European Review for Medical and Pharmacological Sciences 2020; 24: 9908-9914

LncRNA PCAT6 aggravates the progression of bladder cancer cells by targeting miR-513a-5p

W. XIA[1], C. CHEN[1], M.-R. ZHANG[1], L.-N. ZHU[2]

[1]Department of Urinary Surgery, the First People's Hospital of Changzhou, Changzhou, China
[2]Department of Oncology, the First People's Hospital of Changzhou, Changzhou, China

Abstract. – OBJECTIVE: Long non-coding RNAs (lncRNAs) have been demonstrated to play critical roles in tumorigenesis of bladder cancer (BC). Our research aimed to explore the underlying mechanisms of lncRNA prostate cancer-associated transcript 6 (PCAT6) in BC.

PATIENTS AND METHODS: Real Time-quantitative Polymerase Chain reaction (RT-qPCR) was used to measure the levels of PCAT6 and miR-513a in BC tissues and cells. The Kaplan-Meier analysis was utilized to evaluate the overall survival time of BC patients. Besides, cell viability was detected by Cell Counting Kit-8 (CCK-8) assay. Cell migration and invasion were evaluated by wound healing and transwell assays. Furthermore, starBase and Dual-Luciferase reporter assay were used to determine the interaction between PCAT6 and miR-513a in BC cells.

RESULTS: PCAT6 expression was upregulated, while miR-513a was downregulated in BC tissues and cell lines. BC patients with high expression of PCAT6 exhibited a shorter overall survival time compared with those patients with low expression of PCAT6. Moreover, PCAT6 knockdown notably suppressed cell progression. In addition, PCAT6 inhibited miR-513a expression through direct interaction, and the silencing of PCAT6 remarkably increased the expression of miR-513a. Finally, the knockdown of miR-513a partly abolished PCAT6 silencing-induced inhibitory effects on BC progression.

CONCLUSIONS: Our study illustrated that PCAT6 knockdown inhibited cell progression of BC by regulating miR-513a, suggesting that PCAT6 might act as a prognostic biomarker and therapeutic target for BC patients.

Key Words:
PCAT6, MiR-513a-5p, Bladder cancer.

Introduction

Bladder cancer (BC) is considered the most common genitourinary malignancy in the world[1,2]. Despite great progress has been made in the treatment for BC, patients in the advanced stage still face poor outcomes in the last decade[3,4]. Poor understanding of the pathologic mechanism of BC progression is the major limitation of BC treatment[5,6]. Therefore, it is pivotal to understand the pathogenesis of BC and improve patient prognosis.

Long noncoding RNAs (lncRNAs) are a class of non-coding RNAs longer than 200 nucleotides[7]. The dysregulation of lncRNA played a key regulatory role in tumor development[8-10]. Huang et al[11] indicated that lncRNA LINC00511 accelerated the development of gastric cancer by promoting EZH2 expression *via* sequestering miR-124-3p. Li et al[12] reported that ZEB2-AS1 accelerated colon cancer progression by regulating miR-188/TAB3 axis. Liu et al[13] demonstrated that DLX6-AS1 aggravated laryngeal cancer progression by targeting miR-26a. LncRNA prostate cancer-associated transcript 6 (PCAT6) was indicated as an oncogene in cervical cancer[14], gastric cancer[15] and hepatocellular carcinoma[16]. However, the exact mechanisms of PCAT6 in BC progression have not been fully explored.

MicroRNAs (MiRNAs) are a family of small non-coding RNAs of approximately 22 nucleotides in length[17,18]. A series of miRNAs are involved in the pathogenesis and development of BC. Ning et al[19] revealed that miR-425 targeted DKK3 to promote metastasis of BC cells. Guo et al[20] illustrated that miR-22 impeded cell growth and invasion of BC by targeting E2F3. Peng et al[21] showed that miR-4500 negatively regulated cell proliferation and migration by targeting CCR7 in BC. However, the regulation of miR-513a in BC is still unclear.

In our study, we found that PCAT6 promoted BC tumorigenesis by sponging miR-513a. These

Corresponding Author: Lina Zhu, MD; e-mail: linazhu@fphcz.com

findings will provide a novel theoretical basis for BC treatment.

Patients and Methods

Human Tissue Samples

A total of 21 paired BC tissues and adjacent normal tissues were obtained from the First People's Hospital of Changzhou. This research was approved by the Ethics Committee of the First People's Hospital of Changzhou and all participants signed informed consent forms in this study.

