

## DNA METHYLATION AND EXPRESSION OF *p16<sup>INK4A</sup>* GENE IN PULMONARY ADENOCARCINOMA AND ANTHRACOSIS IN BACKGROUND LUNG

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**The *p16* (CDKN2/MTS-1/INK4A) tumor-suppressor gene is frequently inactivated by DNA methylation in lung carcinomas. To clarify whether background anthracosis may play a role in DNA methylation and inactivation of the *p16* gene, we examined DNA methylation of the *p16*-promoter region by methylation-specific polymerase chain reaction, and *p16* expression immunohistochemically, and compared the results with the level of background anthracosis which was measured by an original quantitative method. At autopsy, DNA methylation of the *p16* gene was observed in 6/19 tumors (32%) from patients who had died of pulmonary adenocarcinoma. The degree of background anthracosis (the effect of extrinsic carcinogenic factors) (mean absorbance value,  $A = 0.715$ ) of the cases with *p16*-gene methylation was significantly higher than that without methylation (mean  $A$  value = 0.298). *p16* expression was inactivated in all tumors with *p16*-gene methylation. The mean  $A$  value of black dust matter deposition in cases with normal expression of *p16* ( $A = 0.151$ ) was significantly lower than cases with abnormal expression of *p16* ( $A = 0.531$ ). These results indicate that the level of background anthracosis is closely associated with inactivation of *p16* expression and also DNA methylation of the *p16*-gene promoter region in pulmonary adenocarcinogenesis. *Int. J. Cancer (Pred. Oncol.)* 84:609–613, 1999.**

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Adenocarcinoma of the lung is the most common form of lung cancer and is increasing in Japan, the US and European countries (Thun *et al.*, 1997). Genetic alterations in these tumors including point mutations of dominant oncogenes such as the *ras* genes, and point mutations of tumor-suppressor genes such as *p53* and the retinoblastoma (*rb*) gene have been well characterized (Jonson and Kelley, 1993). The *p16* tumor-suppressor gene is also closely associated with lung carcinogenesis (Hayashi *et al.*, 1994; Okamoto *et al.*, 1995). *p16* encodes a cell-cycle protein which is a potent inhibitor of CDK4 and CDK6 (Serrano *et al.*, 1993, 1995) and negatively regulates the cyclin-D-dependent phosphorylation of the *Rb*-gene product, thereby inhibiting cell-cycle progression  $G_1$  to S-phase by sequestration of E2F (Lukas *et al.*, 1995). Therefore, its genetic alteration results in de-regulation of cell proliferation through loss of  $G_1$  arrest control. It is commonly accepted that loss of function of tumor-suppressor genes usually occurs through allelic loss and point mutation (Tamura *et al.*, 1997). However, in some tumor-suppressor genes, including *p16*, loss of function is mediated mainly by DNA methylation, which is thought to be an important epigenetic alteration in lung carcinogenesis (Merlo *et al.*, 1995; Otterson *et al.*, 1995).

The lung may be exposed to numerous DNA-damaging chemical carcinogens, such as mycotoxins, plant alkaloids, nitrosamines, heterocyclic amines and polycyclic aromatic hydrocarbons (PAHs) (Phillips, 1983; Fraga *et al.*, 1990; Appel *et al.*, 1990; Sun *et al.*, 1982, 1984). Many reports have indicated that DNA can be modified by tobacco and diesel exhaust. However, there are no good methods for evaluating the grade of exposure to chemical carcinogens, and little work has been done to analyze the relationship between exposure to chemical carcinogens and genetic alterations of the lung tumor and background region. We have developed an objective method for the quantitative evaluation of black-dust-matter deposition (anthracosis). We have demonstrated that well-differentiated pulmonary adenocarcinoma tends to develop more frequently than poorly differentiated ones in lung showing less deposition, and that patients with severe anthracosis

tended to have a poorer prognosis than those with mild anthracosis (Hou *et al.*, 1998).

The aim of this study was to examine whether the degree of background anthracosis was associated with the methylation and inactivation of the *p16* gene in lung adenocarcinogenesis.

### MATERIAL AND METHODS

#### Patients

We examined 47 patients who had died of pulmonary adenocarcinoma at the University Hospital of Tsukuba (Japan) between 1988 and 1997. Their autopsied lungs were fixed with 10% formalin. Both lungs were sliced along the largest cut surface and paraffin-embedded blocks were made from all the sliced tissues. These were cut into 20- $\mu$ m-thick sections.

