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Aberrant promoter methylation of multiple genes in sputum from individuals exposed to smoky coal emissions

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Summary

Aberrant methylation in the promoter region of cancer-related genes leads to gene transcriptional inactivation and plays an integral role in lung tumorigenesis. Recent studies demonstrated that promoter methylation was detected not only in lung tumors from patients with lung cancer but also in sputum of smokers without the disease, suggesting the potential for aberrant gene promoter methylation in sputum as a predictive marker for lung cancer. In the present study, we investigated promoter methylation of 4 genes frequently detected in lung tumors, including p16, MGMT, RASSF1A and DAPK genes, in sputum samples obtained from 107 individuals, including 34 never-smoking females and 73 mostly smoking males, who had no evidence of lung cancer but who were exposed to smoky coal emission in Xuan Wei County, China, where lung cancer rate is more than 6 times the Chinese national average rate. Forty nine of the individuals showed evidence of chronic bronchitis while the remaining 58 individuals showed no such a symptom. Promoter methylation of p16, MGMT, RASSF1A and DAPK was detected in 51.4% (55/107), 17.8% (19/107), 29.9% (32/107), and 15.9% (17/107) of the sputum samples from these individuals, respectively. There were no differences in promoter methylation frequencies of any of these genes according to smoking status or gender of the subjects or between individuals with chronic bronchitis and those without evidence of such a symptom. Therefore, individuals exposed to smoky coal emissions in this region harbored in their sputum frequent promoter methylation of these genes that have been previously found in lung tumors and implicated in lung cancer development.

Keywords

Smoky coal emissions; Gene promoter methylation; Lung cancer

Introduction

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

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cancers [1], including lung cancer, the most common cause of cancer death worldwide. Although much attention has been paid to understand 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-third of patients at the time of diagnosis [3]. Detection of lung cancer at earlier stages could potentially increase survival rates by 10–50 folds [4]. Recently, gene promoter methylation has become a target for the development of screening methods for early detection, diagnosis, and treatment of lung cancer [5,6]. Results from several studies have indeed suggested the potential for gene promoter methylation in sputum as a predictive marker for lung cancer [7–11].

Most studies of gene promoter methylation in sputum involved so far lung cancer patients or smokers from Europe and the United States [7–11]. 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, a low sulfur, medium volatility bituminous 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 are 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, including cell cycle regulation, DNA repair, signal transduction and apoptosis, respectively [19–23]. In the present study, we examined aberrant promoter methylation of these genes 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. We analyzed the results in relation to the smoking status, gender, and the presence or absence of symptoms of chronic bronchitis in these individuals.

Methods

Subject enrollment and sputum collection and processing

All sputum samples were collected from XWC, China. Individuals who donated sputum samples analyzed in this study were part of the subjects involved in a previous study [13]. These individuals showed a minimum of clinical symptoms and chest X-ray analysis at the Xuan Wei Hospital and were found with no evidence of lung cancer. 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 human subjects, this study was conducted according to recommendations of the World Medical Association Declaration of Helsinki (1989) [24]. The research protocol met the requirements for protection of human subject certification by the US EPA.

The demographic and clinical information of the 107 individuals involved in this study is shown in Table 1. All the male subjects (n = 73) with the exception of one subject are smokers and all the female subjects are 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.

Sputum was collected first-morning on five consecutive mornings. Each subject was instructed to rinse his/her mouth with water to remove extraneous material, to take a deep breath, and cough deeply and expectorate into a plastic cup. Each morning sputum sample was stored in 40 ml of Saccomanno's fluid (39% ethanol, 3% polyoxyethylene, and 2% isopropanol; Lerner Laboratories, Pittsburgh, PA) to fix and preserve the cells. The sputum samples were stored at 4°C and transported to the U.S. by air. To collect cells, each sputum sample in Saccomanno's fluid was blended for 8–15 seconds in a blender to break the mucus and free the cells. The sample was then centrifuged at 600-g for 10 minutes. The supernatant was discarded and the cell pellet was resuspended in fresh Saccomanno's fluid by vortexing to achieve a cell concentration of approximately 10^6 cells per ml. The cells were subjected to cytological examination using the method described by Saccomanno in order to determine whether the sputum samples were derived from the lower respiratory tracts and also to confirm the presence of bronchial epithelial cells.

DNA extraction and promoter methylation analysis—Genomic DNA was extracted from each sputum sample, using the method combining Proteinase digestion and phenol-chloroform extraction, and recovered by ethanol precipitation and suspended in deionized water.

