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Aberrant Promoter Methylation of Multiple Genes in Bronchial Brush Samples from Former Cigarette Smokers[1]

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Abstract

Promoter hypermethylation plays an important role in the inactivation of tumor suppressor genes during tumorigenesis. Recent data suggest that such epigenetic abnormality may occur very early in lung carcinogenesis. To determine the extent of promoter hypermethylation in early lung tumorigenesis, we analyzed promoter methylation status of the *p16*, *death-associated protein kinase* (*DAPK*) and *glutathione S-transferase P1* (*GSTP1*) genes using methylation-specific PCR in bronchial brush samples obtained from 100 former smokers enrolled in a chemoprevention clinical trial. We found that 17% of the samples showed methylation for *p16* and 17% the same for *DAPK*, whereas only 6% of the samples displayed methylation for *GSTP1*. A total of 32% of the samples had methylation in at least one of the three genes tested, and 8% of the samples had methylation in two genes. The methylation status of *p16* was correlated with that of *DAPK* ($P = 0.04$, Fisher's exact test). *p16* methylation was higher in former smokers with a history of previous cancer than in former smokers without a history of cancer ($P = 0.04$, Fisher's exact test), and methylation of *DAPK* was detected more frequently in older patients than it was in younger patients ($P = 0.01$, Wilcoxon rank-sum test). Surprisingly, no correlation was found between methylation in any of these genes and the smoking characteristics of the individuals analyzed (packs per day, pack-years, smoking years, quitting years). The precise meaning of methylated genes in the bronchial brush samples of former smokers must be sought by means of careful follow-up of these individuals.

Introduction

Lung cancer is the most common cause of cancer death in Western countries, accounting for more deaths than those caused by prostate, breast, and colorectal cancer combined (1). The prognosis for patients with lung cancer is strongly correlated with the stage of the disease at the time of diagnosis. Whereas patients with clinical stage I disease have a 5-year survival rate of about 60%, the clinical stage II-IV disease 5-year survival rate ranges from 40% to less than 5% (2). This poor prognosis is attributable largely to lack of efficient diagnostic methods for early detection and the inability to cure metastatic disease. Thus, efforts aimed at early identification and interventions in lung cancer are of the highest importance. Currently, 50% of all new lung cancers are diagnosed in former smokers, individuals who have followed the only established advice for decreasing their risk of lung cancer (3). Former smokers are, thus, an ideal target population in whom to implement early diagnostic procedures and chemoprevention strategies.

Inactivation of tumor suppressor genes and of genes important in metabolizing carcinogens is essential for lung tumorigenesis. One of the major mechanisms to inactivate these genes is promoter hypermethylation (4). The use of aberrant gene methylation as a molecular marker system seems to offer a potentially powerful approach to population-based screening for the detection of lung cancer. In particular, hypermethylation of normally unmethylated CpG islands in the promoter regions of many tumor suppressor genes correlates with a loss of gene expression (4). Recently, the development of the sensitive MSP[3] has simplified the study of genes inactivated by promoter hypermethylation in human cancer (5). The MSP technique has multiple advantages: simplicity, specificity for each gene, high sensitivity, and rapidity, making it possible to study multiple markers.

Recent data have demonstrated that aberrant promoter methylation of the *p16* tumor suppressor gene, which plays a key role in cell cycle regulation, is an early and frequent event in NSCLC (6). Methylation of the apoptosis-associated gene *DAPK* has been recently described in stage I NSCLC, in which it portends a poor prognosis (7). GSTP1 is a multifunctional enzyme associated with cellular detoxification of hydrophobic electrophilic compounds including carcinogens and exogenous drugs. Inactivation of the *GSTP1* gene by promoter hypermethylation has been reported in human neoplasia including prostate, breast, renal, and lung tumors (8). Thus, the *p16*, *DAPK*, and *GSTP1* genes are strong candidate biomarkers for early detection of lung cancer. In this study, we examined the frequency of CpG-island methylation status of these three genes in 100 consecutive prospectively collected bronchial brush samples from former smokers.

