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PCAT1 promotes the proliferative and migratory potentials of ovarian cancer *via* targeting NEK2

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Abstract. – OBJECTIVE: The aim of this study was to clarify the potential role of PCAT1 in the occurrence and development of ovarian cancer (OC).

PATIENTS AND METHODS: Expression levels of PCAT1 and NEK2 in OC tissues and cell lines were detected by quantitative Real-time polymerase chain reaction (qRT-PCR). Correlation between PCAT1 expression with tumor stage and prognosis of OC patients was analyzed. Knockdown or over-expression of PCAT1 and NEK2 were achieved by siRNA or lentivirus transfection, respectively. Subsequently, cell viability, apoptosis, cell cycle progression and migration were determined by cell counting kit-8 (CCK-8), flow cytometry and transwell assay, respectively. Furthermore, the protein levels of relative genes in Wnt pathway were detected by Western blot.

RESULTS: PCAT1 was highly expressed in OC tissues and cell lines, especially in tumor tissues with stage III-IV compared with stage I-II. The prognosis of OC patients with higher expression of PCAT1 was significantly worse than those with lower expression. *In vitro* experiments confirmed that PCAT1 knockdown obviously inhibited proliferative and migratory potentials, whereas induced apoptosis of OC cells. No significant changes were observed in cell cycle progression of OC cells after knockdown or overexpression of PCAT1. Meanwhile, overexpression of PCAT1 remarkably upregulated the expression level of NEK2, which was the target gene of PCAT1. Interestingly, NEK2 knockdown could obviously suppress cell migration. Furthermore, Western blot results elucidated that PCAT1 knockdown could inhibit the protein levels of relative genes in Wnt pathway in OC cells.

CONCLUSIONS: PCAT1 was highly expressed in OC tissues than adjacent normal tissues. PCAT1 overexpression significantly promoted proliferative and migratory potentials, whereas inhibited apoptosis of OC cells through upregulating NEK2 expression via Wnt pathway.

Key Words:
PCAT1, NEK2, Migration, Ovarian cancer.

Introduction

Ovarian cancer (OC) is one of the most common malignancies in female reproductive system, whose incidence is second only to cervical cancer and endometrial cancer. Subtype of epithelial OC accounts for the first place in ovarian malignant tumors. It is characterized by occult onset, high rate of distant metastasis, and drug resistance[1,2]. Therefore, OC patients, especially those in advanced stage, are prone to pelvic and abdominal cavity diffusion accompanied by a large amount of ascites. Currently, cytoreductive surgery combined with paclitaxel and platinum-based chemotherapy has been widely applied. However, the 5-year survival rate of OC remains only 30%[3]. Lack of specific diagnostic methods and effective treatments in the early stage may explain the high mortality of OC. Cai et al[4] have demonstrated that the tumorigenesis of OC is a long-term process involving multiple factors. Early diagnosis contributes to improve the sensitivity and effectiveness of treatment of OC. Therefore, it is still an urgent problem to be solved. Long non-coding RNA (lncRNA) is a type of RNA with larger than 200 nt in length. LncRNA does not have protein-coding function[5,6]. The important roles of lncRNA in the regulation of cell cycle, gene expression and cell apoptosis have been widely reported. The functional mechanisms of lncRNAs depend on their subcellular localization. Therefore, lncRNAs exhibit different nuclear localization patterns to regulate gene transcription through transcriptional interference and chromatin remodeling. By comparison, lncRNAs distributed in the cytoplasm exert their biological functions through mediating RNA processing, mRNA stability or directly influencing protein functions[7-9]. In recent years, the relationship between lncRNAs and malignant tumors has been well concerned. LncRNA HOTAIR induces methylation of PTEN

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(gene of phosphate and tension homology deleted on chromosome 10), thereby promoting the spread and metastasis of human laryngeal squamous cell carcinoma[10]. LincRNA-p21 regulates gene expression relative to DNA damage by activating p53. Furthermore, downregulation of lincRNA p21 suppresses expressions of hundreds of genes through cooperation with hnRNP-K, thereby promoting cell apoptosis and inhibiting tumorigenesis[11]. PCAT1 is a lncRNA that is highly expressed in metastatic prostate cancer tissues. Studies[12,13] have shown that PCAT1 can form a complex with chromosomal remodeling protein polycomb repressor complex 2 (PRC2). Due to tissue specificity, PCAT1 is believed to be associated with tumor progression. In addition, PCAT1 participates in transcriptional regulation and controls tumor development by acting as a tumor-suppressor gene[14,15]. However, the specific role of PCAT1 in OC still needs to be further investigated.

