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Original Article

Methylation of *PRDM2, PRDM5* and *PRDM16* genes in lung cancer cells

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Abstract: Aims: To investigate the changes of expression and methylation status of PRDM2, PRDM5, PRDM16 in lung cancer cells after treatment with demethylation agent. Methods: A549 (lung adenocarcinoma cell line), HTB-182 (lung squamous cell carcinoma cell line) and HBE (normal bronchial cell line) were treated with 5-aza-2dC. The methylation state of PRDM2, PRDM5, PRDM16 was detected by MSP. The expression of PRDM2, PRDM5, PRDM16 was detected by RT-PCR and Western blot analysis. Cell growth was detected by MTT assay. Results: 5-aza-2-dC reduced the methylation of PRDM2, PRDM5, PRDM16 gene in A549 and HTB-182 cells but not in HBE cells. Consistently, 5-aza-2dC increased mRNA and protein expression of PRDM2, PRDM5, PRDM16 in A549 and HTB-182 cells but not in HBE cells. Furthermore, 5-aza-2dC inhibited the growth of A549 and HTB-182 cells but not HBE cells. Conclusions: PRDM2, PRDM5, PRDM16 promoters are methylated and their expression is suppressed in lung cancer cells. Demethylation drug 5-aza-2dC could upregulate the expression of PRDM2, PRDM5, PRDM16 and suppress lung cancer cell growth. 5-aza-2dC has potential to be used for lung cancer therapy by epigenetic mechanism.

Keywords: PRDM, methylation, lung cancer, epigenetics, gene expression

Introduction

Lung cancer is one of the malignant tumors that are harmful to human health in the world today. Currently, lung cancer is the most common cancer and leading cause of cancer death in China [1]. Several studies have shown that the methylation of tumor suppressor genes leads to gene expression inactivation and presents an important mechanism of tumor development [2-4]. PR (PRDI-BF1 and RIZ) domain proteins (PRDM) are a family of kruppel-like zinc finger transcription factors Seventeen family members are known currently in the human body, named PRDM1 to PRDM17 [5]. Current evidence suggests that PRDM family members play an important role in cell differentiation and malignant transformation [6, 7].

PRDM2 gene is located on human chromosome 21 and its transcription product is 5166 bp [8]. *PRDM2* expression is reduced or lost in breast cancer and gastrointestinal cancer [9]. *PRDM5* gene is located on human chromosome 4. The expression levels of PRDM5 mRNA and PRDM5 protein are reduced in breast, ovarian, liver, lung tumors [10]. In addition, epigenetic silencing of PRDM5 is a frequent event in gastrointestinal cancer and could be a useful molecular target for diagnosis and therapy [11]. *PRDM16* gene is located on human chromosome 1, encoding a protein of 1275 amino acids. *PRDM16* gene is known to be rearranged in acute myeloid leukaemia (AML) and myelodysplastic syndrome (MDS) [12]. However, the role of PRDM16 in solid tumors remains largely unclear. Taken together, while PRDM2, PRDM5, and PRDM16 are implicated in tumorigenesis, their expression and role in lung cancer have not been reported.

In this study, we speculated that PRDM2, PRDM5, and PRDM16 gene methylation may be involved in the pathogenesis of lung cancer. Therefore, we used cultured A549 (lung adenocarcinoma cell line) and HTB-182 (lung squamous cell carcinoma cell line) as in vitro models. We treated the cells with different concentration of demethylation agent 5-aza-2'-deoxycitydine (*5-aza-2dC*) and evaluated the

