Knockdown long non-coding RNA PCAT7 inhibits proliferation, migration and invasion of luminal B breast cancer cells via cell cycle arrest

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Research Article

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Abstract

Breast cancer is the most commonly diagnosed cancer and one of the leading causes of cancer death. In recent years, an increasing number of studies have shown that the dysregulation of long non-coding RNAs has vital effects on the progression of breast cancer. But the function of PCAT7 in luminal B breast cancer has not been explored. This study aims to investigate the expression of PCAT7 in luminal B breast cancer tissues and cell lines and the effects of PCAT7 knockdown in luminal B breast cancer cells. We found that PCAT7 was dramatically up-regulated in luminal B breast cancer tissues and related cell lines. Knockdown of PCAT7 significantly inhibited cell proliferation, migration and invasion of luminal B breast cancer cells. Cell cycle assay showed that PCAT7 knockdown induced G0/G1 phase arrest of the luminal B breast cancer cells. Moreover, the relative expression of p27 protein significantly increased after PCAT7 knockdown. In conclusion, knockdown of PCAT7 inhibits the proliferation, migration, and invasion of luminal B breast cancer cells via cell cycle arrest.

Introduction

In women, breast cancer is the most commonly diagnosed cancer [1]. Despite treatments including surgery, chemotherapy, targeted therapy, endocrine therapy, and radiotherapy [2], breast cancer is the leading cause of cancer death in women [1]. Breast cancer is a heterogeneous disease, it can be divided into four subtypes according to molecular surface markers, including luminal A, luminal B, human epidermal growth factor receptor 2-positive (HER-2+) and triple negative breast cancer (TNBC). Both luminal A and luminal B breast cancers are estrogen receptor-positive and can be treated with endocrine therapies [2]. However, luminal B breast cancer has lower progesterone receptor expression, an earlier relapse after endocrine therapy, and a higher rate of distant metastasis [3]. Therefore, it is desperately needed to find novel biomarker, which helps to find new therapeutic targets in luminal B breast cancer.

With the development of the sequencing of the human genome, we find that 70–90% of our genome has been transcribed, but only 2% of these transcripts are translated into proteins. The rest of the transcripts are defined as non-coding RNAs (ncRNAs) [4]. Long non-coding RNAs (lncRNAs) are defined as ncRNAs with transcript lengths exceeding 200 nucleotides [5]. LncRNAs play crucial roles in numerous cellular processes, including the cell cycle, differentiation, metabolism, as well as in disease including malignant tumor [6]. LncRNA BCRT1 was found to be overexpressed in breast cancer and has been linked to tumor progression by attenuating the repressive effect of miR-1303 on PTBP3 and promoting M2 polarization [7]. LncRNA TINCR was found to be upregulated in trastuzumab-resistant cells when compared with sensitive cells. Furthermore, H3K27-activated TINCR regulates the miR-125b-HER-2/Snail-1 pathway, which contributes to trastuzumab resistance and EMT [8]. However, the roles and biological mechanisms of lncRNAs in the regulation of luminal B breast cancer remain largely unknown.

The prostate cancer-associated transcript 7(PCAT7) has been reported to be significantly overexpressed in nasopharyngeal carcinoma [9], prostate cancer [10], non-small-cell lung cancer [11] and is associated with poor prognosis. In our previous study, we screened lncRNA expression profiles in luminal B breast

cancer tissues and found that PCAT7 was significantly overexpressed in luminal B breast cancer tissues. Although Zhou et al. [12] revealed a pro-oncogenic role for PCAT7 in breast cancer, little is known about PCAT7's regulatory role in luminal B breast cancer. So, we conducted this study to explore the biological function of PCAT7 in luminal B breast cancer.

Material And Methods

Human specimens

After all patients signed the informed consent, 7 fresh luminal B breast cancer tissues and the matched adjacent non-tumor breast tissues were collected during surgery at the People's Hospital of Zhengzhou University. The fresh tissues were collected and immediately snap-frozen in liquid nitrogen and stored at -80°C until further use. All breast cancer tissue specimens were pathologically tested by two separate pathologists and confirmed to be luminal B breast cancer. None of the patients had received preoperative chemotherapy, endocrine therapy, radiotherapy or any other therapies. Approval was given by Medical Ethics Committee of Henan Provincial People's Hospital.

