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Predicting Lung Cancer by Detecting Aberrant Promoter Methylation in Sputum[1]

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Abstract

Despite the promise of using DNA markers for the early detection of cancer, none has proven universally applicable to the most common and lethal forms of human malignancy. Lung carcinoma, the leading cause of tumor-related death, is a key example of a cancer for which mortality could be greatly reduced through the development of sensitive molecular markers detectable at the earliest stages of disease. By increasing the sensitivity of a PCR approach to detect methylated DNA sequences, we now demonstrate that aberrant methylation of the *p16* and/or *O*^{6}*-methylguanine-DNA methyltransferase* promoters can be detected in DNA from sputum in 100% of 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. 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, and possibly the other common forms of human cancer.

Introduction

The goal of the present investigation was to assess the potential of a molecular-based marker approach for the early detection of human lung cancer. Lung cancer is the leading cause of cancer-related death in the United States and is projected to reach epidemic levels in the world during the 21st century (1). Mortality from this disease could be reduced greatly through the development of molecular markers that identify individuals at the earliest stages of lung cancer in which curative resection is feasible. Candidate biomarkers should have high sensitivity and specificity and appear early enough in the course of disease for medical intervention to improve prognosis. Finally, the markers must be present in a biological fluid that can be obtained noninvasively, making its collection feasible for population-based screening.

Because current and even former cigarette smokers have increased bronchial secretions that contain exfoliated cells from the bronchial tree, the analysis of sputum from these individuals has been an active area of research for marker development (2). The late Dr. Geno Saccomanno, pioneer of sputum cytology, demonstrated that premalignant cytological changes could be detected several years before a clinical diagnosis of lung cancer in high-risk subjects (3). Unfortunately, these studies were difficult to replicate, most likely because of the skills required for identifying subtle nuclear changes in cells that often comprise <5% of the sputum slide. Subsequent studies suggested that molecular assays could be used to enhance the predictive value of sputum samples. Mutations within the *K-ras* gene have been detected in sputum specimens collected before tumor resection (4); identical microsatellite alterations have been detected in primary tumors and corresponding sputum samples (4, 5). However, methods to detect both alterations lack sensitivity, and the overall prevalence of these changes in NSCLC[3] is <25%.

We have pursued another approach for developing DNA-based assays to aid in the early diagnosis of lung and other cancers. Our approach involves the detection of gene promoter regions that are aberrantly hypermethylated in human tumors. This change is associated with an epigenetically mediated gene silencing that constitutes an alternative to coding region mutations for loss of tumor suppressor gene function in cancer (6). Recent studies (7) in our laboratory demonstrated that aberrant promoter methylation of the *p16* tumor suppressor gene, which plays a key role in cell cycle regulation, is an early and very frequent event in SCC of the lung. Another gene frequently inactivated by aberrant promoter methylation in NSCLC (8) is *MGMT*. *MGMT* is a DNA repair enzyme that protects cells from the carcinogenic effects of alkylating agents by removing adducts from the *O*^{6} position of guanine. Thus, the *p16* and *MGMT* genes are strong candidate biomarkers for early detection of lung cancer.

Our recent observations that aberrant methylation of the *p16* promoter region can be detected in DNA from exfoliated cells in sputum of patients with this disease further support this approach in the early diagnosis of cancer. In that study, aberrant methylation of the *p16* gene promoter was detected in sputum from 3 of 7 patients with lung cancer, and 5 of 26 cancer-free individuals at high-risk (7). We have modified the sensitive MSP assay (9) used initially, to achieve an even higher detection efficiency. This new modification has now been used not only to extend analyses of the *p16* promoter but also to study the methylation status of a similar region of *MGMT*. Aberrant methylation of one of these two promoter regions was detected in the sputum of 100% of patients with proven SCC, not only at the time of diagnosis, but also in all sputum samples taken from patients 5–35 months before clinical tumor detection. Furthermore, these sputum markers were detected in a subset of cancer-free individuals with very high risk for developing lung tumors. Our results suggest that detection of aberrant promoter region methylation constitutes a promising approach for using DNA-based markers for the early detection of lung and other common human cancers.

