Inhibition of Nonsmall Cell Lung Cancer Cell Migration by Protein Arginine Methyltransferase 1-small Hairpin RNA Through Inhibiting Epithelial-mesenchymal Transition, Extracellular Matrix Degradation, and Src Phosphorylation *In Vitro*

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Abstract

Background: Protein arginine methyltransferases 1 (PRMT1) is over-expressed in a variety of cancers, including lung cancer, and is correlated with a poor prognosis of tumor development. This study aimed to investigate the role of PRMT1 in nonsmall cell lung cancer (NSCLC) migration *in vitro*.
Methods: In this study, PRMT1 expression in the NSCLC cell line A549 was silenced using lentiviral vector-mediated short hairpin RNAs. Cell migration was measured using both scratch wound healing and transwell cell migration assays. The mRNA expression levels of matrix metalloproteinase 2 (MMP-2) and tissue inhibitor of metalloproteinase 1, 2 (TIMP1, 2) were measured using quantitative real-time reverse transcription-polymerase chain reaction. The expression levels of protein markers for epithelial-mesenchymal transition (EMT) (E-cadherin, N-cadherin), focal adhesion kinase (FAK), Src, AKT, and their corresponding phosphorylated states were detected by Western blot.
Results: Cell migration was significantly inhibited in the PRMT1 silenced group compared to the control group. The mRNA expression of MMP-2 decreased while TIMP1 and TIMP2 increased significantly. E-cadherin mRNA expression also increased while N-cadherin decreased. Only phosphorylated Src levels decreased in the silenced group while FAK or AKT remained unchanged.
Conclusions: PRMT1-small hairpin RNA inhibits the migration abilities of NSCLC A549 cells by inhibiting EMT, extracellular matrix degradation, and Src phosphorylation *in vitro*.

Key words: Epithelial-mesenchymal Transition; Extracellular Matrix Degradation; Migration; Nonsmall Cell Lung Cancer; Protein Arginine Methyltransferases 1; Src

Introduction

Recently, protein arginine methyltransferases 1 (PRMT1), one of the most important types of protein in PRMT family member in mammals, was reported to be over-expressed in a variety of cancers,[1-5] including lung cancer,[1,2] and is correlated with a poor prognosis of tumor development.[2-4,6] Elakoum *et al*.[1] showed that siRNA silencing of PRMT1 reduced the proliferation of lung cancer cell lines A549 and H1299 by decreasing the colony formation on soft agar. Yoshimatsu *et al*.[2] suppressed the expression of PRMT1 and PRMT6 with siRNA, which significantly suppressed the growth of lung and bladder cancer cells. The dysregulation of PRMT1 and PRMT6 is believed to be involved in human carcinogenesis.[2] However, the role of PRMT1 in tumor, especially in nonsmall-cell lung carcinoma (NSCLC) progression, particularly metastasis, remains largely unknown.

Family members of PRMT play a crucial role in gene transcription regulation, thereby affecting several physiological and pathological processes such as cell growth, DNA repair, signal transduction, and tumorigenesis.[7,8] PRMT1 is reported to methylate

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histone H4 arginine 3 side chain, ω-N, which is used to form asymmetric double methyl and monomethyl and plays a critical role in regulating protein function.[9] As a transcriptional co-activator, PRMT1 is required by several transcription factors to bind to promoters and activate transcription.[10,11]

There are several important mechanisms involved in tumor metastasis, such as epithelial-mesenchymal transition (EMT),[12,13] extracellular matrix degradation,[14-18] and some signaling pathways activation, such as focal adhesion kinase (FAK),[19] Src,[20] and AKT.[21]

To determine the role of PRMT1 in NSCLC migration, we down-regulated the PRMT1 expression in the NSCLC cell line A549 via a lentiviral vector-mediated small hairpin RNAs (shRNAs). Cell migration, transcription, and protein expression of several targets were analyzed after PRMT1 silencing.