Materials and Methods

Study Population. One hundred former smokers enrolled in an ongoing chemoprevention trial performed at M. D. Anderson Cancer Center were included in this study. All of the 100 former smokers had at least a 20-pack-year history of smoking and had quit for at least 12 months before study entry. All of the subjects were clinically free of any cancer at enrollment. However, patients with a prior history of cancer (head and neck squamous cell carcinoma, esophageal cancer, NSCLC, or transitional cell carcinoma of the bladder of stages I, II, or III) were included if they were free of disease for $\geq$6 months after treatment of their initial cancer. Prior history of other cancer was also allowed as long as the patients were free of cancer for $>$3 years. In the 100 former smokers studied here, 9 had prior cancers including 3 with lung cancer, 3 with skin cancer, 1 with breast cancer, 1 with ovarian cancer, and 1 with cervical cancer. All of the patients underwent a bronchoscopy at baseline, and bronchial brushes were performed at the carina during the procedure.

Bronchial Brush Processing and DNA Extraction. On bronchoscopy, brushes were placed in 3 ml of plain DMEM culture (Life Technologies, Inc., Gaithersburg, MD) in sterile tissue culture tubes and stored at 4°C for processing the same day. The brushes were vortexed lightly to detach attached

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[3] The abbreviations used are: MSP, methylation-specific PCR; NSCLC, non-small cell lung cancer; DAPK, death-associated protein kinase; GSTP1, glutathione *S*-transferase P1; hTERT, human telomerase reverse transcriptase.

Table 1 *PCR primers sequences, annealing temperatures, and PCR product sizes used for MSP*

Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Annealing temperature (°C)	Size (bp)
p16 = M[a]	TTATTAGAGGGTGGGGCGGATCGCGTGC	ACCCGACCCCGAACCGCGACCGTAA	64	150
p16 = U	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	60	151
DAPK = M	GGATAGTCGGATCGAGTTAACGTC	CCCTCCCAAACGCCGA	56	98
DAPK = U	GGAGGATAGTTGGATTGAGTTAATGTT	CAAATCCCTCCCAAACACCAA	61	108
GSTP1 = M	TTCGGGGTGTAGCGCTCGTC	GCCCCAATACTAAATCACGACG	58	91
GSTP1 = U	GATGTTTGGGGTGTAGTGGTTGT	CCACCCCAATACTAAATCACAACA	59	97

[a] M, methylated-specific primers; U, unmethylated-specific primers.

material. After removal of the brush from the tube, the cell suspension was centrifuged at 2,500 rpm for 5 min. Cell pellets were then washed with 2 ml of 1× PBS twice, and an aliquot of material was saved for DNA extraction. Cell pellets were resuspended in TE9 and digested with 10× proteinase K-SDS solution [5 mg/ml proteinase K (Roche Molecular Biochemicals, Indianapolis, IN), 10% SDS (Life Technologies, Inc.)] at 42°C overnight. DNA was extracted twice with PC9 (a 1:1 ratio of phenol:chloroform), and then precipitated with three volumes of 100% ethanol, a one-third volume of 10 M NH_4OAc, and 2 μl of glycogen (Roche Molecular Biochemicals) at −20°C. Precipitated DNA was pelleted at 14,000 rpm for 20 min and then washed with 500 μl of 70% ethanol. DNA was air-dried and dissolved in 20 μl of distilled water.

MSP. At least 100 ng of sample DNA, mixed with 1 μg of salmon sperm (Life Technologies, Inc.), were submitted to chemical modification following the protocol of Herman *et al.* (5). Briefly, DNA was denatured with 2 M of NaOH, followed by treatment with 10 mM of hydroquinone and 3 M of sodium bisulfite (Sigma Chemical Co., St. Louis, MO). After purification in a Wizard SV Plus kit column (Promega, Madison, WI), the DNA was treated with 3 M of NaOH and precipitated with three volumes of 100% ethanol, a one-third volume of 10 M NH_4OAc, and 2 μl of glycogen (Roche Molecular Biochemicals) at −20°C. The precipitated DNA was washed with 70% ethanol and dissolved in distilled water. PCR was conducted with primers specific for either the methylated or the unmethylated versions of the *p16*, *DAPK*, or *GSTP1* promoter regions (see Table 1). The 12.5-μl total reaction volume contained 25 ng of modified DNA, 3% DMSO, all four deoxynucleoside triphosphates (each at 200 μM), 1.5 mM of $MgCl_2$, 0.4 μM of PCR primers, and 0.625 units of HotStar Taq DNA polymerase (Qiagen, Valencia, CA). Water was substituted for DNA as a negative control, and NCI-H460 cell line [American Type Culture Collection (Manassas, VA)] DNA treated with SssI Methylase (New England Biolabs, Beverly, MA) was used as a positive control. DNA was amplified by an initial cycle at 95°C for 15 min as required for enzyme activation followed by 40 cycles of 95°C for 30 s, annealing for 45 s, and 72°C for 1 min, and ending with a 5-min extension at 72°C in a thermocycler (Applied Biosystems, Foster City, CA). PCR products were separated on 2% agarose gels and visualized after staining with ethidium bromide.