Materials and Methods

Human Tissue Samples. Sputum samples and matched SCCs were obtained from 21 people previously enrolled in a Lung Cancer Surveillance Study conducted through St Mary's Hospital, Grand Junction, CO. The SCCs were obtained through biopsy or surgical resection. Sputum was collected by standardized procedures (3) at Johns Hopkins Medical Institutions from 32 patients being evaluated for possible lung cancer through referral from their primary

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[3] The abbreviations used are: NSCLC, non-small cell lung cancer; MSP, methylation-specific PCR; SCC, squamous cell carcinoma; *p16*, *p16*^{INK4a}; *MGMT*, *O*^{6}*-methylguanine-DNA methyltransferase.*

care physician and from 91 cancer-free, former uranium miners from Grants, New Mexico, participating in a cancer surveillance study. This study was approved by the respective Institutional Review Boards; all of the participants gave written informed consent. Sputum was considered unsatisfactory for evaluation if alveolar lung macrophages were absent or if a marked inflammatory component was present that diluted the concentration of pulmonary epithelial cells.

Microdissection of Tumors and DNA Isolation. SCC tumors were obtained as frozen or formalin-fixed specimens. Frozen tumors were not microdissected. Sequential sections (5 μm) were prepared from tumors or biopsies, deparafinized, and stained with toluidine blue to facilitate dissection. A 25-gauge needle attached to a tuberculin syringe was used to remove the lesions under a dissecting microscope. Because some SCCs are extensively contaminated with normal tissue or very small in size (the situation for the biopsy), it was essential to include normal-appearing cells to ensure that, after bisulfite modification and column clean-up of the DNA template, enough sample remained to conduct the MSP assay as described below. Thus, because the goal of the study was to determine whether *p16* methylation was present and not to quantitate methylation levels, microdissection was used to enrich the samples examined. DNA was isolated from frozen tumors, microdissected tumors, and sputum by digestion with Pronase in SDS (1%), followed by standard phenol-chloroform extraction and ethanol precipitation.

MSP. The methylation status of the *p16* and *MGMT* genes was determined by the method of MSP (9, 10) with the following modifications. We developed a nested, two-stage PCR approach, which improved the sensitivity to detect methylated alleles by >50-fold over the original method (one methylated allele in >50,000 unmethylated alleles). DNAs were subjected to bisulfite modification, and PCR was performed to amplify a 280-bp and 289-bp fragment of the *p16* and *MGMT* genes, respectively, including a portion of their CpG-rich promoter region. The primers recognize the bisulfite-modified template but do not discriminate between methylated and unmethylated alleles. The stage-1 PCR products were diluted 50-fold, and 5 μl was subjected to a stage-2 PCR in which primers specific to methylated or unmethylated template were used. Primer sequences used in the stage 1 amplification of the *p16* and *MGMT* genes are as follows: *p16*-Forward, 5′-GAAGAAAGAGGAGGGGTTGG-3′; *p16*-Reverse, 5′-CTACAAACCCTCTAC CCACC-3′; *MGMT*-Forward, 5′-GGATATGTTG GGATAGTT-3′; and *MGMT*-Reverse, 5′-CCAAAAAC-CCCAAACCC-3′. Taq Gold polymerase (Perkin-Elmer) in a 50-μl volume was used in all of the PCRs. The PCR amplification protocol for stage 1 was as follows: 95°C for 10 min, then denature at 95°C for 30 s, anneal at 60°C (*p16*) or 52°C (*MGMT*) for 30 s, extension at 72°C for 30 s for 40 cycles followed by a 10-min final extension. Primers used to selectively amplify unmethylated or methylated alleles of the *p16* and *MGMT* genes in the stage 2 PCR have been described previously (8, 9). Annealing temperatures were increased to 70°C (*p16*) and 62°C (*MGMT*), and all of the cycling times were reduced to 15 s for a total of 40 cycles. Normal human tissue collected from autopsy of never smokers and cell lines positive for *p16* (Calu6) and *MGMT* (SkuLu1) methylation served as negative and positive controls. All of the assays were conducted in at least duplicate. Sensitivity for detecting methylated alleles was determined by mixing DNA isolated from either Calu6 or SkuLu1 cells with DNA isolated from lung tissue of a never smoker to achieve dilutions up to 1 in 100,000. The mixed DNA sample was then subjected to bisulfite modification and subsequent analysis by the two-stage MSP approach.