Cell Culture

Human bladder epithelial immortalized cell (SV-HUC-1) and BC cells (T24, EJ, 253j, and 5637) and 293T cells were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) and supplemented with 10% fetal bovine serum (FBS; Gibco, MD, USA) at 37°C in a humidified atmosphere with 5% CO_2.

Cell Transfection

Short hairpin RNA shRNAs targeting PCAT6 (shPCAT6) and shNC synthesized by Sangon (Shanghai, China). MiR-513a mimics with control (NC mimics) and miR-513a inhibitor with control (NC inhibitor) were obtained from Genechem (Shanghai, China). The transfection was performed using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Real Time-quantitative Polymerase Chain reaction (RT-qPCR)

Total RNA was extracted from tissues and cells using TRIzol Reagent (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA). The RNAs were reverse transcribed to cDNAs through a reverse transcriptase kit (TaKaRa, Dalian, Liaoning, China). RT-qPCR was performed using the SYBR-Green PCR Master Mix kit (TaKaRa, Dalian, China). The level of genes was assessed using the $2^{-\Delta\Delta Ct}$ method.

Cell Counting Kit-8 (CCK-8)

T24 and EJ cells were seeded into 96-well plates with 5×10^5 cells/well. Cell viability was detected at 0, 24, 48, and 72 hours. 10 μL CCK-8 solution (Dojindo, Tokyo, Japan) was added into each well for another 4 h. The absorbance at 450 nm was recorded by a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Wound Healing Assay

The transfected cells (1×10^4/well) were cultured in 6-well plates until growing to about confluent of 90%. Then, the cells were scratched with a pipette tip. Wound closure was viewed and photographed at 0 and 24 hours.

Transwell Assay

BC cell invasion was measured by using transwell chambers 8.0 μm pore size; BD Biosciences, Franklin Lakes, NJ, USA) and Matrigel (BD, Franklin Lakes, NJ, USA). Briefly, free-medium contained cells were added into the upper chamber coated with Matrigel, and 600 ul Dulbecco's Modified Eagle's Medium (DMEM; Corning Life Sciences, Costar, CA, USA) contained 10% FBS (Gibco, Grand Island, NJ, USA) was added into the bottom chamber. After 48 h, cells in the upper chamber were removed, and cells in the lower membrane were stained with 0.1% crystal violet. Invaded cells were counted under a microscope (Zeiss, Jena, Germany).

Luciferase Reporter Assay

The wild-type (WT) and mutant (Mut) target of miR-513a in PCAT6 sequence was sub-cloned into the pmirGLO Dual-Luciferase vector (Promega, Madison, WI, USA) to establish PCAT6-Wt/Mut. Later, PCAT6-Wt/Mut reporters were co-transfected with miR-513a mimics into 293T cells by using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The activity of Luciferase was evaluated by Dual-Luciferase reporter system (Promega, Madison, WI, USA).

Statistical Analysis

Data were presented as the mean ± standard deviation (SD). Each experiment was performed at least 3 times. Statistical analysis was carried out with SPSS 16.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism (La Jolla, CA, USA). Comparisons between two groups were performed by a Student's *t*-test. Comparisons among three groups were analyzed using one-way ANOVA followed by Tukey's test. $p<0.05$ was defined as statistically significant.

Results

LncRNA PCAT6 Was Highly Expressed in BC Tissues and Cells

RT-qPCR was used to measure the expression of PCAT6 in BC, and the results indicated that PCAT6 expression was upregulated in BC tissues compared with the adjacent normal tissue (Figure 1A). In addition, PCAT6 level was higher in BC cell lines, especially T24 and EJ cell lines, than that in normal bladder epithelial cells (SV-HUC-1) (Figure 1B). Furthermore, Kaplan-Meier analysis revealed that high level of PCAT6 was associated with poor prognosis of BC patients (Figure 1C). In conclusion, these results suggested that PCAT6 was upregulated in BC and the upregulation of PCAT6 predicted poor prognosis in BC.

LncRNA PCAT6 Knockdown Inhibited the Progression of BC Cells

To determine the biological function of PCAT6 in the development of BC cells, T24 and EJ cells were transfected with shNC and shPCAT6. RT-qPCR was applied to confirm the transfection efficiency (Figure 2A). Moreover, CCK-8 assay revealed that PCAT6 knockdown inhibited the viability of T24 and EJ cells (Figure 2B). Wound healing and transwell assays indicated that the silencing of PCAT6 markedly suppressed the migration and invasion of BC cells (Figure 2C and D). These data demonstrated that PCAT6 depletion inhibited the viability, migration and invasion of BC cells.

