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Original Article

LncRNA PCAT7 promotes non-small cell lung cancer progression by activating miR-486-5p/CDK4 axis-mediated cell cycle

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Abstract: Objective: Lung cancer remains one of the common cancers worldwide. Both LncRNA PCAT7 and miR-486-5p are tightly correlated with NSCLC. However, the relationship between PCAT7 and miR-486-5p and the detailed mechanisms underlying the effect of PCAT7 on NSCLC are not discovered yet. Methods: GEPIA and ENCORI databases were used to determine the expression of PCAT7 in different cancers. CCK8, colony formation and Transwell assay were used to confirm the ability of cells. Luciferase reporter gene assay was employed to estimate the luciferase activity of the gene. Flow cytometry was used to compare cell cycle of NSCLC cells after indicated treatment. Results: GEPIA combined ENCORI database illustrated that LncRNA PCAT7 was upregulated dramatically in NSCLC. The mRNA level of PCAT7 cells was higher than that in normal cells. Silencing PCAT7 inhibited the progression of NSCLC cells significantly. Data from ENCORI website showed that miR-486-5p was the target of PCAT7 and was negatively controlled by it. The data also showed that CDK4 could be bound and negatively regulated by miR-486-5p. MiR-486-5p inhibitor or CDK4 could partly restore the inhibitory effect of PCAT7 in NSCLC cells. In addition, silencing PCAT7 could arrest cell cycle to S in addition to G2 stage while transfecting miR-486-5p inhibitor or CDK4 could partially eliminate the retarding effects. Conclusion: In our study, we elaborated that LncRNA PCAT7 could promote the development of NSCLC cells by accelerating cell cycle *via* miR-486-5p/CDK4 axis.

Keywords: Cell cycle, LncRNAs, miR-486-5p, non-small cell lung cancer (NSCLC), PCAT7

Introduction

Carcinoma of the lungs, the leading cause of cancer-associated death, comprises of two primary types: NSCLC (non-small cell lung cancer) and SCLC (small cell lung cancer). Research shows that NSCLC, as the uppermost category of lung cancer, accounts for no less than 80% of the cancer cases [1, 2]. Emerging therapies for NSCLC including radiotherapy, surgery and chemotherapy have come into service, but there remain many limits in individual treatment and prognosis [3]. Lacking an adequate understanding of the pathogenesis of NSCLC is one of the main reasons that result in the present situation. Therefore, exploring the nosogenesis and revealing potent therapeutic targets are crucial for patients with NSCLC.

Long non-coding RNAs, also named LncRNAs, possess the potential to be expressed into transcripts more than 200 nt nucleotides while they cannot be transcribed as proteins [4]. More and more evidence manifests that disordered LncRNAs are involved in cancer induction [5]. Lately, multiple LncRNAs have been discovered to be correlated with NSCLC. TUG1, PANDAR and HOTAIR, for example, are able to impact homeostasis procedure in cells and then play a part in the carcinogenesis as well as the progress of NSCLC [6, 7]. It can be seen that most LncRNAs have the potential to be therapeutic targets in various cancers. Prostate cancer-associated transcript 7 (PCAT7), which is located on chromosome 9q22.32, is a 1937-bp long non-coding RNA [8]. Recently, PCAT7 has been found to be involved in a variety of tumorigene-

sis. For example, Liu et al. revealed that PCAT7 could promote the progression of nasopharyngeal carcinoma by targeting miR-134-5p [8]. Horie et al. found that PCAT7 is up-regulated in NSCLC, and Wu et al. uncovered that PCAT7 could accelerate tumorigenesis by inhibiting miR-134-5p in NSCLC [3, 9]. However, the in depth and accurate mechanism of PCAT7 on the progression of NSCLC has not been explored adequately yet.

MiRNAs, small non-coding RNAs with ~22 nucleotides, possess the capacity to bind to various target genes and regulate a series of biological procedures [10-12]. MiRNAs are often expressed in an abnormal manner in different cancers [13]. Interestingly, depressing the expression of multiple miRNAs is likely to promote the progress of tumors such as accelerating tumor cell proliferation, migration and invasion and inhibiting apoptosis [14]. Thus, most miRNAs can act as antigens to suppress cancer development and progression [15]. MiR-486-5p has been discovered to be one of tumor suppressors in multiple cancers, and this microRNA maps at chromosome 8 and is cloned from liver tissue [16-18]. Recently, LncRNAs have been found to combine with miRNAs to control target genes in biological progressions [19]. Xing et al. revealed that miR-486-5p could be one of the targets of LINC01194 and regulate the development of NSCLC [20]. Nevertheless, other potent LncRNA of miR-486-5p had not been expounded until now. Our current study aimed to reveal a novel upstream LncRNA of miR-486-5p, which could promote the procedure of NSCLC by triggering miR-486-5p axis mediated cell cycle.

Materials and methods

Cell culture

NSCLC cell lines PC5, A549, H1975 H1299, H460 and control cell line BES-2B were bought from ATCC (American Type Culture Collection, USA). A549 and H1299 cells were cultured in RPMI 1640 medium (Gibco), while the other cells were cultured in DMEM (Gibco) at a 37°C incubator with 95% O_2 as well as 5% CO_2.

Transfection

The miR-486-5p mimic (UCCUGUACUGAGCUGCCCCGAG) and miR-486-5p inhibitor (CUCGGGGCAGCUCAGUACAGGA) as well as miR-NC (UUCUCCGAACGUGUCACGUTT) were bought from RiboBio (China). The vectors with shRNA sequences targeting PCAT7 (sh-PCAT7-1# target 5'-GAACATGCAGTCTAGGAACCGGCAT-3' (sense), 5'-ATGCCGGTTCCTAGACTGCATGTTC-3' (antisense); sh-PCAT7-2# target 5'-AGTCTAGGAACCGGCATGCGCATAA-3' (sense), 5'-TTATGCGCATGCCGGTTCCTAGACT-3' (antisense); sh-PCAT7-3# target 5'-AGCAACATGAAGAGAGATGCCAGGA-3' (sense), 5'-TCCTGGCATCTCTCTTCATGTTGCT-3' (antisense) and sh-NC target 5'-CCCATAAGAGTAATAATAT-3' (sense), 5'-ATATTATTACTCTTATGGG-3' (antisense) were bought from Genepharma (China). The mimic, inhibitor or vectors were transiently transfected into cells using Lipofectamine 2000 combined with Plus Reagent (Invitrogen) according to the manufacturer's protocols.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was collected by employing TRIzol reagent (Invitrogen) and reverse transcribed to cDNA. QRT-PCR was conducted using ViiATM 7 real-time PCR software (Life Technologies) according to the instruction. The load of mRNA was analyzed by the $2^{-\Delta\Delta CT}$ method. The levels of LncRNA/genes and microRNA were normalized by GAPDH and U6 separately. The sequence of corresponding primers is listed in **Table 1**.

CCK8 assay

Cells after transfecting were planted in 96-well plates at concentration of 1×10^3 cells each well. After 24 hours, 10 μL CCK8 (Sigma) reagent with 90 μL medium was added into each well in the form of changing medium. After incubating for 1 h, the value of optical density (OD) of every well was detected by a microplate reader (BioTek) at the wavelength of 450 nm.

Colony formation assay

The transfected cells were seeded into 6-well plates at the concentration of 300 cells each well. Cells were incubated for 7-10 days at 37°C. After that, the medium was thrown away, and cells were tinged using 1% crystal violet for 30 min at room temperature. After washing, colonies were photographed and counted with a microscope.