Sputum samples that gave positive methylation products were also analyzed by a second method using restriction enzyme digestion of the resulting PCR product. Second-stage PCRs were performed in duplicate for each sample. Then one of each sample pair was incubated with the restriction enzyme *FNU*4HI that cuts (G/CGGCG**)** at two sites within the amplified region of the methylated *p16* gene promoter. Thus, this restriction enzyme will only cut template that is methylated at the two CpG sites because the unmethylated cytosines would be modified by the bisulfite treatment to uracils. Because *FNU*4HI cuts at two different sites within the promoter, we confirmed that four CpGs were methylated, which verified the methylation status of the sample. An identical approach was used for the *MGMT* gene using the *Taq*I and *BstU*1 restriction enzymes to assay three different CpG sites. All of the sputum samples that scored as positive for methylation were confirmed by both procedures.

Data Analysis. Statistical comparisons were done by Fisher's exact test.

Results

Aberrant Methylation of *p16* and/or *MGMT* Is Detected in the Sputum of All Tested Lung Cancer Patients at the Time of Diagnosis. Sputum samples and matched SCCs were obtained from 21 people previously enrolled in a Lung Cancer Surveillance Study conducted through St Mary's Hospital, Grand Junction, CO. All of the subjects had a history of smoking, and approximately 50% were exposed to radon through uranium mining at the Colorado Plateau. At sufficiently high concentrations, radon (^{222}Ra) and the associated α-particle-emitting decay products polonium-214 and -218 cause an excess of lung cancers in smokers who mined uranium (11). Sputum was collected from 10 of the above 21 individuals at the time of diagnosis of SCC; however, only four samples were diagnostic of cancer by cytological criteria (Table 1). In marked contrast to cytology findings, one or both of the gene promoters tested were abnormally methylated in all of these sputum samples. Abnormal *p16* gene methylation was present in sputum from all eight patients whose tumors were also positive for this marker, but not in sputum from the two individuals whose tumors were negative for this change (Table 1). Four of the six patients with abnormal methylation of *MGMT* in their tumors also had this change detected in their sputum, including the two patients whose tumors lacked the *p16* change (Table 1). Methylation of *p16* was present in sputum of the two individuals whose sputum was negative for *MGMT* (Table 1, cases 4 and 5). Aberrant *MGMT* methylation was not detected in sputum or tumor from three cases. In case 8 (Table 1), *MGMT* methylation was detected in the sputum, but not in the tumor.

Detection of *p16* and *MGMT* Promoter Methylation Precedes Clinical Cancer. For the other 11 of 21 original individuals with SCC, sputum samples were obtained well before the diagnosis of SCC at times ranging from 5 to 35 months (Table 2). In only one patient (Table 2, case 3) was the sputum sample thought to have unequivocal signs of cancer by cytological criteria. However, abnormal methylation of the *p16* promoter region was detected in DNA from sputum of all 11 subjects, with the longest time to tumor diagnosis being 35 months. In those cases in which multiple sputum samples were available either as replicate specimens (case 2) or as temporal samples

Table 1 *Methylation[a] of the p16 and MGMT genes in tumor/sputum pairs at time of diagnosis*

Case	Exposure	Sample	Cytology	Methylation	
				p16	*MGMT*
1	Tob[b]/Radon	SCC	NA	M	U
		Sputum	Mild Atypia	M	U
2	Tob/Radon	SCC	NA	U	M
		Sputum	SCC	U	M
3	Tob/Radon	SCC	NA	U	M
		Sputum	SCC	U	M
4	Tob	SCC	NA	M	M
		Sputum	SCC	M	U
5	Tob	SCC	NA	M	M
		Sputum	SCC	M	U
6	Tob/Radon	SCC	NA	M	U
		Sputum	Mod. Dyspl.	M	U
7	Tob	SCC	NA	M	M
		Sputum	Marked Dyspl.	M	M
8	Tob	SCC	NA	M	U
		Sputum	Mod. Dyspl.	M	M
9	Tob	SCC	NA	M	U
		Sputum	Mod. Dyspl.	M	U
10	Tob/Radon	SCC	NA	M	M
		Sputum	Marked Dyspl.	M	M

[a] Methylation state of the *p16* and *MGMT* genes in tumors and sputa obtained at the time of diagnosis.