Patients and Methods

Patients

Forty paired OC tissues and adjacent normal tissues were collected from OC patients who underwent surgical resection in Qingdao Women and Children's Hospital from June 2014 to July 2017. Among all enrolled patients, 15 and 25 cases were in stage I-II and stage III-IV, respectively. Samples were pathologically diagnosed by two pathologists independently. Then, they were immediately frozen in liquid nitrogen and stored at -80°C until use. None of patients received preoperative chemotherapy or radiotherapy. The study was approved by the Ethics Committee of Qingdao Women and Children's Hospital. Informed consent was obtained from each subject prior to sample collection.

Cell Culture

Human OC cell line (SKOV3) and normal ovarian epithelial cell line (HOSE) were preserved in our laboratory. Human OC cell lines (A2780, OVCA429, OVCAR3 and OVCA433) were purchased from KeyGentec Biotechnology Co., Ltd. (Nanjing, China). HOSE cells were cultured in M199/MCDB105 containing 5% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA). SKOV3 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) (HyClone, South Logan, UT, USA) containing 10% FBS. Meanwhile, A2780, OVCA429, OVCAR3, OVCA433 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% FBS. All cells were maintained in an incubator at 37°C with 5% CO_2.

Cell Transfection

For transient transfection, cells in logarithmic growth phase were first seeded into 6-well plates with serum-free medium. Until 50% of confluence, cells were incubated with diluted Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and transfection plasmids (si-PCAT1, si-NEK2 and negative control). Subsequently, the culture medium was replaced at 8 h. For lentivirus transfection, diluted lentivirus (MOI=80) and Polybrene were added in each well for overnight culture. Fresh medium was replaced at the other day. All transfection plasmids and lentiviruses were provided by GenePharma (Shanghai, China).

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA in cells or tissues was extracted according to the instructions of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The concentration of extracted RNA was detected by a spectrophotometer. Complementary deoxyribose nucleic acid (cDNA) was obtained according to the instructions of TaKaRa reverse transcription kit (Otsu, Shiga, Japan). Specific qRT-PCR procedure was: 94°C for 3 min, 94°C for 3 s, 60°C for 20 s and 72°C 1 min, for a total of 40 cycles. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was utilized as an internal reference. Primers used in this study were as follows: PCAT1, F: TGCTTCGTGAACTGAAACATCC, R: CCAGAGTCAACTGAGTCATCAC; NEK2, F: CTGGATGGCAAGCAAAACGTC, R: CCAGCGAGTTCTTTCTGGCTA.

Cell Counting Kit-8 (CCK-8)

100 μL of cell suspension containing 2000 cells was added to each well of 96-well plates. At 24 h, 48 h, 72 h, and 96 h, 10 μL of CCK-8 reagent (Dojindo Molecular Technologics, Kumamoto, Japan) was added to each well, respectively. After culture for another 2 h in dark, the absorbance of each well at the wavelength of 450 nm was measured by a microplate reader. Finally, a growth curve was plotted.

Transwell Assay

Cell suspension with 2×10^5 cells/mL was prepared using serum-free medium. 100 μL of cell suspension were added to the upper chamber, and 600 μL of complete medium were added to the basolat-

eral chamber. On the other day, un-penetrating cells above the chamber were wiped off. Subsequently, the chamber was fixed in 4% paraformaldehyde for 30 min and dyed with 1% crystal violet for another 30 min. Five fields were randomly selected for each sample. Finally, migrating cells were captured using an inverted microscope (magnification 20×).

Flow Cytometry

Cells were digested with Ethylene Diamine Tetraacetic Acid (EDTA)-free trypsin (Thermo Fisher Scientific, Waltham, MA, USA), centrifuged and suspended in 200 μL of 1× binding buffer. Subsequently, the cells were incubated with 5 μL of Annexin V-FITC (fluorescein isothiocyanate) and 5 μL of Propidium Iodide (PI) for 15 min in dark. Cell apoptosis was determined within 1 h using flow cytometry (Partec AG, Arlesheim, Switzerland). For cell cycle determination, cells were incubated with 1 mg/mL RNase A and 5 μL of PI for 30 min in dark. Cell cycle progression was determined using flow cytometry.

Western Blot

Total protein in cells was first extracted using cell lysate. The concentration of extracted protein sample was quantified by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Subsequently, extracted proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis and transferred onto membranes. After blocking with 5% skim milk, the membranes were incubated with primary antibody and corresponding secondary antibody. Immuno-reactive bands were developed by enhanced chemiluminescence (ECL).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) and GraphPad Prism 6 software (La Jolla, CA, USA) were utilized for statistical analysis. Quantitative data were represented as mean ± standard deviation ($\bar{x}\pm s$). *t*-test was used to compare the differences between two groups. Overall survival was analyzed by Gehan-Breslow-Wilcoxon test. $p<0.05$ was considered statistically significant.

