

Circulating UCA1 is highly expressed in patients with systemic lupus erythematosus and promotes the progression through the AKT pathway

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Abstract. – **OBJECTIVE:** To investigate the role of Urothelial Carcinoma Associated 1 (UCA1) during the progression of systemic lupus erythematosus (SLE) and the underlying mechanism.

PATIENTS AND METHODS: UCA1 expression in peripheral blood of SLE patients, as well as the expression of protein kinase B (AKT) in the peripheral blood mononuclear cell (PBMC), was detected by qRT-PCR. Expression differences in UCA1 and AKT between different groups were compared by t-test or univariate analysis. Through correlation analysis, the correlation between UCA1, AKT and clinical indicators of patients was analyzed. After overexpression and knockout of UCA1, the effect on phenotypes of BaF3 cell was examined. Finally, we analyzed the correlation between AKT and UCA1, and the effect on AKT pathway after overexpression and knockout of UCA1.

RESULTS: We found that plasma level of UCA1 and AKT was significantly enhanced in SLE patients. By analyzing the clinical data, a higher UCA1 level was observed in female patients than in males. In addition, UCA1 level in SLE patients with active stage and pathological lesions was higher than those in a stable stage without organ involvement. Correlation analysis showed that there was a positive correlation between UCA1 and C3, anti-ds-DNA, ESR and Systemic Lupus Erythematosus Disease Activity Index (SLEDAI). Similarly, there was a positive correlation between AKT and C3, anti-ds-DNA, erythrocyte sedimentation rate (ESR) and SLEDAI, respectively. After overexpression and knockdown of UCA1, it was found that overexpression of UCA1 significantly enhanced cell proliferation, while the interference with UCA1 significantly inhibited cell proliferation. Western blot revealed increased expressions of PI3K and AKT after overexpressing UCA1, whereas knockdown of UCA1 led significantly decreased expressions of PI3K and AKT.

CONCLUSIONS: UCA1 expression was significantly increased in SLE, which promoted the progression of SLE by activating AKT pathway.

Key Words:

SLE, lncRNA, AKT, Circulating lncRNA.

Introduction

Systemic lupus erythematosus (SLE) is a common immune system disease, with high incidence especially among young women. Characteristic symptoms of SLE are alopecia, fever, fatigue, oral ulcers, etc. The development of the disease would affect the patient's skin, mucous membranes, bones, blood system, respiratory system and other systems, which have a serious impact on the patient's physical and mental health. Therefore, it is noteworthy to find out timely and effective treatment in order to improve life quality of patients¹⁻³.

Nowadays it is well recognized that only less than 2% of the human genome exerts the protein coding function, while the remaining 98% were non-protein coding genes, including microRNA and long non-coding RNA^{4,5}. The regulatory role of microRNAs and long noncoding RNAs (lncRNAs) in important molecules was widely concerned. Different from the high conservatism and unique mechanism of microRNA, lncRNA has a low conservation with various regulatory mechanisms⁶.

lncRNA urothelial carcinoma associated antigen (UCA1) locates at 19p13.12 is found differentially expressed in multiple malignancies^{7,8}. For instance, Wang et al⁹ found that UCA1 was highly expressed in bladder cancer by using bioinformatics methods. In addition, UCA1 was also found to be highly expressed in the villus, placenta and fetal bladder. In most of the cancerous tissues, the expression of UCA1 was higher than that in the corresponding paracancerous tissues. However, its mechanism in SLE has not been studied.

Therefore, to investigate the mechanism of UCA1 in SLE can further clarify the pathogenesis of SLE and provide a theoretical basis for drug development and clinical treatment.

Patients and Methods

Patients

45 plasma samples from SLE patients treated in the First People's Hospital of Jining City from June 2014 to June 2015 were collected; 5 were males and 40 were females. All subjects met the 1997 American College of Rheumatology revised SLE classification criteria. SLE activity was assessed using the standard lupus erythematosus disease activity index (SLEDAI). 20 healthy subjects in the same period were selected as the control group, including 4 males and 16 females. This study was approved by the Ethics Committee of the First People's Hospital of Jining City. Signed written informed consents were obtained from all participants before the research.

