

RETRACTED ARTICLE: LncRNA MCM3AP-AS1 Promotes Cell Proliferation and Invasion Through Regulating miR-543-3p/SLC39A10/PTEN Axis in Prostate Cancer

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Objective: Long-chain noncoding RNAs (lncRNAs) are key players in a wide range of biological processes, especially the pathogenesis and development of tumors. LncRNA MCM3AP-AS1 has been demonstrated to be involved in the invasion of various tumors including prostate cancer (PCa). However, its functions in PCa have not been fully elucidated.

Methods: qRT-PCR was conducted to measure the expression levels of lncRNA MCM3AP-AS1 and miR-543-3p in PCa tissue samples and cell lines. The expression levels of E-cadherin and SLC39A10 proteins were detected by Western blots. CCK-8 test, cell scratch test and trans-well test were used to evaluate the proliferation, invasion and migration abilities of PCa cells, respectively. Annexin V-FITC/PI experiments were carried out to determine the status of apoptosis. Bioinformatics analysis and Luciferase assay were used to explore the relationship between lncRNA MCM3AP-AS1, miR-543-3p and SLC39A10.

Results: In PCa tissue samples and cell lines, lncRNA MCM3AP-AS1 was up-regulated while miR-543-3p was down-regulated. Over-expression of MCM3AP-AS1 could promote the proliferation and invasion of PCa cells. Correlation analysis showed that the expression of MCM3AP-AS1 and miR-543-3p was significantly and inversely correlated. We further verified that miR-543-3p inhibitor was able to reverse si-MCM3AP-AS1-mediated inhibitory effects on the PCa cell proliferation, migration and invasion through regulating the downstream protein axis SLC39A10/PTEN/Akt. Finally, in vivo experiments indicated that knocking down of MCM3AP-AS1 could largely reduce tumor volumes, and decreased the ratio of Ki67-positive cells and the expression of SLC39A10 in tumor samples.

Conclusion: LncRNA MCM3AP-AS1 can promote the proliferation, migration and invasion abilities of PCa cells through regulating the miR-543-3p/SLC39A10/PTEN axis, which suggests that lncRNA MCM3AP-AS1 might be a potential target for prostate cancer therapy.

Keywords: lncRNA MCM3AP-AS1, miR-543-3p, SLC39A10, PTEN, prostate cancer

Introduction

Prostate cancer (PCa) is the one of most common heterogeneous tumors occurring among males worldwide.¹ Also, PCa is the second most fatal malignant disease in men, of which the incidence increases with age.² Early-stage PCa patients can usually benefit from tumor resection and radiotherapy.³ However,

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the 5-year survival rate and life quality of other patients at late-stage PCa are markedly affected.⁴ Thus, carrying out in-depth research on PCa is of great significance to the health and survival rate. Previous studies have established that the occurrence of PCa is largely related with the dysregulation of multiple molecules, including proteins, microRNAs (miRNAs) and long non-coding RNAs (lncRNAs).⁵

In recent years, extensive studies have investigated the functions of non-coding RNAs.⁶ It is reported that non-coding RNAs are a class of RNA molecules that do not encode proteins and can regulate gene expression at different levels.^{7,8} Among non-coding RNAs, lncRNA transcripts are over 200 nucleotides in length and accounting for 98% of total RNAs.⁹ However, biological functions of most lncRNAs are complex, which are involved in various regulatory processes (eg, genomic imprinting, chromatin modification and intranuclear transport).¹⁰ Moreover, it has been discovered that some lncRNAs play important roles in the occurrence and development of PCa, such as PCAT29,¹¹ GAS5,¹² PCGEM1¹³ and MCM3AP. LncRNA MCM3AP is located on human chromosome 21q22.3 and regulates protein expression in various human diseases. Besides, lncRNA MCM3AP-AS1 is an RNA molecule with the reverse sequence of MCM3AP gene.¹⁴ One study reported that the expression of lncRNA MCM3AP-AS1 was elevated in glioma microvascular endothelial cells and knocking down MCM3AP-AS1 could suppress the viability and migration of glioblastoma cells.¹⁵ Furthermore, MCM3AP-AS1 was reported to facilitate PCa development in vivo and knock-down of MCM3AP-AS1 was able to inhibit the proliferation, invasion and migration of PCa cells via methylation of DNMT1/DNMT3.¹⁶ Similarly, in our preliminary experiments, we found that the expression of lncRNA MCM3AP-AS1 was dysregulated in PCa cells. However, detailed exploration of the functions of MCM3AP-AS1 and the underlying regulatory mechanism are urgently needed.

MicroRNAs (miRNAs), a group of non-coding RNAs that are 19–24 nt in length, play regulatory roles in the process of basic metabolism, differentiation, proliferation, survival and cell death.¹⁷ MiR-543-3p functions as a tumor growth inhibitory effector in lung cancer cells and pancreatic cancer cells.¹⁸ Our previous bioinformatics experiments revealed that lncRNA MCM3AP-AS1 might bind with miR-543-3p, and miR-

543 could bind with its downstream regulatory factor SLC39A10, which is a zinc transporter essential for cell survival in PCa.¹⁹ However, the roles that MCM3AP-AS1/miR-543-3p/SLC39A10 axis play in PCa are elusive. Therefore, our study focuses on the interactions among lncRNA MCM3AP-AS1, miR-543-3p and SLC39A10, as well as possible downstream signaling pathways involved in PCa. Our findings may provide a new therapeutic target for PCa.

Methods

Tissue Samples

Sixty-four pairs of PCa tissue samples and adjacent normal tissues were collected from the First Affiliated Hospital and College of Clinical Medicine of Henan University of Science and Technology between March 2016 and June 2018. The study was approved by the Ethics Committees of the First Affiliated Hospital and College of Clinical Medicine of Henan University of Science and Technology, and was carried out in accordance with the principles of the Declaration of Helsinki. All patients signed the written informed consent. Tissue samples were stored at -80°C before use.

Cells and Transfection

Human PCa cell lines (PC-3, DU145, 22RV1, LNCaP) and normal follicular epithelial cell line (WPMY-1) were provided by ATCC, USA. Cells were incubated in RPMI 1640 with 10% FBS at 37°C. Control siRNA (si-NC), si-MCM3AP-AS1, miR-543-3p mimics, miR-543-3p inhibitor, pcDNA3.1, pcDNA3.1- MCM3AP-AS1, control miRNA, lentivirus-sh-MCM3AP-AS1 (LV-sh-MCM3AP-AS1) and control lentivirus-sh (LV-shRNA-NC) were made by GenePharma, China, and transfected to cells using Lipofectamine 2000 (Invitrogen, USA).

