European Review for Medical and Pharmacological Sciences 2019; 23: 8230-8238

LncRNA PCAT6 promotes occurrence and development of ovarian cancer by inhibiting PTEN

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Abstract. – OBJECTIVE: The purpose of this study was to explore the expression and function of long non-coding RNA (lncRNA) PCAT6 in ovarian cancer.

PATIENTS AND METHODS: Quantitative Real-Time-Polymerase Chain Reaction (qRT-PCR) was used to detect the expression of lncRNA PCAT6 in 42 pairs of ovarian cancer tissues and adjacent normal tissues. Then, the relationship between PCAT6 expression and pathological indicators of ovarian cancer was analyzed. Subsequently, the transfection efficiency of PCAT6 in ovarian cancer cells was verified, and the PCAT6 knockdown model was constructed using lentiviruses in SKOV3 and CAOV3 ovarian cancer cell lines. In addition, Cell Counting Kit-8 (CCK-8) test, wound healing assay and transwell invasion and migration experiments were performed to estimate the effect of PCAT6 on the biological function of ovarian cancer cells, to further explore the possible potential mechanisms.

RESULTS: QRT-PCR results showed that the expression level of PCAT6 in ovarian cancer was higher than that in the adjacent normal tissues. The incidence of distant metastasis and lymph node metastasis in patients with high expression of PCAT6 was higher than those with low PCAT6 expression. Compared with the NC group, the proliferation, metastasis and invasion ability of ovarian cancer cells in si-PCAT6 group decreased significantly. QRT-PCR results demonstrated that the PTEN expression was increased after the knockdown of PCAT6. In addition, the recovery experiment also revealed that PCAT6 and PTEN have a mutual regulation, which can jointly regulate the development of ovarian cancer.

CONCLUSIONS: LncRNA PCAT6 was up-regulated in ovarian cancer tissues and was closely related to distant metastasis or lymph node metastasis. Additionally, lncRNA PCAT6 might promote the proliferation, migration and invasion of ovarian tumor cells by inhibiting PTEN.

Key Words:
Long-chain non-coding RNA, PCAT6, PTEN, Ovarian cancer.

Introduction

Ovarian cancer is one of the three major malignant tumors in the female reproductive system, and more than 90% are epithelial ovarian cancers[1,2]. Ovarian epithelial cancer (hereinafter referred to as ovarian cancer) has a high mortality rate and has been considered the "number one killer" of women's health today[3,4]. Although the surgical techniques for breast cancer have become more mature in recent years and new drugs have emerged, the 5-year survival rate of ovarian cancer patients is still unsatisfying through 30 years, lingering around 30%-40%. The main reason is that the onset of ovarian cancer is concealed, and there are no specific symptoms in the early stage. More than 70% of patients are in an advanced stage at the time of diagnosis, and metastasis and extensive dissemination of the abdominal cavity have occurred[5,6]. At present, the etiology and pathogenesis of ovarian cancer remain elusive. Therefore, the research on the pathogenesis of ovarian cancer is particularly important, which may contribute to a better understanding of the pathogenesis of ovarian cancer, thus providing new ideas for clinical diagnosis and treatment[7,8].

Depending on the length of the transcript, non-coding RNA (ncRNA) can be divided into short-chain ncRNAs represented by piRNA, siRNA, miRNA, and lncRNA with a transcript length of more than 200 nt[9,10]. According to genome sequencing, only about 2% of human genome sequences have protein-coding functions, and the remaining 98% of ncRNAs lack an open reading frame[11,12]. Following extensive studies on the role of lncRNAs with transcripts greater than 200 nt in tumors has received considerable attention[13,14]. LncRNA is transcribed by RNA polymerase II and its expression is tissue-specific and highly conserved during mammalian evolution. At first, people considered it a "noise"

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in the process of gene transcription without any function[14]. However, many studies in recent years have shown that lncRNA participates in a series of life processes, such as X chromosome silencing, chromatin modification, transcriptional interference and activation, and intranuclear transport by regulating the expression of genes in RNA form. LncRNAs are involved in almost all human physiological and pathological processes. Abnormal expression of lncRNA is closely related to the development of many human tumors[11,12]; however, the biological functions of most lncRNAs have not been studied. Previous studies have indicated that lncRNA PCAT6 can promote the growth and metastasis of tumor cells, which may affect the tumor development. But the function of PCAT6 in the development of ovarian cancer has not been reported[15-17].

