Controls. The correlation between controls, whereas RASSFIA promoter methylation was only seen in 2 methylation. Methylation of the MGMT gene was seen in sputum from 9 sputum from 16 controls, and 8 of these subjects were positive for p16 never-smokers. Methylation of the epithelium from current smokers as from former smokers. No promoter control study of lung cancer. The relationship between loss of heterozy- epithelial cells from current and former smokers in a hospital-based, case control study of lung cancer. The relationship between loss of heterozygosity, p16 and p16 methylation in bronchial epithelial and the prev- alence for methylation of these four genes in sputum from cancer-free, current and former smokers were also determined. Aberrant promoter methylation of p16 was seen in at least one bronchial epithelial site from 44% of cases and controls. Methylation of the DAP kinase gene was seen in only 1 site from 5 cases and 4 controls, whereas methylation of the RASSFIA was not detected in the bronchial epithelium. Promoter methylation for p16 and DAP kinase was seen as frequently in bronchial epithelium from current smokers as from former smokers. No promoter methylation of these genes was detected in bronchial epithelium from never-smokers. Methylation of the p16 gene was detected in sputum from 23 of 66 controls. DAP kinase gene promoter methylation was also seen in sputum from 16 controls, and 8 of these subjects were positive for p16 methylation. Methylation of the MGMT gene was seen in sputum from 9 controls, whereas RASSFIA promoter methylation was only seen in 2 controls. The correlation between p16 status in the bronchial epithelium obtained from lung lobes that did not contain the primary tumor and the tumor itself was examined. Seventeen of 18 tumors (94%) showed an absolute concordance, being either methylated in the tumor and at least 1 bronchial epithelial site, or unmethylated in both tumor and bronchial epithelium. These results indicate that aberrant promoter hypermethylation of the p16 gene, and to a lesser extent, DAP kinase, occurs frequently in the bronchial epithelium of lung cancer cases and cancer-free controls and persists after smoking cessation. The strong association between p16 methylation in the bronchial epithelium and corresponding primary tumor substantiates that inactivation of this gene, although not transform- ing by itself, is likely permissive for the acquisition of additional genetic and epigenetic changes leading to lung cancer.

INTRODUCTION

Lung cancer is the most frequent cause of cancer-related mortality in the United States (1). The 171,000 new cases every year and nearly equivalent number of fatalities illustrate the lack of effective thera-peutic alternatives for a disease that is largely diagnosed at an ad- vanced stage. The development of biomarkers that can detect this disease at an early stage should greatly improve survival by allowing tumor resection before metastasis. We have pursued gene-specific aberrant promoter hypermethylation as an approach to aid in the early diagnosis of lung cancer (2, 3). Aberrant promoter hypermethylation is associated with an epigenetically mediated gene silencing that constitutes an alternative to coding region mutations for loss of gene function in cancer (4). The MSP4 approach developed by Herman et al. (5) allows one to specifically assay for methylation at a common region within a gene promoter that correlates with loss of tran- scription.

Recent studies (2) 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 inactivated frequently by aberrant promoter methylation in NSCLC (6) is MGMT. MGMT is a DNA repair enzyme that protects cells from the carcinogenic effects of alkylating agents by removing adducts from the O6 position of gua- nine. Thus, the p16 and MGMT genes are strong candidate biomarkers for early detection.

Many current and former smokers have increased bronchial secre- tions that result in the regular production of sputum containing exfoliated cells from the lower respiratory tract. This has made the cytological and molecular analysis of sputum an active area of re- search for marker development (7–9). By increasing the sensitivity of the MSP approach to detect methylated sequences, we demonstrated that aberrant methylation of the p16 and MGMT promoters can be detected in DNA from sputum in 100% of patients with SCC up to 3 years before clinical diagnosis (3). The methylation changes detected in the sputum were also present in the SCC. Moreover, the prevalence of these markers in sputum from cancer-free, high-risk subjects approximates lifetime risk for lung cancer (10). These findings indicate that aberrant gene methylation could be a powerful molecular marker approach to population-based screening for detecting lung cancer and monitoring the efficacy of chemopreventive agents.

