CANCER
CYTOPATHOLOGY

Anthracotic Index and DNA Methylation Status of Sputum Contents can be Used for Identifying the Population at Risk of Lung Carcinoma

Sato Konno, C.T.[1]
Yukio Morishita, M.D.[2]
Masakatsu Fukasawa, C.T.[3]
Yujian Shu, M.D.[1]
Daye Wang, M.D.[1]
Ryota Tanaka, M.D.[1,4]
Yuko Minami, M.D.[1]
Tatsuo Iijima, M.D.[1]
Masayuki Noguchi, M.D.[1]

[1] Department of Pathology, Institute of Basic Medical Sciences, University of Tsukuba, Ibaraki, Japan.

[2] Department of Clinical Pathology, Institute of Clinical Medicine, University of Tsukuba, Ibaraki, Japan.

[3] Department of Pathology, University Hospital of Tsukuba, Ibaraki, Japan.

[4] Department of Surgery, Kyorin University, School of Medicine, Tokyo, Japan.

Address for reprints: Masayuki Noguchi, M.D., Department of Pathology, Institute of Basic Medical Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba-shi, Ibaraki 305-8575, Japan; Fax: (011) 81-29-853-3150; E-mail: nmasayuk@md.tsukuba.ac.jp

Received April 12, 2004; revision received July 22, 2004; accepted August 4, 2004.

BACKGROUND. Sputum cytology for the mass screening of lung carcinoma is a noninvasive, repeatable, and useful examination, but the detection rate is usually $< 0.05\%$ and the reliability is not high.

METHODS. The anthracotic index (AI) and methylation status of the promoter regions of the *p16*, *adenomatous polyposis coli* (*APC*), and *retinoic acid receptor-beta* (*RARβ*) genes were examined in 356 sputum specimens after routine cytologic examination.

RESULTS. The mean AI of specimens from males was significantly higher than that from females. AI increased with increasing age and smoking index. The mean AI of patients with lung carcinoma was significantly higher than that of the nonaffected population. Furthermore, the mean AI of the specimens with or without cancer cells from patients with cancer was significantly higher than that of the nonaffected population. Abnormal methylation of the *p16*, *APC*, and *RARβ* genes was detected in 21.7%, 28.2%, and 26.9% of specimens from patients with cancer, respectively. These ratios were significantly higher than those of the nonaffected populations (0%, 3.9%, and 7.6%, respectively). The incidences of abnormal methylation of the three genes were not associated with histologic classification, smoking index, gender, age, or occupation.

CONCLUSIONS. These findings suggested that the AI and abnormal methylation status were useful for identifying a population at risk of lung carcinoma using mass screening of cytology specimens. ***Cancer (Cancer Cytopathol)* 2004;102:348–54.**

KEYWORDS: lung, anthracosis, methylation, sputum, *p16*, *adenomatous polyposis coli* gene, *retinoic acid receptor*-beta gene, biomarker.

Black matter deposition (anthracosis) is correlated closely with exposure to chemical carcinogens such as polycyclic aromatic hydrocarbons.[1] Hou et al.[2,3] and Wang et al.[4] examined background anthracosis in pulmonary adenocarcinoma using autopsy cases and surgically treated patients, respectively, and demonstrated a strong correlation between the degree of black dust matter deposition and histologic differentiation of the adenocarcinomas and/or histologic subtypes of small-sized adenocarcinoma. They indicated that patients with severe anthracosis tended to have a poorer prognosis than those with mild anthracosis and that adenocarcinoma that develops in heavily anthracotic lungs readily progresses to an advanced stage or that adenocarcinoma with a less favorable prognosis tends to develop in severely anthracotic lungs. These findings suggest that the degree of background anthracosis is a very useful risk factor for estimating the prognosis of pulmonary adenocarcinoma. However, it is not easy

DOI 10.1002/cncr.20643
Published online 12 October 2004 in Wiley InterScience (www.interscience.wiley.com).

to measure the degree of background anthracosis in the lung before open biopsy or surgical treatment.