Materials, Reagents, and Equipment

Mouse original B cell line BaF3 was purchased from iCell Bioscience, Inc. (Shanghai, China). Dulbecco's Modified Eagle Medium (DMEM) high glucose medium, and fetal bovine serum (FBS) were purchased from HyClone Corporation (South Logan, UT, USA). TRIzol reagent (Invitrogen, Carlsbad, CA, USA), reverse transcription kit, and qRT-PCR kit, were purchased from TaKaRa (Otsu, Shiga, Japan). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), dual luciferase (GeneCopoeia, Rockville, MD, USA), RNeasy Micro Kit Trace RNA Extraction Kit (Qiagen, Hilden, Germany), rabbit anti-mouse PTEN antibody, and rabbit anti-mouse PI3K antibody, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The pMD18-T vector (TaKaRa, Otsu, Shiga, Japan), UCA1, protein kinase B (AKT), PI3K, β -actin primer sequences were synthesized by Gene Pharma (Shanghai, China).

Detection of mRNA Expression of UCA1 in Plasma by RT-PCR

2 mL of EDTA anticoagulant peripheral blood were taken, centrifuged at 3000 r/min for 20 min at 4°C to collect the supernatant. RNA was extracted according to the instructions of RNeasy Micro Kit Micro RNA Extraction Kit, and reverse transcribed into cDNA. The relative expression of UCA1 in each sample was calculated following

the formula: $\text{Folds} = 2^{-\Delta Ct}$. Each experiment was performed in triplicate.

Detection of mRNA Expression of AKT in PBMCs Detected by qRT-PCR

5 mL of EDTA anticoagulant peripheral blood were harvested, centrifuged at 1500 r/min for 10 min at room temperature. After centrifugation, the supernatant was removed, and twice volume of phosphate-buffered saline (PBS) buffer was added to resuspend the pellet. The cell suspension was slowly transferred to an Eppendorf tube (EP) (Hamburg, Germany) containing 3 ml of lymphatic fluid, then centrifuged at 2500 r/min for 20 min at room temperature. The supernatant was discarded and the pellet was washed with 3 mL of PBS buffer and centrifuged again at 2,500 rpm for 20 min at room temperature. Total RNA of the peripheral blood mononuclear cell (PBMC) was extracted from PBMC using RNA fast 2000 total RNA extraction kit. Reverse transcription kit was used to reverse transcribe protein kinase B (AKT) mRNA into cDNA. β -actin was used as an internal reference for amplification and qRT-PCR was performed to analyze results. Each experiment was performed in triplicate.

Cell Culture and Transfection

BaF3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) high glucose medium supplemented with 10% fetal bovine serum (FBS) at 37°C, 5% CO₂ incubator. BaF3 cells were seeded in 6-well plates and grown into 50% confluence. 100 pmol of si-NC, si-UCA1, pcDNA-NC and pcDNA-UCA1 were then mixed with 5 μ L of lipofectamine and incubated for 30 min at room temperature. After incubation, the transfection mixture was added to the 6-well plate and maintained in an incubator for 24 h. Total RNA was extracted from the cells post-transfection to detect UCA1 expression level. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control.

Cell Counting kit-8 (CCK8) Assay for Cell Proliferation

The transfection time point was 0 h, then the medium was changed 6 h later. Cells were inoculated in 96-well plates with a density of $3 \times 10^3/100 \mu\text{L}$ per well at 24 h. Cell counting kit-8 (CCK8) assay was performed after cells were cultured for 24, 48, 72 and 96 h. The serum-free medium was replaced at the time of detection. 10 μ L of CCK8 were added to each well, followed by 1 h incubation.

tion at 37°C and 5% CO₂. Finally, the OD value was measured at 450 nm. Each measurement was performed in quintuplicate.

