



Effect of silencing CITED1 gene to regulate PI3K/AKT pathway on the biological function of PTC cells and its mechanism

Wanzhi Chen, Jichun Yu, Rong Xie, Shuyong Zhang, Tao Zhou, Chenfeng Xiong, Da Huang, Meijun Zhong*

Department of Thyroid Surgery, The Second Affiliated Hospital of Nanchang University, Nanchang, Jiangxi, 330006, China

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ABSTRACT

This study aims to investigate the effect of silencing the CITED1 gene to regulate the PI3K/AKT pathway on the biological function of papillary thyroid carcinoma (PTC) cells and its mechanism of action. Human PTC cells SW1736 were divided into 4 groups: control group, siCITED1 group, LY294002 group and siCITED1+LY294002 group. CITED1 was silenced by transfection with siCITED1 plasmid. The PI3K/AKT pathway was inhibited by LY294002 (5 μmol/L). Each group was determined for cell proliferation, apoptosis and invasion capabilities, as well as PI3K/AKT transcription and protein expression levels. CITED1 mRNA and protein levels in the siCITED1 group and the siCITED1+LY294002 group were significantly lower than those in the control group ($P < 0.05$), and the two levels were not significantly different between the LY294002 group and the control group ($P > 0.05$). Compared with the control group, the siCITED1 group showed remarkably lower proliferation and invasion capabilities, and remarkably higher apoptosis rate ($P < 0.05$). There was no significant difference in proliferation, apoptosis and invasion capabilities between the LY294002 group and the siCITED1+LY294002 group ($P > 0.05$), both of which had significantly lower proliferation and invasion capabilities but significantly higher apoptosis rate than the siCITED1 group ($P < 0.05$). PI3K and AKT protein levels in the siCITED1 group were significantly lower than those in the control group ($P < 0.05$). The PI3K and AKT protein levels in the LY294002 group and the siCITED1+LY294002 group were not significantly different ($P > 0.05$), and were significantly lower than those in the siCITED1 group ($P < 0.05$). In conclusion: CITED1 silence may inhibit the progression of PTC cells by inhibiting the PI3K/AKT pathway.

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Introduction

Thyroid carcinoma (TC) accounts for 1.7% of all tumors, among which papillary TC (PTC) is the most common type, and surgical resection is the most frequently used and effective method for PTC treatment (1). Although the 5-year survival rate of patients with TC is above 90%, a lot of factors may lead to tumor metastasis and recurrence (2). The PI3K/AKT pathway, which promotes the transcription of proto-oncogenes, is one of the most critical pathways triggering the occurrence and development of PTC, and inhibition of the pathway has become a target for PTC treatment; however, the mechanism of PI3K/AKT pathway regulation in PTC remains unclear (3). The CITED1 gene is located on chromosome Xq13.1 and encodes the 27 kD nuclear protein CITED1; as a transcriptional activator, the CITED1 protein can induce transcription and translation of protein kinase, thus promoting carcinoma metastasis (4). Recent studies have revealed that CITED1 is elevated in PTC tissues and can promote PTC progression, but the effects and molecular mechanisms of CITED1 on the biological behavior of PTC cells remain unclear (6). At present, immune-targeted therapy through a molecular perspective has become a new research direction for various tumor diseases (such as PD-1 inhibitors, etc.), and if we can fully understand and master the effect of CITED1 on PTC cells, we may be looking for a new PTC treatment option. And this will not only achieve the

effect of non-invasive treatment of PTC, but also may be more effective in inhibiting the prognosis of PTC recurrence and provide more reliable safety for patients (5,6). In summary, this study mainly analyzes the effects of silencing the CITED1 gene to regulate the PI3K/AKT pathway on the biological function of PTC cells and its mechanism, to provide reference and guidance for subsequent studies.