PCAT6 Acted As MiR-513a Sponge in BC

Bioinformatics analysis was used to predict the binding sites between PCAT6 and miR-513a (Figure 3A). Moreover, the Luciferase activity was weakened after the co-transfection of PCAT6-WT and miR-513a mimics in 293T cells (Figure 3B), which confirmed the binding ability between PCAT6 and miR-513a. In addition, miR-513a level was decreased in BC tissues and cells (Figure 3C and D). Moreover, there was a negative correlation between the expression of PCAT6 and miR-513a in BC tissues (Figure 3E). RT-qPCR indicated that PCAT6 knockdown enhanced the level of miR-513a in T24 and EJ cells (Figure 3F). In sum, our results revealed that PCAT6 directly interacted with miR-513a in BC.

Inhibition of MiR-513a Abolished PCAT6 Knockdown-Induced Suppressive Effects on BC Cells

To further investigate whether PCAT6 promoted BC progression by regulating miR-513a, T24 and EJ cells were transfected with shNC, shPCAT6, shPCAT6+miR-513a inhibitor. RT-qPCR assay showed that miR-513a inhibitor abolished the inhibitory effect of PCAT6 knockdown on PCAT6 expression (Figure 4A). Furthermore, the silencing of miR-33a partially restored shPCAT6-attenuated viability, migration and invasion of BC cells (Figure 4B-D). Taken together, these data elucidated that PCAT6 facilitated BC progression by absorbing miR-513a.

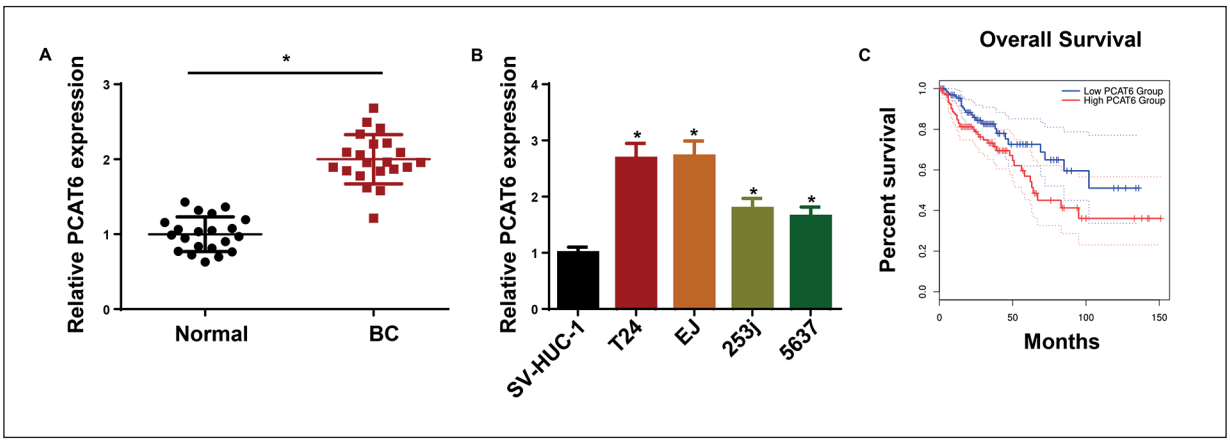

Figure 1. LncRNA PCAT6 was highly expressed in BC tissues and cells. **A,** The expression of PCAT6 was measured in BC tissues and normal tissues by qRT-PCR. **B,** The level of PCAT6 was detected in human bladder epithelial immortalized cell (SV-HUC-1) and BC cells (T24, EJ, 253j, and 5637) by RT-qPCR. **C,** The survival rate of patients was analyzed in high or low PCAT6 expression group. *$p < 0.05$.