Therefore, we selected lncRNA PCAT6 as the research object, focused on the differential expression of PCAT6 in ovarian cancer, and further explored its biological function and the molecular mechanism involved in tumorigenesis as well as the malignant progression of ovarian cancer.

Patients and Methods

Patients and PCa Samples

Tumor tissues identified as ovarian cancer tissues by hematoxylin and eosin (HE) staining (Solarbio, Beijing, China) and corresponding paracancerous tissues of 42 patients with ovarian cancer were collected, frozen and stored in a refrigerator at -80°C. According to the 8th edition of the UICC/AJCC ovarian cancer tumor node metastasis (TNM) staging criteria, all patients were diagnosed with ovarian cancer by postoperative pathological analysis. No anti-tumor treatment such as radiotherapy or chemotherapy was performed before surgery. The study was approved by the Ethics Committee of the Affiliated Hospital of Jining Medical University and all patients signed informed consent.

Cell Lines and Reagents

Human ovarian cancer cells including SKOV3, OVCAR3, PEO1, A2780, 3AO, CAOV3 and one normal human ovarian surface epithelial cells (HOSEPiCs) were all acquired from the American Type Culture Collection (ATCC; Manassas, VA, USA). Both Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were acquired from Life Technologies (Gaithersburg, MD, USA). The cells were cultured in an incubator with 5% CO_2 at 37°C with medium containing 10% fetal bovine serum (FBS).

Transfection

The control group (NC) and the lentivirus containing the PCAT6 knockdown sequence (si-PCAT6) were purchased from Shanghai Jima Company (Shanghai, China). Cells were cultured in 6-well plates and transfected at a cell density of 40% according to the instructions. 48 hours later after transfection, cells were collected for quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) analysis and cell function experiments.

Transwell Assay

After transfection for 48 hours, the cells were digested, centrifuged and resuspended in medium without FBS to make the cell density 5×10^5 cells/mL. 200 uL (1×10^5 cells) of cell suspension was added to the upper chamber, and the lower chamber was added with 700 uL of medium containing 20% FBS. The chamber was clipped and the cells were washed 3 times with 1 × Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA) and then fixed in methanol for another 15 min. After the chamber was stained in 0.2% crystal violet for 20 min, the cells on the upper surface of the chamber were carefully wiped off with water and a cotton swab. The chamber was placed under a microscope to observe, and 10 fields of view were randomly selected for counting and statistical analysis.

Cell Wound Healing Assay

After 48 hours of transfection, the cells were digested, centrifuged and resuspended in medium without FBS to make the cell density 5×10^5 cells/mL. The density of the cells was determined according to the size of the cells (the majority of the number of cells plated was set to 50,000 cells/well), and the confluence of more than 90% per day was optimal. The cells were then gently rinsed 2-3 times with PBS, cultured in medium with low concentrations of serum (e.g. 1% FBS) for 24 h and again observed. Scratch pre-experiment and migration area were used to judge cell-healing ability.

Quantitative Real Time-Polymerase Chain Reaction

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA from ovarian

cancer tissues and cells, and Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan) was used to reverse RNAs into cDNAs. QRT-PCR reactions were performed using SYBR® Premix Ex TaqTM (TaKaRa, Otsu, Shiga, Japan), and StepOne Plus Real Time-PCR System (Applied Biosystems, Foster City, CA, USA). The primers following were designed for qRT-PCR reaction: PCAT6: forward: 5'-ACTCGGGACATACCTGCTCT-3', reverse: 5'-GCCCTAGGGGAGTGACTACA-3'; PTEN: forward: 5'-AAGGACAGCAAGAAGAAGG-3', reverse: 5'-GAGGAACAGGTGGAGAGT-3'; β-actin: forward: 5'-CCTGGCACCCAGCACAAT-3', reverse: 5'-GCTGATCCACATCTGCTGGAA-3'. Data analysis was performed using ABI Step One software (Applied Biosystems, Foster City, CA, USA), and the $2^{-\Delta\Delta Ct}$ method was used to calculate the relative mRNA expression.

