADD2 (Figure 4B). Subsequently, Western blotting was employed to access the protein expression level of ADD2. Result indicated that ADD2 was downregulated in the mimics group (Figure 4C). In sum, our findings validated that ADD2 was a direct downstream target of miR-218 in EC.

MiR-218 Suppressed the Migration and Invasion of EC by Negatively Regulating ADD2

To further investigate the relevance between miR-218 and ADD2, we subsequently performed the rescue assay. We co-transfected cells with ADD2 over-expressed plasmid pcDNA-3.1 or ADD2 siRNA in the mimics group and the inhibitor group, respectively. As shown in Figure 4D, compared with the negative control group, ADD2 expression level in the mimics+ADD2 group was significantly increased. However, the ADD2 expression was significantly downregulated in the inhibitor+si-ADD2 group. Rescue assay demonstrated the wound healing ability of ECC-1 cells was recovered after transfection with ADD2 over-expressed plasmid, whereas the wound healing ability was remarkably impaired after transfection with ADD2 siRNA (Figure 5A). Consistently, similar results were obtained in the transwell assay (Figure 5B). Taken together, the above results suggested that miR-218 suppressed the migration and invasion ability of EC by negatively regulating ADD2.

Discussion

The incidence and mortality rate of EC remains high worldwide[19]. Due to evident symptoms, the prognosis of endometrial adenocarcinoma is favorable. However, the mortality rate of uterine cancer is growing rapidly, which is mainly related to poor prognosis of EC patients in the advanced stage[20]. The rapid increase in EC mortality is primarily caused by the lack of efficient treatment for EC patients. Therefore, it is of vital importance to seek for novel treatment and biomarkers for EC.

MiRNAs are a class of small endogenous non-coding RNAs, which are associated with various disease, especially malignant tumors[21]. It's reported that miRNAs participate in multiple cellular processes, including cell proliferation, migration, invasion, and cell self-renewal[22-24].

