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LncRNA CCAT1 promotes the progression of preeclampsia by regulating CDK4

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Abstract. – OBJECTIVE: Preeclampsia is one of the leading causes of maternal and perinatal deaths. This study mainly explored the mechanism of long non-coding RNA (lncRNA) CCAT1 expression in the placenta of preeclampsia patients and its effect on the progression of preeclampsia.

MATERIALS AND METHODS: We used quantitative reverse transcription PCR (qRT-PCR) to detect the lncRNA CCAT1 expression in 40 preeclampsia and 40 normal pregnancy placenta samples. CCAT1 expression and its relationship with the clinicopathological parameters of preeclampsia was statistically analyzed. The specific small interfering RNA (si-CCAT1) and plasmid (pcDNA-CCAT1) targeting lncRNA CCAT1 were synthesized and transfected into Bew and JEG-3 cells. The CCAT1 expression in Bew and JEG-3 cells was determined by qRT-PCR. The effect of overexpression and interference of lncRNA CCAT1 on the proliferation of Bew and JEG-3 cells was observed. The effect of CCAT1 on cell cycle was examined by cell cycle assay. The protein expression was accessed by Western blot.

RESULTS: Higher lncRNA CCAT1 expression was found in preeclampsia patients. The systolic blood pressure, diastolic blood pressure and urine protein in preeclampsia patients were significantly higher than those in normal pregnant women. The birth weight of fetus was significantly lower than that of normal pregnant women. However, there was no significant difference in weight and age of patients. According to the CCAT1 expression, preeclampsia patients were assigned into high expression group and low expression group. Higher systolic blood pressure, diastolic blood pressure, and urinary protein levels in CCAT1 high expression group were observed comparing to those in low expression group, while the birth weight in low expression group was significantly higher than the high expression group. In addition, we found that after interference with CCAT1, trophoblast proliferation was significantly increased and cell cycle was significantly accelerated, whereas overexpression of CCAT1 led to the contrary. Western blotting indicated that the expressions of E2F1, cyclin D, CDK2 and CDK4 in BeWo cells were increased after CCAT1 was knocked down. The expressions of E2F1, cyclin D, CDK2 and CDK4 in JEG3 cells were decreased after CCAT1 was overexpressed.

CONCLUSIONS: LncRNA CCAT1 was highly expressed in preeclampsia and can promote the progression of preeclampsia by inhibiting the expression of CDK4.

Key Words:
LncRNA CCAT1, Preeclampsia, CDK4.

Introduction

Preeclampsia is a multifactorial and multi-systemic disease unique to the third trimester of pregnancy. With a high prevalence of 7-10%, it is one of the major causes of maternal and perinatal death[1]. Current studies[2] suggested that the pathogenesis of preeclampsia may be related to genetic, immune, oxidative stress, placental ischemia and hypoxia, vascular endothelial dysfunction and other factors, but it has not yet been fully elucidated. Among all the potential pathogenic factors, inadequate infiltration of the uterine spiral artery due to insufficient infiltration of trophoblast cells was one of the main incentives for preeclampsia[3,4].

Long non-coding RNA (lncRNA) is non-coding RNA with approximately 200 nucleotides (nt) in length. The discovery of lncRNAs has opened entirely new fields for gene expression regulation and epigenetics, and lncRNAs have now been proved to be closely associated with various diseases, including tumors[5]. Additionally, more and more studies confirmed that lncRNA can participate in the deve-