Conversely, abnormal methylation of the CpG islands of the promoter region is one of the mechanisms responsible for the loss of function of several tumor suppressor genes.[5,6] High incidences of abnormal methylation of the promoter regions in the *p16*, *adenomatous polyposis coli* (*APC*), and *retinoic acid receptor-beta* (*RARβ*) genes have been reported in lung carcinomas.[7–10] They are located on chromosomal regions 9p21, 5q21, and 3q24, respectively, and high incidences of loss of heterozygosity in these regions also have been reported.[11–13] Therefore, several reports have indicated that the expressions of these genes decrease according to stepwise progression of lung carcinoma due to the abnormal methylation of the CpG islands of their promoter regions. Practically, cytology materials are useful specimens for making the primary diagnosis of lung carcinoma. Palmisano et al.[14] applied the study of abnormal promoter methylation of the *p16* gene in sputum specimens of patients with lung carcinoma and found extremely high incidences of abnormal methylation. They indicated that analysis of the abnormal methylation of *p16* in sputum specimens is a useful molecular marker.

Sputum cytology for the mass screening of lung carcinoma is a noninvasive, repeatable, and useful examination but the detection rate is usually $< 0.05\%$[15] and the reliability is not high. The aim of the current study is to apply anthracotic index (AI) analysis and abnormal methylation analyses of the promoter regions of several tumor suppressor genes to sputum cytology specimens and to estimate the usefulness of this approach for detecting lung carcinoma or selecting a group at high risk for lung carcinoma.

MATERIALS AND METHODS

Patients

To examine the AI, sputum samples from 210 patients (152 males and 58 females) were identified from the files of the University Hospital of Tsukuba (Ibaraki, Japan). The cohort included 87 patients with lung carcinoma, 22 patients who had undergone previous surgery for lung carcinoma, and 101 individuals without a history of lung carcinoma. The histologic subtypes of the 87 patients with lung carcinoma included adenocarcinoma ($n = 41$), squamous cell carcinoma ($n = 33$), small cell carcinoma ($n = 8$), and other subtypes ($n = 5$). The histologic subtypes of the 22 patients whose status was determined after surgery included adenocarcinoma ($n = 5$) and squamous cell carcinoma ($n = 17$). The mean age of the patients was 67.6 years (range, 22–91 years). There were 126 smokers, 55 nonsmokers, and 29 whose smoking history was unknown. The smoking index (SI) (the number of cigarettes smoked per day $\times$ years of smoking) of 32 smokers was < 600, and the SI of 94 smokers was ≥ 600. According to a previous report,[16] a smoker with an SI of ≥ 600 was considered to be a heavy smoker.

For the analysis of abnormal methylation of the three tumor suppressor genes, we selected 78 patients with lung carcinoma (59 males and 19 females) from the patients examined for background anthracosis. The mean age of the patients was 70.6 years (range, 42–86 years). Fifty-five patients were smokers and 15 patients were nonsmokers. The smoking history of eight patients was unknown. The SI of 10 smokers was < 600, and that of 45 smokers was ≥ 600. We selected 37, 51, and 52 patients without a history of lung carcinoma for the study of abnormal methylation of the *p16*, *APC*, and *RARβ* genes, respectively.

Extraction of Black Dust Matter and DNA from Sputum

Black dust matter and DNA samples were extracted according to the method reported previously, with some modifications.[2–4] After morphologic examination and diagnosis, the remaining sputum specimen was stored in Saccamano's solution[17] until black dust particles and DNA specimens were extracted. Sputum samples were unsatisfactory for evaluation if alveolar lung macrophages were absent. Sputum samples were washed 3 times with mucus liquefacient solution (0.05% [weight/volume] N-acetyl-L-cysteine/50% [volume/volume] alcohol). After rehydration, the sputum specimen was digested with 100 μg/mL Proteinase K for 2 or 3 days at 48 °C. Black dust matter was separated by centrifugation at 10,000 revolutions per minute for 15 minutes. DNA samples were extracted twice with saturated phenol-chloroform and precipitated using ethanol. The black dust matter was dot blotted onto a nitrocellulose membrane (Schleicher & Schuell, Inc., Dassel, Germany) using Hybri-Slot (Gibco, BRL, Gaithersburg, MD). The density of the blotted black dust matter was calculated using a GS-700 imaging densitometer (BioRad, Hercules, CA). The absolute absorbance detected by densitometry was defined as the AI.