Cell lines and culture

Normal human breast epithelial cell line MCF-10A and luminal B breast cancer cell lines ZR-75-1, ZR-75-30, and BT-474 were purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences. The human luminal B breast cancer cell line MDA-MB-415 cell line was purchased from Procell.

MCF-10A cells were cultured in the MEGM kit (Lonza/Clonetics) supplemented with 10 ng/ml cholera toxin. BT-474 cells were maintained in RPMI-1640 (Biological Industries) medium supplemented with 10% fetal bovine serum (FBS) (Biological Industries), 10 μg/mL insulin (Yeasen Biotechnology, Shanghai, China), and 1% penicillin-streptomycin solution (P/S) (Sangon Biotech, Shanghai, China). MDA-MB-436 cells were kept in Leibovitz's L-15 medium (Procell, Wuhan, China), supplemented with 10 μg/mL insulin, 10 μg/mL glutathione, 15% FBS, and 1% P/S. All cells were placed in an incubator at 37°C with 5% CO_2.

RNA Isolation and real-time PCR

Total RNA was extracted from luminal B breast cancer tissues, matched adjacent non-tumor breast tissues, luminal B breast cancer cell lines, and human normal breast epithelial cell line using the Ultrapure RNA Kit (CW0581, ComWin Biotech Co., Ltd, China), following the manufacturer's instructions. The purity and concentration of the total RNA were detected by Nano Drop 2000. RNA was reversely transcribed into cDNA by the reverse transcription kit (Vazyme Biotech Co., Ltd) according to the manufacturer's protocol. Real-time PCR was performed using the Vazyme Real-Time PCR Kit. $2^{-\Delta\Delta CT}$ method was applied to calculate the relative expression of target genes, with GAPDH as the internal reference. The primer sequences used are displayed in Table 1.

Table 1
Primer sequences and siRNAs used in this study

Gene	Primer sequence
GAPDH-F	5′-AAGACCTTGGGCTGGGACTG−3′
GAPDH-R	5′-ACCAAATCCGTTGACTCCGA−3′
PCAT7-F	5′-AAACAAGCCAACCGCACAAT−3′
PCAT7-R	5′-CCTGCTTGCTGTGTTACTGC−3′
siRNA1	5′-GUGGCAGAUACCACCUUAAATT−3′
siRNA2	5′-CCCGUCUUUACUAAAUAUATT−3′
siRNA3	5′-GUGCCAAGGAGACUCAAUATT−3′
NC	5′-UUCUCCGAACGUGUCACGUTT−3′

Small interfering RNA (siRNA) synthesis and transfection

GenePharma was entrusted with designing and synthesizing specific interference siRNA sequences targeting PCAT7 (siRNA1, siRNA2 and siRNA3), as well as the negative control sequence (si-NC). The sequences used are displayed in Table 1. Cells were seeded in 6-well plates with 3×10^3 cells per well and cultured at 60%−80% confluence. Then the cells were transfected using siRNA-Mate (GenePharma, Shanghai, China). Real-time PCR was used to determine the knockdown efficiency at 48 h after transfection. And the most effective siRNA was used for the following experiments.

Colony formation assay

After being transfected for 48 h, the cells were inoculated in 6-well plates. After 14 days of incubation, the plates were photographed under the microscope and the cells were washed with phosphate buffered saline (PBS). Then the cells were fixed with 4% paraformaldehyde for 30 min, followed by washing with PBS twice and staining with 0.1% crystal violet for 30 min. The plates were photographed and the number of colonies was counted.

Wound healing assay

Transfected BT−474 cells were inoculated in 6-well plates, when cells were grown to approximately 80% confluence, a 200 μL plastic tip was used to make horizontal lines. Then, the well were washed twice by PBS to remove floating cells and cellular fragments. Photos were taken at 0 and 48 h to measure the distance.

Transwell invasion assay

After being transfected for 24 h, the cells were digested and washed with PBS twice. Next, cells were re-suspended in serum-free medium and seeded in the upper chamber. The lower chamber was filled with

complete medium containing 20% FBS. After being cultured for 48 h, the upper chambers were washed twice with PBS. Then, cotton swabs were used to wipe off the non-invaded cells in the upper chamber. The invaded cells were fixed for 1 hour with paraformaldehyde. Next, the cells were stained with 0.1% crystal violet for 30 min. Finally, a microscope was used to count the number of stained cells in randomly selected fields.

