ORIGINAL ARTICLE

Investigation of the effect of P14 promoter aberrant methylation on the biological function of human lung cancer cells

Bing-Yang Jia, Rong-Hua Yang, Wen-Jie Jiao & Kai-Hua Tian [ID]

Department of Thoracic Surgery, The Affiliated Hospital of Qingdao University, Qingdao, China

Abstract

Background: This study was conducted to investigate the effect of P14 promoter aberrant methylation on the biological function of human lung adenocarcinoma cells.

Methods: We used nested methylation-specific PCR (NMSP) to detect the methylation status of the p14ARF promoter region in SPCA1 and BEAS2B cell lines. The experimental groups were treated with 5-aza-2′-deoxycytidine (5-Aza). Quantitative real-time PCR, Western blot, flow cytometry, and Cell Counting Kit 8 were used to detect the expression of p14ARF messenger RNA and protein in each group, apoptosis, and cell proliferation inhibition, respectively.

Results: NMSP detected that the p14 promoter region of SPCA1 cells has abnormal methylation status. After treatment with 5-Aza, the expression of p14ARF messenger RNA and protein in SPCA1 cells ($P < 0.05$) and the inhibition rate of cell proliferation ($P < 0.05$) were significantly increased, while the apoptosis rate was markedly increased ($P < 0.05$). However, no differences were observed in BEAS2B cells ($P > 0.05$).

Conclusion: Abnormal methylation of the p14ARF promoter region plays an important role in the development of lung cancer cells. Our results suggest the use of P14 promoter aberrant methylation as a therapeutic target for drug research or to improve the sensitivity of other drugs.

Introduction

In recent years, lung cancer has become the most common respiratory malignancy with the highest mortality rate (26%) of all cancer types.[1] With the continuous advancement of science and technology, numerous therapeutic marker genes have emerged, which have significantly contributed to the treatment of lung cancer.[2] According to the pathological types, lung cancer can be divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC includes lung adenocarcinoma, lung squamous cell carcinoma, and large cell lung cancer. Despite several treatment options for NSCLC, surgery is recommended for the treatment of early lung cancer.[3] Therefore, early diagnosis and treatment options for lung cancer have become the goals of medical researchers.

Changes in epigenetics play a significant role in the occurrence of lung cancer.[4] In particular, abnormal hypermethylation of the promoter region in the CpG island,[5] which involves the silencing of tumor suppressing gene transcription, is considered one of the earliest and most common changes in cancer.[6]

As a tumor suppressor gene, the *P14ARF* gene locus on the short arm 2 region 1 of human chromosome 9 (9p21), plays a major role in cell cycle regulation by regulating the *p53* gene.[7] Abnormal methylation of the *P14ARF* gene promoter region is significantly different between lung cancer and paracancerous tissues, which suggests that abnormal methylation of the *P14ARF* gene promoter region is related to the occurrence and development of lung cancer.[8] SPCA1 is a lung adenocarcinoma cell line. In previous studies, this cell line has been shown to be abnormally methylated in the *p16* gene promoter, whose locus is same as *p14*;[9]

therefore, we used this cell line to study *p14* gene methylation. Beas2b cells are normal human lung bronchial epithelial cells, and according to their cell description, there is no relevant genetic alteration, therefore they were applied as the control.

In this study, we used nested methylation-specific PCR (NMSP) to analyze methylation of the SPCA1 cell line and removed abnormal methylation of the *P14ARF* gene promoter region to detect its effect on ARF protein expression and tumor cell apoptosis. From our results, we can conclude the effect of P14 promoter aberrant methylation on the biological function of lung adenocarcinoma. The objective of this study was to determine whether methylation of the *P14ARF* gene promoter region can be used as a potential target gene for NSCLC diagnosis and therapy.