Colony Formation Assay

The transfection time point was considered as 0 h; next, the medium was changed 6 h later. After incubation for 24 h, 400 cells were inoculated into the medium plate and incubated at 37°C, 5% CO₂. The medium was replaced every 4 d and the culture was terminated after 14 d. Next, the medium was removed and cells were washed with PBS twice, fixed with 4% paraformaldehyde for 30 min. After fixation, remaining liquid was removed, and 1 mL of 0.1% crystal violet solution was added per well for 30 min. After staining, cells were washed and visible colonies were counted.

Western Blotting

3 groups of transfected cells were lysed with radioimmunoprecipitation assay (RIPA), and the total cellular proteins were extracted and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis. Rabbit anti-mouse AKT antibody (1:300) and rabbit anti-mouse PI3K antibody (1:400) were used to incubate overnight at 4°C. Horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (1:4000) was used as secondary antibody. The chromogenic reagent was used to expose the protein bands. GeneTools software was used to analyze protein expressions of AKT and PI3K.

Statistical Analysis

We used statistic package for social science (SPSS) 22.0 statistical software (IBM, Armonk, NY, USA) for data analysis. All measurement data were expressed as mean \pm standard deviation ($\bar{x} \pm s$). The comparison between groups was done using One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). The correlation was analyzed by Pearson test; $p < 0.05$ indicated statistically significant differences.

Results

mRNA Expressions of AKT in Plasma UCA1 and PBMC of SLE Patients

Plasma level of UCA1 in SLE patients was detected by qRT-PCR. The results indicated that there was a higher expression of plasma UCA1 in SLE patients compared with that in the con-

trol group. At the same time, we also found that there was a higher mRNA expression of AKT in PBMC of SLE patients than that of the control group (Figure 1).

Relationship Between Expressions of UCA1, AKT and Clinical Data in SLE Patients

The relationship between expressions of UCA1, AKT in SLE patients and age, gender, disease progression, organ involvement and other indicators, was analyzed. The results showed that the expression of plasma UCA1 in women, high SLEDAI score and organ involvement of SLE patients was higher than those in men, low SLEDAI score and no organ involvement of SLE patients. AKT expression in PBMC from SLE patients with high SLEDAI score and organ involvement was lower than those with SLEDAI score but without organ involvement. The correlation of UCA1, AKT and SLE clinical markers, such as C3, anti-ds-DNA, erythrocyte sedimentation rate (ESR) and SLEDAI was analyzed by Spearman method. The results showed that UCA1 was positively correlated with C3, anti-dsDNA, ESR, and SLEDAI. AKT was positively correlated with C3, anti-ds-DNA, ESR, SLEDAI by the same method (Figure 2).

UCA1 Promoted the Proliferation of BaF3 Cells

Next, we focused on the biological function of UCA1 in PBMCs from SLE patients. BaF3 cell line was transfected with the UCA1 interference sequence, UCA1 overexpression plasmid and their controls, respectively. After overexpression and interference of UCA1 in BaF3 cells, the qRT-PCR result indicated that UCA1 expression changed significantly. By CCK8 assay, we found that overexpression of UCA1 can promote proliferation of BaF3 cells, whereas interference of UCA1 can inhibit it. The consistent results were obtained with colony formation assay (Figure 3).

Expressions of AKT and PI3K After UCA1 Transfection

Based on correlation analysis, we found that UCA1 expression was positively correlated with AKT expression; therefore, we speculated that UCA1 may promote the progression of SLE by regulating AKT pathway. Western blot showed that after UCA1 transfection, we observed increased expressions of PI3K and AKT. In contrast, after interference with UCA1, expressions of PI3K and AKT were significantly