#### Extraction of black dust matter and DNA

Black dust matter and DNA were separately extracted from sections of background lung and tumorous lesions, and the black dust matter was blotted onto a nitrocellulose membrane, as reported (Hou *et al.*, 1998). Briefly, all the sections were de-paraffinized and digested with proteinase K (100  $\mu$ g/mL). After separation of black dust matter from the extraction buffer, DNA was extracted with phenol and chloroform. The black dust matter was suspended in a TE buffer, and an appropriate volume of the suspension was dot-blotted onto a nitrocellulose membrane. The amount of sample loaded on each lane corresponded to tissue from which 5  $\mu$ g of DNA had been extracted. The density of the blotted black dust matter was analyzed using a GS-700 imaging densitometer (Bio-Rad, Hercules, CA).

#### Methylation analysis

DNA methylation of the *p16* gene was examined by methylation-specific polymerase chain reaction (MS-PCR), as reported (Herman *et al.*, 1996). This assay entails initial modification of DNA by sodium bisulfite, converting all unmethylated, but not methylated, cytosines to uracil, and subsequent amplification with primers specific for methylated vs. unmethylated DNA. MS-PCR requires only small quantities of DNA, is sensitive to 0.1% methylated alleles of a given CpG island locus, and can be performed on DNA extracted from paraffin-embedded samples. Briefly, 30  $\mu$ l of 10 mM hydroquinone (Sigma, St. Louis, MO) and 520  $\mu$ l of 3 M sodium bisulfite (Sigma) at pH 5, both freshly prepared, were added and mixed with de-natured DNA (1  $\mu$ g) and samples were incubated under mineral oil at 50°C for 16 hr. Modified DNA was purified and eluted into 50  $\mu$ l of water. Modification was completed by NaOH (final concentration, 0.3 M) treatment for 5 min at room temperature, followed by ethanol precipitation. DNA was re-suspended in water and used immediately. Three primer pairs, wild-type primers (*p16*-W), methylated-specific primers (*p16*-M) and unmethylated-

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specific primers (*p16-U*) were as follows; *p16-W*, 5'-CAGAGGGTGGGGCGGACCGC-3' and 5'-CGGGCCGCG-GCCGTGG-3'; *p16-M*, 5'-TTATTAGAGGGTGGGGCG-GATCGC-3' and 5'-GACCCCGAACCGCGACCGTAA-3'; *p16-U*, 5'-TTATTAGAGGGTGGGGTGGATTGT-3' and 5'-CAACCC-CAAACCAACCCATAA-3'. PCR was carried out in a TAKARA PCR Thermal Cycler MP (Takara, Tokyo, Japan). The PCR mixture contained 1× PCR buffer (16.7 mM ammonium sulfate/67 mM Tris, pH 8.8/6.7 mM MgCl<sub>2</sub>/10 mM 2-mercapto-ethanol), dNTPs (each at 1.25 mM), primers (300 ng each per reaction) and bisulfite-modified DNA (50 ng) or unmodified DNA (50–100 ng). Reaction was hot-started at 95°C for 5 min before the addition of 1.25 units of Taq polymerase (Takara). Amplification was carried out for 35 cycles of 30 sec at 94°C, 30 sec at 65°C (for primer sets of *p16-W* and *p16-M*) or 60°C (for primer set of *p16-U*) and 30 sec at 72°C, followed by a final 4-min extension at 72°C. PCR products (10 μl) were separated by 1% agarose-gel electrophoresis, stained with ethidium bromide and directly visualized under UV illumination.

To verify further the specificity of the primers for the methylated alleles and to check specific cytosines for methylation within the region amplified, we examined the differences in sequence at a methylation-sensitive restriction site between methylated/modified DNA and unmethylated/modified DNA. For the restriction analysis, PCR mixture was digested with BstUI in conditions specified by the manufacturer (New England Biolabs, Beverly, MA). PCR products of methylation-specific PCR and unmethylation-specific PCR are 150 base pair (bp) and 151 bp respectively. The BstUI recognition site, CGCG, will remain CGCG if both Cs are methylated after bisulfite treatment and amplification, but will become TGTG if unmethylated. Therefore digestion of the amplified products with BstUI can distinguish these 2 products. Only methylated/modified products retain CGCG site and are cleaved by BstUI, while products amplified with unmethylated/modified primers are not cleaved.