Western Blot Assay

After treated correspondingly, the cells were harvested and washed twice with PBS when the density reached 90%, and the cell lysate radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) was added. Then, the cells were centrifuged at 4°C, 14000 r/min for 15 min, and the supernatant was collected as total protein. The protein lysate was mixed in a 5:1 ratio in 5× loading buffer and boiled for 5-10 min. 20 μg of protein was mixed with 2 ×sodium dodecyl sulphate (SDS) loading buffer at a ratio of 1:1, and heated at 95°C for 10 min. After 2 min on ice, the protein was subjected to 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis. The protein on the PAGE gel was electrotransferred onto the polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) after electrophoresis. The PVDF membrane was incubated with a blocking solution for 1 h; then, the primary antibody (PTEN, GAPDH) was added for co-incubation with the membrane at 4°C overnight. The next day, after washing the membrane, horse reddish peroxidase (HRP)-labeled secondary antibody was added, and the luminescence was detected by enhanced chemiluminescence (ECL) detection kit (Thermo Fisher Scientific, Waltham, MA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference.

Statistical Analysis

The *t*-test was used to analyze the continuous variables, and the χ^2-test or Fisher's exact probability method was performed for the categorical variables. The survival time of patients was evaluated by the Kaplan-Meier method, and the difference between different curves was compared by the Log-rank test. The program was processed using the Statistical Product and Service Solutions (SPSS) 22.0 program (IBM, Armonk, NY, USA) and the data were expressed as mean ± standard deviation. $p<0.05$ was considered to be statistically significant.

Results

PCAT6 Was Expressed Highly in Ovarian Cancer Tissues and Cell Lines

We used qRT-PCR assay to explore PCAT6 expression in 42 pairs of ovarian cancer tissues and paracancerous tissues or cancer cell lines. The results of qRT-PCR demonstrated that PCAT6 expression in ovarian cancer tissues was higher than that in the paracancerous tissues, and the difference was statistically significant (Figure 1A). Meanwhile, PCAT6 was also highly expressed in ovarian cancer cells, compared to HOSEPiCs cells (Figure 1B).

PCAT6 Expression Was Related to Lymph Node or Distance Metastasis in Ovarian Cancer Patients

According to the results of qRT-PCR, ovarian cancer tissues were divided into PCAT6 high expression group and PCAT6 low expression group. The number of each group was analyzed and the relationship between PCAT6 expression and age, clinical stage, distant metastasis and lymph node metastasis of ovarian cancer patients was analyzed, respectively. The results demonstrated that high expression of PCAT6 was positively related to lymph node metastasis and distant metastasis of ovarian cancer (Table I).

Knockdown of PCAT6 Inhibited Cell Invasion and Migration

To explore the effects of PCAT6 on the proliferation, invasion and migration ability of ovarian cancer cells, we first successfully constructed a PCAT6 knockdown model and validated it using qRT-PCR assay (Figure 2A). Subsequently, we used the CCK-8 test to explore the proliferation rate of those cells. The results of CCK-8 showed that the cell proliferation rate in the si-PCAT6 group was lower than that in the NC group (Figure 2B). In addition, the transwell migration