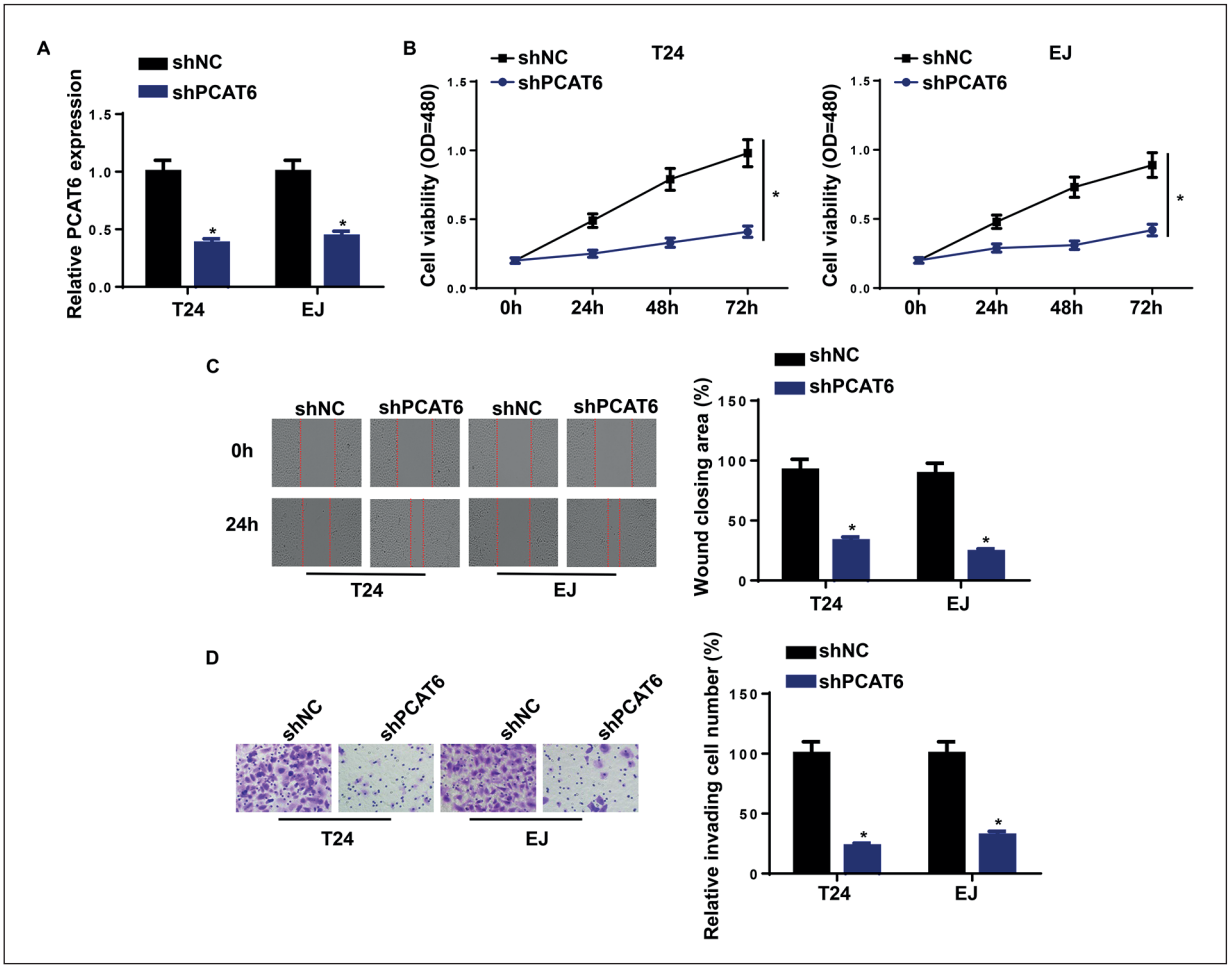

Figure 2. LncRNA PCAT6 knockdown inhibited the progression of BC cells. **A,** RT-qPCR verified the interference efficiency of sh-PCAT6 in T24 and EJ cells. **B,** CCK-8 assay detected proliferation of T24 and EJ cells after transfection of sh-PCAT6. **C,** Wound healing assay measured migration of T24 and EJ cells after transfection of sh-PCAT6 (magnification ×200). **D,** Transwell assay detected invasion of T24 and EJ cells after transfection of sh-PCAT6 (magnification ×200). *$p < 0.05$.

Discussion

BC is the fourth leading cause of cancer-related death among men worldwide[22]. In 2017, estimated 440,000 new cases and 130,000 deaths from BC occurred worldwide[23]. Though multiple therapeutic methods have been conducted in medical treatment, patients with BC still have poor outcomes[24]. Therefore, it is particularly crucial to develop novel therapeutics for the treatment of BC.