BLAST

Alignment searches were conducted with NCBI's BLAST suite. Top result with value less than 0.01 was recorded. Through analysis, we selected miR-543-3p, which was highly bound to MCM3AP-AS1, for subsequent experiments.

qRT-PCR

The miRNeasy Mini Kit (Qiagen, USA) was used to extract RNAs from tissue samples and cells. The concentration and quality of RNAs were evaluated by

NanoDrop 2000 (Thermo Fisher, USA). First-strand cDNA was synthesized by SuperMix (TransGen, China) following the manufacturer's instructions. RT-qPCR analysis was conducted by SYBR SuperMix (Applied Biosystems, USA). The conditions were: 40 cycles at 55°C for 10 min, 95°C for 30s, 55–59°C for 30 s, and 72°C for 42 s. The fold changes were calculated by $2^{-\Delta\Delta Ct}$ method. The expression of MCM3AP-AS1 and miR-543-3p was normalized to GAPDH and U6, respectively. Primer sequences were as follows: MCM3AP-AS1, 5'-TGGGATTCAGACGCTAACGC-3' (forward) and 5'-TCCACAGCATCTTTGGCACC-3' (reverse); miR-543-3p, 5'-ATGCCTCGACCACAATCAGA-3' (forward) and 5'-AGGATGCCGGTACTACTCGAT-3' (reverse); GAPDH, 5'-TCGACAGTCAGCCGCATCTTCTTT-3' (forward) and 5'-ACCAAATCCGTTGACTCGACCTT-3' (reverse); U6, 5'-GCTTCGGCAGCACATATACTAAAAT-3' (forward) and 5'-CGCTTCACGAATTTGCGTGTTCAT-3' (reverse).

RNA Pull-Down Assay

For miRNA pull-down assay, PC-3 and LNCaP cells were transfected with biotinylated miR-543-3p (miR-543-3p probe) or control probe (Genescript, Nanjing, China) and harvested in lysis buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM MgCl₂, 0.5% NP-40 and 1 mM DTT) with 100 μL Recombinant RNase inhibitor (TaKaRa). Total RNAs were pretreated with DNaseI and then heated at 65°C for 5 min, followed by an instant ice bath. Afterwards, the RNAs were incubated with streptavidin-coated magnetic beads (New England BioLabs, S1420S) at 4°C for 4 h. After incubation, beads were washed twice with lysis buffer and RNAs were extracted with Trizol (Invitrogen, CA, USA). The expression of lncRNA MCM3AP-AS1 was detected by RT-qPCR.

Luciferase Reporter Assays

MCM3AP-AS1 cDNA with miRNA were amplified and cloned into pmirGLO plasmids (Promega, USA) to construct pmirGLO-MCM3AP-AS1. MiR-543-3p mimics or control mi-RNA were co-transfected with pmirGLO-MCM3AP-AS1 or mutant pmirGLO-MCM3AP-AS1 (pmirGLO-MCM3AP-AS1-MUT) into PC-3 or LNCaP cells by Lipofectamine 2000 (Invitrogen). After transfection for 48 h, luciferase activities were detected through Dual-Luciferase Assay.

RNA Binding Protein

Immunoprecipitation Assay

The interaction between miR-543-3p and SLC39A10 was assessed using RIP kit (Merck Millipore Darmstadt, Germany). The cells were washed with pre-cooled PBS and the supernatant was discarded. Cells were then lysed using equal volume of RIPA lysis in ice bath for 5 min and centrifuged at 2000 × g for at 4°C 10 min to collect the supernatant. Subsequently, the supernatant was incubated with an SLC39A10 antibody for co-precipitation. Specifically, 50 μL magnetic beads of each co-precipitation system were washed with cold PBS, and resuspended in 100 μL RIP Wash Buffer, and incubated with 5 μg SLC39A10 antibody (1:50) and IgG (1:100) separately. Next, the bead-antibody complex was rinsed and resuspended in 900 μL RIP Wash Buffer, and incubated with 100 μL cell extract at 4°C overnight. The samples were then placed on the bead pedestal to collect the complex of bead protein. After the samples were treated with protease-K, RNAs were extracted for RT-PCR.

Cell Proliferation Assay

Cell proliferation rate was detected by Cell Counting Kit-8 (CCK-8; Dojindo, JPN). PC-3 and LNCaP cells were transfected with si-NC, si-MCM3AP-AS1, miR-543-3p inhibitor or si-MCM3AP-AS1 + miR-543-3p inhibitor, harvested and seeded to 96-well plates (1×10^3 cells/well). After 24, 48, 72 or 96 h, 10 μL of CCK-8 assay reagent was added to each well, and cells were incubated for 2 h and an enzyme immunoassay analysis (Bio-rad, Hercules, CA, USA) was performed. For cell colony formation assay, cells (500 cells/well) were plated in 6-well plates and incubated in DMEM with 10% of bovine calf serum at 37°C. After two weeks, cells were fixed and stained with 0.1% of crystal violet. Finally, the number of visible colonies was manually counted.

Wound Healing Assays and Trans-Well Assays

In wound healing assay, cells were seeded in 6-well plate. The culture medium was removed and cells were scraped by a pipette tip. After incubation for 24 h, the wound widths were measured and calculated as percentage. In trans-well assay, chambers were placed with Matrigel. After incubation, cells on the upper chamber were taken off by cotton. The remaining cells were fixed and stained by violet crystalline.

Western Blots

Cells and tissues were lysed in ice-cold RIPA lysis buffer (Sigma, USA). Cell or tissue lysates were centrifuged and the concentration of proteins was determined by BCA assay. Total proteins were separated by SDS-PAGE and then transferred to PVDF membranes (Millipore, USA). The membranes were blocked by skim milk and incubated with anti-SLC39A10 (1:1000, Abcam) or anti-GADPH (1:1000, Abcam). After primary antibody incubation for overnight, membranes were incubated with secondary goat anti-rabbit IgG (Abcam) at room temperature for 1 h. Signals were detected by ECL.

Cell Cycle Analysis by Flow Cytometry

Cells were transfected with the indicated siRNA for 72 h, washed with PBS, fixed in 50% ethanol, incubated with RNAase cocktail (Ambion) for 30 min, and stained with propidium iodide (100 $\mu\text{g/mL}$). The stained cells were subjected to flow cytometry.

Flow Cytometric Analysis of Cell Apoptosis

Apoptotic rate was examined using Annexin V-FITC apoptosis detection kit (Beyotime Biotechnology, China) following the manufacturer's instructions. Briefly, the PCa cells were washed twice with PBS, and harvested by Trypsin without EDTA. Afterwards, the cells were resuspended in 195 μL binding buffer, 5 μL Annexin V-FITC reagent and 10 μL propidium iodide (1 mg/mL) reagent, and incubated for 15 min at room temperature in the dark. The cell apoptosis was detected by flow cytometry (BD FACSCalibur cytometer, Becton Dickinson, San Jose, CA, USA).

Lentivirus Constructions

Lentiviral vector of MCM3AP-AS1-shRNA was made by Genechem, China. MCM3AP-AS1-siRNA was sub-cloned to pFU-GW-RNAi vector. PC-3 cells were seeded into 6-well plates. After incubation at 37°C with 5% CO₂ for 12 h, PC-3 cells were infected with LV-siRNA-NC or LV-siRNA-MCM3AP-AS1.

In vivo Experiments

In vivo experiments were approved by the Institutional Animal Care and Use Committee of the First Affiliated

Hospital and College of Clinical Medicine of Henan University of Science and Technology, and all procedures were in accordance with Council Directive 2010/63 of the European Union. Cells infected with LV-si-MCM3AP-AS1 or LV-si-NC were administered into 6-week-old BALB/c mice. Tumor growth and tumor volume were measured every 3 d.