Results

PCAT1 was Highly Expressed in OC Tissues and Cells

First, we examined the expression of PCAT1 in OC tissues and adjacent normal tissues. PCAT1 was highly expressed in tumor tissues when compared with adjacent normal tissues (Figure 1A). Moreover, the expression of PCAT1 in OC patients with stage III-IV was remarkably higher than those with stage I-II (Figure 1B). Similarly, PCAT1 expression was significantly up-regulated in OC cell lines when compared with normal ovarian cells. Among them, OVCAR3 and A2780 cells exhibited the highest expression level of PCAT1, which were selected for subsequent experiments (Figure 1C). Survival analysis showed that the overall survival of OC patients with higher expression of PCAT1 was remarkably worse than those with lower expression (Figure 1D). These results suggested that abnormal expression of PCAT1 might be associated with the development of OC.

PCAT1 Knockdown Suppressed Proliferative and Migratory Potentials, Whereas Induced Apoptosis of OC Cells

To further explore the biological function of PCAT1, we first constructed si-PCAT1 and transfected into OVCAR3 and A2780 cells. Transfection efficacy was verified by qRT-PCR (Figure 2A). CCK-8 assay showed significantly decreased proliferative rate after PCAT1 knockdown, with significant differences at 72 h (Figure 2B). Flow cytometry was conducted to evaluate the changes in cell apoptosis and cell cycle progression. Results demonstrated that transfection of si-PCAT1 markedly accelerated the apoptosis of OVCAR3 and A2780 cells. However, no significant difference was found in cell cycle progression (Figure 2C and 2D). Transwell assay revealed that the migratory potential of OC cells was significantly inhibited by PCAT1 knockdown (Figure 2E). The above data indicated that PCAT1 knockdown suppressed proliferative and migratory potentials, whereas induced apoptosis of OC cells.

PCAT1 Overexpression Promoted Proliferative and Migratory Potentials, whereas inhibited apoptosis of OC cells

Subsequently, cellular behaviors of OVCAR3 and A2780 cells overexpressing PCAT1 were evaluated as well. Firstly, lentivirus transfection of PCAT1 efficiently up-regulated PCAT1 expression in both OVCAR3 and A2780 cells (Figure 3A). CCK-8 data revealed that the proliferative rate of cells was remarkably enhanced after overexpression of PCAT1 (Figure 3B). Flow cytometry data showed a significantly inhibited apoptotic rate of OVCAR3 and A2780 cells overexpressing

