Abstract. Lung adenocarcinoma (LUAD), a major subtype of lung cancer, is the leading cause of cancer-related mortality worldwide. Previous studies have determined the role of the protein arginine methyltransferases (PRMTs) in the physiology and pathology of LUAD. However, to the best of our knowledge, no empirical studies have been performed determining the association between protein arginine methyltransferase 6 (PRMT6) and LUAD. The present study aimed to determine the expression levels of PRMT6 in LUAD and its association with the clinicopathological characteristics. The effect of PRMT6 knockdown on cell growth was analyzed and chromatin immunoprecipitation (ChIP) assay was used to investigate the regulatory mechanisms of PRMT6 on downstream gene expression. In addition, a xenograft model was used to determine whether the PRMT6-regulated expression levels of p18 in vitro could be validated in vivo. PRMT6 overexpression in LUAD is associated with high clinical stage, lymph node metastasis and poor clinical outcomes. Furthermore, the silencing of PRMT6 significantly reduced the enrichment of Histone H3 asymmetric demethylation at arginine 2 in the promoter region of the p18 gene, thereby activating the expression of the gene. This, in turn, induced G1/S phase cell cycle arrest, resulting in the inhibition of cell proliferation. The xenograft model also suggested that PRMT6 suppressed LUAD development by activating p18 expression in vivo. In conclusion, the findings of the present study suggested that PRMT6 may serve as an oncogene in the progression of LUAD through epigenetically suppressing p18 expression. Thus, PRMT6 may represent a novel potential therapeutic target for LUAD.

Introduction

Non-small cell lung carcinoma (NSCLC) accounts for ~85% of all lung cancer cases (1); lung adenocarcinoma (LUAD) is the main pathological type of NSCLC (2). The prognosis of lung cancer is related to the rate of recurrence, metastasis and chemotherapy resistance (3). The 5-year survival rate is 51.4% for patients with adenocarcinoma (4). The poor prognosis of lung cancer highlights the requirement for the development of novel biomarkers for the early diagnosis of the disease (5,6). Thus, there is an urgent need to improve the diagnosis and management of NSCLC.

Protein arginine methyltransferases (PRMTs) can be divided into three types: Asymmetrically, symmetrically and monomethylate protein arginines (type I/II/III, respectively) (7-9). Histone protein arginine methylation, catalyzed by PRMTs serves a crucial role in gene regulation (10). In addition, several non-histone substrates have also been discovered to be involved in gene transcription and protein translation (11). PRMTs were revealed to be widely expressed and activated in gastric and prostate cancer, as well as myeloid leukemia, where they were involved in cell growth, differentiation and apoptosis (12-15). In fact, the disruption of the modification catalyzed by PRMTs suppressed tumor development, indicating that PRMTs may be used as a potential therapeutic target for cancer (16). However, to the best of our knowledge, only a few studies have reported the dysregulation of PRMTs in lung cancer. For example, PRMT1 and PRMT4 were identified to be involved in the regulation of proliferation in lung cancer (17); and PRMT1 and PRMT5 were discovered to regulate apoptosis induced by doxorubicin or pemetrexed by affecting cellular FADD-like IL-1β-converting enzyme-inhibitory protein in NSCLC cells (18). In addition, enolase 1 methylation by PRMT5 was discovered to be critical for lung cancer cell invasion (19). Interestingly, to the best of our knowledge, no other PRMT members and their dysregulation were reported to be associated with lung cancer.

PRMT6, a type I arginine methyltransferase, has high affinity for the arginine-2 of Histone H3, specifically catalyzing Histone H3 asymmetric demethylation at arginine 2 (H3R2me2a) (10). PRMT6 was first identified to modify the glycine-and arginine-rich motifs (13), and subsequently reported to target histones and non-histones (20). However, the role of PRMT6 in human cancer remains controversial.
The downregulation of PRMT6 expression levels has been reported in melanoma (21), while the upregulation of PRMT6 expression levels was reported in the bladder (13), liver (22) and prostate (14). Interestingly, in one study, PRMT6 upregulation contributed to global DNA hypomethylation in colorectal and lung adenocarcinoma (23).