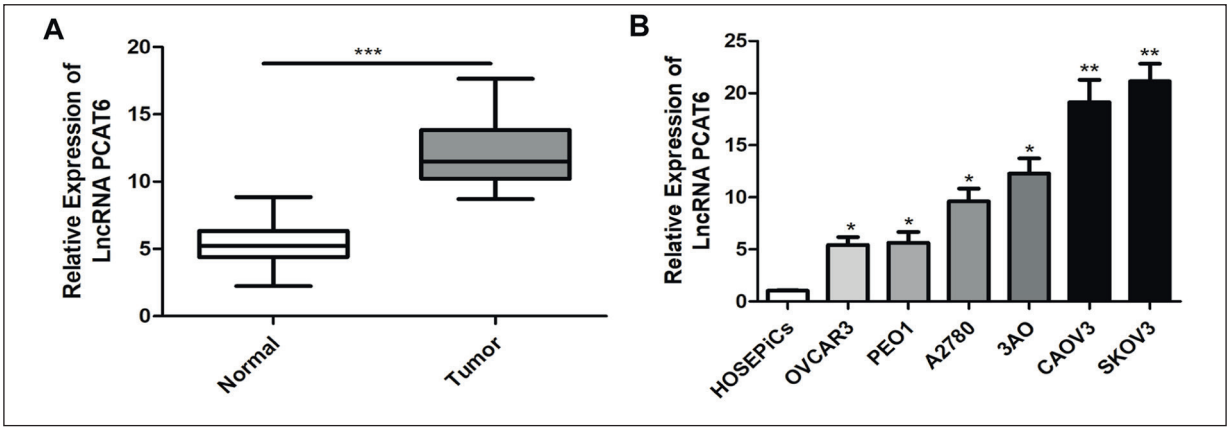

Figure 1. LncRNA PCAT6 was highly expressed in ovarian cancer tissues and cell lines. **A,** qRT-PCR was used to detect the differential expression of lncRNA PCAT6 in ovarian cancer tumor tissues and paracancerous tissues. **B,** qRT-PCR was used to detect the expression of lncRNA PCAT6 in ovarian cancer cell lines. Data are mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

assay and cell wound healing test were used to detect the migration invasive ability of those cells. The results of the transwell migration assay showed that the number of ovarian cancer cells transfected with si-PCAT6 in the transwell chamber was significantly reduced compared with the NC group. Those results suggested that the migration invasive ability is inhibited (Figure 2D). The cell wound healing test also revealed that the cells in the si-PCAT6 group had a poor healing ability (Figure 2C).

Knockdown of PCAT6 Increased PTEN Expression

To analyze the potential mechanisms of PTEN and PCAT6 affecting the malignant progression of ovarian cancer, we examined the PTEN expression in ovarian cancer tissues and paracancerous tissues. We found that the expression of PTEN in ovarian cancer tissues was lower than that in paracancerous tissues (Figure 3A). PTEN was also less expressed in ovarian cancer cells than in HOSEPiCs (Figure 3B). At the same time, we detected the PTEN expression changes after knockdown of PCAT6 by qRT-PCR and Western blot. We found that both mRNA and the protein expression of PTEN were increased (Figure 3C and 3D).

PTEN Could Modulate PCAT6 Expression in Human Ovarian Cancer Cells

To explore the interaction between PCAT6 and PTEN in ovarian cancer cells, we reduced the PTEN expression by disrupting the fragment in knockdown of PCAT6 ovarian cancer cell

Table I. Association of lncRNA PCAT6 expression with clinicopathologic characteristics of ovarian cancer.

Parameters	Number of cases	PCAT6 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.710
<60	15	8	7	
≥60	27	16	11	
T stage				0.941
T1-T2	26	15	11	
T3-T4	16	10	7	
Lymph node metastasis				0.047
No	28	19	9	
Yes	14	5	9	
Distance metastasis				0.020
No	31	21	10	
Yes	11	3	8	