Methods

Lentiviral-mediated small hairpin RNA silencing of A549 cells

Double-stranded oligos were inserted into the BamHI/EcoRI site of pUCTP vector, which contains a red fluorescent protein (tdTomato) marker for cell tracking. Eight shRNA primers (A-H) were designed based on the PRMT1 gene to ensure that each gene has a minimum of 1–2 shRNA primers to inhibit its expression. PRMT1-shRNA primers are the following: A: CCGGCAGTACAAAGACTACAA; B: GTGTTCCAGTATCTCTGATTA; C: CCAGGCGGAAAGCAGTGAGAA; D: GCAAGTGAAGCGGAATGACTA; E: TGTTCTACATGGAGGACTACC; F: TGGAGAAGGTGGACATCATCA; G: TTGACTCCTACGCACACTTTG; H: CTTACCGCAACTCCATGTTTC. shRNA primers were annealing with 10 μl of primers (10 μm), 10 μl × 10 annealing buffer, and 70 μl ddH_2O. shRNA was integrated into lentiviral vectors with connection system as follows: shRNA 4 μl, vectors 2 μl, ddH_2O 2 μl, ×10 T4 buffer 1 μl, Roche T4 ligase 1 μl, 24°C for 2 h. GeneHogs, a chemically competent bacterium, was transformed using 5 μl of connection products. Plasmids were validated by colony polymerase chain reaction (PCR) and restriction enzyme digestion. At the screening stage, the plasmid mixtures A-D and E-H were used. Human embryonic kidney (293T) cells (ATCC, Manassas, VA, USA) were co-transfected with lentiviral and helper plasmids and cultured with serum-free Dulbecco's modified eagle medium (Cat: 10313–021, DMEM, Life Technologies, Invitrogen, USA). At a logarithmic growth phase, cell suspensions were seeded into 96 well plates (Thermo Scientific, USA) at 10×10^5 cells/well the day before transfection. Cells were transfected according to transfection reagent instructions 24 h after seeding into 96 well plates. Total DNA (0.125 μg, containing lentiviral expression plasmids) was added to each well; 0.25 μl transfection reagent was also added to each well. The viral solution was diluted using 4 μl × 2. A-D were in serum-free DMEM. shRNA lentiviral plasmid mixture and E-H mixture were used. Each gene corresponded to two hybrid viruses. Crude viral solution was collected at 48 and 72 h, respectively, after transfection, and kept at − 80°C. Human lung carcinoma A549 cells (ATCC, Manassas, VA, USA) were seeded into 96 well plates with 2500 cells per well. The lentivirus particles produced from the transfected 293T cells were used to infect A549 cells in the presence of 8 μg/ml polybrene (Sigma-Aldrich, St. Louis, MO, USA). The shControl group was constructed by A549 cells transfected with empty vector (without shRNA targeted PRMT1). Ninety-six hours after infection, the knockdown efficiency was validated by quantitative PCR (qPCR).

Quantitative real-time polymerase chain reaction

Protein arginine methyltransferases 1 knockdown efficiency was validated by qPCR. qPCR was carried out using CFX Connect Real-Time PCR Detection System (Bio-Rad, 185-5200, USA). The primers of PRMT1 (Forward: TCAGCGCATCCGGTAGTC, Reverse: TCAAAGCCAACAAGTTAGACCA) and β-actin (housekeeping gene) (Forward: GCATCCCCCAAAGTTCACAA, Reverse: GGACTTCCTGTAACAACGCATCT) were designed using primer analysis software Primer Premier 6.0 (PREMIER Biosoft International, Palo Alto, CA, USA). Total RNA was extracted from cell lines using TRIzol reagent (Invitrogen, Life Technologies GmbH, Darmstadt, Germany,). cDNA was synthesized from 2 μg of RNA using a reverse transcriptase (RT) reagent kit (Takara Biotechnology Co. Ltd., Dalian, China), in the following reaction system: 4 μl of × 5 Primescript Buffer-2, 1 μl of Primescript RT Enzyme Mix, and 1 μl of RT Primer Mix. The reaction mixture was treated as follows: DNase (Takara Biotechnology Co. Ltd.) and ddH_2O up to 20 μl and incubated at 37°C for 15 min and then 85°C for 5 s. qRT-PCR reaction system: ×2 SYBR Green qPCR Mix (DSTM) 7.5 μl, Primer/10 μmol/L 0.2 μl, ddH_2O 1.3–2 μl, cDNA 0.3–1.0 μl, 15 μl in total. qPCR protocol was performed as follows: 94°C for 2 min, 95°C for 15 s, 60°C for 30 s, 72°C for 30 s at 40 cycles. At the extension stage, the fluorescence was measured at 72°C. All the experiments were repeated in triplicate. In order to quantify gene expression, the mRNA level of the target genes was normalized by the β-actin mRNA level and the relative gene expression was calculated adjusting the target gene expression in the silenced shPRMT1 group with the expression in the shControl group.

