H3K27ac-induced IncRNA PAXIP1-AS1 exhibits oncogenic property in ovarian cancer by targeting miR-6744-5p/PCBP2 axis

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Research

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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 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 effect of silenced PAXIP1-AS1 on cell proliferation, apoptosis, migration and EMT. Overall, lncRNA 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.

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 that 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]. Besides, highly-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 indicated 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 was widely reported as competing endogenous RNA (ceRNA) that involved in lncRNA/miRNA/mRNA network [14]. This is a new model which proposes that lncRNAs regulate expression of target gene 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). 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. Cultured for 48 h, RT-qPCR validated the transfection efficiency.

RT-qPCR

Firstly, Trizol reagent (Invitrogen) was used to isolate total RNAs from OC cell lines. Then, the reverse transcription of miRNA or IncRNA/mRNA was performed by miRNA First-Stand cDNA Synthesis Kit (GeneCopoeia) or 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 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 culturing for two weeks, 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, number of visible colonies were manually counted.

EdU assay

Transfected cells of SKOV3 or OVCAR3 were collected and planted into 96-well plates at the density of 1 x 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, fluorescence microscope (Olympus, Tokyo, Japan) was applied to observe proliferative cells.

**Flow cytometry analysis**

SKOV3 or OVCAR3 cells were seeded in a 6-well plate. After centrifugalization, the binding buffer, resuspended with residue, was added with 5-μL Annexin V-FITC and 5-μL propidium (PI). Then, 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 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, enhanced chemiluminescence (ECL) Plus kit (Beyotime, Shanghai, China) visualized the protein bands.

**Transwell assay**

The migration ability was assessed by the use of transwell chambers without matrigel. In brief, cells (1 × 10^5) were seeded into upper chambers contained 200ul serum-free media. The lower chambers were added with 600 ul of 20% FBS media. Incubated for 48 h, cells without invasion in the upper chambers were wiped away by cotton wool. Fixed with methanol, cells were stained with 0.5% crystal violet in the lower chamber. Lastly, five random fields were photographed under an inverted microscope.

**ChIP assay**

ChIP assays were carried out by ChIP Assay Kit (Thermo Fisher Scientific) with SKOV3 or OVCAR3 cells. For DNA-protein cross-links, SKOV3 or OVCAR3 cells were incubated with 1% formaldehyde for 10 minutes, 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 analyzed the ChIP samples.

**Subcellular fractionation assay**

Firstly, the nuclear and cytoplasmic fractions of SKOV3 and OVCAR3 cells were obtained. Then, with Cytoplasmic & Nuclear RNA Purification Kit (Norgen), the fractions were separated and purified as per the manual. At last, RT-qPCR analyzed 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 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, dual-luciferase reporter assay system (Promega) testified the relative luciferase activity.

**RNA pull down 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 used to incubate with the cell lysates for 4 h at 4 °C. Using precooled 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, 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 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) (Figure 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 (Figure 1B). Through colony formation assay, we found silenced PAXIP1-AS1 significantly decreased colonies of SKOV3 and OVCAR3 cells (Figure 1C). EdU assay further confirmed the inhibitive effect of PAXIP1-AS1 deficiency on cell proliferation (Figure 1D). In addition, flow cytometry analysis suggested that cell apoptosis was remarkably promoted by sh-PAXIP1-AS1 transfection (Figure 1E). To further confirm this, levels of apoptosis-related proteins after PAXIP1-AS1 knockdown was tested by western blot analysis. The results showed that silenced PAXIP1-AS1 reduced Bcl-2 expression and lifted Bax, caspase 3 and caspase 9 levels (Figure 1F). Transwell assay denoted that PAXIP1-AS1 knockdown obviously lessened migrated cells (Figure 1G). Moreover, expressions of migration-related proteins (MMP2, MMP9) and EMT-relevant
proteins (E-cadherin, N-cadherin) with sh-PAXIP1-AS1 transfection was testified. As we observed, MMP2, MMP9 and N-cadherin protein levels were decreased while E-cadherin protein level was induced by PAXIP1-AS1 silencing (Figure 1H). Taken together, PAXIP1-AS1 was upregulated in OC and exhibited 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 [17, 18]. Afterwards, high density of H3K27ac enrichment was predicted in PAXIP1-AS1 promoter region through genome bioinformatics analysis (Figure 2A). To further validate this, ChIP assay was performed. As demonstrated in Figure 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 with comparison of HOSEpiC cell line. Interestingly, we found that PAXIP1-AS1 expression was significantly downregulated when OC cells were treated C646, an inhibitor of histone acetyltransferase (HAT) (Figure 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 [19]. To test this, RT-qPCR was used to evaluate CBP expression in OC cells. As expected, compared to HOSEpiC cell line, CBP was upregulated in OC cells (Figure 2D). Subsequently, results of ChIP assay uncovered that CBP precipitates was obviously enriched by PAXIP1-AS1 promoter (Figure 2E). Then, CBP was stably silenced by sh-CBP and the transfection efficiency was validated by RT-qPCR (Figure 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 (Figure 2G). Furthermore, CBP knockdown also led to an obvious decrease on PAXIP1-AS1 expression in OC cells (Figure 2H). To be concluded, 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. Subcellular fractionation assay implied that PAXIP1-AS1 was mainly localized in the cytoplasm of OC cells (Figure 3A). Increasing reports indicated that cytoplasmic lncRNAs regulated cancer progression by sequestering miRNAs [20]. Hence, we aimed to find the potential miRNAs for PAXIP1-AS1. Through DIANA tool, five miRNAs (binding score >0.9) were predicted with binding sites to PAXIP1-AS1 (Figure 3B). Through RT-qPCR analysis, miR-6744-5p demonstrated a low level in OC cells, while miR-3942-3p, miR-6505-5p, miR-6796-5p and miR-1976 didn’t show expression differences (Figure 3C). Later, we obtained the binding sequence of PAXIP1-AS1 on miR-6744-5p, and mutated the site to conduct luciferase reporter assay (Figure 3D). Meanwhile, miR-6744-5p was overexpressed with transfection of miR-6744-5p mimics (Figure 3E). As observed, luciferase activity of PAXIP1-AS1-WT, but not PAXIP1-AS1-Mut, exhibited an overt reduction upon miR-6744-5p overexpression (Figure 3F). Furthermore, RNA pull down assay revealed a great enrichment of
PAXIP1-AS1 in wide type miR-6744-5p pellets (Figure 3G). Data above highlighted the interaction between PAXIP1-AS1 and miR-6744-5p.