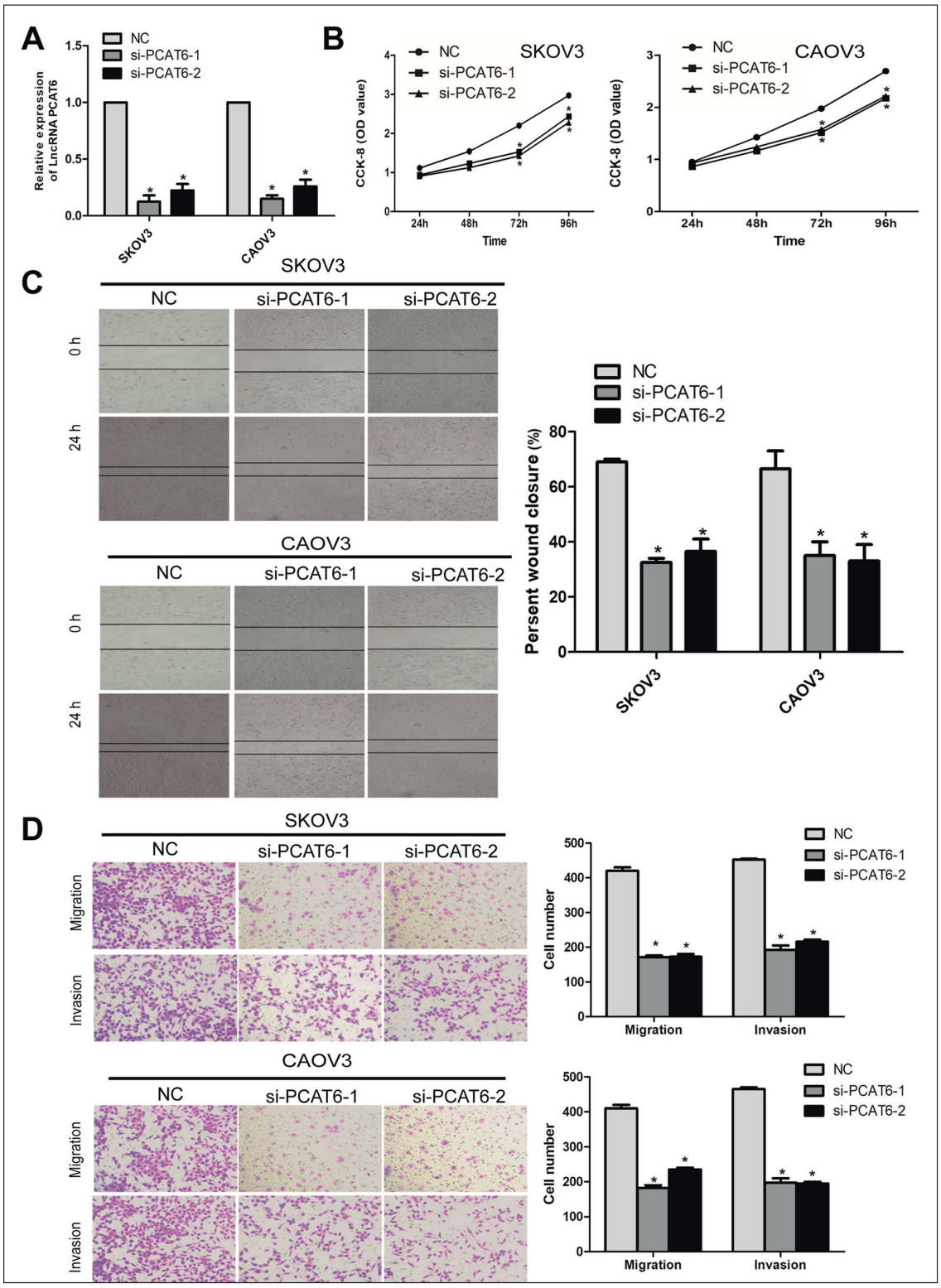

Figure 2. The knockdown of lncRNA PCAT6 reduces the proliferation and invasion and migration of ovarian cancer cells. **A,** qRT-PCR verified the interference efficiency of lncRNA PCAT6 after transfection of lncRNA PCAT6 knockout in SKOV3 and CAOV3 cell lines. **B,** CCK-8 assay to detect the effects of SKOV3 and CAOV3 cell lines on ovarian cancer cell proliferation. **C,** Cell scratch assay to test the effect of SKOV3 and CAOV3 cell lines on ovarian cancer cell healing (magnification: 10×). **D,** The transwell migration invasion assay to detect the invasion and migration of ovarian cancer cells by SKOV3 and CAOV3 cell lines (magnification: 40×). Data are mean ± SD, *$p<0.05$.