Bisulfite Modification and Methylation-Specific Polymerase Chain Reaction

Bisulfite modification of genomic DNA was performed as reported previously.[18] Modified DNA was purified using Wizard DNA purfication resin (Promega, Madison, WI) followed by ethanol precipitation. Treatment with 5.5 μL of 3 M NaOH for 20 minutes at 37 °C was followed by ethanol precipitation. DNA was resuspended in water and stored at −20 °C. The methyl-

TABLE 1
Summary of Primer Sequences

Gene	Primer sequences	Gene location	Annealing temperature (°C)	Cycle no.
p16—Stage1-F	GAAGAAAGAGGAGGGGTTGG	9p21	60	40
p16—Stage1-R	CTACAAACCCTCTAC CCACC			
p16—Stage2-U-F	TTATTAGAGGGTGGGGTGGATTGT		55	35
p16—Stage2-U-R	CAACCCCAAACCACAACCATAA			
p16—Stage2-M-F	TTATTAGAGGGTGGGGCGGATCGC		60	35
p16—Stage2-M-R	GACCCCGAACCGCGACCGTAA			
APC-W-F	GTGCCCCACTGCGGAGTGCGGGTC	5q21	60	35
APC-W-R	TCGGCGGGCTCCCGACGG			
APC-U-F	GTGTTTTATTGTGGAGTGTGGGTT		60	35
APC-U-R	CCAATCAACAAACTCCCAACAA			
APC-M-F	TATTGCGGAGTGCGGGTC		55	35
APC-M-R	TCGACGAACTCCCGACGA			
RAR-W-F	CCGAGAACGCGAGCGATCCG	3p24	64	35
RARβ-W-R	GGCCAATCCAGCCGGGGCGG			
RARβ-U-F	GTGTTTTATTGTGGAGTGTGGGTT		54	35
RARβ-U-R	CCAATCAACAAACTCCCAACAA			
RARβ-M-F	TCGAGAACGCGAGCGATTCG		64	35
RARβ-M-R	GACCAATCCAACCGAAACGA			

F: forward primer sequences (5′-3′); R: reverse primer sequences (5′-3′); U: unmethylated sequence-specific primers; M: methylated sequence-specific primers; *APC: adenomatous polyposis coli* gene; W: unmodified or wild-type sequence primers; RAR/*RARβ: retinoic acid receptor/RAR-beta* gene.

specific polymerase chain reaction (PCR) primer sequences are listed in Table 1. Abnormal methylation of *p16* was specially determined by nested methyl-specific PCR.[14] Abnormal methylations of the other genes were determined by standard methyl-specific PCR.[10,18,19] Stage 1 of the nested methyl-specific PCR protocol was as follows: 95 °C for 10 minutes, denaturing at 95 °C for 30 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 30 seconds (40 cycles) followed by a 10-minute final extension. We used 1 μL of Stage 1 products for Stage 2 PCR. The Stage 2 protocol was as follows: 95 °C for 10 minutes, denaturing at 95 °C for 15 seconds, annealing at 55 °C (for unmethylated-type primers), 60 °C (for methylated type primer) for 15 seconds, and extension at 72 °C for 30 seconds (35 cycles) followed by a 10-minute final extension. The methyl-specific PCR protocol for *APC* was as follows: 97 °C for 5 minutes, denaturing at 95 °C for 40 seconds, annealing at 60 °C (wild-type primer and unmethylated-type primer), 55 °C (methylated type primer) for 40 seconds, and extension at 72 °C for 50 seconds (35 cycles) followed by a 10-minute final extension. The methyl-specific PCR protocol for *RARβ* was as follows: 95 °C for 10 minutes, denaturing at 94 °C for 1 minute, annealing at 64 °C (wild-type primer and methylated-type primer), 54 °C (unmethylated-type primer) for 1 minute, and extension at 72 °C for 1 minute (35 cycles) followed by a 10-minute final extension. PCR products (10 μL) were loaded onto 2% (weight/volume) agarose gels, stained with ethidium bromide, and visualized under ultraviolet illumination.

To confirm the methylation status, restriction enzyme analysis with *BstUI* was performed for stage 2 PCR products that were positive for *p16* methylation.

RESULTS

Anthracosis

Morphologic examination showed that the black dust materials were detectable mainly in the cytoplasm of alveolar macrophage (dust cells; Fig. 1). Representative AI values are shown in Figure 2. The mean AI of the male group (0.110 ± 0.015) was significantly higher than the female group (0.050 ± 0.014; $P < 0.01$). The values of mean AI increased in proportion to the patient's age and the mean AI of patients ≥ 50 years was greater than that of patients < 50 years ($P < 0.01$; Table 2).

The relation between the degree of anthracosis and smoking history is shown in Table 2. The mean AI increased in proportion to the value of the SI. Although the mean AI of heavy smokers was significantly higher than that of nonsmokers ($P < 0.05$), the correlation between the SI and AI was not significant.