Flow cytometry analysis

The cell apoptosis rate was analyzed according to the protocol of Annexin V-PE apoptosis as-

Table 1. The sequences of primers

Gene	RT-PCT Primers
PCAT7	Forward: 5' GCGTTGCAAAAAGACGATGGGG-3'
	Reverse: 5'-CCCCATCGTCTTTTTGCAACGC-3'
MiR-486-5p	Forward: 5'-CATTGTGCTGTTCGTGCAGTTAA-3'
	Reverse: 5'-CCCTCCAGGAATTGGCCTGTCTT-3'
CDK4	Forward: 5'-GCAGCGACTATGCACAACGA-3'
	Reverse: 5'-CCAGAGTGGTGACGGAGACA-3'
GAPDH	Forward: 5'-CCTTCCGTGTCCCCACT-3'
	Reverse: 5'-GCCTGCTTCACCACCTTC-3'
U6	Forward: 5'-GTGCTCGCTTCGGCAGCACATATAC-3'
	Reverse: 5'-AAAAATATGGAACGCTCACGAATTTG3'

say kit (KGA1011, KeyGEN, BioTECH, China). Briefly, A549 or PC9 cells were seeded in 6-well plates (1×10^5 cells/well) and transfected with indicated vectors. After 24 h, the cells were washed, digested and collected in 500 μl binding buffer containing 5 μl Annexin V-fluorescein isothiocyanate (FITC) reagent and 5 μl propidium iodide (PI) reagent. The mixture was incubated at 37°C for 20 min in dark. Finally, a flow cytometer (CytoFLEX, Beckman, USA) was used to detect apoptotic cells.

Transwell assays

For migration assay, the cell suspension was seeded in the upper chamber. After 12 hours, upper chamber was washed with PBS 3 times. Then 90% formaldehyde was used to fix cells, and crystal violet was used to stain cells for 15 min at room temperature. Finally, the chamber was observed and photographed using a microscope. For the invasion assay, the upper chamber was pre-treated with Matrigel (20 μg) overnight, and the other procedures were the same as the migration assay.

Nuclear-cytoplasmic fractionation assay

Nuclear as well as cytoplasmic RNA was harvested by employing Ne-per™ Nuclear and cytoplasmic extraction reagent (78833, Thermo Scientific, USA) according to the protocol. Then qRT-PCR assay was utilized to determine the expression level of PCAT7 in nucleus and cytoplasm, respectively, as described above. U6 was regarded as the nuclear control, whereas actin was regarded as the cytoplasmic control. The sequences of primers are provided in **Table 1**.

Luciferase reporter assay

The 3'UTR sections of wildtype PCAT7 and CDK4 were cloned by PCR technology taking human genomic DNA as the template. The binding sequences were mutated using the method of overlapping PCR, and the products were constructed into pGL3 plasmid (Promega). Next, cells were co-transfected with the above-mentioned reporter vectors in addition to miR-486-5p mimics or inhibitors separately. After 36 hours, luciferase activity was estimated by dual-luciferase detection software (Promega).

Western blot

After cells were cultured and harvested, total proteins were collected, and the concentration of protein was calculated by BCA protein assay kit (Invitrogen). Then specific protein was separated by 10% SDS-PAGE and incubated with primary antibodies against GAPDH (60004-1-Ig, Proteintech, 1:1000), E-cadherin (20874-1-AP, Proteintech, 1:1000), N-cadherin (ab18203, Abcam, 1:1000), CyclinD1 (orb77046, Biorbyt, 1:1000), CDK4 (12790, Cell Signaling Technology, 1:1000) and CyclinB2 (ABIN3017744, Antibodies online, Germany, 1:1000). Subsequently, a secondary antibody was employed at room temperature for 1 h, and protein bands were visualized using a gel imaging analysis system (Bio-rad).

Cell-cycle analysis

Transfected cells were washed by PBS. Trypsin was used to trypsinize cells and stopped by medium containing 10% FBS. After that, cells were fixed with pre-cooled 70% ethanol on ice for half an hour. Then the cells were washed 3 times by PBS containing 0.5% Tween-20, and they were re-suspended with PBS containing 5 μg/μL PI (Propidium Iodide) and 0.25 μg/μL RNase A. The mixture was incubated at room temperature for 10 min in dark. Subsequently, a flow cytometer was used to analyze every sample, and data were analyzed using FlowJo software.

Statistical methods

All data were analyzed using software SPSS and expressed in the manner of mean ± stan-

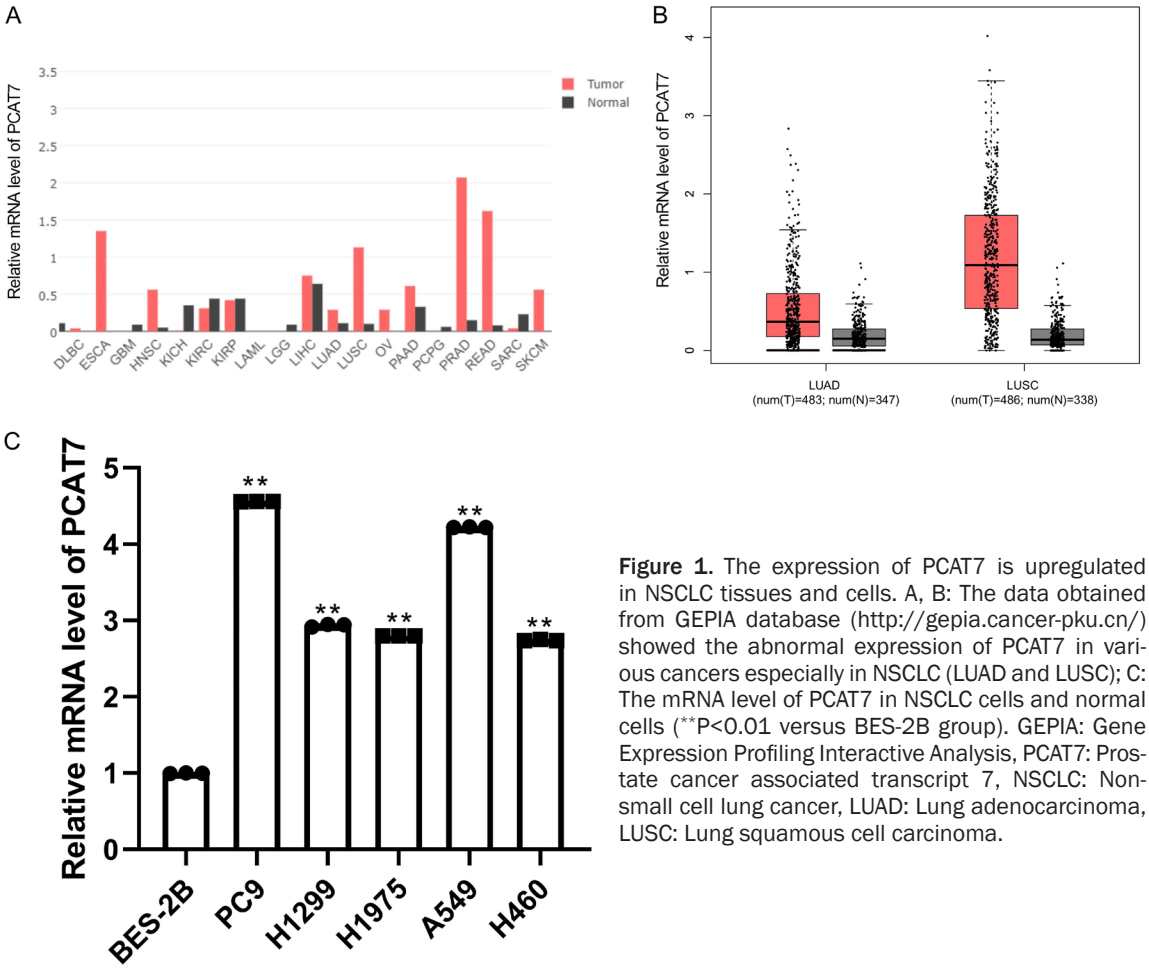

Figure 1. The expression of PCAT7 is upregulated in NSCLC tissues and cells. A, B: The data obtained from GEPIA database (http://gepia.cancer-pku.cn/) showed the abnormal expression of PCAT7 in various cancers especially in NSCLC (LUAD and LUSC); C: The mRNA level of PCAT7 in NSCLC cells and normal cells (**P<0.01 versus BES-2B group). GEPIA: Gene Expression Profiling Interactive Analysis, PCAT7: Prostate cancer associated transcript 7, NSCLC: Non-small cell lung cancer, LUAD: Lung adenocarcinoma, LUSC: Lung squamous cell carcinoma.

dard deviation ($\bar{x}$ ± sd). Significance analysis was conducted by unpaired Student's t test between two groups or by one-way ANOVA followed by Tukey post hoc tests among multiple groups. P<0.01 was considered statistically significant.