[b] Tob, tobacco; NA, not applicable; M, methylated; U, unmethylated; Mod. Dyspl., moderate dysplasia.

Table 2 *Methylation[a] of the p16 and MGMT genes in sputum precedes clinical diagnosis of SCC*

Case	Exposure	Sample	Mon prior to cancer	Cytology	Methylation	
					p16	*MGMT*
1	Tob[b]/Radon	SCC	0	NA	U	M
		Sputum	5	Mod. Dysp.	M	M
2	Tob/Radon	SCC	0	NA	M	M
		Sputum	5	Suspicious	M	M
		Sputum	5	Suspicious	M	M
		Sputum	5	Marked Dysp.	M	M
3	Tob	SCC	0	NA	M	M
		Sputum	6	SCC	M	M
4	Tob/Radon	SCC	0	NA	M	U
		Sputum	10	Mod. Dysp.	M	U
5	Tob/Radon	SCC	0	NA	M	M
		Sputum	8	Suspicious	M	M
		Sputum	13	Suspicious	M	M
6	Tob/Radon	SCC	0	NA	M	M
		Sputum	2	SCC	M	M
		Sputum	9	Mod. Dysp.	M	M
		Sputum	15	Mild Atypia	M	U
7	Tob/Radon	SCC	0	NA	M	M
		Sputum	5	Mod. Dysp.	M	M
		Sputum	15	Mod. Dysp.	M	M
8	Tob/Radon	SCC	0	NA	M	M
		Sputum	8	Suspicious	M	M
		Sputum	20	Suspicious	M	M
9	Tob/Radon	SCC	0	NA	M	U
		Sputum	0	SCC	M	U
		Sputum	20	CIS	M	U
10	Tob	SCC	0	NA	M	M
		Sputum	34	Severe Dysp.	M	U
11	Tob	SCC	0	NA	M	M
		Sputum	35	Severe Dysp.	M	U

[a] Methylation state of the *p16* and *MGMT* genes in tumors and sputa obtained from 11 different cases.

[b] Tob, tobacco; NA, not applicable; U, unmethylated; M, methylated; Mod. Dysp., moderate dysplasia; Suspicious, suspicious for malignancy; CIS, carcinoma in situ.

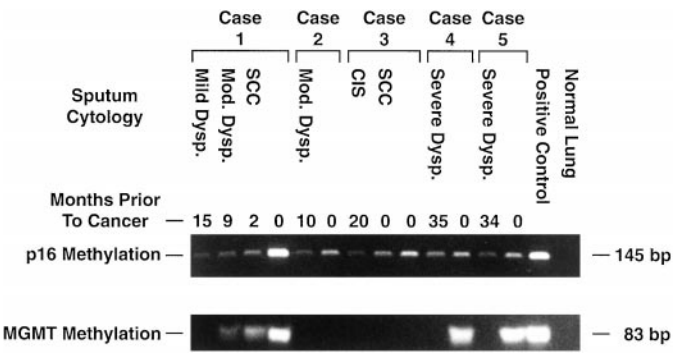

Fig. 1. *p16* and *MGMT* methylation in sputum are biomarkers for SCC. Results of the stage-2 PCR using methylation-specific primers for the *p16* and *MGMT* gene. Sputum (samples collected months before cancer) and primary SCC (time zero) are shown for five cases. The presence of a 145-bp and 83-bp PCR product indicates that the sputum or tumor was positive for *p16* and *MGMT* methylation, respectively. The CaLu6 and SKuLu1 cell lines are the positive controls for *p16* and *MGMT* methylation, respectively, and normal lung served as a negative control. Sputum cytology varied by case and is indicated for each specimen.