Statistical Analysis. Statistical analysis was performed using the χ^2 test or Fisher's exact test for correlation among genes and for correlation between gene status and gender, race, and previous cancer; the Wilcoxon rank-sum test was used for differences in median of age, packs per day, smoking years, pack-years, and quitting years between groups. All of the tests were two-sided. $P < 0.05$ was considered statistically significant.

Results

Frequency of Methylation in Bronchial Brush Samples from Former Smokers. We determined the frequency of methylation of *p16*, *DAPK*, and *GSTP1* in 100 consecutive bronchial brush samples obtained from people who had been heavy smokers (Fig. 1). The unmethylated form of each of the genes was detected in 100% of the samples. We found that 17% of the bronchial brush samples were methylated for *p16* and 17% likewise for *DAPK*, whereas only 6% of the samples displayed methylation for *GSTP1*. A total of 32% of the samples had methylation in at least one of the three genes tested, and 8% of the samples had methylation in two genes. No sample had methylation in all of the three genes. These results are summarized in Fig. 2. A statistically significant correlation was found for the methylation status between *p16* and *DAPK* ($P = 0.04$, Fisher's exact test), whereas the other genes were independent of each other when their methylation status was compared.

Clinicopathological Correlations. We analyzed the methylation changes in the bronchial brush samples and the clinical data obtained from the former smokers (see Table 2). Overall, we found that methylation of *DAPK* was detected more frequently in older patients than it was in younger patients (the median age was 63 years in the *DAPK* methylated group *versus* 55 years in the unmethylated group; $P = 0.01$, Wilcoxon rank-sum test). *p16* methylation was higher in former smokers with a history of previous cancer (4 of 9) than it was in former smokers without a cancer history (13 of 91; $P = 0.04$, Fisher's exact test); this trend was also observed for *DAPK* but did not reach statistical significance ($P = 0.18$, Fisher's exact test). Finally, *DAPK* methylation tended to be more frequently observed in nonwhite former smokers (4 of 11) than in white former smokers (13 of

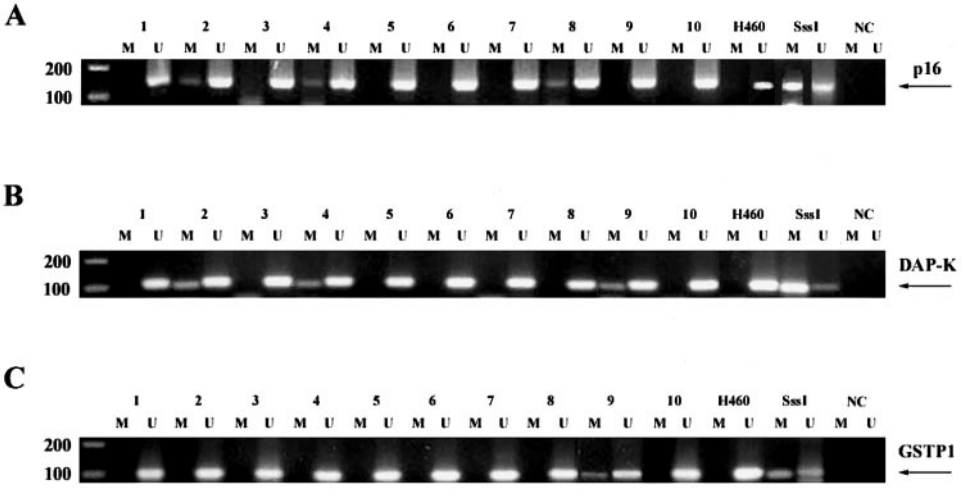

Fig. 1. Methylation analysis of three genes in bronchial brush samples from former cigarette smokers. *At the right of each panel*, the gene studied. *Lanes 1–10*, samples from different former smokers. *H460*, NSCLC cell line NCI-H460. *SssI*, NCI-H460 DNA treated by SssI methylase (positive control). *NC*, negative control; *Lanes M*, amplified product with primers recognizing methylated sequence; *Lanes U*, amplified product with primers recognizing unmethylated sequence.