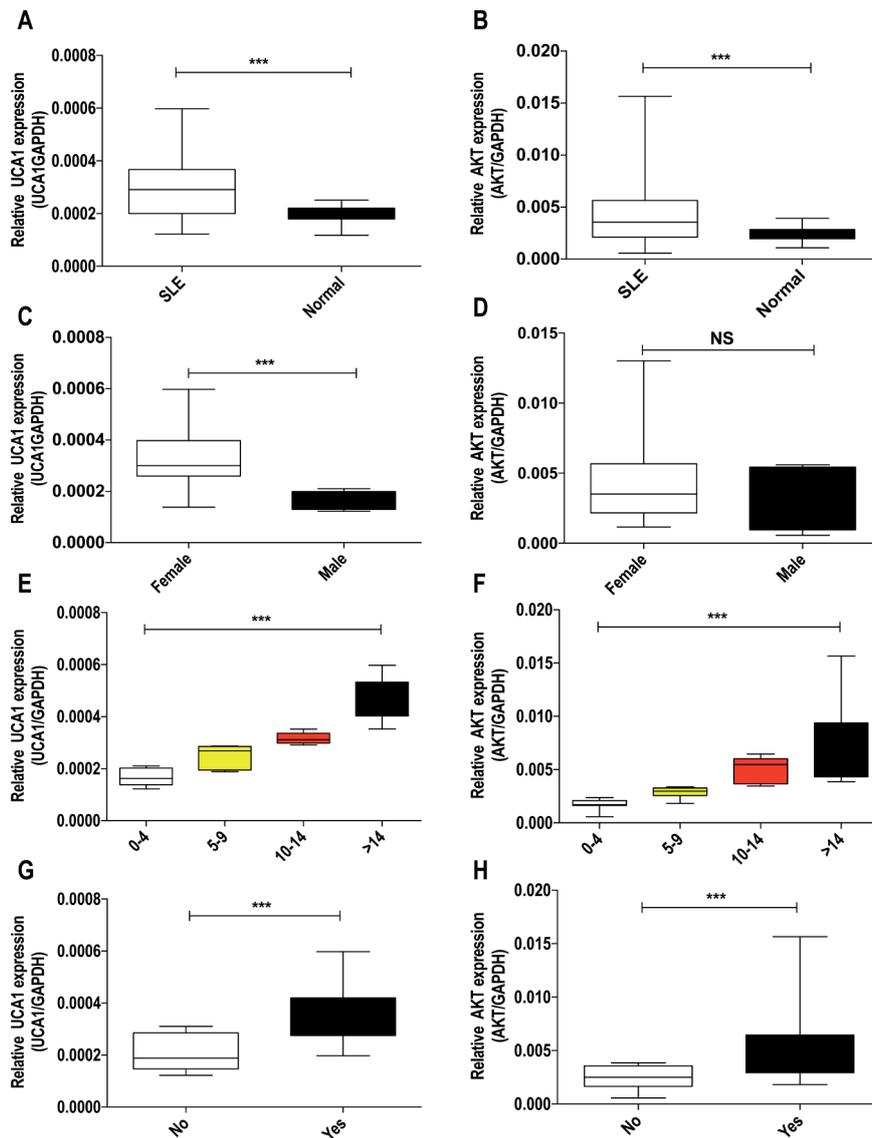


Figure 1. UCA1 is highly expressed in SLE patients and is closely related to the patient's condition. **A**, UCA1 was highly expressed in the patient's plasma. **B**, AKT expression was significantly increased in PMBC of patients. **C**, UCA1 expression in female patients was significantly higher than that in male patients. **D**, There was not significant different in AKT expression of PMBC between female patients and male patients. **E**, UCA1 expression was significantly associated with the patients' SLEDAI score. **F**, AKT expression was significantly correlated with the patients' SLEDAI score. **G**, UCA1 expression in organ involvement group was significantly higher than that in uninvolved group. **H**, AKT expression in organ involvement group was significantly higher than that in uninvolved group.

reduced. It suggested that UCA1 may regulate the progression of SLE through the AKT pathway (Figure 4).