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lopment of preeclampsia by affecting the function of trophoblast cells. Gao et al[6] found that lncRNA H19 was significantly down-regulated in the placenta of preeclampsia, and the promoter region of its coding gene presented hypermethylation. H19 can lead to the excessive proliferation of trophoblast cells through the H19/miR-675/NOM01/Nodal signaling pathway, and then participate in the development of preeclampsia. Oudejans et al[7] found that lncRNA STOX2-IT3 can attenuate the differentiation and invasion of trophoblast cells through the regulation of STOX2 gene expression, thus leading to PE. CCAT1, a 2628 nt non-coding RNA molecule, was first found in colon cancer. CCAT1 is located at 8q24.21, which is a transcription factor near C-Myc. Studies have shown that this was the genome region where high incidence of genetic mutations occured[8]. C-Myc binds to cis-acting element E-box upstream of CCAT1, resulting in the high expression of CCAT1 in cells[9]. c-Myc also promotes DNA synthesis and transcription, accelerates cell entry from G1 phase to S phase, as well as cell proliferation, while inhibiting apoptosis[10]. At present, the correlation between CCAT1 and preeclampsia has not been reported yet. CDK4 functions as a regulator in the cell proliferation cycle transmitted from G1 phase to S phase. Specifically, CDK4 binds to the regulatory sub-unit Cyclin D1 to form a complex of Cyclin D1-CDK4 to be activated, resulting in phosphorylation of Rb, release of nuclear factor E2F1 and promotion of DNA replication. Some studies have confirmed that CDK4 in some tumors and cell lines were abnormally expressed.

Our investigation was focused on the functions of lncRNA CCAT1 on proliferation and cell cycle of preeclampsia and to explore the regulatory mechanism of lncRNA CCAT1 in preeclampsia.

Patients and Methods

Patients

40 preeclampsia and 40 normal pregnancy placenta samples were selected. Sterile placenta after cesarean section was taken, and placental tissue sized 4×3 cm from the proximal umbilical cord of the placental matrix was extracted. The extracted tissues were repeatedly rinsed with phosphate-buffered saline (PBS) until no blood remained and then placed in liquid nitrogen, and transferred to liquid nitrogen for further use. This study was approved by the Ethics Committee of Jining First People's Hospital.

Cell Culture and Transfection

Trophoblastic HTR-8/SVneo, JEG-3, Wish and Bewo cell lines bought from American Type Culture Collection (ATCC, Manassas, VA, USA) were cultured in F12K medium (Gibco, Rockville, MD, USA) supplemented with 5% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), and maintained in an incubator at 37°C and 5% CO_2. Cells were passaged and digested by trypsin until cells were grown to over 90%. Cells were inoculated in 6-well plates (2×10^5/well) for 24 h incubation until the cells were grown to 70%. The cells were transfected with si-NC, si-CCAT1, pcDNA-NC and pcDNA- CCAT1, respectively, following the instructions.

Quantitative Reverse Transcription PCR (qRT-PCR)

Total RNA was extracted and reverse transcribed to cDNA. QRT-PCR was carried out on a 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) with primers and RNA mixed by SYBR Premix Ex Taq II kit (TaKaRa, Otsu, Shiga, Japan). The sequences of the primers were as follows: CCAT1 (F): 5'-CATTGGGAAAGGTGCCGAGA-3', CCAT1 (R): 5'-ACGCTTAGCCATACAGAGCC-3'; CDK4 (F): 5'-CTTCCCGTCAGCACAGTTC-3'; CKD4 (R): 5'-GGTCAGCATTTCCAGTAGC-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (F): 5'-AGGAGCGAGATCCCGCCAACA-3', GAPDH (R): 5'-CGGCCGTCACGCCACATCTT-3'. The entire experiment was independently repeated three times.

Cell Counting Kit-8 (CCK8) Assay

The transfected JEG-3, Bewo cells, were seeded in 96-well plates. After cell was cultured for 24, 48, 72 and 96 h, culture medium was replaced with serum-free medium. 10 μL of CCK8 were added per well and incubated at 37°C, 5% CO_2 for 1 h. OD values at 450 nm were measured. Each group had 5 replicated wells.

Cell Cycle Assay

Cells were seeded in 6-well plates, and transfection was performed when cells were adherent. After 24 h of transfection, 1×10^6 cells were harvested from each group, washed twice with phosphate-buffered saline (PBS) with the supernatant discarded. 70% ethanol (precooling at -20°C) was added for fixation, followed by incubation with RNase for 15 min. At last, 50 μg/mL of propidium iodide (PI) was added in each sample for 30

min, and the stained cells were detected by flow cytometry. Four replicates were included for each group in the experiment.