Each genomic DNA sample was treated with sodium bisulfite (Sigma, Saint Louis, MO) and purified by using a Wizard DNA Clean-Up System (Promega Corporation, Madison, WI), as described previously [25]. Universal methylated human genomic DNA (Chemicon International, Temecula, CA) 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)—Two-step MSP was used for analysis of all 4 genes promoter methylation. The methylation status of the p16 and MGMT were determined by the methods as described before [25]. The methylation status of the RASSF1A and DAPK were determined by using the primers and a protocol modified from that described by Belinsky et al [21]. During the stage-I PCR, the PCR amplification was carried out in a 25-μl reaction mixture containing 10 mM Tris-HCL (pH 8.3), 50 mM KCL, 2 mM $MgCl_2$, 100 μM each dNTP, and 0.2 μM each primer. The reaction was heated at 95°C for 10 min., then amplified for 40 cycles [95°C/30 sec., 64°C (for RASSF1A) or 58°C (for DAPK) / 30 sec., and 72°C/30 sec.], followed by a 10-min.-final extension at 72°C. Three microliters of each stage-I PCR product was diluted 10-fold and 1-μl was used for stage-II PCR, using the same reagents and conditions as for stage-I, except that the $MgCl_2$ concentration was reduced to 1 mM and each sample was amplified in duplicated reaction, with one reaction containing primers specific for a methylated C and the other reaction containing primers specific for unmethylated C. Each reaction was heated at 95°C for 10 min., then amplified for 40 cycles each consisting, for the reaction containing methylated primers, of 95°C/30 sec., 68°C (for RASSF1A) or 66°C (for DAPK)/30 sec., and 72°C/30 sec., and, for the reaction containing unmethylated primers, of 95°C /30 sec., 64 °C (for RASSF1A) or 68°C (for DAPK)/30 sec., and 72°C/30 sec., followed by a 10-min.-final extension at 72°C. Five microliters of each stage-II PCR product was separated on an 8% polyacrylamide gel. The gel was stained with ethidium bromide and photographed under UV illumination. Promoter methylation of p16, MGMT and DAPK were further confirmed by 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 involved in this study, p16, MGMT, RASSF1A and DAPK promoter methylation was detected in 51.4% (55/107), 17.8% (19/107), 29.9% (32/107), 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 2, there were no differences in promoter methylation frequencies between the smoking group and nonsmoking group for either the 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 (18.3% vs. 11.4%, p = 0.364) gene, 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 = 0.710).

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 3, only age showed significant effect on DAPK methylation status (odds ratio (OR) = 1.072; 95% confidence interval (CI) = 1.008 – 1.141, p = 0.027, table 3), while these variables did not have any effect on the promoter methylation status of the other 3 genes.

Discussion

In this study, we demonstrated that gene promoter methylation occurred frequently in DNA extracted from sputum of individuals exposed to smoky coal emissions in XWC, with a frequency varying from 51.4% (55/107) for p16 gene, to 29.9% (32/107) for RASSF1A gene, 17.8% (19/107) for DAPK gene, and 15.9% (17/107) for MGMT gene. Furthermore, 3 individuals (2.8%) showed the co-occurrence of promoter methylation of all four 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, respectively. The clinical implication of the co-occurrence of promoter methylation on lung cancer risk remains unclear since these subjects 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, Palmisano et al reported that aberrant methylation of the 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 lifetime risk for lung cancer [7]. A study by Kersting et al of p16 gene promoter methylation, and p53 and K-ras mutations in exfoliated cells from 51 NSCLC patients and 25 chronic smokers showed that 8 of the chronic smokers harbored a genetic and/or epigenetic alteration, and 3 of whom were subsequently diagnosed with lung cancer [8]. Two recent studies, both from the Belinsky's group, showed that the co-occurrence of promoter methylation of three genes and of more than 3 genes in sputum was associated with a 3.6- and 6.5-fold increased risk of lung cancer, respectively [10,11]. Therefore, individuals with sputum positive for aberrant promoter methylation, especially those showing such an alteration in multiples genes, may be at high risk for lung cancer.

In this study, the gene promoter methylation frequencies found in sputum of the XWC population are higher than those reported previously in sputum from smokers from Europe and the United States [7,8,26,27]. For instance, Destro et al reported that only 4 cases (4%) among 100 heavy smokers (age>60, >20 cigarettes/day last at least 20 years) showed p16 promoter methylation [26]. This discrepancy was unlikely attributable to technical problems because similar detection methods were used in both our present study and these studies. Ethnic differences might play a factor to account for this difference. It has been reported that there is a relationship between gene methylation and geography [28] and the Chinese lung cancer patients harboured higher frequency of gene hypermethylation in their tumor tissues, plasma and sputum [29] and bronchoaleolar lavage than Western cases [30]. Furthermore, in this study, there were no differences in promoter methylation frequencies for any of the genes in sputum between the nonsmokers and the smokers, by using either univariate (table 2) or logistic (table 3) analysis. This result is in disagreement with the significantly higher promoter methylation frequencies observed for p16 and RASSF1A genes in sputum samples from smokers, compared with nonsmokers [31]. Taken together, the higher promoter methylation frequencies found in sputum of the XWC individuals, compared with those found in sputum of the European and American smokers, and the similar promoter methylation frequencies between smokers, mostly men, and nonsmokers, mostly women, in the XWC population, may be due to the exposure of the XWC subjects to smoky coal combustion. These emissions contained 81% of organic matter, of which 43% were PAHs [32] and were previously associated with the detection of a higher level of benzo(a)pyrene-adducted guanine in urine from nonsmokers exposed smoky coal emissions, compared with urine from smokers not exposed to these emissions [33]. Furthermore, exposure to these emissions has been associated with high frequencies of p53 and K-ras mutations in lung tumors from lung cancer patients [17,18] and in sputum from individuals who showed no evidence of lung cancer [34] in XWC.