Discussion

Researches¹⁰ reported that the incidence of SLE in China was about 70-100/100, 000 peo-

ple, with a 1:10 ratio in male and female. SLE patients were normally given anti-DS-DNA and other autoantibodies during treatment, leading to cellular and humoral immune dysfunction, as well as tissue damage and organ failure in kidney, skin, joints and other organs^{11,12}. Although the specific pathogenesis of SLE was not clear, immune cells, DNA methylation, histone modification and environmental factors were con-

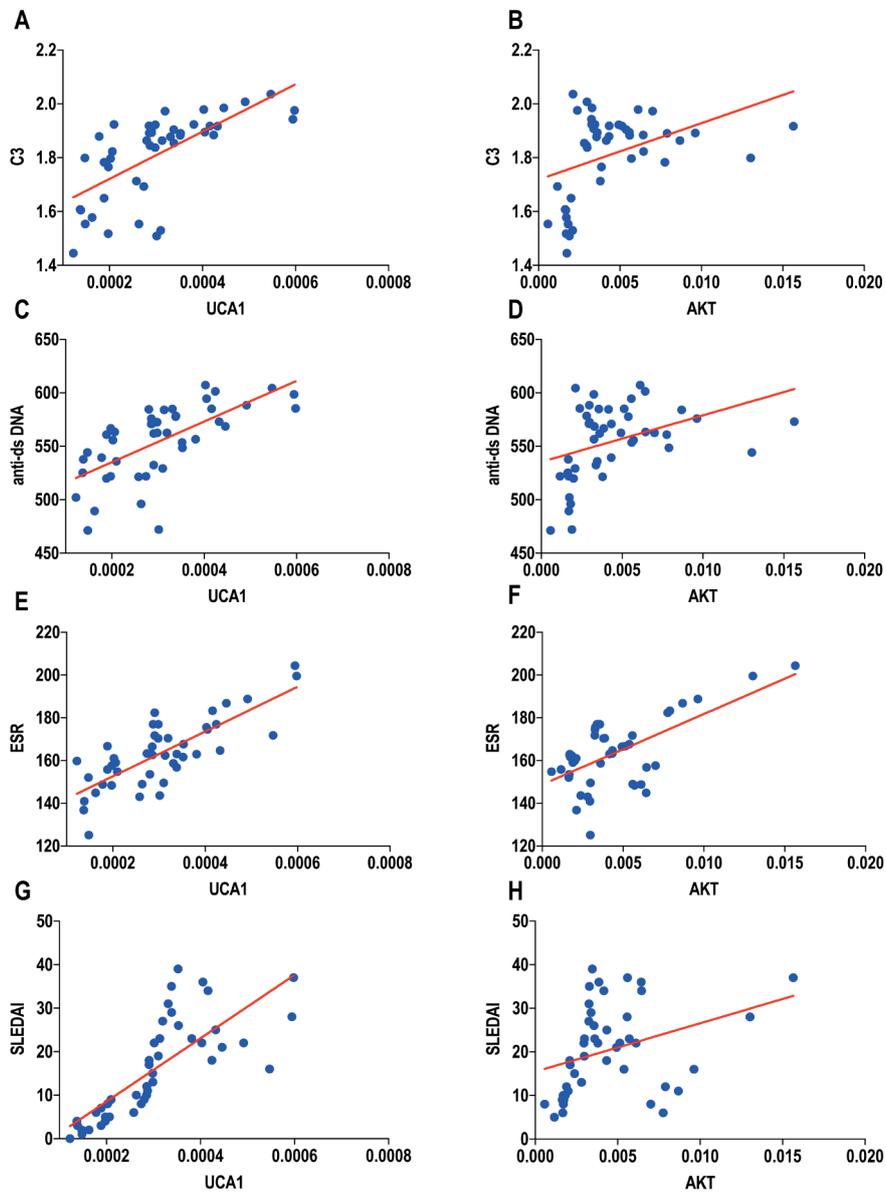


Figure 2. Correlation of expressions of UCA1, AKT and patient's condition. UCA1 expression was positively correlated with C3 complement, anti-ds-DNA, ESR and SLEDAI score. AKT expression was positively correlated with C3 complement, anti-ds-DNA, ESR and SLEDAI score.