#### Immunohistochemical analysis

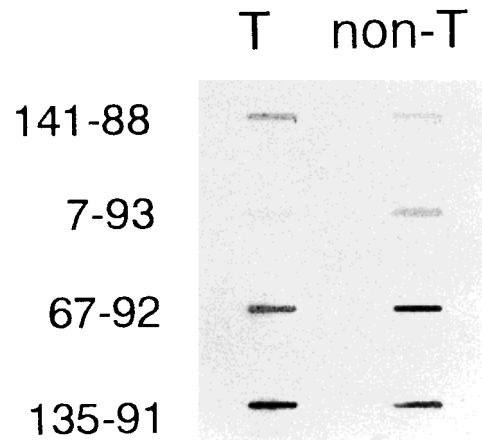
Immunostaining of p16 protein was performed using the ABC method. After treatment with 0.3% H<sub>2</sub>O<sub>2</sub>-methanol solution and digestion with pepsin, non-specific Ig G binding was suppressed with 5% normal swine serum. The primary antibody against human p16/INK4 was rabbit polyclonal Ig G (PharMingen, San Diego, CA) and was applied to the slides and incubated at 4°C overnight. Human lung-cancer cell lines A549 and Lu141 were used as negative and positive controls respectively (Okamoto *et al.*, 1995) (data not shown). For scoring the p16 staining pattern as normal (+) or abnormal (–), we used criteria described by Geradts and Wilson (1996), with some modification. Cytoplasmic reactivity was disregarded, and only nuclear staining above any cytoplasmic background was considered evidence of expression of the *p16* gene. The nuclear staining pattern of p16 protein were classified as: +, entire tumor cells exhibit distinct nuclear staining (normal pattern); ±, unstained clusters of tumor-cell nuclei are intermingled with stained cells or occupy certain areas of the same tumor (abnormal pattern); –, entire tumor cells are unstained (abnormal pattern).

#### Statistical analysis

The 2-tailed Fisher's exact test was used to analyze the significance of differences between the patient groups for each analysis.

## RESULTS

The background lungs of the 47 patients were evaluated for black-dust-matter deposition and the representative results of the dot blotting are shown in Figure 1. The patients were sub-divided into 3 groups based on absolute absorbance (A) value of background lung: (1) mild anthracosis (A value < 0.3, 20 patients), (2)



**FIGURE 1** – Representative results of the black-dust-matter dot blotting. T, tumor; non-T, non-tumor. 141-88, 7-93, 67-92 and 135-91 are case numbers.

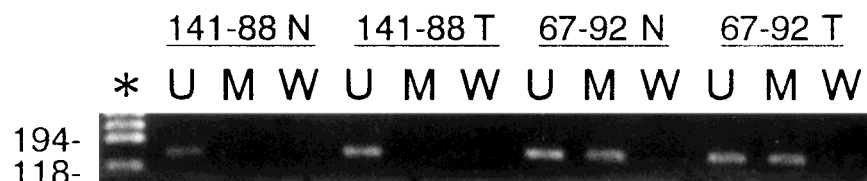
moderate anthracosis ( $0.3 \leq A \text{ value} \leq 0.6$ , 8 patients), and (3) severe anthracosis (A value > 0.6, 19 patients).

A total of 47 tumor samples and matched background-lung-tissue samples was analyzed by MS-PCR (Figs. 2, 3). Nineteen sets of tumor and background region were successfully amplified, and methylation of the *p16* gene was detected in 6 (32%) out of 19 tumors (Tables I, II), and in background lung in one case (case 67–92, Fig. 2). There were no correlation between methylation state and clinicopathological characteristics of the tumors, such as histological differentiation, cytological sub-types or pathological stage (Table I). However, the patients with methylated tumor *p16* had a poorer prognosis than those with unmethylated tumor *p16* DNA. The incidence of smoking history was also compared in tumors with *p16*-gene methylation and without methylation. Smokers comprised 5 (83%) of 6 patients with methylated tumor *p16* and 8 (62%) of 13 patients with unmethylated tumor *p16* ( $p = \text{NS}$ ) (Table I).

