Original Article

LncRNA PCAT-1 plays an oncogenic role in epithelial ovarian cancer by modulating cyclinD1/CDK4 expression

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Abstract: Epithelial ovarian cancer is one of the most common causes of gynecological cancer deaths. The knock-down of LncRNA PCAT-1 has been reported to suppress tumor growth in various kinds of cancers, including esophageal cancer, breast cancer, bladder cancer, and hepatocellular carcinoma. However, its function in epithelial ovarian cancer (EOC) is still unclear. In the present study, the expression of LncRNA PCAT-1 was investigated. The results indicate that the expression of LncRNA PCAT-1 is up-regulated in EOC tissues compared with non-cancer controls by reverse transcription-quantitative polymerase chain reaction analysis (RT-qPCR), and its higher expression is always associated with larger tumor sizes and advanced tumor grades in patients with EOC. In addition, silencing PCAT-1 in the EOC cell lines SKOV3 and OVCAR3 significantly inhibits cell proliferation, migration and invasion, which is also shown by cell cycle assays, as the proportion of cells in G0/G1 phase is dramatically increased after knocking down PCAT1. Finally, it is observed that PCAT-1’s knockdown significantly decreased the levels of cyclin D1 and CDK4 protein expression. Taken together, LncRNA PCAT-1’s oncogenic role in EOC by mediating cyclin D1/CDK4 is demonstrated, indicating it is a potential target for EOC treatment.

Keywords: LncRNA PCAT-1, epithelial ovarian cancer, cyclinD1/CDK4

Introduction

Epithelial ovarian cancer (EOC) is one of the leading causes of cancer-associated deaths in women, because a large proportion of patients are diagnosed with it at the advanced stages [1]. Currently, EOC is mainly treated using platinum-based chemotherapy; however, despite the sensitivity of EOC to the chemotherapy, most patients eventually develop a recurrence within 18 months after treatment [2]. Therefore, it is of great significance and urgency to seek new therapeutic targets for EOC treatment.

Long non-coding RNAs (lncRNAs) are defined as a type of RNA molecules longer than 200 nucleotides that have no protein coding function. It has been demonstrated in recent studies that lncRNAs play critical roles in physiological and pathological processes, including organ development, chromatin remodeling, cell differentiation and gene regulation [3-5]; and their abnormal expression is associated with cancer proliferation, metastasis and recurrence [6]. This mechanism is explained by lncRNAs’ interaction with chromatin DNA, mRNA, or proteins, which regulate chromatin stability and protein activity [7, 8].

Prostate cancer associated transcripts 1 (PCAT-1) was first discovered in patients with prostate cancer by transcript sequencing. However, its up-regulation is not only observed in patients with prostate cancer, but also in patients with esophageal squamous carcinoma, colorectal cancer, osteosarcoma, hepatocellular carcinoma and multiple myeloma. Usually, LncRNA PCAT-1 plays an oncogenic role in these cancers and is correlated with advanced clinical stages and poor prognosis, but its functional role in EOC has not been investigated until now [6, 9-17].

In this study, LncRNA PCAT-1 was found to be significantly upregulated in EOC tissue samples and cell lines, compared to the levels in the
controls. Similarly, the knocking down of LncRNA PCAT-1 impaired cell proliferation and inhibited cell migration and invasion. In addition, a cell cycle analysis indicated that the EOC cells were arrested at G0/G1 after silencing LncRNA PCAT-1. The potential mechanism for this is that the knocking down LncRNA PCAT-1 will decrease the protein expression of cyclin D1 and CDK4, thereby exerting an impact on the cell cycle. Our findings suggest that LncRNA PCAT-1 may serve as an oncogene in EOC and inhibiting its expression could be a potential therapeutic target.

**Materials and methods**

**Patients and samples**

EOC tissues (formalin-fixed and paraffin-embedded) from 32 patients (age, 48.3 ± 8.6 years) and controls (postoperative pathological examination confirmed benign ovarian tumors) from 20 patients (age, 47.2 ± 9.5 years) were collected from Hospital Torrecárdenas (Almería, Spain) between August 2015 and August 2017 according to the protocol approved by the Ethical Committee of Hospital Torrecárdenas. It was performed in compliance with the principles of the Declaration of Helsinki. Written consents from all the participants in the study were obtained. The patients were divided into the high LncRNA PCAT-1 expression group and the low-expression group according to the median LncRNA PCAT-1 expression level. All participants underwent surgery, and their diagnoses were conducted based on immunohistochemical staining and morphological criteria. Moreover, patients who had ever received any therapy were excluded from the study.