Results

The expression of PCAT7 is upregulated in NSCLC tissues and cells

The data on GEPIA website showed that PCAT7 level was dysregulated in a variety of tumors and was dramatically higher in NSCLC (LUAD and LUSC) tissues than that in normal tissues (**Figure 1A** and **1B**). Moreover, we conducted qRT-PCR to measure the mRNA level of PCAT7 in five NSCLC cell lines (PC9, H1299, H1975, A549 and H460) and human normal lung epithelial cell line BES-2B. The data showed that the expression level of PCAT7 in NSCLC cells was increased notably (**P<0.01, **Figure 1C**). And the load of PCAT7 in A549 in addition to PC9 was higher than that in other NSCLC cell lines, so we chose PC9 and A549 to conduct the subsequent experiments. However, data from starBase showed that low expression of PCAT7 was not significantly associated with better prognosis in patients with LUAD or LUSC (Figure S1). On the basis of these data, we hypothesized that PCAT7 was related to the progression of NSCLC.

Knockdown of PCAT7 suppresses the biological progression of NSCLC cells

Vector with si-NC or si-PCAT7 was transfected into PC9 and A549 cells to further confirm the impact of PCAT7 on tumorigenesis. As shown in **Figure 2A**, compared to the control group, the mRNA level of PCAT7 in si-PCAT7 group was decreased significantly, which meant that the transfection was successful. Given that the expression level of PCAT7 was the lowest in PC9-si-PCAT7-2# as well as A549-si-PCAT7-1#,

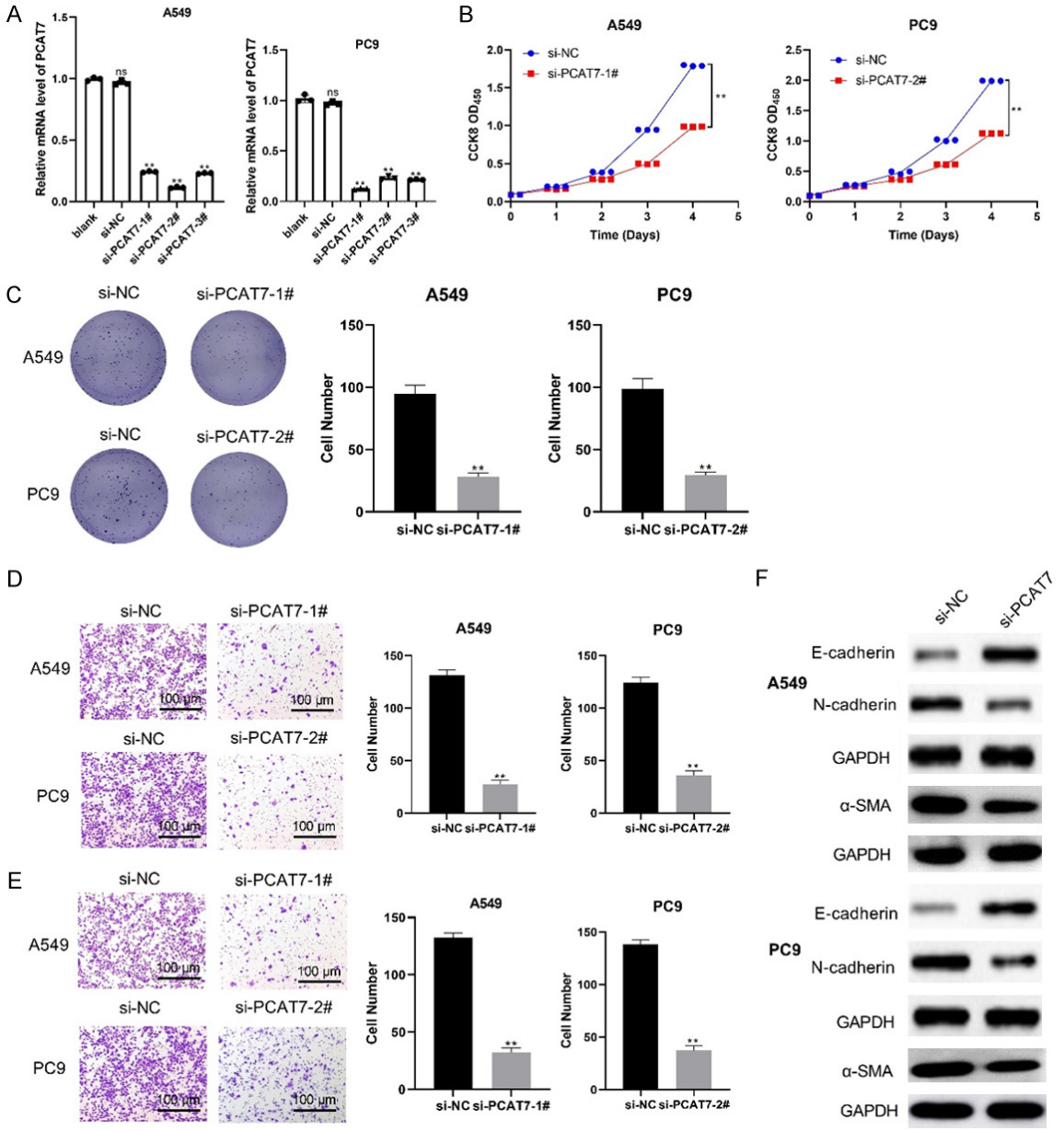

Figure 2. Knockdown of PCAT7 suppresses the biological progression of NSCLC cells. A: The load of PCAT7 was decreased dramatically by siPCAT7 in PC9 and A549 cells (**P<0.01); B: CCK8 assay showed that siPCAT7 inhibited the proliferative ability of NSCLC cells (**P<0.01); C: Colony formation assay showed that siPCAT7 inhibited the proliferative ability of NSCLC cells (**P<0.01); D, E: Transwell assay illustrated that knockdown of PCAT7 suppressed the migration and invasion of NSCLC cells, scale bar × 100 (**P<0.01); F: Proteins involved in EMT progress was measured. PCAT7: Prostate cancer associated transcript 7, CCK8: Cell counting kit 8, NSCLC: Non-small cell lung cancer, EMT: Epithelial-mesenchymal transition. **P<0.01 versus si-NC group.

we chose the above two cell lines to finish following exploration (**P<0.01, **Figure 2A**). Both CCK8 and colony formation assay showed that silencing PCAT7 depressed the proliferative activity of PC9 as well as A549 (**P<0.01, **Figure 2B** and **2C**). Transwell assay illustrated that knockdown of PCAT7 obviously down-regulated the migration and invasion ability of NSCLC (**P<0.01, **Figure 2D** and **2E**). In addition, by analyzing proteins related to EMT progression, we found that decreasing PCAT7 decelerated the progress of EMT (**Figure 2F**). However, silencing PCAT7 did not induce apoptosis of NSCLC cells (Figure S2). These data