Chemoprevention studies in current and former cancer-free subjects have largely been focused on the effect of defined interventions on cytological and genetic changes in bronchial epithelium obtained through biopsy (11, 12). This approach is predicated by the fact that virtually the entire lower respiratory tract is exposed to inhaled carcinogens within cigarette smoke. The resulting field cancerization involves the generation of multiple, independently initiated sites throughout the lungs of persons with a long history of smoking (13).
Cytological and genetic changes have been detected throughout the bronchial tree (14–22). However, many of the genetic changes detected in the bronchial epithelium may have limited utility for assessing lung cancer risk and response to intervention therapy, e.g., assays for LOH in cytologically normal tissue have limited sensitivity, and quite often, subjects will not be informative for the microsatellite marker used to assess LOH at a specific chromosome locus. Moreover, some LOH changes appear less frequently in former smokers compared with current smokers, suggesting they may be associated more with exposure (20). In addition, the conduct of large population-based prevention trials will necessitate the use of biological fluids, such as sputum, that can be obtained noninvasively for monitoring drug response. Neither PCR- nor FISH-based assays will have the sensitivity to detect LOH in sputum. At present, there is no extensive data on methylation in the bronchial epithelium in either persons with prevalent lung cancer or cancer-free smokers. Moreover, the effect of smoking cessation on these markers has not been evaluated.

The purpose of our current study was to determine the prevalence for aberrant promoter methylation in nonmalignant BECs from current and former smokers in a hospital-based, case control study of lung cancer. In addition to p16 and MGMT, the DAP kinase and RASSF1A genes were evaluated in this study because they have been inactivated by promoter hypermethylation (prevalence of 25–40%) in NSCLC (23–25) and because of their involvement in two important cellular regulatory pathways, apoptosis and ras signal transduction, respectively. The relationship between LOH at 9p and p16 methylation in bronchial epithelium and the prevalence for methylation of these four genes in sputum from cancer-free current and former smokers were also determined.

### MATERIALS AND METHODS

**Subject Enrollment.** This study was approved by our Institutional Review Board, and all participants gave written informed consent. Cases (n = 52) and controls (n = 89) were recruited from veterans who use the NMVHCS for their primary care. Current or former cigarette smokers (>100 cigarettes smoked during their lifetime) were eligible for the study. In addition, subjects could not have had prior diagnosis of cancer of the aerodigestive tract, have undergone chemotherapy or radiotherapy to the chest within the last year, or be unable to tolerate all procedures required to obtain tissue samples (described below).

Cases were recruited from patients referred to the Multispecialty Chest Clinic at NMVHCS, where >90% of the NMVHCS lung cancer cases are evaluated for diagnosis and treatment recommendations. Patients are referred to this clinic because of signs or symptoms suggestive of lung cancer. All patients referred for diagnostic evaluation for lung cancer were recruited for participation in the study. However, only those patients with histological confirmation of lung cancer and who gave consent were enrolled into the study population as cases. All stages and histological types of lung cancer were included. Controls were enrolled from the population of lung cancer-free individuals who received their primary care at the NMVHCS. Patients were approached randomly during primary care clinic visits and asked to participate in the study. All subjects were asked to volunteer for a bronchoscopy; however, their participation was not dependent on agreeing to this procedure. In addition, patients undergoing bronchoscopy for reasons other than a diagnosis of lung cancer (e.g., interstitial lung disease) were included as controls.

All subjects completed a standardized respiratory questionnaire based on an adult questionnaire from the American Thoracic Society (26) that describes in detail each subject’s complete smoking history, including variability in smoking intensity over time and periodic intervals of cessation. The questionnaire also documents respiratory health (cough, dyspnea, etc.), family history, and occupational exposures. Information from this questionnaire was then entered into a database developed for this study.

**Pathology and Exposure History.** The distribution of tumor histology among the 52 cases was 44% SCC, 31% adenocarcinoma, 2% large cell carcinoma, 8% small cell carcinoma, and 15% NSCLC (subtype not specified). Selected demographic variables by case control status are summarized in Table 1. The only significant differences seen between groups were for smoking history where duration and pack-years were greater in cases, whereas years since quitting for former smokers were greater in the control group.

**Bronchoscopy and Tumor Tissue Collection.** All participants enrolled for this study were approached to undergo bronchoscopy. A high proportion (>85%) of cases underwent bronchoscopy because it is performed routinely as a diagnostic or staging procedure in lung cancer patients, and 46% of controls consented to undergo this procedure. BECs were obtained during bronchoscopy as described previously (21). Briefly, BECs were harvested from up to four different sites at anatomically normal appearing bifurcations in the lungs, usually within each upper and lower lobe, using a standard cytology brush. These sites were sampled for three reasons: (a) they are high-deposition areas for particles; (b) they are associated frequently with histological changes in smokers; and (c) they represent common sites of tumors (27, 28). Sites selected for brushing were first washed with saline to remove nonadherent cells. Sites were not brushed if a tumor was visualized within 3 cm of the site. After brushing, the brush was placed in serum-free medium and kept on ice until processed. Up to three brushings were performed at each site. In addition, BECs from never-smokers were obtained from two different sources: (a) cells derived at autopsy by Clonetics, Inc. (San Diego, CA) from four never-smokers and (b) cells from three never-smokers recruited through the NMVHCS.