Western Blotting

Radioimmunoprecipitation assay (RIPA) lysis solution (Beyotime, Shanghai, China) was used to lyse cells to extract total proteins. After routine electrophoresis, E2F1, cyclin D, CDK4 and CDK2 primary antibodies (1:1000, Abcam, Cambridge, MA, USA) were used for incubation overnight at 4°C. After washed with phosphate-buffered saline (PBS), membranes were incubated with horseradish peroxidase (HRP)-labeled secondary antibodies (Cell Signaling, Danvers, MA, USA) for 2 h at room temperature. Next, the membranes were developed with enhanced chemiluminescence (ECL) imaging (Beyotime, Shanghai, China). The integral optical density (IOD) value of each band was recorded by a gel imaging analysis system using GAPDH (Abcam, Cambridge, MA, USA) as an internal reference.

Statistical Analysis

We used statistical product and service solutions (SPSS) 22.0 statistical software (IBM, Armonk, NY, USA) for data analysis, and GraphPad Prism 5.0 (Version X; La Jolla, CA, USA) for image editing. Comparisons between groups were analyzed using *t*-test. Measurement data were expressed as mean ± standard deviation ($\bar{x}\pm s$), and classification data was analyzed using x^2-test. $p<0.05$ was considered statistically significant. $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$.