Materials and Methods

Materials

Materials used in the study included human PTC cells SW1736 (ATCC, USA), 96-well and 24-well tissue culture plates (Corning, USA), RPMI 1640 medium and antibodies (Gibco, USA), siCITED1 plasmid and corresponding negative control (NC) (GenePharma, PRC), Lipofectamine® 2000 (Invitrogen, USA), PI3K/AKT inhibitor LY294002 (Selleck, USA), apoptosis kit (Annexin V-FITC/PI) (Yesen, China), flow cytometry (Becton, USA), Transwell device and Matrigel (Corning, USA), Trizol and RNeasy Mini kits (QIAGEN GmbH, Germany), SYBR Premix Ex Taq™ Kit (TaKaRa, China), PCR instrument (ABI7900, ABI, USA), rabbit monoclonal CITED1 primary antibody (ab87978), rabbit monoclonal PI3K primary antibody (ab32089), rabbit polyclonal AKT primary antibody (ab8805) and goat anti-IgG secondary antibody (ab6721) (Abcam, USA), PDVF membrane (EMD Millipore, USA). and ECL chromogenic Kit (Thermo Fisher,

* Corresponding author. Email: Zhongmeijun0523@163.com

USA).

Cell culture and grouping

Human PTC cells SW1736 were cultured in RPMI 1640 complete medium containing 10% fetal bovine serum (FBS), 0.1 mg/mL streptomycin and 100 U/mL penicillin. Cells were cultured in an incubator containing 5% CO₂ at 37°C and 95% humidity. The cells were divided into 4 groups: control group, siCITED1 group, LY294002 group and siCITED1+LY294002 group. CITED1 in the siCITED1 group and siCITED1+LY294002 group were silenced by transfection of siCITED1 plasmid. Cells were cultured in 6-well plates; when 60% confluence was reached, Opti (100 μ L) and LipofectamineTM 2000 (5 μ L) were added for incubation for 5 min, and Reagent A was therefore obtained. Opti (100 μ L) and siCITED1 plasmid (20 ng/ μ L) was added for incubation for 5 min, and Reagent B was therefore obtained. Reagents A and B were mixed and incubated for 20 min. After 16 h, the medium was replaced and the cells were harvested. Cells in the control group were transfected with the same amount of NC as the control. Cells in the LY294002 group and the siCITED1+LY294002 group were added with LY294002 (5 μ mol/L) to inhibit the PI3K/AKT pathway, and the duration of pre-incubation was 24 h [6].

Test methods

Cell proliferation viability by CCK-8

One hundred μ L of cell suspension was added to a 96-well plate and incubated for 48 h; then, 10 μ L of CCK-8 solution was added to each well for 2-hour incubation. The optical density (OD) value of each well was measured at 450 nm with a microplate reader.

Apoptosis by flow cytometry

Cells were washed with 1 \times PBS and suspended in 100 μ L of binding buffer, added with 5 μ L of Annexin V-FITC and 10 μ L of PI, incubated at room temperature in the dark for 10–15 min, added with 400 μ L of binding buffer, and determined for cell apoptotic rate by flow cytometry within 1 h.

Invasion by Transwell

Matrigel (1:8 dilution) was added to the upper chamber of a Transwell permeable support and incubated at 37°C for 30 min, and 600 μ L of complete medium was added to the lower chamber of the 24-well permeable support. Cells were starved by incubation in a serum-free medium at 37°C for 24 h. After digestion, 100 μ L of cell solution (5 \times 10⁵ cells/mL) was added to the hydrated Transwell upper chamber. After 24 h, non-invaded cells were washed away. Cells penetrating the lower chamber were fixed with 95% ethanol and stained with 0.1% crystal violet for 20 min at room temperature. The number of cells was counted in 5 random fields in a 400 \times microscope field.

RT-qPCR

Total RNA was extracted from cell cultures using the TRIzol reagent and quantified using the Nanodrop 2000. miRNAs were isolated by the RNeasy Mini Kit. RT-qPCR reaction was performed with the SYBR Premix Ex TaqTM: 95°C for 2 min, 58°C for 20 s, followed by a reaction at 72°C for 20 s (40 cycles for the step). Using GAPDH as the internal reference, the relative expression

levels of CITED1, PI3K and AKT mRNA were calculated by the 2- $\Delta\Delta$ Ct method.

Western blot

After cell lysis, the total protein was collected by centrifugation (4°C, 12000 rpm, 5 min). Forty μ g of protein was separated by 10% SDS-PAGE, transferred to a nitrocellulose membrane, blocked with 5% skimmed milk (room temperature, 2 h), added with CITED1, PI3K and AKT primary antibodies (1:800 dilution, 4°C, overnight), washed, and added with secondary antibodies (1:5000 dilution, room temperature, 2 h). The bands were visualized using the ECL, and GAPDH was used as the internal reference for the analysis of relative expression levels of CITED1, PI3K and AKT.