At present, studies on PRMT6 are mainly focused on its function in the nucleus, while the biological function and important target proteins of PRMT6 in human cancer remain unclear. The present study aimed to determine the association between PRMT6 expression levels and clinicopathological characteristics of LUAD via analyzing the putative oncogenic role and the potential underlying mechanism of PRMT6 in LUAD. The present study demonstrated that PRMT6 expression levels were markedly upregulated LUAD.

Materials and methods

Tissue microarray. The LUAD tissue microarray (cat. no. HuLGa180Su05), including 85 pairs of LUAD tissues and matched normal adjacent tissues (NAT) with clinicopathological data, was provided by Shanghai Outo Dotec Biotech Co. Ltd. (Outo Dotec). All patients were classified according to the tumor-node-metastasis (TNM) classification by the American Joint Commission of Cancer (24). Lymph node metastasis and the depth of invasion were classified using the 7th edition of the International Union Against Cancer TNM staging system (25). The survival time was set as the time from the day of pathological diagnosis to the day of last contact or the date of death.

Patient studies. Fresh LUAD tissues and matched NAT from 7 LUAD patients [3 male and 4 female; age, 47.4±5.2 years (mean ± SD)] were obtained from The Second Affiliated Hospital of Nanjing University of Chinese Medicine (Nanjing, China) from March 2018 to February 2019. Patients included in the study had neither received chemotherapy nor undergone surgery. Combined with lung disease already known, other tumor and autoimmune diseases as exclusion criteria. The study was approved by The Ethics Committee of The Second Affiliated Hospital of Nanjing University of Chinese Medicine (Nanjing, China). Written informed consent was obtained from the patients for the use of fresh lung tissues.

Hematoxylin & Eosin (H&E) staining and immunohistochemistry. Lung tissues were fixed in 4% paraformaldehyde for 24 h at room temperature before paraffin embedding. Sections (3.5 µm) cut from paraffin-embedded specimens were deparaffinized in xylene and rehydrated through graded alcohol series. The sections were first processed for hematoxylin and eosin staining according to standard method (26). Then, antigen retrieval was conducted using the 1X Diva Decloaker antigen retrieval solution (Biocare Medical, LLC). at 95°C for 15 min, and then blocking non-specific sites with 10% goat serum (cat. no. C0265; Beyotime Institute of Biotechnology) in PBS for 1 h at room temperature. Subsequently, the sections were incubated with an anti-PRMT6 primary antibody (1:250; cat.no. 14641; Cell Signaling Technology, Inc.), anti-p18 (1:250; cat. no. A8751; Abclonal Biotech Co., Ltd.) and anti-Ki67 (1:500; cat. no. A2094; Abclonal Biotech Co., Ltd.) at 4°C for overnight. Following the primary antibody incubation, the slides were then incubated with a horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (1:1,000; cat. no. AS038; Abclonal Biotech Co., Ltd.) at 25°C for 1 h. The slides were subsequently stained with a DAB substrate kit (Dako; Agilent Technologies, Inc.) and counterstained with hematoxylin at 25°C for 20 sec. The immunostaining was detected using an Aperio Digital Pathology Slide scanner (Leica Biosystems, Wetzlar, Germany).

The nuclear staining of PRMT6/p18/Ki67 was analyzed using the H-score system. Nuclear staining results were analyzed using Hscore using Zeiss microscope at a ×100 magnification. Positive cells were analyzed according to the staining intensity on a scale of 0-3 (0 = negative, 1 = weak, 2 = moderate, 3 = strong). H-scores were calculated as the sum of the intensity score (I) multiplied by the percentage of cells at each intensity (P). H-score =Σ [I(P)] ×100. Score values range between 0 and 300.