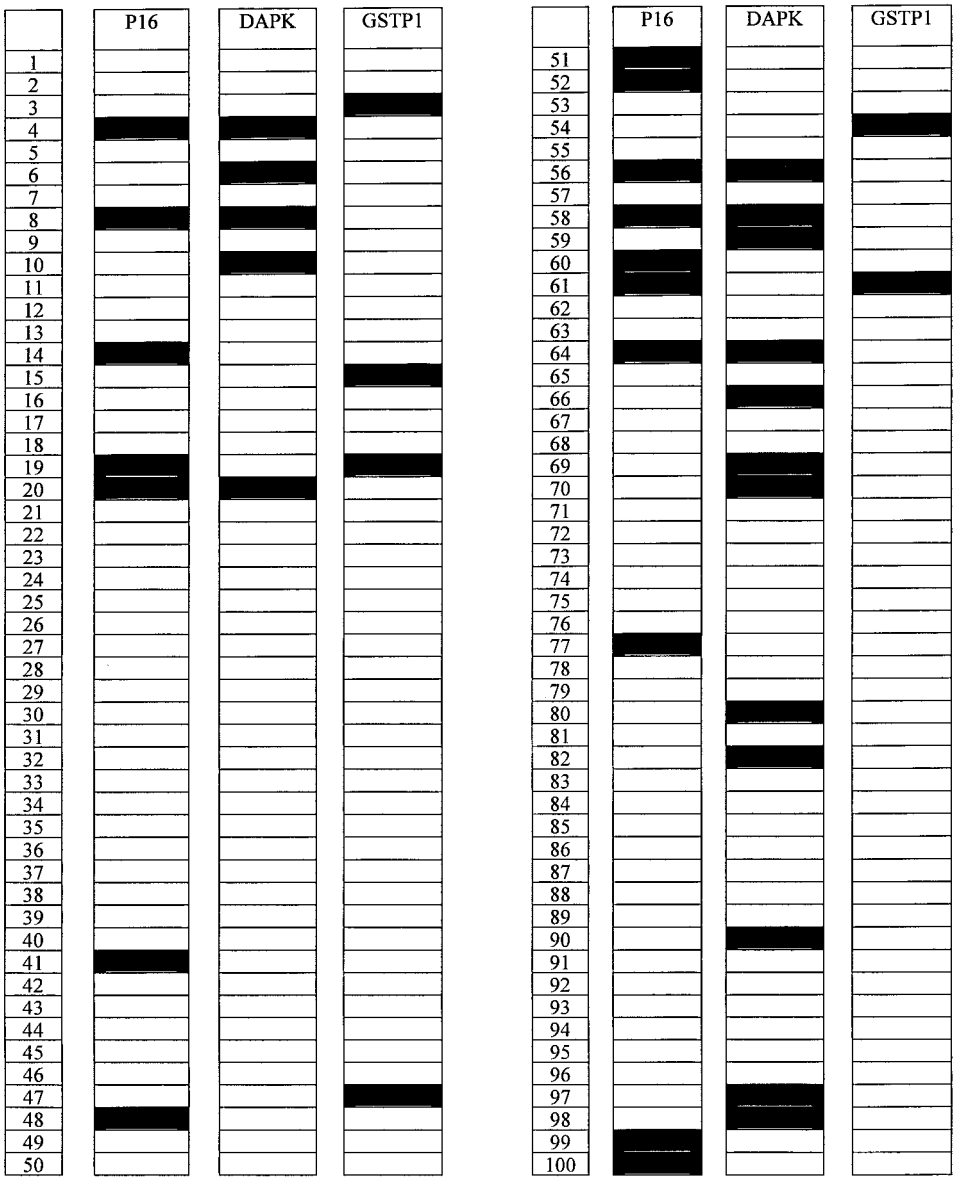

Fig. 2. Summary of methylation of *p16*, *DAPK*, and *GSTP1* in 100 consecutive bronchial brush samples obtained from former heavy cigarette smokers. □, samples that are not methylated; ■, samples that are methylated.

89; $P = 0.09$, Fisher's exact test). No relationship was found between gender and the methylation status of any of the three genes. We analyzed the methylation status of each of these genes with regard to the smoking characteristics of the patients: packs per day, pack-years, smoking years, and quitting years. Surprisingly, there was no statistically significant difference in any of these variables when comparing methylated groups with unmethylated groups for *p16*, *DAPK*, or *GSTP1*. Because only three cases had a prior history of cancer in the upper aerodigestive tract, the association between the prior cancer history and aberrant methylation pattern was not analyzed.

