Abberrant promoter hypermethylation in serum DNA from patients with silicosis

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It is well established that patients with silicosis are at high risk for lung cancer; however, it is difficult to detect lung cancer by chest radiography during follow-up treatment of patients with silicosis because of preexisting diffuse pulmonary shadows. The purpose of this study is to evaluate the usefulness of detection of serum DNA methylation for early detection of lung cancer in silicosis. Serum samples from healthy controls (n = 20) and silicosis patients with (n = 11) and without (n = 67) lung cancer were tested for aberrant hypermethylation at the promoters of the DNA repair gene O6-methylguanine-DNA methyltransferase (MGMT), p16(INK4a), ras association domain family 1A (RASSF1A), the apoptosis-related gene death-associated protein kinase (DAPK) and retinoic acid receptor β (RARb) by methylation-specific polymerase chain reaction. Aberrant promoter methylation in at least one of five tumor suppressor genes was detected more frequently in the serum DNA of silicosis patients with lung cancer than in that of patients without it (P = 0.006). Furthermore, the odds ratio of having lung cancer was 9.77 (P = 0.009) for those silicosis patients with methylation of at least one gene. Extended exposure to silica (>30 years) was correlated with an increased methylation frequency (P = 0.017); however, methylation status did not correlate with age, smoking history or radiographic findings of silicosis. These results suggest that testing for aberrant promoter methylation of tumor suppressor genes using serum DNA may facilitate early detection of lung cancer in patients with silicosis.

Introduction

Occupational exposure to silica occurs in a large number of industries and circumstances, including mines and stone quarries as well as in the production of granite, ceramics, pottery and steel. In the USA, it is estimated that of more than one million workers occupationally exposed to free crystalline silica dust each year, 59,000 will eventually develop silicosis and ~300 will die from the disease (World Health Organization Fact sheet N°28, 2000. http://www.who.int/mediacentre/factsheets/fs28/en/). Growing evidence indicates that the association between silicosis and lung cancer is causal (1–4). In 1997, the International Agency for Research on Cancer concluded that inhaled crystalline silica is a human lung carcinogen (5). Chan et al. (6) reported that 33 (2.3%) of 1490 workers diagnosed with silicosis in Hong Kong died from lung cancer. Lung cancer spreads gradually throughout the body and has a poor prognosis because it is largely unresponsive to chemotherapy. Only those patients diagnosed at an early stage of the disease can be cured by complete resection. However, it is difficult to detect lung cancer by chest radiography or computed tomography during the follow-up treatment of patients with silicosis because of preexisting diffuse pulmonary shadows (7,8). A reliable clinical marker for early detection of lung cancer in patients with silicosis is thus urgently needed.

Epigenetic changes such as hypermethylation increasingly appear to play a role in carcinogenesis. DNA methylation is one form of epigenetic variability in mammalian cells. As aberrant hypermethylation in the CpG-rich promoter regions of many tumor suppressor genes interferes with transcription, hypermethylation may contribute to the development and progression of various cancers by abolishing tumor suppressor gene function (9–11). Aberrant hypermethylation of tumor suppressor genes has been observed in various specimens from lung cancer patients, including serum, plasma, sputum, lavage fluid and epithelial brushing (12,13). These findings raise the possibility that methylation analysis of such materials may be a useful tool for cancer detection. A number of studies have reported increased methylation of several tumor suppressor genes in lung cancer cells, including the DNA repair gene O6-methylguanine-DNA methyltransferase (MGMT) (14,15), p16(INK4a) (16), ras association domain family 1A (RASSF1A) (17), apoptosis-associated genes such as death-associated protein kinase (DAPK) (18) and retinoic acid receptor β (RARb) (19); in contrast, methylation of these genes is rarely reported in non-malignant lung tissue (14). We demonstrated previously that screening for promoter methylation in these five genes using serum (20) and pleural fluid (21) DNA can be useful for early detection of lung cancer. Thus, detection of tumor suppressor gene promoter hypermethylation in serum DNA may facilitate early detection of lung cancer in patients with silicosis. Here, we examined whether promoter hypermethylation could identify lung cancer in a clinical setting by testing the promoter methylation status of five tumor suppressor genes in serum DNA from 78 silicosis patients with and without lung cancer.