Statistical treatment

Statistical analysis was performed using SPSS 19. Data were presented as mean \pm standard deviation (SD). Analysis of variance was used for comparison among multiple groups, and the SNK-q test was used for pairwise comparison. Statistical significance was expressed as $P < 0.05$.

Results

Comparison of CITED1 mRNA and protein levels in cells after transfection

CITED1 mRNA and protein levels in the siCITED1 group and the siCITED1+LY294002 group were significantly lower than those in the control group ($P < 0.05$), and the two levels were not significantly different between the LY294002 group and the control group ($P > 0.05$). (Figure 1).

Effect of regulation of PI3K/AKT by CITED1 on PTC cell proliferation viability

The differences in cell proliferation viability were statistically significant among the four groups ($P < 0.05$). The OD value of the siCITED1 group (0.63 \pm 0.06) was significantly lower than that of the control group (0.74 \pm 0.06) ($P < 0.05$). The OD values of the LY294002 group (0.55 \pm 0.06) and the siCITED1+LY294002 group (0.53 \pm 0.07) were not significantly different ($P > 0.05$), and were significantly lower than those of the siCITED1 group ($P < 0.05$). (Figure 2)

Regulation of PI3K/AKT by CITED1 on PTC cell apoptosis

The differences in cell apoptotic rate were statistically

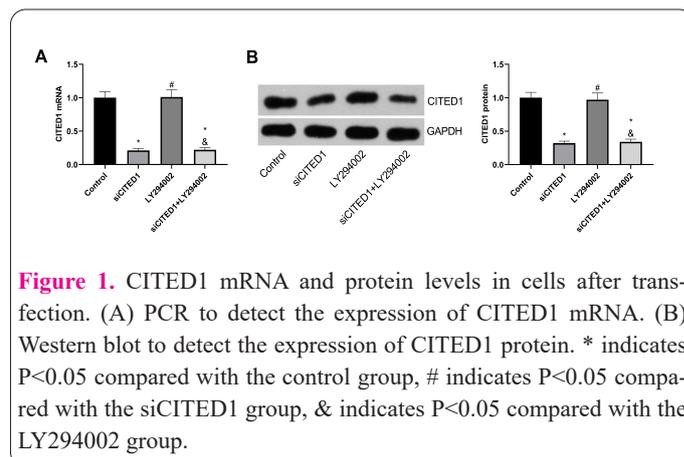


Figure 1. CITED1 mRNA and protein levels in cells after transfection. (A) PCR to detect the expression of CITED1 mRNA. (B) Western blot to detect the expression of CITED1 protein. * indicates $P < 0.05$ compared with the control group, # indicates $P < 0.05$ compared with the siCITED1 group, & indicates $P < 0.05$ compared with the LY294002 group.

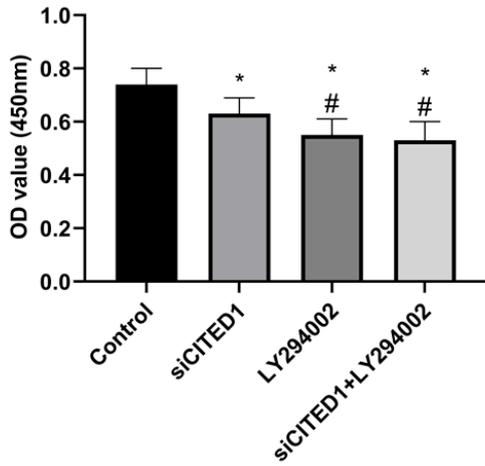


Figure 2. Effect of regulation of PI3K/AKT by CITED1 on PTC cell proliferation viability. * indicates $P < 0.05$ compared with the control group, # indicates $P < 0.05$ compared with the siCITED1 group.

significant among the four groups ($P < 0.05$). The apoptotic rate in the siCITED1 group ($8.92 \pm 0.75\%$) was significantly higher than that in the control group ($3.21 \pm 0.27\%$) ($P < 0.05$). The apoptotic rate in the LY294002 group ($15.62 \pm 1.04\%$) and the siCITED1+LY294002 group ($16.08 \pm 1.12\%$) were not significantly different ($P > 0.05$), and were significantly higher than those in the siCITED1 group ($P < 0.05$). (Figure 3).