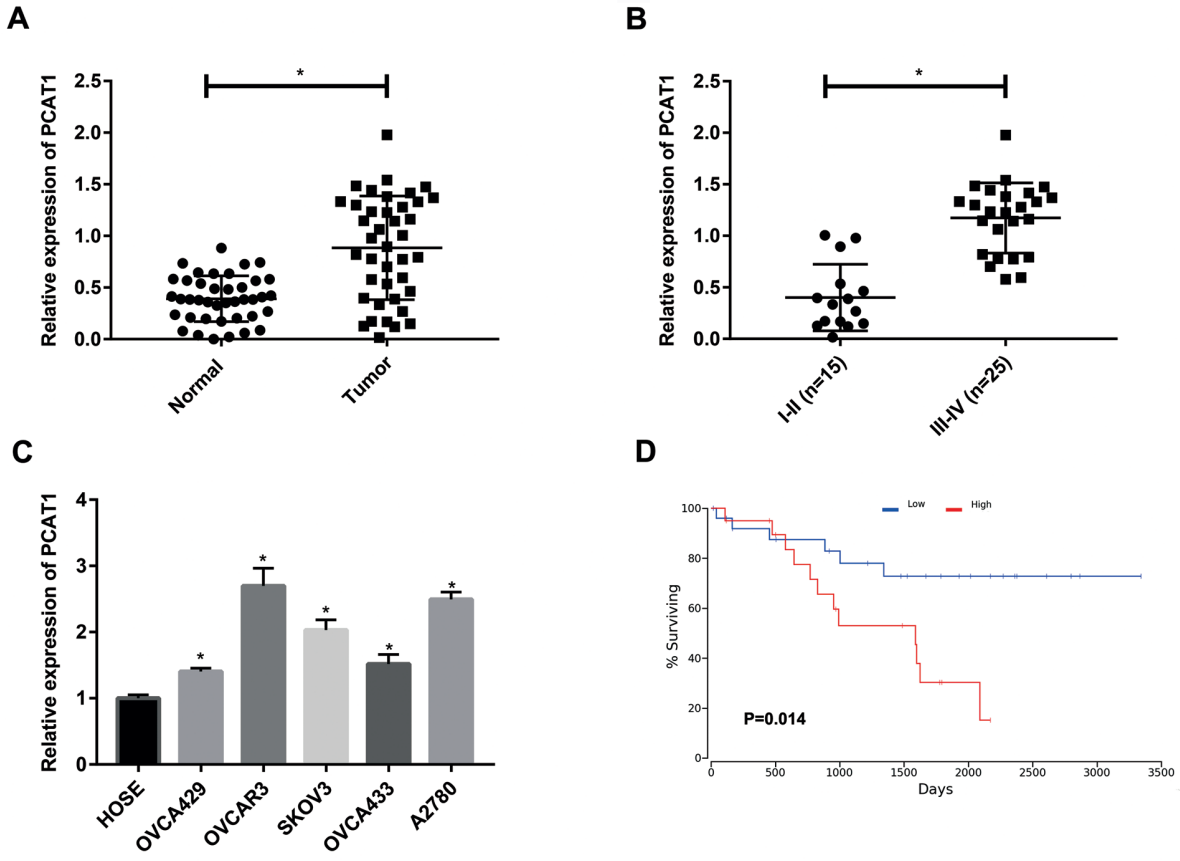

Figure 1. PCAT1 was highly expressed in ovarian cancer. **A,** PCAT1 was highly expressed in tumor tissues compared with adjacent normal tissues. **B,** PCAT1 expression in ovarian cancer patients with stage III-IV was remarkably higher than those with stage I-II. **C,** PCAT1 expression was highly expressed in ovarian cancer cell lines compared with normal ovarian cells. **D,** Survival analysis showed that the overall survival of ovarian cancer patients with higher expression of PCAT1 was worse than those with lower expression. *p<0.05.

PCAT1. Subsequent Western blot analyses verified that Bax was down-regulated and Bcl-2 was up-regulated (Figure 3C and 3D). Enhanced migratory rate was observed in OC cells after overexpression of PCAT1 (Figure 3E). The above findings indicated that PCAT1 overexpression promoted proliferative and migratory potentials, whereas inhibited apoptosis of OC cells.

PCAT1 Upregulated NEK2 Expression

Bioinformatics predicted that NEK2 might be a potential target of PCAT1 (data not shown). We then examined the expression of NEK2 after overexpressing PCAT1 in OC cells. Both the mRNA and protein levels of NEK2 were significantly upregulated after PCAT1 overexpression in OVCAR3 and A2780 cells (Figure 4A and 4B). Subsequently, we silenced the expression of NEK2 by siRNA transfection to explore the function of NEK2 (Figure 4C). Transwell assay revealed that NEK2 knockdown significantly inhibited migratory ability of OC cells (Figure 4D). Previous studies have suggested that Wnt pathway is closely related to cell migratory ability. Here, we demonstrated that the protein levels of β-catenin, Cyclin D1 and C-myc were significantly upregulated in OC cells transfected with PCAT1, suggesting the activation of Wnt pathway. Meanwhile, knocking down the expression of NEK2 could abolish the activation of Wnt pathway induced by PCAT1 (Figure 4E).

Discussion

With the in-depth study of lncRNAs in recent years, relative lncRNAs in OC have been well explored. LncRNA H19 was the first lncRNA found

to be related to OC[16]. Gao et al[17] have found that HOST2 contains a binding site of miR-let-7b. HOST2 serves as a ceRNA to sponge miR-let-7b, thereby inhibiting the anti-tumor effect of miR-let-7b[17]. OVAL is involved in the development and progression of malignant tumors through amplification of somatic chromosome copy number[18]. HOTAIR overexpression accelerates the oncogenic behaviors of OC cells. HOTAIR knockdown upregulates the expression level of miRNA-373, thereby inhibiting the viability, migration and proliferation of OC cells. Furthermore, HOTAIR overexpression inhibits the activity of miRNA-373 and reverses behaviors of cancer cells by acting as an endogenous sponge to Ras-associated protein RAB-22 (RAB22A)[19]. Zhao et al[20] have shown that LINC00092 is involved in glycolytic regulation during cancer-associated fibroblasts-mediated tumorigenesis of OC. Glycolytic regulatory molecule PFKFB2 maintains the micro-environment of tumor by controlling glycolytic products. This allows to promote tumor metastasis by binding to