Methods

Cell culture and 5-Aza

SPCA1 cell lines were obtained from the cell bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and Beas2b cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). SPCA1 cells were maintained in RPMI-1640 and supplemented with 10% fetal bovine serum (FBS, BI, Beit Ha'emek, Israel) containing 1% penicillin-streptomycin, while BEAS2B cells were maintained in BEGM (Lonza, Walkersville, MD, USA). The cell culture was maintained in a humidified incubator at 37°C in an atmosphere of 5% CO_2. The experimental group cells were treated with 10 μM 5-Aza (MCE Co., Monmouth, NJ, USA) for four days. The control group cells were treated with the same amounts of phosphate buffered saline (PBS, BI) for the same time periods. Culture medium and 5-Aza were replaced daily.

DNA extraction and bisulfite conversion

The cells were washed with PBS. DNA was collected and extracted using the TIANamp Genomic DNA kit (Tiangen Biotech [Beijing] Co., Ltd., Beijing, China) according to the protocol. The extracted DNA was then treated with a DNA Bisulfite Conversion Kit (Tiangen Biotech [Beijing] Co., Ltd.). All bisulfite converted DNA samples were stored at −20 °C.

Nested methylation-specific PCR

NMSP was performed as previously described.[10] The p14ARF external primers were: 5-TGGGTTTTAGTTTG TAGTTAA-3 (forward) and 5-CTCCTCAATAACATCAA CAC-3 (reverse). The methylation-specific p14ARF

primers were: 5-TTTTTGGTGTTAAAGGGTGGTGTA GT-3 (unmethylated forward), 5-ACCACACACACACCA AATCCA-3 (unmethylated reverse), 5-GTGTTAAAGGGC GGCGTAGC-3 (methylated forward), and 5-CGCGACG AACCGCACGCGCG-3 (methylated reverse).[10] Three rounds of PCR were performed on bisulfite-converted DNA. For the first round, the bisulfite-converted DNA was used, and the external primer was used to obtain a product larger than the target DNA and containing the promoter region of the *p14ARF* gene. The products from the first round were then used in the next two rounds of PCR, and methylated and unmethylated primers were used to identify the methylated and unmethylated regions of the gene, respectively.

For the first round of PCR, the volume of the reaction system was 20 μl, and its main components were 2 μl of bisulfite-treated DNA, 1.6 μl of nucleoside triphosphate (dNTP, 2.5 mM), 2 μl of 10 × MSP PCR buffer, 0.4 μl of Taq genomic DNA polymerase (Tiangen Biotech [Beijing] Co., Ltd.), 1 μl of forward external primer, and 1 μl of reverse external primer. The amplification conditions for the first PCR were: 5 minutes at 95°C, denaturation at 94°C for 20 seconds and annealing at 60°C for 30 seconds, extension at 72 °C for 20 seconds for 35 cycles, and a final 5 minute extension step. We used first round of PCR product to dilute 100 times and then took 2 μl of it for the second round of PCR. The volume and composition of the reaction system in the second round were the same as those used in the first round. In the second round, the different primers were divided into two groups. The cycle parameter was 94°C for 3 minutes, then 94°C denaturation for 30 seconds, 58 °C annealing for 30 seconds (for the unmethylated primer, the annealing temperature was 57°C), extended at 72°C for 30 seconds for 40 cycles, and finally extended at 72°C for 7 minutes; 5 μl of the PCR product was electrophoresed on 2% agarose gel. The experiment was repeated three times.

Quantitative real-time PCR

An RNAprep Pure Kit was used to extract total RNA and a Quant One Step qRT-PCR Kit was then used to detect p14 messenger RNA (mRNA) expression (Tiangen Biotech [Beijing] Co., Ltd.). The primers were: 5'-CCCTCGTGC TGATGCTACTG-3′(forward) and 5'-CCATCATCATGAC CTGGTCTTCT-3′(reverse) with a FAM-labeled Taqman MGB probe AATTCATGTATAAAACTAAGGGCGT (Sangon Biotech [Shanghai] Co.,Ltd., Shanghai, China). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control reagents were used as an internal reference. The volume of the reaction system was 50 μl, and contained the following: Quant One Step RT-qPCR Mix (Probe) 25 μl, Hotmaster Taq Polymerase 2.5 U/μl 2.5 μl, Quant

RTase 0.5 μl, RNA template 20 ng, 1 μl of forward primer, and 1 μl of reverse primer, probe 0.2 μM, and RNase-Free ddH2O 15 μl. The protocol of the Tiangen Quant One Step qRT-PCR Kite was followed. The quantity was then calculated using Applied Biosystems Prism Sequence Detection software version 2.3. The experiment was repeated three times.