Table 1. Primers used for methylation-specific PCR

Gene		Primer sequences (5'-3')	Amplicion (bp)	Annealing temperature (°C)	Cycle number
PRDM2	Methylation primer	TCCAAACAAACAAATACCACAAT CCCTAAAACTAAAATCCTACGTA	206	55.37	35
	Unmethylation primer	TTAGGGTAGTAAATAAATTTAGTAGTTGTG CACCCTAAAACTAAAATCCTACATA	212	55.99	35
PRDM5	Methylation primer	TTTTATAGGGAGTAATGGTTTAGCG GCTAATTAACCCGAAATTAACGAC	107	56.84	35
	Unmethylation primer	TTTATAGGGAGTAATGGTTTAGTGG CACTAATTAACCCAAAATTAACAAC	106	53.78	35
PRDM16	Methylation primer	AAATCGTAGTCGTCGTTTTATTTTC TAACCCTTTAAAAAAACATTCCGTA	101	54.60	35
	Unmethylation primer	GGAAAATTGTAGTTGTTGTTTTATTTT TAACCCTTTAAAAAAACATTCCATA	103	52.68	35

Table 2. Primers used for RT-PCR

Gene	Primer sequence (5'-3')		Amplicion (bp)	Annealing temperature (°C)	Cycle number
PRDM2	Upstream	GCTCAAACAACTTCTTCAAACC	518	56.7	35
	Downstream	TGCCTTCAGAGTCACTACAATG			
PRDM5	Upstream	GGAGGTTCGTGGGAGTAAGG	319	52.5	35
	Downstream	TTTACAGCCAAGGCGATCTT			
PRDM16	Upstream	AAATACTGACGGACGTGGAAGT	555	59.1	35
	Downstream	GACACTGGTCGCATTTGTACTC			
β-actin	Upstream	CACGATGGAGGGGCCGGACTCATC	225	62.9	35
	Downstream	TAAAGACCTCTATGCCAACACAGT			

effects on cell growth, and the changes of methylation state, mRNA and protein expression levels of PRDM2, PRDM5 and PRDM16.

Materials and methods

Cell lines

Lung adenocarcinoma cell line A549, lung squamous cell carcinoma cell line HTB-182, and normal bronchial cell line HBE were provided by Central South University XiangYa Medical Center. All cell lines were grown in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Invitrogen, Carlsbad, CA) at 37°C and 5% CO_2. Cells were treated with different concentrations of 5-aza-2dC (0 μmol/L, 1 μmol/L, and 5 μmol/L, 10 μmol/L) for 72 h, and then were collected and used for subsequent experiments.

MTT assay

Cells were seeded into 96-well culture plates at a density of 10,000 cells/well, and cultured in a humidified chamber at 37°C overnight. Next the cells were treated with different concentrations of 5-aza-2dC. Each day for six consecutive days, viable cells were evaluated with MTT assay kit (Sigma, St Louis, MO, USA) according to the manufacturer's instructions. 20 μL of MTT (5 mg/mL) solution were added to each well in 96-well plates and the plates were incubated at 37°C for 4 h, then 150 μL of DMSO were added to each well in 96-well plates and the plates were incubated at room temperature for 10 min. The absorption value of every well (A) was read at 490 nm using a microplate reader (ELX800, Bio-Tek, USA).

Methylation-specific PCR

Genomic DNA was extracted from the cells using Universal Genomic DNA Extraction Kit (Takara, Tokyo, Japan). Genomic DNA (1 μg) was modified with sodium bisulfiteusing EZ-DNA methylation kit (Zymo research, Orange, CA, USA). Bisulfite-treated DNA was used for methylation-specific PCR (MSP). MSP primers

