

Original Article

Deoxyribonucleic acid (DNA) methyltransferase contributes to p16 promoter CpG island methylation in lung adenocarcinoma with smoking

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Abstract: In this study, the relationship between CpG island methylation and smoking and DNA methyltransferase in the occurrence and development of lung adenocarcinoma was explored by detecting p16 promoter methylation status. Protein and mRNA levels of p16 were detected by immunohistochemistry and in situ hybridization assays. p16 gene promoter and exon 1 CpG island locus Hap II sites methylation status was analyzed with the methylation-specific PCR. Only 4 of 40 p16-positive cases were detected to methylate on CpG islands with 10% methylating rate whereas 18 of p16-negative cases were methylated up to 36.73% of methylating rate. The methylating rates of both p16-positive and p16-negative groups were significantly different. 17 of 50 cases with smoking from total 89 lung adenocarcinoma cases were detected to methylate on CpG islands while only 5 of the remaining 39 non-smokers to methylate. The difference of the methylating rates in both smokers and non-smokers was significant to suggest the closely association of CpG island methylation of p16 with smoking. Furthermore, p16 promoter CpG islands were detected to methylate in 15 of 35 cases with higher DNA methyltransferase activity whereas only 7 detected to methylate in the remaining 54 cases with lower DNA methyltransferase activity. p16 promoter CpG island methylation likely made p16 expressing silence thus contributed to the tumorigenesis of lung adenocarcinoma. Smoking is likely to promote p16 CpG island methylation or by its effect of the activity and metabolism of DNA methyltransferase 1 (DNMT) on CpG island methylation status.

Keywords: p16 tumor suppressor gene, methylation, DNA methyltransferase, smoking, lung adenocarcinoma

Introduction

Lung cancer is one of the fastest growing in morbidity and mortality and the most increasingly life-threatening malignant tumors. The past 50 years many countries have reported that lung cancer was significantly increasing in morbidity. The morbidity and mortality of the male lung cancer are in the first one accounting for all malignancies. Long-term smoking, deterioration of environment and air pollution are closely related to the tumorigenesis of lung cancer although its cause and pathogenesis are yet unclear entirely [1]. Studies have shown that scattered CpG are usually modified by methylation in the normal genome whereas CpG islands non-methylating modification. CpG islands aberrant methylation are often associated with neoplastic diseases and also closely associated with some tumor suppressor genes

transcriptional inactivation [2, 3]. CDKN2/p16, as a tumor suppressor gene reported firstly by Kamb et al in 1994 is found deletion mutations in many tumors involved in tumorigenesis [4]. Some studies support the involvement of deoxyribonucleic acid (DNA) methylation in p16 inactivation. But it is unclear whether smoking and DNMT are involved in the CpG island methylation of p16 [3, 5]. In this study, p16 promoter and exon 1 CpG islands methylation status was detected to analyze whether smoking or DNA methyltransferase 1 (DNMT) related to p16 CpG islands methylation.

Materials and methods

Materials

89 cases of lung adenocarcinoma samples (all through bronchoscopy, biopsy or clinical diag-

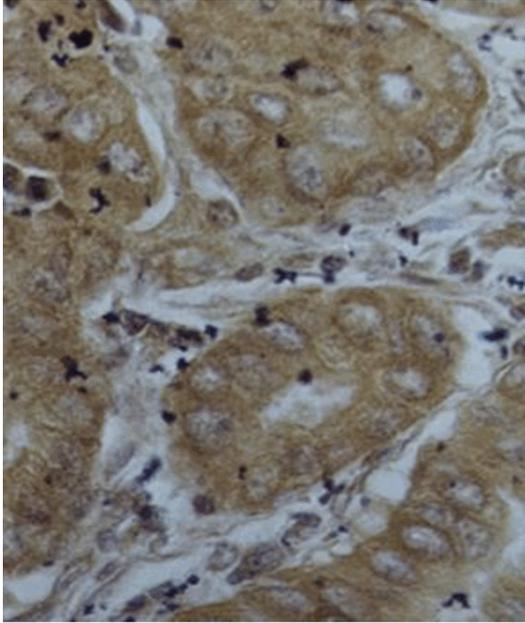


Figure 1. Representative result that p16 protein positive expression with typical dark brown particles in cytoplasm and nucleus in lung adenocarcinoma (DAB stained, 400×).