Western blot analysis

The cells were scraped and added into radio-immunoprecipitation assay lysis buffer containing 1% phenylmethanesulfonyl fluoride. The product was then incubated on ice for 30 minutes and centrifuged to remove cell debris. A protein quantification kit (Tiangen Biotech [Beijing] Co., Ltd.) was used to determine protein concentration; 50 μg of the protein was transferred to a polyvinylidene fluoride membrane at 250 mA on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The membrane was blocked in 0.05% tris-buffered saline plus tween 20 (TBST) and 5% skim milk powder for two hours at room temperature, 1:500 anti-ARF and 1:1000 β-actin antibodies (Abcam, Shanghai, China) were added, and the membrane was then maintained overnight at 4°C and washed with TBST. The membrane was incubated three times and then incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Abcam) for one hour at 37°C. The protein of interest was visualized using an enhanced electrochemiluminescence detection system (Beyotime Biotechnology, Shanghai, China). The densitometry of the bands was quantified by ImageJ software. The experiment was repeated three times.

Flow cytometry analysis

To evaluate cell apoptosis, the cells were collected and adjusted at a density of 1×10^6/ml, washed twice with PBS, stained with Annexin V-fluorescein isothiocyanate and propidium iodide (Solarbio Life Sciences, Beijing, China) for 15 minutes at room temperature, and then determined by flow cytometry (FCM, Becton-Dickinson, Franklin Lakes, NJ, USA). The experiment was repeated three times.

Cell Counting Kit-8

All cells were seeded in 96-well plates, with 2×10^3 cells in 100 μl of the medium. Cell proliferation was determined using Cell Counting Kit 8 (Solarbio Life Sciences). There were four groups: SPCA1 5-Aza treatment and control groups, and Beas2b 5-Aza treatment and control groups. After culturing for 24, 48, 72, and 96 hours, we measured the optical density (OD) at 450 nm wavelengths and

calculated the proliferation inhibition rate of each. The experiment was repeated three times.

Statistical analysis

Data are shown as mean values ± standard deviation (x̄ ± SD). Differences between the two independent groups were analyzed by the Student's *t*-test. $P < 0.05$ was considered significant. SPSS version 22.0 was used for statistical analyses.

Results

Methylation status of the p14ARF promoter region

We detected the methylation status of the *p14* gene promoter region in SPCA1 and BEAS2B cell lines by NMSP. The results showed that the *p14* gene promoter region of the SPCA1 cell line was methylated, while BEAS2B was not (Fig 1).

Restoration of p14ARF expression by treatment with 5-Aza demethylation

To test whether methylation directly induced p14ARF gene silencing, we used 5-Aza to demethylate the *p14ARF* gene in SPCA1 and BEAS2B cells. Figure 2a shows that after treatment with 10μM 5-Aza for 96 hours, the mRNA of p14 in SPCA1 cells appeared. Expression of p14 mRNA in the 5-Aza group was 0.63-fold higher than in the control
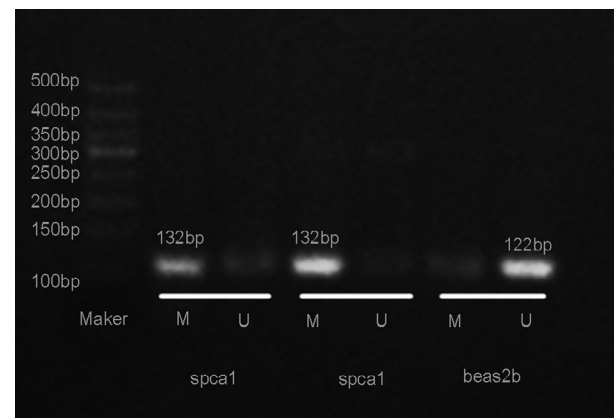