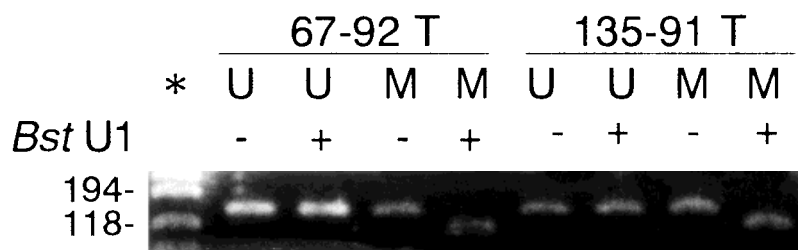
We compared the degree of black-dust-matter deposition (anthracosis) and methylation of the *p16* gene (Table II). Mean A value of the 19 cases examined by MS-PCR ( $A = 0.431 \pm 0.082$ ) was equivalent to that of the total of 47 cases examined in this study ( $A = 0.450 \pm 0.057$ ). The mean A value of the 6 cases with *p16*-gene methylation ( $A = 0.715 \pm 0.091$ ) was significantly higher than that of the remaining 13 cases without methylation ( $A = 0.298 \pm 0.088$ ) ( $p < 0.05$ ). None of the 8 cases with mild anthracosis ( $A < 0.3$ ) showed methylation of the *p16* gene. However, in 2 of 4 tumors (50%) with moderate anthracosis ( $0.3 \leq A \leq 0.6$ ) and 4 of 7 tumors (57%) with severe anthracosis ( $A < 0.6$ ), the *p16* gene was methylated.

Using an immunohistochemical approach, we studied p16 expression in 47 cases evaluated for black-dust-matter deposition, including 19 tumors for which the methylation status of the *p16*-gene-promoter region was successfully determined. In the 19 tumors, immunohistochemical analysis revealed that p16 protein was expressed in 6 tumors (32%) (Tables I, III). Both nucleus and cytoplasm of the tumor cells were stained in the tumors which were positive for p16 protein (Fig. 4). Of the 6 tumors that expressed p16 protein, 4 were stained diffusely (+) and 2 were stained heterogeneously (±). All the tumors with *p16*-gene methylation were negative for p16 protein, whereas p16 was expressed in 6 out of 13 tumors without *p16*-gene methylation (46%). Table IV shows the relationship between the degree of anthracosis and the incidence of p16 protein expression in the total of 47 patients. p16 inactivation was observed in 11 (55%) of 20 tumors, 7 (87.5%) of 8 tumors and 19 (100%) of 19 tumors with mild, moderate and severe anthracosis respectively. Cases with tumors which expressing p16 protein normally (+) had a significantly lower mean A value ( $A = 0.151$ )

**FIGURE 2** – Methylation-specific polymerase chain reaction of *p16* gene. 141-88 and 67-92 are case numbers. \*, molecular-weight marker  $\phi$ X174-HaeIII digest. Primer sets used for amplification are designated as unmethylated (U), methylated (M), or unmodified/wild-type (W). N, non-tumor; T, tumor. Both tumorous and non-tumorous regions are methylated in case 67-92 but not in case 141-88.



**FIGURE 3** – Methylation-specific polymerase chain reaction of *p16* gene and restriction analysis with BstU1. The amplified *p16* products were restricted with BstU1 (+) or were not restricted (-). \*, molecular-weight marker  $\phi$ X174-HaeIII digest. Primer sets used for amplification are designated as unmethylated (U) and methylated (M). T, tumor. 67-92 and 135-91 are case numbers.



**TABLE I** – CLINICOPATHOLOGICAL FINDINGS, EXPRESSION AND METHYLATION STATUS OF *p16<sup>INK4A</sup>* AND BACKGROUND ANTHRACOSIS IN CASES EXAMINED BY METHYLATION-SPECIFIC PCR ANALYSIS

Case number	Diff-erentiation <sup>1</sup>	Pathological stage	Smoking history	Survival time (months)	Immunohisto-chemistry <sup>4</sup>	Anthra-cosis <sup>5</sup>
<b>Patients with methylated tumor p16</b>						
134-84	W	II	S	<6	-	1.019
67-92	P	I	S	≧6	-	0.937
63-87	Mo	III	S	<6	-	0.709
141-90	Mo	I	S	<6	-	0.624
63-93	Mo	III	S	<6	-	0.552
135-91	Mo	IV	N	≧6	-	0.447
<b>Patients with unmethylated tumor p16</b>						
7-93	Mo	III	S	≧6	+	0.160
141-88	W	II	N	≧6	+	0.053
25-86	Mo	II	S	≧6	+	0.071
104-85	W	III	N	≧6	+	0.028
36-93	W	I	N	≧6	±	0.461
22-85	W	I	N	≧6	±	0.022
86-90	Mo	I	S	<6	-	0.834
54-92	P	IV	S	<6	-	0.718
158-88	P	II	S	<6	-	0.638
81-88	W	IV	S	≧6	-	0.481
38-92	P	III	S	<6	-	0.234
44-90	W	I	N	≧6	-	0.134
122-88	Mo	III	S	≧6	-	0.045