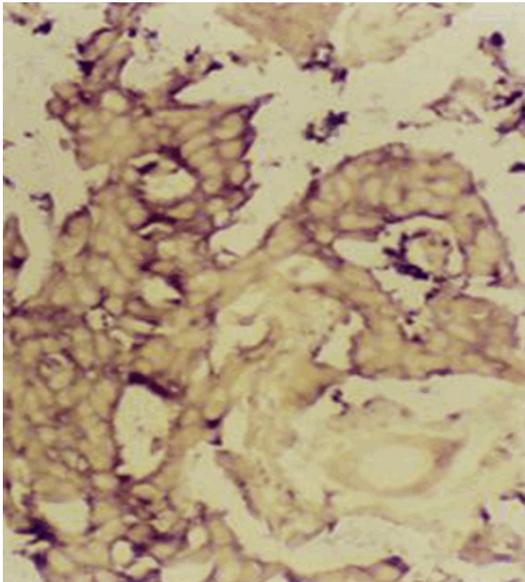


Figure 2. Representative result of in situ hybridization that p16 mRNA positive signals with dark blue particles in the cytoplasm in lung cancer (NBT-BCIB stained, 400×).

nosis and operation, TNM of lung cancer between T1N0M0-T1-3N1M0). All were male patients, including 50 smokers, 20 cases with regional lymph node metastasis. Blood test was prepared for p16 methylation and DNMT

activity. 30 cases of survival more than 5 years postoperative follow-up. 16 cases of benign diseases as control group. Rabbit anti-human p16ink4a polyclonal antibody (c-20, Santa Cruz), Streptavidin-peroxidase immunohistochemical staining kit (SP9001, Zymed, CA). p16 gene promoter and exon 1 primer, sense: 5'-AAT TCG GCA CGA GGC AGC ATG GA-3', antisense: 5'-AAG AGC CAG TCT CTG GCC CCA GCC A-3'. In situ hybridization: probe: A-CAAGCTGGCGCT-GCCCCGA labeled 3' end, B-CAGACAGGTTCA-CGCCCTT labeled 5' end of digoxin antibody labeled probes were synthesized by Beijing Institute of Microbiology, Chinese Academy of Sciences. DNMT activity/inhibition assay kit (EpiQuik DNMT activity assay kit) (AP3009, Epigentek, NY). This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of General Hospital of PLA. Written informed consent was obtained from all participants.

p16 gene expression p16 protein level was detected by immunohistochemical technique in lung cancer samples, messenger ribonucleic acid (mRNA) level of p16 was detected by in situ hybridization with digoxigenin labeled probes. Briefly, specific probes were prepared and labeled as the following sequences: probe: A-CAAGCTGGCGCTGCCCCGA labeled 3' end, B-CAGACAGGTTACGCCCTT. The tissue sections were hybridized with the labeled probes and colored with NBT-BCIP under a dark environment. The positive cells were ones with dark blue granules in cytoplasm. The percentage of positive cells was counted and staining intensity were calculated with computer-aided scanning/image analyze.

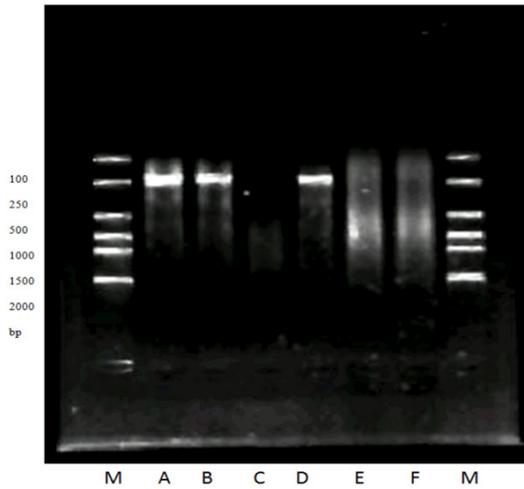
Methylation-specific polymerase chain reaction (MS-PCR) normally, both Hap II and Msp I endonucleases can cut CpGs sequences. When the presence of methylated CpGs modifications, Hap II cannot recognize and cut DNA sequence and 233 bp specific chips can be obtained after PCR amplification while Msp I can smoothly cut methylated DNA due to its not sensitive. No bands can be obtained after PCR as a control. About 1 µg DNA plus 20-30 units Hap II or Msp I digested overnight, 50 ng digested DNA for PCR amplification. PCR reaction conditions were: 94°C for 1 min, 55°C for 70 s, 72°C for 80 s and repeated 21 cycles, then extended last cycle 5 to 10 min. 1.2% agarose