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

It was commonly recognized that lncRNAs released downstream genes by competitively combining with miRNAs [21]. 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 (Figure 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 (Figure 4B). Then, miR-6744-5p site on PCBP2 sequence was identified through TargetScan website, and the mutation was designed (Figure 4C). Through luciferase reporter assay, miR-6744-5p overexpression attenuated the luciferase activity of PCBP2-WT reporter rather than PCBP2-Mut reporter (Figure 4D). Later, biotinylated miR-6744-5p-WT was revealed to show significant enrichment of PCBP2 by RNA pull down assay (Figure 4E). RIP assay validated the enrichments of PAXIP1-AS1, miR-6744-5p, and PCBP2 in the precipitates of anti-Ago2 (Figure 4F). We then inhibited the expression of miR-6744-5p in SKOV3 and OVCAR3 cells by using miR-6744-5p inhibitor (Figure 4G). Subsequently, we confirmed that miR-6744-5p inhibition counteracted the inhibitory role of silenced PAXIP1-AS1 in PCBP2 mRNA and protein expressions (Figure 4H). Collectively, PAXIP1-AS1 sequestered miR-6744-5p to positively regulated PCBP2 expression.

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

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 (Figure 5A). Colony formation and EdU assays depicted that cell proliferation hampered by PAXIP1-AS1 knockdown was recovered by PCBP2 overexpression (Figure 5B-C). Moreover, the apoptosis of OC cells was promoted by PAXIP1-AS1 silencing, and such promotion was rescued by PCBP2 upregulation (Figure 5D). Furthermore, overexpressed PCBP2 also reserved the effect of PAXIP1-AS1 knockdown on levels of apoptosis-relevant proteins (Figure 5E). Transwell assay demonstrated that cell migration inhibited by silenced PAXIP1-AS1 was counteracted by pcDNA3.1/PCBP2 transfection (Figure 5F). Additionally, the role of PAXIP1-AS1 downregulation in levels of proteins associated with migration and EMT process was countervailed via overexpressing PCBP2 (Figure 5G). Conclusively, PAXIP1-AS1 accelerated OC cellular process through regulating PCBP2.

**Discussion**

Over the past decades, significant attention has been payed 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 [22]. LncRNA WDFY3-AS2 acts as a tumor
suppressor to inhibit tumor growth in OC via delaying miR-18a [23]. LncRNA HAND2-AS1 represents anti-oncogenic property in OC by targeting BCL2L11 [24]. 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 IncRNA 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 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 [25, 26]. Previous researches have suggested that H3K27ac at promoter regions led to the overexpression of some carcinogenic IncRNAs, such as PLAC2 and Inc-SLC4A1-1 [27, 28], 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 IncRNA [19, 27]. Thus, we firstly validated that CBP interacted with PAXIP1-AS1 promoter to trigger H3K27ac and upregulated PAXIP1-AS1 expression.

In mechanism, it was widely proved that IncRNAs play the role of ceRNAs by which IncRNAs released mRNAs from posttranscriptional silence through sponging miRNAs [20, 29]. Our study first found that miR-6744-5p could be potentially targeted by PAXIP1-AS1. Moreover, we conducted luciferase reporter assay and RNA pull down assay to confirm the strongest affinity of miR-6744-5p with PAXIP1-AS1. Formerly, miR-6744-5p was reported to accelerate anoikis by directly targeting NAT1 enzyme in breast cancer [30]. Furthermore, we identified that PCBP2 was the target gene for miR-6744-5p. Former studies have showed the oncogenic role of PCBP2 in cancers. As reported, PCBP2 was involved in cell proliferation and migration in bladder cancer [31]. PCBP2 enhanced cell viability through regulating CDK2 in gastric cancer [32]. PCBP2 was overexpressed in glioblastoma and associated with unfavorable prognosis [33]. In our study, we first found that miR-6744-5p combined with PCBP2 to repress its expression, and that PAXIP1-AS1 upregulated PCBP2 through miR-6744-5p. Rescue assay delineated that PAXIP1-AS1 aggravated cell growth and migration by targeting PCBP2 in OC.

In conclusion, our study first depicted that H3K27ac-induced IncRNA PAXIP1-AS1 promoted cellular process through miR-6744-5p/PCBP2 axis, suggesting PAXIP1-AS1 as an underlying novel biomarker for improving research on OC molecular therapy.

**Declarations**

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Availability of data and materials

None.

Conflicts of interest

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

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