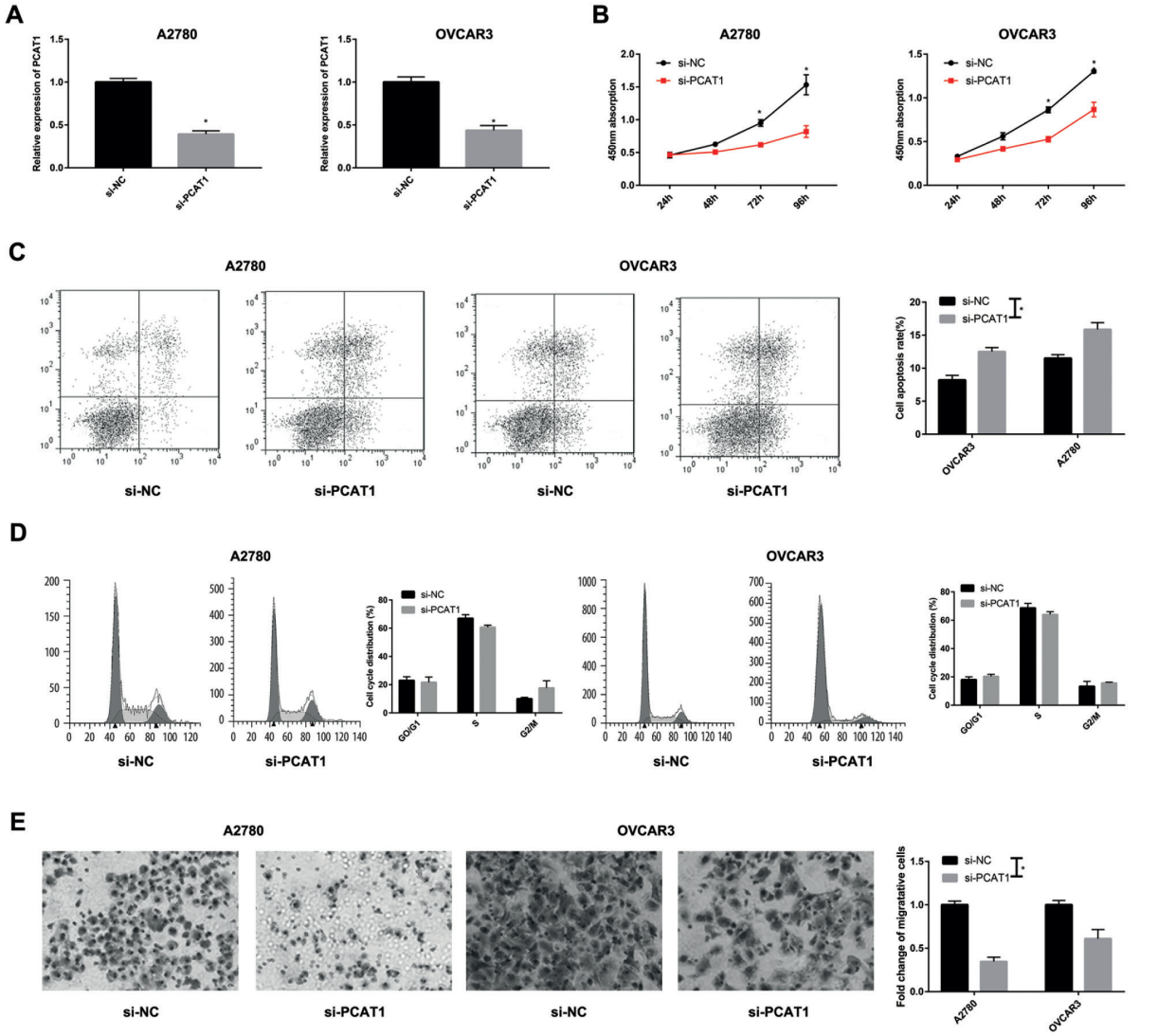

Figure 2. PCAT1 knockdown suppressed proliferative and migratory potentials, whereas induced apoptosis of ovarian cancer cells. **A,** Transfection efficacy of si-PCAT1 in OVCAR3 and A2780 cells. **B,** CCK-8 assay showed significantly decreased proliferative rate after PCAT1 knockdown, with significant differences at 72 h. **C,** Transfection of si-PCAT1 markedly accelerated apoptosis of OVCAR3 and A2780 cells. **D,** Transfection of si-PCAT1 showed no significant difference in cell cycle progression. **E,** Transwell assay revealed a significantly inhibited migratory potential of ovarian cancer cells after PCAT1 knockdown (magnification: 40×) **p*<0.05.