Matrix metalloproteinase/tissue inhibitor of metalloproteinase mRNA expression by quantitative polymerase chain reaction

Quantitative polymerase chain reaction primers of matrix metalloproteinase 1 (MMP-1) (Forward: TCGATGCTGCTCTTTCTGAG, Reverse:

GATAACCTGGATCCATAGATCGTT), MMP-2 (Forward: TGCTGGAGACAAATTCTGGA, Reverse: GATGGCATTCCAGGCATC), tissue inhibitor of metalloproteinase 1 (TIMP1) (Forward: TTTGTGGCTCCCTGGAACAG, Reverse: CATTCCTCACA GCCAACAGTGT), TIMP2 (Forward: GAAGGAGCCCCATCAATCCT, R: CTCCCATTTCTACAAGGCTCAGA) were also designed using Primer Premier 6.0. The mRNA expression was evaluated using the qPCR protocol described above.

Migration of A549 cells infected by protein arginine methyltransferases 1-shRNA

Scratch wound healing assay

A549 cells infected by PRMT1-shRNA and empty vector were grown in DMEM supplemented with 10% FBS. Cells were seeded into 24-well tissue culture plate at a density of 1×10^5 cells/ml, reaching 80% confluence as a monolayer after 24 h. The monolayer was gently and slowly scratched creating a straight line with a new 1 ml pipette tip across the center of the well. The medium was then discarded, and fresh medium was added to the well. Cells were incubated for additional 48 h, then washed twice with × 1 PBS, fixed with 3.7% paraformaldehyde for 30 min and finally stained with 1% crystal violet in 2% ethanol for 30 min. Photos of the stained monolayer were taken on a microscope (Axio Vert A1 FL, Carl Zeiss, Germany) and gap distance at 0 h and 24 h was quantitatively evaluated using image analyzing software (Axio CSM 700, Carl Zeiss). Each experimental group was repeated 3 times.

Transwell cell migration assay

A549 cells infected by PRMT1-shRNA and the empty vector were trypsinized and centrifuged, discarding the supernatant. The cells were resuspended in serum-free medium, counted and diluted to a density of 5×10^5/ml. A total of 500 μl medium was added to each well of a 24 well cell culture insert (8.0 μm pore; BD FALCON, Franklin Lakes, NJ, USA). The cell suspension (100–200 μl) was transferred into the upper compartment of the cell culture insert and incubated at 37°C (Thermo FORMA, 3111) for 12–48 h. The migrated cells in the lower compartment were then fixed using 4% paraformaldehyde for 15–20 min. Paraformaldehyde was removed, and the cells were washed with PBS for 1 min. PBS was removed, and the chambers were inverted and stained with 0.1% crystal violet solution (HT90132, Sigma, USA) for 15 min. The dye solution in excess was washed out with distilled water. The chambers were stored at 4°C after drying. Five visual fields per chamber were selected for imaging. The number of migrated cells in the lower compartment was counted within each field; the number of cells in shPRMT1 group was calculated adjusting with the number in the shControl group.

Western blotting

A549 cells infected by PRMT1-shRNA and the empty vector were cultured in 6-well plates and lysed with RIPA lysis buffer and phosphatase inhibitors (Sigma-Aldrich, USA). Cell lysates were collected by scraping and centrifuged, protein concentration was measured using a bicinchoninic acid protein assay reagent (Cat: 23227, Pierce, Thermo Scientific, USA) and diluted with lysis buffer to obtain a protein concentration of 5–6 μg/ml. 10–30 μg of total protein from each sample was loaded onto a 8–10% SDS-PAGE gel for electrophoresis at 200 V (Bio-Rad, USA) and transferred onto nitrocellulose membranes (Millipore, USA) at 100 V for 60 min. Membranes were blocked in 5% blocking buffer (Bio-Rad) in TBS containing 0.1% Tween-20 (TBST) for 1 h at 25°C and then incubated with a specific monoclonal antibody for 1–2 h at 25°C. Primary monoclonal antibody was incubated in 1% BSA in PBS for 1 h at 25°C. The primary monoclonal antibodies used were E-cadherin (CST, USA), N-cadherin (CST), FAK (CST), phospho-FAK (CST), Src (CST), phospho-Src (CST), AKT (CST), phospho-AKT (CST) and GAPDH (CST), each of them used at a dilution of 1:1000. After washing, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (CST, dilution: 1:2000), and washed again in PBS with 0.1% Tween-20, for three times of 10 min each, The membrane was transferred to a shallow tray and treated with DAB solution (10 ml of 0.05% chromomeric substrate 3,3'-diaminobenzidine in PBS + 10 μl H_2O_2 30%). The membrane was incubated at 25°C under gentle shaking for 1–2 min in a darkroom, then washed in PBS and dried. Images were acquired on ChemiDoc XRS + System (Bio-Rad) in a darkroom and analyzed by Image Lab software (Bio-Rad). Ratios of target gene to housekeeping gene (GAPDH) were calculated to normalize the expression of target proteins.