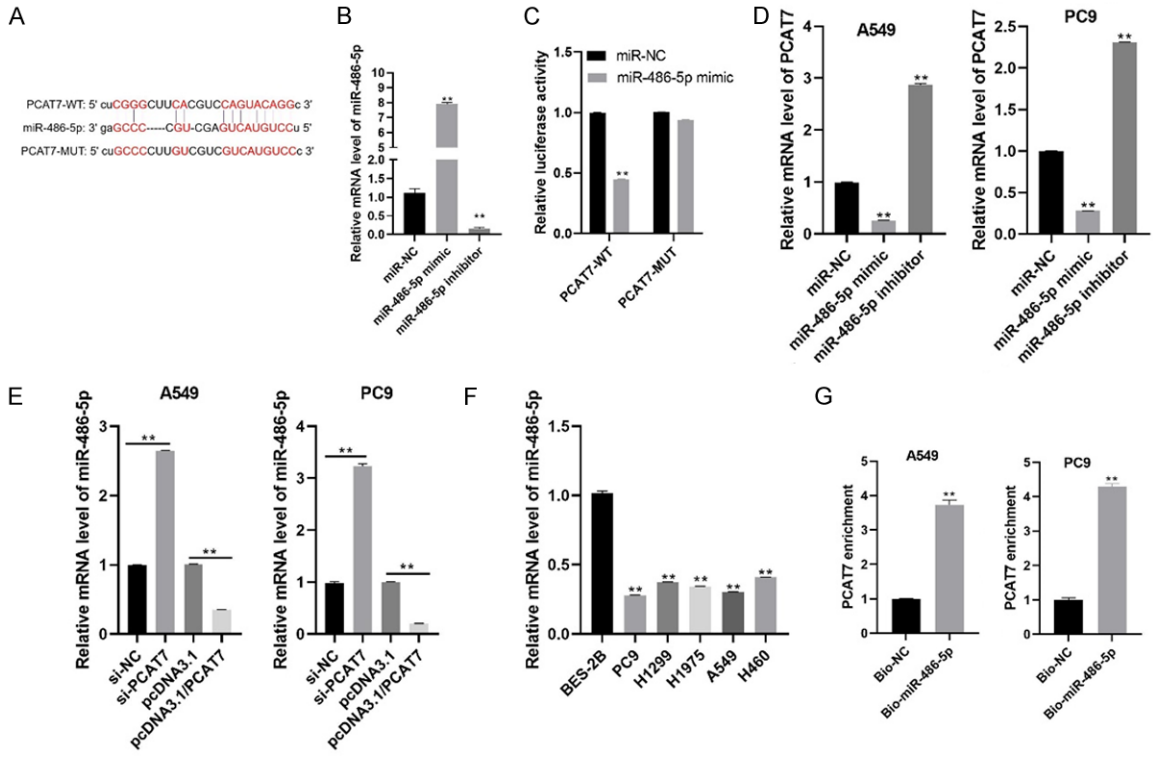

Figure 3. MiR-486-5p is the direct target of PCAT7 and it is negatively correlated with PCAT7. A: MiR-486-5p was the direct target predicted by ENCORI database (http://starbase.sysu.edu.cn/agoClipRNA.php?source=lncRNA&flag=miRNA&clade=mammal&genome=human&assembly=hg19&miRNA=hsa-miR-337-3p&clipNum=&deNum=&panNum=&target=); B: The expression level of miR-486-5p detected by qRT-PCR (**P<0.01 versus miR-NC group); C: MiR-486-5p could depress the expression of wildtype PCAT7 (**P<0.01 versus miR-NC group), but no obvious effect was found on mutant PCAT7 (P>0.01); D: The mRNA level of PCAC7 was decreased by transfecting miR-486-5p mimic, while it was increased by transfecting miR-486-5p inhibitor (**P<0.01 versus miR-NC group); E: The mRNA level of miR-486-5p was increased by silencing PCAT7, but it was decreased by overexpression of PCAT7 (**P<0.01); F: The mRNA level of miR-486-5p in NSCLC and normal cells (**P<0.01 versus BES-2B group); G: The correlation between PCAT7 and miR-486-5p detected by RNA pull down experiment (**P<0.01 versus Bio-NC group). PCAT7: Prostate cancer associated transcript 7, NSCLC: Non-small cell lung cancer.

indicated that PCAT7 was able to facilitate NSCLC cells to proliferate, migrate, invade and epithelial-mesenchymal transit.

MiR-486-5p is the direct target of PCAT7 and it is negatively correlated with PCAT7

According to ENCORI database, we discovered that miR-486-5p was the direct target of PCAT7 (**Figure 3A**). Nuclear-cytoplasmic fractionation assay illustrated that PCAT7 was chiefly located in the cytoplasm of A549 and PC9 cells, indicating that PCAT7 might be a competing endogenous RNA (ceRNA) (Figure S3). Next, we detected the successful efficiency of miR-486-5p mimic and inhibitor (**P<0.01, **Figure 3B**). To further explore the relationship between PCAT7 and miR-486-5p, we constructed wild-type (WT)-PCAT7 or mutant (MUT)-PCAT7 vector with luciferase reporter and employed luciferase reporter gene experiments. The results demonstrated that luciferase activity of WT-PCAT7 was depressed by miR-486-5p mimic significantly (**P<0.01), but only a slight difference occurred in the luciferase activity of MUT-PCAT7 (P>0.01, **Figure 3C**). Additionally, the mRNA level of PCAT7 was decreased by miR-486-5p mimic while increased by miR-486-5p inhibitor notably (**P<0.01, **Figure 3D**). Consistently, the expression level of miR-486-5p was dramatically up-regulated by knockdown of PCAT7 but down-regulated by overexpression of PCAT7 (**P<0.01, **Figure 3E**), which verified the negative correlation between PCAT7 and miR-486-5p. Furthermore, we measured the mRNA level of miR-486-5p and found the distinct reduced expression of miR-486-5p in NSCLC cells as compared with normal human

lung epithelial (BES-2B) cells (**P<0.01, **Figure 3F**). Furthermore, the RNA pull-down experiment also confirmed the negative correlation between PCAT7 and miR-486-5p (**P<0.01, **Figure 3G**). Collectively, these findings suggested that PCAT7 negatively regulated the expression of miR-486-5p.

The impacts of PCAT7 on NSCLC cells are recovered partly by miR-486-5p inhibitor

For validating whether PCAT7 affects NSCLC cells by targeting miR-486-5p, we transfected miR-486-5p inhibitor into PC9 cells which had been transfected with si-PCAT7. Knockdown of PCAT7 could suppress proliferative ability of PC9, while transfecting miR-486-5p inhibitor counteracted the effect induced by silencing PCAT7 based on the data from CCK8 (**P<0.01, **Figure 4A**). As shown in **Figure 4B**, **4C**, co-transfecting siPCAT7 as well as miR-486-5p inhibitor eliminated the inhibitory effects of decreasing PCAT7-induced migration and invasion of PC9 cells (**P<0.01, **Figure 4B** and **4C**). In addition, the EMT progression depressed by knockdown of PCAT7 was renovated after transfecting miR-486-5p inhibitor according to the change of proteins related to EMT progress (**Figure 4D**). These results confirmed that PCAT7 regulated NSCLC cell progression *via* sponging miR-486-5p.

CDK4 is the target of miR-486-5p and it is negatively regulated by miR-486-5p

CDK4 was predicted as the direct target of miR-486-5p and negatively correlated with miR-486-5p according to ENCORI website (**Figure 5A** and **5B**). WT-CDK4 and MUT-CDK4 plasmids with luciferase reporter were constructed and used in luciferase reporter gene assay combined with miR-486-5p mimic. The results revealed that the luciferase activity of WT-CDK4 was down-regulated by miR-486-5p mimic (**P<0.01). However, it had no obvious impact on the activity of MUT-CDK4 (P>0.05, **Figure 5C**). Consistently, transfecting miR-486-5p mimic decreased the mRNA and protein levels of CDK4 while miR-486-5p inhibitor increased those in PC9 cells (**P<0.01, **Figure 5D**). Additionally, si-PCAT7 notably down-regulated CDK4 expression level, and transfection of miR-486-5p inhibitor was able to restore the inhibitory impact of si-PCAT7 on the CDK4 mRNA level (**P<0.01, **Figure 5E**).

CDK partially eliminates the effects of PCAT7 on the progression of NSCLC cells

To explore whether PCAT7 regulated NSCLC cells by targeting miR-486-5p/CDK4 axis, we conducted subsequent experiments. As shown in **Figure 6A** and **6B**, PCAT7 was effectively silenced by si-PCAT7, and the expression of CDK4 was increased dramatically by transfecting vector with CDK4 (**P<0.01). In addition, si-PCAT7 dramatically depressed the proliferation of PC9 cells, while co-transfecting CDK4 in addition to si-PCAT67 partially restored the impact of si-PCAT7 on cell viability (**P<0.01, **Figure 6C**). Subsequently, Transwell assay verified that expressing CDK4 ectopically partially restored the inhibitory functions of si-PCAT7 on cell migration as well as invasion (**P<0.01, **Figure 6D** and **6E**). Additionally, CDK4 also partially eliminated the effect of si-PCAT7 on the EMT progress of PC9 cells (**Figure 6F**).