sidered to be the main causes of SLE¹³. In recent years, the biological function of lncRNA research has attracted widespread attention. Studies found that lncRNAs were involved in development and progression of tumors, autoimmune diseases and other diseases¹⁴⁻¹⁶. There is also extracellular lncRNA, which is resistant to RNase in the body fluid such as serum, plasma and urine, namely circulating lncRNA. It is of great value, therefore, to evaluate the effects of these lncRNAs in the pathogenesis of SLE^{17,18}. We found that in SLE patients, the plasma UCA1 and AKT expression was significantly increased. By analyzing the clinical data, we found that UCA1 level in female was significantly higher than that in male patients. Also, UCA1 expression in SLE patients with active stage and pathological lesions was higher than those in stable stage with no organ involvement, indicating that upregulation of plasma UCA1 may be related to the occurrence and development of SLE. To further clarify the relationship between plasma UCA1 and activity of SLE disease, the correlation between UCA1 and C3, anti-ds-DNA, ESR

and SLEDAI was respectively analyzed. The results showed that UCA1 was associated with C3, anti-dsDNA, ESR and SLEDAI, which further illustrated that overexpression of plasma UCA1 was associated with the development of SLE. After that, we over-expressed and/or interfered with UCA1 for further experiments. After overexpression of UCA1, cell proliferation ability was significantly enhanced, while after UCA1 interference, cell proliferation decreased significantly. This suggested that UCA1 mainly promoted the progress of SLE. Scholars have reported that UCA1 could affect the cell cycle through regulating PI3K/AKT pathway and promote proliferation of tumor cells^{19,20}. AKT is an important pathway that is commonly activated in tumor activation process^{21,22}. AKT pathway also plays an important role in SLE^{23,24}. In our study, Western blot showed increased expressions of PI3K and AKT after overexpression of UCA1, and decreased expressions of PI3K and AKT after knockdown of UCA1. These findings indicated that UCA1 can promote the progression of SLE by regulating AKT pathway.