Long non-coding (lncRNAs) have been widely proved to be abnormally expressed in human cancers, including BC[25]. Besides, lncRNAs can exert its effect on the biological process by regulating downstream gene expression[26]. For example, Liu et al[27] indicated that the knockdown of LINC00675 deteriorated the progression of BC, and low expression of LINC00675 was associated with worse survival rates. Wang et al[28] reported that lncRNA BCAR4 expression was upregulated in BC cell lines, and it could promote tumor development *via* modulating miR-644a/TLX1 axis. Lou et al[29] reported that TMPO-AS1-mediated miR-98 repression accelerated BC progression by upregulating EBF1. It has been indicated that the dysregulation of PCAT6 results in an imbalance between cell proliferation and apoptosis. Huang et al[30] showed that the upregulation of PCAT6 promoted cell growth and inhibited cell apoptosis in colon cancer. To the best of our knowledge, this is the first study to show the function of PCAT6 in BC. We demonstrated that PCAT6 was upreg-

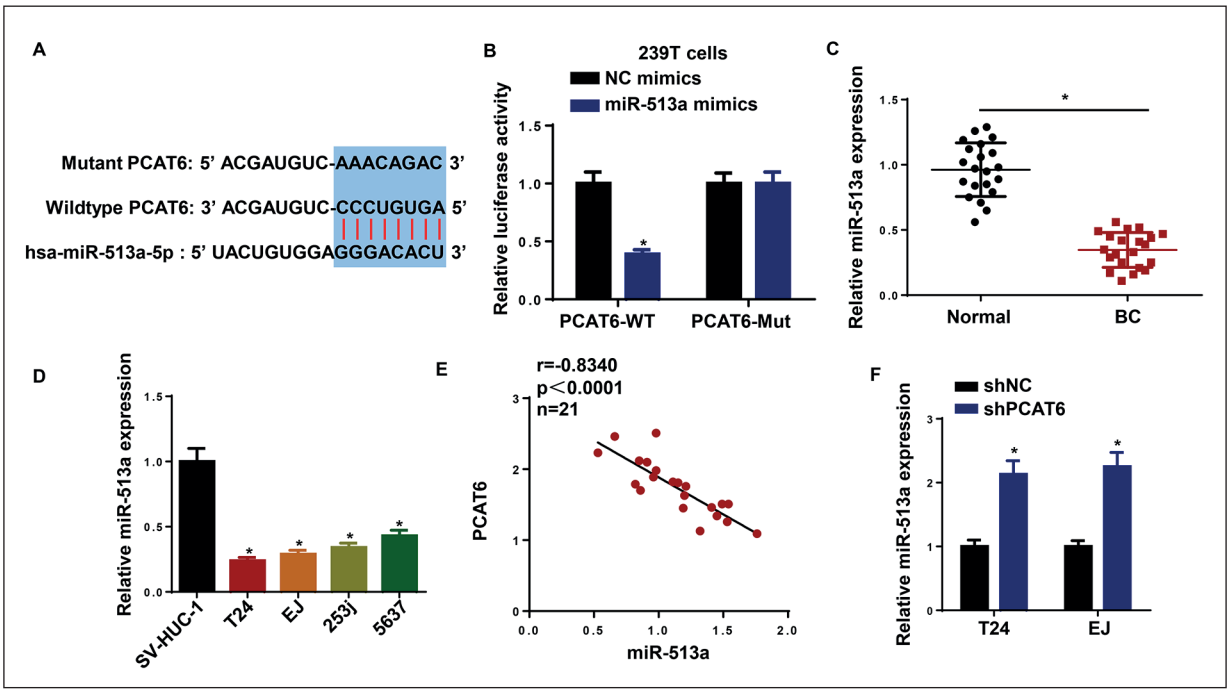

Figure 3. PCAT6 acted as miR-513a sponge in BC. **A,** The potential binding sites of PCAT6 and miR-513a. **B,** Luciferase activity was measured in 293T cells co-transfected with miR-513a mimics or NC mimics and PCAT6-WT or PCAT6-Mut. **C,** The expression of miR-513a was detected in BC tissues and normal tissues by RT-qPCR. **D,** The expression of miR-513a was detected in in human bladder epithelial immortalized cell (SV-HUC-1) and BC cells (T24, EJ, 253j, and 5637) by RT-qPCR. **E,** The levels of miR-513a were negatively correlated with PCAT6 in BC tissues. **F,** RT-qPCR showed the expression of miR-513a in BC cells transfected with shNC and shPCAT6. *$p < 0.05$.

ulated in BC tissues and cells, and the high level of PCAT6 was associated with poor prognosis in BC. In addition, we found that PCAT6 knockdown inhibited the proliferation, migration and invasion of BC cells. In sum, these results illustrated that PCAT6 acted as an oncogene in the progression of BC.