PCAT7 regulates the progression of NSCLC cells by activating cell cycle mediated by miR-486-5p/CDK4 axis

CDK4 has been found to serve as a crucial protein involved in the cell cycle. Flow cytometry was used to verify whether PCAT7 could promote NSCLC cell development by influencing cell cycle. The results showed that silencing PCAT7 arrested cell cycle to S as well as G2 phases. Nevertheless, co-transfecting si-PCAT7 with miR-486-5p inhibitor or CDK4 significantly increased the proportion of PC9 cells in the G1 phase and meanwhile decreased that in the S and G2 phases (**Figure 7A**). Furthermore, proteins related to cell cycle were affected by si-PCAT7, and the effect was partially eliminated by transfecting miR-486-5p inhibitor or CDK4 (**Figure 7B**). These results demonstrated that PCAT7 promoted NSCLC cells' progression by accelerating cell cycle which was regulated by miR-486-5p/CDK axis.

Discussion

For the first time, this investigation revealed that LncRNA PCAT7 was upregulated in NSCLC tissues and cells. Additionally, PCAT7 knockdown significantly inhibited NSCLC cell progression including cell proliferation, migration, invasion and EMT progress. Moreover, miR-486-5p was discovered to be a direct target of PCAT7 and was negatively regulated by PCAT7. As

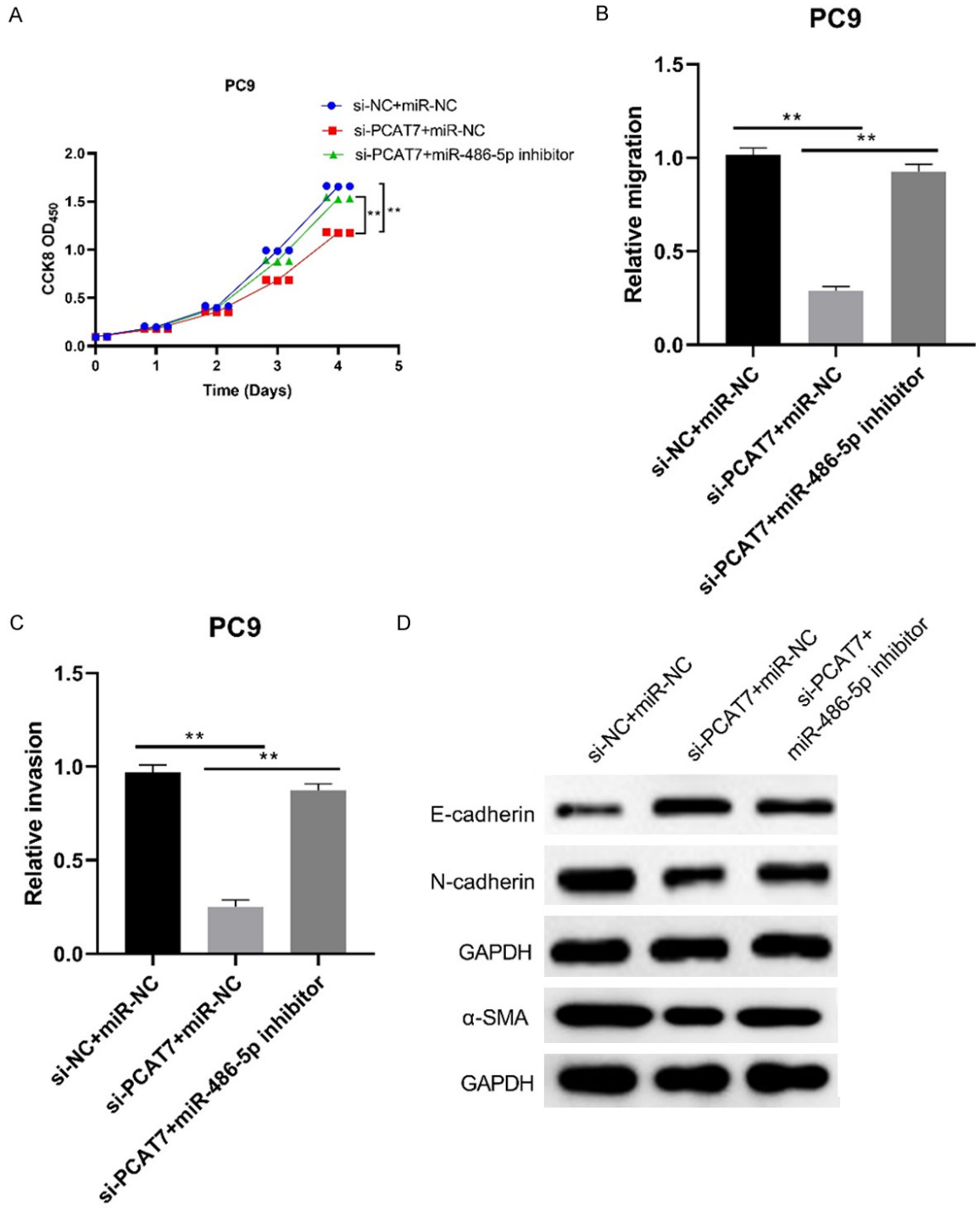

Figure 4. The impacts of PCAT7 on NSCLC cells are recovered by miR-486-5p inhibitor. A: CCK8 assay showed that miR-486-5p could partially restore proliferative ability of PC9 cells (**P<0.01); B, C: Transwell assay revealed the effects of miR-486-5p on PC9 cells after silencing PCAT7 (**P<0.01); D: Proteins involved in EMT progress were measured. CCK8: Cell counting kit 8, NSCLC: Non-small cell lung cancer, EMT: Epithelial-mesenchymal transition.

expected, inhibiting miR-486-5p dramatically eliminated the inhibitory effect of PCAT7 knockdown on NSCLC progression. Furthermore, our findings confirmed that PCAT7 mediated miR-486-5p by CDK4, and overexpressing CDK4 effectively counteracted the effects of PCAT7 on NSCLC development by modulating the cell cycle.