Immunohistochemistry

Tumor tissues were dried, dewaxed and rehydrated. After washing with PBS, tissues were blocked and incubated with Ki67 antibody (Bioss, 1:200) at 4°C overnight. And then, the tissues were treated with HRP DAB (ZSGB-BIO, China) at 25°C for 20 min followed by staining with hematoxylin.

Statistical Analysis

The data were expressed as mean \pm SD. Student's *t*-test (two-tailed) was used for comparisons between two groups. One-way analysis of variance (ANOVA) was used for multi-group comparisons followed by Bonferroni post hoc analysis. The correlation of the measured data was analyzed by Pearson correlation analysis. P value less than 0.05 was regarded as statistically significant.

Results

LncRNA MCM3AP-AS1 was Upregulated in Patients with PCa

Tumor tissue samples and adjacent healthy tissue samples from 64 PCa patients were selected for comparison. It was shown that the expression levels of lncRNA MCM3AP-AS1 were markedly increased in PCa patient samples compared to that in healthy tissue samples (Figure 1A). Based on the average expression levels of lncRNA MCM3AP-AS1, all patients were divided into high expression group and low expression group (Figure 1B). Long-term follow-up showed that the long-term survival rate of the high expression group was markedly decreased than that of the low expression group (Figure 1C). In our further analysis, the baseline data suggested that there were great differences between the two groups in Gleason scores for N staging (Table 1). Cox regression analysis also showed similar results (Table 2).

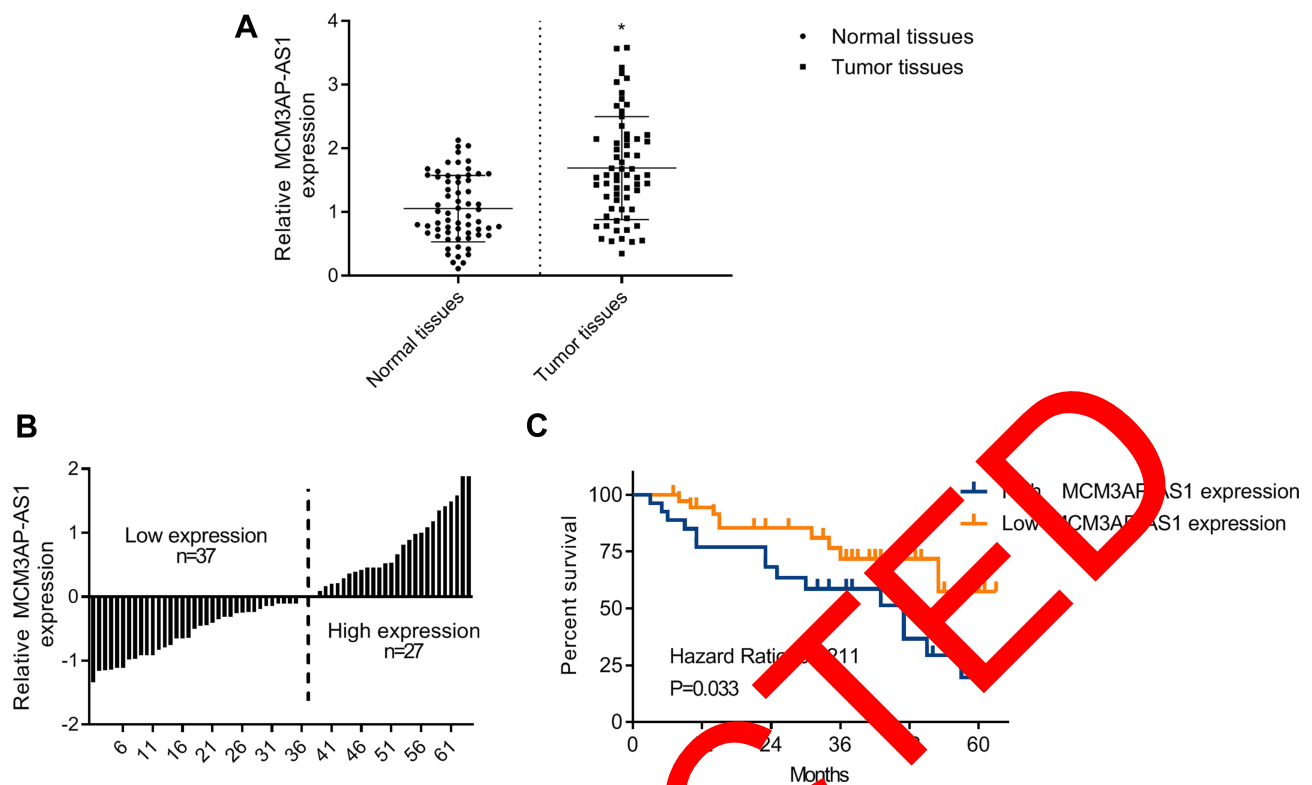


Figure 1 LncRNA MCM3AP-AS1 markedly upregulated in patients with PCa. **(A)** LncRNA MCM3AP-AS1 was highly expressed in PCa patient tumor tissues than that in normal tissues. **(B)** Histogram of PCa with high LncRNA MCM3AP-AS1 expression group and low LncRNA MCM3AP-AS1 expression group. A total of 37 cases were subgrouped into low LncRNA MCM3AP-AS1 expression group, and 27 cases were subgrouped into high LncRNA MCM3AP-AS1 expression group. **(C)** Survival curve of PCa in high LncRNA MCM3AP-AS1 expression group and low LncRNA MCM3AP-AS1 expressions group. Patients with high LncRNA MCM3AP-AS1 expression had poorer survival. * $p < 0.05$.

Knockdown of LncRNA MCM3AP-AS1 Markedly Inhibited Tumor Proliferation and Metastasis

In PCa cell lines (PC-3, DU145, LNCaP and LNCaP), the expression level of LncRNA MCM3AP-AS1 were significantly increased compared to that in normal human prostate epithelial cells (HPME1) (Figure 2A). PC-3 and LNCaP cells were selected for subsequent experiments because they had the greatest increase. Three siRNAs were designed for knockdown of LncRNA MCM3AP-AS1, and si-MCM3AP-AS1-1 had the highest knockdown efficiency (Figure 2B). In addition, si-MCM3AP-AS1 could inhibit tumor cell proliferation which was detected by CCK-8 assay (Figure 2C) and colony formation assay (Figure 2D), and migration was evaluated by wound healing assay (Figure 2E) and invasion was determined by transwell assay (Figure 2F).