Figure 1 Methylation of the p14ARF gene promoter in each group. Marker: DNA molecular weight marker, M: p14ARF promoter methylation, U: p14ARF promoter unmethylation. Two groups of SPCA1 cells and one group of BEAS2B cells were included. The quantity of PCR production for the methylation statue is 132 bp, while the unmethylation statue is 122 bp. Obviously, the p14 gene promoter region of the SPCA1 cell line was methylated, whereas that of the BEAS2B cell was unmethylated.
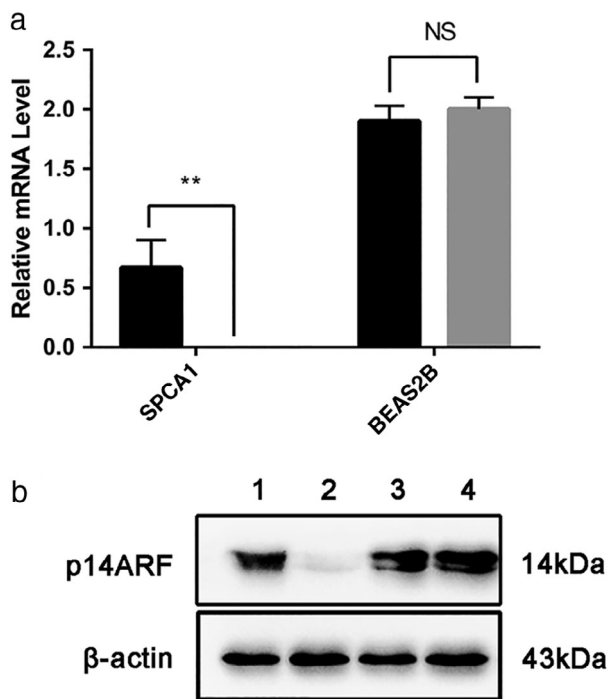
Figure 2 The expression of p14ARF messenger RNA (mRNA) and protein in each group. (**a**) Quantitative real-time PCR. The level of p14 mRNA was normalized to glyceraldehyde 3-phosphate dehydrogenase. *******P* < 0.05. NS, no significance (■) 5 Aza, and (■) Control. (**b**) Western blot: lane 1: SPCA1 cells treated with 10 μM 5-Aza; lane 2: SPCA1 cells in the control group; lane 3: BEAS2B cells treated with 10 μM 5-Aza; lane 4: BEAS2B cells in the control group. p14ARF proteins in the SPCA1 cells were restored, while there was no change in the BEAS2B cells.

group. Meanwhile, Figure 2b shows that p14ARF proteins in the SPCA1 cells were restored. However, there was no obvious change in BEAS2B cells (the expression of mRNA was 0.05-fold lower). These results indicate that methylation directly mediates the silencing of p14ARF in NSCLC.

Biological function is affected by p14ARF demethylation with 5-Aza

We used FCM to investigate the effect of p14ARF promoter region demethylation on apoptosis. The experimental group of each cell type was treated with 10 μM 5-Aza for 96 hours. After FCM, the apoptosis rate of SPCA1 cells in the experiment group was significantly higher at 22.36 ± 2.04% compared to 10.96 ± 0.63% in the SPCA1 control group (*P* < 0.05). As for the BEAS2B cells, there were no significant differences in the experimental 7.71 ± 0.24% and control groups 7.31 ± 0.45% (*P* > 0.05). Therefore, it is apparent that p14ARF promoter region demethylation induces the apoptosis of SPCA1 cells (Fig 3).

To investigate cell proliferation, the OD value of each group was measured after treatment with CCK-8. The results after 96 hours showed that the OD values of the SPCA1 cells in the experimental group (0.57 ± 0.06) were obviously lower than in the control group (1.02 ± 0.23; *P* < 0.05). After treatment with 5-Aza for 24, 48, 72, and 96 hours, the SPCA1 cell proliferation inhibition rates were 39%, 40%, 42%, and 52%, respectively, but there was no significant inhibition in the BEAS2B cells. To conclude, cell proliferation was markedly inhibited in SPCA1 cells when the p14 promoter region was demethylated (Fig 4).