Cell culture. A549 cells were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences and cultured in high glucose DMEM (Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin streptomycin combination (Gibco; Thermo Fisher Scientific, Inc.). Cells were maintained at 37°C in an atmosphere containing 5% CO₂.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the adherent cells and tissues using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and cDNA was synthesized using HiScript III 1st Strand cDNA Synthesis Kit for qPCR (cat. no. R312; Vazyme Biotech Co., Ltd.). The thermocycling conditions of the RT were as follows: Remove genomic DNA at 42°C for 2 min; first strand cDNA synthesised at 25°C for 5 min, 37°C for 45 sec and 85°C for 5 sec. qPCR was subsequently performed using the ChamQ Universal SYBR qPCR Master Mix (cat. no. Q711 Vazyme Biotech co., ltd.) and the primers provided in Table I on an ABI 7500 Real-Time PCR machine (Applied Biosystems Inc.). The thermocycling conditions of the qPCR were as follows: Denaturation at 95°C for 5 min; 40 cycles at 95°C for 10 sec and 60°C for 30 sec; and a final dissociation stage (95°C for 15 sec, 60°C for 60 sec and 95°C for 15 sec) was added at the end of the amplification procedure. The data were analyzed using the ABI 7500 SDS software (Version 2.0.6, Applied Biosystems Inc.). The relative mRNA expression levels were calculated using the 2^[-ΔΔCt] method (27) and normalized to the GAPDH reference gene.

Western blotting. Total protein was extracted from tissues and LUAD cells using RIPA lysis buffer (Beyotime Institute of Biotechnology), supplemented with protease and phosphatase inhibitor cocktails (CST Biological Reagents Co., Ltd.). Nuclear protein extracts were obtained using the Nuclear Extract kit (cat. no. P0027; Beyotime Institute of Biotechnology). Core histones were extracted from the nuclear extracts of the LUAD cells using an acid-extraction method as previously described (28). Total protein was quantified using a bicinchoninic acid assay kit (cat. no. 23227; Pierce; Thermo Fisher Scientific, Inc.) and 25 µg total protein extracts/lane and 10 µg nuclear extracts/lane were separated
via 12% SDS-PAGE. Subsequently, the separated proteins were transferred onto PVDF membranes (Roche Diagnostics) and probed with specific primary antibodies in 5% skimmed milk in PBST (PBS with 0.1% Tween-20) overnight at 4°C. The membranes were then incubated with rabbit-or mouse-specific HRP-conjugated secondary antibodies for 2 h at room temperature. The following primary antibodies were used: Anti-GAPDH (1:10,000; cat. no. M171-3; MBL Co., Ltd.), anti-PRMT6 (1:1,000; cat. no. A7814; Abclonal Biotech Co., Ltd.), anti-p18 (1:1,000; cat. no. A8751; Abclonal Biotech Co., Ltd.), anti-Lamin B1 (1:1,000; cat. no. A11495 Abclonal Biotech Co., Ltd.), anti-Histone H3 (1:1,000; cat. no. A2348; Abclonal Biotech Co., Ltd.) and anti-Histone H3R2me2a (1:1,000; cat. no. A3155; Abclonal Biotech Co., Ltd.). Goat HRP-conjugated anti-rabbit immunoglobulin G (1:1,000; cat. no. a3155; abclonal Biotech co., ltd.) secondary antibodies were used. Lamin B1 was used as a loading control for nuclear proteins and GAPDH was used as a loading control for total proteins. Antibody binding was detected using an ECL detection system (cat. no. 32106; Thermo Fisher Scientific, Inc.).

Chromatin immunoprecipitation (ChIP). The ChIP assay was performed as previously described (29). Briefly, cells were crosslinked by 1% formaldehyde (Sigma-Aldrich) in PBS for 10 min at 25°C. Formaldehyde was quenched by the addition of glycine (Beijing Solarbio Science & Technology Co., Ltd.) to a final concentration of 125 µM. Then, 1x10⁶ cells were collected by centrifugation at 300 x g for 3 min at 25°C and washed with pre-cooled PBS twice. The immunoprecipitation of crosslinked 100 µg DNA (using a spectrophotometer at 260 nm) was performed using 2 µg anti-H3R2me2a (1 µg/µl, H3R2me2a; cat. no. A3155; Abclonal Biotech Co., Ltd.), anti-histone H3 lysine 4 trimethylation (1 µg/µl, H3K4me3; cat. no. A2357; Abclonal Biotech Co., Ltd.) or anti-mouse/rabbit IgG (1 µg/µl, cat. no. A7028 and A7016; Beyotime Institute of Biotechnology) antibodies for 2 h incubation at 4°C. The immunoprecipitated DNA was purified using a ChIP DNA purification kit (cat. no. D0033; Beyotime Institute of Biotechnology) and amplified by qPCR as described above. The chip primers for the detection of H3R2me2a/H4K4me3 enrichment on p18 promoter as follows: Forward, 5'-GTCTTAATAACAAACCCCTGTGTC-3' and reverse, 5'-CTCCTCCCGTCAGTCTCCTCGC-3'.

