H3K27ac-induced IncRNA PAXIP1-AS1 promotes cell proliferation, migration, EMT and apoptosis in ovarian cancer by targeting miR-6744-5p/PCBP2 axis

Yimin Ma* and Wei Zheng²

Abstract

We aimed to explore role of lncRNA PAX-interacting protein 1-antisense RNA1 (PAXIP1-AS1) in ovarian cancer (OC). RT-qPCR analysis identified upregulation of PAXIP1-AS1 in OC cell lines. Functionally, PAXIP1-AS1 knockdown inhibited cell proliferation, accelerated cell apoptosis, and suppressed cell migration and epithelial-mesenchymal transition (EMT) process. Upregulation of PAXIP1-AS1 was induced by CBP-mediated H3K27 acetylation (H3K27ac) via bioinformatic analysis and ChIP assay. Furthermore, PAXIP1-AS1 served as a competing endogenous RNA (ceRNA) to regulate PCBP2 expression by sponging microRNA-6744-5p (miR-6744-5p). Restoration experiments showed that overexpressed PCBP2 rescued effects of silenced PAXIP1-AS1 on cell proliferation, apoptosis, migration and EMT. Overall, IncRNA PAXIP1-AS1 activated by H3K27ac functioned as a tumor promoter in OC via mediating miR-6744-5p/PCBP2 axis, which provided promising insight into exploration on OC therapy.

Keywords: PAXIP1-AS1, H3K27ac, ceRNA, Ovarian cancer

Introduction

As a female gynecologic malignancy, ovarian cancer (OC) is one of the most common type of lethal tumors with higher mortality rate worldwide [1]. Each year, there is a continuous increase in the newly-diagnosed OC cases and in OC-related deaths [2]. In recent decades, therapeutic approaches, such as surgery, chemotherapy and radiotherapy, have achieved great progress. However, the long-term survival is still poor due to the imperceptible symptoms at early stage and the distant metastasis at advanced stage [3, 4]. Thus, it is quite imperative to explore the underlying mechanisms and develop novel methods for the treatment of OC.

Long noncoding RNAs (lncRNAs), a new group of noncoding RNAs with over 200 nucleotides in length, are recently identified [5]. Although no potential ability in protein-coding, lncRNAs have been found to exert important effect on multiple biological processes [6, 7]. Extensive studies have suggested that lncRNAs could modulate survival, cell proliferation, stemness, differentiation, and epithelial-mesenchymal transition (EMT) [8–10]. For example, lncRNA CRNDE shows a high level in tongue squamous cell carcinoma tissues and inhibits miR-384 to facilitate cell proliferation and metastasis [11]. In addition, high-expressed LINC01296 is revealed to predict poor prognosis in lung cancer patients and enhances tumor growth via modulating miR-598/Twist1 pathway [12]. Importantly, mounting reports indicate that lncRNAs regulate gene expression to mediate cancer progression through multiple ways, such as histone modification, transcriptional and post-transcriptional...
regulation [13]. Among which, lncRNAs were widely reported as competing endogenous RNAs (ceRNAs) that involved in lncRNA/miRNA/mRNA network [14]. This is a new model which proposes that lncRNAs regulate expression of target genes via miRNA response elements [15]. Because of the importance of lncRNAs in cancers, much more research should be conducted to depict their functions in OC.

LncRNA PAX-interacting protein 1-antisense RNA1 (PAXIP1-AS1) has been reported as a tumor promoter in glioma by aggravating cell invasion and angiogenesis [16]. However, its functional role in other cancers, especially in OC remains obscure. This study focused on the biological role and underlying mechanism of PAXIP1-AS1 in OC. The results demonstrated that PAXIP1-AS1 induced by H3K27ac was upregulated in OC cells and promoted cellular process in OC by sponging miR-6744-5p and targeting PCBP2, providing a new molecular mechanism implicated in OC cellular development.

Materials and methods

Cell lines
Human ovarian epithelial cell line (HOSEpiC) and OC cell lines (SKOV3, A2780, OVCAR3 and COV362) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The four OC cell lines were identified for study since functional experiments and examination of gene expression were successfully conducted using these cell lines in previous studies [17–20]. All above cell lines were cultured at 37 °C in RPMI-1640 medium with a supplement of 100 U/ml penicillin, 100 mg/ml streptomycin and 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) in a humidified atmosphere containing 5% CO2. C646, an inhibitor of acetylation, was commercially provided by Sigma Chemical (St. Louis, MO).