Regulation of PI3K/AKT by CITED1 on PTC cell invasion capability

The differences in cell invasion capability were statistically significant among the four groups ($P < 0.05$). The number of invaded cells in the siCITED1 group (61.76 ± 3.35) was significantly lower than that of the control group (98.44 ± 5.27) ($P < 0.05$). The number of invaded cells in the LY294002 group (16.75 ± 2.51) and the siCITED1+LY294002 group (15.92 ± 2.85) were not significantly different ($P > 0.05$), and were significantly lower than that in the siCITED1 group ($P < 0.05$). (Figure 4)

Effect of CITED1 on PI3K and AKT mRNA levels

PI3K and AKT mRNA levels in the siCITED1 group and the siCITED1+LY294002 group were significantly lower than those in the control group ($P < 0.05$), and the two levels were not significantly different between the LY294002 group and the control group ($P > 0.05$). (Figure 5)

Effect of CITED1 on PI3K and AKT protein levels

PI3K and AKT protein levels in the siCITED1 group were significantly lower than those in the control group ($P < 0.05$). The PI3K and AKT protein levels in the LY294002 group and the siCITED1+LY294002 group were not significantly different ($P > 0.05$), and were significantly lower than those in the siCITED1 group ($P < 0.05$). (Figure 6).

Discussion

PTC is the most common malignant tumor in the endocrine system, and the majority of patients are women; China has the largest number of PTC patients, and relevant survey results show that the incidence of PTC is on the rise

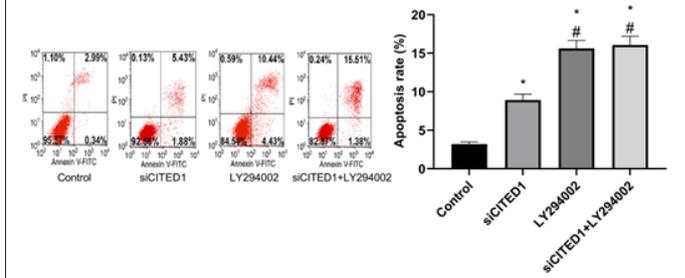


Figure 3. Regulation of PI3K/AKT by CITED1 on PTC cell apoptosis. * indicates $P < 0.05$ compared with the control group, # indicates $P < 0.05$ compared with the siCITED1 group.

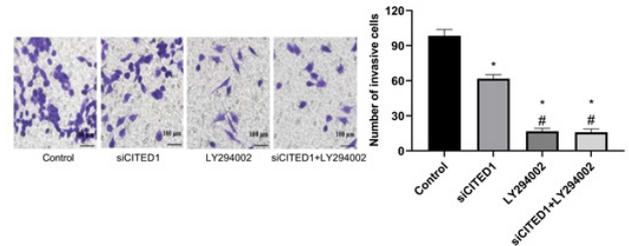


Figure 4. Regulation of PI3K/AKT by CITED1 on PTC cell invasion capability. * indicates $P < 0.05$ compared with the control group, # indicates $P < 0.05$ compared with the siCITED1 group.

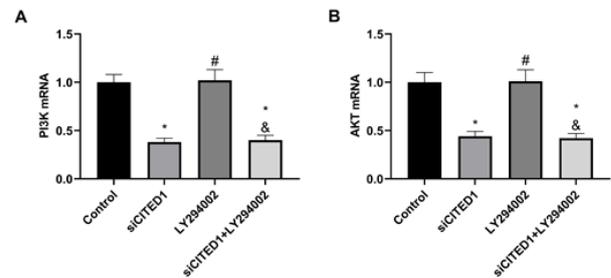


Figure 5. Effect of CITED1 on PI3K and AKT mRNA levels. A: PI3K mRNA. B: AKT mRNA. * indicates $P < 0.05$ compared with the control group, # indicates $P < 0.05$ compared with the siCITED1 group, & indicates $P < 0.05$ compared with the LY294002 group.

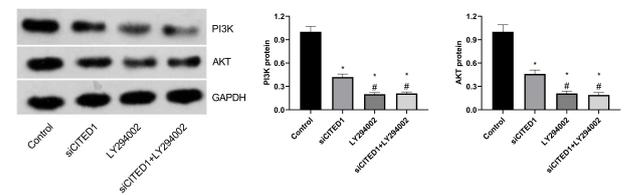


Figure 6. Effect of CITED1 on PI3K and AKT protein levels. * indicates $P < 0.05$ compared with the control group, # indicates $P < 0.05$ compared with the siCITED1 group.