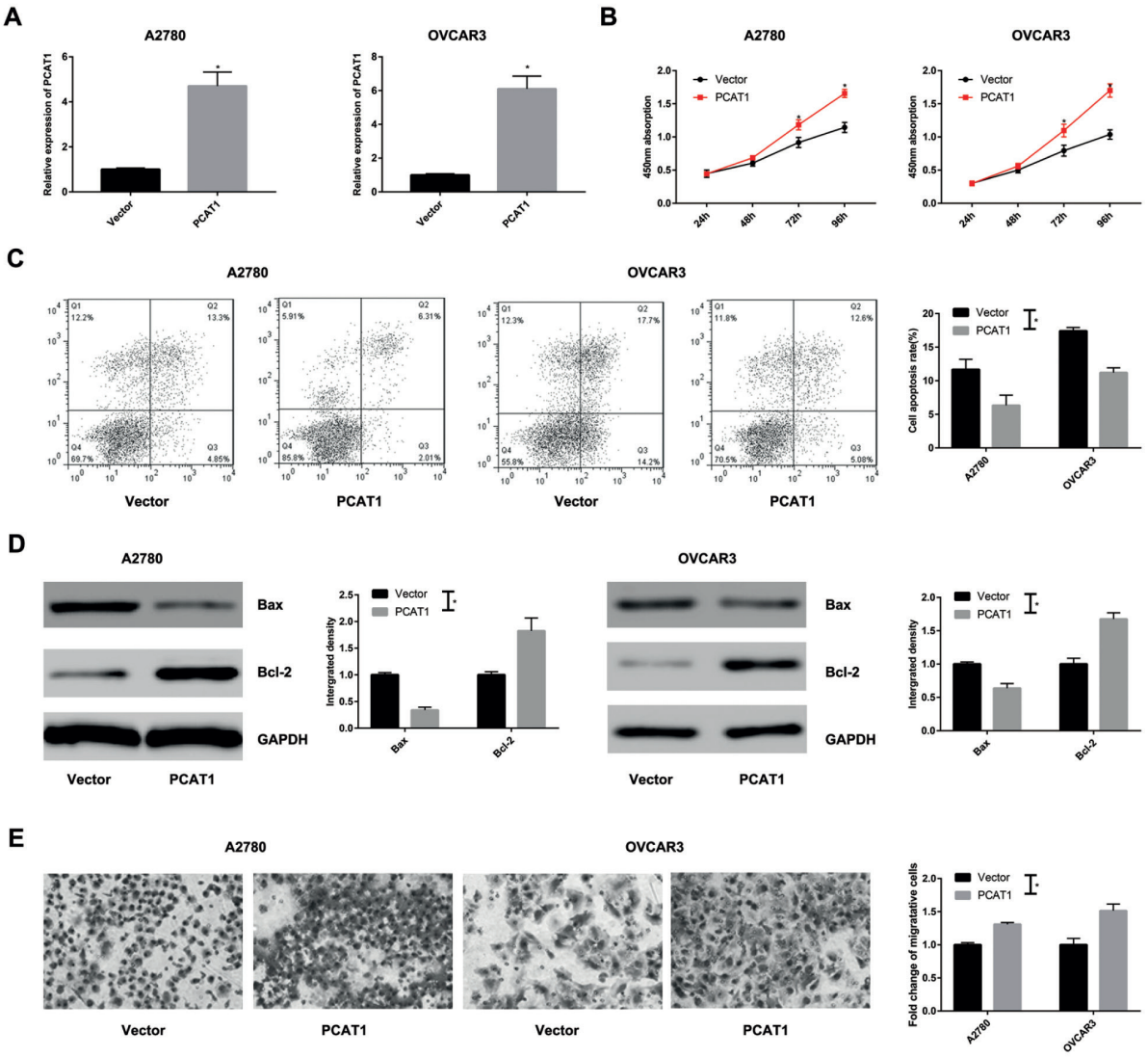

Figure 3. PCAT1 overexpression promoted proliferative and migratory potentials, whereas inhibited apoptosis of ovarian cancer cells. **A,** Lentivirus transfection of PCAT1 efficiently enhanced PCAT1 expression in both OVCAR3 and A2780 cells. **B,** CCK-8 showed that the proliferative rate was remarkably enhanced after overexpression of PCAT1. **C,** Flow cytometry data showed a significantly inhibited apoptotic rate in OVCAR3 and A2780 cells overexpressing PCAT1. **D,** Western blot analyses showed that PCAT1 overexpression remarkably downregulated Bax, while upregulated Bcl-2 in ovarian cancer cells. **E,** Transwell assay showed that overexpression of PCAT1 enhanced migratory rate of ovarian cancer cells (magnification: 40×) *$p<0.05$.