Statistical analysis

Data were expressed as mean ± standard error and analyzed by *t*-test using SPSS 19.0 statistical software (IBM, Armonk, NY, USA). $P < 0.05$ was considered statistically significant.

Results

Selection and validation of optimal protein arginine methyltransferases 1-small hairpin RNA target sites for RNAi

Protein arginine methyltransferases 1 expression in the NSCLC cell line A549 was silenced using lentiviral vector-mediated short hairpin RNAs. Plasmids were validated by colony PCR [Figure 1a], restriction enzyme digestion [Figure 1b], 293T cells infection [Figure 1c], and PRMT1 silencing efficiency in A549 cells was evaluated by qPCR. The expression of PRMT1 mRNA in A549 cells infected by shPRMT1-EH was remarkably lower than the shControl group, with an expression reduction of 87.89% ($P < 0.05$) [Figure 1d]. Therefore, shPRMT1-EH shRNA was selected to infect A549 cells in the subsequent experiments to obtain A549 silenced cells.

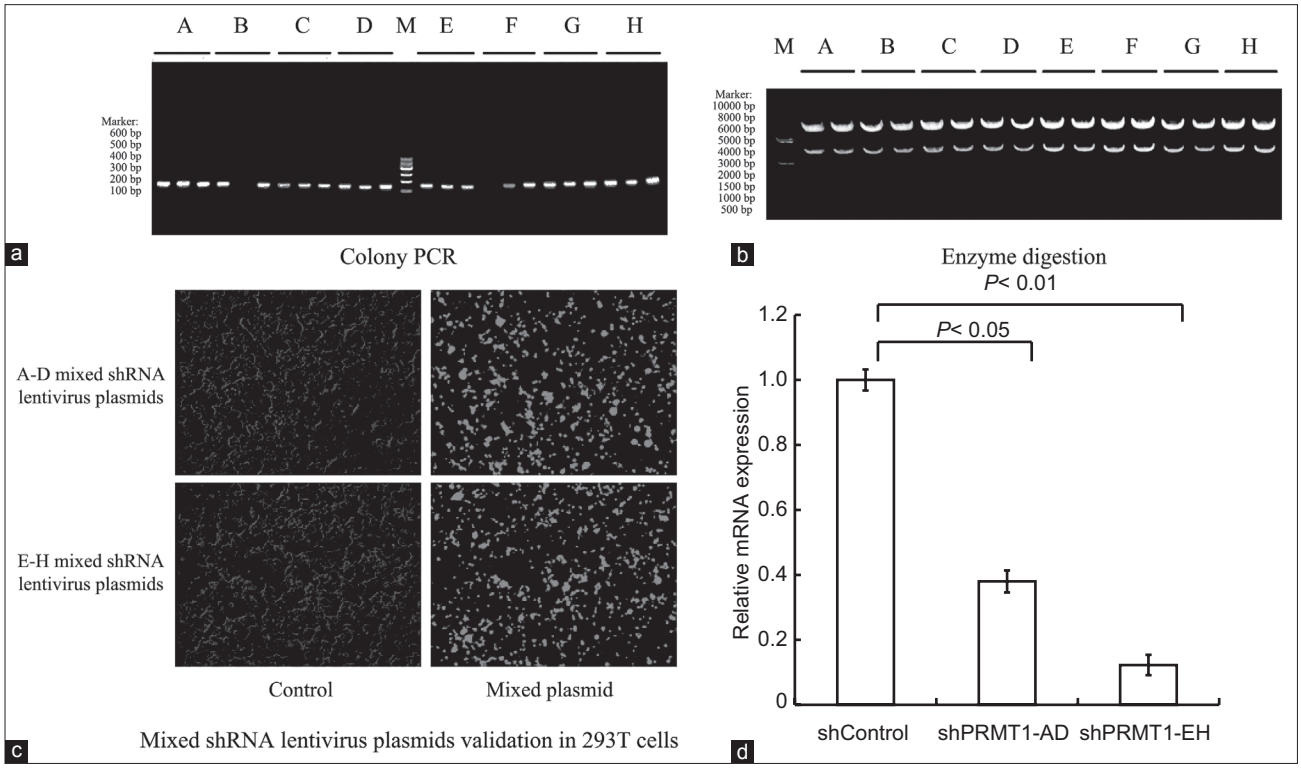

Figure 1: Selection and validation of optimal protein arginine methyltransferases 1 (PRMT1)-small hairpin RNA (shRNA) target sites for RNAi. (a) shRNA was integrated into lentiviral vectors with a connection system where a chemically competent bacterium (GeneHogs) was transformed using connection products. Plasmids were validated by colony polymerase chain reaction (PCR) and the positive clones showed amplified bands with a size of approximately 170 bp; (b) Plasmids restriction enzyme digestion: Each selected fragment had two positive clones, which were digested with KPNI restriction enzyme producing a 2.95 and 7.5 kb fragment; (c) Lentiviral and helper plasmid validation in 293T cells. 293T cells were transfected with lentiviral plasmid and helper plasmid. shRNA lentiviral plasmids containing mixed primers (A-D and E-H) were used for viral packaging, thus each shRNA corresponded to two hybrid viruses; (d) PRMT1 silencing efficiency in A549 cells by quantitative PCR: The expression of PRMT1 mRNA in A549 cells infected by shPRMT1-E was reduced by 87.89% respect the shControl group ($P < 0.05$).