Tumor tissue was available either by resection or biopsy from 18 persons diagnosed with lung cancer. Tumor tissue was fixed in formalin and embedded in paraffin for subsequent analysis for methylation of the p16 gene.

**BEC Culture.** Cells recovered from the Airways were cultured to expand the number of cells available for analysis. Cultures were established using serum-free BEGM (Clonetics, Inc.), which is selective for the growth of BECs (21, 22). Cells were removed from brushes by vigorous shaking in BEGM, washed, and resuspended in BEGM. Cells were then seeded onto 100-mm fibronectin-coated tissue culture dishes and grown at 37°C in 3.5% CO2 until 80% confluent. Before passage, aliquots of cells were cryopreserved. Cells were fixed in methanol/acetic acid (3:1) or flash frozen in liquid nitrogen at passage 1 or 2 for FISH assays and DNA isolation, respectively.

Cytology. Cells from one brush from each bronchial collection site were prepared for cytological analysis by smearing the cells across a microscope slide. The cells were then fixed with 96% ethanol and stained according to the Pap procedure (29) to facilitate morphological evaluation by a cytopathologist.

**Sputum Collection and Processing.** On enrollment, patients were asked to provide an uninduced sputum specimen. If unsuccessful, participants underwent sputum induction. A variation of the ultrasonic nebulization technique described by Saccomanno (30) was used for sputum induction. Subjects used water or saline to gently brush tongue, buccal surfaces, teeth, and gingival to remove superficial epithelial cells and bacteria, followed by gargling and rinsing with tap water. Patients then inhaled a nebulized 3% saline solution from an ultrasonic nebulizer for 20–30 min. Sputum was collected in a sterile specimen cup, and an equal volume of Saccomanno solution was added immediately. Sputum samples were processed for methylation analysis by extensive mixing by vortex, washed once with Saccomanno solution, and stored at room temperature until analyzed. In addition, at least two slides from sputum samples were prepared and underwent Pap staining for morphological examination by a certified cytopathologist.

### Table 1 Summary of selected demographic variables by study group

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cases (n = 52)</th>
<th>Controls (n = 89)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>68 (44, 79)</td>
<td>67 (45, 81)</td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>96</td>
<td>99</td>
</tr>
<tr>
<td>Ethnicity (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>67</td>
<td>69</td>
</tr>
<tr>
<td>Hispanic</td>
<td>21</td>
<td>27</td>
</tr>
<tr>
<td>African-American</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Native-American</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Smoking status (% current)</td>
<td>28</td>
<td>32</td>
</tr>
<tr>
<td>Smoking, duration (yrs)</td>
<td>50 (18, 66)</td>
<td>36 (3, 64)</td>
</tr>
<tr>
<td>Smoking, pack-years</td>
<td>66 (18, 177)</td>
<td>48 (1, 183)</td>
</tr>
<tr>
<td>Smoking, time quit (yrs)</td>
<td>3 (1, 34)</td>
<td>20 (1, 42)</td>
</tr>
</tbody>
</table>

*Statistical comparisons are between cases and controls.

*Median (minimum and maximum).

P < 0.005.
Nucleic Acid Isolation and MSP. DNA was isolated from BECs, tumors, and sputum by digestion with Pronase in 1% SDS, followed by standard phenol-chloroform extraction and ethanol precipitation.