Cell cycle essay

Transfected cells were collected 24 h later. Then the cells were digested, centrifuged at 1500 rpm for 5 min, and washed twice with pre-cold PBS. Next, the cells were fixed with 75% pre-cold ethanol at 4°C for 4 h. After that, the fixed cells were washed with PBS once and incubated with 400 μL of propidium iodide (PI) and 100 μL of RNase for 30 min at 4°C in the dark. Then the cell cycle was evaluated by flow cytometry.

Western blotting

After being transfected for 48 h, the cells were washed three times with PBS. Then cellular proteins were extracted. Protein samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PCDF) membranes. After being blocked with skim milk (5%), the membranes were incubated overnight with primary antibodies at 4°C. Then the membranes were incubated with secondary antibodies. The chemiluminescent substrate kit was used to determine the expression of targeted proteins.

Statistical analysis

Data Analysis was performed using GraphPad Prism 9.0 (GraphPad Software, Inc., La Jolla, CA, USA). Experimental data was represented as the mean ± standard deviation. Independent samples t-tests were performed to compare differences between two group. $P < 0.05$ was considered to be statistically significant.

Results

PCAT7 expression is significantly upregulated in luminal B breast cancer tissues and cell lines.

According to the real-time PCR results, the expression level of PCAT7 was higher in luminal B breast cancer tissues than in adjacent non-tumor breast tissues ($Z = 2.37$, $P < 0.05$) (Fig. 1A). The level of PCAT7 in four luminal B breast cancer cell lines (MDA-MB-415, BT474, ZR-75-1, ZR-75-30) was considerably higher than that of the normal breast cell line (MCF-10A). The expression level of PCAT7 was highest in BT-474 cells ($t = 13.29$, $P < 0.05$). So BT474 cells were used for the following experiments (Fig. 1B).

Downregulation of PCAT7 inhibits luminal B breast cancer cell proliferation.

To investigate the role of PCAT7 in BT474 cells, we constructed three PCAT7 siRNAs (siRNA1, siRNA2, and siRNA3). PCAT7 expression was significantly suppressed in BT474 cells transfected with siRNA3

(t=-80.62, P < 0.001, Fig. 1C), so siRNA3 was used for knockdown experiments. Colony formation assays were conducted to detect the role of PCAT7 in luminal B breast cancer proliferation, and it was revealed that the proliferation ability of BT474 cells transfected with siRNA3 was markedly inhibited compared with the si-NC group (t = 4.01, P < 0.05, Fig. 1D).

Downregulation of PCAT7 inhibits luminal B breast cancer cell migration and invasion.

Since cellular migration and invasion are key steps in cancer metastasis, we conducted wound healing assay and transwell invasion assay to detect the influence of PCAT7 knockdown on luminal B breast cancer cell migration and invasion. In the wound healing assay, cell migration was noticeably decreased with the transfection of siRNA3 (t = 3.72, P < 0.05, Fig. 2A). Transwell invasion assay revealed that invasion ability was suppressed in PCAT7 knockdown BT474 cells as compared to the si-NC group (t = 9.17, P < 0.001, Fig. 2B).

Downregulation of PCAT7 induces G0/G1 phase arrest

According to the cell cycle assay, downregulation of PCAT7 remained BT474 cells in the G0/G1 phase compared to those cells transfected with si-NC (t = 5.18, P < 0.01, Fig. 2A). These finding suggested that downregulation of PCAT7 induced G0/G1 phase arrest. Then we conducted western blot assays to further explore the consequences of PCAT7 loss on key cell cycle regulators. We found that the level of p27 protein was significantly increased after PCAT7 knockdown as compared to control cells (t = 6.28, P < 0.05, Fig. 3B).

Discussion

Breast cancer is the most common malignancy tumor and the leading cause of cancer death [1]. In recent years, a growing number of studies have shown that the lncRNA dysregulation has vital effects on the progression of various malignancy tumors [13, 14, 15], including breast cancer [16]. But the function of PCAT7 in luminal B breast cancer has not been explored.