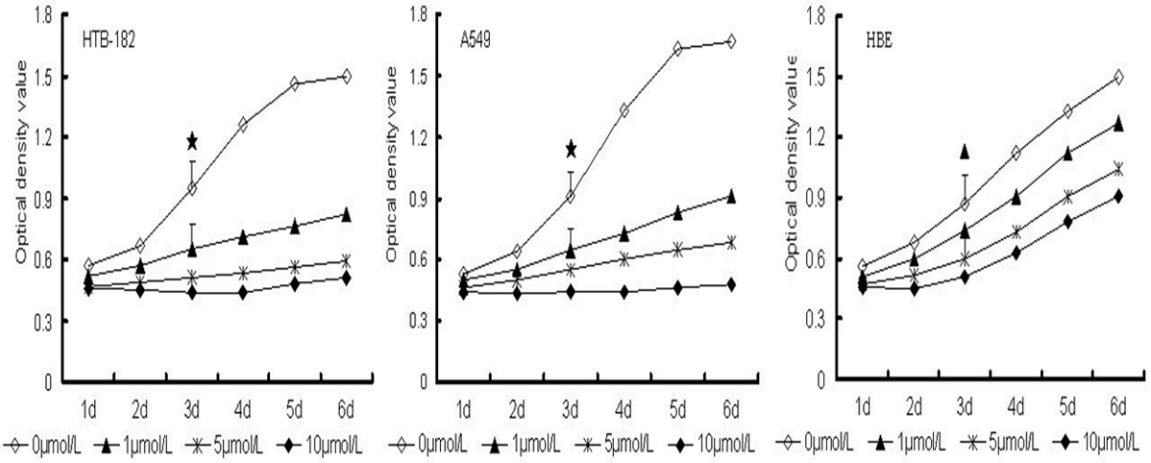

Figure 1. The growth curves of HBE, A549 and HTB-182 cells after treatment with 5-aza-2dC. HBE, normal bronchial epithelial cell line; A549, lung adenocarcinoma cell line; HTB-182, lung squamous cell carcinoma cell line.

were designed by online software (http://www.urogene.org/methprimer/index1.html) and listed in **Table 1**. PCR amplification system (25 μL) includes 10 × Buffer 2.5 μL, dNTP 1.0 μL, 1 μL each methylation or unmethylation primers, DNA template 2 μL, and $MgCl_2$ 2 μL. PCR parameters include 95°C for 5 min, then 95°C degeneration for 30 s, annealing for 30 s, and 72°C extensions for 30 s. PCR products were electrophoresed on 2% agarose gel, and the images were scanned using the uv gel imaging system.

RT-PCR

Total RNA was extracted from the cells using TRIzol (Invertrogen, USA) following the manufacturer's manual. cDNA was synthesized by reverse transcription using a RT kit (Promega, Madison, WI, USA) following the manufacturer's manual. PCR amplification of PRDM2, PRDM5, PRDM16 and β-actin was performed with Taq Master Mix (Promega, Madison, WI, USA) and the primers listed in **Table 2**. Amplification conditions were as follows: 5 min at 94°C (one cycle); 30 sec at 94°C, 30 sec at the annealing temperature, and 30 sec at 72°C (35 cycles); and 72°C for 5 min (one cycle). PCR products were electrophoresed on 1% agarose gel, and the images were scanned using the uv gel imaging system.

Western blot analysis

Total protein was isolated from the cells and quantitated by BSA method. 50 μg protein was loaded and separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Next, the membranes were incubated with specific antibody for PRDM2 (Abcam, 1:500), PRDM5 (Abcam, 1:800), and PRDM16 (Abcam, 1:750) at 4°C o/n. The membranes were washed and then incubated with secondary antibody for 1 h at room temperature. Finally, the membranes were developed using ECL kit (Pierce, Rockford, IL, USA) and exposed to X-ray film for quantifying with Image.plus 5.1 software.

Statistical analysis

All data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using SPSS16.0 software (SPSS, Inc., Chicago, IL, USA). The comparison was performed by using t-test or Q test. The correlation was analyzed by correlation analysis. $P<0.05$ was considered significant.

Results

5-aza-2dC inhibits lung cancer cell growth

MTT assay showed that 5-aza-2dC inhibited the growth of A549 and HTB-182 lung cancer cells in a time and dose dependent manner. In contrast, 5-aza-2dC had no significant effects on the growth of HBE normal bronchial epithelial cells (**Figure 1**). These data suggest that 5-aza-2dC may relieve the suppression of the expression of tumor suppressor genes, which then function to inhibit lung cancer cell growth.