Cell transfection
The miR-6744-5p mimics/inhibitor and their matched negative control (NC mimics/inhibitor), short hairpin RNAs (shRNAs) against PAXIP1-AS1 or CBP (sh-PAXIP1-AS1#1/2 or sh-CBP#1/2) and their negative control (sh-NC), and overexpressing plasmids pcDNA3.1/PCBP2 (PCBP2) and its negative control pcDNA3.1 (Vector) were synthesized by GenePharma (Shanghai, China). Using Lipofectamine 2000 (Invitrogen), cell transfection was conducted with manufacturer’s instructions. After 48 h of cell culture, RT-qPCR was conducted to validate the transfection efficiency.

RT-qPCR
First, TRIzol reagent (Invitrogen) was used to isolate total RNAs from OC cell lines. Next, the reverse transcription of miRNA or lncRNA/mRNA was performed by the miRNA First-Stand cDNA Synthesis Kit (GeneCopoeia) or the cDNA Synthesis SuperMix Kit (TransGen, Beijing, China). Afterwards, RT-qPCR was performed on Applied Biosystems 7500 Real-time PCR Systems (Thermo Fisher Scientific). Gene expression was quantified by 2−ΔΔCt method and normalized to U6 or GAPDH.

Colony formation assay
Cells at a density of 600 cells/well were grown in 6-well plates with RPMI-1640 medium containing 10% FBS. After 2 weeks of cell culture, methanol was used for fixing colonies for 15 min at room temperature, and then the cells were stained with 0.1% crystal violet (Invitrogen) for 15 min. Finally, the number of visible colonies was manually counted.

EdU assay
After transfection, SKOV3 or OVCAR3 cells were collected and plated to 96-well plates at the density of 1 × 10⁴ cells each well. Later, the plates were added with EdU assay kit (Ribobio) at 37 °C for 2 h. Cell nuclei was stained by DAPI solution. Finally, a fluorescence microscope (Olympus, Tokyo, Japan) was applied to observe proliferative cells.

Flow cytometry analysis
SKOV3 or OVCAR3 cells were seeded in 6-well plate. After centrifugalization, the binding buffer, resuspended with residue, was added with 5-μL Annexin V-FITC and 5-μL propidium (PI). Next, a flow cytometer (BD, Franklin Lake, NJ, USA) was used to measure cell apoptotic rate, and results were analyzed by the software WinMDI 2.9 (Invitrogen).

Western blot analysis
Cell lysis was conducted in the RIPA lysis buffer, and then the lysate was separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore). Blocked with 5% nonfat milk, the membranes were incubated with primary antibodies overnight at 4 °C. After an incubation with secondary antibody at 37 °C for 1 h, the enhanced chemiluminescence (ECL) Plus kit (Beyotime, Shanghai, China) was used to visualize the protein bands.

Transwell assay
The migratory ability of SKOV3 and OVCAR3 cells was assessed using transwell chambers without matrigel. In brief, the cells (1 × 10⁵) were seeded to upper chambers containing 200 ul serum-free media. The lower chambers were added with 600 ul of 20% FBS media. Incubated for 48 h, the cells without invasion in the upper chambers were wiped away by cotton wool. Fixed with
methanol, the cells were stained with 0.5% crystal violet in the lower chamber. Finally, five random fields were photographed under an inverted microscope.

**ChIP assay**

ChIP assays were carried out by ChIP Assay Kits (Thermo Fisher Scientific) with SKOV3 or OVCAR3 cells. For DNA-protein cross-links, SKOV3 or OVCAR3 cells were incubated with 1% formaldehyde for 10 min, and then an ultrasound machine was utilized to break the cross-linked chromatin DNAs into segments sized 200 to 1000 bp. The chromatin lysate was precipitated by anti-H3K27ac (Abcam), anti-CBP (Abcam) or anti-IgG (Abcam). Finally, RT-qPCR was performed to analyze the ChIP samples.

**Subcellular fractionation assay**

First, the nuclear and cytoplasmic fractions of SKOV3 and OVCAR3 cells were obtained. Next, with the Cytosplasmic & Nuclear RNA Purification Kit (Norgen), the fractions were separated and purified as per the manual. At last, RT-qPCR was performed to analyze the isolated RNA (GADPH, U6, PAXIP1-AS1). GAPDH was the internal control for cytoplasm, and the distribution of PAXIP1-AS1 in nucleus was normalized to U6.

**Luciferase reporter assay**

To conduct the luciferase reporter assay, pmirGLO vector (Promega, Madison, WI) was used. Briefly, the wild-type (WT) PAXIP1-AS1/PCBP2 or mutant (Mut) PAXIP1-AS1/PCBP2 binding element in the sequence of miR-6744-5p was cloned into pmirGLO vector for the construction of PAXIP1-AS1-WT/Mut or PCBP2-WT/Mut. Then, the reporters were separately co-transfected with miR-6744-5p mimics in SKOV3 or OVCAR3 cells. After 48 h, the relative luciferase activity was testified by the dual-luciferase reporter assay system (Promega).