Prostate cancer-associated transcript 7 (PCAT7) is a 1937-bp lncRNA that maps at chromosome 9q22.32 [9]. Liu et al. revealed that PCAT7 was over-expressed in nasopharyngeal carcinoma and contributed to tumor progression via the miR-134-5p/ELF2 signaling pathway [9]. Moreover, PCAT7 promoted non-small cell lung cancer development by inhibiting miR-134-5p [11]. Zhou et al. demonstrated that PCAT7 was up-regulated in breast cancer and promoted the malignant progression of breast cancer by regulating the ErbB/PI3K/Akt pathway [12]. However, the expression and biological function of PCAT7 in luminal B breast cancer remain unclear. So we conducted this study to investigate the expression of PCAT7 in luminal B breast cancer and its potential biological roles in luminal B breast cancer cells.

In this study, we found that PCAT7 was highly expressed in luminal B breast cancer tissues compared to paired normal tissues. And PCAT7 was up-regulated in luminal B breast cancer cell lines. In biological function studies, the colony formation assay demonstrated that PCAT7 significantly promoted the

proliferation of BT-474 cells. The wound healing assay and the transwell invasion assay demonstrated that PCAT7 siRNA inhibited the migration and invasion of luminal B breast cancer cells (BT-474).

It has been demonstrated that dysregulation of the cell cycle and cell-cycle regulatory proteins leads to uncontrolled cell proliferation in several malignant tumors, including breast cancer [17, 18, 19]. Qiu et al. [20] demonstrated that LINC00668 can promote the progression of breast cancer by accelerating cell cycle progression. Wang et al. found that lncRNA MIR100HG promoted triple-negative breast cancer progression by regulating the p27 gene to control the cell cycle. So we conducted cell cycle assays to examine the influence of PCAT7 knockdown on the luminal B breast cancer cell cycle. We found that PCAT7 knockdown significantly arrested the cell cycle in the G0/G1 phase compared with that of the control group. Then we performed western blot assay to elucidate the possible mechanism of PCAT7 in cell cycle regulation. We found that compared to the si-NC group, the level of p27 protein significantly increased in PCAT7 knockdown cells. p27 is known as a cell cycle regulatory protein preventing cell cycle progression in the G1 phase [22]. PCAT7 promoted luminal B breast cancer cell proliferation, migration and invasion by regulating cell cycle and survival proteins, according to these findings.

Conclusions

In conclusion, PCAT7 was dramatically up-regulated in luminal B breast cancer tissues and related cell lines. And PCAT7 knockdown significantly inhibited cell proliferation, migration, and invasion. Moreover, PCAT7 mediated oncogenic effects partially through inducing cell cycle arrest. Our findings provide a potential target for the treatment of luminal B breast cancer. We will conducting more assays to explore the specific regulatory pathway of PCAT7.

Declarations

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Author contributions All authors contributed to conceiving and designing the study, drafting and revising the manuscript.

Competing Interests The authors have no relevant financial or non-financial interests to disclose.

Ethical approval The experimental protocol was established according to the ethical guidelines of the Helsinki Declaration and was approved by the Medical Ethics Committee of Henan Provincial People's Hospital

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Figures

Figure 1

PCAT7 overexpression in luminal B breast cancer tissues and cell lines. Downregulation of PCAT7 inhibits BT474 cells proliferation. **a** Real-time PCR of PCAT7 in luminal B breast cancer tissues and normal adjacent tissues. **b** Real-time PCR of PCAT7 in luminal B breast cancer cell lines and normal breast cancer cell lines. **c** The knockdown efficiency of siRNAs on PCAT7 mRNA in BT474 cells. **e** The images of colony formation assays were illustrated. **f** Histogram shows the clone number. Data are presented as the mean ± SD. **P<0.05*, ***P<0.01, ***P<0.001*

Figure 2

Downregulation of PCAT7 inhibited BT474 cells migration and invasion. Wound healing assays (**a**) and transwell invasion assays (**c**) were performed to determine the influence of PCAT7 knockdown on luminal B breast cancer cell migration and invasion. Histogram shows the proportion of wound width (**b**) and the invasion cell number (**d**) in each group. Downregulation of PCAT7 inhibited BT474 cells migration and invasion. Data are presented as the mean ± SD. **P<0.05*, ***P<0.01, ***P<0.001.*