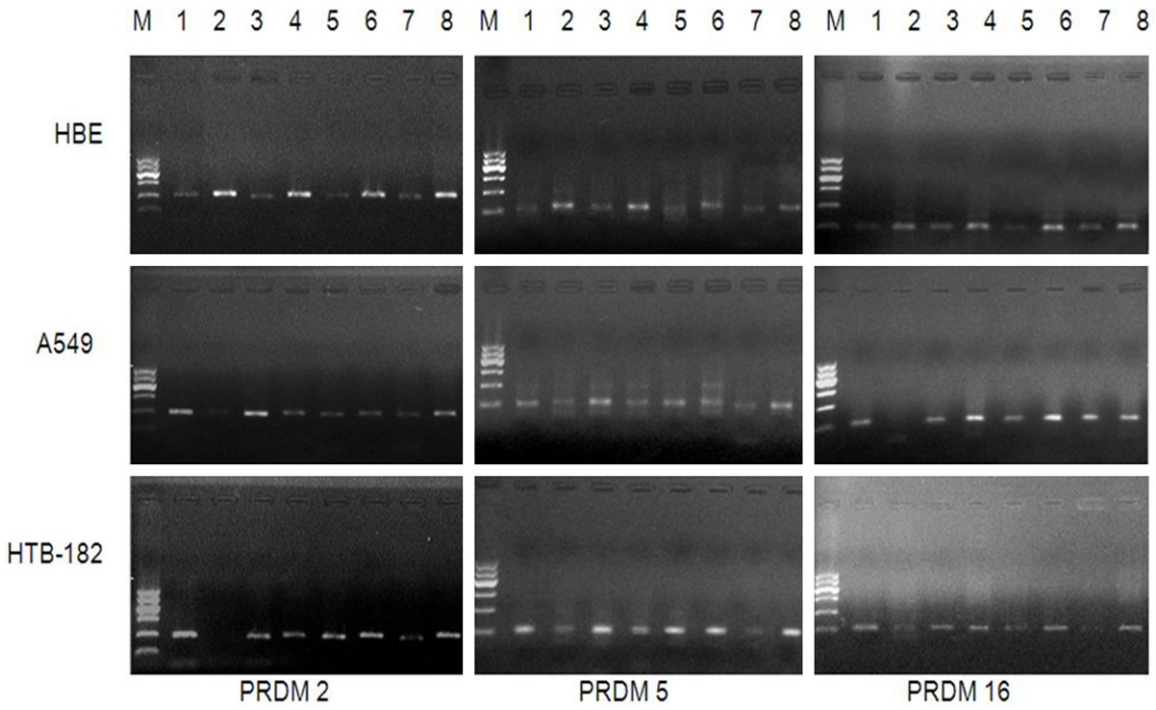

Figure 2. 5-aza-2dC inhibits promoter methylation of *PRDM2, PRDM5* and *PRDM16* in lung cancer cells. Electrophoresis patterns of products of MSP are showed. Lanes 1, 3, 5, 7 represent methylation primer amplification products; lanes 2, 4, 6, 8 represent unmethylation primer amplification products. Lanes 1, 2: cells treated with 0 μmol/L 5-aza-2dC; lanes 3, 4: cells treated with 1 μmol/L 5-aza-2dC; lanes 5, 6: cells treated with 5 μmol/L 5-aza-2dC; lanes 7, 8: cells treated with 10 μmol/L 5-aza-2dC. M: 100 bp DNA ladder.

5-aza-2dC inhibits promoter methylation of PRDM2, PRDM5 and PRDM16 in lung cancer cells

MSP assay showed that 5-aza-2dC inhibited high methylation of promoter regions of *PRDM2, PRDM5* and *PRDM16* in A549 and HTB-182 lung cancer cells in a dose dependent manner. In contrast, 5-aza-2dC had no significant effects on the methylation of promoter regions of *PRDM2, PRDM5* and *PRDM16* in HBE normal bronchial epithelial cells (**Figure 2**). These data demonstrate that 5-aza-2dC could relieve the promoter methylation of tumor suppressor genes *PRDM2, PRDM5* and *PRDM16* in lung cancer cells.