As Table 3 shows, the mean AI of patients with lung carcinoma (0.134 ± 0.023) was significantly

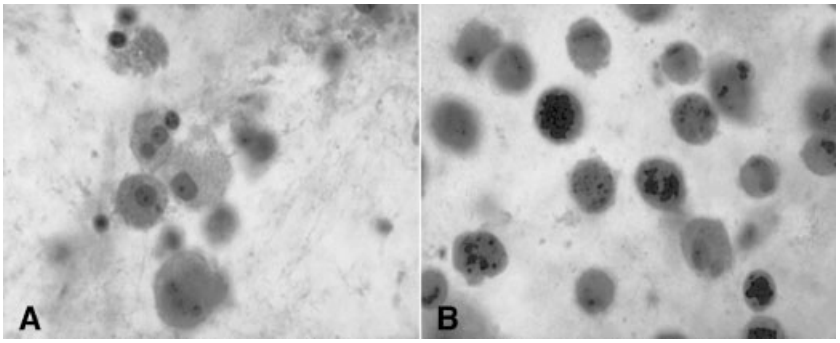

FIGURE 1. Black dust matter was detected microscopically mainly in the cytoplasm of dust cells. (A) Sputum sample anthracotic index (AI) = 0.053. (B) Sputum sample AI = 0.786 (Papanicolaou stain, × 1000).

FIGURE 2. Representative results of the blotting of the black dusty material. Class II, V: Papanicolaou classification. N: no patients with cancer; sp: sputum sample; Ad: adenocarcinoma; Sq: squamous cell carcinoma; SCLC: small cell carcinoma.

TABLE 2
Summary of Mean AI[a] of Age and SI[b]

	AI					
	No.	All	No.	Male	No.	Female
Age (yrs)						
<50	12	0.018 ± 0.008	8	0.005 ± 0.003	4	0.044 ± 0.017
≥50	198	0.099 ± 0.012	144	0.114 ± 0.016	54	0.050 ± 0.015
SI						
Nonsmoker[c]	55	0.022 ± 0.006	15	0.010 ± 0.005	40	0.026 ± 0.008
Light smoker (SI < 600)	32	0.056 ± 0.021	24	0.039 ± 0.020	8	0.105 ± 0.066
Heavy smoker (SI ≥ 600)	94	0.149 ± 0.023	90	0.150 ± 0.024	4	0.121 ± 0.112

AI: anthracotic index; SI: smoking index.

[a] Absolute absorbance measured with a densitometer was defined as the anthracotic index.

[b] Smoking index was defined as the number of cigarettes smoked per day × smoked years.

[c] Statistical analysis of mean anthracotic index of the heavy smoker group versus that of the nonsmoker group was performed by using the Student *t* test ($P < 0.05$).

higher than that of patients without lung carcinoma (0.058 ± 0.012; $P < 0.01$). We subdivided the patients with lung carcinoma according to tumor histologic subtypes. The mean AI value of patients with squamous cell carcinoma and small cell carcinoma was significantly higher than that of patients with adenocarcinoma ($P < 0.05$). The sputum specimens of patients with lung carcinoma also were subdivided into two groups, one including specimens in which cancer cells were not detected by microscopic examination, and the other including specimens in which cancer cells were detected. There were no significant differences in the mean AI between the group in which lung carcinoma cells were detected (0.140 ± 0.036) and the group in which lung carcinoma cells were not detected (0.127 ± 0.029). However, the values of both groups were significantly higher than those of the group without lung carcinoma. Even among heavy smokers, the mean AI of the sputum samples from patients with lung carcinoma without cancer cells was 0.194 ± 0.043 and that of the sputum specimens with lung carcinoma cells was 0.238 ± 0.072, and there was no significant difference between them. However, the mean AI of the specimens of patients with lung carcinoma without lung carcinoma cells in their specimens was significantly higher than that of patients without lung carcinoma ($P < 0.05$).