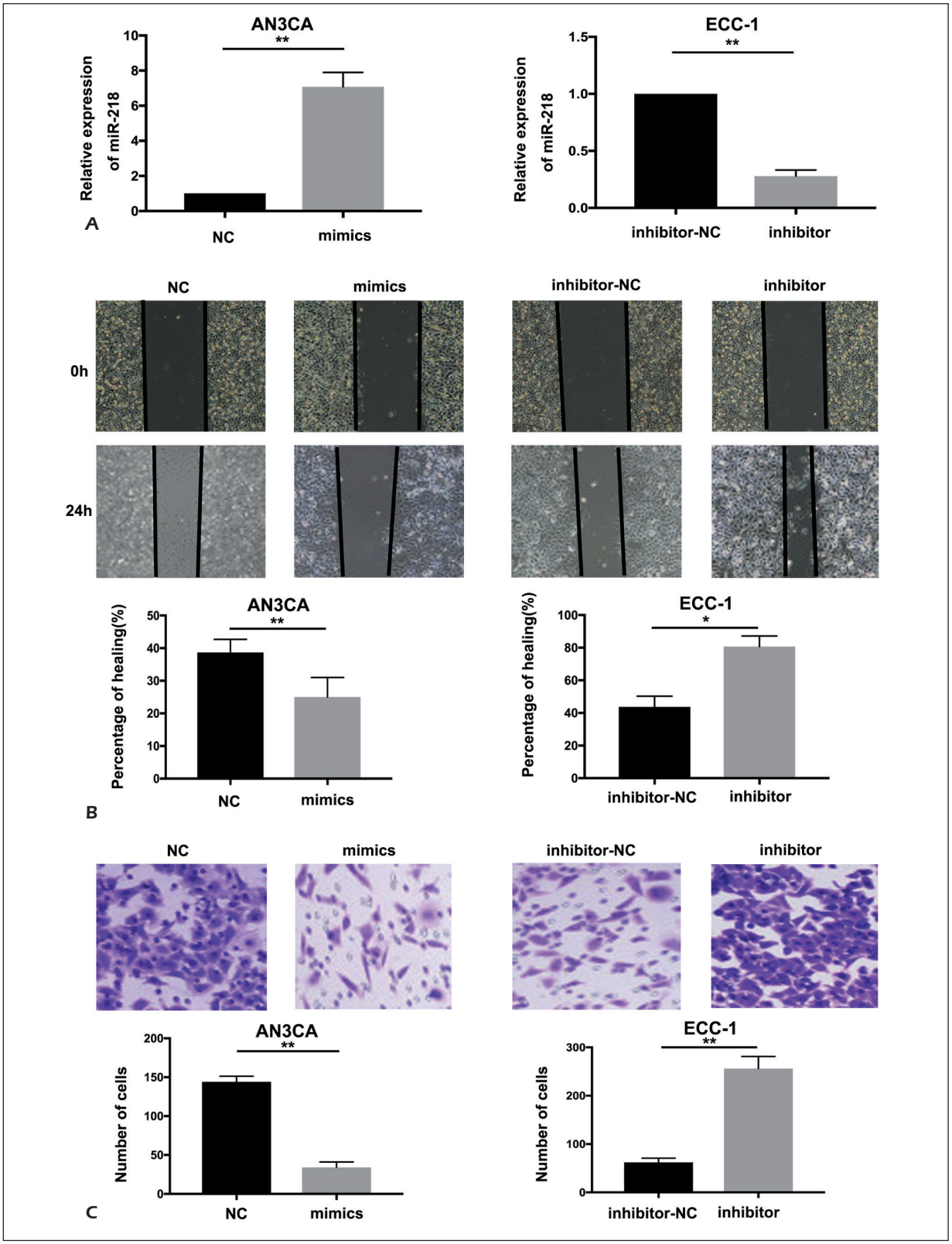

Figure 2. MiR-218 suppressed the migration and invasion of EC cells. **A**, Relative expression of miR-218 in ECC-1 or AN3CA cells transfected with mimics or inhibitor. **B**, Wound healing assay was performed in transfected cells. C, Invasive cells were detected by matrigel assay. *p<0.05, **p<0.01, compared with the control group. Data were expressed as mean ± SD.