Discussion

DNA methylation is an important component of epigenetics, which play a prominent role in cell regulation and the development of cancer.[12] In addition, *p14ARF* has different biological functions in cells. Previous studies have shown that *p14* acts as a tumor suppressor gene to stabilize and activate *p53* by neutralizing the inhibitory effect of E3 ubiquitin ligase hMdm2 on cancer cells.[13] In addition, *p14* plays an important role in tumor suppression by promoting cell cycle arrest or apoptosis through a p53-independent pathway.[14,15] Abnormal methylation of the p14 promoter region plays a significant role in the development of cancer, and is manifested in liver, bladder, and intestinal cancers.[16]

In this study, methylation of the p14ARF promoter region in the lung cancer SPCA1 cell line was detected by NMSP, while the BEAS2B cell line did not show abnormal methylation. Therefore, it can be concluded that methylation in this region has a certain influence on the development of lung cancer. These results are consistent with findings by Zemaitis *et al.* who reported that most lung cancer patients exhibited methylation of the tumor suppressor gene, which might be associated with the development of cancer.[17] Previous studies have shown that ARF expression can inhibit the occurrence of cancer.[18] Therefore, we used RT-PCR to detect p14ARF mRNA expression and Western blot to detect ARF protein expression. No p14ARF mRNA or protein expression was detected in the SPCA1 cell line, while the beas2b cell line exhibited normal expression, which may indicate that methylation of the p14ARF promoter region contributes to the occurrence of lung cancer.