(7). The occurrence of PTC is generally related to genetic abnormalities and environmental factors; although new drugs and surgical strategies have been applied in clinics in recent years, some patients with PTC still have poor prognoses (8).

CITED1 is a transcriptional co-activator of p300/CBP mediated transcription complex; as a non-DNA binding transcriptional co-activator, CITED1 induces transcriptional activation by enhancing DNA-binding transcription factor SMAD and stimulates estrogen-dependent activa-

tion mediated by estrogen receptor signal (9, 10). Recent studies have shown that CITED1 has pro-oncogenic effects and that inhibition of CITED1 expression reduces the proliferation of melanoma cells (11). It is also suggested that RUNX1 facilitates the occurrence and progression of breast cancer cells by increasing the expression level of the CITED1 protein (12). CITED1 plays an important role in PTC, and histological studies have indicated that CITED1 is elevated in PTC tissues and is associated with malignant clinical features and poor prognosis in PTC patients (13). To preliminarily analyze the impacts of CITED1 on PTC cells, a CITED1-silenced PTC cell model was established through transfection. According to the results of cell-based experiments, when the levels of CITED1 mRNA and protein were reduced, cell proliferation and invasion capabilities were inhibited, while the apoptotic rate increased significantly. Combined with relevant literature, it was confirmed at the cellular level that silencing CITED1 could inhibit the proliferation and invasion and promote apoptosis of PTC cells, suggesting that CITED1 is involved in PTC occurrence and progression.

To further analyze the mechanism by which CITED1 suppresses PTC, the level of the PI3K/AKT pathway was determined. The PI3K protein transmits the signal to the AKT protein, and the activated AKT protein enters the nucleus to regulate the transcription, which facilitates the transcription of genes related to the cell cycle, anti-apoptosis and metastasis, so that PTC progression is suppressed (14,15). As indicated by the study, when CITED1 was silenced, the levels of PI3K and AKT mRNA and protein in PTC cells were significantly reduced. To preliminarily verify that regulation of PTC cell biological behavior by CITED1 is related to the PI3K/AKT pathway, a validation experiment was conducted using LY294002 to inhibit the level of the PI3K/AKT pathway, followed by CITED1 silence by transfection. LY294002 does not affect the PI3K and AKT transcription levels but inhibits the protein level (16). The results showed that when the PI3K/AKT pathway was inhibited by LY294002, cell proliferation and metastatic capabilities were reduced while the apoptotic rate was elevated; CITED1 silence on such basis would not further inhibit the malignant biological behavior of PTC cells, suggesting that the regulatory effect of CITED1 on PTC cells is closely related to PI3K/AKT. Combined with relevant literature, the results of the study suggest that in PTC, CITED1 promotes the proliferation and invasion band inhibit apoptosis of PTC cells through promoting PI3K/AKT pathway transcription and protein expression.

However, certain shortcomings still exist in the study. First, the effect of over-expressed CITED1 on PTC cells and the PI3K/AKT pathway should be explored. Then, the role and mechanism of CITED1 in suppressing PTC by regulating the PI3K/AKT pathway should be confirmed by animal experiments. In addition, the molecular mechanism by which CITED1 regulates the PI3K/AKT pathway still needs to be further studied.

Silencing CITED1 may inhibit the progression of PTC cells by inhibiting the PI3K/AKT pathway, suggesting that CITED1 may become a potential target for PTC diagnosis and treatment.

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Not applicable.

Interest conflict

The authors report no conflict of interest.

Consent for publications

The author read and proved the final manuscript for publication.

Availability of data and material

All data generated during this study are included in this published article

Authors' contribution

Wanzhi Chen and Jichun Yu conceived and designed the project, and wrote the paper. Rong Xie and Shuyong Zhang generated the data. Tao Zhou, Chenfeng Xiong and Da Huang analyzed the data. Meijun Zhong modified the manuscript. All authors gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Ethics approval and consent to participate

The study was approved by the Ethics Committee of The Second Affiliated Hospital of Nanchang University.

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