It is well known that lncRNA could serve as a competing endogenous RNA (ceRNA) of miRNA and suppress the miRNA function. To further identify the potential mechanisms of PCAT6 in the pathogenesis of BC, miR-513a was predicted as a potential target of PCAT6 through starBase. Yang et al[31] showed that miR-513a overexpression remarkably suppressed the proliferation of retinoblastoma cells. Dai et al[32] showed that miR-513a stimulated cell apoptosis by inhibiting APE1 expression in osteosarcoma. However, the function of miR-513a in BC is still unknown. In the present study, the miR-513a level was downregulated in BC tissues and cell lines. Moreover, we showed that PCAT6 interacted with miR-513a and inhibited miR-513a expression in BC cells. Functional experiments indicated that PCAT6 knockdown inhibited cell viability, migration and invasion, whereas miR-513a inhibitor abolished these effects. These results indicated that the miR-513a axis contributed to PCAT6 driven stimulation of BC cell progression.

Conclusions

Our study demonstrated that knockdown of PCAT6 inhibited BC cell progression by targeting miR-513a. Our findings indicated that lncRNA PCAT6 might act as a valid prognostic indicator and novel therapeutic target of BC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

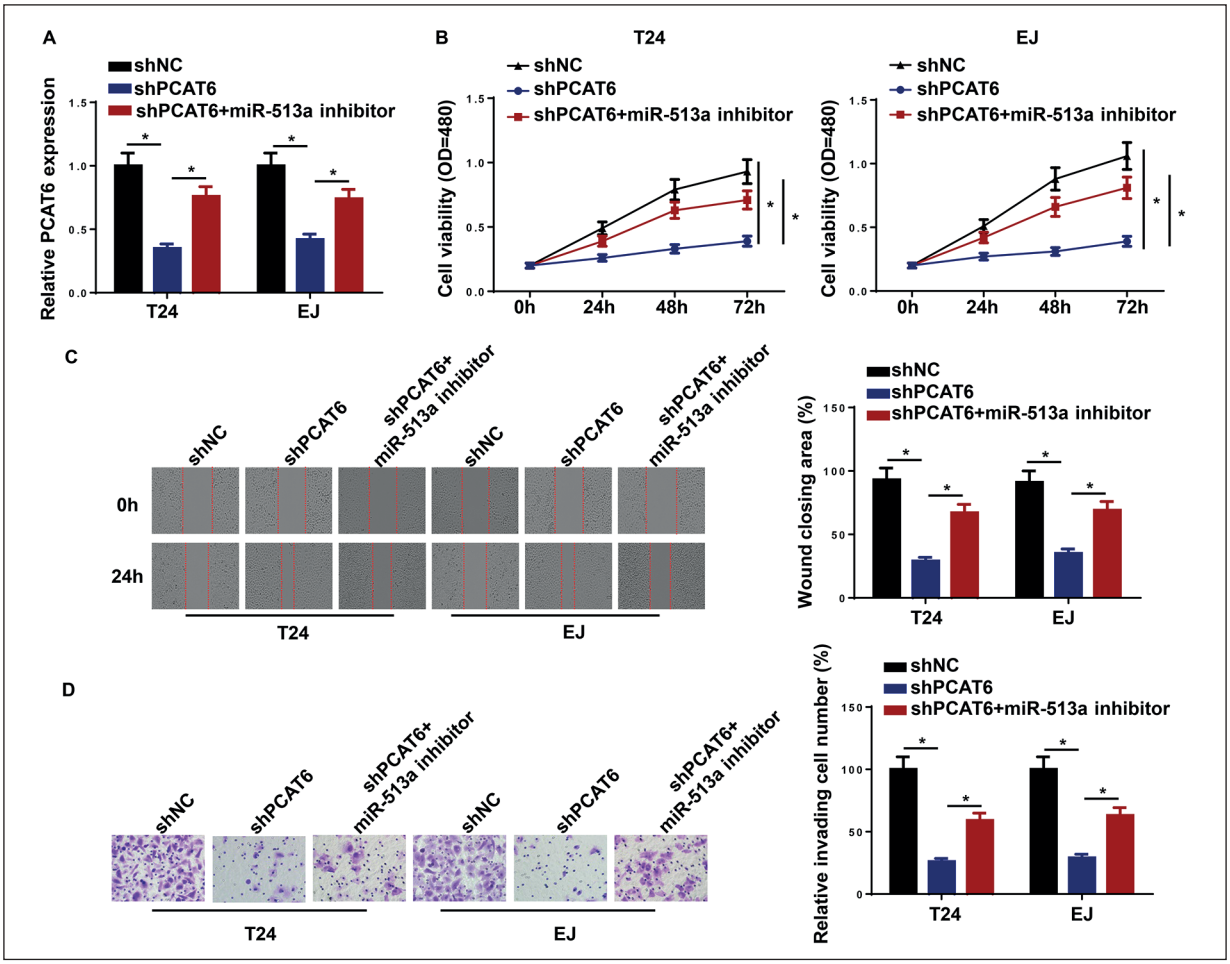

Figure 4. Inhibition of miR-513a abolished PCAT6 knockdown-induced suppressive effects on BC cells. **A,** RT-qPCR analysis tested the expression of miR-513a in T24 and EJ cells. **B,** CCK-8 assay evaluated cell viability among shNC, shPCAT6, shPCAT6+miR-513a inhibitor groups in T24 and EJ cells. **C,** and **D,** Wound healing and transwell assays detected cell migration and invasion among shNC, shPCAT6, shPCAT6+miR-513a inhibitor groups in T24 and EJ cells (magnification ×200). *$p < 0.05$.

References

1) Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018; 68: 394-424.
2) Berdik C. Bladder cancer: 4 big questions. Nature 2017; 551: S51.
3) Homma I, Kitamura H, Torigoe T, Tanaka T, Sato E, Hirohashi Y, Masumori N, Sato N, Tsukamoto T. Human leukocyte antigen class I down-regulation in muscle-invasive bladder cancer: its association with clinical characteristics and survival after cystectomy. Cancer Sci 2009; 100: 2331-2334.
4) Su H, Jiang H, Tao T, Kang X, Zhang X, Kang D, Li S, Li C, Wang H, Yang Z, Zhang J, Li C. Hope and challenge: Precision medicine in bladder cancer. Cancer Med 2019; 8: 1806-1816.