The methylation status of the p16, MGMT, DAP kinase, and RASSF1A genes was determined by the method of MSP (2, 5) with the following modifications. We conducted our recently developed (3) 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 bisulfate modification, and PCR was performed to amplify a 280-, 289-, 209-, and 260-bp fragment of the p16, MGMT, DAP kinase, and RASSF1A genes, respectively, including a portion of their CpG-rich promoter region. The primers recognize the bisulfate-modified template but do not discriminate between methylated and unmethylated alleles. The stage I PCR products were diluted 50-fold, and 5 μl were subjected to a stage II PCR in which primers specific to methylated or unmethylated template were used. Primer sequences and conditions used in stage I and II amplification of the p16 and MGMT promoters have been described (3). Primer sequences used in the stage I amplification of the DAP kinase and RASSF1A genes are as follows: DAP kinase-Forward, 5′-GGTTTTTTCGGATGTGGAGG-3′, DAP kinase-Reverse, 5′-GCTATCGA-AAAACCGGATTAAC-3′, RASSF1A-Forward, 5′-GGAGGGAGGACGGATTAAGG-3′, RASSF1A-Reverse, 5′-CAACCTAAATGCCTACCTCC-3′. Taq Gold polymerase (Perkin-Elmer) in a 50-μl volume was used in all PCRs. The PCR amplification protocol for stage I was as follows: 95°C for 10 min, then denature at 95°C for 30 s, anneal at 58°C (DAP kinase) or 60°C (RASSF1A) for 30 s, extension at 72°C for 30 s for 40 cycles, followed by a 5-min final extension. Primers used to selectively amplify unmethylated or methylated alleles of the DAP kinase gene are as follows: forward unmethylated, 5′-GGGAGGATGGTGATTGTTAAGTGT-3′, reverse unmethylated, 5′-CAAACTCTCCCAACACACAA-3′, forward methylated, 5′-ATAGTCGCTGCTGTTAAGCTC-3′, and reverse methylated, 5′-AAACCTCAACCAAGGGACAC-3′. Primers used to selectively amplify unmethylated or methylated alleles of the RASSF1A gene are forward unmethylated, 5′-GGGTTTGTGAGAGTGTTTAG-3′, reverse unmethylated, 5′-ACATCAAAACAAACCAACAC-3′, forward methylated, 5′-GGGTTTTTCGGAGCGGC-3′, and reverse methylated, 5′-CCCGATTAAACCCGTACTCCG-3′. Annealing temperatures were increased to 70°C and 62°C (DAP kinase) and 68°C and 62°C (RASSF1A) to amplify methylated and unmethylated sequences, respectively, and all cycling times were reduced to 15 s for a total of 40 cycles. Product sizes for each gene promoter were as follows: 153 and 106 bp for methylated and unmethylated DAP kinase and 204 and 170 bp for methylated and unmethylated RASSF1A. Normal human tissue collected from autopsy of never-smokers and cell lines positive for p16 (Calu6), MGMT (SKL-U1), DAP kinase (H92009), and RASSF1A (AS549) metylation served as positive and negative controls. These cell lines were purchased from American Type Culture Collection (Manassas, VA) and cultured using the suggested protocols.

Samples that gave positive methylation products were also analyzed by methylation-sensitive restriction enzyme digestion of the resulting PCR products. Second-stage PCRs were performed in duplicate for each sample. Then for p16, one of each sample pair was incubated with the restriction enzyme FNU-4HI that cuts (G/C)CGCGG at two sites within the amplification 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 bisulfate treatment to uracils. Because FNU-4HI cuts at two different sites within the portion of the p16 promoter being examined, we confirmed that four CpGs were methylated, which verified the methylation status of the sample. An identical approach was used for the DAP kinase and RASSF1A genes using the BstUI and HinfI restriction enzymes to assay 2 and 1 CpG site, respectively. The TaqI and BstUI restriction enzymes were used to assay three different CpG sites within the MGMT gene (3). All samples scored as positive for methylation were confirmed by restriction analysis.

FISH for Detecting LOH at Chromosome 9p21 and 9p23 in BECs. Two regions on the short arm of chromosome 9, p21 and p23, were examined for LOH in BECs. A YAC containing ∼900 kb of DNA localized to the D9S157 marker at 9p23 was obtained from the Centre d’Etude du Polymorphisme Humain, Paris, France. A COSp16 probe (comprised of eight cosmids that encompass the p16 gene on 9p21) was obtained from Dr. Olopade (University of Chicago, Chicago, IL). An α-satellite probe to the centromere of chromo-

some 9 was obtained from the American Type Culture Collection. The α-satellite probe was labeled directly by nick translation with Fluorogenic Green. The YAC and COSp16 probes were labeled by random priming with biotin-14-dUTP and detected with cy3 streptavidin. BECs were dropped on precoated glass slides (5–10,000 cells/slide) and dehydrated; DNA was denatured and hybridized first with the centromere probe for ∼3 h. Slides were rinsed, then hybridized overnight with either the YAC or COSp16 probe. All hybridizations were conducted at 37°C in a humidified chamber. Nonspecific hybridization was blocked by the inclusion of placentan DNA and herring sperm DNA in the hybridization solution. BECs were then washed under stringent conditions and incubated with cy3 streptavidin, and nuclei were counterstained with 4′,6-diamidino-2-phenylindole/FTIC/hexamethyly-
lyrhodamine isothiocyanate triple bandpass filter. Metaphase spreads of human fibroblasts were hybridized overnight with each probe, and localization to chromosome 9 was verified. Details of these FISH procedures are available on request from the corresponding author.