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Table 1. The relationship of p16 promoter CpG island methylation to p16 protein expression

| p16 expression | Hap II sites methylation state | | Total |
|----------------|--------------------------------|-----------------|-------|
| | Methylation | Non-methylation | |
| Positive | 4 | 36 | 40 |
| Negative | 18 | 31 | 49 |
| Total | 22 | 67 | 89 |

$\chi^2 = 8.4586$, $P = 0.006$.



Note: Methylated representative case (A, B and C); Non-methylated representative case (D, E and F)

Figure 3. CpG island methylation analysis that gel electrophoresis with PCR products for genomic DNA completely digested by both endonuclease Hap II and Msp I. This is a representative result. M, low molecular weight marker; A and D, parallel control; B and E, Hap II enzyme digested, lane B but not lane E showed specific 233 bp bands meaning that p16 promoter CpG islands methylation locu Hap II sites; C and F, Msp I enzyme digested, no specific bands appeared.

gel electrophoresis analysis and statistical methylation percentage.

DNA methyltransferase activity analysis Epi-Quik DNMT activity/inhibition assay kit (AP3-009, Epigentek), including 10 \times wash buffer, DNMT assay buffer, AdoMet (8 mM), DNMT positive control, capture antibody, detection antibody (200 μ g/mL), developing solution, stop solution, 8-well substrate-coated strip. DNMT activity was measured as fluorescence intensity, its excitation wavelength of 530 \pm 20 nm, an emission wavelength of 580 \pm 20 nm. Compared with the negative control, DNMT activity is proportional to the relative fluorescence units (RFU). To detect total DNMT activity in blood of 89 cases of lung adenocarcinoma, 50 μ L/sample was taken in 96-well plate to

measure the OD and RFU and calculate the rate (pmol/h). DNMT relative activity is stratified that the relative activity of more than 5 is regard as high DNMT activity while the relative activity of less than 5 is regard as low DNMT activity. The relationship between DNMT activity and p16 promoter methylation status was analyzed by the chi-square test.

Statistical analysis

All data was analyzed using the chi-square test or Fisher's exact test or corrected chi-square test. * $P < 0.05$ was considered significant and ** $P < 0.01$ was considered highly significant. Test level $\alpha = 0.05$.

Results

p16 expression was probably transcription inhibited by the promoter CpG islands aberrant methylation

p16 protein and mRNA levels were detected respectively using immunohistochemistry and in situ hybridization in 89 diagnosed cases of lung adenocarcinoma. In immunohistochemistry assay, p16 antibody was instead of PBS as a negative control, samples that p16 clearly expressed in lung cancer was as positive control. p16 protein positive signal was brown, mainly in both nuclear and plasma or only in plasma (**Figure 1**). p16 protein was regarded as positive as at least 10% positive tumor cells counted in 200 cells randomly. p16 mRNA positive signal was dark blue particles in cytoplasm (**Figure 2**). p16 expressed positively in 40 of 89 cases with a 44.94% positive percentage whereas 14 of 16 cases of the benign control group with a 87.5% positive percentage. The difference of p16 expression between lung adenocarcinoma and control groups was significant ($\chi^2 = 9.8324$, $P = 0.002$). DNA extracted from lung cancer tissues of 89 lung adenocarcinoma cases and 16 benign lung disease cases was performed to PCR to detect p16 promoter CpG islands methylation states using methylation-specific PCR assay. The results showed that 22 of 89 lung cancer cases were detected to p16 promoter and exon 1 CpG islands methylation with a 24.72% of methylation rate whereas no methylation was detected in control group. The difference of CpG islands methylation rate was strongly significant compared to that of control group ($P = 0.016$) (**Table 1**). The positive representative result of p16