Discussion

The goal of the present investigation was to evaluate the presence of aberrant promoter methylation in bronchial brush samples from former smokers. In this study, we demonstrated that methylation of three different genes (related to three different pathways: cell cycle, apoptosis, and detoxification) can be detected in exfoliated cells from the bronchial trees of former smokers. Our results demonstrate a significant frequency of abnormal methylation (32% overall) in people with a past smoking history of greater than 20 pack-years, who are considered to be at high risk of developing lung cancer. There are few previous reports that have tried to address the frequency of biomarker abnormalities in chronic current or former smokers. By studying *p16* promoter hypermethylation, *p53* mutation, and K-*ras* mutation in exfoliate material (sputum, bronchial lavage, and brushings) of 25 symptomatic current chronic smokers, Kertsing *et al.* reported an overall 32% frequency of genetic alterations (9). This is in line with our results. However, Kersting *et al.* reported a 28% rate (higher than ours) of *p16* hypermethylation in the 25 symptomatic chronic smokers analyzed. Palmisano *et al.* have recently reported that *p16* methylation could be detected in the sputum of 6 (18.5%) of 32 cancer-free high-risk category smokers (10). Finally Belinsky *et al.* have reported *p16* methylation in 5 (19.2%) of 26 cancer-free smokers (6). These percentages are similar to our 17% positivity rate. To our knowledge, no previous reports have evaluated the frequency of *DAPK* or *GSTP1* methylation in cancer-free smokers. In contrast, many reports have evaluated the frequency of aberrant promoter methylation of *DAPK*, *GSTP1*, and *p16* in invasive NSCLC lesions or their exfoliate material. Hypermethylation of *DAPK* has been reported in 18–44% of invasive NSCLCs (7, 11–13), whereas hypermethylation of *GSTP1*

Table 2 *Demographic and smoking data compared with methylation status of the different genes*

		p16		*DAPK*		*GSTP1*	
	All subjects	M[a]	U	M	U	M	U
Subjects, *n*	100	17	83	17	83	6	94
Age (yr)							
Median	56	58	56	63	55	55	56
Range	35–78	45–70	35–78	48–71	35–78	40–60	35–78
Mean ± SD	56.6 ± 9.6	58.6 ± 7	56.2 ± 10	61.5 ± 6.9	55.6 ± 9.8	53 ± 7.7	56.8 ± 9.7
Gender							
Male	54	9	45	8	46	2	52
Female	46	8	38	9	37	4	42
Race							
White	89	16	73	13	76	6	0
Others	11	1	10	4	7	83	11
Cancer history							
Yes	9	4	5	3	6	0	9
No	91	13	78	14	77	6	85
Smoking status, median (range)							
Packs per day	2 (0.8–4)	1.5 (1–2.5)	2 (0.8–4)	1.5 (0.8–3)	2 (1–4)	1 (1–2.5)	2 (0.8–4)
Smoking years	26.5 (11–50)	26 (15–49)	27 (11–50)	32 (15–49)	26 (11–50)	26 (20–38)	26.5 (11–50)
Pack-years	42 (20–136)	35 (20–95)	42 (20–136)	42 (22–120)	42 (20–136)	29 (20–95)	42 (20–136)
Quitting years	10.2 (1–48.1)	10 (1.2–35.2)	10.2 (1–48)	9.7 (1.5–35.2)	10.2 (1–48.1)	2.6 (1.1–23.2)	10.2 (0.9–48.1)

[a] M, methylated-specific primers; U, unmethylated-specific primers.

has been found in 7–9% of NSCLCs (8, 11, 12). Methylation of *p16* has been described in 25–58% of NSCLCs, most often in tumors with squamous cell histology (up to 80%; Refs. 9–15). The molecular detection of *p16* methylation has also been reported in exfoliated material (bronchoalveolar lavage, bronchial brush, or sputum) of patients with invasive NSCLC, at rates of between 24 and 61%, again with the highest frequency for the squamous cell subtype (6, 9, 10, 15). Interestingly, data from Palmisano *et al.* and Kersting *et al.* strongly suggest that sputum is a good alternative to bronchial brush samples, obtaining sputum samples does not require an invasive bronchoscopy, and sputum seems to offer a level of detection comparable with other sample types (9, 10).