**Cell culture conditions**

Human ovarian epithelial adenocarcinoma cell lines, including SKOV-3, OVCAR-3, HEY-A8, and HO8910-PM were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The human ovarian immortalized non-tumorigenic ovarian surface epithelial (IOSE-80) cell line was supplied by the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in the RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS; Sigma-Aldrich Co., St Louis, MO, USA). The cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C.

**Cell transfection**

The SKOV-3 cells (1 × 10⁶/well) and OVCAR3 cells (1 × 10⁶/well) were seeded in 6-well plates and a 75% confluence was reached the next day. Then, they were transfected with small interfering (si)-LncRNA PCAT-1-1 (si-LncPCAT-1-1), si-LncRNA PCAT-1-2 (si-LncPCAT-1-2) and negative control (NC), using Lipofectamine 3000 (Thermo Fisher Scientific, Inc.) as the transfection reagent in OptiMEM media. The sequence of si-LncPCAT-1-1 is 5'-UUAAGAGAGGCUCCACUGAUU-3'. The sequence of si-LncPCAT-1-2 is 5'-GCGAACACACCAUGGAAU-3'. The sequence of the negative control is: Sense, 5'-UUC UCC GAA CGU GUC ACG UTT-3' and antisense, 5'-ACG UGA CAC GUU CGG AGA ATT-3'. 48 hours post transfection, the cells were collected for further analysis. The cells were used for further experiments after the 48h transfection.

**RT-qPCR analysis**

RT-qPCR was performed using the Applied Biosystems 7500 Fast Real-Time PCR system (Thermo Fisher Scientific, Inc.). Total RNA was extracted from the human samples and cell lines mentioned above using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. A reverse transcription kit (Takara 047a; Takara Bio, Inc.) was used to transform RNA into cDNA. A qPCR reaction was performed using a SYBR Premix kit (Takara 420a; Takara Bio, Inc.). 20 μl PCR reaction mix was comprised of 10 μl SYBR Green I mix, 3 μl of cDNA, 1 μl of forward primer, 1 μl of reverse primer and 5 μl of RNase-free H₂O. The reaction was performed at 95°C for 3 min, followed by 40 cycles at 95°C for 15 sec, and then 60°C for 1 min. The LncRNA PCAT-1 expression level was calculated using the comparative Cq (2-ΔΔCq) method. Primers for LncRNA PCAT-1: Forward, 5'-GCTGGCATTGGTCAACATAAC-3', and Reverse, 5'-GTGAATATGGCGGATGAGGAA-3'. Primers for GAPDH: Forward, 5'-GTGAATATGGCGGATGAGGAA-3' and Reverse, 5'-CAGCCTTCTCCATGGGTGGA-3'.

**In situ hybridization (ISH)**

ISH was performed using an ISH Detection Kit III (AP; Boster Biological Technology, CA, USA). The samples were digested using proteinase K for 5 min and then fixed in 4% paraformalde-
LncRNA PCAT-1 plays an oncogenic role in epithelial ovarian cancer

hyde for 1.5 h at 37°C. Afterward, the samples were hybridized with a 5’-digoxin-labeled probe for LncRNA PCAT-1 with a sequence of 5’-GTCCATTAGCGCTATTGA-3’ at 55°C overnight. Then, paraffin-embedded sections with a thickness of 5 μm were deparaffinized using xylene and washed with ethanol at concentrations of 75, 85, 95, and 100%, respectively. After that, the samples were incubated with horseradish peroxidase at 4°C for 0.5 h, and then hematoxylin and eosin were added. Finally, the results were read using a microscope (magnification, × 200).

Transwell assay

After 48 hours transfection, the SKOV-3 and OVCAR-3 cells were harvested and were then seeded in the upper chamber with a diameter of 8 μm (Corning Incorporated, Corning, NY, USA) (Density: SKOV-3: 2.5 × 10^4 cells/well, OVCAR-3: 5 × 10^4 cells/well), but the lower chamber was filled with 600 μl of culture medium containing 10% FBS. For the invasion assay, Matrigel-coated chambers (Corning Incorporated) containing 8 μm pores were used. After incubation for 12 hours at 37°C, the cells were fixed with methanol for 10 min at 4°C at first and then stained with crystal violet for 30 min. Finally, the results were quantified under a light microscope (TCS SP2; Leica Microsystems, Germany).