Vectors, transfections and infections. pLKO lentiviral vectors for gene knockdown and the pLKO-scrambled (Scr) short hairpin (sh)RNA vector (control) were obtained from Sigma-Aldrich; Merck KGaA. The shRNA sequences for the pLKO lentiviral vector constructions are listed in Table II. A total of 6x10⁵ 293T cells were seeded into 100-mm cell culture dishes and incubated at 37°C and 5% CO₂ for 16 h. When the cultured cells reached 85% confluence, cells were co-transfected with 3 µg of lentiviral expression constructs pLKO.1-shRNA, pMD2.G and psPAX2 (Sigma- Aldrich; Merck KGaA) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Viral supernatants were collected by centrifugation at 800 x g for 5 min at 25°C and filtered through a 0.22-µm membrane filter 48 h post-transfection and stored at -80°C. A549 cells (5x10⁶ cells per 100-mm culture dish) were seeded and incubated overnight at 37°C prior to infection. Medium was then replaced with 1:1 diluted viral supernatant supplemented with 8 µg/ml polybrene and incubated for 24 h at 37°C, followed by replacement with normal growth medium. Stable cell lines with shRNA were selected by puromycin (Clontech Laboratories, Inc.) at a final concentration of 2 µg/ml in A549 cells.

Cell proliferation assay. For the cell proliferation assay, 2x10³ A549 cells/well were seeded into 96-well plates in triplicate. Cell proliferation was analyzed using a Cell Counting Kit-8 (CCK-8; cat. no. A311; Vazyme Biotech Co., Ltd.) assay, according to manufacturer's protocol. Briefly, cells were plated and incubated for 24 h in 96-well plates prior to test, 10 µl CCK8 solution was added to each well and incubated at 37°C for 2 h. The optical density (OD) was read at an absorbance of 450 nm using a multifunction microplate reader (Safire, TECAN) for 4 continuous days.

For the colony formation assay, 500 viable A549 cells per well were seeded into 6-well plates in triplicate. Following incubation at 37°C for 10 days, the colonies were fixed with methanol at room temperature for 30 min, stained with 0.05% crystal violet (Beyotime Institute of Biotechnology) for 60 min at 25°C, washed with running water to remove the excessive dye and imaged with an Epson Perfection V550 Photo scanner (Seiko Epson Corporation). Number of colonies (>50 cells) was calculated using ImageJ software version 1.45 (National Institutes of Health, Bethesda, MD, USA).

Cell cycle assay. A total of 1x10⁴ cells were collected by centrifugation at 300 x g for 3 min at 25°C and permeabilized with ice-cold 70% ethanol overnight at 4°C. Then, the cells were collected by centrifugation at 300 x g for 3 min at 4°C and stained with 50 µg/ml propidium iodide in ice-cold PBS supplemented with 0.25 mg/ml RNase A at 4°C for 30 min (cat. no. KG214-10; Nanjing KeyGen Biotech Co., Ltd.). The cells were analyzed using a FACSCalibur (BD Biosciences) flow cytometer and FlowJo X V10.0.7 software (FlowJo LLC) was used to analyze the data.

In vivo tumor models. The animal studies were performed according to the guidelines of the National Institutes of Health for the Care and Use of Laboratory Animals (30). The protocols were approved by The Institute of Animal Care and Use Committee of Nanjing University of Chinese Medicine (Nanjing, China).