TABLE 3
Summary of Mean AI

Patient and tumor category	No.	AI
Patients		
Patients without lung carcinoma	101	0.058 ± 0.012[b,d]
Past patients with lung carcinoma	22	0.090 ± 0.033
Patients with lung carcinoma	87	0.134 ± 0.023[b]
Histologic subtypes		
Ad	41	0.075 ± 0.019[c]
Sq	33	0.186 ± 0.049[c]
SCLC	8	0.227 ± 0.081[c]
Other types	5	0.152 ± 0.048
Patients with lung carcinoma[a]		
Cytology (−)/lung carcinoma	43	0.127 ± 0.029[d]
Cytology (+)/lung carcinoma	44	0.140 ± 0.036[d]
Heavy smoker (smoking index ≥ 600)		
Nonlung carcinoma	33	0.082 ± 0.025[e]
Cytology (−)/lung carcinoma	23	0.194 ± 0.043[e]
Cytology (+)/lung carcinoma	20	0.238 ± 0.072

AI: anthracotic index; Ad: adenocarcinoma; Sq: squamous cell carcinoma; SCLC: small cell lung carcinoma; −: negative; +: positive.

[a] Cytology (−) indicates lung carcinoma: the sputum specimen of patients with lung carcinoma that contained no tumor cells; cytology (+) indicates lung carcinoma: the sputum specimen of patients with lung carcinoma that contained tumor cells.

[b] Statistical analysis of the mean anthracotic index of patients with lung carcinoma versus that of patients without lung carcinoma was performed using the Student *t* test ($P < 0.01$).

[c] Statistical analysis of the mean anthracotic index of the squamous cell carcinoma and small cell lung carcinoma patient group versus that of the adenocarcinoma group was performed using the Student *t* test ($P < 0.05$).

[d] Statistical analysis of the mean anthracotic index of patients with lung carcinoma with negative cytology and/or positive cytology versus that of patients without lung carcinoma was performed by using the Student *t* test, respectively, ($P < 0.05$).

[e] Statistical analysis of the mean anthracotic index of patients with lung carcinoma who were heavy smokers in the negative cytology group versus that of patients without lung carcinoma who were heavy smokers was performed using the Student *t* test ($P < 0.05$).

Abnormal Methylation of Suppressor Genes

We examined abnormal methylation of the *p16*, *APC*, and *RARβ* genes in 78 sputum samples obtained by methyl-specific PCR from patients with lung carcinoma (Fig. 3). We found that 56% (44 of 78) of the patients with lung carcinoma showed abnormal methylation of at least one of these genes. The frequencies of abnormal methylation in patients with and without lung carcinoma were 21.7% (17 of 78) and 0% (0 of 37) for *p16* ($P < 0.01$), 28.2% (22 of 78) and 3.9% (2 of 51) for *APC* ($P < 0.01$), and 26.9% (21 of 78) and 7.6% (4 of 52) for *RARβ* ($P < 0.05$; Table 4). No significant correlation was found between abnormal methylation in any of these genes and histologic types, age, and SI (Tables 3, 4). The frequencies of abnormal methylation in the three genes in specimens from patients with lung carcinoma without and with cancer cells were 25.7% (9 of 35) and 18.6% (8 of 43) for *p16*, 25.7% (9 of 35) and 30.2% (13 of 43) for *APC*, and 25.7% (9 of 35) and 27.9% (12 of 43) for *RARβ*. There were no correlations between the AI of the sputum specimens and promoter hypermethylation of tumor suppressor genes (*p16*, *APC*, and *RARβ*).

DISCUSSION

In the current study, we demonstrated the usefulness of analysis of AI in sputum cytology specimens to select the population at high risk of lung carcinoma. The number of dust cells in the sputum specimens containing vacuolated black dust materials is believed to reflect the background anthracosis of the patient's lung tissue. AI was correlated with the patient's age but not with tobacco smoking. Therefore, AI is be-