<sup>1</sup>W, well differentiated; Mo, moderately differentiated; P, poorly differentiated. –<sup>2</sup>N, non-smoker; S, smoker. –<sup>3</sup>Time between tumor detection and death. –<sup>4</sup>+, all tumor cells show distinct nuclear staining; ±, unstained clusters of tumor cell nuclei are intermingled with stained cells or occupy certain areas of the same tumor; -, tumor cells show no staining. –<sup>5</sup>Mean A value.

than those with tumors expressing p16 protein abnormally (± and -) (A = 0.531) ( $p < 0.05$ ).

**DISCUSSION**

Inactivation of the *p16*-gene locus is thought to be a frequent event in non-small-cell carcinoma through distinct mechanisms including rare point mutations, homozygous deletions and promoter methylation (Hayashi *et al.*, 1994; Okamoto *et al.*, 1995; Merlo *et al.*, 1995; Otterson *et al.*, 1995). In this study, we tested the hypothesis that black-dust-matter deposition may be a good marker of hypermethylation and inactivation of the *p16* gene; we used a new quantitative method for the evaluation of anthracosis in autopsy cases.

**TABLE II** – ANTHRACOSIS AND METHYLATION OF THE *p16* GENE

Methylation state of tumors	Number examined	Black-dust-matter deposition			
		A < 0.3	0.3 ≧ A ≧ 0.6	0.6 < A	Mean ± SE
Methylated	6	0	2	4	0.715 ± 0.091*
Unmethylated	13	8	2	3	0.298 ± 0.088
Total	19	8	4	7	0.431 ± 0.082

<sup>1</sup>A, absorbance \*,  $p < 0.05$ .

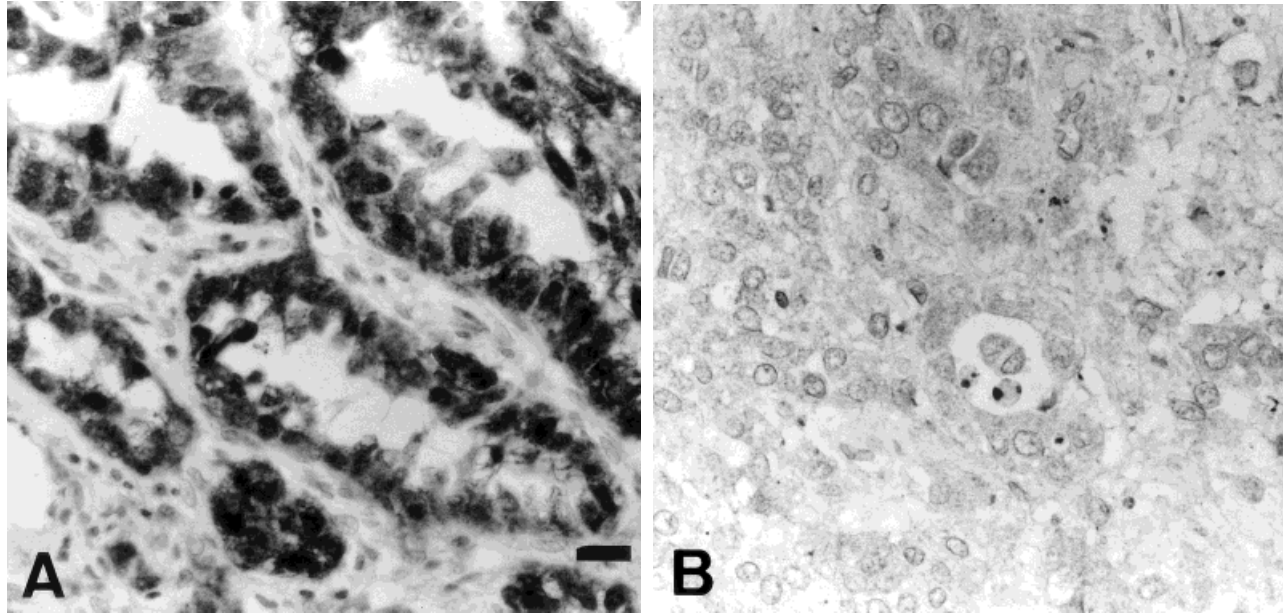
**TABLE III** – IMMUNOHISTOCHEMICAL STAINING OF p16 PROTEIN AND METHYLATION STATE

	Number examined	Immunohistochemistry of p16 protein		
		(+)	(±)	(-)
Methylated	6	0	0	6 (100%)
Unmethylated	13	4	2	7 (54%)
Total	19	4	2	13

Methylated, tumors with *p16*-gene methylation; unmethylated, tumors without *p16*-gene methylation; (+) all tumor cells show distinct nuclear staining; (±) unstained clusters of tumor cell nuclei are intermingled with stained cells or occupy certain areas of the same tumor; (-), tumor cells show no staining.