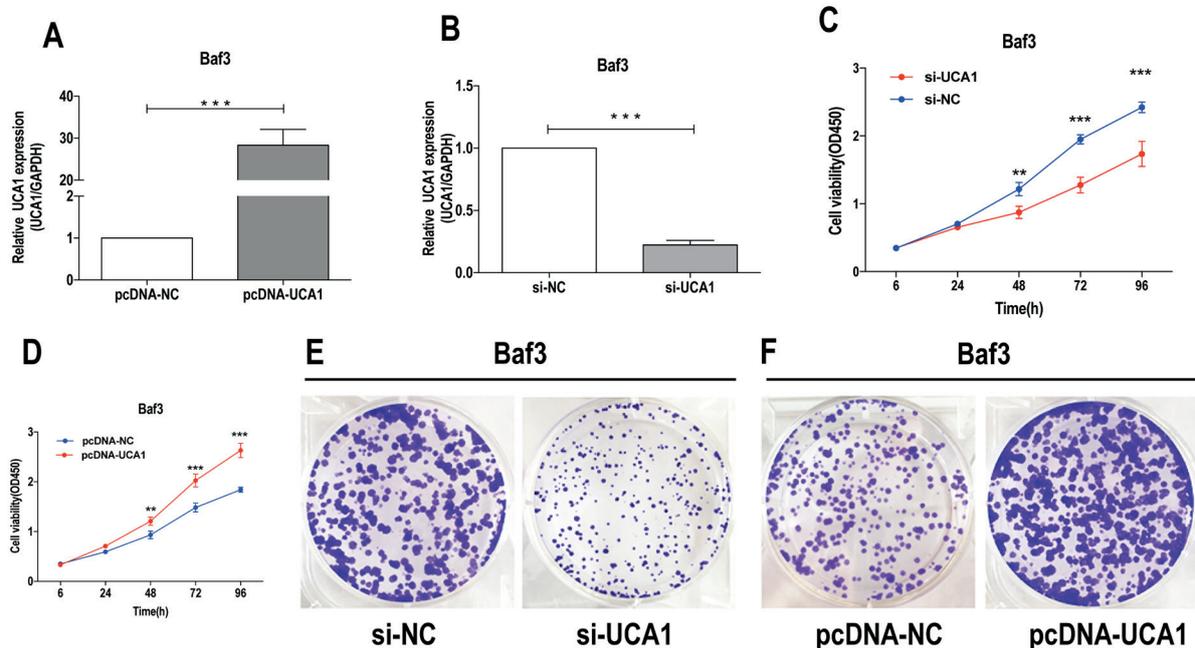


Figure 3. UCA1 promotes proliferation of BaF3 cells. **A**, UCA1 expression in BaF3 cells was significantly increased after overexpressing UCA1. **B**, UCA1 expression in BaF3 cells was significantly reduced after knockdown of UCA1. **C**, CCK8 assay showed that cell proliferation increased significantly after overexpressing UCA1. **D**, CCK8 assay showed that cell proliferation ability was significantly decreased after knockdown of UCA1. **E**, Colony formation ability of BaF3 cells was significantly enhanced after overexpressing UCA1. **F**, Colony formation ability of BaF3 cells was significantly weakened after knockdown of UCA1.

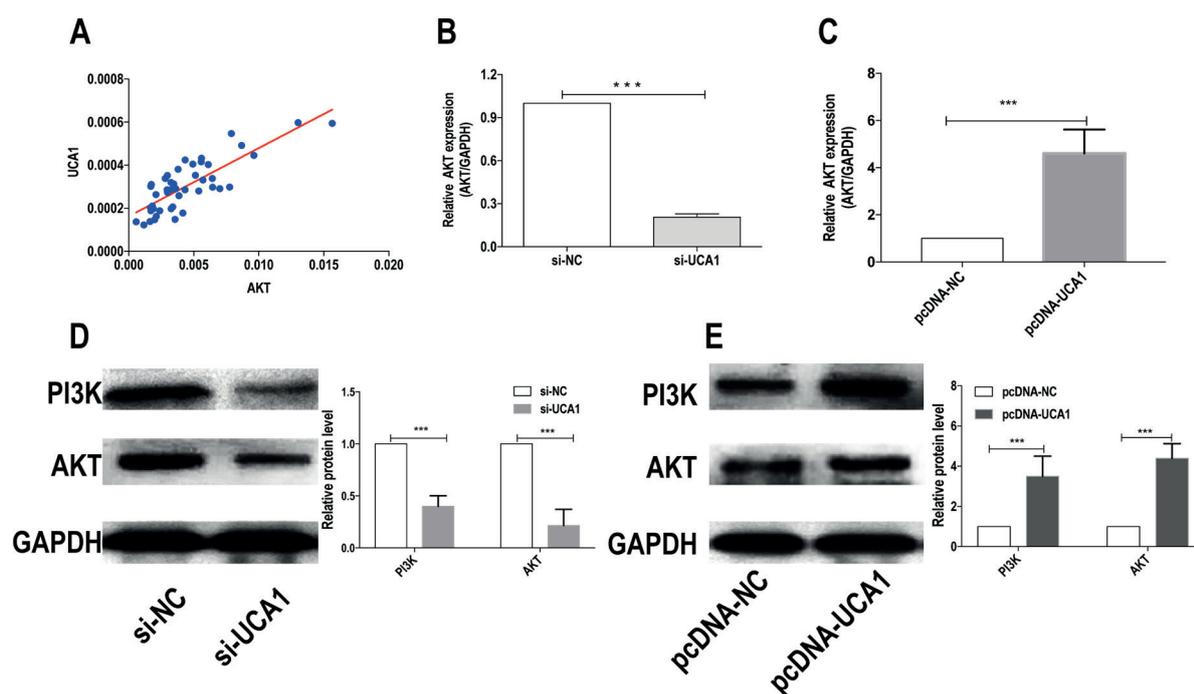


Figure 4. UCA1 regulates the progression of SLE through the AKT pathway. **A**, PCR results showed that UCA1 expression was positively correlated with AKT. **B**, The mRNA level of AKT was significantly reduced after knockdown of UCA1. **C**, The mRNA level of AKT was increased significantly after overexpressing UCA1. **D**, Protein expressions of PI3K and AKT increased significantly after overexpressing UCA1. **E**, Protein expressions of PI3K and AKT decreased significantly after knockdown of UCA1.

Conclusions

We found that UCA1 was significantly increased in SLE, which promoted the disease progression by activating AKT pathway.

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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