Studies have shown that 5-Aza has a demethylation function by inhibiting DNA methyltransferase I, which can restore gene function.[19] Therefore, were treated the SPCA1 cell line with 5-Aza to remove *p14* methylation, which is abnormally methylated in the promoter, and restored the expression of the *p14* gene. RT-PCR and Western blot analysis showed that 5-Aza restored p14ARF mRNA and protein expression in the treatment group. Apoptosis of
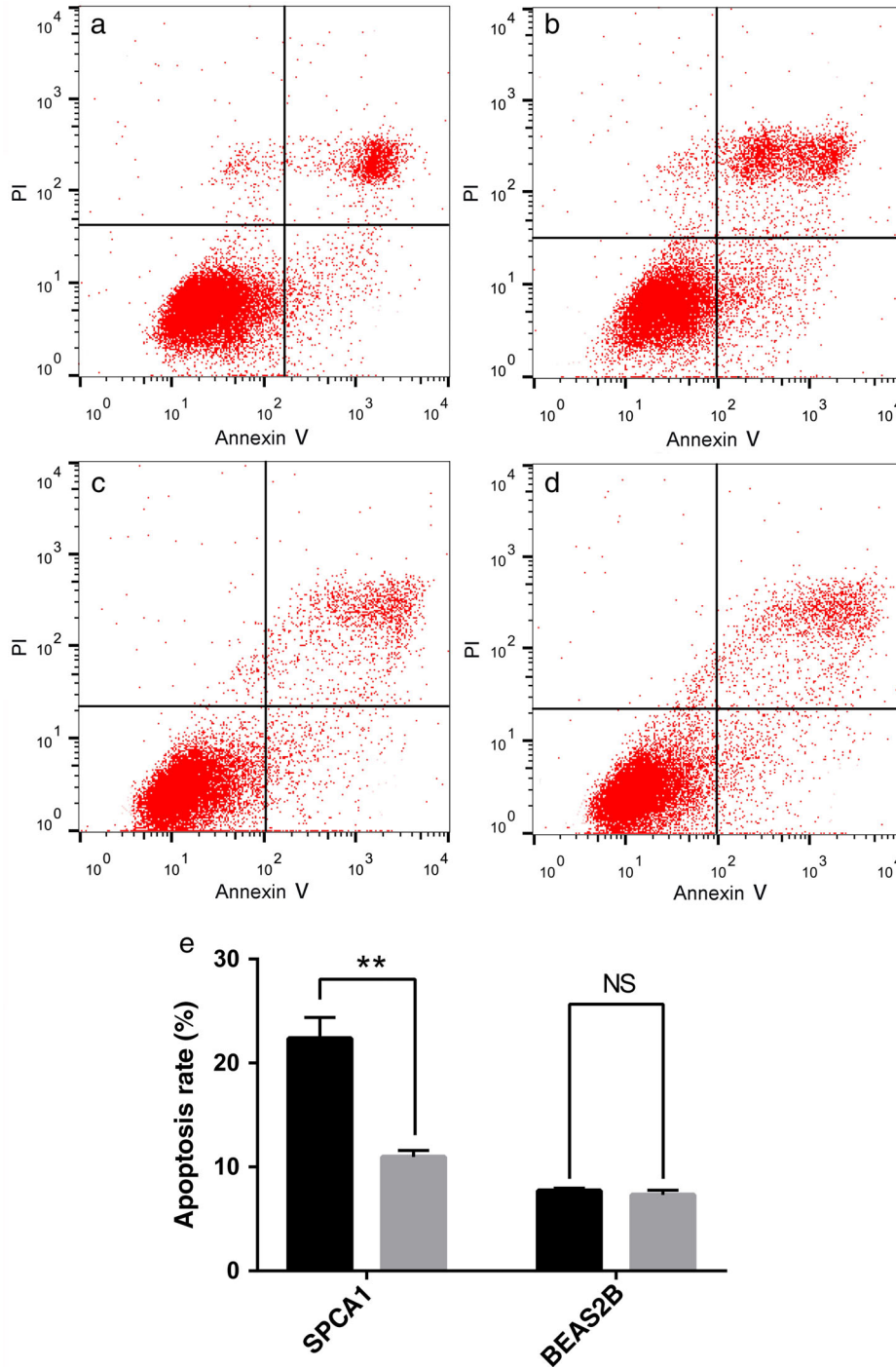
Figure 3 P14 promoter methylation decreases the apoptosis rate of lung cancer cells. (**a**) SPCA1 control group, (**b**) SPCA1 10μM 5-Aza group, (**c**) BEAS2B control group, (**d**) BEAS2B 10μM 5-Aza group and (**e**) the apoptosis rate in each group (■) 5-Aza, and (■) Control. The apoptosis rate in SPCA1 cells was significantly higher than in BEAS2B cells. **P < 0.05. NS, no significance.

cells is an important factor regulating the normal growth and proliferation of cells, but abnormal apoptosis in cancer cells often leads to aberrant proliferation.[20,21] Heo *et al.* used all-trans retinoic acid (ATRA) to remove *P14* gene methylation in cancer cells and activate the *p14* gene. ATRA upregulated the p53 levels via the p14-MDM2-p53 pathway and then induced apoptosis via activation of *p53*.[22] Therefore, we performed FMC to determine the effect of apoptosis. FCM showed that the apoptosis rate of the SPCA1 cell line was increased, while CCK8 showed that cell proliferation was inhibited, and there was no significant difference in the detection results in the BEAS2B cell line. These differences are most likely caused by the following reasons: restoring the expression of the *p14* gene upregulates protein p53, enhances the stability of p53-induced cell apoptosis, and inhibits proliferation.[23]
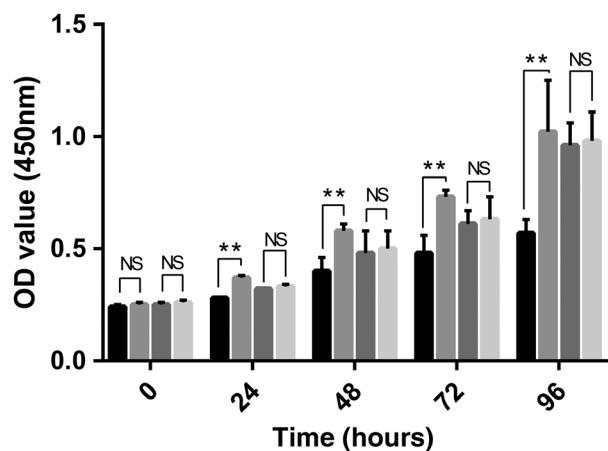
Figure 4 P14 promoter methylation increases the cell proliferation of lung cancer cells. Cell growth curves were delineated and the inhibition rate of cell proliferation was calculated by measuring absorbance at 450 nm. The changes in optical density (OD) value in SPCA1 cells were significantly higher than in BEAS2B cells. **$P < 0.05$. NS, no significance. (■) spca1 5-Aza, (■) spca1 control, (■) beas2b 5-Aza, and (■) beas2b control.