In order to establish a subcutaneous tumor model, 18 female BALB/c nu/nu mice (6-week-old; weight, 18-20 g) were obtained from the Model Animal Research Center of Nanjing University of Chinese Medicine, and maintained under specific pathogen-free conditions at The Animal Experiment Center of The Second Affiliated Hospital of Nanjing University of Chinese Medicine (Nanjing, China). Mice were housed 6 per cage at 25±2°C with 50±10% humidity and on a 12-hour light–dark cycle with free access to pellet food and water. They were given a minimum acclimation period of 1 week before subcutaneous tumor implantation. A549 cells were first transfected
with either Scr, PRMT6 sh1 or combined PRMT6 sh1 and p18 sh1 lentiviruses to establish stable cell lines for in vivo studies. Subsequently, 2x10^6 cells in 200 µl DMEM supplemented with 50% Matrigel (2 mg/ml; BD Biosciences) were inoculated subcutaneously into the right flank of 8-week-old BALB/c nude mice (6 mice/group). Tumor growth rate was monitored by measuring tumor diameters every 4 days. Both length and width (W) of the tumor were measured using a slide caliper, and the tumor volume was calculated as \(\text{Volume (cm}^3) = \frac{\text{length } \times \text{width }^2}{2}\).

The sizes of the tumors were measured every 3 days from injection and tumor volumes were calculated using the formula: \(\text{Volume (cm}^3) = 0.52 \times (\text{length } \times \text{width}^2)\). All animals were euthanized under general anesthesia with carbon dioxide when max tumor volumes reached humane endpoints (~1,000 mm^3). The flow rate displaced 10-30% of the chamber volume/minute. The animals that lost consciousness and muscle activity were identified as deceased.

**Statistical analysis.** Data are presented as the mean ± SD of three independent experiments. Statistical significances were determined using a two-tailed Student’s paired t-test, or a one-way ANOVA, followed by a Bonferroni’s multiple comparisons post hoc test. Receiver operating characteristic (ROC) curve analysis was performed to determine the diagnostic value of PRMT6 expression levels in LUAD. Kaplan-Meier estimates for the primary end point were calculated and compared using a log-rank (Mantel-Cox) test of equality. The correlation between PRMT6 and p18

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### Table I. Sequences of the primers used for reverse transcription-quantitative PCR.

| Gene                                      | Primer sequence (5’→3’) |
|--------------------------------------------|-------------------------|
| Protein arginine methyltransferase 6       | F: ACGAGTGGCTACTCGGAGCTTTT G:
| p21                                        | R: AGTTCCGAAAGATACCGAG |
| p27                                        | R: TACGGGTTGCGCTCGAG |
| p18                                        | R: CGGGGTTTGGAGAGCTGAAG |
| CDK inhibitor 3                            | R: CACGCAATACACGAGCAT |
| CDK4                                       | R: CGTCGGCAATGGGAAACCTC |
| CDK6                                       | R: GACGACATCAAACACCTGACC |
| Cyclin D1                                  | R: CCTTCTTGACACATTGGA |
| Cyclin E1                                  | R: ACTCAAGCTGGCAAGCTCG |
| S-phase kinase-associated protein 2        | R: GCCTCAAGAAATGTGCTGATCCC |
| GAPDH                                      | R: GAGGCACATCGCTCAAGCAC |

F, forward; R, reverse.

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### Table II. shRNA sequence used for pLKO lentiviral vectors construction.

| shRNA   | Sequence (5’→3’)                  |
|---------|-----------------------------------|
| PRMT6 sh1 | CCGGCACCGGCATTTCTGAGCATCTCTCGAGAAGATGCTCAGAACATGCCCGGTGTTTTT |
| PRMT6 sh2 | CCGGCACCGGCAGTTTCTAGAGAATCTCGAGATCTCTCTGAGAACGTCCCGGTGTTTTT |
| p18 sh1  | CCGGTGGATTTGGAAGGAGCTCGACTCAGACAG CAGCTCCTGCCAACATCCCATTTT |
| p18 sh2  | CCGGACTGTTTTCGCTGTTCATTCATCTCGAGAAGAGTACAGCAGAACACAGGTGTTTTT |
| Scr      | CCTAAGGGTTAAGTCGCCCTCGCTCGAGGGGAGAGGAGGACCTAACCTTTAG |

sh/shRNA, short hairpin; PRMT6, protein arginine methyltransferase 6; Scr, scramble.
expression levels was analyzed using Pearson's correlation analysis. All the statistical analyses were conducted using GraphPad Prism 7 software (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

