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Original article

# Upregulation of miR-582-5p regulates cell proliferation and apoptosis by targeting AKT3 in human endometrial carcinoma

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ABSTRACT

The human endometrial carcinoma is one of the most common female malignancies, and there is an urgent requirement to explore new therapeutic strategies. There is accumulating evidence that microRNAs (miRNAs) can serve as potential diagnostic and prognostic biomarkers for various types of cancer, but the significance of miR-582-5p still remains largely unknown in the endometrial carcinoma. The aims of this study were to understand and identify the influence of miR-582-5p on the proliferation and apoptosis of human endometrial carcinoma and its relevant mechanism. First, quantitative real-time PCR (qRT-PCR) was used to detect miR-582-5p and AKT3 expression in human tissue samples and cells. Then, CyQuant assay and 2D colony assay were employed to evaluate cell proliferation. Western blotting was used to determine protein expression. Subsequently, the luciferase reporter assay was used to identify the target of miR-582-5p. Finally, Annexin V assay was used to detect cell apoptosis. We found that miR-582-5p expression was significantly decreased in human endometrial carcinoma tissues, and miR-582-5p upregulation in human endometrial carcinoma cells inhibit cell proliferation and promote apoptosis. Moreover, AKT3 was validated as a target of miR-582-5p and AKT3 expression was inversely correlated with miR-582-5p expression. Besides, AKT3 upregulation efficiently abrogates the effect of miR-582-5p on the cells. These results demonstrated that miR-582-5p regulates cell proliferation and apoptosis in human endometrial carcinoma via AKT3. Thus, miR-582-5p represents a potential therapeutic target in human endometrial carcinoma meriting further investigation.



## 1. Introduction

The human endometrium is a highly dynamic tissue and undergoes complex dynamic changes under the control hormones (Yin et al., 2015). Endometrial cancer has become the most common gynecological malignancy and the fourth most common diagnosed malignancy among women. Recently, despite a great improvement in endometrial cancer treatment and diagnosis, advanced stages of the disease are still difficult to manage (Bansal et al., 2009). The incidence of endometrial cancer increases, and the lack of powerful treatment strategies calls for the need of developing novel and effective therapeutic strategies for this malignancy. MicroRNAs (miRNAs) have become as one of the novel class of diagnostic biomarkers in human cancers (Koturbash et al., 2011; Shah et al., 2014). MiRNAs are small non-coding RNAs that negatively regulate gene expression. Over the last decade, extensive research has demonstrated the importance of miRNAs in cancer biology as powerful regulators of cellular processes in cancer initiation, progression and metastasis (Croce et al., 2009; Shah et al., 2014). Many miRNAs have been identified to be involved in the cancer development, such as miR17, miR-34 and miR-145 (Ma et al., 2012; Wu et al., 2012; Pagliuca et al., 2013). MiR-582-5p has been shown to be associated with various biological progression of cancer cells. For example, miR-582-5p has been reported to inhibit tumor proliferation in bladder cancer (Uchino et al., 2013). MiR-582-5p overexpression was also found to inhibit cell proliferation, cell cycle progression and invasion in human colorectal carcinoma (Zhang et al., 2015). However, its biological role and underlying molecular mechanism are still poor understand in human endometrial carcinoma.

AKT belongs to the most hyperactivated signaling pathways in human cancer to regulate various cellular functions including cell growth, proliferation, invasion, and survival (Altomare et al., 2005; Gonzalez et al., 2009; Khan et al., 2013). AKT1, AKT2, and

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AKT3 share roughly 80% overall sequence identity (Cheng et al., 2008). Among these isoforms, AKT2, and possibly AKT3, are important for disease progression and maintenance, but not AKT1 (Michiue et al., 2009; Mure et al., 2010). Akt3 has been also shown to contribute to the regulation of cell proliferation, migration, and invasion in many cancers such as thyroid cancer (Lin et al., 2017). However, the significance of AKT3 is still not clear in the miRNA regulatory network of human endometrial carcinoma.

In this study, the biological role of miR-582-5p in human endometrial carcinoma were explored. Additionally, we obtained evidence indicating miR-582-5p regulate the cell proliferation and apoptosis via AKT3.

## 2. Material and methods

### 2.1. Tissue collection

The human endometrial carcinoma and normal endometrial tissue samples for use in research were obtained according to the operational and ethical guidelines of Luoyang central hospital affiliated to Zhengzhou university. All patients are female. Tissues were collected and frozen in liquid nitrogen immediately after mincing on ice and then stored at −80 °C until analyze.