Cell proliferation assay

A cell proliferation assay was conducted using a Cell Counting Kit-8 (CCK8; Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer’s instructions. After transfection with si-LncPCAT-1-1, si-LncPCAT-1-2 or NC, EOC cell lines, SKOV-3 and OVCAR-3 were seeded into 96-well plates with a density of 3000-6000 cells/well. Then, the cells were incubated for 24 h, 48 h, 72 h, and 96 h, respectively. After that, 10 μl of CCK-8 was added to each well prior to the reading of the results with a microplate reader at 450 nm and a frequency of every 0.5 h for 3 h in total.

Cell cycle analysis

The SKOV-3 and OVCAR-3 cells were digested using trypsin and then fixed in 70% ethanol. Subsequently, the cells were incubated with 25 μg/ml of PI (Propidium Iodide), 25 μl/ml of RNase A and Triton X-100 for 30 min at 4°C. Flow cytometry was carried out in the end so as to detect the cell cycle.

Flow cytometry

A flow cytometry analysis was performed for the apoptosis analysis, which was completed using an Annexin V-Fluorescein Isothiocyanate/Propidium Iodide Apoptosis Detection Kit (cat. No. 556547; BD Biosciences, NJ, USA) following the manufacturer’s instructions. Specifically, the cells were first stained using 5 μl Annexin V-FITC and 5 μl PI for 15 min in the dark so as to detect the cell cycle using FACSCelesta (BD Biosciences), and finally the results were analyzed with FlowJo software (version 10.9; FlowJo LLC, OR, USA).

Western blotting

After transfection, the cells were lysed in a radioimmunoprecipitation assay lysis buffer (Beyotime Biotechnology) on ice. Afterward, 20 μg to 30 μg of protein samples were separated on 8%-12% SDS-PAGE gels, and the bands were transferred onto nitrocellulose membranes. Then the membranes were blocked in Tris-buffered saline with Tween-20 and 5% non-fat milk for 1 h at room temperature. Next, the membranes were incubated overnight at 4°C with various primary antibodies, including anti-Cyclin D1 (cat. no. ab134175; 1:1000; Abcam), anti-CDK4 (cat. no. ab199728; 1:500; Abcam), anti-Cyclin E1 (cat. no. ab3927; 1:1000; Abcam), anti-CDK2 (cat. no. ab32147; 1:1000; Abcam) and anti-GAPDH (cat. no. ab9485; 1:2500; Abcam). Then the membranes were incubated with HRP-conjugated secondary antibody (Goat Anti-Rabbit IgG H&L, cat. no. ab150077; 1:2000; Abcam) for 1 h at room temperature after being washed 3 times. Finally, the protein expression level was detected using an enhanced chemiluminescence substrate (Beyotime Institute of Biotechnology).

Statistical analysis

Data expressed as the mean ± standard deviation (SD) on the basis of triplicate experiments and were analyzed using SPSS (SPSS Inc., Chicago, USA). To analyze the significance of the differences between groups, a two-way ANOVA analysis was conducted, and if there was a significant difference, Scheffe’s post hoc
LncRNA PCAT-1 plays an oncogenic role in epithelial ovarian cancer

Results

LncRNA PCAT-1 was upregulated in EOC patient samples and cell lines

Compared to the non-tumor ovarian tissues, the expression levels of LncRNA PCAT-1 in the EOC patient tissues was significant higher. Specifically, the transcription levels of LncRNA PCAT-1 was 2-fold higher in the EOC group compared to the control group, as shown in Figure 1A, which is in accordance with the results found in the cell lines. In all the EOC cell lines (SKOV-3, HEY-A8, HO8910-PM, and OVCAR-3), the expression of LncRNA PCAT-1 was much higher than it was in the IOSE-80 non-tumor cell line, of which the OVCAR-3 cells exhibited the highest expression (a 3-fold increase), followed by the SKOV-3 cells (a 2.6-fold increase), the HO8910-PM cells (2.5-fold increase) and the HEY-A8 cells (a 2.3-fold increase) (Figure 1B). Subsequently, the patients were divided into two groups according to the median expression level of LncRNA PCAT-1, and it was found that the higher expression of LncRNA PCAT-1 was closely related to the advanced International Federation of Gynecology and Obstetrics stage (P = 0.015) and larger tumor size (P = 0.014). The relationship between the LncRNA PCAT-1 expression level and the clinical characteristics is provided in Table 1.