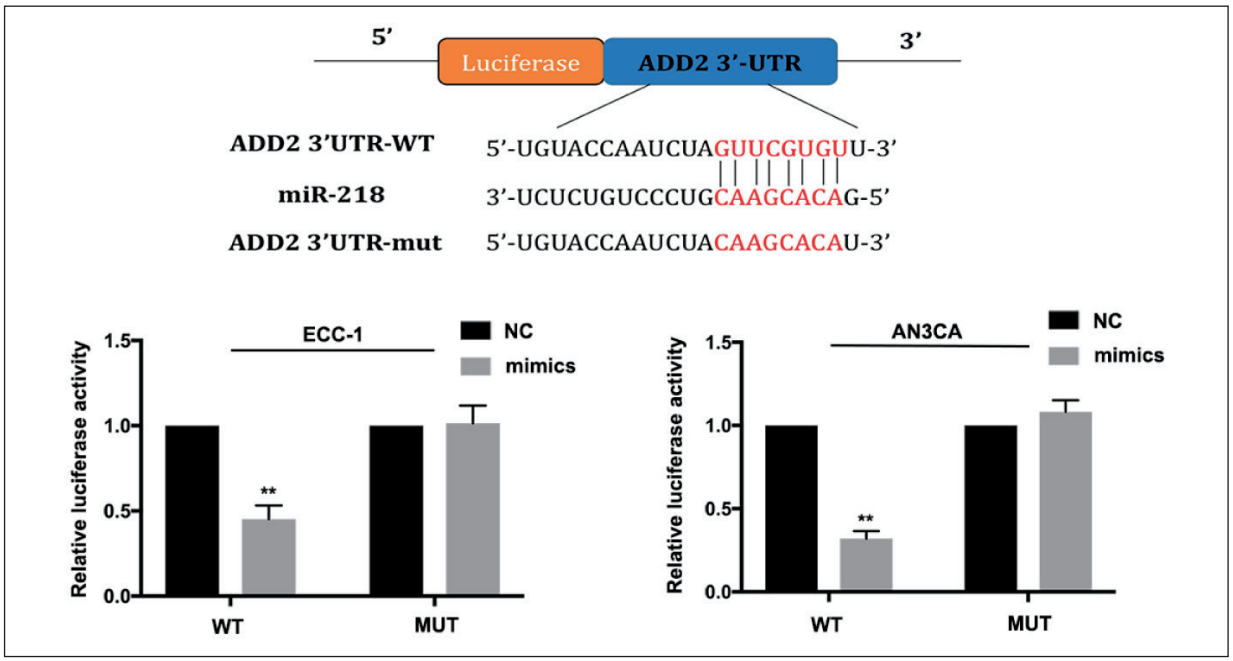

Figure 3. ADD2 was a down-stream target of miR-218. Luciferase reporter gene assay proved that miR-218 directly bond to the 3'-UTR of ADD2. The sequence of wild-type and mutant-type of ADD2 3'-UTR was shown. After co-transfection with wild-type or mutant 3'-UTR reporter plasmids, the relative ADD2 luciferase activity was calculated and recorded by histogram. $^{**}p<0.01$, compared with the control group. Data were expressed as mean ± SD.

According to the diverse functions of miRNAs, they have been reported to be potential biomarkers or molecular therapeutic targets in various cancers. For example, miR-17-5p is reported to be a novel predictor for recurrent breast cancer[25]. MiR-266 is considered as a potential biomarker for renal cell carcinoma prognosis[26]. Meanwhile, anti-miR-155 has been used for colorectal cancer therapy[27]. Based on the role of miRNAs in cancer, it is important to figure out the physiology of miRNAs in EC, thereby searching for novel biomarkers and molecular therapeutic targets for EC. Plenty of studies[12,28,29] validated that miR-218 is dysregulated in various cancer types. However, the physiological role of miR-218 in EC remains unknown. In the present study, we validated that miR-218 was significantly down-regulated in EC. Besides, wound healing assay and transwell assay revealed that miR-218 over-expression could remarkably suppress the migration and invasion of EC cells. All these results indicated that miR-218 played as a carcinoma suppressor in EC.

As known, miRNAs act as oncogenes or tumor suppressor genes by directly binding to their down-stream target genes. We then used bioinformatics analysis to predict the target gene of miR-218. Result indicated that ADD2 had a potential binding site with miR-218 in 3'-UTR. Subsequently, Luciferase reporter gene assay validated the hypothesis and showed that miR-218 had a binding site with ADD2. ADD2 belongs to the adducin family which is limited reported in cancer. Several studies[18,30,31] have explored the role of the adducin family in cancer. However, the relevance between miRNAs and ADD2 is rarely reported. Meanwhile, the specific role of ADD2 in EC remains unclear. Thus, we performed qRT-PCR to detect the expression of ADD2 in EC tissues. Results found that ADD2 was significantly up-regulated in EC tissues. Pearson correlation analysis elucidated that miR-218 expression is negatively correlated with ADD2. Besides, the protein expression of ADD2 was significantly down-regulated in the mimics group. Therefore, we wondered whether miR-218 exerted its role in EC by regulating ADD2 expression. Subsequently, rescue assay indicated that ADD2 over-expression could partially abolish the inhibitory effects of miR-218 on cell migration and invasion. Taken together, all these findings indicated that miR-218 acted as a tumor suppressor in EC through negatively regulating ADD2 expression.