In this study, the frequency of *p16*-gene methylation was 32%, similar to data reported by Merlo *et al.* (1995) (7/27, 26%), Herman *et al.* (1996) (8/15, 53%) and Kashiwabara *et al.* (1998) (10/29, 34%). We found a high frequency of *p16*-gene methylation in tumors with severe anthracosis (4/6 cases, 67%), and the mean A value for background anthracosis was significantly higher in tumors with *p16*-gene methylation. There was no significant correlation between methylation of the *p16* gene and pathological stage, suggesting that *p16*-gene methylation might occur at an early stage of pulmonary adenocarcinogenesis. We suggest that severely anthracotic background is necessary for occurrence of *p16*-gene methylation, although 3 of 13 tumors (23%) without *p16*-gene methylation showed a background of severe anthracosis.

Methylation is one of the most important epigenetic events in human carcinogenesis, and is thought to be closely associated with inactivation of tumor-suppressor genes. Eguchi *et al.* (1997) reported DNA hypermethylation at the D17S5 at which a candidate tumor-suppressor gene, *h1c* (hypermethylated in cancer), occurred not only in tumorous lesions but also in non-tumorous lung tissues (31%), and the incidence was significantly correlated with smoking history. It is very interesting that we found *p16*-gene methylation also in the background region of one severely anthracotic lung (case 67-92, Figs. 1, 2). This may be due to infiltrating tumor cells



**FIGURE 4** – Immunostaining of p16 protein. (a) All tumor cells show strong immunoreactivity (case 141-88); (b) no tumor cells show immunoreactivity (case 67-92). Scale bar = 50  $\mu$ m.

**TABLE IV** – ANTHRACOSIS AND IMMUNOHISTOCHEMISTRY OF p16 EXPRESSION

Immunostaining <sup>1</sup>	Number examined	Black-dust-matter deposition			Mean $\pm$ SE
		A < 0.3	0.3 $\leq$ A $\leq$ 0.6	0.6 < A	
(+)	10	9	1	0	0.151 $\pm$ 0.0691*
( $\pm$ )	3	1	2	0	0.330 $\pm$ 0.155
(-)	34	10	5	19	0.548 $\pm$ 0.065
Total	47	20	8	19	0.450 $\pm$ 0.057

<sup>1</sup>(+), all tumor cells show distinct nuclear staining; ( $\pm$ ), unstained clusters of tumor cells nuclei are intermingled with stained cells or occupy certain areas of the same tumor; (-), tumor cells show no staining. A, absorbance. \* $p < 0.05$ .

in background lung, or to some pre-cancerous or precursor cells of the lung adenocarcinoma which already have DNA methylation of *p16* gene.

We used immunohistochemistry to confirm abnormal p16 expression caused by DNA methylation of the promoter region of the *p16* gene (Table III) and examine the relationship between the expression of p16 protein and background anthracosis (Table IV). p16

protein was positively stained in 10 out of 47 cases (21.3%). This rate of p16-protein expression was lower than that reported earlier (Geradts *et al.*, 1995; Kinoshita *et al.*, 1996; Sakaguchi *et al.*, 1996), but our specimens were autopsy cases, which may have poorer preservation of antigenicity than frozen samples or biopsy or surgical specimens. Interestingly, all cases that showed *p16*-gene methylation were negative for p16 protein, suggesting that hypermethylation of the *p16* promoter region is closely associated with gene inactivation. On the other hand, the mean A value of black-dust deposition of cases positive for p16 protein (A = 0.151) was significantly lower than that of cases negative for p16 protein (A = 0.531). These results indicate that inactivation of p16 protein is closely associated with background anthracosis.

In summary, we have demonstrated that the degree of background anthracosis was significantly higher in cases with *p16*-gene methylation than in those without *p16*-gene methylation, and in cases with abnormal p16 expression than in those with its normal expression. The level of background anthracosis is thus closely associated with DNA methylation of the *p16* promoter region and with inactivation of p16 expression in pulmonary adenocarcinogenesis.

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