MiR-543-3p is a Regulatory Target for LncRNA MCM3AP-AS1

Bioinformatics analysis showed that LncRNA MCM3AP-AS1 had a binding site with miR-543-3p (Figure 3A). To validate that miR-543-3p suppressed the expression of MCM3AP-AS1 by directly binding to the 3'-UTR of MCM3AP-AS1, RNA pull down assay and luciferase reporter assay were conducted. Firstly, a biotin-avidin pull-down assay was conducted to evaluate the direct binding of miR-543-3p to MCM3AP-AS1. After transfection with miR-543-3p probe into PC-3 or LNCaP cells for 48 h, streptavidin-coated magnetic beads were used to pull down biotinylated miR-543-3p and the expression of co-precipitated LncRNA MCM3AP-AS1 was measured by RT-qPCR. LncRNA MCM3AP-AS1 could only be precipitated by miR-543-3p probe and was undetectable in the product precipitated by control probe (Figure 3B), indicating that miR-543-3p could

Table 1 Correlation Between lncRNA MCM3AP-AS1 Expressions and Prostate Cancer Patients

Variables	Low lncRNA MCM3AP-AS1	High lncRNA MCM3AP-AS1	P value
Age (yrs)	57.8±7.6	56.4±8.8	0.814
Tumor TNM stage			0.018
I	17 (53.1%)	7 (21.9%)	
II	10 (31.3%)	16 (50.0%)	
III	5 (15.6%)	9 (28.1%)	
T stage			0.341
T1	9 (28.1%)	7 (21.9%)	
T2	19 (59.3%)	18 (56.3%)	
T3	3 (9.4%)	5 (15.6%)	
T4	1 (3.1%)	2 (6.3%)	
N stage			0.019
N0	22 (68.8%)	12 (37.5%)	
N1	8 (25.0%)	16 (50.0%)	
N2	2 (6.3%)	4 (12.5%)	
Gleason score			0.027
2–5	15 (46.9%)	10 (31.3%)	
6–8	13 (40.6%)	17 (53.1%)	
9–10	4 (12.5%)	5 (15.6%)	

directly interact with lncRNA MCM3AP-AS1 in PC-3 and LNCaP cells.

To evaluate whether miR-543-3p can directly bind to lncRNA MCM3AP-AS1, two luciferase reporter plasmids containing *MCM3AP-AS1* 3'UTR or *MCM3AP-AS1* mutant cDNA were constructed and co-transfected with miR-543-3p mimic or miR-NC mimics into PC-3 or LNCaP cells for 48 h. As expected, overexpression of miR-543-3p induced approximately 70% reduction in luciferase reporter activity in PC-3 and 60% reduction in LNCaP compared to cells transfected with the control

mimic (Figure 3C), whereas miR-543-3p did not affect the mutated reporter activity (Figure 3C).

In order to determine whether miR-543-3p can affect the transcription of *MCM3AP-AS1*, RT-qPCR was used to evaluate the expression levels of lncRNA MCM3AP-AS1 in PCa cells transfected with control mi-RNA, miR-543-3p mimics or miR-543-3p inhibitor. The results showed that miR-543-3p mimics could decrease *MCM3AP-AS1* transcription to lncRNA MCM3AP-AS1, while miR-543-3p inhibitor reversed this effect (Figure 3D). In addition, knockdown of *MCM3AP-AS1* strongly induced the expression of miR-543-3p in PCa cells (Figure 3E). It was also found that the expression of *MCM3AP-AS1* and miR-543-3p were inversely correlated (Figure 3F).

According to bioinformatics analysis, miR-543-3p might bind to SLC39A10 protein (Figure 3G). RIP assay results suggested that miR-543-3p could directly interact with SLC39A10 (Figure 3H), and RT-qPCR results exhibited a significant reduction in the expression of SLC39A10 by knockdown of MCM3AP-AS1 (Figure 3I). The correlation between MCM3AP-AS1 and SLC39A10 was also analyzed and a positive correlation with each other was observed (Figure 3J).

lncRNA MCM3AP-AS1 Regulates the Proliferation and Migration of PCa Cells Through Targeting miR-543-3p

Further cellular experiments discovered the inhibitory effects of si-MCM3AP-AS1 on tumor cell proliferation by CCK-8 assay (Figure 4A) and colony formation assay (Figure 4B), migration by cell scratches (Figure 4C), and invasion by Trans-well experiment (Figure 4D). However, those inhibitory effects could be reversed by 543-3p-inhibitor. Interestingly, the same was true for the expression of SLC39A10, and the regulatory signaling pathway involved in this mechanism might be PTEN/Akt (Figure 4E). On the other hand, MCM3AP-AS1 was overexpressed to detect the above-mentioned PCa cell behaviors. The results showed that overexpression of MCM3AP-AS1 significantly promoted PCa cell proliferation (Supplementary Figure A), migration (Supplementary Figure B) and invasion (Supplementary Figure C).

Si-MCM3AP-AS1 Affects Cell Cycle but Not Cell Apoptosis

To further clarify the function of lncRNA MCM3AP-AS1 /miR-543-3p in PCa cells, si-MCM3AP-AS1 or/and 543-

Table 2 Cox Multivariate Regression Analysis

Factors	P value	HR	95% CI
lncRNA MCM3AP-AS1 expression	0.023	1.538	1.050–2.274
Age	0.212	1.765	0.603–4.656
TNM stage	0.008	1.652	1.108–3.052
T stage	0.437	1.324	0.587–2.832
N stage	0.042	1.572	0.999–2.384
Gleason Score ≥6	0.032	1.427	0.875–2.547

Abbreviations: HR, hazard ratio; CI, confidence interval.