Another aim of this study was to investigate whether methylation of *p16*, *DAPK*, and *GSTP1* in former smokers is associated with clinicopathological parameters, particularly smoking data. *DAPK* methylation was more frequent in older smokers. Such finding is in line with the concept of age-related methylation, which affects normal cells initially and is magnified in neoplastic cells (16). Nevertheless, it is important to point out that Zochbauer-Muller *et al.* did not find this age difference in patients with stage I-III NSCLC who analyzed for *DAPK* methylation (12). The methylation status of *p16* and *DAPK* were statistically correlated. This is consistent with previous reports that suggested a possible pathway of global hypermethylation in cancer, which is characterized by the simultaneous methylation of multiple CpG islands, including several known genes, such as *p16*, *hMLH1*, and *THBS1* (17). Interestingly, in our study, *p16* methylation occurred more frequently in smokers with a history of previous cancer than it did in smokers without a cancer history ($P = 0.04$); this trend was also observed for *DAPK* but did not reach statistical significance. This finding can be considered in line with the concept of field cancerization. Surprisingly, we found no correlation between methylation at any of these genes and the smoking characteristics of the individuals analyzed. A previous report suggested that methylation of *p16* was associated with pack-years smoked, duration of smoking, and, negatively, with the time since quitting smoking in patients with primary NSCLC (18). Therefore, all of the clinical correlations here reported or negated need to be confirmed in other independent studies. Indeed, when making multiple comparisons of clinical data with multiple biomarkers like the three methylated genes, as was done in this study, caution must be used with conservative statistical corrections (such as the Bonferroni, Turkey, and Newman-Kauls post tests) before deciding that significant correlations exist.

The exact risk of developing lung cancer for individuals with methylated genes in the bronchial brush samples is unclear. Longitudinal studies are necessary to reveal potential clinical values of testing such aberrant methylation in smoking damaged lungs. Nevertheless, hypermethylation-associated inactivation of *p16* is an early and frequent event in NSCLC (5, 6); and in experimental models, such loss appears to act as a "gatekeeper" in permitting cells to pass through the early steps of cellular immortalization (19). Thus, the presence of *p16* methylation in the bronchial brush samples of former smokers may indicate an increased risk of lung cancer developing. This conclusion is supported by our finding of *p16* and *DAPK* methylation in the cancer-free high-risk population at a prevalence that approximates lifetime risk for lung cancer in smokers (20). Furthermore, a recent report has shown that it is possible to predict lung cancer by detecting aberrant promoter methylation of *p16* in the sputum of individuals at high-risk (10). Of potential importance, in this report, is the finding that concomitant methylation of two genes (most frequently *p16* and *DAPK*) was observed in 8% of the patients tested. Patients with such a methylation pattern might be even at increased risk for lung cancer than patients with only one methylated gene, if a dose-effect should be considered. Therefore, we suggest that these biomarkers may also aid the sensitivity and accuracy of other diagnostic and screening procedures, particularly imaging techniques. The use of low-dose spiral computed tomography for the screening of occult lung cancer in high-risk populations has been evaluated recently. This procedure can detect very small nodules that are invisible in chest X-rays films. However, in terms of the actual presence of lung cancer, many false-positive results are the rule, and actual cancer detection rates using spiral computed tomography are below 1% (21). This diagnostic imaging approach might be enhanced by combining it with the determination of hypermethylation of specific genes in exfoliated cells (sputum, bronchial brush, and bronchoalveolar lavage).

Although the detection rate of methylation for any of the markers investigated in this study was somewhat low, with frequencies ranging between 6 and 17%, combining the three markers increased the detection rate of aberrant methylation to 32%. There is increasing evidence that clonal evolution of tumors from premalignant lesions is a complex process that involves multiple molecular abnormalities, which do not necessarily have a linear progression. Thus, effective detection of preneoplasia or identification of individuals at high-risk is likely to mandate the use of a panel of molecular markers, spanning the various genetic alterations that might be present, rather than being

feasible with single markers alone. In that regard, we have recently demonstrated that expression of hTERT is a very common phenomenon in the bronchial epithelium of smokers (22). Thus, combining MSP for specific biomarkers along with other genetic alterations (loss of heterozygosity, hTERT expression, and so forth) might be the way to progress in the identification of specific biological subgroups among smokers with higher risks of lung cancer.

Finally, our results emphasize the need to closely monitor the changes in methylation profiles in former smokers who are enrolled in chemopreventive approaches. Indeed, such biomarkers may serve as surrogate end points to judge the efficacy of the chemopreventive agents used in such settings.

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