Aberrant Gene Promoter Methylation in Sputum from Individuals Exposed to Smoky Coal Emissions

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Abstract. Background: Recent studies suggested the potential for aberrant gene promoter methylation in sputum as a predictive marker for lung cancer. Here, the promoter methylation of p16, MGMT, RASSF1A and DAPK genes was investigated in sputum of individuals exposed to smoky coal emissions in Xuan Wei, China, where the lung cancer rate is more than 6 times the Chinese national average. Materials and Methods: Sputum DNA of 107 noncancer individuals and 58 lung cancer patients was screened for promoter methylation using methylation-specific PCR. Results: Promoter methylation of the p16 gene was detected in about half (51.4% (55/107)) of sputum DNA from noncancer individuals, a frequency higher than that observed for the RASSF1A (29.9%), MGMT (17.8%) and DAPK (15.9%) genes. Furthermore, the p16 gene was affected by promoter methylation at a frequency even higher among the lung cancer group, compared with the noncancer group [70.7% (41/58) versus 51.7% (55/107), p=0.017]. Conclusion: Individuals exposed to smoky coal emissions in this region harbored frequent promoter methylation of these genes in their sputum and some of such alterations may be involved in lung tumor development.

Aberrant promoter methylation of tumor suppressor genes is an important mechanism of gene transcriptional inactivation and has been associated with the development of many kinds of cancer (1), including lung cancer, the most common cause of cancer death worldwide. Although much attention has been paid to understanding the molecular and cellular mechanisms of lung cancer, the 5-year overall survival rate for all stages combined is only 15% (2), due primarily to the presence of metastatic tumors in approximately two-thirds of patients at the time of diagnosis (3). Detection of lung cancer at earlier stages could potentially increase survival rates by 10 to 50-fold (4). Recently, gene promoter methylation has become a target for the development of screening methods for early detection, diagnosis and treatment of lung cancer (5-11).

In Xuan Wei County (XWC), Yunnan Province, China, lung cancer rates for women, who were mostly nonsmokers, and for men, who were mostly smokers, were eight times and four times the Chinese national average rates for women and men, respectively. Several studies demonstrated a strong association between the high lung cancer rate in this region and the use of smoky coal for cooking and heating in homes without chimneys (12-14). These emissions contained a high level of polycyclic aromatic hydrocarbons (PAHs), among which methylated PAHs have higher tumorigenic potency than the parent PAHs (15). PAHs in XWC smoky coal emission were found to be more carcinogenic than cigarette smoke in a murine skin-tumor assay (16). Furthermore, these emissions have been associated with high frequencies of p53 and K-ras mutations in lung tumors and in sputum from lung cancer patients from XWC (17, 18). However, the effects of exposure to smoky coal emissions on epigenetic alterations, specifically gene promoter methylation, in this population remain unclear.
Promoter methylation of the p16, MGMT, RASSF1A and DAPK genes has been commonly found in lung tumors and implicated in different pathways of lung tumorigenesis (19-23). In the present study, aberrant promoter methylation of these genes was examined in sputum samples obtained from 107 individuals who were exposed to smoky coal emissions in XWC and who showed no evidence of lung cancer but were at high risk for developing the disease. In addition, sputum from 58 lung cancer patients was examined for promoter methylation of the p16 gene. The results were analyzed in relation to the smoking status, gender, the presence or absence of symptoms of chronic bronchitis and the diagnosis of lung cancer.

Materials and Methods

**Subject enrolment and sputum collection and processing.** All sputum samples were collected from XWC, China. Individuals who donated sputum samples analyzed in this study had also taken part in a previous study (13). Sputum from 165 individuals were examined in this study, including 107 individuals who showed a minimum of clinical symptoms, and chest X-ray analysis at the Xuan Wei Hospital found with no evidence of lung cancer, while 58 individuals were diagnosed with lung tumors. Each individual who provided informed consent to participate in this study also answered a standardized closed questionnaire on demographic information, smoking history, family and personal medical history, as well as information on other variables. For the protection of the participants, this study was conducted according to recommendations of the World Medical Association Declaration of Helsinki (1989) (24). The research protocol met the requirements for the protection of human subject certification by the US EPA.