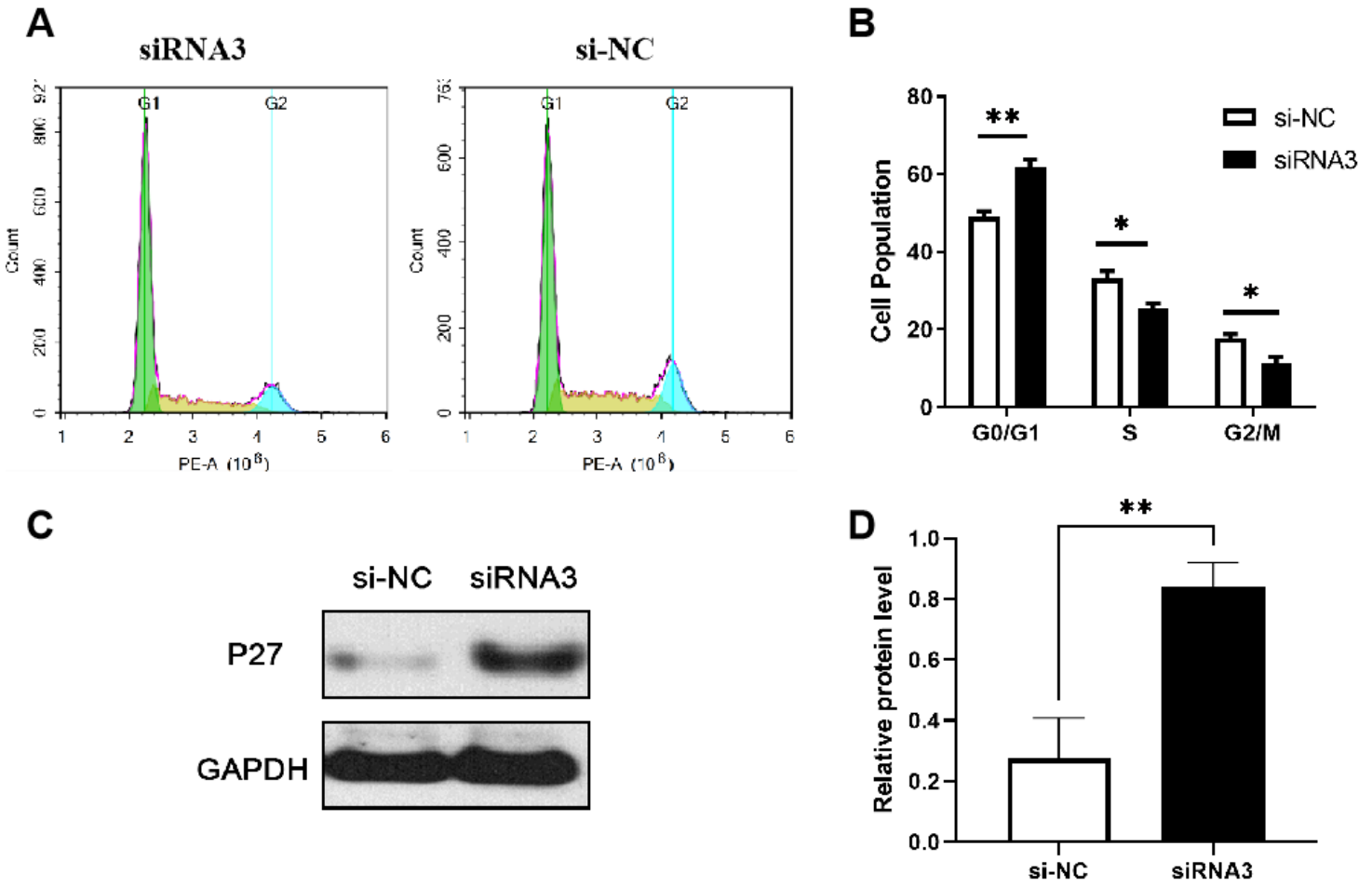

Figure 3

Downregulation of PCAT7 arrested cell cycle in G0/G1 phase. **A.** Images of fow cytometry assays of BT474 cell cycle was graphically praphically presented. **B.** Histogram shows the proportion of G0/G1, S, and G2/M phase of BT474 cells in each group. **C.** Representative western blot images of P27. **D.** Histogram shows the relative protein level of P27. Data are presented as the mean ± SD. **P<0.05*, ***P<0.01.*