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Table 2. p16 promoter CpG island methylation is related to smoking in lung cancer

| Smoking | CpG islands methylation state | | Total |
|-------------|-------------------------------|-----------------|-------|
| | Methylation | Non-methylation | |
| Exposed | 17 | 33 | 50 |
| Non-exposed | 5 | 34 | 39 |
| Total | 22 | 67 | 89 |

$\chi^2 = 5.2815, P = 0.02.$

Table 3. The relationship of smoking to DNMT activity in lung adenocarcinoma

| Smoking | DNMT relative activity (Ratio) | | Total |
|-------------|--------------------------------|--------------|-------|
| | High activity | Low activity | |
| Exposed | 26 | 24 | 50 |
| Non-exposed | 9 | 30 | 39 |
| Total | 35 | 54 | 89 |

$\chi^2 = 7.6816, P = 0.008.$

promoter methylation by MS-PCR was detected to present specific 233bp band after Hap II enzyme digestion (**Figure 3**). Of all 89 cases, only 4 of 40 cases that p16 expressed positively were detected to promoter CpG islands methylation while 18 of the remaining 49 cases that p16 expressed negatively detected to methylation of CpG islands. The difference was clearly significant suggesting that promoter CpG island methylation states significantly inhibited the expression of p16 ($\chi^2 = 8.4586, P = 0.006$) (**Table 1**).

Smoking probably affects p16 promoter CpG islands methylation state in lung cancers

Of all 89 cases of lung adenocarcinoma, 17 of 50 smokers were detected to promoter CpG island methylation whereas only 5 of the remaining 39 non-smokers detected to methylation. The difference was significant ($\chi^2 = 5.2815, P = 0.02$) suggesting that smoking probably inhibits p16 promoter CpG island methylation in lung cancer (**Table 2**).

DNMT activity affects p16 promoter CpG island methylation and may be related to smoking

Preoperative peripheral blood samples were collected from 89 cases of lung adenocarcinomas to detect DNMT activity and analyze the relationship of DNMT, smoking with p16 promoter CpG island methylation. 35 of 89 cases showed high DNMT activity while only 1 of 16 benign controls showed high DNMT activity,

suggesting that DNMT activity of lung adenocarcinomas were much higher than that of benign controls ($\chi^2 = 6.5852, P = 0.012$). In all 89 lung cancers, 26 of 50 smokers were detected to have high DNMT activity whereas 9 of the remaining 39 non-smokers had high DNMT activity. The difference was significant ($\chi^2 = 7.6816, P = 0.008$), suggesting that DNMT activity of lung adenocarcinomas with smoking was higher than that of non-smokers (**Table 3**). Furthermore, 15 of 35 cases with high DNMT activity were detected to have p16 promoter CpG islands methylation whereas only 7 of the remaining 54 cases with low DNMT activity had p16 promoter methylation. The difference was significant, suggesting a closely association between DNMT activity and p16 promoter CpG island methylation ($\chi^2 = 10.1983, P = 0.001$) (**Table 4**).

Discussion

DNA methylation is closely related to human tumorigenesis. CpG island methylation leading to inactivation of tumor suppressor genes play an important role in cancer development and progression. Environmental factors such as exogenous carcinogens can make DNA abnormal methylation and inhibit its binding transcription factors and block gene transcription and silence gene expression and directly affect the functions performed by proteins. About 26% of the 5'CpG island methylation associated with transcriptional silence occurred in the events of primary lung cancers. It is estimated that, in the several types of primary malignant tumors, the frequency of occurrence of the tumor suppressor gene methylation may be 0%-75% [6-10].

In the past 10 years, DNA methylation as epigenetic modification occurs often in the promoter region of tumor suppressor gene and is associated with transcriptional silencing of the genes [11, 12]. p16INK4A gene, known as multiple tumor suppressor 1 is directly involved in cell cycle regulation and negative regulation of cell growth and division [4]. The p16INK4A is frequently inactivated by de novo promoter hypermethylation in many cancer types including lung cancer [13, 14]. In this paper, 89 male cases lung adenocarcinomas data from north China region were analyzed to focus on the regional characteristics of p16 methylation leading to its inactivation and its relation to smoking and DNMT activity.