Table 1. The association between LncRNA PCAT-1 expression and clinical features in 32 patients with EOC

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients (n = 32)</th>
<th>LncRNA PCAT-1 expression level</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>&lt; 55</td>
<td>18</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>≥ 55</td>
<td>14</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>FIGO stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-II</td>
<td>15</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>III-IV</td>
<td>17</td>
<td>3</td>
<td>14</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 5</td>
<td>14</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>&gt; 5</td>
<td>18</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>CA125 (U/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 35</td>
<td>10</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>&gt; 35</td>
<td>22</td>
<td>13</td>
<td>9</td>
</tr>
</tbody>
</table>

The knockdown of LncRNA PCAT-1 impaired cell proliferation, migration and invasion in EOC cell lines

To investigate the functional role of LncRNA PCAT-1, OVCAR-3 and SKOV-3 cells transfected (Knockdown efficiency was confirmed by RT-
LncRNA PCAT-1 plays an oncogenic role in epithelial ovarian cancer

qPCR) with LncRNA PCAT-1 siRNAs and NC were employed for further analysis. As shown in Figure 1C, the transcription levels of LncRNA PCAT-1 were significantly decreased after transfection with si-LncPCAT-1-1 and si-LncPCAT-1-2 in comparison to the NC group in both cell lines (OVCAR-3: si-LncPCAT-1-1 vs NC, P < 0.001, si-LncPCAT-1-2 vs NC, P < 0.001; SKOV-3: si-LncPCAT-1-1 vs NC, P < 0.001, si-LncPCAT-1-2 vs NC, P < 0.001).

The results of the CCK-8 assays performed after 24, 48, 72, and 96 hours transfection showed that there was no significant difference in the cell proliferation rate between the siRNA and NC groups in both cell lines in the first 24 h (P > 0.05). However, cell proliferation was significantly inhibited in si-LncPCAT-1 groups (P < 0.05) at 48, 72 and 96 h. The results are presented in Figure 2A, 2B.

By implementing the Transwell assay, the role of LncRNA PCAT-1 in migration and invasion was studied, and the results showed that the migratory and invasive abilities of the cells were visually decreased after transfection with si-LncPCAT-1s in both the SKOV-3 and OVCAR-3 cell lines (Figure 2C-F). Specifically, the migratory ability decreased by up to 41.3% (si-LncPCAT-1-1), 44.8% (si-LncPCAT-1-2) in the SKOV-3 cell line and 60.6% (si-LncPCAT-1-1), 45.5% (si-LncPCAT-1-2) in the OVCAR-3 cell line; the invasive ability was inhibited by up to 57.1% (si-LncPCAT-1-1), 54.3% (si-LncPCAT-1-2) in the SKOV-3 cell line and 60.5% (si-LncPCAT-1-1), 55.3% (si-LncPCAT-1-2) in the OVCAR-3 cell line (Figure 2C, 2D). Taken together, it is believed that LncRNA PCAT-1 could promote the growth and metastasis of EOC cells.

LncRNA PCAT-1 alters the cell cycle of the EOC cell lines and its downregulation arrested the cell cycle at the G0/G1 phase.

The cell cycle analysis was carried out in the SKOV-3 cell line to investigate the effects of LncRNA PCAT-1 on the cell cycle by silencing LncRNA PCAT-1, and the results suggested that, after treatment with LncRNA PCAT-1 siRNAs, the proportion of cells in the G0/G1 phase was significantly increased compared to the proportion in the NC group (P < 0.05) (Figure 3). In other words, cell cycle progression was arrested in the G0/G1 phase after the LncRNA PCAT-1 knockdown.
LncRNA PCAT-1 plays an oncogenic role in epithelial ovarian cancer

Figure 3. The role of LncRNA PCAT-1 in the cell cycle. The cell cycle was measured by flow cytometry in the SKOV-3 cell line (*P < 0.05).
LncRNA PCAT-1 plays an oncogenic role in epithelial ovarian cancer

The knockdown of LncRNA PCAT-1 may arrest the cell cycle by downregulating cyclin D1/CDK4

To investigate the mechanisms of silencing the LncRNA PCAT-1-induced cell cycle arrest, the expressions of the proteins that are associated with the cell cycle were detected using a western blot analysis. As shown in Figure 4, the expressions of cyclin D1 and CDK4, which are key regulators of the G1 phase, were significantly downregulated after the si-LncPCAT-1 transfection in the SKOV-3 cell line. However, such changes were not observed with the expressions of cyclin E1 and CDK2, indicating that knocking down LncRNA PCAT-1 may arrest the cell cycle at the G1 phase through cyclin D1/CDK4. The same result appears in the OVCAR-3 cell line (Figure 4B). We wonder whether the overexpression of LncRNA PCAT-1 can upregulate the expression of cyclin D1 and CDK4.