(*e. g.*, case 6), methylation of *p16* was always present (Table 2). A 90% concordance was also observed between *p16* methylation in the primary SCC and paired sputum samples (Table 2; Fig. 1). Sputum from 7 of 11 cases (Table 1; Fig. 1) also showed methylation of the *MGMT* gene. A 78% concordance was noted between *MGMT* methylation in the primary SCC and the paired sputum sample. For the two discordant samples, *MGMT* methylation was detected in the tumor but not in the single sputum sample obtained 34 or 35 months before cancer (Table 2).

Methylation of *p16* and *MGMT* in Cancer-free, High-Risk Subjects. The excellent concordance between detecting *p16* and/or *MGMT* methylation in sputum samples before and at the time of SCC diagnosis warranted defining whether these two markers are present in any sputum samples from cancer-free subjects who are at high risk for lung cancer development. This information could be invaluable for the ultimate development of risk estimates for lung cancer. Sputum samples were collected from 123 cancer-free subjects. People were divided into three groups based on exposure to: (*a*) tobacco; (*b*) tobacco + radon; and (*c*) radon alone. Approximately 50% of these people were considered heavy smokers with >30-pack-year history. Radon exposure ranged from 3 to 577 working-level months; 75% of the people had >100 working-level months. These former uranium miners worked in Grants, New Mexico, where exposures were much lower than in Colorado. Thus, there is some additional risk for lung cancer in these cancer-free people, although not nearly of the magnitude initially reported for workers in Colorado (11). Sputum cytology was not diagnostic for cancer in any of these individuals and ranged from normal to marked dysplasia in this population. The frequency for detecting aberrant methylation of the *p16* or *MGMT* genes was similar across all of the groups, occurring in 12–19% and 16–36%, respectively (Fig. 2). Smoking status was available for a subset ($n = 35$) of the uranium miners, and the methylation markers were present in sputum from both current and former smokers, which is consistent with the continued risk for lung cancer in former smokers. Bisulfite sequencing of sputum samples ($n = 5$ for each gene) positive for *p16* or *MGMT* revealed methylation at all of the CpG sites between the PCR primers (not shown). Direct comparison of sputum from current or former smokers to never smokers is technically difficult because never smokers produce very little sputum, and composition of the sample also differs from that of a current or former smoker. If a low background for methylation of this gene existed in normal lung tissue, it should have been detected by our improved MSP assay in the majority of the sputum samples, and this was not the case. Furthermore, we have examined DNA isolated from the lungs of autopsy cases, bronchial epithelial cells obtained by bronchoscopy, and lymphocytes (a component of the sputum) from never smokers, and all have proven negative for the methylation markers (Ref. 9; not shown).

Methylation of both *p16* and *MGMT* was detected in only 4 (3%) of 123 cancer-free subjects as opposed to 10 (48%) of 21 of the patients with SCC. The much lower incidence ($P < 0.001$) of detecting both sputum markers in cancer-free subjects rather than in the 21 individuals studied who had proven lung cancer emphasizes two facts. First, hypermethylation changes in sputum DNA do not simply reflect exposure to risk factors for lung cancer but rather track either with a very high-risk status or with the actual presence of cancer. Second, the

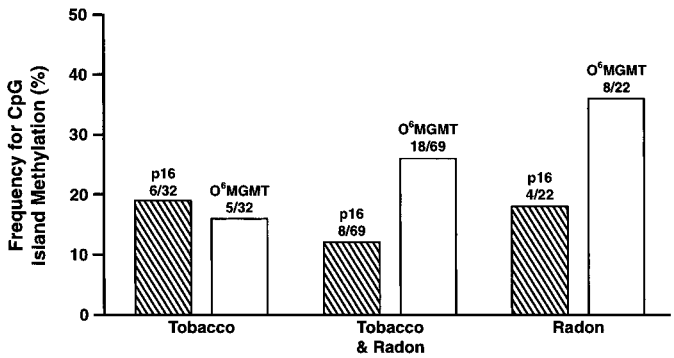

Fig. 2. Prevalence for *p16* and *MGMT* methylation in sputum from cancer-free subjects. The frequency for *p16* and *MGMT* methylation in cancer-free subjects in sputum is expressed as a percentage. *Above the bar graphs*, the total number of positive samples per total sample population.

average incidence of ~25% for the detection of either of the sputum markers in the cancer-free individuals is approximately equal to the known risk of lung cancer development in the populations studied (11, 12). This indicates that these hypermethylation markers may potentially identify those patients at high risk who are most likely to actually get the disease.