Protein arginine methyltransferases 1 enhancing A549 cells migration

Scratch wound healing assays of protein arginine methyltransferases 1-silenced A549 cells

A scratch wound healing assay was conducted 96 h after A549 transfection with the hybrid plasmids which targeted PRMT1 and with an empty vector (without shRNA, the shControl group). The results showed that migration distance of A549 cells infected by shPRMT1-EH (184.50 ± 44.55 μm) was shorter than the shControl group (288.00 ± 5.66 μm) ($P < 0.05$), while A549 cells infected by shPRMT1-AD (272.00 ± 50.91 μm) were similar with the shControl group [Figure 2] ($P > 0.05$). shPRMT1-EH was the optimal shRNA and would be applied in the following assay.

Transwell cell migration assays of protein arginine methyltransferases 1-silenced A549 cells

The migrated number of A549 cells infected by either PRMT1-EH (0.536 ± 0.183) ($P < 0.02$) or PRMT1-AD (0.371 ± 0.136) ($P < 0.05$) decreased than the shControl group (1.000 ± 0.01) [Figure 3]. The results showed that shPRMT1-EH was the optimal shRNA and would be applied in the following assay. Since PRMT1 expression is associated with an increase A549 migration, the transwell experiment indicated that the migration ability of A549 cells decreased after infected with shPRMT1.

Expression of epithelial-mesenchymal transition markers

Western blotting analysis revealed that the expression of E-cadherin increased while the expression of N-cadherin decreased in A549 cells after PRMT1 silencing [Figure 4a]. The ratio of E-Cadherin to GAPDH in shPRMT1 group (0.909 ± 0.07) was increased significantly than the shControl group (0.624 ± 0.09) ($P < 0.01$), while N-Cadherin in shPRMT1 group (0.860 ± 0.07) was decreased significantly than the shControl group (0.709 ± 0.04) ($P < 0.01$) [Figure 4b]. The results suggesting that PRMT1 facilitates the cell migration of A549 cells via EMT.

Degradation of extracellular matrix proteins

Matrix metalloproteinase 2 mRNA expression levels (0.60 ± 0.15) decreased significantly in PRMT1-silenced A549 cells compared to the shControl group ($P < 0.05$), while the expression