**RNA pulldown assay**

NC-miRNA and miR-6744-5p-WT/Mut were labeled with biotin and then transfected into SKOV3 and OVCAR3 cells. Streptavidin magnetic beads were incubated with the cell lysates for 4 h at 4°C. Using pre-cooled lysis buffer and salt buffer, the beads were rinsed. After that, PAXIP1-AS1 or PCBP2 level was detected following the extraction of pull-down RNAs.

**RIP assay**

For RNA immunoprecipitation, the EZMagna RIP kit (Millipore) was applied. Cells were lysed in RIP lysis buffer after being harvested. Then, the cell lysate was incubated with magnetic beads absorbed anti-IgG (Millipore) or anti-Ago2 antibody (Millipore). Finally, RT-qPCR analyzed the purified RNA.

**Statistical analysis**

Three biological repeats were applied to all experimental procedures. Shown as the mean ± SD, data were statistically analyzed through GraphPad Prism 6 (GraphPad). Differences between two groups were analyzed by Student’s t test or one-way ANOVA for multiple groups with $p < 0.05$ as a cut-off value.

**Results**

**Upregulated PAXIP1-AS1 in OC enhanced cell proliferation, migration and inhibited cell apoptosis**

First, PAXIP1-AS1 expression pattern in OC cells was assessed by RT-qPCR for the investigation of its biological role. Compared with HOSEpiC cell line, high expression of PAXIP1-AS1 was observed in OC cell lines (SKOV3, A2780, OVCAR3 and COV362) (Fig. 1a). Then, SKOV3 and OVCAR3 cells presenting higher PAXIP1-AS1 expression were chose to probe functional role of PAXIP1-AS1 in OC. Accordingly, we designed and conducted loss-of-function assays by transfecting sh-PAXIP1-AS1 (sh-PAXIP1-AS1#1/2) into SKOV3 and OVCAR3 cells. The results of RT-qPCR analysis confirmed that PAXIP1-AS1 expression was apparently downregulated after sh-PAXIP1-AS1 transfection (Fig. 1b). Through colony formation assay, we found silenced PAXIP1-AS1 significantly decreased colonies of SKOV3 and OVCAR3 cells (Fig. 1c). EdU assay further confirmed the inhibitory effect of PAXIP1-AS1 deficiency on cell proliferation (Fig. 1d). In addition, flow cytometry analysis suggested that cell apoptosis was remarkably promoted by sh-PAXIP1-AS1 transfection (Fig. 1e). To further confirm this, levels of apoptosis-related proteins after PAXIP1-AS1 knockdown were tested by western blot analysis. The results showed that silenced PAXIP1-AS1 reduced Bcl-2 protein level and lifted Bax, caspase 3 and caspase 9 levels (Fig. 1f). Transwell assay denoted that PAXIP1-AS1 knockdown obviously lessened migrated cells (Fig. 1g). Moreover, expression levels of migration-related proteins (MMP2, MMP9) and EMT-related proteins (E-cadherin, N-cadherin) with sh-PAXIP1-AS1 transfection were testified. As we observed, MMP2, MMP9 and N-cadherin protein levels were decreased while E-cadherin protein level was induced by PAXIP1-AS1 silencing (Fig. 1h). Overall, PAXIP1-AS1 was upregulated in OC and exhibited an oncogenic role by facilitating cell proliferation, migration, EMT and suppressing cell apoptosis.