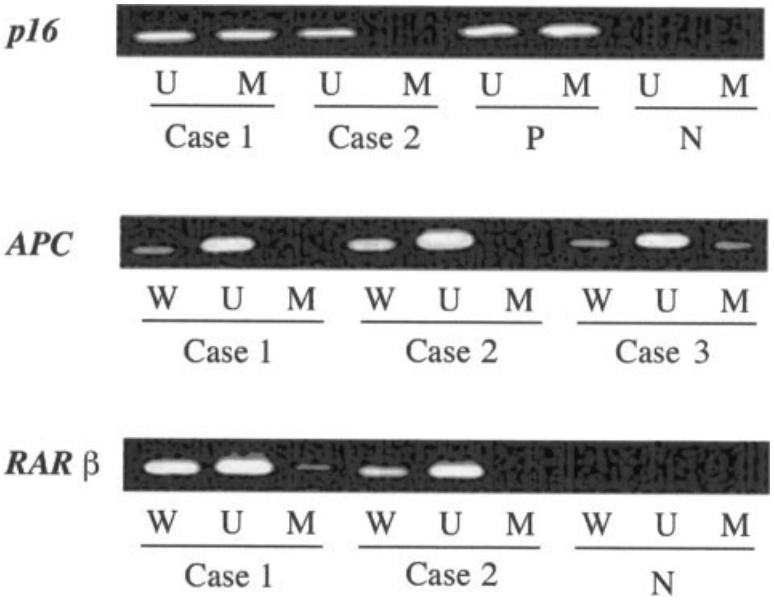

FIGURE 3. Representative results of methylation-specific polymerase chain reaction analysis for the *p16*, *adenomatous polyposis coli*, and *retinoic acid receptor-beta* genes. Primer sets used for the amplification are designated as unmodified or wild type (W), unmethylated (U), and methylated (M; see Table 1). P: positive control; N: negative control.

TABLE 4
Frequency of Aberrant Methylation of the *p16, APC,* and *RAR-β* Gene Promoter

	Abnormal methylation		
Characteristics	***p16* (%)**	***APC* (%)**	***RARβ* (%)**
Age (yrs)			
< 70	6/30 (20.0)	6/30 (20.0)	6/30 (20.0)
≥ 70	11/48 (22.9)	16/48 (33.3)	15/48 (31.2)
SI			
Nonsmoker	3/15 (20.0)	7/15 (46.6)	7/15 (46.6)
Light smoker (SI < 600)	2/10 (20.0)	2/10 (20.0)	3/10 (30.0)
Heavy smoker (SI ≥ 600)	10/45 (22.2)	11/45 (24.4)	9/45 (20.0)
Patients[a]			
Patients with nonlung carcinoma	0/37 (0)	2/51 (3.9)	4/52 (7.6)
Patients with lung carcinoma	17/28 (21.7)	22/78 (28.2)	21/78 (26.9)
Histologic subtypes			
Ad	9/37 (24.3)	2/51 (3.9)	9/37 (27.3)
Sq	7/28 (25.0)	12/37 (32.4)	9/28 (32.1)
SCLC	0/8 (0)	5/28 (17.8)	2/8 (25.0)
Other types	1/5 (20.0)	3/8 (37.5)	1/5 (20.0)
Patients with lung carcinoma			
Cytology (−)/lung carcinoma	9/35 (25.7)	9/35 (25.7)	9/35 (25.7)
Cytology (+)/lung carcinoma	8/43 (18.6)	13/43 (30.2)	12/43 (27.9)

APC: adenomatous *polyposis coli gene; RARβ: retinoic acid receptor-beta* gene; SI: smoking index; Ad: adenocarcinoma; Sq: squamous cell carcinoma; SCLC: small cell lung carcinoma; −: negative; +: positive.

[a] Statistical analysis of abnormal methylation rates of *p16, APC,* and *RARβ* in the patients with lung carcinoma versus those of patients without lung carcinoma was performed by using the chi-square test ($P < 0.01$).

lieved to be an independent risk factor for lung carcinoma, especially squamous cell carcinoma and small cell carcinoma. It is noteworthy that the AI of sputum specimens with or without cancer cells from patients with lung carcinoma was higher than the AI of sputum specimens from patients without lung carcinoma. This result has been confirmed in the population of heavy smokers. Morphologic examination of the sputum specimens in mass screening for lung carcinoma can find only 5 positive persons per 1000 population. However, if the AI was examined in a first screening, the high-risk population could be selected and it is expected that a higher proportion of affected persons would be indentified from a second screening of the high-risk group.

Abnormal methylation status in the sputum specimens is also important for selecting the high-risk population. If we were to screen for abnormal methylation status of sputum specimens, then a high-risk group could be selected. It is noteworthy that the abnormal methylations of the three genes were detected more commonly in the sputum specimens from the patients with lung carcinoma that lacked cancer cells than in those from patients with lung carcinoma that contained cancer cells. There are several possibile explanations. First, the specimen for morphologic examination did not contain cancer cells, but the cancer cells existed in the remaining materials. Second, there were cancer cells that could not be diagnosed by morphologic examination. Third, there were no cancer cells but fragments of the DNA of cancer cells. Finally, normal cells showed abnormal methylation in the sputum specimens from patients with lung carcinoma. Both the specificity and sensitivity values in the current study were less than the values reported in the study by Palmisano et al.[14] but the detection rate was significantly high in the sputum specimens from patients with lung carcinoma even if the specimens contained no cancer cells. The abnormal methylation examination for the three genes is believed to be a new molecular marker to identify persons at high risk of lung carcinoma.