The number of FISH signals was evaluated in 400 BECs/site. BECs were considered positive for loss of chromosome 9p21 or 9p23 if the number of cells that contained only one p21 or p23 signal was greater than the average values seen in BECs from never-smokers plus 3 SDs. Only cells in which two copies of the centromere probe were present were evaluated for chromosome loss. For quality assurance, two technicians both scored 20% of the slides. Their results differed by <0.4%.

Statistical Methods. Data were summarized using percentages for discrete variables and medians with ranges for continuous variables. Differences between groups were assessed using Fisher’s exact test for discrete variables and the Wilcoxon rank-sum test for continuous variables. Results from pairs of markers were examined using Fisher’s exact test to assess the association between markers and MacNemar’s test to assess differences in proportions positive for the markers. Logistic regression models with case control status as the outcome were used to control for multiple predictor variables simultaneously. All analyses were conducted in SAS software (SAS Institute, Inc., Cary, NC).

RESULTS

Cytological Changes in BECs. Cytological changes were characterized in BECs obtained from ≤4 sites in 51 cases (cytology not available for 1 case) and 41 controls. A total of 166 and 150 sites were evaluated in cases and controls, respectively. Metaplasia (reactive) and mild dysplasia (atypia) were the predominant cytological abnormalities observed, and their distribution did not differ among groups. One case had severe dysplasia within a bronchial epithelial site. Cytological changes were seen in ≥1 site in ∼54% of both cases and controls (Table 2). The number of cases with ≥2 or 3 sites positive for cytology exceeded those observed in controls: 33 versus 20% (P = 0.24) and 25 versus 3% (P = 0.007), respectively. These cytological changes were observed in <10% of cells recovered from the diagnostic brush. There was no apparent association between the presence of cytology and tumor histology or smoking status (current versus former); however, cases with cytological changes in their bronchial epithelium smoked more than those without cytological changes (median pack-years, 78 versus 59; P = 0.10).

Ablarrant Promoter Hypermethylation in BECs. BEC cultures were successfully established from 94% of the collected sites allowing the methylation state of the p16, MGMT, DAP kinase, and RASSF1A genes to be examined in 160 and 137 sites from cases and controls, respectively. Ablarrant promoter methylation of the p16 gene was seen in ≥1 site from 44% of both cases and controls, and 14 and 15% of cases and controls, respectively, had 2 sites positive (Fig. 1 and Table 2). Furthermore, 2 cases and 1 control had 3 sites positive, but no subjects had methylation of p16 in all 4 BEC sites (Table 2). The prevalence for methylation of the DAP kinase gene in bronchial epithelium was significantly less than observed for p16, irrespective of case control status (P = 0.001). Only 5 cases (10%) and 4 controls

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HYPERMETHYLATION IN BRONCHIAL EPITHELIUM AND SPUTUM

Table 2 Cytology, aberrant promoter hypermethylation, and LOH at 9p23 in nonmalignant BECs from lung cancer cases and controls

<table>
<thead>
<tr>
<th>End point</th>
<th>One</th>
<th>Two</th>
<th>Three</th>
<th>Four</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytologya</td>
<td>27/51 (54)</td>
<td>22/41 (54)</td>
<td>17/51 (33)</td>
<td>8/41 (20)</td>
</tr>
<tr>
<td>p16 methylation</td>
<td>23/52 (44)</td>
<td>18/41 (44)</td>
<td>8/52 (14)</td>
<td>6/41 (15)</td>
</tr>
<tr>
<td>DAP kinase methylation</td>
<td>5/52 (10)</td>
<td>4/41 (10)</td>
<td>0/52 (0)</td>
<td>0/41 (0)</td>
</tr>
<tr>
<td>9p23 LOH</td>
<td>25/55 (71)</td>
<td>19/26 (73)</td>
<td>18/55 (51)</td>
<td>8/26 (31)</td>
</tr>
</tbody>
</table>

* Summary data are presented as the total number of cases and controls who were positive per total sample population for cytology, methylation, or LOH at 9p23 in at least one, two, three, or four bronchial epithelial sites. Because only two sites were collected from some cases and controls, the number of persons who could be evaluated for positivity at three or four sites was fewer than the total sample size. Values in parentheses refer to the percentage of persons positive.