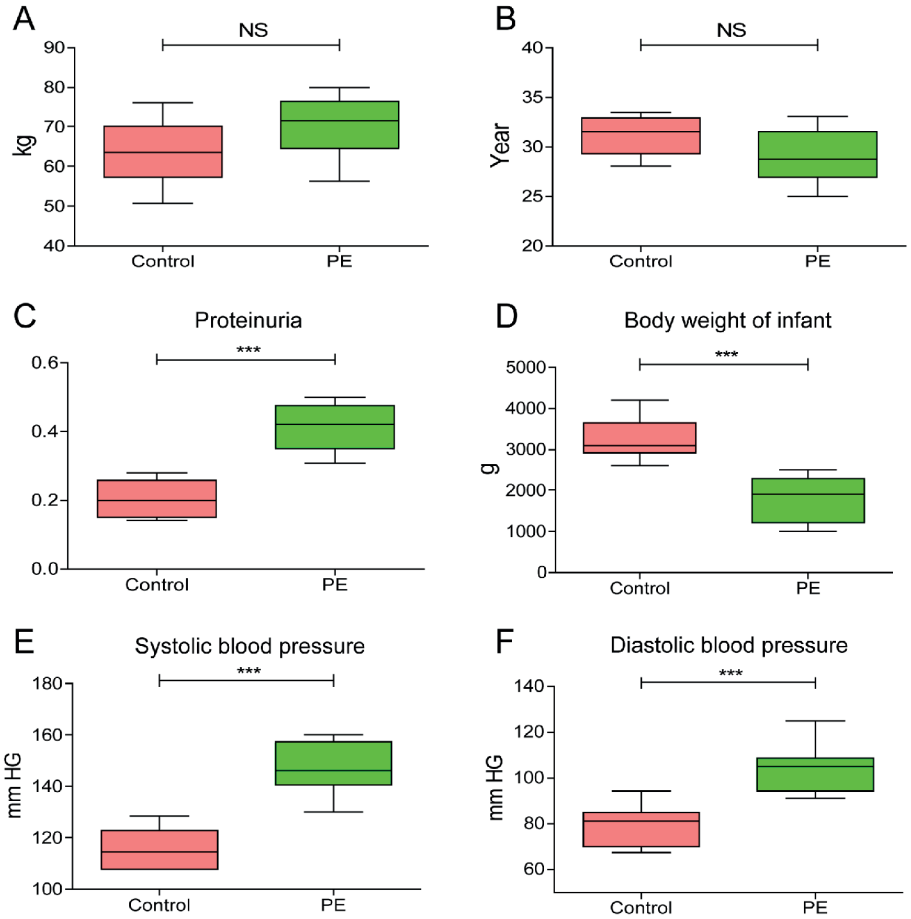

Figure 1. Similarities and differences between preeclampsia and normal pregnant women. ***A,*** There is no significant difference in body weight between preeclampsia and normal pregnant women. ***B,*** There was no significant difference in maternal age between preeclampsia and normal pregnancy. ***C,*** Urinary protein level in patients with preeclampsia was significantly higher than normal pregnant women. ***D,*** Neonatal weight of preeclampsia patients was significantly lower than that of normal pregnant women. ***E,*** Systolic blood pressure of patients with preeclampsia was significantly higher than normal pregnant women. ***F,*** Diastolic blood pressure in patients with preeclampsia was significantly higher than normal pregnant women.

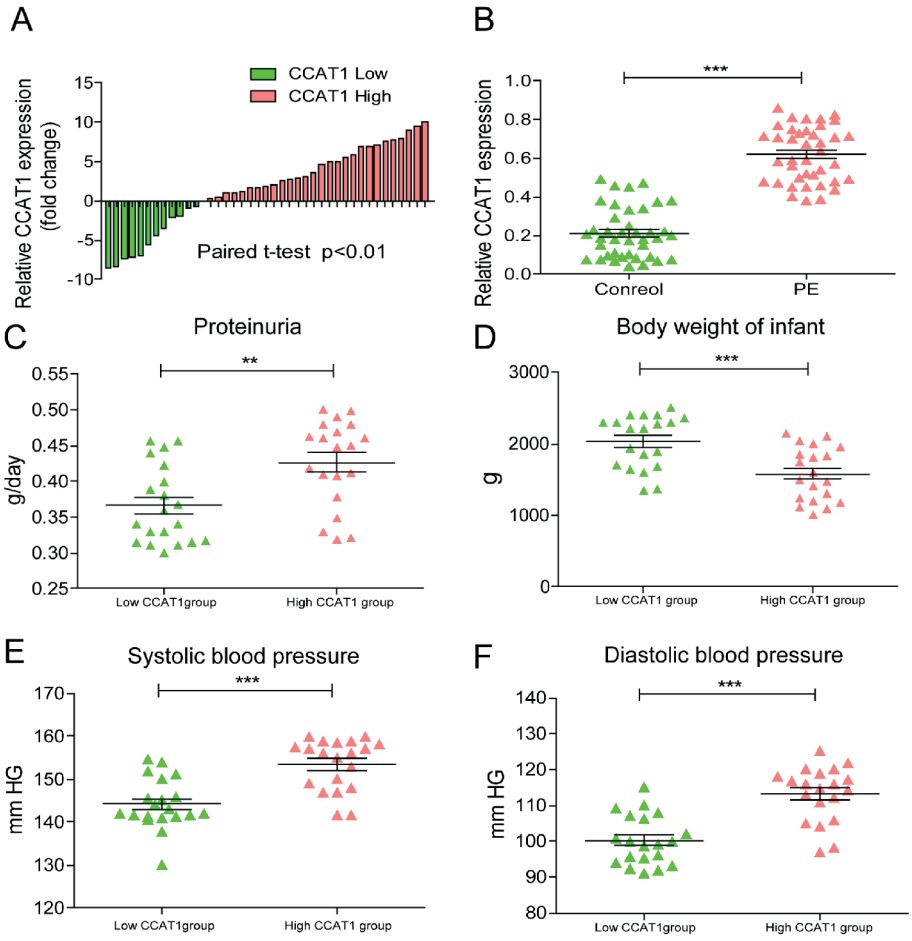

Figure 2. CCAT1 is highly expressed in preeclampsia patients and the relationship with the clinical data. ***A*** and ***B,*** The expression of CCAT1 in placenta of preeclampsia patients was significantly higher than that in normal controls. ***C,*** Urine protein in patients with high CCAT1 expression was significantly higher than that in patients with low expression. ***D,*** Newborn weight in patients with high CCAT1 expression was significantly lower than in patients with low expression. ***E,*** Systolic blood pressure in patients with high CCAT1 expression was significantly higher than the low expression group. ***F,*** Diastolic blood pressure in patients with high CCAT1 expression was significantly higher than in patients with low expression.