In the current study, we demonstrated that examinations of AI and abnormal methylation status in sputum cytology materials are reliable, independent markers for identifying persons at high risk of lung carcinoma. Sputum cytology is a noninvasive, repeatable, and simple method for identifying persons with lung carcinoma. Furthermore, analysis of AI and abnormal methylation using sputum specimens is believed to be a supportive and powerful protocol.

REFERENCES

1. Seto H, Ohkubo T, Watanabe N, et al. Black dust matter in human lung and causes of the deposition. *Annu Rep Tokyo Metrop Res Lab P H.* 1994;45:139–148.
2. Hou M, Morishita Y, Iijima T, et al. The implication of anthracosis in the development of pulmonary adenocarcinoma. *Jpn J Cancer Res.* 1998;89:1251–1256.
3. Hou M, Morishita Y, Iijima T, et al. DNA methylation and expression of p16(INK4A) gene in pulmonary adenocarcinoma and anthracosis in background lung. *Int J Cancer.* 1999;84:609–613.
4. Wang D, Minami Y, Shu Y, et al. The implication of background anthracosis in the development and progression of pulmonary adenocarcinoma. *Cancer Sci.* 2003;94:707–711.
5. Baylin SB, Herman JG, Graff JR, Vertino PM, Issa JP. Alterations in DNA methylation: a fundamental aspect of neoplasia. *Adv Cancer Res.* 1998;72:141–196.
6. Fearon ER. Human cancer syndromes: clues to the origin and nature of cancer. *Science.* 1997;278:1043–1050.
7. Swafford DS, Middleton SK, Palmisano WA, et al. Frequent abnormal methylation of *p16INK4a* in primary rat lung tumors. *Mol Cell Biol.* 1997;17:1366–1374.
8. Belinsky SA, Nikula KJ, Palmisano WA, et al. Aberrant methylation of *p16* (*INK4a*) is an early event in lung cancer and a potential biomarker for early diagnosis. *Proc Natl Acad Sci USA.* 1998;95:11891–11896.
9. Virmani AK, Rathi A, Sathyanarayana UG, et al. Abnormal methylation of the *adenomatous polyposis coli* (*APC*) gene promoter 1A in breast and lung carcinomas. *Clin Cancer Res.* 2001;7:1998–2004.

10. Virmani AK, Rathi A, Zochbauer MS, et al. Promoter methylation and silencing of the *retinoic acid receptor-beta* gene in lung carcinomas. *J Natl Cancer Inst*. 2000;92:1303–1307.
11. Shimizu T, Sekiya T. Loss of heterozygosity at 9p21 loci and mutations of the *MTS1* and *MTS2* genes in human lung cancers. *Int J Cancer*. 1995;63:616–620.
12. Wieland I, Bohm M. Frequent allelic deletion at a novel locus on chromosome 5 in human lung cancer. *Cancer Res*. 1994;54:1772–1774.
13. Picard E, Seguin C, Monhoven N, et al. Expression of retinoid receptor genes and proteins in non-small-cell lung cancer. *J Natl Cancer Inst*. 1999;91:1059–1066.
14. Palmisano WA, Divinee KK, Saccomano G, et al. Predicting lung cancer by detecting abnormal promoter methylation in sputum. *Cancer Res*. 2000;60:5954–5958.
15. Okamoto N. Cost-effectiveness of lung cancer screening in Japan. *Cancer*. 2000;89:2489–2493.
16. Shiba M, Kohno H, Kakizawa K, et al. Ki-67 immunostaining and other prognostic factors including tobacco smoking in patients with resected nonsmall cell lung carcinoma. *Cancer*. 2000;89:1457–1465.
17. Saccomanno G, Archer V, Auerbach O, Saunders RP, Brennan LM. Development of carcinoma of the lung as reflected in exfoliated cells. *Cancer*. 1974;33:256–270.
18. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA*. 1996; 93:9821–9826.
19. Tsuchiya T, Tamura G, Sato K, et al. Distinct methylation patterns of two *APC* gene promoters in normal and cancerous gastric epithelia. *Oncogene*. 2000;19:3642–3646.