Finally, there were no differences in the frequencies of promoter methylation in any of the genes between the groups of individuals diagnosed with chronic bronchitis and the group of those without such a symptom, by using either univariate (table 2) or logistic (table 3) analysis. This result is in line with our previous study of these same sputum samples showing no differences in p53 mutation frequencies between these two groups of individuals [34]. These results suggests that promoter methylation of these genes, like p53 mutations, was associated primarily with exposure to smoky coal emissions.

Taken together, these results suggest that chemicals in the smoky coal emissions, particularly the high concentration of PAHs and other chemicals [35], may play a primary role in the formation of genetic and epigenetic alterations found in sputum from individuals without evidence of lung cancer in XWC.

Conclusion

In this study, we investigated gene promoter methylation in sputum of 107 individuals who had no evidence of lung cancer but were at high risk for developing lung cancer because of their history of long-term exposure to smoky coal emissions in XWC. We found that promoter methylation of these genes was relatively frequent in this population. This alteration was not associated with the smoking status, gender, or chronic bronchitis diagnosis of the subjects, indicating a dominant role of chemicals present in smoky coal emissions in the formation of promoter methylation in sputum of these individuals. Weaknesses of this study include the relatively small number of subjects and the lack of individuals not exposed to smoky coal emissions who might serve as a control group and that fact that these individuals were not followed up. Nevertheless, 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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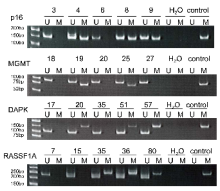

Figure 1.

Detection of p16, MGMT, RASSF1A and DAPK promoter methylation by MSP. M indicates the presence of methylated p16, MGMT, RASSF1A or DAPK. U indicates the presence of unmethylated p16, MGMT, RASSF1A or MGMT. All samples were performed twice and representative data are shown.

Table 1

Demographic and clinical information of individuals exposed to smoky coal emission in Xuan Wei, China (n=107)

Variables	Cases (%)
Sex	
Male	73 (68.2%)
Female	34 (31.8%)
Age (mean ± s.d.)	57.2 ± 10.8
Smoking status	
Smoking	72 (67.3%)
Non-smoking	35 (32.7%)
Bronchitis status	
Bronchitis	49 (45.8%)
Non-bronchitis	58 (54.2%)

Table 2

Promoter methylation of p16, MGMT, RASSF1A and DAPK according to gender, smoking and bronchitis status in Xuan Wei, China

	P16	MGMT	RASSF1A	DAPK
Gender				
Male (73)	49.3% (36/73)	17.8% (13/73)	31.5% (23/73)	18.1% (13/72)
Female (34)	55.9% (19/34)	17.6% (6/34)	26.5% (9/34)	11.8% (4/34)
Bronchitis status				
Bronchitis (49)	51.0% (25/49)	14.3% (7/49)	30.6% (15/49)	14.6% (7/48)
Non-bronchitis (58)	51.7% (30/58)	20.7% (12/58)	29.3% (17/58)	17.2% (10/58)
Smoking status				
Smoking M (72)	50.0% (36/72)	18.1% (13/72)	31.9% (23/72)	18.3% (13/71)
Nonsmoking (35)	54.3% (19/35)	17.1% (6/35)	25.7% (9/35)	11.4 % (4/35)

Table 3

Logistic Regression Models of p16, MGMT, RASSF1A and DAPK Promoter Methylation

	P16			MGMT			RASSF1A			DAPK		
	OR	**95% CI**	**P**	**OR**	**95% CI**	**P**	**OR**	**95% CI**	**P**	**OR**	**95% CI**	**P**
Sex	0.646	(0.133–3.135)	0.588	0.822	(0.093–7.243)	0.859	0.983	(0.170–5.688)	0.985	1.130	(0.085–14.999)	0.926
Age	0.999	(0.963–1.036)	0.960	1.031	(0.980–1.085)	0.234	1.012	(0.971–1.054)	0.583	1.072	(1.008–1.141)	0.027
Smoking	1.219	(0.255–5.835)	0.804	1.230	(0.140–10.812)	0.852	1.355	(0.236–7.790)	0.734	1.537	(0.115–20.454)	0.745
Bronchitis	0.954	(0.432–2.107)	0.908	0.540	(0.187–1.559)	0.255	0.999	(0.421–2.369)	0.998	0.588	(0.193–1.792)	0.350