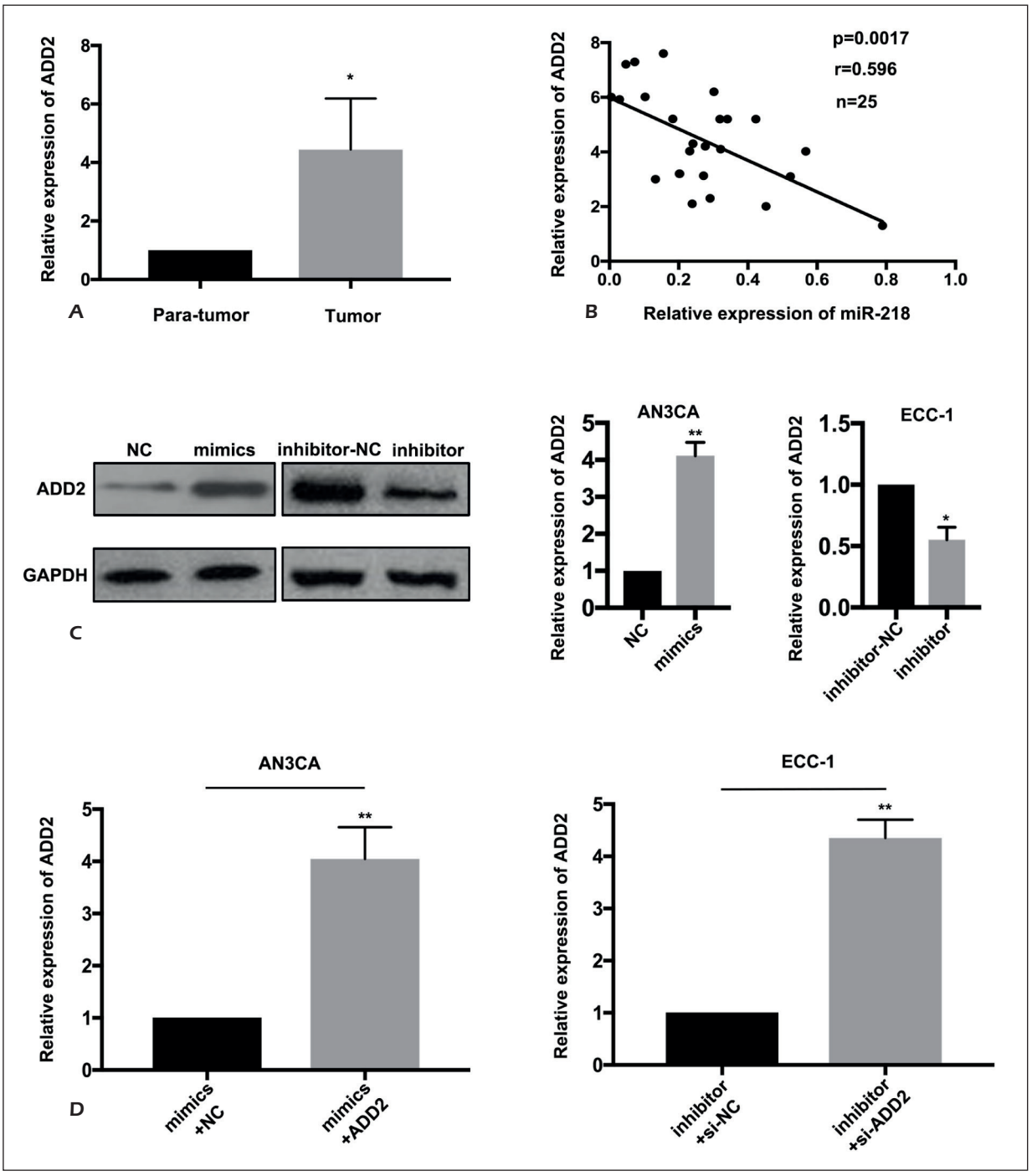

Figure 4. MiR-218 negatively regulated the expression of ADD2 in EC. **A**, The expression of ADD2 in EC tumor tissues and adjacent normal tissues. **B**, Pearson correlation analysis was used to analyze the correlation between ADD2 expression and miR-218 expression (p=0.0017). **C**, Western blot was used to detect the protein expression level of ADD2 in transfected cells. **D**, QRT-PCR was used to measure the mRNA expression of ADD2 in co-transfected cells. *p<0.05, **p<0.01, compared with the control group. Data were expressed as mean ± SD.