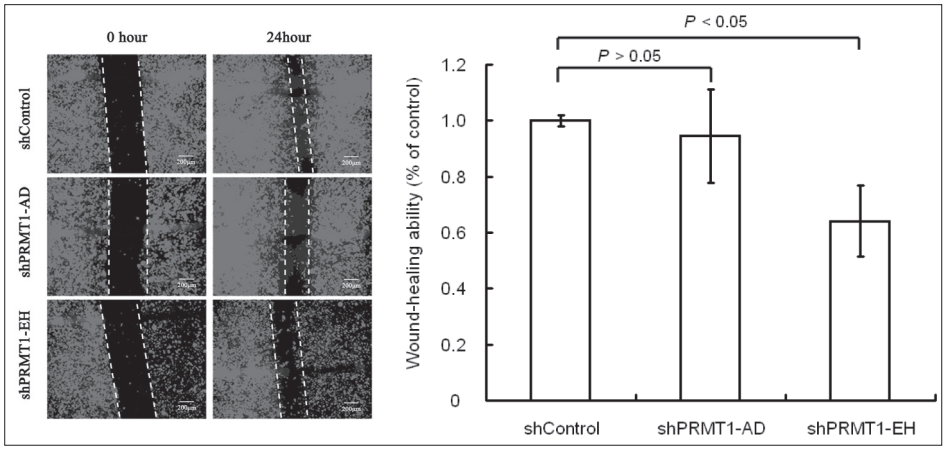

Figure 2: Cell migration of protein arginine methyltransferases 1 (PRMT1)-silenced A549 cells (scratch wound healing assay). The results showed that migration distance of A549 cells infected by shPRMT1-EH was shorter than the shControl group ($P < 0.05$), while A549 cells infected by shPRMT1-AD was similar with the shControl group ($P > 0.05$). shPRMT1-EH was the optimal small hairpin RNA and would be applied in the following assay.

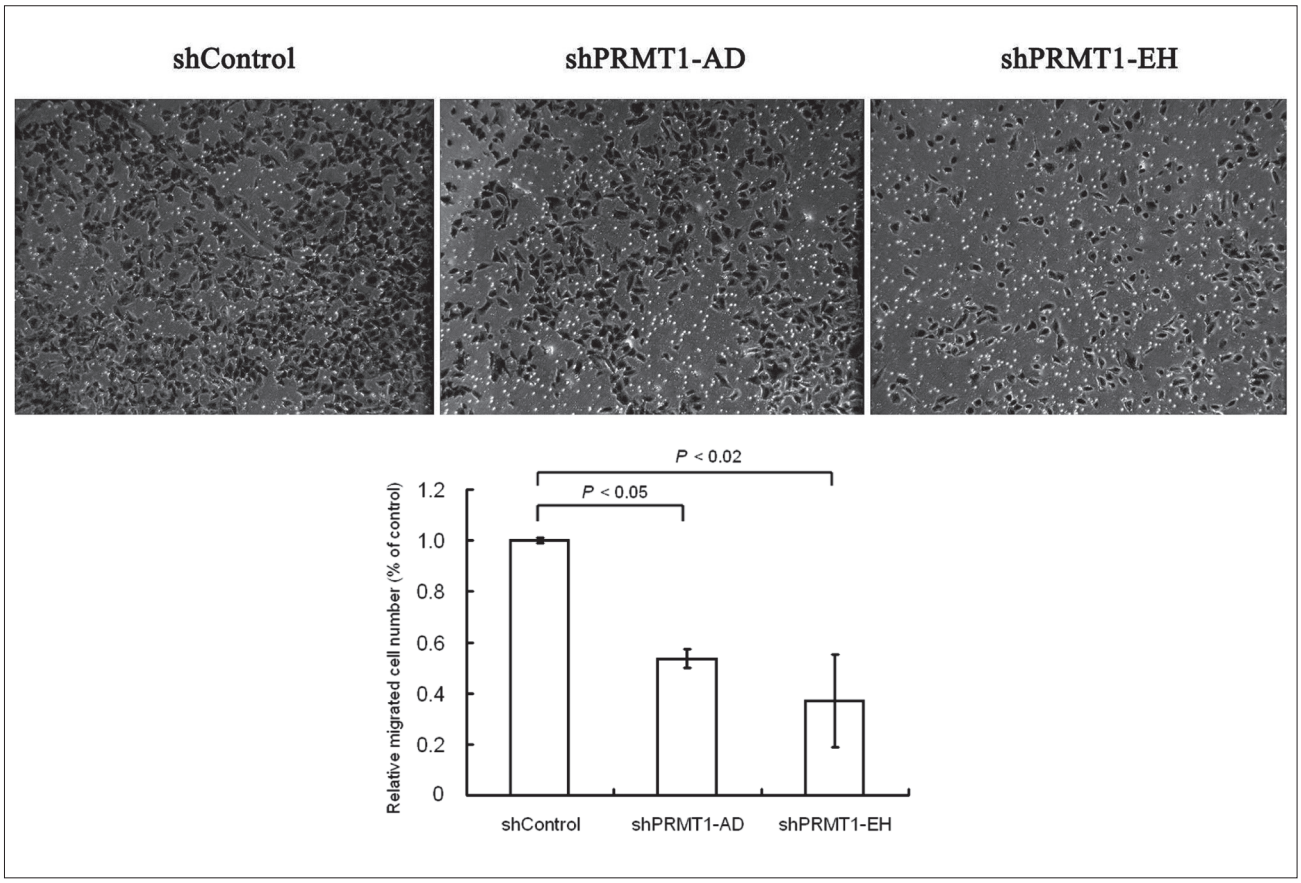

Figure 3: Cell migration of protein arginine methyltransferases 1 (PRMT1)-silenced A549 cells (Transwell cell migration assay). The number of migrated cells in the lower compartment was counted within each field; the number of cells in shPRMT1 group was calculated adjusting with the number in the shControl group. The migrated number of A549 cells infected by either PRMT1-EH ($P < 0.02$) or PRMT1-AD ($P < 0.05$) decreased than the shControl group. The results showed that shPRMT1-EH was the optimal small hairpin RNA and would be applied in the following assay.