### 2.2. Cell culture

The endometrial carcinoma cell line ECC1 was cultured in the DMEM/F12 medium (Life Technologies, Carlsbad, CA) with 10% fetal bovine serum (FBS), penicillin and streptomycin (100 U/mL and 100 μg/mL). Cells were then maintained at 37 °C in the humidified chamber with 5% $CO_2$.

### 2.3. mi-582-5p mimic transfection

The cells were seeded in to 12-well plate at a density of 1 X 105 per well. The has-miR-582-5p and its negative control (miR-NC) were commercially available at Genepharma Co. (Shanghai, China). The cells were transfected with 25 nmol/L of miR-582-5p or miR-NC by RNAiMAX (life technologies) according to the manufacturer's instructions. The effect of miR-582-5p mimic transfection was determined by quantitative real time-PCR.

### 2.4. Quantitative real time-PCR (qRT-PCR)

The total RNA was extracted from human tissues or cells by mirVanaTM miRNA isolation kit (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's protocol. The quantitative real time-PCR (qRT-PCR) was used to determine the mRNA and miRNA expression. For specific gene expression determination, the complement DNA (cDNA) was generated by RNA-to-cDNA kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The gene specific probes were purchased from Applied Biosystems (Foster City, CA). GAPDH was used as an internal reference gene.

The Applied Biosystems TaqMan Advanced miRNA cDNA Synthesis Kit (Applied Biosystems) was used to generate the cDNA template of miR-582-5p. The U6 snRNA was used as an internal control. U6 cDNA was prepared by the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems).

### 2.5. Cell proliferation assay

The Cyquant assay was employed to monitor the cell viability (Thermo Fisher Scientific, Waltham, MA). The untransfected cells and transfected cells were plated into a 96-well plate at a density of 5000 cells per well. The plate was frozen at indicated time for the 1, 2, 3, 4 and 5 days incubation. Subsequently, the fresh prepared 100 μl CyQuant solution was added to the well to read with excitation at 485 nm and emission at 530 nm.

### 2.6. 2D colony formation assay

The colony formation assay is an in vitro assay to detect the ability of a single cell to grow into a colony. The suspended ECC1 cells were seeded in the 6-well plate (BD Biosciences, Bedford, MA) at a density of 1000 cells per well. The colonies were fixed with 80% ethanol and stained with crystal violet (0.5% w/v) (Millipore, Temecula, CA).

### 2.7. 3′-UTR luciferase assay

The ECC1 cells were seeded in the 6-well plate at the density of 0.2 M cells per well. After being incubated for 24 h, the cells were co-transfected with 20 nM miR-582-5p or miR-NC and 1 μg AKT3 3′-UTR luciferase reporter construct (GeneCopoeia, Rockville, MD) by lipofectamine 2000 (Invitrogen). Cells were lysed 48 h after transfection, and the renilla and firefly luciferase activity was detected by the Premega dual luciferase assay kit. Data was expressed as the ratio of firefly luciferase activity/renilla luciferase activity. The control luciferase reporter construct (Ctrl) was also available from GeneCopoeia.

### 2.8. AKT3 overexpression

To overexpress AKT3 in vitro, we used AKT3 vector construct (OriGene Technologies, Inc., Rockville, MD) and followed the manufacturer's instructions. The negative control vector (VC) was also commercially available at OriGene Technologies.

### 2.9. Apoptosis assay

The Annexin V Fluorescein Isothiocyanate kit (BD Pharmingen, San Diego, CA) was used to detect cell apoptosis. The cells were collected after 48hrs transfection with miRNA mimic or gene construct. After then, cells were suspended in 100 ml binding buffer ($1 \times 106$ cells/ml) and incubated with Annexin V-FITC and propidium iodide for 15 min. The apoptosis was detected on a BD FACSCalibur™ system (Becton-Dickinson, Franklin Lakes, NJ, USA). miR-NC or VC were used as the negative controls for miRNA or gene, respectively.

### 2.10. Western blot analysis

The total protein was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes (Millipore). After blocked with 5% non-fat milk, the membranes were then incubated with the anti-AKT3 antibody (Abcam, Cambridge, MA) or anti-GAPDH antibody (Abcam), which was used as the internal control. Then, goat anti-mouse or anti-rabbit horseradish peroxidase secondary antibody (Sigma, St. Louis, MO) was used for further incubation. The enhanced chemiluminescence reagents was introduced to detect the protein complex (Thermo Fisher Scientific).

### 2.11. Statistical analysis

Results were expressed as the means ± SE from 3 independent measurements. Data from 2 treatments were analyzed using the Student's t-test. One-way analysis of variance (ANOVA) test, followed by Tukey's multiple comparison for multiple groups were used to compare the significance of differences. $P < 0.05$ were

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