Results

CCAT1 Expression Detection and Clinical Data Analysis

Clinical data analysis indicated that systolic blood pressure, diastolic blood pressure, and proteinuria in preeclampsia patients were higher than those in normal pregnancy placenta. The birth weight of fetus was lower than that of fetus born in normal pregnancy. No significant differences between pregnancy age and pregnancy weight were found (Figure 1). Patients with preeclampsia were further assigned into CCAT1 high expression group and low expression group (Figure 2A). We detected mRNA level in placental tissues from 40 preeclampsia and 40 normal pregnancy patients. Our results demonstrated that CCAT1 expression in PE placenta was higher in comparison with that in normal pregnancy ($p< 0.05$) (Figure 2B). Proteinuria in the high expression group was significantly higher than in the low expression group (Figure 2C). Newborns with high expression had significantly lower birth weights than those with low expression (Figure 2D). In addition, results illustrated that systolic blood pressure (Figure 2E) and diastolic blood pressure (Figure 2F) were higher in patients with high expression of CCAT1 than those with low expression of CCAT1. These data suggested that CCAT1 may be involved in the progression of preeclampsia.

CCAT1 Promotes the Progression of Preeclampsia

QRT-PCR results revealed that CCAT1 expression was the lowest in JEG-3 cells and highest in Bewo cells (Figure 3A). JEG-3 cells were selected for overexpression of CCAT1 experiments, while Bewo cells were used for interference experiments (Figure 3B, C). CCK-8 assay showed that the viability of Bewo cells was significantly increased after CCAT1 knockdown, and whereas the viability of JEG-3 cells was significantly decreased after CCAT1 was overexpressed (Figures 3D, G). By accessing the cell cycle through flow cytometry, we found that JEG-3 cells were signifi-