levels of TIMP1 (1.69 ± 0.12) ($P < 0.01$) and TIMP2 (1.38 ± 0.07) ($P < 0.05$) increased, respectively [Figure 5]. These results may indicate that PRMT1 promotes the degradation of extracellular matrix proteins to facilitate A549 cell migration. MMP-1 mRNA expression (0.67 ± 0.31) was not significantly changed in PRMT1-silenced A549 cells respect to the shControl group.

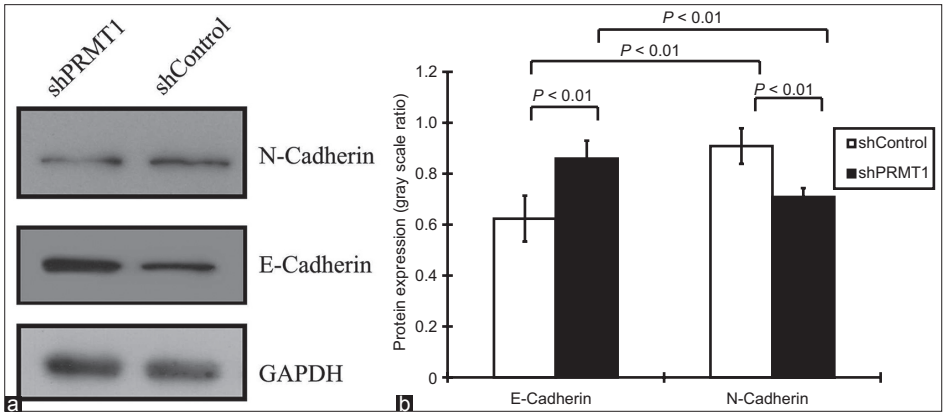

Figure 4: Epithelial-mesenchymal transition marker proteins expression in protein arginine methyltransferases 1 (PRMT1)-silenced A549 cells. (a) Western blot analysis showing the increased expression of E-cadherin and decreased expression of N-cadherin in A549 cells after PRMT1 silencing; (b) Ratios of E-cadherin and N-cadherin to housekeeping gene (GAPDH) were calculated to normalize the expression of proteins. Figure shows the ratio of E-cadherin to GAPDH in shPRMT1 group was increased significantly than the shControl group ($P < 0.01$), while N-cadherin in shPRMT1 group was decreased significantly than the shControl group ($P < 0.01$).

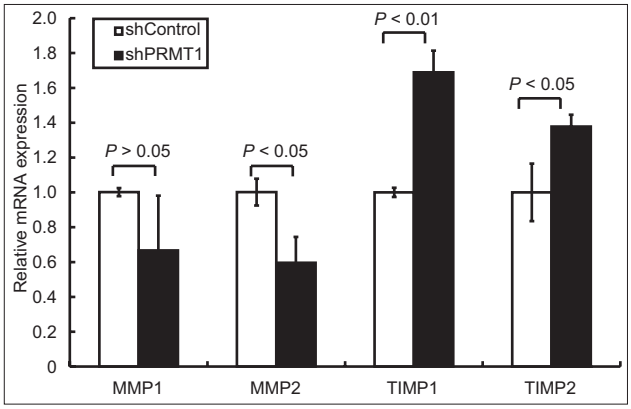

Figure 5: mRNA expression of matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) in protein arginine methyltransferases 1 (PRMT1)-silenced A549 cells. MMP2 mRNA expression levels decreased significantly in PRMT1-silenced A549 cells compared to the shControl group ($P < 0.05$), while the expression levels of TIMP1 ($P < 0.01$) and TIMP2 ($P < 0.05$) increased, respectively. These results may indicate that PRMT1 promotes the degradation of extracellular matrix proteins to facilitate A549 cell migration. MMP1 mRNA expression was not significantly changed in PRMT1-silenced A549 cells respect to the shControl group.

Migration activity of A549 cells

The expression of phospho-Src was reduced in PRMT1-silenced A549 cells, while phospho-FAK and phospho-AKT levels remained unchanged, indicating that PRMT1 promotes A549 cell migration by activating the oncogene Src [Figure 6].