Badal *et al.* reported that 5-Aza can increase ARF protein expression in p14ARF promoter region methylated cells.[11] Using p53 activity assays, they observed an increase in p53 protein activity after 5-Aza treatment, which also induced apoptosis. Liu *et al.* showed that the overexpression of wild-type c-MYC obviously upregulates p14 expression, which induces apoptosis.[24] Our results are consistent with these findings. Therefore, we can conclude that abnormal methylation of the promoter region of the *p14ARF* gene can inhibit ARF mRNA and protein synthesis, which subsequently inhibits lung cancer cell apoptosis. As a result, lung cancer cell proliferation cannot be controlled.

At present, the treatment methods for lung cancer are diverse, from traditional chemotherapy to immunotherapy.[25] However, debate over the choice of treatment method for lung cancer continues.[26] Our study demonstrated the effect of p14 promoter methylation on the biological function, and we found that demethylation can restore *p14* gene expression via a demethylating agent. To increase the cure rate of lung cancer and prolong the survival time of patients, our future studies will investigate the sensitivity of chemotherapy, targeted therapy, or immunotherapeutic drugs after demethylation. Our research was limited to a single lung cancer cell line; therefore, we will include more types of lung cancer cells in future studies.

Overall, abnormal methylation of the promoter region of the *p14ARF* gene affects mRNA and protein expression, cell proliferation, and apoptosis of lung cancer. Our results suggest the use of P14 promoter aberrant methylation as a therapeutic target for drug research or to improve the sensitivity of other drugs.

Disclosure

No authors report any conflict of interest.

References

1 Siegel RL, Miller KD, Jemal A. Cancer statistics, 2017. *CA Cancer J Clin* 2017; **67** (1): 7–30.

2 Bracht JWP, Mayo-de-Las-Casas C, Berenguer J, Karachaliou N, Rosell R. The present and future of liquid biopsies in non-small cell lung cancer: Combining four biosources for diagnosis, prognosis, prediction, and disease monitoring. *Curr Oncol Rep* 2018; **20** (9): 70.

3 Shimada Y, Saji H, Yoshida K *et al.* Pathological vascular invasion and tumor differentiation predict cancer recurrence in stage IA non-small-cell lung cancer after complete surgical resection. *J Thorac Oncol* 2012; **7** (8): 1263–70.

4 Jones PA, Baylin SB. The epigenomics of cancer. *Cell* 2007; **128** (4): 683–92.

5 Klutstein M, Nejman D, Greenfield R, Cedar H. DNA methylation in cancer and aging. *Cancer Res* 2016; **76** (12): 3446–50.

6 Mehta A, Dobersch S, Romero-Olmedo AJ, Barreto G. Epigenetics in lung cancer diagnosis and therapy. *Cancer Metastasis Rev* 2015; **34**: 229–41.

7 Ito T, Nishida N, Fukuda Y, Nishimura T, Komeda T, Nakao K. Alteration of the p14(ARF) gene and p53 status in human hepatocellular carcinomas. *J Gastroenterol* 2004; **39**: 355–61.

8 Mascaux C, Bex F, Martin B *et al.* The role of NPM, p14arf and MDM2 in precursors of bronchial squamous cell carcinoma. *Eur Respir J* 2008; **32** (3): 678–86.