**Results**

**PRMT6 is overexpressed in LUAD.** To determine the clinical significance of PRMT6 expression in LUAD, the expression levels of PRMT6 in the lung tissue from a cohort of
85 patients with LUAD were investigated using immunohistochemistry and a specific anti-PRMT6 antibody. The protein was discovered to be predominantly localized in the nucleus of the glandular epithelium of malignant tissues (Fig. 1A). Notably, significantly upregulated expression levels of PRMT6 were observed in the tumor tissues of patients with LUAD compared with the matched NAT (Fig. 1B). Western blotting results confirmed that the expression levels of PRMT6 in tumor tissues from 7 patients with LUAD were significantly upregulated compared with in NAT (Fig. 1C and D). Thus, these findings suggested that PRMT6 expression levels may be significantly upregulated in LUAD.

Association between PRMT6 expression levels and clinicopathological features. The association between PRMT6 expression levels and clinicopathological features of LUAD was further investigated. The expression levels of PRMT6 protein were significantly upregulated in patients with advanced clinical stages (III and IV) and lymph node metastasis compared with the patients with non-advanced clinical stages (I and II) and no lymph nodes metastasis, respectively (Fig. 2A and B). Notably, the expression levels of PRMT6 were not related to the age, sex, tumor size, differentiation or local invasion of patients with LUAD (data not shown). Kaplan-Meier survival analysis indicated that high expression levels of PRMT6 protein were linked to a significantly poorer prognosis in patients with LUAD compared with patients with low expression levels of PRMT6 (Fig. 2C). Furthermore, the predictive value of PRMT6 was evaluated using ROC curve analysis. The results indicated that the area under the curve (AUC) was 0.88 [95% confidence interval (CI), 0.83-0.93] between LUAD tissues and NAT (Fig. 2D). In the ROC curve...
analysis, H-score 97 was set as the cut-off for the expression levels, based on which, the tumor tissues were discriminated from NAT with high sensitivity (84.71%) and specificity (76.47%; Fig. 2D). These findings suggested that PRMT6 may be used as a novel diagnostic biomarker for LUAD.

Knockdown of PRMT6 suppresses LUAD cell growth in vitro through G1/S phase arrest. A549 cells are the most frequently used cells to study LUAD (31), thus, the present study used the A549 cell line to represent LUAD. The expression of PRMT6 in mRNA (Fig. 3A) and protein (Fig. 3B) levels was reduced in A549 cell lines using shRNAs (sh1 and sh2) mediated by lentivirus. PRMT6 was previously demonstrated to mediate the H3R2me2a modification (9). Herein, the expression levels of global H3R2me2a were markedly downregulated in PRMT6 sh1/2-transfected cells compared with Scr-transfected cells (Fig. 3B). Subsequently, the effects of PRMT6 on the proliferation of A549 cells in vitro were analyzed using a CCK-8 assay. Notably, the stable knockdown of PRMT6 significantly suppressed the proliferation of A549 cells compared with the Scr cells (Fig. 3C). In addition, the number of cell colonies formed were significantly decreased in the knockdown cells compared with in the Scr-transfected cells (Fig. 3D and E).

To further investigate the molecular mechanism underlying the action of PRMT6 in the proliferation of LUAD cells, the cell cycling patterns of Scr- and PRMT6 sh1/2-transfected
cells were determined using flow cytometry (Fig. 3F and G). The number of PRMT6 knockdown cells in the G0/1 phase was significantly increased compared with the Scr-transfected cells. By contrast, the number of PRMT6 knockdown cells in the S and G2/M phases was significantly decreased compared with the Scr-transfected cells (Fig. 3F and G). These results suggested that the downregulation of PRMT6 may induce G1/S phase arrest in A549 cells, which may subsequently inhibit PRMT6-mediated proliferation.

p18 is a direct target of PRMT6 and interferes with G1/S phase arrest in LUAD. To investigate the mechanism underlying cell cycle arrest induced by the knockdown of PRMT6 in LUAD cells, the mRNA expression levels of important regulatory genes involved in the G1/S transition or switch, including p21, p27, p18, CDK inhibitor 3 (CDKIN3), CDK4, CDK6, cyclin D1 (CCND1), cyclin E1 (CCNE1) and S-phase kinase-associated protein 2 (SKP2), were analyzed. RT-qPCR results demonstrated that the expression levels of p18 were significantly upregulated in PRMT6 knockdown or double knockdown of PRMT6 and p18 in A549 cells. (G) Colony formation assay was used to evaluate the effect on proliferation of PRMT6 knockdown or double knockdown of PRMT6 and p18 in A549 cells. (H) Number of colonies formed in part (G) was counted using Image J software. P<0.05, **P<0.01.