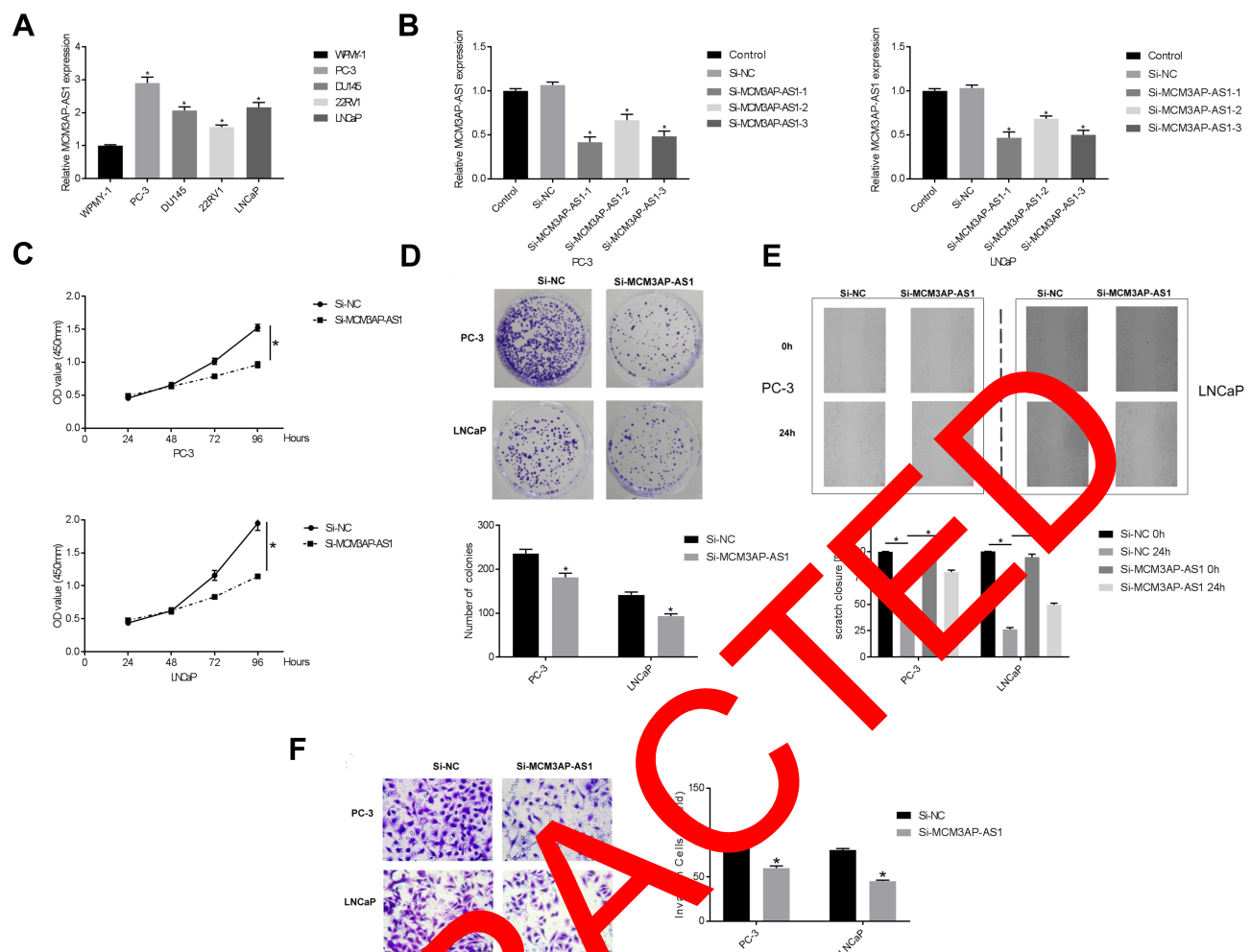


Figure 2 Knockdown of lncRNA MCM3AP-AS1 significantly inhibits proliferation and metastasis. (A) The expression of MCM3AP-AS1 in PC-3, DU145, 22RV1, LNCaP and WPMY-1 cell lines. PC-3 and LNCaP are two types of PCa cell lines with the highest expression levels of MCM3AP-AS1. (B) The expression of MCM3AP-AS1-1 in PC-3 and LNCaP with the transfection of control (untransfected cells), si-NC, si-MCM3AP-AS1-1, si-MCM3AP-AS1-2 and si-MCM3AP-AS1-3. si-MCM3AP-AS1-1 had the highest knockdown efficiency and then was used to conduct the subsequent experiments. (C) CCK-8 assay was carried out to detect cell viability under the transfection with si-NC or si-MCM3AP-AS1. Knocking-down of si-MCM3AP-AS1 significantly inhibit the proliferation of both PC-3 and LNCaP. (D) Colony formation assay of PCa cells was conducted under the transfection of si-NC or si-MCM3AP-AS1. The number of colonies was largely decreased after the transfection of si-MCM3AP-AS1 compared to si-NC. (E) Cell scratch tests applied for the evaluation of cell migration ability under si-NC or si-MCM3AP-AS1 transfection after 24 h of culture. Compared to si-NC, si-MCM3AP-AS1E markedly prohibited cell migration. (F) Trans-well experiments were conducted to assess cell invasion ability under the transfection with si-NC or si-MCM3AP-AS1. The number of invasive cells in si-MCM3AP-AS1 transfection group was much smaller than that in si-NC group. * $p < 0.05$.

3p-inhibitor were transfected/co-transfected into PC-3 or LNCaP. Next, we investigated the influence of silencing of lncRNA MCM3AP-AS1 or miR-543-3p inhibition on the cell cycle of above two types of PCa cells according to the cell cycle stage distribution (G1, S, G2/M). As shown in Figure 5A, there was an upregulation of the cell percentage at G1 phase and less S phase-arrested cells were detected, suggesting that suppression of MCM3AP-AS1 arrested cell cycle at G1 phase. Secondly, in order to verify whether lncRNA MCM3AP-AS1 has any effects on PCa cell apoptosis, Annexin V-FITC/PI test and flow cytometry were conducted. The proportions of apoptotic cells in 543-

3p-inhibitor group and si-MCM3AP-AS1+543-3p-inhibitor group were not markedly different from that in the control group (Figure 5B), indicating that lncRNA MCM3AP-AS1 did not affect PCa cell apoptosis.

Knocking Down lncRNA MCM3AP-AS1 Can Reduce Tumor Volume of PCa and the Expression of SLC39A10

The LV-sh-Control and LV-sh-MCM3AP-AS1 were constructed, and then intratumorally injected into mice. After 8 weeks, mice were sacrificed, and tumors