The demographic and clinical information of the 165 individuals involved in this study is shown in Table I. Among the 107 noncancer individuals, all the males (n=73) with the exception of one were smokers and all the females were nonsmokers. Of these individuals, 49 had symptoms of chronic bronchitis, with excessive bronchial mucus and a chronic cough for three months or more in at least three consecutive years and without any other disease that could account for these symptoms, and 58 had no such symptoms. The 58 lung cancer patients consisted of 36 males, including 31 smokers and 5 nonsmokers, and 22 female nonsmokers. Information on the tumor types was not available because most of the patients did not undergo tumor resection.

The collection and processing of sputum samples, cytological examination of sputum cells and genomic DNA extraction from sputum samples were described in previous studies (13, 25).

**Promoter methylation analysis.** Each genomic DNA sample was treated with sodium bisulfite (Sigma, Saint Louis, MO, USA) and purified using a Wizard DNA Clean-Up System (Promega Corporation, Madison, WI, USA), as described elsewhere (25). Universal methylated human genomic DNA (Chemicon International, Temecula, CA, USA) was treated the same way and was used as a positive control DNA, while water was used as a negative control.

**Methylation-specific polymerase chain reaction (MSP).** Nested two-step MSP was used for analysis of promoter methylation of all four genes. The methylation status of p16, MGMT, RASSF1A and DAPK was determined by the methods described elsewhere (25, 26). The reproducibility of the results was confirmed by repeating the MSP analysis for each sample and those which showed methylation products for both MSP were scored as positive for methylation. Promoter methylation of p16, MGMT and DAPK was further confirmed using digestion of the resulting PCR products with the restriction enzymes Fnu4HI, TaqI and BstUI, respectively. Promoter methylation of RASSF1A was confirmed by direct sequence.

**Statistical analysis.** Chi-square test was used in univariate analysis. Logistic regression models were used to assess the effect of multiple variables on methylation status.

## Results

Figure 1 shows a representative example of MSP analysis of sputum DNA. Of the 107 individuals without lung cancer, p16, RASSF1A, MGMT, and DAPK promoter methylation was detected in 51.4% (55/107), 29.9% (32/107), 17.8% (19/107), and 15.9% (17/107), respectively. Seventy-three (68.2%) of the 107 individuals showed promoter methylation of at least one of the genes, including 3 (2.8%), 8 (7.5%), 21 (19.6%) and 41 (38.3%) individuals showing the alteration in all 4 genes, 3 genes, 2 genes and 1 gene, respectively.

As shown in Table II, there were no differences in promoter methylation frequencies between the smoking group and nonsmoking group for either p16 (50.0% vs. 54.3%, p=0.677), MGMT (18.1% vs. 17.1%, p=0.908), RASSF1A (31.9% vs. 25.7%, p=0.509), or DAPK (18.1% vs. 11.4%, p=0.369), or between the group of individuals with chronic bronchitis and the group of those without this symptom (51.0% vs. 51.7% for p16, p=0.938; 14.3% vs. 20.7% for MGMT, p=0.377; 30.6% vs. 29.3% for RASSF1A, p=0.899; 14.3% vs. 17.2% for DAPK, p=0.677).

Multivariate logistic regression models were employed to control for potential confounding effects of variables such as gender, age, smoking status and bronchitis. As shown in
Table III, only age showed a significant effect on DAPK methylation status \(\text{odds ratio (OR)}=1.072; \text{95\% confidence interval (CI)}=1.008 – 1.141, p=0.027, \text{Table III}\), while these variables did not have any effect on the promotor methylation status of the other three genes.

Following the observation of frequent promotor methylation of these genes, and particularly the \(\text{p16}\) gene in normal individuals, we investigated whether such gene alteration could be detected in sputum from lung cancer patients. However, because of the limited amount of DNA available from sputum of the cancer patient group, we focused on only the \(\text{p16}\) gene that was found to be the most frequently affected gene by promotor methylation. \(\text{p16}\) promoter methylation was detected in 70.7\% (41/58) of the sputum samples from these patients and was not significantly different between the smokers, who were all male, and the nonsmokers, who were mostly female \[61.3\% (19/31) \text{vs. } 81.5\% (22/27), p=0.093\].