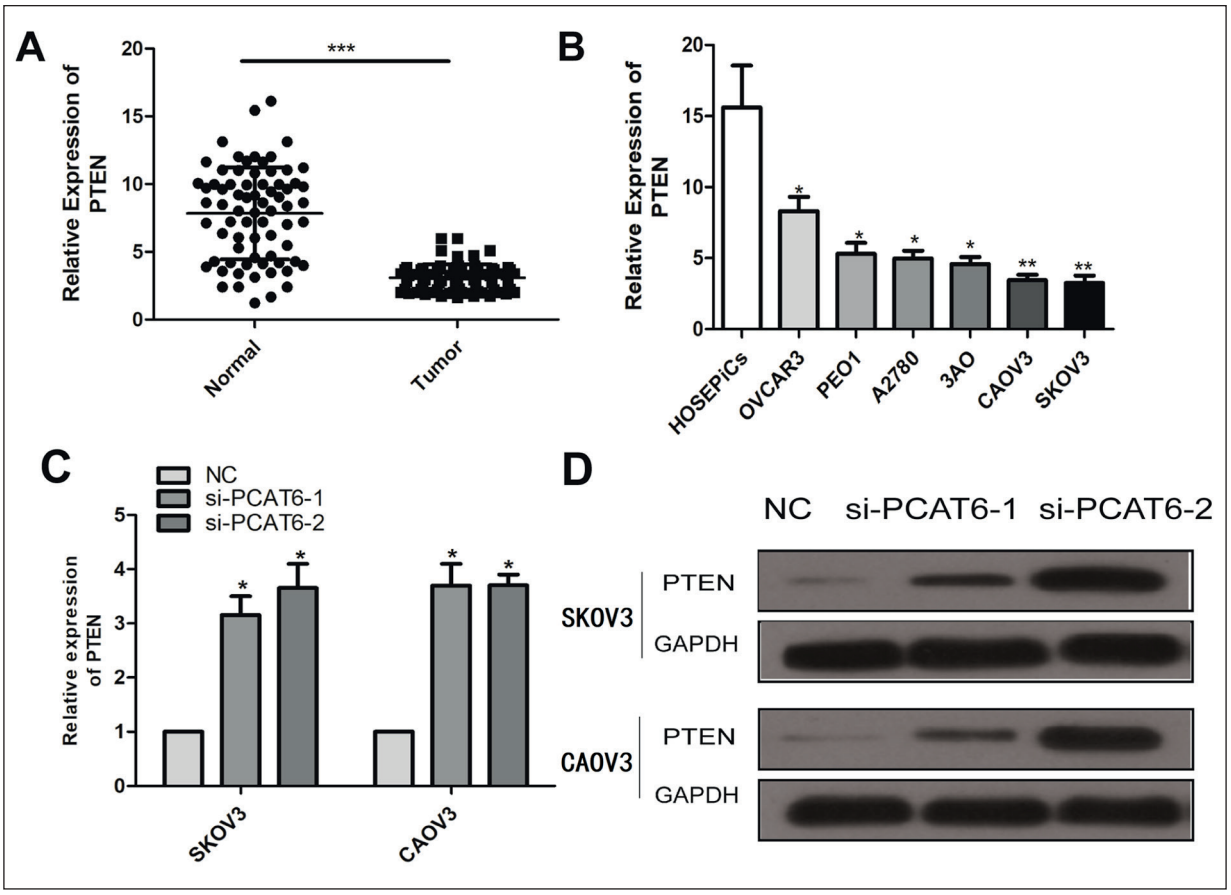

Figure 3. Low expression of PTEN in ovarian cancer tissues and cell lines. **A,** qRT-PCR detection of PTEN expression in ovarian cancer tumor tissues and adjacent non-tumor tissues. **B,** qRT-PCR detection of PTEN expression in ovarian cancer cell lines. **C,** qRT-PCR validation in SKOV3 and mRNA expression level of PTEN after knockdown of lncRNA PCAT6 in CAOV3 cell line. **D,** Western blot verified the protein expression of PTEN after knockdown of lncRNA PCAT6 in SKOV3 and CAOV3 cell lines. Data are mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

lines. The expression of PTEN was explored by qRT-PCR and Western blot. We found that the knockdown of PTEN restored the role of knockdown of PCAT6 in ovarian cancer (Figure 4A and 4B). Subsequently, we revealed that the knockdown of PTEN counteracted the effect of the decreased PCAT6 on the metastasis by transwell experiment and wound healing assay (Figure 4C and 4D).

Discussion

The invasion and metastasis of malignant tumors is the most important biological characteristic of tumor cells distinguished from normal cells during special evolution[18]. Invasion refers to the direct spread of tumor cells, and metastasis refers to the discontinuous spread of tumor cells. There is a series of extremely complex processes involving multiple factors, pathways, and the transmission of signals related to metastasis. Invasion is also associated with the transmission of metastasis-related signals, the ability of tumor cells to proliferate and migrate, the activation of tumor metastasis-related genes, and the inactivation of metastasis suppressor genes[18-21]. Li et al[21] have shown that long-non-coding RNA (lncRNA) with a transcript length of more than 200 nt is closely related to many malignant tumors in humans, which lacks a clear reading frame during transcription.