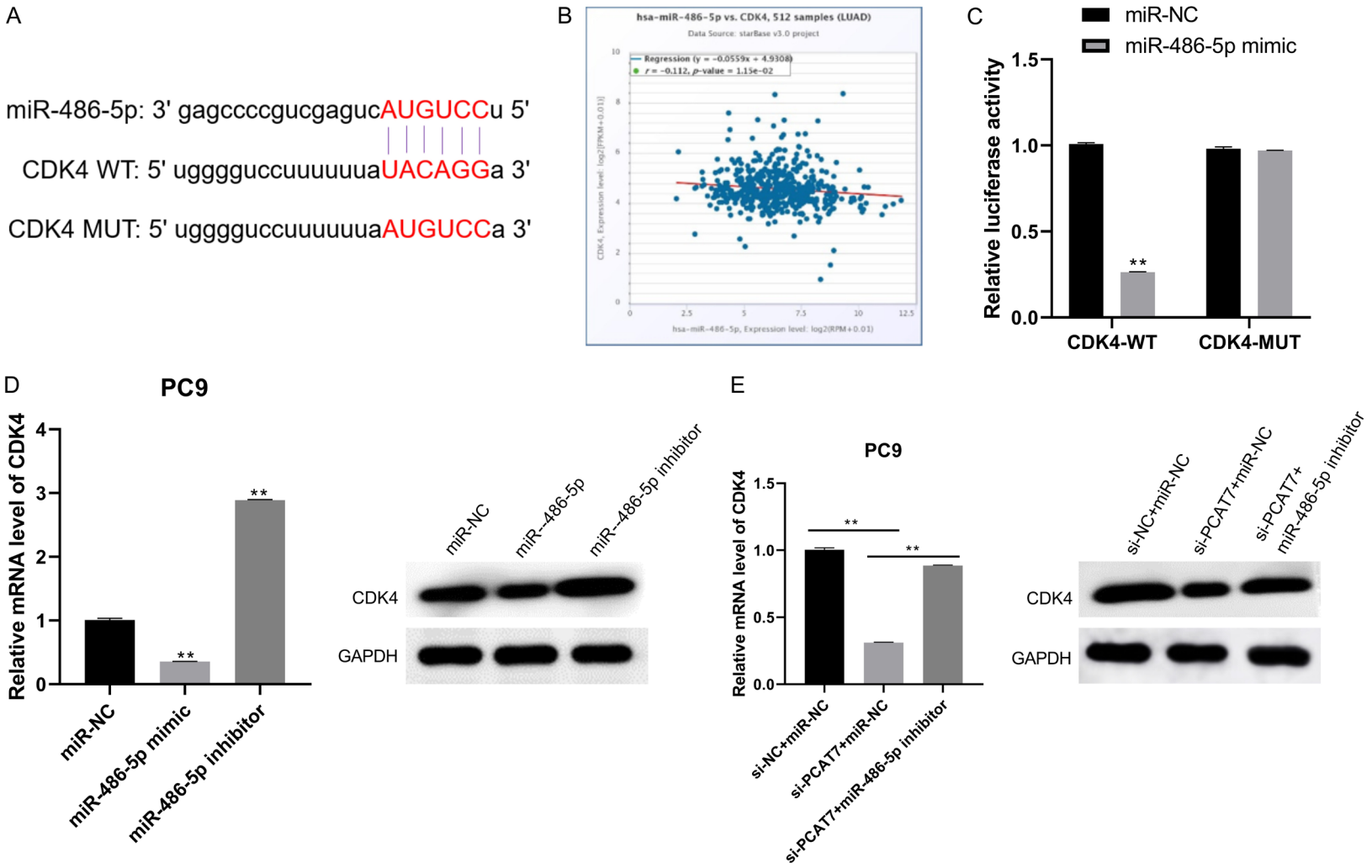

Figure 5. CDK4 is the target of miR-486-5p and it is negatively regulated by miR-486-5p. A, B: CDK4 was the target of miR-486-5p and showed negative correlation with miR-486-5p (http://starbase.sysu.edu.cn/agoClipRNA.php?source=lncRNA&flag=miRNA&clade=mammal&genome=human&assembly=hg19&miRNA=hsa-miR-337-3p&clipNum=&deNum=&panNum=&target=); C: MiR-486-5p mimic down-regulated the activity of WT-CDK4 (**P<0.01 versus miR-NC group) but not MUT-CDK4 (P>0.01); D: The mRNA and protein levels of CDK4 were decreased by miR-486-5p mimic, but they were increased by miR-486-5p inhibitor (**P<0.01 versus miR-NC group); E: The mRNA and protein levels of CDK4 after transfecting siPCAT7 and miR-486-5p inhibitor (**P<0.01). CDK4: Cyclin dependent kinase 4, WT: Wild type, MUT: Mutant, PCAT7: prostate cancer associated transcript 7, NSCLC: Non-small cell lung cancer.

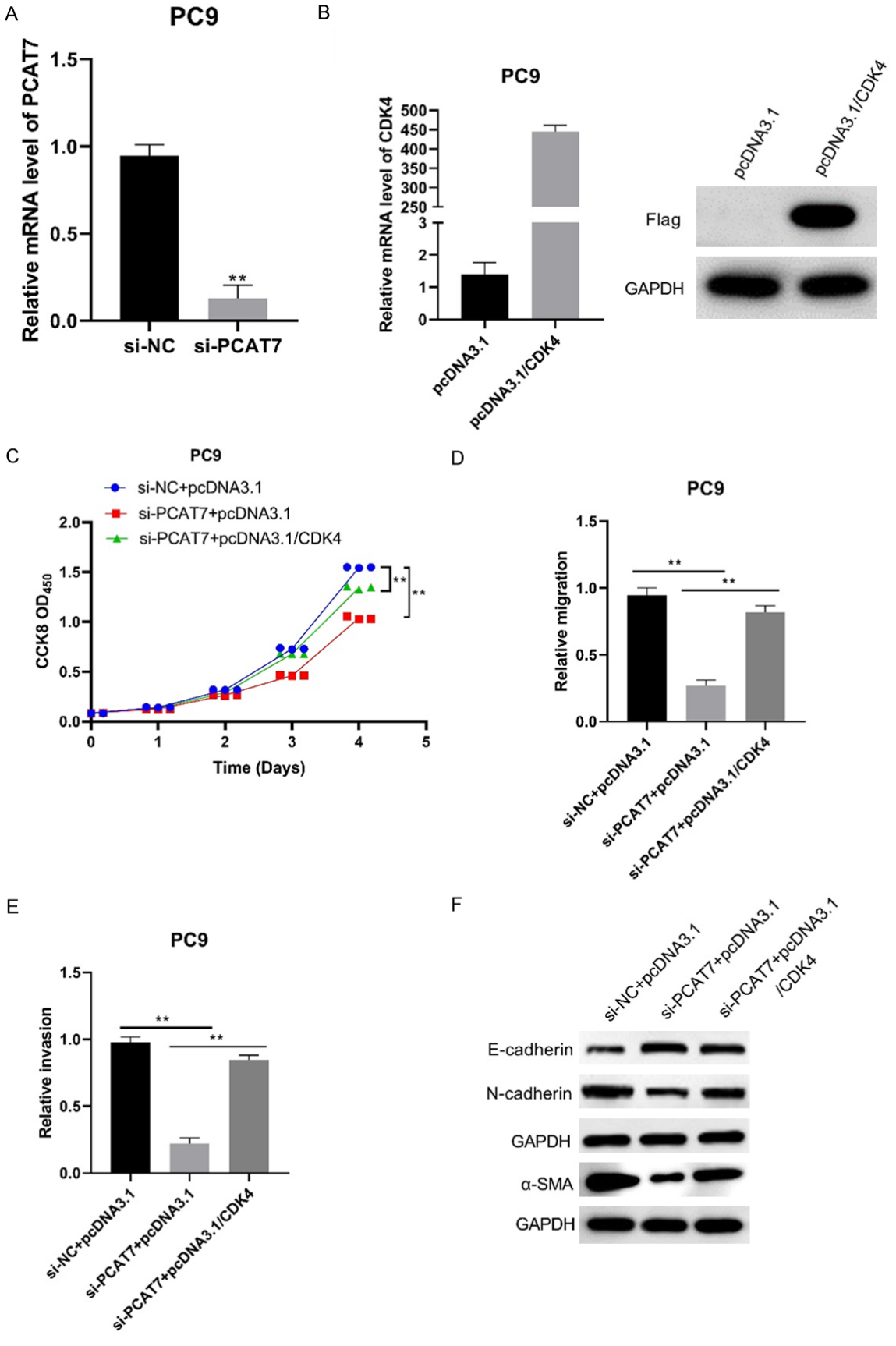
A
PC9
Relative mRNA level of PCAT7
si-NC
si-PCAT7
B
PC9
Relative mRNA level of CDK4
pcDNA3.1
pcDNA3.1/CDK4
Flag
GAPDH
C
PC9
si-NC+pcDNA3.1
si-PCAT7+pcDNA3.1
si-PCAT7+pcDNA3.1/CDK4
CCK8 OD450
Time (Days)
D
PC9
Relative migration
E
PC9
Relative invasion
F
E-cadherin
N-cadherin
GAPDH
α-SMA
GAPDH

Figure 6. CDK partially eliminates the effects of PCAT7 on the progression of NSCLC cells. A: The expression level of PCAT7 (**P<0.01 versus si-NC group); B: The expression of CDK4 at mRNA and protein levels (**P<0.01 versus pcDNA3.1 group); C: CDK4 partially increased the viability of PC9 cells, which was decreased by siPCAT7 according to CCK8 assay (**P<0.01); D, E: CDK4 partially restored the ability of migration as well as invasion of PC9 cells, which was suppressed by silencing PCAT7 (**P<0.01); F: Proteins related to EMT progress were estimated. CDK4: Cyclin dependent kinase 4, PCAT7: Prostate cancer associated transcript 7, NSCLC: Non-small cell lung cancer, CCK8: Cell counting kit 8, EMT: Epithelial-mesenchymal transition.