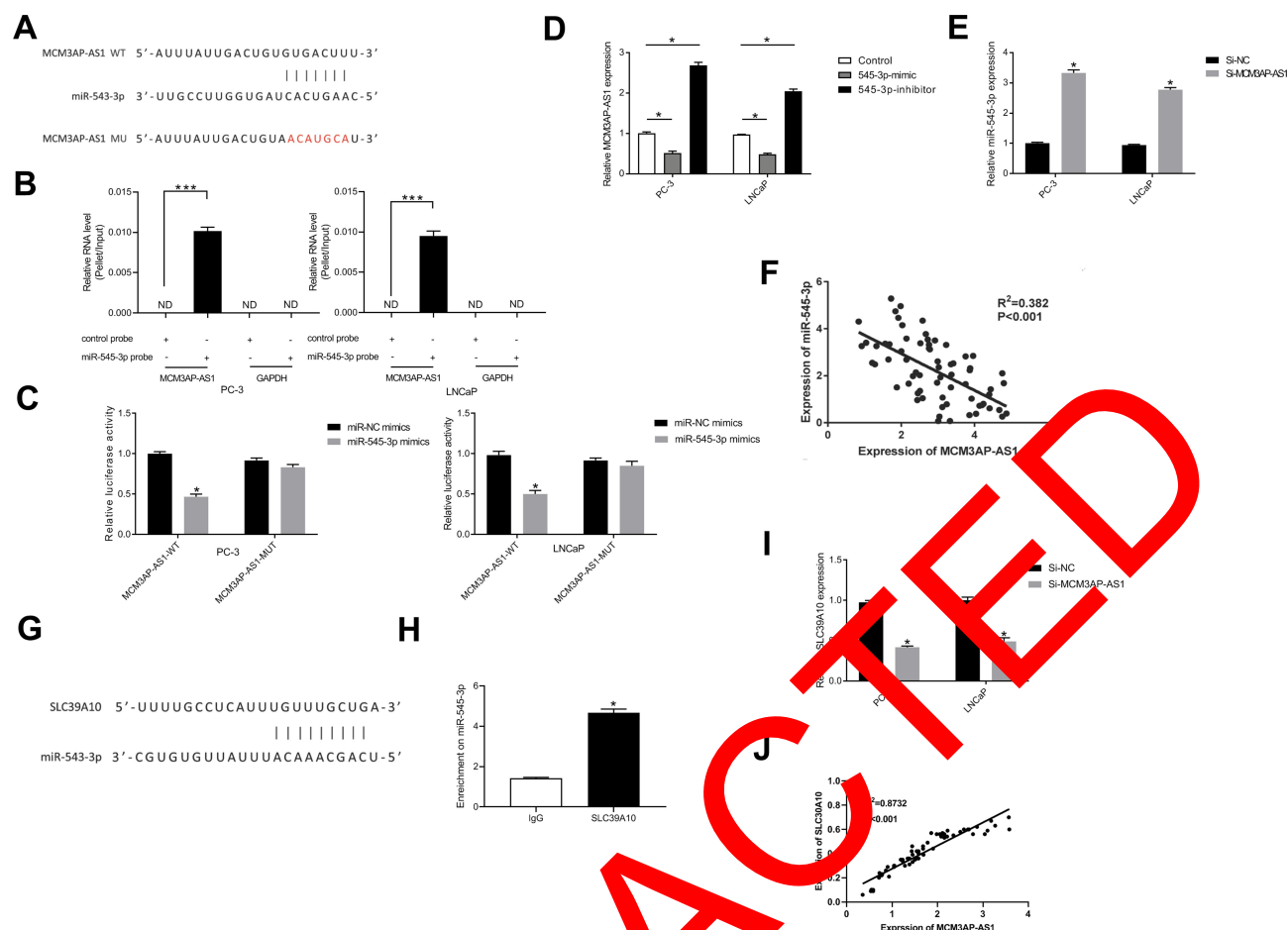


Figure 3 MiR-543-3p was a regulatory target for lncRNA MCM3AP-AS1. (A) Bioinformatics analysis showed that lncRNA MCM3AP-AS1 had a binding site with miR-543-3p but not MUT construct. (B) RNA pull-down was conducted to detect the interaction between lncRNA MCM3AP-AS1 and RNA level of miR-543-3p could be measured by RT-qPCR after immunoprecipitation by miR-543-3p probe or not control probe. (C) In luciferase experiments, 543-3p-inhibitor was discovered to markedly reduce luciferase activities around WT lncRNA MCM3AP-AS1 but not effect on the MUT group. (D) RT-qPCR was applied to detect the expression levels of MCM3AP-AS1 in PC-3 and LNCaP with the transfection of miR-543-3p mimic and miR-543-3p inhibitor. Overexpression of miR-543-3p significantly decreased the expression levels of MCM3AP-AS1. However, miR-543-3p inhibitor had the opposite effect. (E) RT-qPCR was also conducted to evaluate the expression of miR-543-3p in both PC-3 and LNCaP with the transfection of si-NC and si-MCM3AP-AS1. Knockdown MCM3AP-AS1 promoted the expression of MCM3AP-AS1 in both cell lines. (F) lncRNA MCM3AP-AS1 is negatively correlated with the expression of miR-543-3p in tumor samples. (G) MiR-543-3p has a binding site with SLC39A10, which was found by bioinformatics analysis. (H) RNA binding protein immunoprecipitation assay was used to detect the interaction of miR-543-3p and SLC39A10. IgG and anti-MCM3AP-AS1 were applied for immunoprecipitating SLC39A10 protein and then miR-543-3p expression was detected by RT-qPCR. (I) The expression levels of SLC39A10 were determined by RT-qPCR in PC-3 and LNCaP with the transfection of si-NC and si-MCM3AP-AS1. Knockdown of MCM3AP-AS1 significantly decreased the expression levels of SLC39A10 in both types of cells. (J) The correlation between MCM3AP-AS1 and SLC39A10 was also analyzed and a positive correlation with each other was observed. * $p < 0.05$; *** $p < 0.001$.

were removed ($n = 5$). From tumor samples, it was found that the volumes of transplanted tumors in LV-sh-MCM3AP-AS1 model were much lower than that in negative control group (LV-siRNA-NC) (Figure 6A). Next, those tumor samples were used to test for the expression of lncRNA MCM3AP-AS1 and miR-543-3p, which showed that the expression levels of lncRNA MCM3AP-AS1 were decreased (Figure 6B), and the expression levels of miR-543-3p were promoted in LV-sh-MCM3AP-AS1 group (Figure 6C). Furthermore, LV-sh-MCM3AP-AS1 could induce

decreases in both the expression of Ki67 and SLC39A10 (Figure 6D and E).

Discussion

It has been well established that lncRNA MCM3AP-AS1 plays important roles in the regulation of gene expression, as well as the tumorigenesis and tumor development in hepatocellular carcinoma,²⁰ glioblastoma,¹⁵ and thyroid cancer.²¹ lncRNA MCM3AP-AS1 can affect apoptosis, signaling pathways, tumor metastasis and invasion, which has