**PAXIP1-AS1 was transcriptionally activated by CBP-mediated H3K27ac**

Thereafter, we explored the cause of PAXIP1-AS1 upregulation in OC. Existing reports highlighted that lncRNAs could be activated through H3K27ac at transcriptional level [21, 22]. Afterwards, high density of
H3K27ac enrichment was predicted in PAXIP1-AS1 promoter region through genome bioinformatics analysis (Fig. 2a). To further validate this, ChIP assay was performed. As demonstrated in Fig. 2b, PAXIP1-AS1 promoter region was enriched with H3K27ac in both OC cells and HOSEpiC cell line. Importantly, H3K27ac enrichment level was significantly increased in SKOV3 and OVCAR3 cell lines compared with that in HOSEpiC cell line. Interestingly, we found that PAXIP1-AS1 expression was significantly downregulated when OC cells were treated with C646, an inhibitor of histone acetyltransferase (HAT) (Fig. 2c). Thus, we wondered whether there were other vital enzymes involved in H3K27ac process. Previous studies have confirmed that CBP is crucial for chromatin acetylation and responsible for the promotive acetylation [23]. To test this, RT-qPCR was
used to evaluate CBP expression in OC cells. As expected, compared with CBP in HOSepiC cell line, CBP was upregulated in OC cells (Fig. 2d). Subsequently, results of ChIP assay uncovered that CBP precipitates was obviously enriched by PAXIP1-AS1 promoter (Fig. 2e). Then, CBP was stably silenced by sh-CBP and the transfection efficiency was validated by RT-qPCR (Fig. 2f). For assessing the effect of CBP on H3K27ac enrichment, ChIP assay was conducted. As expected, CBP knockdown significantly decreased the enrichment of H3K27ac at PAXIP1-AS1 promoter (Fig. 2g). Furthermore, CBP knockdown also led to an obvious decrease on PAXIP1-AS1 expression in OC cells (Fig. 2h). In conclusion, PAXIP1-AS1 upregulation in OC was caused by CBP-mediated H3K27ac at its promoter region.

**PAXIP1-AS1 sponged miR-6744-5p in OC**

Next, downstream molecular mechanism of PAXIP1-AS1 in OC was explored. The subcellular fractionation assay implied that PAXIP1-AS1 was mainly localized in the cytoplasm of OC cells (Fig. 3a). Increasing reports indicated that cytoplasmic lncRNAs regulated cancer progression by sequestering miRNAs [24]. Hence, we aimed to find the potential miRNAs for PAXIP1-AS1. Through DIANA tool, five miRNAs (binding score > 0.9) were predicted with binding site to PAXIP1-AS1 (Fig. 3b). Through RT-qPCR analysis, miR-6744-5p demonstrated a low level in OC cells, while miR-3942-3p, miR-6505-5p, and miR-1976 failed to show expression differences (Fig. 3c). Later, we obtained the binding sequence of PAXIP1-AS1 on miR-6744-5p and
mutated the site to conduct luciferase reporter assay (Fig. 3d). Meanwhile, miR-6744-5p was overexpressed in SKOV3 and OVCAR3 cells with transfection of miR-6744-5p mimics (Fig. 3e). As observed, the luciferase activity of PAXIP1-AS1-WT, but not PAXIP1-AS1-Mut, exhibited an overt reduction upon miR-6744-5p overexpression (Fig. 3f). Furthermore, RNA pulldown assay revealed a great enrichment of PAXIP1-AS1 in wide type miR-6744-5p pellets (Fig. 3g). Data above highlighted the interaction between PAXIP1-AS1 and miR-6744-5p.

**PAXIP1-AS1 positively regulated PCBP2 expression through miR-6744-5p**

It was commonly recognized that lncRNAs release downstream genes by competitively combining with miRNAs [25]. Therefore, we explored the potential downstream targets of miR-6744-5p. Combining the prediction results of miRDB, miRTarBase and TargetScan (three bioinformatics websites), nine candidate genes were found (Fig. 4a). Considering that CCND2, CKS2, RNF187 and YWHAZ have been investigated in OC, the
rest five mRNAs (PCBP2, YBX1, EPHA4, FAM102A and NMNAT2) were selected for further study. Through RT-qPCR analysis, we found only PCBP2 was apparently downregulated by miR-6744-5p overexpression (Fig. 4b). Then, miR-6744-5p site on PCBP2 sequence was identified through TargetScan website, and the mutation was designed (Fig. 4c). Through the luciferase reporter assay, miR-6744-5p overexpression attenuated the luciferase activity of PCBP2-WT reporte r rather than PCBP2-Mut reporter (Fig. 4d). Later, biotinylated miR-6744-5p-WT was revealed to show significant enrichment of PCBP2 by RNA pulldown assay (Fig. 4e). An RIP assay validated the enrichments of PAXIP1-AS1, miR-6744-5p, and PCBP2 in the precipitates of anti-Ago2 (Fig. 4f). We then inhibited the expression of miR-6744-5p in SKOV3 and OVCAR3 cells by using miR-6744-5p inhibitor (Fig. 4g). Subsequently, we confirmed that miR-6744-5p inhibition counteracted the inhibitory role of silenced PAXIP1-AS1 in PCBP2 mRNA expression and protein levels (Fig. 4h). Collectively, PAXIP1-AS1 sequestered miR-6744-5p to positively regulate PCBP2 expression.