9 Fang HL, Yu ZC, Zhu HB, Jin YT: [Effects of 5-Aza-2-deoxycytidine on DNA methylation of anti-oncogenes in non-small cell lung cancer cells]. *Zhonghua Zhong Liu Za Zhi* 2012;**34**:658–663. (In Chinese.)

10 Li L, Shen Y, Wang M *et al.* Identification of the methylation of p14ARF promoter as a novel non-invasive biomarker for early detection of lung cancer. *Clin Transl Oncol* 2014; **16**: 581–9.

11 Badal V, Menendez S, Coomber D, Lane DP. Regulation of the p14ARF promoter by DNA methylation. *Cell Cycle* 2008; **7** (1): 112–9.

12 Koch A, Joosten SC, Feng Z *et al.* Analysis of DNA methylation in cancer: Location revisited. *Nat Rev Clin Oncol* 2018; **15** (7): 459–66.

13 Sharpless NE. INK4a/ARF: A multifunctional tumor suppressor locus. *Mutat Res* 2005; **576** (1–2): 22–38.

14 Müer A, Overkamp T, Gillissen B *et al.* p14(ARF)-induced apoptosis in p53 protein-deficient cells is mediated by BH3-only protein-independent derepression of Bak protein through down-regulation of Mcl-1 and Bcl-xL proteins. *J Biol Chem* 2012; **287**: 17343–52.

15 Weber JD, Jeffers JR, Rehg JE *et al.* p53-independent functions of the p19(ARF) tumor suppressor. *Genes Dev* 2000; **14**: 2358–65.

16 Ozenne P, Eymin B, Brambilla E, Gazzeri S. The ARF tumor suppressor: Structure, functions and status in cancer. *Int J Cancer* 2010; **127** (10): 2239–47.

17 Zemaitis M, Rieger N, Fischer JR, Sakalauskas R, Malakauskas K, Lahm H. [Aberrant promoter methylation of tumor suppressor genes in serum from lung cancer patients: Frequency and correlation with clinicopathological characteristics]. *Medicina (Kaunas)* 2005. **41**(2): 123–31. (In Lithuanian.)

18 Zindy F, Williams RT, Baudino TA *et al.* Arf tumor suppressor promoter monitors latent oncogenic signals in vivo. *Proc Natl Acad Sci U S A* 2003; **100** (26): 15930–5.

19 Karahoca M, Momparler RL. Pharmacokinetic and pharmacodynamic analysis of 5-aza-2′-deoxycytidine (decitabine) in the design of its dose-schedule for cancer therapy. *Clin Epigenetics* 2013; **5** (1): 3.

20 Fuchs Y, Steller H. Programmed cell death in animal development and disease. *Cell* 2011; **147** (4): 742–58.

21 Shivapurkar N, Reddy J, Chaudhary PM, Gazdar AF. Apoptosis and lung cancer: A review. *J Cell Biochem* 2003; **88** (5): 885–98.

22 Heo SH, Kwak J, Jang KL. All-trans retinoic acid induces p53-depenent apoptosis in human hepatocytes by activating p14 expression via promoter hypomethylation. *Cancer Lett* 2015; **362** (1): 139–48.

23 Matallanas D, Romano D, Al-Mulla F *et al.* Mutant K-Ras activation of the proapoptotic MST2 pathway is antagonized by wild-type K-Ras. *Mol Cell* 2011; **44** (6): 893–906.

24 Liu XJ, Li FN, Jiang DD, Wang XG, Liu XP, Zhang DL, Meng CH [Regulation of p14(ARF) expression and induction of cell apoptosis with c-myc in a p53-independent pathway]. *Zhonghua Yi Xue Za Zhi* 2012. **92**(30): 2140–3. (In Chinese.)

25 Hirsch FR, Scagliotti GV, Mulshine JL *et al.* Lung cancer: Current therapies and new targeted treatments. *Lancet* 2017; **389** (10066): 299–311.

26 Zugazagoitia J, Guedes C, Ponce S, Ferrer I, Molina-Pinelo S, Paz-Ares L. Current challenges in cancer treatment. *Clin Ther* 2016; **38** (7): 1551–66.