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Table 4. p16 promoter CpG islands methylation related to DNMT activity in lung adenocarcinoma

| RFU of DNMT activity | CpG island Hap II methylation states | | Total |
|----------------------|--------------------------------------|-----------------|-------|
| | Methylation | Non-methylation | |
| More than 5 of RFU | 15 | 20 | 35 |
| Less than 5 of RFU | 7 | 47 | 54 |
| Total | 22 | 67 | 89 |

$\chi^2 = 10.1983, P = 0.001.$

In the 89 male lung adenocarcinoma cases randomly selected from northern China, 40 cases were detected to express p16 protein positively with a 44.94% of positive percentage. Under the same laboratory conditions, 14 of 16 benign lung diseases were detected to express p16 protein with a 87.5% of positive percentage. p16 protein expression of lung adenocarcinomas was much lower than that of control group. 22 of 89 cases were detected to CpG island Hap II sites methylation in the p16 promoter and exon 1 region with a 24.74% of methylating percentage whereas no methylation detected in CpG island in the p16 promoter region of all benign control group, suggesting that the regulatory sequences upstream of p16 promoter region methylation is likely to be characteristic abnormal changes of lung cancer cells different from non-tumor cells. Furthermore, 18 of 22 cases with CpG island methylation in the promoter of p16 gene were detected to negative expression of p16 protein whereas only 4 of 44 cases with p16 protein positive expression were detected to CpG island methylation in the promoter and exon 1 region, namely the majority cases of p16 promoter CpG island methylation were negatively expressed of p16 protein in lung adenocarcinomas, strongly suggesting that CpG island methylation in the promoter regulatory region significantly inhibits the expression of p16 protein.

In all 55 smokers of 89 male lung adenocarcinoma cases, 17 smokers were detected to CpG island methylation in the promoter and exon 1 of p16 gene. Only 5 cases of the remaining 34 non-smokers of 89 lung cancers were detected to CpG island methylation. The results suggested that smoking exposure was closely associated with p16 promoter CpG island methylation. Long-term exposed smoking is likely an important risk factor to induce p16 promoter methylation. DNA methylation is catalyzed by DNA methyltransferase enzyme, thus aberrant methylation of tumor suppressor gene is likely related to DNA methyltransferase activity, me-

tabolism and/or abnormal regulation [15, 16]. Furthermore, DNA methyltransferase activity was detected paralleled at the same time p16 promoter methylation detected in all peripheral blood samples of 89 lung cancer cases. The results showed that 15 of 35 (42.86%) cases with higher DNMT activity (RFU more than 5) were detected to p16 CpG island methylation whereas only 7 of 54 (12.96%) cases with lower DNMT activity (RFU less than 5) were detected to p16 promoter methylation, namely p16 CpG island methylation easily occurred in the cases with higher DNMT activity, suggesting that DNMT activity was closely related to p16 promoter CpG island methylation.

Although the exact mechanism of smoking induced lung tumorigenesis is unclear entirely, Smoking has strong association with lung cancer and smoking, at least partially contributes to the tumorigenesis of lung cancer [17-19]. In this paper, a likely link was proposed that smoking closely related to p16 promoter methylation probably through DNMT in male lung adenocarcinomas with smoking history. p16 promoter and exon 1 region CpG islands is probable one of targets of smoking-induced lung cancer. Smoking, especially its some components induces DNMT activity by regulating DNMT itself or its certain metabolic processes and finally targets CpG islands methylation in the p16 promoter regulating upstream regions by regulating methylation metabolic and modifications. CpG islands methylation leads expressing silence of p16 gene.

Transcriptional inhibition is an important mechanism of gene methylation leading to expressing inactivation. In this process, the amount of generated mRNA decrease or is deleted and the gene sequence itself is not changed. Although the 5'CpG islands aberrant methylation, p16 gene has still expression potentials [15]. Methylation modification is a reversible dynamic process, when the factors leading to methylation once released, genes can be restored partially from methylation modifications. Methylation inhibitors such as 5-aza-z'-deoxycytidine can make CpG islands hypermethylation be corrected, and guide tumor suppressor genes to re-express functional proteins [16, 20-23]. This provides a theoretical basis and clinical ideas for the application of demethylation drugs to treat cancers.

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Disclosure of conflict of interest

None.

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