5) Chakraborty A, Dasari S, Long W, Mohan C. Urine protein biomarkers for the detection, surveillance, and treatment response prediction of bladder cancer. Am J Cancer Res 2019; 9: 1104-1117.
6) Eapen LJ, MacRae RM. Management considerations after cystectomy for bladder cancer. J Clin Oncol 2016; 34: 3228.
7) Wang SH, Wu XC, Zhang MD, Weng MZ, Zhou D, Quan ZW. Erratum: upregulation of H19 indicates a poor prognosis in gallbladder carcinoma and promotes epithelial-mesenchymal transition. Am J Cancer Res 2016; 6: 876-877.
8) Lim LJ, Wong SYS, Huang F, Lim S, Chong SS, Ooi LL, Kon OL, Lee CG. Roles and regulation of long noncoding rnas in hepatocellular carcinoma. Cancer Res 2019; 79: 5131-5139.
9) Wang Y, Chen W, Lian J, Zhang H, Yu B, Zhang M, Wei F, Wu J, Jiang J, Jia Y, Mo F, Zhang S, Liang X,

Mou X, Tang J. The lncRNA PVT1 regulates nasopharyngeal carcinoma cell proliferation via activating the KAT2A acetyltransferase and stabilizing HIF-1alpha. Cell Death Differ 2020; 27: 695-710.

10) Zheng S, Lv P, Su J, Miao K, Xu H, Li M. Silencing of the long non-coding RNA RHPN1-AS1 suppresses the epithelial-to-mesenchymal transition and inhibits breast cancer progression. Am J Transl Res 2019; 11: 3505-3517.

11) Huang HG, Tang XL, Huang XS, Zhou L, Hao YG, Zheng YF. Long noncoding RNA LINC00511 promoted cell proliferation and invasion via regulating miR-124-3p/EZH2 pathway in gastric cancer. Eur Rev Med Pharmacol Sci 2020; 24: 4232-4245.

12) Li YL, Zhang XX, Yao JN, Gao B, Gao SL, Wang CF, Zhou HN, Zhang LF. ZEB2-AS1 regulates the expression of TAB3 and promotes the development of colon cancer by adsorbing microRNA-188. Eur Rev Med Pharmacol Sci 2020; 24: 4180-4189.

13) Dong Y, Wan G, Yan P, Qian C, Li F, Peng G. Long noncoding RNA LINC00324 promotes retinoblastoma progression by acting as a competing endogenous RNA for microRNA-769-5p, thereby increasing STAT3 expression. Aging (Albany NY) 2020; 12: 7729–7746.

14) Ma Z, Gu G, Pan W, Chen X. LncRNA PCAT6 accelerates the progression and chemoresistance of cervical cancer through up-regulating ZEB1 by sponging miR-543. Onco Targets Ther 2020; 13: 1159-1170.

15) Dong D, Lun Y, Sun B, Sun H, Wang Q, Yuan G, Quan J. Silencing of long non-coding RNA PCAT6 restrains gastric cancer cell proliferation and epithelial-mesenchymal transition by targeting microRNA-15a. Gen Physiol Biophys 2020; 39: 1-12.