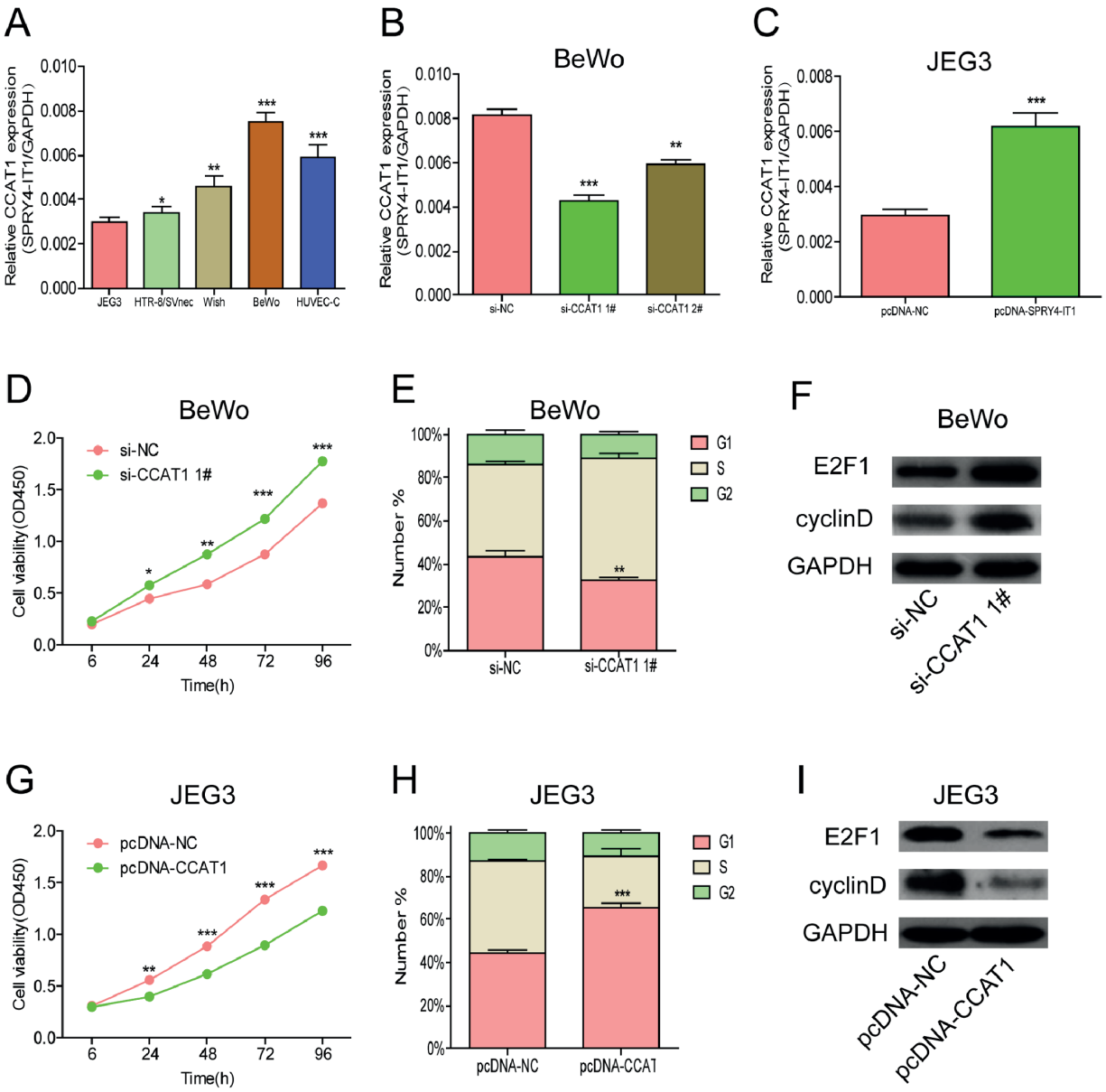

Figure 3. CCAT1 inhibits trophoblast cell proliferation. ***A,*** In trophoblast cells, CCAT1 expression was the lowest in JEG3 and highest in BeWo. ***B,*** Transfection of si-CCAT1 1 # significantly reduced the expression of CCAT1 in BeWo cells, especially in si-CCAT1 1 #. *C,* After CCAT1 overexpression, CCAT1 expression in JEG3 cell lines was significantly increased. ***D,*** After interfering with CCAT1, proliferation of BeWo cells was significantly enhanced. ***E,*** After interfering with CCAT1, cell cycle of BeWo was promoted. ***F,*** After interfering with CCAT1, E2F1, cyclin D expressions in BeWo cells increased. ***G,*** After overexpressing CCAT1, proliferation of JEG3 cells was significantly reduced. ***H,*** JEG3 cell cycle was arrested after CCAT1 overexpression. ***I,*** After overexpression of CCAT1, E2F1, cyclin D expressions in JEG3 cells decreased.