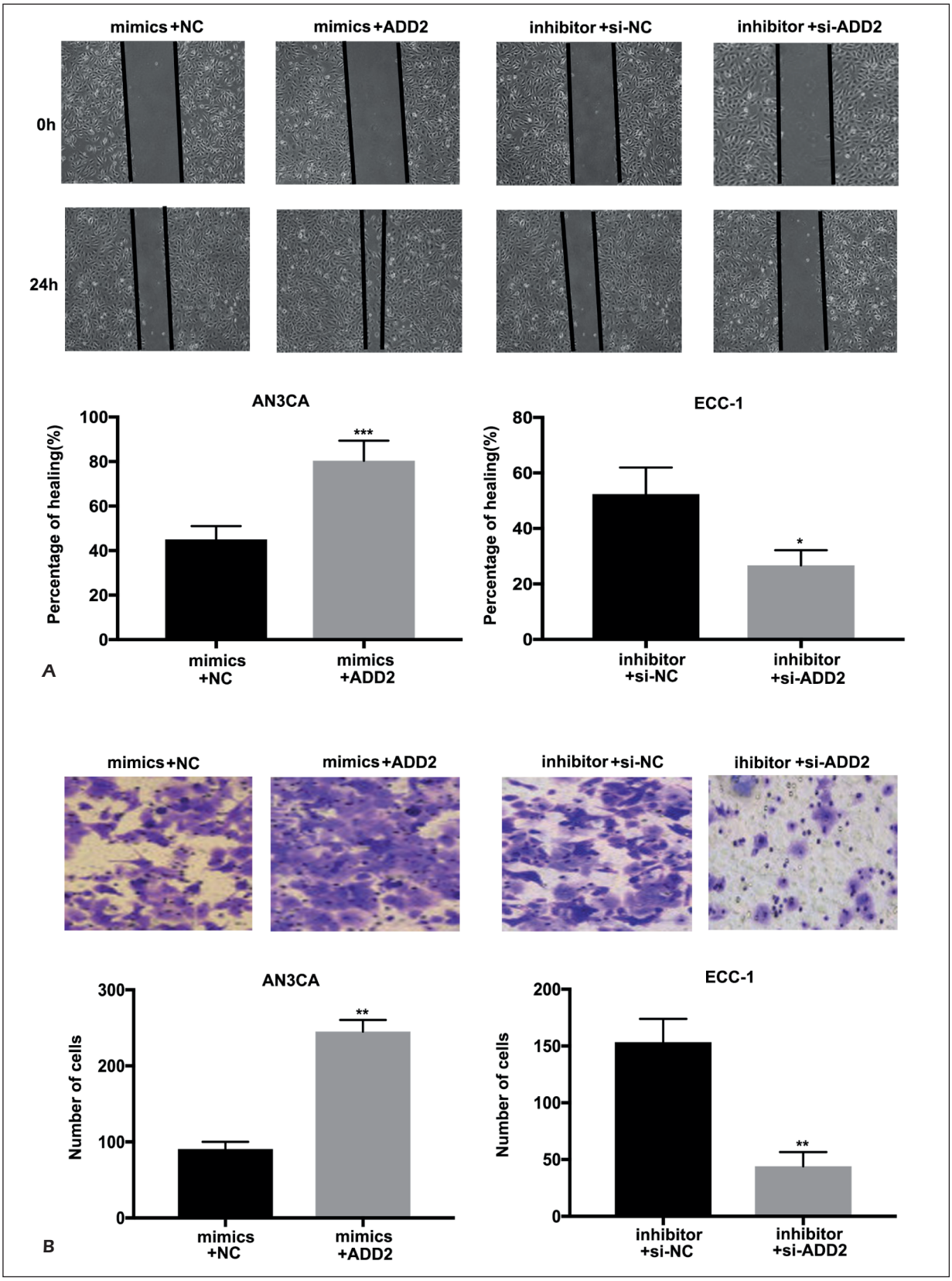

Figure 5. MiR-218 suppressed cell migration and invasion by negatively regulating ADD2. **A**, Wound healing assay was employed to detect the migration ability of co-transfected cells. **B**, Invasive ability of co-transfected cells was detected by transwell assay. *p<0.05, **p<0.01, ***p<0.001, compared with the control group. Data were expressed as mean ± SD.

Conclusions

In the current study, we validated that the expression of miR-218 was significantly downregulated in EC. Wound healing assay and transwell assay revealed that miR-218 significantly suppressed the migration and invasion ability of EC cells. Luciferase reporter gene assay verified that ADD2 was a downstream target of miR-218. Besides, rescue assay confirmed that miR-218 played its role as a carcinoma suppressor in EC by regulating ADD2 expression. In our study, we failed to focus on the physiological mechanism of miR-218 in EC. Thus, the potential molecular mechanism of miR-218 and ADD2 in EC remained to be known. In addition, our study might bring novel insights into biomarkers and molecular therapeutic targets for EC.

Conflict of Interests

The authors declare no conflict of interest.

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