Discussion

In our study, we demonstrated that cell migration was significantly inhibited by silencing PRMT1 in NSCLC cell line A549. PRMT1 expression in A549 cells was silenced using lentiviral vector-mediated short hairpin RNAs. Migration ability of A549 cells infected by PRMT1-shRNA, measured using both scratch wound healing and transwell cell migration assays, was significantly inhibited in the PRMT1 silenced group compared to the control group.

In order to analyze the mechanism of PRMT1 increasing A549 cell migration, we measured the expression level of EMT-related proteins to estimate the relationship between PRMT1 and EMT. EMT represents the transition from an epithelial to a mesenchymal phenotype,[22] and has been considered as one of the hotspot mechanisms regulating tumor invasion and metastasis.[12,13] This transition is characterized by down-regulation of epithelial markers such as E-cadherin and up-regulation of mesenchymal cell markers like vimentin.[23] In this study, E-cadherin expression increased while N-cadherin decreased in PRMT1-silenced A549 cells while expression of E-cadherin and N-cadherin in the shControl groups had opposite change. This suggests that PRMT1-shRNA could inhibit NSCLC migration via inversing EMT.

Extracellular matrix degradation mediated by MMP and TIMP protein families is another key mechanism in tumor metastasis.[14,15] MMPs could proteolytically degrade extracellular matrix proteins leading to tumor metastasis,[16] and this process is proteolytically inhibited by TIMPs.[17] In this study, the mRNA expression of MMP-2 decreased while TIMP1 and TIMP2 increased significantly indicating that PRMT1 may affect expression of MMP and TIMP protein families in A549 cells and regulate the degradation of extracellular matrix.

Previous studies showed that Src can activate MMP-2 via ERK/Sp1 pathway.[24] Src-family kinases are activated in NSCLC and promote the survival of epidermal growth-factor receptor-dependent cell lines.[25] In this study, Src phosphorylation levels increased in A549 cells after PRMT1 silencing by siRNA indicating that PRMT1 affects A549 cell migration through Src activation. However, we did not find any significant phosphorylation of FAK or AKT in PRMT1-silenced A549 cells.

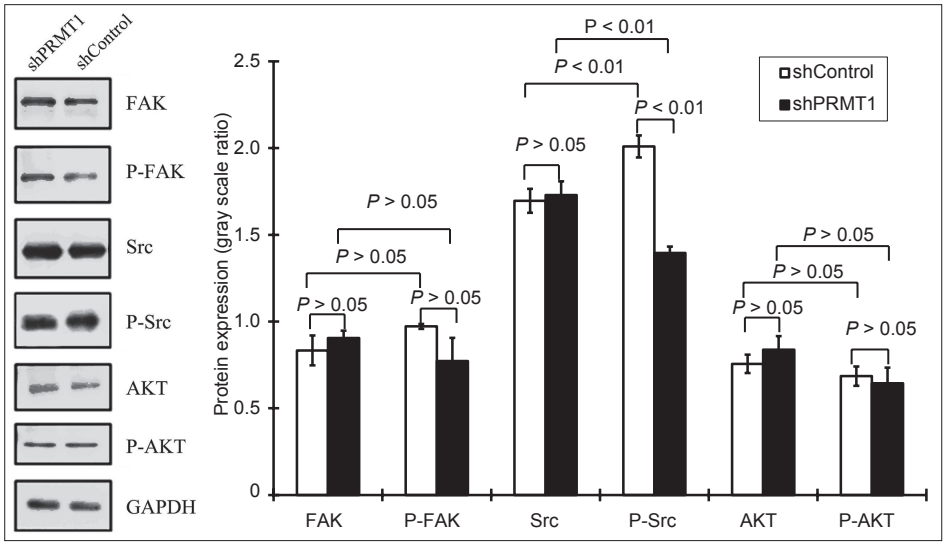

Figure 6: Focal adhesion kinase (FAK), Src, and AKT phosphorylation in protein arginine methyltransferases 1 (PRMT1)-silenced A549 cells. A549 cells infected by shPRMT1 showed a decreased expression of phospho-Src while phospho-FAK and phospho-AKT levels remained unchanged.

In conclusion**,** protein arginine methyltransferases 1-shRNA inhibits the migrated ability of A549 cells *in vitro* by inhibiting EMT, Src phosphorylation, and degradation of extracellular matrix. Our research showed that PRMT1 may be a target gene for the anti-metastatic therapy in lung cancer.

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