5-aza-2dC increases mRNA expression of PRDM2, PRDM5 and PRDM16 in lung cancer cells

We treated A549 and HTB-182 lung cancer cells with different concentration of 5-aza-2dC and performed RT-PCR analysis to detect *PRDM2, PRDM5* and *PRDM16* mRNA levels (**Figure 3A**). Quantitative analysis showed that *PRDM2* mRNA levels gradually increased in A549 and HTB-182 with increasing concentrations of 5-aza-2dC. In contrast, 5-aza-2dC had no significant effects on mRNA expression of *PRDM2* in HBE cells (**Figure 3B**). Similarly, *PRDM5* and *PRDM16* mRNA levels gradually increased in A549 and HTB-182 with increasing concentrations of 5-aza-2dC. In contrast, 5-aza-2dC had no significant effects on mRNA expression of *PRDM5* and *PRDM16* in HBE cells (**Figure 3C** and **3D**).

5-aza-2dC increases protein expression of PRDM2, PRDM5 and PRDM16 in lung cancer cells

To confirm that 5-aza-2dC could relieve the suppression of the expression of tumor suppressor genes *PRDM2, PRDM5* and *PRDM16* in lung cancer cells, we treated cells with different concentration of 5-aza-2dC, and performed Western blot analysis to detect PRDM2, PRDM5 and PRDM16 protein levels (**Figure 4A**). The results showed that 5-aza-2dC had no significant effects on protein expression of PRDM2 in HBE cells, but increased PRDM2 protein levels