In recent studies, LncRNAs have been found to control the transcription of a series of genes combined with sponge microRNA [21]. LncRNAs correlate with cellular progressions such as cell cycle, differentiation and tumorigenesis [22]. Interestingly, the disorder of LncRNA expression is involved in human cancers which contribute to dysregulating the proliferation, migration and invasion of cancer cells. For instance, high regulation of CCAT1 depresses the expression of TP53G1 in ESCC, and some LncRNAs are found to express abnormally in other cells. For example, LncRNA AB074169 regulates papillary thyroid cancer cell progression by modulating CDKN1. LncRNA MIR31HG overexpression obviously suppressed the susceptibility of NSCLC cells to gefitinib by mediating the EGFR/PI3K/AKT axis. LncRNA XIST promotes NSCLC development by mediating miR-449a and Bcl-2 [23-25]. PCAT7 is regarded as an upregulated LncRNA in NSCLC and received more attention recently [8]. In this research, we found that the expression of PCAT7 was increased significantly in NSCLC tissues and cells. In addition, knockdown of PCAT7 could effectively inhibit proliferation, migration, invasion as well as EMT progress of NSCLC cells. These findings effectively associated PCAT7 with NSCLC. Nevertheless, silencing PCAT7 did not significantly affect the apoptosis of NSCLC cells, and how PCAT7 causes cell death still needs to be further studied.

MiRNAs are the well-studied potent targets of LncRNAs which control a series of progression of tumor cells such as proliferation, cell cycle and differentiation, and they possess the capacity to regulate post-transcriptional expression of downstream genes [26]. Recently, increasing studies have revealed that multiple microRNAs are disordered in NSCLC and related to a train of events of NSCLC, among which miR-486-5p is the important leading microRNA in LUAD [27, 28]. The miR-486-5p in peripheral blood and sputum has been regarded as a significant diagnosis marker of NSCLC. Its downregulation is correlated with inhibition of the development of NSCLC [29]. Consistent with previous studies, our research showed that miR-486-5p was the direct target of LncRNA PCAT7 and had a negative relationship with PCAT7. MiR-486-5p was downregulated in NSCLC cells and increased by silencing PCAT7. In addition, miR-486-5p mimic partially restored the inhibitory effects of PCAT7 knockdown on NSCLC cells' progression. For example, miR-486-5p inhibition notably inhibited the viability, migration, invasion and EMT progression upregulated by silencing PCAT7. All these results demonstrated that LncRNA PCAT7 can promote proliferation, migration, invasion as well as EMT of NSCLC cells *via* targeting sponge miR-486-5p.

Studies have reported that LncRNAs could combine with microRNAs to target and regulate downstream genes as well as their corresponding pathway to further affect the progression of cancers [30]. CDK4, an oncogene, has been found to exert vital effects on the cell cycle and miR-486-5p to downregulate the development of NSCLC [31]. Therefore, a series of experiments were conducted to confirm whether CDK4 is involved in the progression of PCAT7-regulated NSCLC cells. In this study, we discovered that CDK4 was the direct target of miR-486-5p and suppressed by transfecting miR-486-5p mimic dramatically. Interestingly, silencing PCAT7 was able to decrease the expression of CDK4, while transfecting miR-486-5p inhibitor could partially eliminate the effects induced by knockdown of PCAT7. Consistently, co-transfecting siPCAT7 and CDK4 could partially restore attenuated proliferation, migration, invasion and EMT of PC9 cells. Given that CDK4 is a crucial protein involved in cell cycle, we measured the effect of PCAT7 combined with miR-486-5p or CDK4 on NSCLC cell cycle. The results indicated that knockdown of PCAT7 arrested cell cycle to S and G2 phases, while overexpression of miR-486-5p inhibitor or CDK4 effectively restored cell cycle progression to G1 phase. Moreover, PCAT7 knockdown obviously reduced CyclinD1 expression but

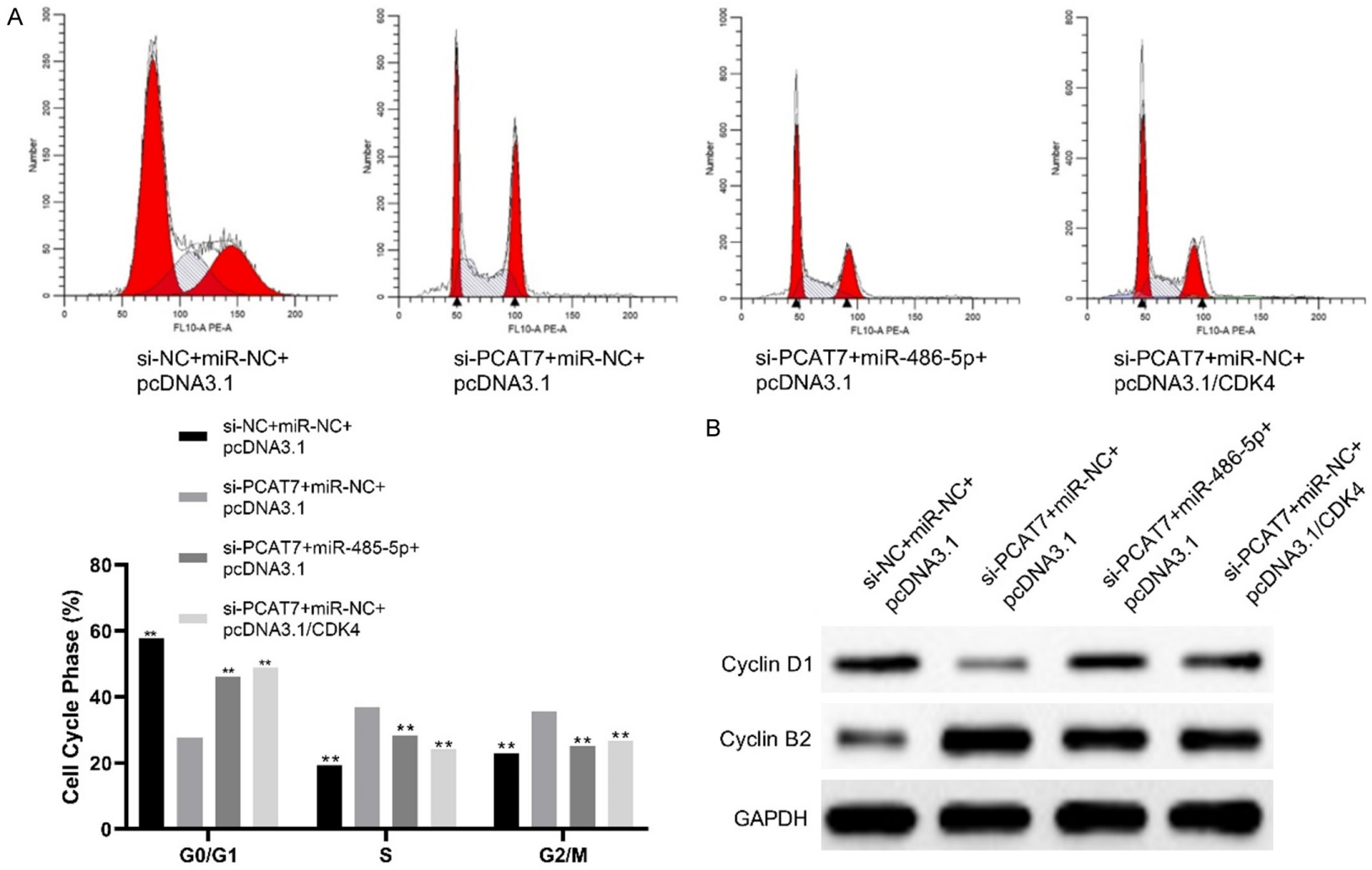

Figure 7. PCAT7 regulates the progression of NSCLC cells by activating cell cycle mediated by miR-486-5p/CDK4 axis. A: Silencing PCAT7 arrested cells to S and G2 phases while transfecting miR-486-5p inhibitor or CDK4 partially eliminated the effect (**P<0.01 versus si-PCAT7+miR-NC+pcDNA3.1 group); B: Proteins involved in cell cycle were measured after indicated transfection. PCAT7: Prostate cancer associated transcript 7, CDK4: Cyclin dependent kinase 4, NSCLC: Non-small cell lung cancer.

increased CyclinB2 expression. All data illustrated that PCAT7 promoted the proliferation, migration, invasion and EMT progression by accelerating NSCLC cell cycle regulated by miR-486-5p/CDK4.