Exactly how high the risk status is for cancer-free patients who have the positive sputum markers remains to be determined. However, the follow-up on this cancer-free population, which now ranges from 2 to 6 years after sputum collection, is intriguing. To date, three lung cancers have been reported: two in former uranium miners who smoked and one in a smoker. Eight deaths have occurred not related to lung cancer. Two lung cancers were diagnosed as SCC, 1 and 3 years after sputum collection. The *MGMT* gene was methylated in sputum samples from both of these subjects. The other tumor was an adenocarcinoma. The incidence of *p16* and *MGMT* hypermethylation is lower in this tumor type as opposed to SCC, and neither marker was present in the sputum sample collected 2 years before diagnosis.

Discussion

Our studies have identified biomarkers with great potential for the early detection of a common, noninherited, cancer. Using two markers, which are frequently found in non-small cell lung tumors, a perfect record for detecting a DNA methylation abnormality in sputum up to 3 years before clinical diagnosis of SCC was obtained in a group of patients. Moreover, with these two biomarkers, 100% of SCCs were also detected through the analysis of sputum collected at the time of clinical diagnosis. These findings offer new markers that should be tested in population-based screening and evaluated as potential targets to monitor in chemoprevention trials. This conclusion is supported by our finding of *p16* and *MGMT* methylation in cancer-free, high-risk subjects at prevalences that approximate lifetime risk for lung cancer (11, 12). Furthermore, in these individuals, sputum samples were positive in both patients in this cohort who developed SCC to date. Our biomarker approach should also aid the sensitivity and accuracy of other diagnostic approaches for lung cancer that are currently receiving much attention. For example, the imaging procedure spiral computerized tomography can detect very small peripheral lung cancers for which routine chest X-rays are negative. However, in terms of the actual presence of lung cancer, many false-positive results are the rule, and actual cancer detection rates using spiral computerized tomography are currently 0.3–0.48% (13, 14). This scanning diagnostic approach might be enhanced by combining it with the determination of hypermethylation biomarkers in the sputum.

Patients with stage I or -II disease have a 5-year cure rate of 60–80%, whereas stage III patients have a median survival of 13 months (15). Lung cancer rates could be reduced through the development of a cost-effective screening approach. Key to this screening approach is the identification of markers of lung cancer that can be detected through sensitive and specific diagnostic assays. It is clear that mutation of the *K-ras* and *p53* genes and microsatellite instability constitute alterations important for lung carcinogenesis (5, 16, 17). However, a recent study examining the concordance of detecting these changes in bronchoalveolar lavage fluid from patients with early-stage lung cancer showed limited sensitivity (18). For example, microsatellite instability was detected in 46% of tumors but in only 14% of the corresponding lavage fluid. Similarly, 56% of tumors contained a *p53* mutation; however, this change was detected in 39% of the corresponding lavage fluid. Both of the genetic alterations are detected through assays that are either limiting in sensitivity (PCR for microsatellite instability) or labor intensive (plaque hybridization assay for *p53*). This study did examine methylation of *p16* using the original assay developed by Herman *et al.* (9) that has a sensitivity for detecting one methylated allele in a background of 1000 unmethylated alleles. The *p16* gene was the best marker, with 63% of the corresponding lavage fluids containing a methylated *p16* gene.

One of the latest markers proposed for noninvasive detection of cancer is mitochondrial DNA mutation (19). Although it is fascinating that mitochondria within a cell accumulate identical cellular mutations, these mutations are dispersed across 16,000 bp and do not show any pattern within tumor types. In fact, across this length of DNA no two tumors of any type appear to contain identical mutations. Furthermore, for lung cancer, mitochondrial mutation was seen in <50% of the 14 tumors examined. Thus, without prior knowledge of the mitochondrial mutation present in a given patient's tumor, an efficient screening system is not possible, and this precludes current use of this marker system for early cancer detection. Our approach in contrast relies on assays that detect a very frequent and early DNA alteration in tumors that occurs in a genomic region identical for all patients. Thus, sputum analyses can be done in a cancer screening setting without knowledge of the methylation changes within the tumor.