Discussion

The clinical outcomes of epithelial ovarian cancer are always disappointing because most patients are diagnosed at the advanced stages. Traditional therapies such as surgery and platinum-based cytotoxic chemotherapy cannot cure this disease, and most patients will finally develop a recurrence after treatment [1]. Therefore, the identification of novel treatment strategies are of great significance and urgently needed. Recent studies reported that LncRNAs may play key roles in cancer development and could serve as prognostic factors or potential treatment targets in various kinds of cancers, including EOC. Martini et al. reported that the expressions of LncRNA PVT1, Lnc-SERTAD2, Lnc-SOX4-1, and Lnc-HRCT1-1 are associated with relapse and poor outcomes in EOC in both univariate and multivariate analyses [18]. Gao et al. indicated that LncRNA-HOST2 promote tumor cell proliferation and invasion in EOC [19].

LncRNA PCAT-1 was first discovered being upregulated in prostate cancer patients, and it shows the ability to promote cell proliferation in prostate cells [20]. In this study, the expression level of LncRNA PCAT-1 was found to be significantly upregulated in EOC tissue and cell lines in comparison to the levels in the normal groups, and a high expression level of LncRNA PCAT-1 always associates with unfavorable prognostic factors, such as larger tumor size and a higher FIGO grade. Additionally, a series of experiments were conducted to investigate the role of LncRNA PCAT-1 in EOC development. Based on these experiments, it was discovered that silencing LncRNA PCAT-1 can impair cell
LncRNA PCAT-1 plays an oncogenic role in epithelial ovarian cancer

2155

proliferation, migration, and invasion in EOC cell lines, and the downregulation of LncRNA PCAT-1 can arrest the cell cycle at the G0/G1 phase. The mechanism involves the downregulation of LncRNA PCAT-1, which may decrease the expression of cyclinD1/CDK4 and then induce cell cycle arrest.

According to current published studies, it is reported that LncRNA PCAT-1 plays oncogenic roles not only in urinary neoplasms, but also in hepatocellular carcinoma [13, 21], colorectal cancer [22], and gynecological cancers, such as cervical cancer [23]. However, the mechanisms through which LncRNA PCAT-1 performs its oncogenic function varies among cancer types. In prostate cancer cells, LncRNA PCAT-1 promotes cell proliferation through cMyc [24] and produces a functional deficiency in homologous recombination by repressing the BRCA2 tumor suppressor [11]. In extrahepatic cholangiocarcinoma, it can regulate tumor progression through the Wnt/β-catenin signaling pathway. We found that the downregulation of LncRNA PCAT-1 can induce cell cycle arrest and inhibit cell proliferation by inhibiting cyclinD1 and CDK4 expression, since CyclinD1 and CDK4 work together to promote the cell cycle transition [25-27]. This finding is similar to that discovered in colorectal cancer cells. Qiao et al. reported that the knockdown of LncRNA PCAT-1 induced the cell cycle arrest of HT-29 and Caco-2 cells and the expression of cyclinD1 was significantly downregulated in both colorectal cancer cell lines. They also found the expressions of cyclin B and cyclin E were significantly reduced after inhibiting LncRNA PCAT-1 [12]. However, the expression of Cyclin E1 did not show a significant difference after silencing LncRNA PCAT-1. The reason may be because the pathways by which LncRNA PCAT-1 regulates the expression of cyclins in EOC and colorectal cancer are different.

In conclusion, the data in this study suggest that LncRNA PCAT-1 plays an oncogenic role in EOC and the downregulation of LncRNA PCAT-1 inhibits the cell proliferation, migration, and invasion in EOC cell lines. Therefore, LncRNA PCAT-1 may serve as a potential therapeutic target for EOC.

Disclosure of conflict of interest

None.

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References

LncRNA PCAT-1 plays an oncogenic role in epithelial ovarian cancer

PCAT-1 induced growth arrest and apoptosis of colorectal cancer cells. Life Sci 2017; 188: 37-44.