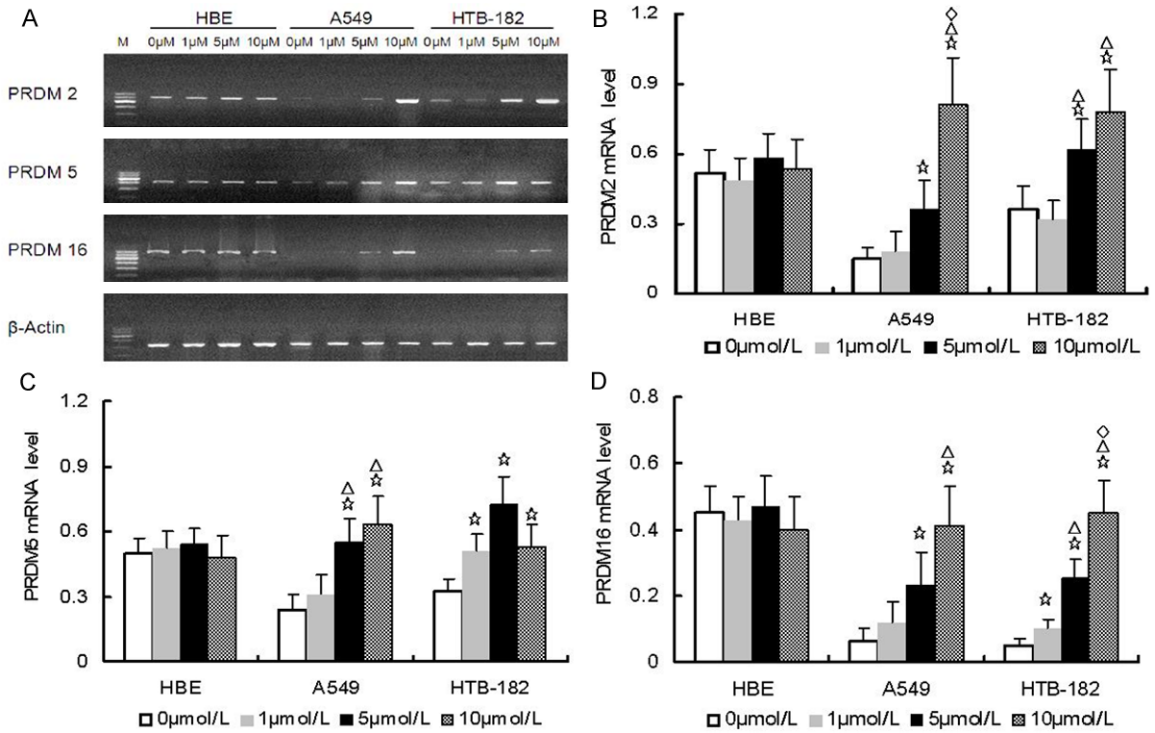

Figure 3. 5-aza-2dC increases the expression of *PRDM2, PRDM5* and *PRDM16* mRNA in lung cancer cells. A: Shown were representative results of RT-PCR analysis. B: PRDM2 mRNA level in cells treated with different concentration of 5-aza-2dC. C: PRDM5 mRNA level in cells treated with different concentration of 5-aza-2dC. D: PRDM16 mRNA level in cells treated with different concentration of 5-aza-2dC. Data were shown as mean ± SD (n=3). ☆P<0.05 compared to 0 μmol/L 5-aza-2dC; △P<0.05 compared to 1 μmol/L 5-aza-2dC; ◇P<0.05 compared to 5 μmol/L 5-aza-2dC.

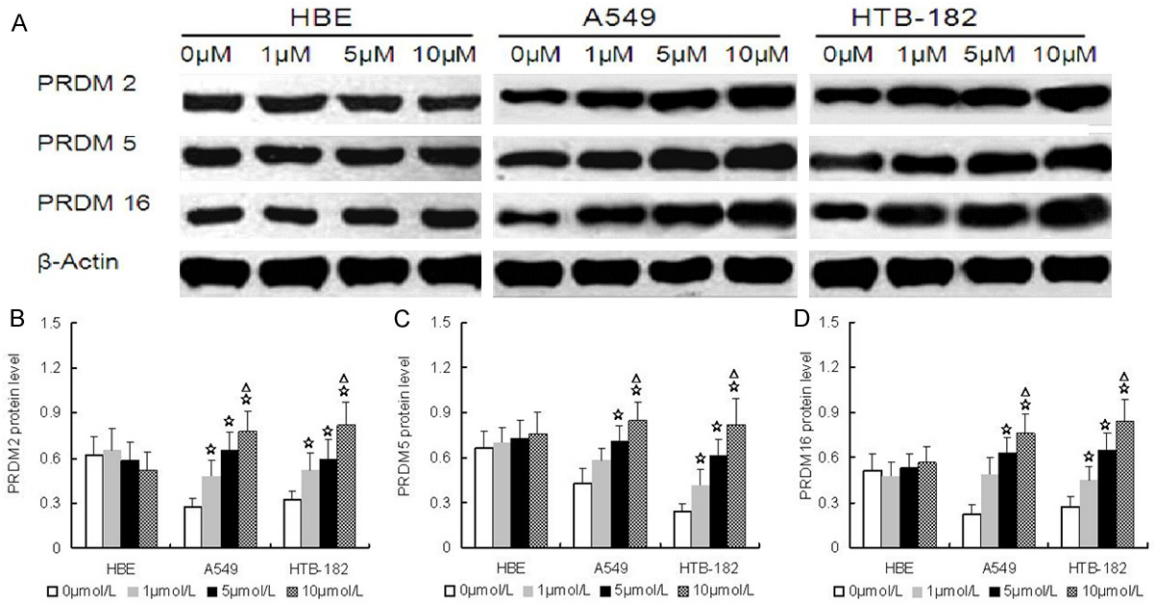

Figure 4. 5-aza-2dC increases the expression of PRDM2, PRDM5 and PRDM16 protein in lung cancer cells. A: Shown were representative blots of Western blot analysis. β-actin was loading control. B: Densitometry analysis of PRDM2 protein level in cells treated with different concentration of 5-aza-2dC. C: Densitometry analysis of PRDM5 protein level in cells treated with different concentration of 5-aza-2dC. D: Densitometry analysis of PRDM16 protein level in cells treated with different concentration of 5-aza-2dC. Data were shown as mean ± SD (n=3). ☆P<0.05 compared to 0 μmol/L 5-aza-2dC; △P<0.05 compared to 1 μmol/L 5-aza-2dC.