### Discussion

In this study, we demonstrated that gene promotor methylation, particularly of the \(\text{p16}\) gene, occurred frequently in DNA extracted from the sputum of individuals without evidence of lung cancer who were exposed to smoky coal emissions in XWC. Furthermore, 3 individuals (2.8\%) showed the co-occurrence of promotor methylation of all 4 genes, while 8 (7.5\%), 21 (19.6\%) and 41 (38.3\%) other individuals showed this alteration in 3 genes, 2 genes and 1 gene in their sputum.
Table III. Logistic regression models of p16, MGMT, RASSF1A and DAPK promoter methylation in 107 noncancer individuals from Xuan Wei, China.

<table>
<thead>
<tr>
<th></th>
<th>p16</th>
<th>OR</th>
<th>95% CI</th>
<th>P</th>
<th>MGMT</th>
<th>OR</th>
<th>95% CI</th>
<th>P</th>
<th>RASSF1A</th>
<th>OR</th>
<th>95% CI</th>
<th>P</th>
<th>DAPK</th>
<th>OR</th>
<th>95% CI</th>
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<tr>
<td>Gender</td>
<td>0.646</td>
<td>0.133-3.135</td>
<td>0.588</td>
<td>0.822</td>
<td>0.093-7.243</td>
<td>0.859</td>
<td>0.983</td>
<td>0.170-5.688</td>
<td>0.985</td>
<td>1.130</td>
<td>0.085-14.999</td>
<td>0.926</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Age</td>
<td>0.999</td>
<td>0.963-1.036</td>
<td>0.960</td>
<td>1.031</td>
<td>0.980-1.085</td>
<td>0.234</td>
<td>1.012</td>
<td>0.971-1.054</td>
<td>0.583</td>
<td>1.072</td>
<td>1.008-1.141</td>
<td>0.027</td>
<td></td>
<td></td>
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<tr>
<td>Smoking</td>
<td>1.219</td>
<td>0.255-5.835</td>
<td>0.804</td>
<td>1.230</td>
<td>0.140-10.812</td>
<td>0.852</td>
<td>1.355</td>
<td>0.236-7.790</td>
<td>0.734</td>
<td>1.537</td>
<td>0.115-20.454</td>
<td>0.745</td>
<td></td>
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<tr>
<td>Bronchitis</td>
<td>0.954</td>
<td>0.432-2.107</td>
<td>0.908</td>
<td>0.540</td>
<td>0.187-1.559</td>
<td>0.255</td>
<td>0.999</td>
<td>0.421-2.369</td>
<td>0.998</td>
<td>0.588</td>
<td>0.193-1.792</td>
<td>0.350</td>
<td></td>
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</tbody>
</table>

OR, odds ratio; CI: confidence interval.

respectively. The clinical implication of promoter methylation in sputum samples on lung cancer risk remains unclear since these individuals were anonymous and were not followed up further. However, results from previous studies of smokers from Europe and the United States suggested that gene promoter methylation in sputum may provide a useful predictive biomarker for lung cancer. For example, Palmasano et al. reported that aberrant methylation of p16 and MGMT was detected in sputum of all patients with squamous cell lung carcinoma up to 3 years before clinical diagnosis. Moreover, the prevalence of these markers in sputum from cancer-free, high-risk subjects approximates the frequency of lung cancer in the United States (7,8,27,28). This discrepancy was unlikely attributable to technical problems because similar detection methods were used in both our present study and these studies.

The gene promoter methylation frequencies in sputum of noncancer population from XWC are higher than those reported previously in sputum from smokers from Europe and the United States (7,8,27,28). This discrepancy was unlikely attributable to technical problems because similar detection methods were used in both our present study and these studies. Ethnic and/or geographical differences might play a role to account for this difference (29-31). However, the p16 promoter methylation frequency (51.4%) in the present study is much higher than the frequency of 6.6% (n=91) in Su et al.’s report (32) and 14% (n=37) in Hsu et al.’s study (33); subjects in both studies were Chinese, lung cancer–free and from other regions of China. Furthermore, in this study, there were no differences in promoter methylation frequencies for any of the 4 genes in sputum between the nonsmokers and the smokers from the noncancer group, by using either univariate (Table II) or logistic (Table III) analysis. These results are in disagreement with the significantly higher promoter methylation frequencies observed for p16 and RASSF1A genes in sputum samples from smokers, compared with nonsmokers (34).