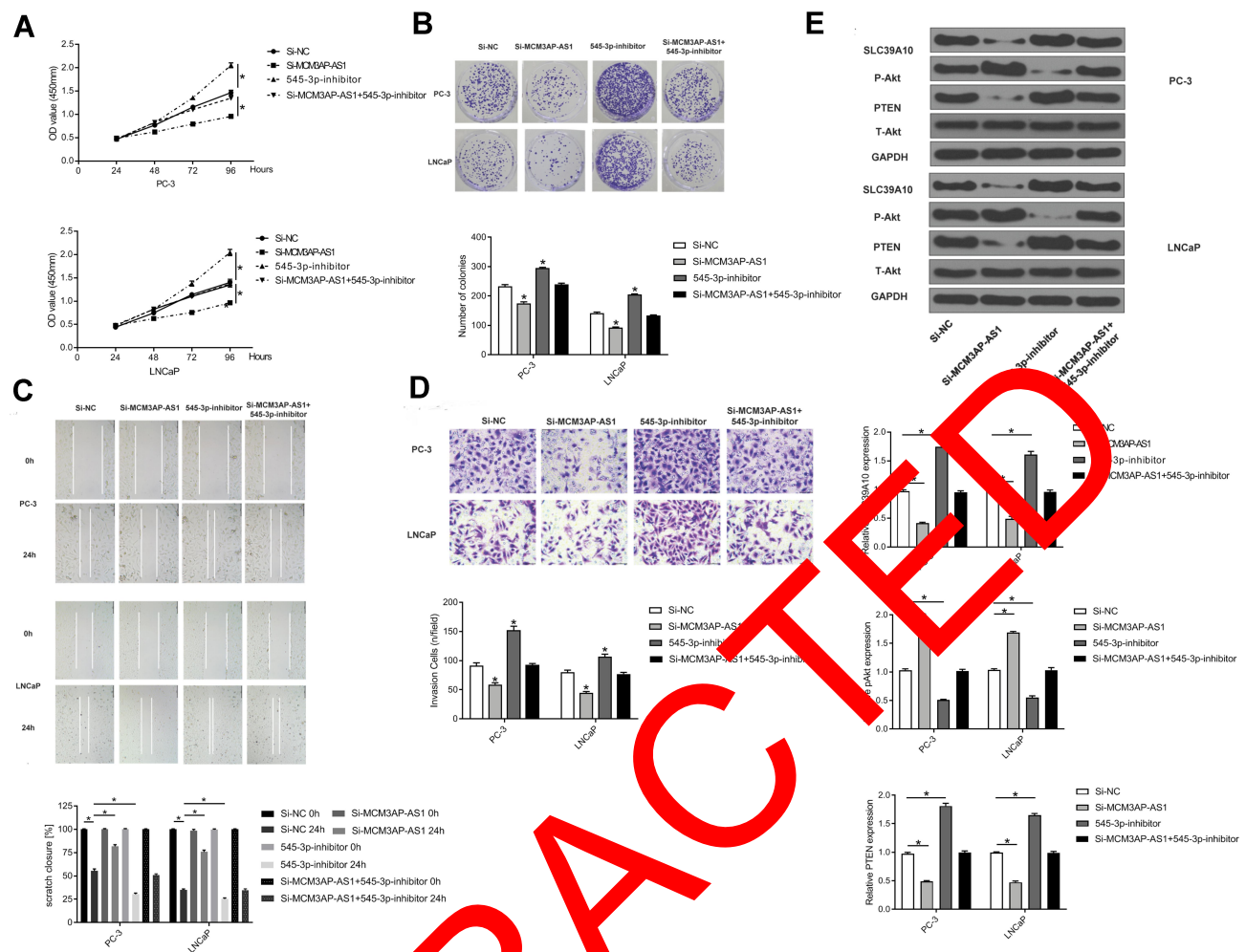


Figure 4 LncRNA MCM3AP-AS1 promotes PCa cell proliferation and invasion through regulating miR-543-3p. (A) CCK-8 experiment was applied to evaluate the proliferation of PC-3 and LNCaP cells with transfection of si-NC, si-MCM3AP-AS1 or/and 543-3p-inhibitor. The proliferation abilities of PC-3 and LNCaP cells are remarkably decreased when si-MCM3AP-AS1 was transfected. However, 543-3p-inhibitor could reverse this effect. (B) Colony formation assay of PCa cells transfected with si-NC or si-MCM3AP-AS1 or/and 543-3p-inhibitor was conducted to detect the effects of MCM3AP-AS1 or 543-3p on the cell proliferation. MCM3AP-AS1 knockdown could significantly decrease the number of colonies compared to si-NC transfection. However, 543-3p-inhibitor was able to reverse this effect. (C) Cell scratch tests were applied to assess cell migration ability under the transfection of si-NC or si-MCM3AP-AS1 or/and 543-3p-inhibitor. Confluent cell layers of PCa cells were transfected with si-NC or si-MCM3AP-AS1 or/and 543-3p-inhibitor for 24 h, scratched and let migrate for 24 h. Prior to imaging (0, 24 h) the detached cells were washed off. Compared to si-NC, si-MCM3AP-AS1 markedly suppressed cell migration. (D) Trans-well assay of PCa cells transfected with si-NC or si-MCM3AP-AS1 or/and 543-3p-inhibitor was used to reflect cell invasion ability. si-MCM3AP-AS1 remarkably perturbed cell invasion; however, 543-3p-inhibitor had opposite effect. (E) As an inhibitory target of miR-543-3p, SLC39A10 transcription level was determined in PC-3 and LNCaP transfected with si-NC or si-MCM3AP-AS1 or/and 543-3p-inhibitor by Western blots. As expected, si-MCM3AP-AS1 largely reduced the expression level of SLC39A10 protein in both types of cells. Besides, si-MCM3AP-AS1 significantly elevated p-Akt and inhibited PTEN expressions. While 543-3p-inhibitor was able to reverse these above effects of si-MCM3AP-AS1. * $p < 0.05$.

provided a new class of clinical early diagnosis and prognostic tool. Our study investigated the regulation roles of lncRNA MCM3AP-AS1 in PCa. Firstly, we found that the expression levels of lncRNA MCM3AP-AS1 significantly increased in PCa patient tissue samples compared to that in healthy tissue samples. Secondly, the long-term survival rate of patients with high expression levels of lncRNA MCM3AP-AS1

markedly decreased than that of patients with low expression levels of MCM3AP-AS1, which might provide new insights into the diagnosis and prognosis of patients with PCa.

The expression of lncRNA MCM3AP-AS1 was elevated in lung cancer cells, and accelerated angiogenesis and progression in lung cancer.²² In our study, we firstly carried out comparison experiments in PCa cell lines to

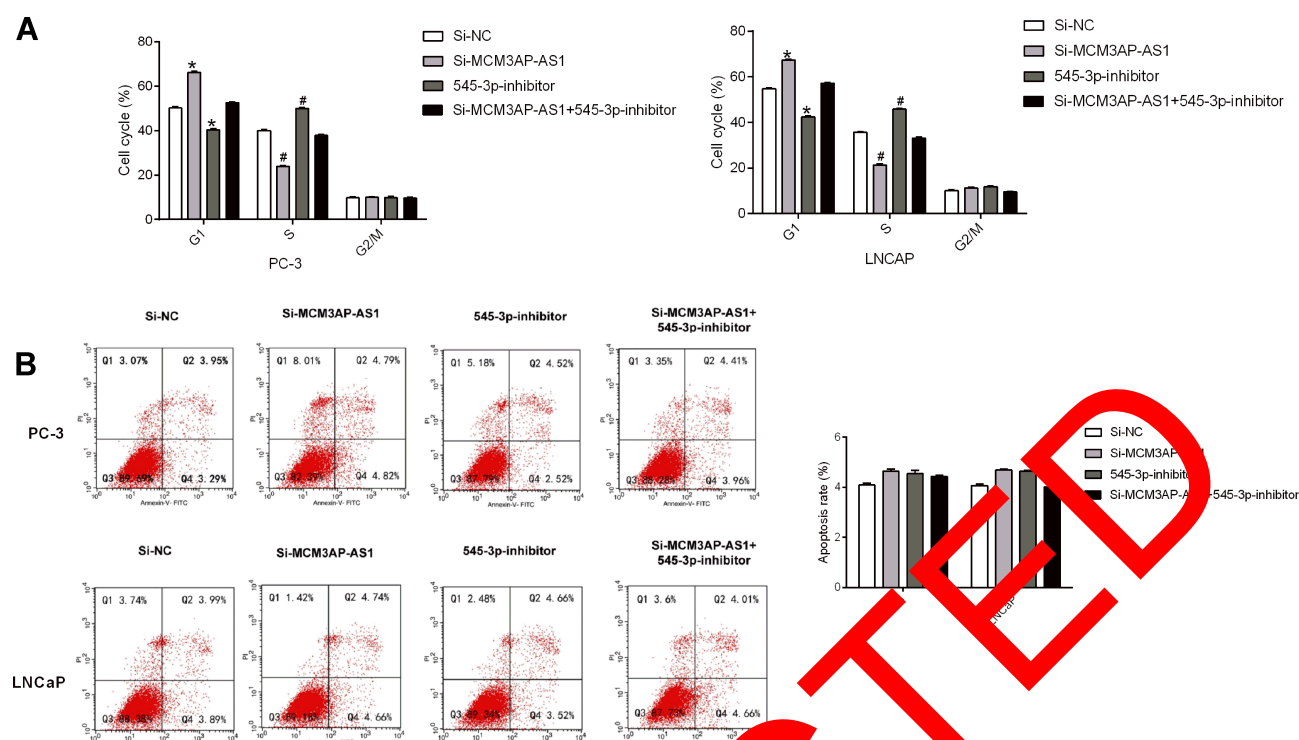


Figure 5 lncRNA MCM3AP-AS1 and miR-543-3p did not affect apoptosis. **(A)** The percentage of apoptotic cells was evaluated by flow cytometry after the transfection with si-NC or si-MCM3AP-AS1 or/and 543-3p-inhibitor for 24 h. There was no any significant in alterations of apoptotic cells percentage among those groups. **(B)** The roles of silencing of lncRNA MCM3AP-AS1 or/and miR-543-3p inhibition in the cell cycle of PC-3 and LNCaP cells were assessed by flow cytometry according to the cell cycle stage distribution (G1, S, G2/M). Si-MCM3AP-AS1 could induce cell to be arrested on G1 phase. However, 543-3p-inhibitor force more cells to roll into S phase. * $p < 0.05$ compared to si-NC in G1 phase; # $p < 0.05$ compared to si-NC in S phase.

investigate the expression levels of lncRNA MCM3AP-AS1 in vitro. It was obvious that the expression levels of lncRNA MCM3AP-AS1 significantly increased in PCa cell lines than that in normal human prostatic epithelial cells. Our study is the first report that lncRNA MCM3AP-AS1 was dysregulated in PCa tissues and cells, and its knockdown could significantly suppress tumor metastasis.