LINC00092. Qiu et al[21] have revealed that ANRIL overexpression promotes cancer cell proliferation, invasion and migration by targeting downstream MET and MMP3 as microarray *in vitro*. Sheng et al[22] have proved that hypermethylation of MEG3 promoter region is involved in the tumorigenesis of epithelial OC. By bioinformatics analyses, Hu et al[23] have found a key oncogene lncRNA FAL1, which serves as a prognostic marker for OC. NEAT1 is highly expressed in OC tissues and cell lines. Meanwhile, its expression is stabilized by HuR, which is a protein-binding RNA highly expressed in OC[24]. In this study, PCAT1 expression in OC tissues and cell lines was remarkably elevated. High expression of PCAT1 was a risk factor indicating poor prognosis of OC patients. *In vitro* overexpression of PCAT1 significantly promoted proliferative and migratory potentials, whereas inhibited apoptosis of OC cells. Subsequently, NEK2 was predicted the potential target gene of PCAT1 by bioinformatics. NEK2 is a member of the NIMA-related kinase (Nek) family, which current-

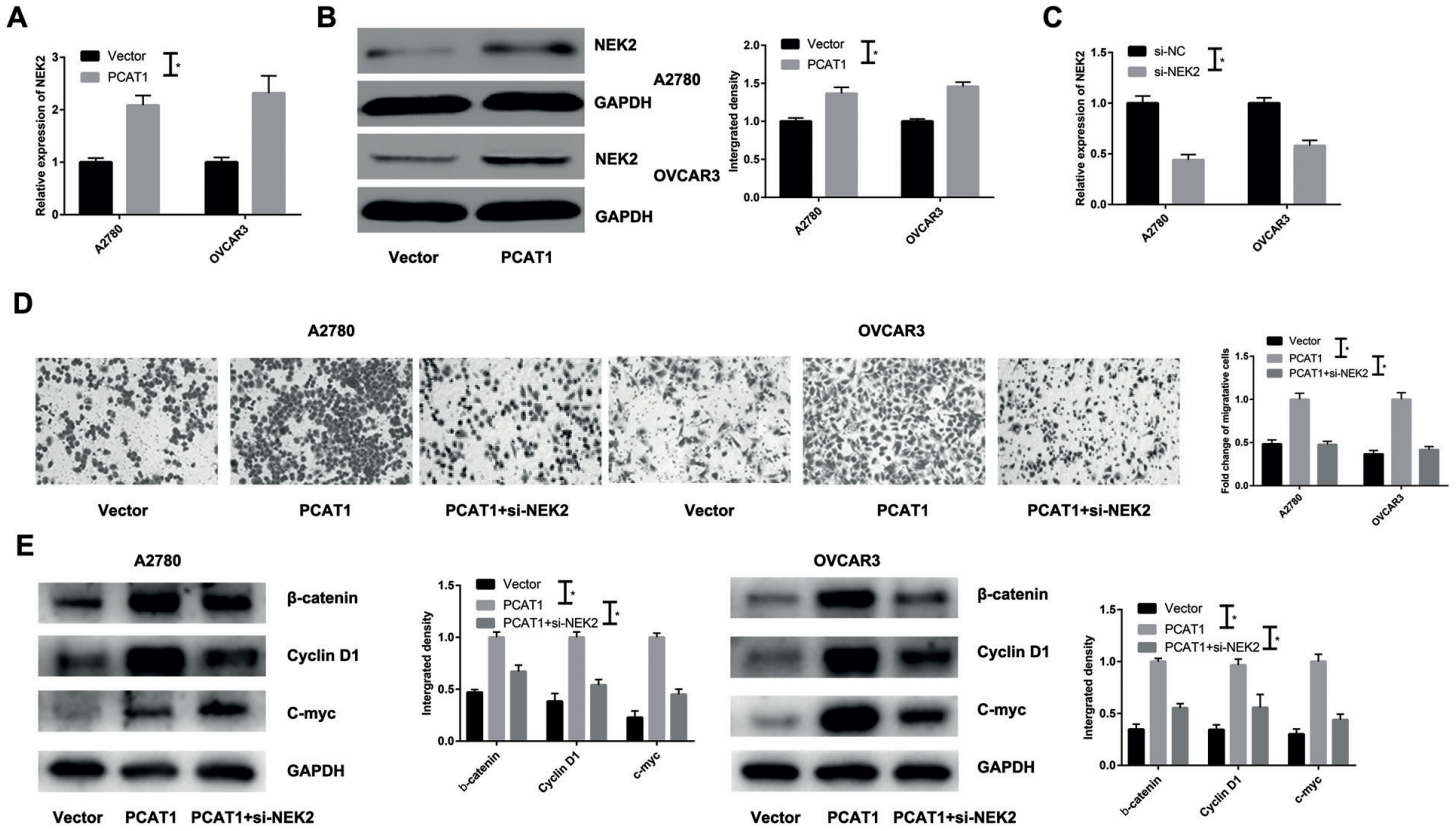

Figure 4. PCAT1 upregulated NEK2 expression. **A-B,** Both the mRNA and protein levels of NEK2 were significantly upregulated by PCAT1 overexpression in OVCAR3 and A2780 cells. **C,** The expression of NEK2 was silenced by si-NEK2. **D,** Transwell assay revealed the migratory ability of ovarian cancer cells was significantly inhibited after NEK2 knockdown (magnification: 40×). **E,** Western blot results demonstrated that the protein levels of β-catenin, Cyclin D1 and C-myc were significantly upregulated in cells transfected with PCAT1. *$p<0.05$.