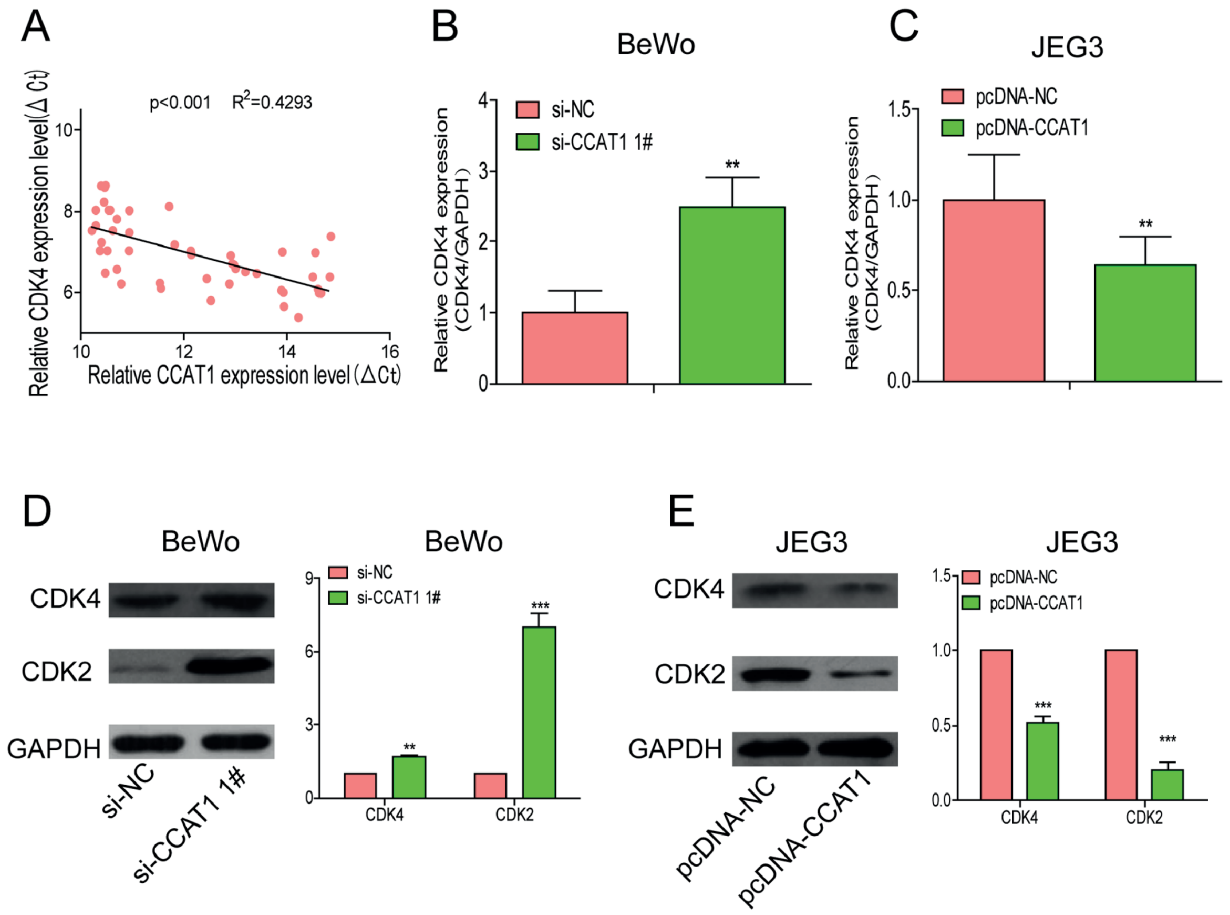

Figure 4. CCAT1 regulates CDK4 and inhibits trophoblast cell cycle. ***A,*** In human samples, the expression of CCAT1 was negatively correlated with the expression of CDK3. ***B,*** CDK4 expression was increased in BeWo cells after interfering with CCAT1. ***C,*** After overexpression of CCAT1, CDK4 expression in JEG3 cells decreased. ***D,*** After interfering with CCAT1, the expression of CDK2 and CDK4 in BeWo cell line was significantly increased. ***E,*** After overexpression of CCAT1, CDK2, CDK4 expressions were significantly reduced.

cantly arrested in GO/G1 phase after CCAT1 was overexpressed, whereas knockdown of CCAT1 significantly accelerated cell cycle of Bewo cells (Figure 3E, H). The protein E2F1 and cyclin D up-regulated while knocking down CCAT1, and the opposite results were observed when over-expression of CCAT1 (Figure 3F, I).