**PAXIP1-AS1 regulated OC cellular process by upregulating PCBP2 expression**

At last, we probed whether PCBP2 was necessary for the regulation of PAXIP1-AS1 on cellular process in OC. First, we transfected pcDNA3.1/PCBP2 into SKOV3 and OVCAR3 cells to overexpress PCBP2 (Fig. 5a). Colony
formation and EdU assays depicted that cell proliferation hampered by PAXIP1-AS1 knockdown was recovered by PCBP2 overexpression (Fig. 5b-c). Moreover, the apoptosis of OC cells was promoted by PAXIP1-AS1 silencing, and such promotion was rescued by PCBP2 upregulation (Fig. 5d). Furthermore, overexpressed PCBP2 also reserved the effect of PAXIP1-AS1 knockdown on levels of apoptosis-relevant proteins (Fig. 5e). Transwell assay demonstrated that cell migration inhibited by silenced PAXIP1-AS1 was counteracted by pcDNA3.1/PCBP2 transfection (Fig. 5f). Additionally, the role of PAXIP1-AS1 downregulation in levels of proteins associated with migration and EMT process was countervailed via overexpressing PCBP2 (Fig. 5g). In conclusion, PAXIP1-AS1 accelerated OC cellular processes through regulating PCBP2 expression.

**Discussion**

Over the past decades, significant attention has been paid on the effect of dysregulated lncRNAs in the progression of cancers, including OC. Previous studies have identified numerous lncRNAs as tumor facilitator or suppressor in OC. For example, lncRNA FLVCR1-AS1 enhances cell migration and EMT process in OC.
through mediating miR-513/YAP1 axis [26]. LncRNA WDFY3-AS2 acts as a tumor suppressor to inhibit tumor growth in OC via delaying miR-18a [27]. LncRNA HAND2-AS1 represents anti-oncogenic property in OC by targeting BCL2L11 [28]. This study was to explore the function of PAXIP1-AS1 in OC. Previously, it has been stated that PAXIP1-AS1 was upregulated and served as an oncogenic lncRNA in glioma [16]. Herein, we found high expression level of PAXIP1-AS1 in OC cell lines. Functional assays revealed that PAXIP1-AS1 accelerated proliferation, restrained apoptosis, and promoted migration and EMT process in OC cells. This suggested that PAXIP1-AS1 played a carcinogenic role in OC.

Histone H3 on lysine 27 acetylation (H3K27ac) has been known as a common type of histone posttranslational regulation, associating with active enhancer modulatory elements to transcriptionally activate gene expression [29, 30]. Previous researchers have suggested that H3K27ac at promoter regions led to the overexpression of some carcinogenic lncRNAs, such as PLAC2 and Inc-SLC4A1-1 [31, 32], thus facilitating tumor development. Herein, we discovered high enrichment of H3K27ac at PAXIP1-AS1 promoter region via UCSC genome browser. Then, H3K27ac high level on the promoter region of PAXIP1-AS1 was validated through ChIP assay. The decreased PAXIP1-AS1 expression by HAT inhibitor (C646) further verified that PAXIP1-AS1 upregulation was attributed to H3K27ac modification. Previously, increasing evidence has implied that CBP is an essential regulator on histone acetylation and gene transcription, including lncRNA [23, 31]. Thus, we first validated that CBP interacted with PAXIP1-AS1 promoter to trigger H3K27ac and upregulated PAXIP1-AS1 expression.

Mechanistically, it was widely accepted that lncRNAs play the role of ceRNAs by which lncRNAs released mRNAs from posttranscriptional silence through sponging miRNAs [24, 33]. Our study first found that miR-6744-5p could be potentially targeted by PAXIP1-AS1. Moreover, we conducted luciferase reporter assay and RNA pulldown assay to confirm the strongest affinity of miR-6744-5p with PAXIP1-AS1. Previously, miR-6744-5p was reported to accelerate anoikis by directly targeting PAXIP1-AS1 as an underlying novel biomarker for improving research on OC molecular therapy.

In conclusion, our study first depicted that H3K27ac-induced IncRNA PAXIP1-AS1 promoted cellular process through miR-6744-5p/PCBP2 axis, suggesting PAXIP1-AS1 upregulated PCBP2 expression through miR-6744-5p. Rescue assays delineated that PAXIP1-AS1 upregulated PCBP2 expression and migration and migration by targeting PCBP2 in OC.

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Authors’ contributions
Both two co-authors participated in the literature search, analysis and interpretation of the data, and the writing of the manuscript. All authors saw and approved the final manuscript.

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Competing interests
The authors declare that there are no competing interests in this study.

Author details
1Department of Gynecology, Ningbo Medical Center Lihuili Hospital, Ningbo 315040, Zhejiang, China. 2Department of Gynecology, Xian Military Industry Hospital, Xi’an 710065, Shaanxi, China.

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