ly has 11 family members (NEK1-11). They have similar catalytic sequences. Particularly, NEK2 is of interest due to the presence of 44% homologous sequences with NIMA. Human NEK2 is a serine/threonine protein kinase consisting of 445 amino acids, which has a relative molecular weight of 48 kD. It has an amino terminus in the kinase domain and a carboxy terminus in the uncatalyzed regulatory region[25]. NEK2, located in the centrosome, is an important centrosome protein kinase. The expression level of NEK2 is cyclically dependent. Specifically, the expression level of NEK2 is very low in G1 phase, but increases rapidly by 3-4 times in G1/S phase and remains high in S and G2 phase. However, NEK2 expression immediately decreases once entering M phase. The expression and activity of NEK2 are highly conserved throughout the cell cycle progression. During this process, NEK2 remains inactive by dephosphorylation of protein phosphatase (PP1). NEK2 is activated by autophosphorylation until PP1 is inhibited by inhibitor-2 or phosphorylated by NEK2[26]. Since NEK2 is involved in chromatin condensation and centrosome separation, it can affect the stability of the entire genome by regulating centrosomes and chromosomes. This can eventually promote malignant transformation of cells[27]. Many studies have shown that NEK2 is highly expressed in a variety of tumor cells, such as breast cancer, cervical cancer, prostate cancer and malignant lymphoma cells. Kokuryo et al[28] have found that NEK2 deficiency prolongs the life of mice in an *in vivo* model of peritoneal dissemination of cholangiocarcinoma. Hayward et al[29] have indicated that NEK2 is highly expressed in invasive breast cancer cells. Tsunoda et al[30] have suggested that NEK2 silence using RNA interference technology inhibits the growth, colony formation and invasion of breast cancer cells. More notably, Suzuki et al[31] confirmed *in vitro* and *in vivo* that NEK2 knockdown enhances the inhibition of cisplatin-induced proliferation and induces apoptosis. The above results suggest that high expression of NEK2 is involved in various biological behaviors of tumor cells. In this study, we found that the expression of NEK2 was regulated by PCAT1. Exogenous knockdown of NEK2 remarkably reduced the migratory ability of OC cells. It is demonstrated that PCAT1 may promote the proliferative and migratory potentials of OC cells by regulating NEK2 expression. Wnt/β-catenin pathway is one of the important signal transduction pathways involved in EMT of tumor cells. Its abnormal activation weakens the adhesion and induces EMT in tumor cells. Eventually, this enhances the invasive and metastatic abilities of tumor cells[32]. A relative study on breast cancer with lung metastasis has pointed out that Wnt pathway is highly activated in the early stage of lung metastasis. LRP6 is capable of inhibiting Wnt pathway. Meanwhile, the expressions of EMT-relative transcriptional factors Slug and Twist are inhibited. As a result, the self-renewal ability and tumorigenicity of tumor cells are attenuated[33]. Immunohistochemistry performed in colon cancer specimens has indicated that the expression of Dickkopf-1 is negatively correlated with expressions of β-catenin and vimentin. However, it is positively correlated with the expression of E-cadherin. Moreover, Dickkopf-1 is able to reverse the epithelial phenotype of colon cancer cells and downregulate the expressions of Snail and Twist[34]. Kanzawa et al[35] have provided direct evidences that Wnt pathway participates in EMT. WNT5A is highly expressed in the periphery of cancer nests in 66% of gastric cancer patients. *In vitro* experiments have demonstrated that WNT5A promotes the invasive and migratory potentials, as well as induces EMT of gastric cancer cells. Therefore, we speculated that PCAT1 might also regulate biological behaviors of OC cells through influencing Wnt pathway. Our results showed that the Wnt pathway was remarkably inhibited after inhibition of PCAT1 expression.

Conclusions

PCAT1 was highly expressed in OC tissues than adjacent normal tissues. PCAT1 overexpression significantly promoted proliferative and migratory potentials, whereas inhibited apoptosis of OC cells through upregulating NEK2 expression *via* Wnt pathway.

Conflicts of interest

The authors declare no conflicts of interest.

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