The current improvement of the MSP procedure incorporates a nested, two-stage PCR approach, which is more sensitive (1 methylated allele in >50,000 unmethylated alleles). The improvement of the MSP procedure resulted in detecting the methylation of the *p16* and *MGMT* genes at frequencies higher than previously reported (7, 8). Our higher frequency for methylation of p16 actually corroborates immunohistochemical studies (20, 21) that have reported loss of p16 protein in 60–80% of SCCs. Because of the inherent difficulties (*e.g.*, DNA degradation) of assaying for methylation in DNA recovered from fixed tissues, previous reports most likely underestimated the actual frequency of methylation. Our previous studies (7, 8) have also shown a strong correlation between the loss of expression of both *p16* and *MGMT* and methylation.

This improvement almost assuredly also accounts for detecting at least one methylation marker in both the sputum and the corresponding tumor from every patient at the time of tumor diagnosis or within 3 years of tumor diagnosis. This is in contrast to our previous study (7), in which, with the one-stage PCR approach, *p16* methylation was detected in sputum from 43% of lung cancer cases. There were a few instances in which there were discordant findings for one of the two markers. In each case, the other marker allowed for a positive sputum change to correlate with presence of a cancer, or the development of one, within 3 years or less. There are at least three likely explanations for the two instances in which a positive marker was found in the sputum but not in the corresponding tumor. First, the presence of promoter hypermethylation for a given gene can be heterogeneous within a tumor, and sampling would then determine whether it is detected or not. Second, the cancer may arise within a widespread process of bronchial epithelial changes, or so-called "field cancerization," in which cells have a very high risk for malignant transformation. Our studies have detected *p16* methylation in nonmalignant bronchial epithelial cells from different lung lobes of the same patient with a defined lung cancer, and such high-risk cell populations could contribute exfoliated cells that contain a hypermethylation marker to the sputum.[4] Finally, the finding that *MGMT* methylation was not present in two sputum samples collected 3 years before clinical diagnosis (cases 10 and 11, Table 2) could be related to the timing for inactivation of this gene in those tumors. Similarly, *MGMT* methylation was not seen in the sputum sample collected in case 6 (Table 1) 15 months before cancer diagnosis but was present at 9 months before diagnosis.

[4] S. A. Belinsky, unpublished observations.

Our data emphasize the functional implications that the loss of gene function associated with promoter hypermethylation has for early stages of lung and other cancers. Hypermethylation-associated inactivation of *p16* is an early and frequent event in NSCLCs (SCC, 60–80%; adenocarcinoma, 30–45%) and other cancers (6, 7, 20). In experimental systems, such loss appears to act as a "gatekeeper" in permitting cells to pass through early steps of cellular immortalization (22, 23). These findings could profoundly affect the area of chemoprevention in which markers are needed to identify high-risk subjects, evaluate efficacy of preventive agents, and identify steps for achieving the prevention itself.

Findings from our present study also strongly support the implementation of longitudinal studies in subjects at high risk for developing lung cancer but also emphasize the need for the development of additional hypermethylation markers for lung and other common human cancers. Although detecting methylation of *p16* and/or *MGMT* methylation in sputum most likely confers a higher risk for lung cancer, the time to tumor is quite variable, presumably because of the necessity for acquiring additional genetic alterations that promote tumor progression. This is quite evident within our cohort of cancer-free subjects in which *p16* methylation was detected in some sputum samples collected up to 6 years ago. Supporting this premise is a marked difference between the prevalence of both markers in sputum from lung cancer cases *versus* cancer-free subjects. Inactivation of genes by methylation is a major mechanism in cancer (6), and new candidate hypermethylation markers will continue to emerge. Thus, longitudinal studies with these methylation markers should facilitate the development of more accurate risk models to incorporate time to tumor and the relationship to multiplicity of biomarkers in the sputum. Our studies have focused on detecting SCC. However, gene promoter hypermethylation changes are now well defined in breast, prostate, colorectal, and other common human cancers (6), and these sensitive biomarkers should prove as useful for these tumors as our results now indicate they are for lung cancer.

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