Materials and methods

Study population

The subjects included 78 patients with silicosis at Okayama Rosai Hospital (n = 76) or Bizen City Yoshinaga Hospital (n = 2) between 2004 and 2006 and 20 healthy controls. Of the 78 patients with silicosis, 11 were diagnosed with lung cancer. The 20 control subjects had no occupational history of silica exposure and they were matched to the cases by gender, age and smoking status. The characteristics of the study population are summarized in Table I. All patients with silicosis were defined as those who have occupational histories for silica exposure in the industries such as stone quarries, granite production, ceramic and pottery industries and steel production and shown profusion rates from one to four on radiographs based on International Labour Organization classification (22). Histological subtypes of lung cancers were based on World Health Organization classification (23). The clinical stage of disease was assessed using the International Staging System (24).

Sample collection and DNA extraction

Peripheral blood samples (6 ml) were collected to investigate the methylation status of the serum DNA. The serum (2 ml) was isolated by centrifugation at 3000 r.p.m. for 10 min and stored at ~80°C until use. Serum DNA was extracted using a QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. We also examined methylation status of the tumor tissues obtained from surgical resection or autopsy. Tumor DNA was extracted from formalin-fixed, paraffin-embedded lung cancer tissues using QIAamp DNA Mini Kit (Qiagen) according to the manufacturer’s instructions. The researchers were unaware of each patient’s diagnosis.
The institutional review board approved the protocols and written informed consent was obtained from the subjects.

**Methylation specific polymerase chain reaction**

Sample DNA was treated with sodium bisulfite using a CpGenome DNA Modification Kit (Intergen, Purchase, NY) as described previously (20, 21). The primers used for MGMT, p16\(^{\text{INK4a}}\), RASSF1A, DAPK and RAR\(\beta\) were described elsewhere (20, 21). DNA from SBC-3 (25), a small-cell lung cancer cell line with promoter methylation of all tested genes, was used as a positive control. The polymerase chain reaction (PCR) mixture contained 10× PCR buffer [100 mM Tris–HCl (pH 8.3), 500 mM KCl and 15 mM MgCl\(_2\)], deoxynucleotide triphosphates (each at 2.5 mM), 0.5 μM of each primer, 0.75 U Hotstar Taq DNA polymerase (Qiagen) and 3 μl of bisulfite-modified DNA in a final volume of 30 μl. An initial denaturation step at 95°C for 15 min was followed by 50 cycles of denaturation at 90–95°C for 20 s, annealing at the appropriate temperature for 30 s and extension at 72°C for 30 s with a final extension at 72°C for 10 min. After amplification, each product was electrophoresed through a 2% agarose gel, stained with ethidium bromide and visualized under ultraviolet illumination. The presence of a band was defined as a methylation-positive result, even if it was faint. Each blood sample was phoresed through a 2% agarose gel, stained with ethidium bromide and visualized under ultraviolet illumination. The presence of a band was defined as a methylation-positive result, even if it was faint. Each blood sample was

**Quantitative real-time PCR**

Quantitative real-time PCR was also performed with locus-specific primers and dual-labeled fluorogenic probes for lung cancer tissue samples. Methylation of p16\(^{\text{INK4a}}\) and RASSF1A was examined using β-actin as the internal control for DNA quantification. The DNA sequences of primers, which are different from those used in above-mentioned methylation specific polymerase chain reaction (MSP), and probes for these genes were based on published data (26, 27). PCR was set up in a reaction volume of 25 μl containing 1× Taqman universal PCR master mix (Applied Biosystems, Foster City, CA), 500 nM of each primer, 150 nM of probe and 3 μl bisulfite-treated DNA samples. After an initial denaturation step at 95°C for 2 min and 95°C for 15 min, 50 cycles of 15 s at 94°C and 1 min at 60–64°C were followed. Amplified data were analyzed using software developed by Applied Biosystems. The methylation ratio was defined as the ratio of the fluorescence emission intensity values for methylated p16\(^{\text{INK4a}}\) and RASSF1A to those of β-actin multiplied by 1000.

**Statistical analysis**

For each patient, the methylation status of the five genes was scored as the total number of methylated genes. The methylation score of those patients with silicosis plus lung cancer was compared with those with silicosis alone using a t-test with unequal variance. An unconditional logistic regression model was applied to estimate the odds ratios and 95% confidence intervals (CIs) for the occurrence of lung cancer. Those patients without methylation were used as a reference group to estimate the odds ratios for those patients with one or more methylated genes. Crude and multivariate models were examined. The factors considered in the multivariate model included age, gender, smoking status, radiological findings and the exposure period. A chi-square or Fisher’s exact test was used to examine the distribution in categorical variables. Statistical significance was defined as \(P < 0.05\). All statistical analyses were conducted using SPSS version 10 (SPSS, Chicago, IL).