There are some limitations in our study. For example, we only explored the role and mechanism of LncRNA PCAT7 on NSCLC cells, so more experiments are needed to investigate whether PCAT7 could exert vital effects on animals. Additionally, whether PCAT7 could serve as a sponge gene of miR-486-5p will be studied in our future experiments. The subsequent studies will perform a series of experiments to verify how PCAT7 affects the NSCLC cell cycle. Whether PCAT7 causes apoptosis and how it causes cell death also need to be studied in the future.

In conclusion, our study, for the first time, demonstrated that PCAT7 could promote the development of NSCLC by triggering the cell cycle mediated by miR-486-5p/CDK4 pathway and uncovered its relevant mechanism. It could be a novel therapeutic target for treating NSCLC.

Disclosure of conflict of interest

None.

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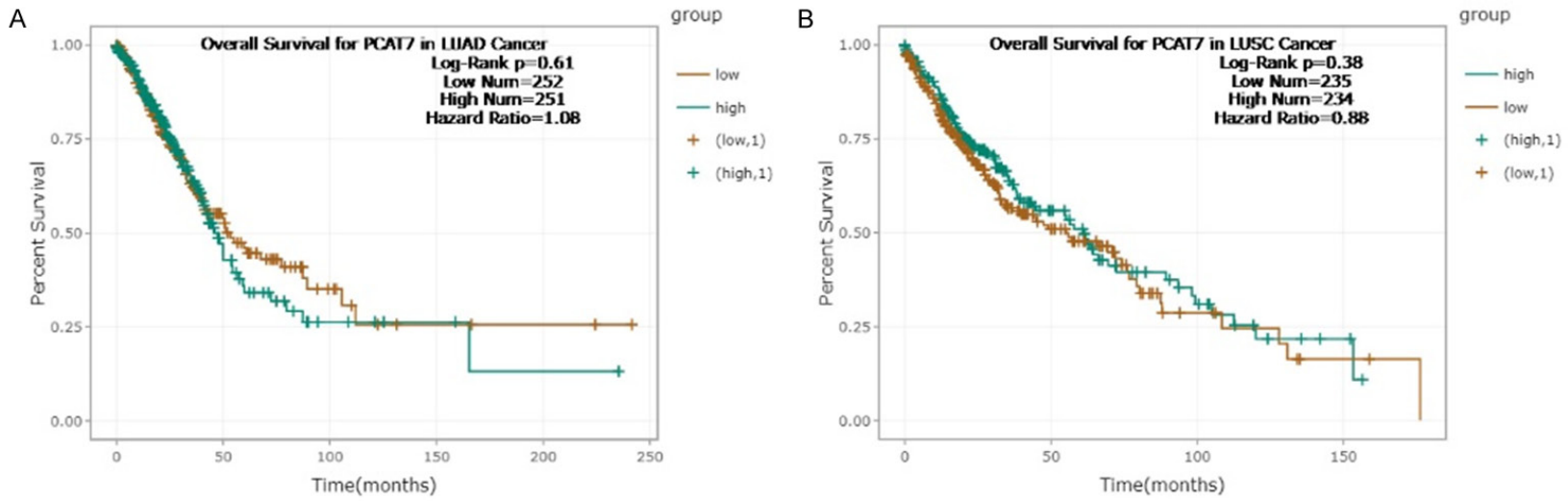

Figure S1. The overall survival for PCAT7 in LUAD or LUSC. A: The overall survival for PCAT7 in LUAD obtained from starBase database (http://starbase.sysu.edu.cn/agoClipRNA.php?source=lncRNA&flag=miRNA&clade=mammal&genome=human&assembly=hg19&miRNA=hsa-miR-337-3p&clipNum=&deNum=&panNum=&target=); B: The overall survival for PCAT7 in LUSC obtained from starBase database (http://starbase.sysu.edu.cn/agoClipRNA.php?source=lncRNA&flag=miRNA&clade=mammal&genome=human&assembly=hg19&miRNA=hsa-miR-337-3p&clipNum=&deNum=&panNum=&target=). PCAT7: Prostate cancer associated transcript 7, LUAD: Lung adenocarcinoma, LUSC: Lung squamous cell carcinoma.

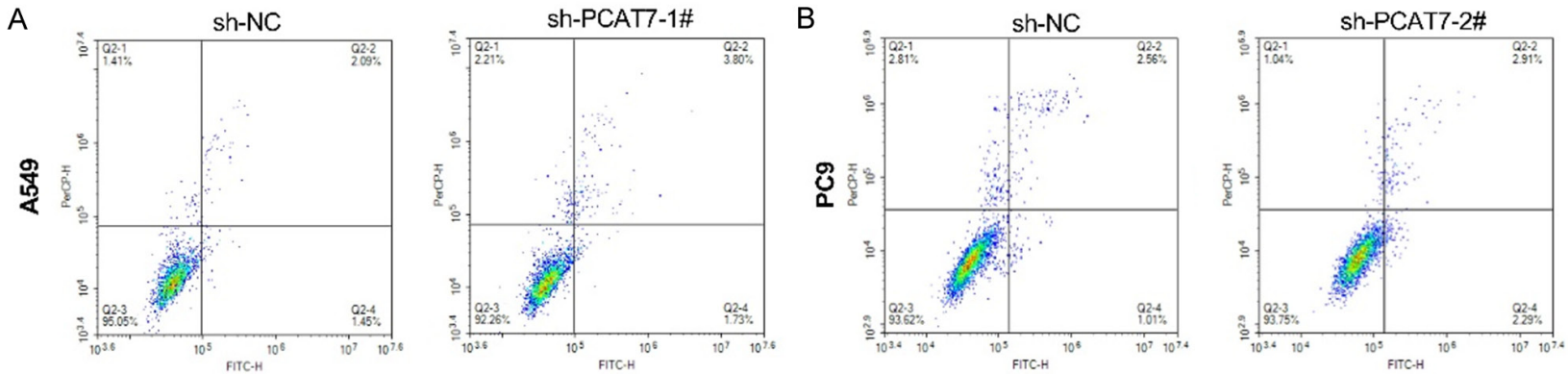

Figure S2. The cell apoptosis rate affected by PCAT7 knockdown. A: The apoptosis rate of A549 cells affected by PCAT7 knockdown; B: The apoptosis rate of PC9 cells affected by PCAT7 knockdown. PCAT7: Prostate cancer associated transcript 7.

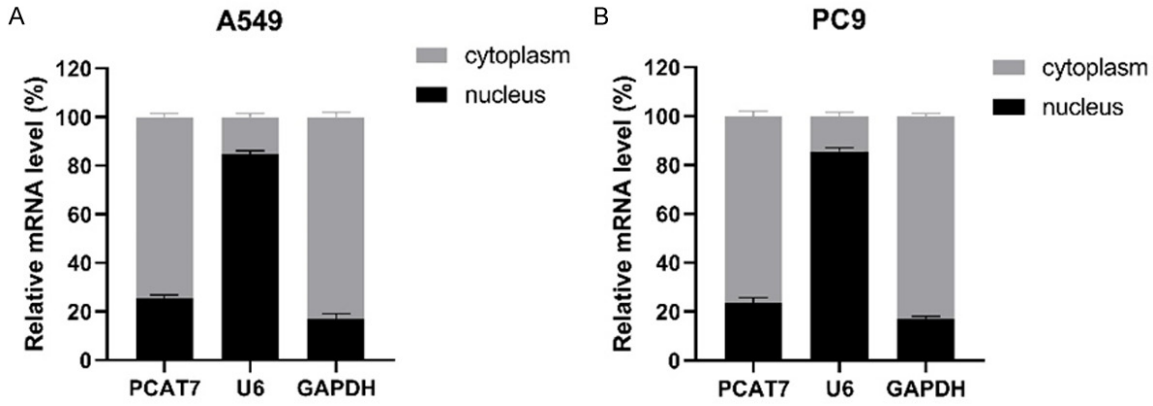

Figure S3. The subcellular localization of PCAT7 in A549 and PC9 cells. A: The subcellular localization of PCAT7 in A549 cells; B: The subcellular localization of PCAT7 in PC9 cells. PCAT7: Prostate cancer associated transcript 7.