Currently, tumor suppressor effects are mediated by their gene products[22]. However, recent studies[9,10] have found that non-coding RNA (ncRNA), a class of RNA molecules that do not encode proteins, accounts for 98% of human transcripts and plays an important role in gene

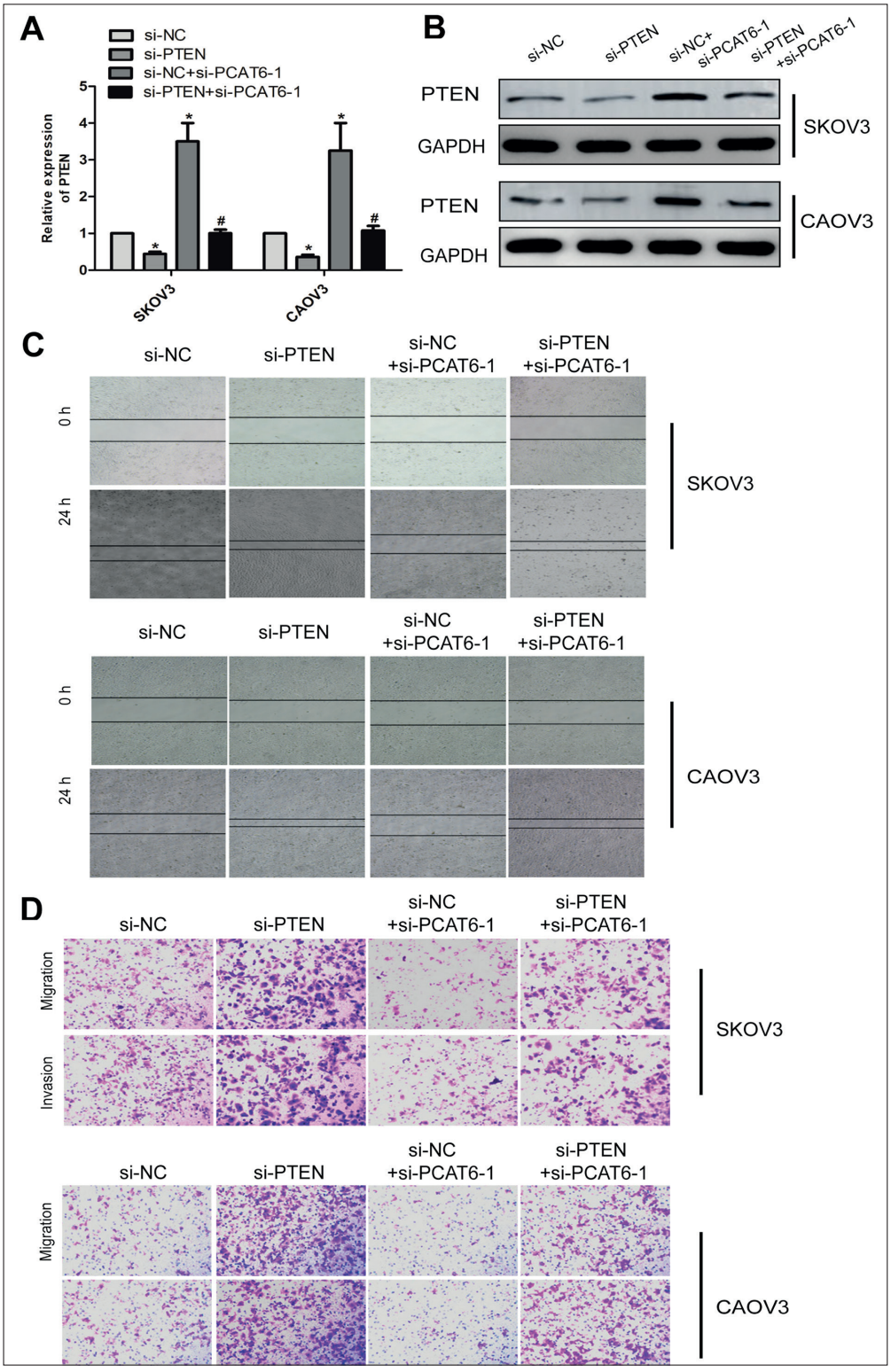

Figure 4. LncRNA PCAT6 regulated the expression of PTEN in ovarian cancer tissues and cell lines. **A,** Detection of PTEN expression in lncRNA PCAT6 and PTEN co-transfected cell lines by qRT-PCR. **B,** Detection of PTEN expression in lncRNA PCAT6 and PTEN co-transfected cell lines by Western blot. **C,** Cell scratch assay to detect the effect of lncRNA PCAT6 and PTEN co-transfection in the regulation of ovarian cancer cell healing (magnification: 10×). **D,** Cell scratch assay and transwell migration assay to detect lncRNA PCAT6 and PTEN co-transfection in the regulation of ovarian cancer cell invasion and migration (magnification: 40×). Data are average ± SD, $*^{\#}p<0.05$.

regulation. Depending on their length, the regulatory non-coding RNAs can be easily divided into two broad categories, namely small non-coding RNAs and long noncoding RNAs (lncRNAs)[11-14]. Unlike small-molecule non-coding RNAs, lncRNA itself does not encode proteins, but regulates gene expression in the form of RNA at various levels[23,24].

Therefore, looking for abnormal expression of lncRNA in ovarian cancer and analyzing its function may help to further understand the pathogenesis and biomarkers of ovarian cancer. LncRNAs associated with ovarian cancer are being discovered gradually, and these lncRNAs are also being applied to the evaluation of clinical diagnosis and prognosis. According to the literature, the lncRNA PCAT6 gene is closely associated with the occurrence and development in small cell lung cancer (SCLC) and gastric cancer, but the relationship with ovarian cancer is still unclear[15-17]. This work explored the lncRNA PCAT6 expression in ovarian cancer and its role in the development of ovarian cancer. Our results demonstrated that PCAT6 expression was markedly up-regulated and positively associated with lymph node metastasis and distant metastasis of ovarian cancer. Therefore, we believed that PCAT6 may