### Results

**Methylation status of five genes and its significance in the detection of lung cancer**

We determined the prevalence of MGMT, p16\(^{\text{INK4a}}\), RASSF1A, DAPK and RAR\(\beta\) methylation in the serum DNA of 78 silicosis patients with or without lung cancer and in 20 healthy controls by MSP. Among the controls, methylation was detected at a frequency of 5.0% for MGMT, 0.0% for p16\(^{\text{INK4a}}\), 0.0% for RASSF1A, 10.0% for DAPK and 5.0% for RAR\(\beta\) (Table I). Among the silicosis patients without lung cancer, methylation was detected at a frequency of 14.9% for MGMT, 3.0% for p16\(^{\text{INK4a}}\), 3.0% for RASSF1A, 11.9% for DAPK and 9.0% for RAR\(\beta\). In contrast, among the silicosis patients with lung cancer, methylation was detected at a frequency of 36.4% for MGMT, 18.2% for p16\(^{\text{INK4a}}\), 9.1% for RASSF1A, 18.2% for DAPK and 0.0% for RAR\(\beta\). The total number of methylations per patient in the five genes was 0.82 [standard deviation (SD), 0.603; 95% CI, 0.41–1.22] for those patients with silicosis plus lung cancer, which was higher than that for the patients with silicosis alone (0.42; SD, 0.721; 95% CI, 0.24–0.59; \(P = 0.066\)). Aberrant promoter methylation in at least one of the five tumor suppressor genes was more frequent in silicosis patients with lung cancer (72.7%) than in those without it (29.9%; \(P = 0.066\)). Among those patients with silicosis and methylation in at least one of the five genes, the sensitivity, specificity, positive predictive values and negative predictive values for the diagnosis of lung cancer were 72.7, 70.1, 28.6 and 94.0%, respectively. Among five patients with stage I disease of lung cancer, four patients had methylation in at least one of the five genes. To quantify these PCR results, methylation value was determined using Scion Image software. Methylation value was higher in silicosis patients with lung cancer than those without in four genes tested (Figure 1).

Tumor tissues were obtained from 8 of 11 cases with lung cancer, four from surgically resection and four from autopsy, and sufficient DNA was extracted in six of the eight cases. All the tumor tissues showed methylation in at least one gene, and in four of these six cases, methylation was also detected in serum DNA in at least one gene (supplementary Figure 2 is available at Carcinogenesis Online).

To validate our results of MSP, methylation of p16\(^{\text{INK4a}}\) and RASSF1A in the tumor tissue was evaluated using quantitative real-time PCR. Methylation of p16\(^{\text{INK4a}}\) was detected in two cases and RASSF1A methylation was detected in five cases. The p16\(^{\text{INK4a}}\) methylation in two cases and RASSF1A methylation in three cases, detected by MSP, were confirmed by this quantitative PCR (supplementary Table 1 is available at Carcinogenesis Online).

**Methylation status and the likelihood of lung cancer**

Given that the silicosis patients with lung cancer exhibited a higher frequency of aberrant methylation than those without it, we analyzed the methylation status and risk of lung cancer in patients with silicosis. Table III shows the results of a crude logistic regression analysis of the correlation between methylation status and the risk of lung cancer. Silicosis patients with at least one methylated gene were 6.26 (95% CI, 1.51–26.07) times more likely to have lung cancer than were silicosis patients with no methylated genes (\(P = 0.012\)). To consider the imbalance in the baseline characteristics, we conducted a similar analysis adjusting for age, gender, smoking status, radiologic findings and the silica exposure period, which were considered associated with the risk of malignancy. After adjusting for these factors,
available at [supplementary Figure 3 is available at Carcinogenesis Online]. Case 2 involved an 82-year-old man who had received segmentectomy for squamous cell carcinoma of the lung. Although no methylation was observed in his serum while he was disease free, *MGMT* methylation was detected in his serum at recurrence. These results indicate that the methylated DNA in the serum of these patients was released from the cancer cells and that methylated DNA in the serum of patients with silicosis may reflect undetected cancerous lesions.