in A549 and HTB-182 cells in a dose dependent manner (**Figure 4B**). Similarly, 5-aza-2dC had no significant effects on protein expression of PRDM5 and PRDM16 in HBE cells, but increased PRDM5 and PRDM16 protein levels in A549 and HTB-182 cells in a dose dependent manner (**Figure 4C** and **4D**). Taken together, these results demonstrate that 5-aza-2dC relieves the suppression of PRDM2, PRDM5 and PRDM16 expression in lung cancer cells.

Discussion

With the completion of the Human Genome Project and the development of gene technology, it becomes apparent that epigenetic information such as DNA methylation, histone covalent modification, and non-coding RNA plays an important role in the regulation of gene function, biological behaviors and diseases [13]. In particular, gene methylation plays a potential role in tumorigenesis and methylation markers have significant implication for cancer diagnostics and treatment [14]. 5-aza-2dC is the first demethylation drug applied in the clinical. At present, 5-aza-2dC has been widely used in the treatment of leukemia and multiple myeloma.

In this study we used A549 (lung adenocarcinoma cancer cell line) and HTB-182 (lung cancer cell line) as the experimental model, with HBE (normal bronchial epithelium cell line) as control, to examine the effects of 5-aza-2dC on *PRDM2, PRDM5, PRDM16* gene methylation status and mRNA and protein expression levels. Our results showed that in lung carcinoma cells, *PRDM2 PRDM5, PRDM16* promoters were highly methylated, correlated with lower mRNA and protein expression levels relative to the control group. After treatment with 5-aza-2dC, lung cancer cell growth was inhibited, the methylation of *PRDM2 PRDM5, PRDM16* promoters was gradually decreased, and mRNA and protein expression levels of PRDM2 PRDM5, PRDM16 gradually increased. These results suggest that demethylation agent 5-aza-2dC could relieve the methylation of *PRDM2, PRDM5, PRDM16* promoters, leading to increased expression of PRDM2, PRDM5, PRDM16, which then function to inhibit lung cancer cell growth.

Shu *et al*. examined PRDM5 expression in multiple tumor tissues and cell lines including nasopharyngeal, esophageal, gastric, hepatocellular and cervical cancers. They reported that *PRDM5* was frequently silenced or downregulated in carcinoma cell lines due to promoter CpG methylation, including 80% nasopharyngeal, 44% esophageal, 76% gastric, 50% cervical, and 25% hepatocellular carcinoma cell lines, but not in normal epithelial cell lines. However, *PRDM5* expression in silenced cell lines was restored by 5-aza-2dC treatment. Furthermore, *PRDM5* methylation was frequently detected in 93% nasopharyngeal, 58% esophageal, 88% gastric and 63% hepatocellular tumors [15]. Consistent with their report, here we showed that *PRDM5* was downregulated in lung cancer cells due to promoter methylated and 5-aza-2dC relieved the silencing of *PRDM5* expression in lung cancer cells.

In summary, in this study for the first time we showed that *PRDM2, PRDM5, PRDM16* promoters are methylated and their expression is suppressed in lung cancer cells. Use of demethylation agent 5-aza-2dC could upregulate the expression of PRDM2, PRDM5, PRDM16 and suppress tumor cell growth. Thus 5-aza-2dC has potential to be used for lung cancer therapy by epigenetic mechanism. However, further studies are needed to elucidate the role of *PRDM2, PRDM5,* and *PRDM16* in the initiation and development of lung cancer.

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Disclosure of conflict of interest

None.

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