exert an influence on promoting the progression of ovarian cancer. To further explore the effects of PCAT6 on the biological function of ovarian cancer cells, we constructed a PCAT6 knockdown expression model using lentivirus. Besides, the results of CCK-8 experiment, transwell assay and wound healing test showed that PCAT6 can promote the development of ovarian cancer, but its specific molecular mechanism remains elusive.

PTEN is a tumor suppressor gene discovered in 1997. Its encoded protein has dual activities of lipid phosphatase and protein phosphatase. It can regulate cell proliferation, apoptosis, migration and adhesion, and genetic stability by regulating the P13K/Akt signaling pathway[25,26]. It has been found that PTEN was inactivated in gastric cancer due to genetic or epigenetic changes such as gene mutations, loss of heterozygosity, promoter methylation, and microRNA regulation[27,28]. In addition, the activity of PTEN is also regulated by phosphorylation, that is, PTEN will lose activity after being phosphorylated, and further lose the tumor suppressor function, enhancing the susceptibility of tumor[29].

In this work, to prove that PCAT6 can promote the development of ovarian cancer by regulating PTEN, we observed the changes of PTEN expression after knockdown of PCAT6. The results illustrated that the protein expression of PTEN was increased after knockdown of PCAT6, suggesting that PCAT6 and PTEN have a mutual regulation. We also demonstrated that the knockdown of PTEN can counteract the role of reduced PCAT6 in ovarian cancer cells through cell recovery experiments. With the deepening of research, further studies on the biological functions of PTEN and the process of tumorigenesis and development will be more helpful for the diagnosis, treatment and prognosis of ovarian cancer.

Conclusions

LncRNA PCAT6 was highly expressed in ovarian cancer cells and tissues. Additionally, the expression of PCAT6 was significantly associated with lymph node metastasis and distant metastasis of ovarian cancer. Furthermore, PCAT6 might promote the malignant progression of ovarian cancer by inhibiting PTEN.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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