### Discussion

It is well established that patients with silicosis are at increased risk for lung cancer (1–4, 28). In the current study, we examined the promoter methylation status of five tumor suppressor genes using serum DNA from 78 silicosis patients with or without lung cancer. Promoter hypermethylation in at least one of the five genes was significantly increased in silicosis patients with lung cancer than in those without it. In addition, among 11 patients with silicosis that developed lung cancer, eight (72.7%) showed methylation in at least one of the five genes tested. In a previous study of the same five genes, almost half of the patients (49.5%) with lung cancer had methylation in their serum (20). These results indicate that lung cancer may be associated with an increased frequency of aberrant methylation if it is accompanied by silicosis, although the present study should be interpreted carefully due to the small sample size. We also examined the methylation status of tumor tissues of these patients with lung cancer and demonstrated that all the tumor tissues examined showed methylation in at least one gene. These results and the altered methylation profile in two described cases indicate that the methylated DNA in the serum was released from the cancer cells, though an unexpected result was also shown that *MGMT* methylation was detected in serum from two patients even though tumors did not show the methylation. We have no clear evidences but speculate two possible explanations of the discrepancy: (i) the tumor DNAs were extracted from formalin-fixed, paraffin-embedded samples and not from fresh-frozen tissues. So DNA pieces of *MGMT* sequences might be fragmented and (ii) the methylated DNA in their serum might come from malignant or premalignant lesions in organs other than the lung. These should be clarified in the future studies.

The increased incidence of DNA methylation in silicosis patients with lung cancer compared with those without it motivated us to evaluate serum DNA methylation as a marker for lung cancer. Our analysis showed that patients with serum DNA methylation were ~10 times more likely to have lung cancer. These findings support the use of DNA methylation status as a marker for lung cancer detection in silicosis patients. Early detection of lung cancer in patients with silicosis is a serious clinical problem (1–4, 8, 28); thus, a reliable marker for rapid and accurate diagnosis is urgently needed. In the current study, methylation in serum DNA was detected even in patients with early stage of lung cancer. These results suggest that aberrant promoter methylation of tumor suppressor genes in serum DNA may be a valuable marker for the early detection of lung cancer during the follow-up treatment of patients with silicosis. Although further evaluation is essential, these results also suggest that serum DNA methylation may be worth examining routinely, particularly before invasive procedures.

Before using serum DNA methylation as a marker for lung cancer during the follow-up of patients with silicosis, several issues must be considered. In the present study, we found that the sensitivity, specificity and positive predictive value of methylation in one or more genes for the diagnosis of lung cancer were 72.7, 70.1 and 28.6%, respectively. These results may not be satisfactory for clinical purposes. One strategy for improving the specificity and sensitivity of testing for lung cancer during follow-up treatment for silicosis is to use a larger number of genes or to apply a quantitative methylation assay (27, 29, 30). The sensitivity of the Taqman method is reported to be 10-fold higher than conventional qualitative MSP (31). In this study, quantitative PCR was performed for limited samples of lung cancer

### Table II. Frequency of methylation in five genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Healthy controls</th>
<th>Silicosis without lung cancer</th>
<th>Silicosis with lung cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 20</td>
<td>n = 67</td>
<td>n = 11</td>
</tr>
<tr>
<td><em>MGMT</em></td>
<td>1 (5.0)</td>
<td>10 (14.9)</td>
<td>4 (36.4)</td>
</tr>
<tr>
<td>p16NGF-α</td>
<td>0 (0.0)</td>
<td>2 (3.0)</td>
<td>2 (18.2)</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>0 (0.0)</td>
<td>2 (3.0)</td>
<td>1 (9.1)</td>
</tr>
<tr>
<td>DAPK</td>
<td>2 (10.0)</td>
<td>8 (11.9)</td>
<td>2 (18.2)</td>
</tr>
<tr>
<td>RARβ</td>
<td>1 (5.0)</td>
<td>6 (9.0)</td>
<td>0 (0.0)</td>
</tr>
</tbody>
</table>

### Table III.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Frequency</th>
<th>n</th>
<th>p-value (Hosmer-Lemeshow test)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>MGMT</em></td>
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<td>0.009</td>
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<tr>
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<th>Frequency</th>
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<th>p-value (Hosmer-Lemeshow test)</th>
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<td>RARβ</td>
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### Fig. 1.