16) Luo Y, Lin J, Zhang Y, Dai G, Li A, Liu X. LncRNA PCAT6 predicts poor prognosis in hepatocellular carcinoma and promotes proliferation through the regulation of cell cycle arrest and apoptosis. Cell Biochem Funct 2020. https://doi.org/10.1002/cbf.3510.

17) Dong Q, Cai N, Tao T, Zhang R, Yan W, Li R, Zhang J, Luo H, Shi Y, Luan W, Zhang Y, You Y, Wang Y, Liu N. An axis involving SNAI1, microRNA-128 and SP1 modulates glioma progression. PLoS One 2014; 9: e98651.

18) Sun Q, Liu T, Zhang T, Du S, Xie GX, Lin X, Chen L, Yuan Y. MiR-101 sensitizes human nasopharyngeal carcinoma cells to radiation by targeting stathmin 1. Mol Med Rep 2015; 11: 3330-3336.

19) Ning JZ, Yu WM, Cheng F, Rao T, Ruan Y. MiR-425 promotes migration and invasion in bladder cancer by targeting Dickkopf 3. J Cancer 2020; 11: 3424-3432.

20) Guo J, Zhang J, Yang T, Zhang W, Liu M. MiR-22 suppresses the growth and metastasis of bladder cancer cells by targeting E2F3. Int J Clin Exp Pathol 2020; 13: 587-596.

21) Peng W, Dong N, Wu S, Gui D, Ye Z, Wu H, Zhong X. MiR-4500 suppresses cell proliferation and migration in bladder cancer via inhibition of STAT3/CCR7 pathway. J Cell Biochem 2019 Dec 1. doi: 10.1002/jcb.29558. Online ahead of print.

22) Soria F, Marra G, Capoun O, Soukup V, Gontero P. Prevention of bladder cancer incidence and recurrence: tobacco use. Curr Opin Urol 2018; 28: 80-87.

23) Jiang F, Qi W, Wang Y, Wang W, Fan L. lncRNA PEG10 promotes cell survival, invasion and migration by sponging miR-134 in human bladder cancer. Biomed Pharmacother 2019; 114: 108814.

24) Wang J, Zhang H, Situ J, Li M, Sun H. KCNQ1OT1 aggravates cell proliferation and migration in bladder cancer through modulating miR-145-5p/PCBP2 axis. Cancer Cell Int 2019; 19: 325.

25) Yu X, Wang ZL, Han CL, Wang MW, Jin Y, Jin XB, Xia QH. LncRNA CASC15 functions as an oncogene by sponging miR-130b-3p in bladder cancer. Eur Rev Med Pharmacol Sci 2019; 23: 9814-9820.

26) Song Y, Pan Y, Liu J. Functional analysis of lncRNAs based on competitive endogenous RNA in tongue squamous cell carcinoma. PeerJ 2019; 7: e6991.

27) Liu X, Ke J, Gu L, Tang H, Luo X. Long non-coding RNA LINC00675 is associated with bladder cancer metastasis and patient survival. J Gene Med 2020: e3210.

28) Wang X, He H, Rui W, Xie X, Wang D, Zhu Y. Long non-coding RNA BCAR4 binds to miR-644a and targets TLX1 to promote the progression of bladder cancer. Onco Targets Ther 2020; 13: 2483-2490.

29) Luo H, Yang L, Liu C, Wang X, Dong Q, Liu L, Wei Q. TMPO-AS1/miR-98-5p/EBF1 feedback loop contributes to the progression of bladder cancer. Int J Biochem Cell Biol 2020; 122: 105702.

30) Huang W, Su G, Huang X, Zou A, Wu J, Yang Y, Zhu Y, Liang S, Li D, Ma F, Guo L. Long noncoding RNA PCAT6 inhibits colon cancer cell apoptosis by regulating anti-apoptotic protein ARC expression via EZH2. Cell Cycle 2019; 18: 69-83.

31) Yang L, Zhang L, Lu L, Wang Y. LncRNA UCA1 increases proliferation and multidrug resistance of retinoblastoma cells through downregulating miR-513a-5p. DNA Cell Biol 2020; 39: 69-77.

32) Dai N, Qing Y, Cun Y, Zhong Z, Li C, Zhang S, Shan J, Yang X, Dai X, Cheng Y, Xiao H, Xu C, Li M, Wang D. miR-513a-5p regulates radiosensitivity of osteosarcoma by targeting human apurinic/apyrimidinic endonuclease. Oncotarget 2018; 9: 25414-25426.