Figure 5. Knockdown of PRMT6 suppresses LUAD cell growth by activating p18 expression levels in vitro. (A) Transfection efficiency of PRMT6 knockdown was analyzed at the mRNA expression level using reverse transcription-quantitative PCR. **P<0.01 vs. Scr group. (B) Transfection efficiency of PRMT6 knockdown was detected at the protein level using western blotting.

**P<0.01 vs. Scr group. (C) Western blotting analysis of p18 protein expression levels following PRMT6 knockdown or the double knockdown of PRMT6 and p18 in A549 cells. (D) Flow cytometric analysis was used to determine the cell cycle distribution of PRMT6 knockdown or the double knockdown of PRMT6 and p18 in LUAD cells. (E) Quantification of the cell cycle distribution of PRMT6 knockdown or double knockdown of PRMT6 and p18 in LUAD cells from part (D). (F) Cell Counting Kit-8 assay was used to determine the effects on proliferation of PRMT6 knockdown or double knockdown of PRMT6 and p18 in A549 cells. (G) Colony formation assay was used to evaluate the effect on proliferation of PRMT6 knockdown or double knockdown of PRMT6 and p18 in A549 cells. (H) Number of colonies formed in part (G) was counted using Image J software. P<0.05, **P<0.01.

PRMT6, protein arginine methyltransferase 6; LUAD, lung adenocarcinoma; sh, short hairpin RNA; Scr, scramble; OD, optical density.
p18 INK4c (commonly referred to as p18) is a member of the INK4 family of CDK inhibitors, which interacts with CDK4/6 and suppresses its activation, functions as a cell growth regulator of G1/S cell cycle progression and serves as a tumor suppressor (32,33). However, to the best of our knowledge, the expression pattern of p18 in LUAD and its association with patient prognosis remains to be determined. Thus, the present study investigated the expression levels of p18 in LUAD tissue arrays using immunohistochemistry. The results demonstrated that p18 was mainly localized in the nucleus of the glandular epithelium of LUAD tissues (Fig. 4C) and the protein expression levels of p18 were significantly downregulated in the LUAD tissues compared with the NAT (Fig. 4D). Western blotting also confirmed that the expression levels of p18 in tumor tissues from seven patients with LUAD were downregulated compared with the NAT (Fig. 4E). Kaplan-Meier survival
PrMT6 functions as a transcriptional repressor by generating H3R2me2a (34). Thus, to determine whether PrMT6 directly regulated p18, the enrichment of H3R2me2a on the p18 promoter was analyzed using a ChIP assay. A prominent enrichment of H3R2me2a was noted in the gene promoter of p18 in Scr-transfected cells, which was significantly decreased when PrMT6 was knocked down in A549 cells (Fig. 4H). The results of ChIP assay are consistent with the inhibitory effect of PrMT6 on p18 gene expression. Also, a significant increase was observed in the enrichment of H3K4me3 in the promoter of p18 when PrMT6 was knocked down in A549 cells (Fig. 4I), indicating a potential crosstalk between H3R2me2a and H3K4me3 to enhance p18 gene expression. These findings indicated that p18 may be a downstream target of PrMT6 and interfere with G1/S phase arrest in LUAD cells.

**Discussion**

The current study aimed to investigate the biological effects of PrMT6 and its potential mechanism of action in LUAD. The results of the present study demonstrated that the proliferation of LUAD cells was significantly suppressed by silencing PrMT6 expression both in vitro and in vivo. In addition, PrMT6 knockdown decreased the enrichment of H3R2me2a in the promoter region of the p18 gene, thereby activating the expression of the gene. G1/S phase arrest was also induced, resulting in the inhibition of cell proliferation. These results strongly indicated that PrMT6 may serve a protooncogenic role in the progression of LUAD through the epigenetic suppression of p18 expression. Thus, these findings may provide a novel potential target for the treatment of LUAD.