Methylation value of five genes. Methylation value was determined as mean value of the ratio: methylated band/positive control quantified with Scion Image.

silicosis patients with methylation in at least one gene were still 9.77 (1.78–53.74; P = 0.009) times more likely to have lung cancer (Table III). These results suggest that methylation of these five tumor suppressor genes is associated with lung cancer in patients with silicosis.

### Methylation status and silicosis

The total number of methylations per patient in the five genes was 0.47 (SD, 0.716; 95% CI, 0.31–0.64) in patients with silicosis, which tended to be higher than in the healthy controls (0.2; SD, 0.523; 95% CI, 0.00–0.44; P = 0.061). We next analyzed methylation status and the risk of silicosis. Table IV shows the results of a crude logistic regression analysis of the correlation between methylation status and the risk of silicosis. Patients with at least one methylated gene were 3.17 (95% CI, 0.855–11.78) times more likely to have silicosis than were patients with no methylation (P = 0.084). We then tested for associations between the methylation status of the five genes and various clinical variables. As shown in Table V, no statistically significant association between methylation status and clinical variables such as age, gender, smoking status or radiologic findings was observed; however, we found a statistically significant association between methylation status and the silica exposure period (P = 0.017). These results suggest that methylation of these five tumor suppressor genes is associated with silica exposure and might be associated with silicosis. Among the patients with long exposure to silica (>30 years), 85.7% of the patients with lung cancer have at least one gene hypermethylation compared with 37.5% of those without lung cancer (P = 0.035) (Table VI).

### The altered serum methylation status of two silicosis patients with lung cancer

We describe two cases that methylation status was examined twice during follow-up treatment for silicosis (supplementary Figure 3 is available at Carcinogenesis Online). Case 1 involved a 69-year-old woman who developed adenocarcinoma of the lung. *DAPK* methylation was detected in her serum at the time of partial resection of the right upper lobe; however, it disappeared 15 months later. The *DAPK* methylation was also detected in resected tumor tissue in the case (sample 4 in supplementary Figure 2 is available at Carcinogenesis Online). Case 2 involved an 82-year-old man who had received segmentectomy for squamous cell carcinoma of the lung. Although no methylation was observed in his serum while he was disease free, *MGMT* methylation was detected in his serum at recurrence. These results indicate that the methylated DNA in the serum of these patients was released from the cancer cells and that methylated DNA in the serum of patients with silicosis may reflect undetected cancerous lesions.
tissues and we found inconsistency between results by non-quantitative MSP and those by quantitative real-time PCR, such as RASSF1A methylation in sample 2. These findings might indicate the higher sensitivity of the Taqman method compared with non-quantitative one. Another strategy is to search for the best combination of genes to use for methylation analysis with or without other diagnostic tests, including low-dose spiral computed tomography (32–34). Additional studies are warranted.

It is of note that a statistically significant association was detected between methylation status and the silica exposure period, although no direct association between methylation status and silica was observed. A previous study using an animal model demonstrated that expression of E-cadherin was significantly reduced by silica-induced chronic inflammation because of promoter hypermethylation (35). Aberrant promoter methylation has also been reported in precancerous lesions, such as in the dysplasias of patients with lung cancer (36). Our finding that long-term silica exposure is correlated with an increased frequency of methylation suggests that silicosis may be a so-called precancerous lesion (1,37). And methylation status did not correlate with age, smoking history or radiographic findings of silicosis. There are numerous reports that show age-related methylation in a subset of genes (38,39) or association between smoking and methylation in the lung (40–42). We have no clear explanation why methylation status did not correlate with age or smoking history in this study, but it might be due to our non-quantitative method for methylation analysis.

We found that the total number of methylations per patient in five genes tended to be higher in patients with silicosis than in healthy controls. These results support the notion that silicosis patients with methylation in some tumor suppressor genes may already be in an early stage of lung cancer development or that some proportion of these patients already has cancerous lesions. Thus, it will be of great interest to investigate whether the methylation-positive patients with silicosis develop lung cancer in the near future.

In conclusion, we showed that aberrant tumor suppressor gene promoter methylation was more frequent in the serum DNA of silicosis patients with lung cancer than in those without it. Our results suggest that testing for aberrant promoter methylation of these genes in serum DNA may aid in the early detection of lung cancer in patients with silicosis. Additional studies are warranted to confirm the value of such analyses and to determine the most informative combination of genes.

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