CCAT1 Promotes the Mechanism of Preeclampsia

To investigate the mechanism of CCAT1 in promoting preeclampsia, correlation analysis indicated that CDK4 was negatively correlated with CCAT1 (Figure 4A). To further verify the changes at transcriptional level, we found that the expression level of CDK4 increased after CCAT1 was disrupted, while it decreased after CCAT1 was overexpressed (Figure 4B, C). These studies suggested that CCAT1 regulated the progression of preeclampsia by modulating CDK4. At last, western bolt results showed that CDK2 and CDK4 were significantly increased after CCAT1 knockdown. After CCAT1 was overexpressed, the expressions of cell cycle promoting proteins, including CDK2 and CDK4, were significantly decreased (Figures 4D, E).

Discussion

Studies confirmed that lncRNAs were involved in the occurrence and development of tumors, affecting the treatment and prognosis of cancer patients. It was gradually recognized that lncRNAs could regulate proliferation, migration, and apoptosis of tumor cells, but the biological functions of lncRNAs and their mechanism are still not very clear. Thus, further investigations

are needed. Colon cancer-associated transcription factor 1 (CCAT1) gene was long non-coding RNA discovered in 2012. Studies[11-13] have shown that CCAT1 was abnormally expressed in colon cancer tissues. The relative expression level of CCAT1 was also associated with proliferative activity and metastasis of colon cancer cells. Many scholars[9,12,14-18] have shown that CCAT1 can also promote the proliferative activity and activation of hepatocytes, cholangiocarcinoma, gastric cancer, liver cancer; it was also considered a classic biomarker for the diagnosis of breast cancer. However, there have been few researches on CCAT1 in preeclampsia.

We observed the function of CCAT1 in the proliferative activity and cell cycle of Bew and JEG-3 cells by interfering and over-expressing CCAT1. We utilized qRT-PCR to verify that si-CCAT1 can effectively inhibit CCAT1 expression, while pcDNA-CCAT1 can increase CCAT1 expression. Effect of CCAT1 on cell proliferation and cell cycle was also confirmed by CCK8 and cell cycle assays. The experimental results demonstrated that, compared with the control group, low expression of CCAT1 can significantly increase cell proliferation and promote cell cycle, whereas overexpression of CCAT1 led to the contrary. All of above indicated that CCAT1 may play an important role in the pathogenesis of PE.

Cyclin-dependent protein kinase 4 (CDK4) is a member of the CDK proteins family. It is also an important serine/threonine protein kinase that functions a lot in DNA synthesis and mitosis. CDK4 exerts kinase activity and regulates cell cycle progression by binding to cyclin D (constituting a CDK/cyclin D complex). After CDK4 binding, retinoblastoma protein (Rb) is further hyper-phosphorylated, resulting in the release of nuclear factor E2F from pRb. The proliferative ability of cells rapidly crosses the G1-S limitation point and enters the S phase, thus enhancing rapid cell proliferation[19]. Therefore, the expression level of CDK4 is closely related to the proliferative activity of cells, and is involved in the occurrence and development of malignant tumors. Cyclin D1-P16-CDK4 pathway is one of the major molecular signal transduction pathways in the G1/S phase of the cell cycle[20] and has an essential barrier effect in the modulation of the cell cycle[21]. A study on the relationship between this pathway and preeclampsia contributes to the development of gene therapy for preeclampsia. In the present study, Western blot revealed that the expressions of E2F1, cyclin D, CDK2 and CDK4 in JEG3 cells were significantly decreased after CCAT1 was overexpressed, while their expressions in BeWo cells were significantly increased after CCAT1 was inhibited. The results demonstrated that CCAT1 may be greatly involved in the regulation of PE placental trophoblasts through the Cyclin D1-P16-CDK4 pathway, which may contribute to the pathogenesis of PE. However, specific regulation mechanism of CCAT1 on PE development is not clear, and further studies are still urgently needed.

Conclusions

We showed that CCAT1 was highly expressed in preeclampsia and can promote the progression of preeclampsia.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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