PMTs, which specialize in methylating both histone and non-histone proteins, have been discovered to be involved in numerous biological processes, such as cell growth, metabolism and signal transduction, among others (35,36). However, to the best of our knowledge, the role of PrMT6 in human LUAD remains unknown. Considering that the detection of protein expression levels in tissue microarrays containing clinical samples are more accurate and reliable than the gene expression levels found in RNA-seq databases, and the observation time of the patients in tissue microarrays was longer compared with the patients with LUAD listed in the RNA-seq database, RNA-seq database analysis was not performed in the present study. In fact, to the best of our knowledge, the investigations of the current study were the first to analyze the expression levels of the proteins in fresh clinical tissue samples. In the present research, the expression levels of PrMT6 were discovered to be negatively associated with the clinical staging, lymph node metastasis and clinical prognosis of patients with LUAD, indicating that PrMT6 may serve an oncogenic role in LUAD development. Furthermore, silencing PrMT6 suppressed the cell proliferation of LUAD cells in vivo and in vitro, which was ascribed to G1/S cell cycle arrest. These data suggested that PrMT6 may serve a pivotal role in the G1/S phase transition of LUAD cells. Interestingly, differences were noted in the shRNA transfection efficiency in A549 cells between Figs. 3B and 4B; in Fig. 3B, sh2 appeared more effective, whereas in Fig. 4B, sh1 exhibited nearly 100% efficiency. The differences in the shRNA transfection efficiency may be related to the semi-quantification of protein samples and/or the transfer efficiency of western blotting. Nevertheless, PrMT6 was effectively knocked down in the present study and the differences in the shRNA transfection efficiencies did not affect the experimental conclusion.

It is well known that cancers are considered to be a disease of cell cycle disorder, which is accompanied by the abnormal
regulation of cell cycle regulatory proteins (37). The arginine methylation of a protein is a post-translational modification, which has been contributed to the disorder of the cell cycle in melanoma (38). PRMTs have been reported to methylate several regulatory proteins of the cell cycle, such as p21, p53, cyclin D1 and phosphorylated Rb (39). Previous studies have also identified that p21 and p27 were direct target genes of PRMT6, which received H3R2me2a modifications and promoted cell cycle progression through CDK1/2 in U2OS and breast cancer cells (34, 40, 41). In the present study, it was hypothesized that the downregulation of PRMT6 expression levels may inhibit the proliferation of LUAD cells through p18 activation. Thus, to investigate whether p18 was a direct target gene of PRMT6. ChIP analysis was performed in LUAD cells, which confirmed that H3R2me2a was significantly enriched in the promoter of the p18 gene. These findings indicated that PRMT6 may serve a prooncogenic function in the development of LUAD via the epigenetic suppression of p18 expression. PRMT6 has been reported to be responsible for H3R2me2a; it has been associated with the inactive promoters of mammalian (42). For example, the H3R2me2a modification has been identified to prevent MLL/SET lysine methyltransferase complexes from binding to H3 (43) and PRMT6 action was discovered to impede the deposition of H3K4me3 (44). In the present study, H3R2me2a was enriched at the p18 gene promoter in the control cells. Correspondingly, the study further confirmed that the enrichment of H3K4me3 on the p18 promoter was significantly increased following the knockdown of PRMT6, indicating crosstalk between H3R2me2a and H3K4me3 and the enhancement of p18 gene repression. In conclusion, the findings of the current study suggested that PRMT6 may serve as a novel potential diagnostic biomarker for LUAD through acting as an oncogene in the disease, epigenetically suppressing p18 expression. Taken together, these findings may offer novel opportunities for the treatment, diagnosis and management of this major subtype of NSCLC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JT performed the experiments; QM and RS analyzed the data; and YX designed the experiments, interpreted the data and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by The Ethics Committee of the Second Affiliated Hospital of Nanjing University of Chinese Medicine (approval no. 2019-010-054). Written informed consent was obtained from the patients for the use of lung tissue. The animal experiments were approved by The Institute of Animal Care and Use Committee of Nanjing University of Chinese Medicine.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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