Taken together, the higher promoter methylation frequencies found in sputum of noncancer individuals in XWC, compared with those found in sputum of the European and American smokers and other regions of China, and the similar promoter methylation frequencies between smokers, mostly men, and nonsmokers, mostly women, in the XWC population, may be due to their exposure to smoky coal combustion. Emissions from smoky coal contained 81% of organic matter, of which 43% were PAHs (35). During the burning of smoky coal for home cooking, the indoor air concentration of benzo(a)pyrene (BaP), an indicator of PAHs, can become as high as 14.7 μg/m³, comparable to exposure levels experienced by coke oven workers (36). Nonsmokers exposed to these emissions have higher levels of PAH metabolites and BaP-adducted guanine in their urine, compared with urine from smokers not exposed to these emissions (12,37), suggesting a higher level of PAHs in smoky coal emission than in tobacco smoke. Furthermore, a study using animal models showed that PAHs taken from XWC smoky coal emissions were more carcinogenic than cigarette smoke (16). Thus, these results suggest that the high frequencies of promoter methylation observed in both smokers and nonsmokers in our study may be associated with exposure of these individuals to PAHs, present at high concentrations in smoky coal emissions.

There were no differences in the frequencies of promoter methylation in any of the genes between the groups of noncancer individuals diagnosed with chronic bronchitis and the group of those without such a symptom, by using either univariate (Table II) or logistic (Table III) analysis. Results from a study of bronchial aspirates from patients with benign lung diseases, including chronic bronchitis, showed only very rare gene methylation among such patients (38). Another study showed that lung disease, including chronic bronchitis and pneumonia, did not affect the prevalence for methylation of any of the p16, MGMT, RASSF1A, and DAPK genes (39).

Results from the present study are in line with our previous study of these same sputum samples showing no differences in p53 mutation frequencies between these two groups of
individually important, suggesting that promoter methylation of the 4 genes investigated, like $p53$ mutations, was associated primarily with exposure to smoky coal emissions. They also suggest that chemicals in the smoky coal emissions, particularly the high concentration of PAHs and other chemicals (36), may play a primary role in the formation of genetic and epigenetic alterations founf in sputum from individuals who had evidence of lung cancer in XWC.

Finally, the $p16$ gene promoter methylation frequency of $70.7\%$ (41/58) of sputum of lung cancer patients from XWC in this study is in agreement with a $76\%$ frequency observed by Liu et al. (30) in sputum of Chinese lung cancer patients from Beijing, China. However, the latter study did not report any information on the gender, environmental/occupational exposure or smoking status of the patients investigated. There were no tumors available from the XWC lung cancer patients investigated in this study so that we could not determine whether the detection of $p16$ gene promoter methylation in sputum of these patients correlated with the presence of this gene alteration in their lung tumors. Nevertheless, results from a previous study of specimens from 50 lung cancer patients by Liu et al. (30) showed a good correlation between the frequency of $p16$ gene promoter methylation in lung tumors and those of this gene alteration in the matched plasma and sputum samples from these patients (84.0% vs. 72.0% and 76%, respectively). Taken together, these results suggest that aberrant promoter methylation of at least the $p16$ gene occur early in individuals exposed to smoky coal emissions in XWC and some of these gene alterations may be involved in lung cancer development.

In conclusion, we showed that promoter methylation of the $p16$, MGMT, RASSF1A, and DAPK genes, was relatively frequent in the sputum of exposed individuals without evidence of lung cancer. Furthermore, $p16$ gene promoter methylation was detected not only in the sputum of noncancer individuals but also at a significantly higher frequency in the sputum of lung cancer patients from the same region. This alteration was not associated with the smoking status, gender, or chronic bronchitis diagnosis of the participants, indicating a dominant role of the chemicals present in smoky coal emissions in the formation of promoter methylation in sputum of these individuals. The results of this study suggest that detection of epigenetic alterations, such as aberrant promoter methylation of these genes or additional genes, in sputum from a larger number of followed-up individuals in XWC who had been exposed to smoky coal emissions may provide a useful means for early detection of lung cancer.

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References


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