Previous studies have indicated that lncRNA MCM3AP-AS1 could bind with miR-211,¹⁵ miR-340-5p,²² and miR-194-5p.²⁰ The interactions between lncRNA MCM3AP-AS1 and these miRNAs exert important biological functions in different tumor types. In this study, we report that miR-543-3p is a regulatory target of lncRNA MCM3AP-AS1 in PCa. Specifically, lncRNA MCM3AP-AS1 can bind with miR-543-3p and there is a negative correlation between them.

Overexpression of lncRNA MCM3AP-AS1 has been shown to promote proliferation, migration and invasion by

sponging miR-138-5p in pancreatic cancer.²³ Similarly, our results demonstrated inhibitory effects of si-MCM3AP-AS1 on the proliferation, invasion, migration and the expression of SLC39A10 in PCa cells, and these effects could be reversed by 543-3p-inhibitor. In order to further confirm the role of lncRNA MCM3AP-AS1 in PCa cell behaviours, MCM3AP-AS1 was overexpressed and PCa cell proliferation, migration and invasion abilities were detected. The results indicate that MCM3AP-AS1 have promotive effects on PCa cell above behaviors, which has confirmed our hypothesis. We also found the potential down-stream signaling pathway involved in the MCM3AP-AS1/miR-54-3p-mediated tumor metabolism might be PTEN/Akt. Moreover, our in vivo experiments demonstrated that tumor proliferation and metastasis abilities in mice with the injection of lncRNA MCM3AP-AS1 became much weaker. Additionally, a reduction in the expression levels of MCM3AP-AS1 and an elevation in the expression levels of miR-543-3p were revealed in tumor samples, which suggested that MCM3AP-AS1 and miR-543-3p might be key regulators in the development of PCa.

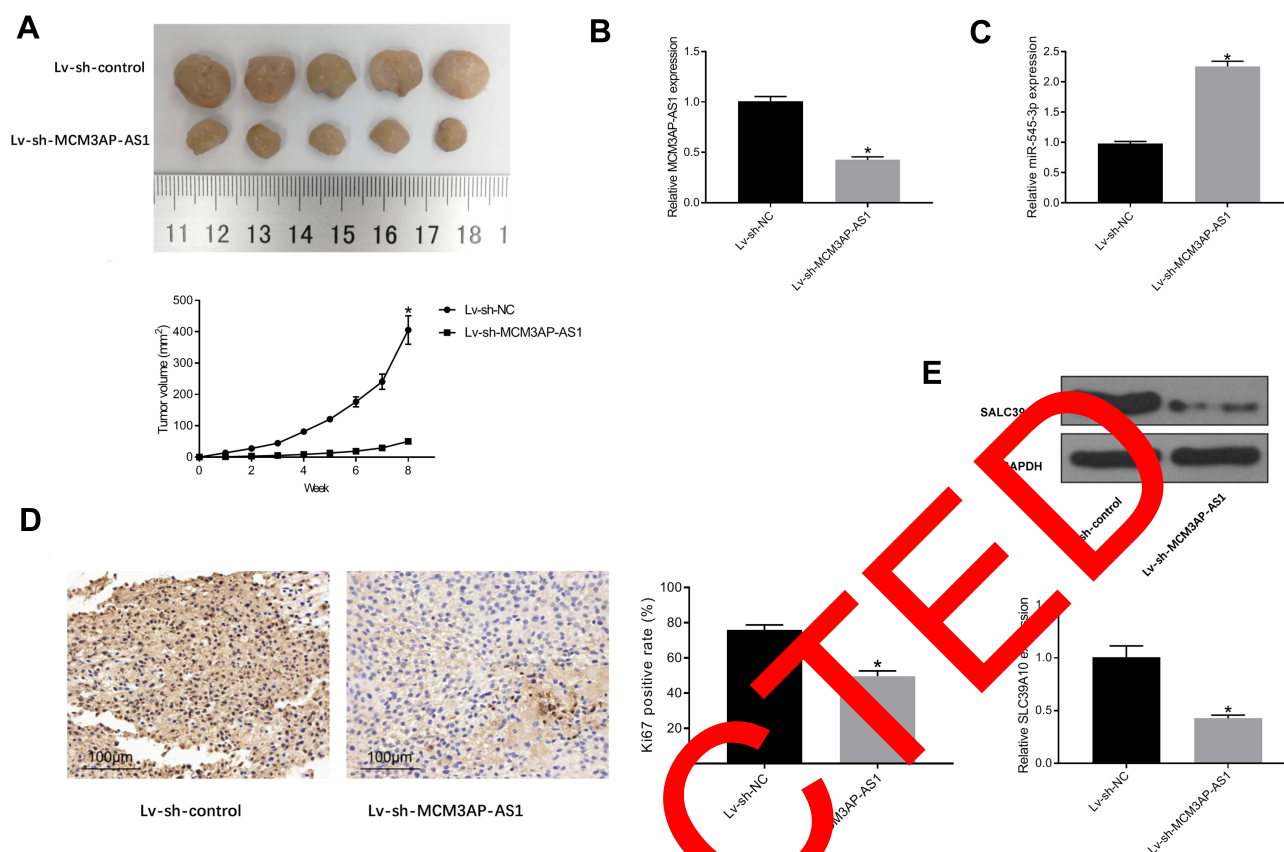


Figure 6 Knocking down MCM3AP-AS1 reduced the tumor volume and inhibited the expression of Ki67 and SLC39A10 in vivo. **(A)** The volume of mice tumors infected with Lv-shRNA-NC or Lv-sh-MCM3AP-AS1 was measured manually. The tumors infected with Lv-sh-MCM3AP-AS1 largely shrank compared to that infected with Lv-sh-control. **(B)** Lv-sh-MCM3AP-AS1 induced a dramatic decrease in the expression of MCM3AP-AS1 in tumor samples. **(C)** The expression of miR-543-3p was elevated in tumors infected with Lv-sh-MCM3AP-AS1. **(D)** Immunohistochemistry was carried out to indicate the ratio of Ki67-positive cells in tumor slice. The number of Ki67-positive cells was significantly decreased in Lv-sh-MCM3AP-AS1 group. **(E)** Western blots were used to show the expression of SLC39A10 in PCa tumor, which indicates there was a significant decrease in the expression level of SLC39A10 in PCa tumor samples infected with Lv-sh-MCM3AP-AS1. * $p < 0.05$.

Conclusion

MCM3AP-AS1 promoted PCa cell proliferation, migration and invasion by regulating miR-543-3p/SLC39A10/PTEN axis, suggesting that it might be a potential and important target for PCa therapy.

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Disclosure

The authors report no conflicts of interest in this work.

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