** Metaplasia (reactive) and mild dysplasia (atypia) were the predominant cytological abnormalities observed.

\* P = 0.007 as compared with cases.

\# P = 0.001 as compared with p16 methylation.

(10%) had a BEC site positive for methylation of the DAP kinase gene; methylation was never seen in >1 site in a subject (Table 2). Methylation of the RASSF1A gene was not detected in any sites. Interestingly, methylation of both p16 and DAP kinase was seen in 2 cases and 1 control. However, only in the control subject was the methylation found at the same site. Methylation of p16, DAP kinase, or RASSF1A was not detected in any of the 17 sites from 7 never-smokers (data not shown), a finding consistent with previous studies that have reported virtually no genetic alterations in bronchial epithelium from this population (19, 20).

In marked contrast to our findings for these three genes in BECs, we observed methylation of the MGMT gene in >75% of BEC sites from cases and controls. In addition, MGMT methylation was seen in 14 of 17 sites from the never-smokers (data not shown). This finding has also been observed in normal human fibroblasts, which displayed extensive methylation of the MGMT CpG island as they became confluent (31). This process was partially reversed when fibroblasts were placed back into logarithmic growth. Thus, in the tissue culture setting, it is apparent that confluence-induced growth constraint must cause transient alterations in epigenetic stability within the MGMT promoter, leading to aberrant methylation. The fact that our BECs are grown to 80% confluence coupled with the use of the sensitive two-stage MSP approach facilitated the detection of this in vitro methylation. This finding does not preclude the use of MGMT as a biomarker in sputum because the high prevalence for the changes seen in vitro occurs in a context quite distinct from that of the airway epithelium. Furthermore, in vitro methylation of MGMT is not recapitulated in either primary NSCLCs or exfoliated cells within sputum where the prevalence for MGMT methylation is 25 and 15%, respectively (3, 6). In vitro methylation does not apply to other genes, such as p16, based on our findings of methylation in only 56 of the 297 BEC sites (19%) examined. Furthermore, when Vertino et al. (32) tried to induce methylation by passage of human fibroblasts that overexpress the cytosine DNA methyltransferase 1 gene, no methylation of p16 was detected after 20 passages.

For the cases, there was no association between tumor histology and methylation of the p16 or DAP kinase genes in the bronchial epithelium. This finding is consistent with the fact that these genes are methylated in both SCC and adenocarcinoma (2, 4, 6). Smoking status had no association with the detection of methylation of either the p16 or DAP kinase gene in the bronchial epithelium. Thus, promoter methylation was seen as frequently in BECs from current smokers as from former smokers. Specifically, for p16, methylation was seen in a bronchial epithelial site from ~43% of either current or former smokers that were cases or controls. In addition, none of the smoking variables categorized (duration, pack-years, and time quit) was associated with the detection of promoter methylation in the bronchial epithelium.

Correlation between p16 Status in BECs and Lung Tumors.

Tumor tissue was available from 18 of the cases diagnosed with lung cancer. To determine whether p16 methylation in the BECs within these cases predicts methylation at this locus in tumors, DNA was isolated from microdissected tumors for MSP analysis of p16. For these 18 cases, all bronchial epithelial sites examined for methylation were from lung lobes that did not contain the primary tumor. Methylation of the p16 gene was detected in four of eight SCCs, four of eight adenocarcinomas, and one of two NSCLCs (data not shown). Seventeen of the 18 tumors showed an absolute concordance (P < 0.001), being either both methylated in the tumor and at least one BEC site (nine pairs) or unmethylated in the tumor and BECs (eight pairs). In the one discordant tumor-BEC pair, the bronchial epithelium was methylated, and the tumor was unmethylated.

Detection of LOH at 9p23 and 9p21.

Allelic loss on the short arm of chromosome 9 extends through 9p23 in most NSCLCs (33, 34). The p16 gene is inactivated largely in lung cancer cells through either promoter hypermethylation of both alleles or by the methylation of one allele and the deletion of the other allele (35). To determine the relationship between p16 methylation and LOH and define the extent of allelic loss in BECs from cases and controls, two areas, p21 and p23, were evaluated using FISH. The 9p23 region was selected for analysis first because this probe was significantly larger than the p21 cosmid contig (900 versus 200 kb), which would facilitate scoring the cells. Background rates for LOH at 9p23 and 9p21 were determined by examining normal BECs from autopsy of never-smokers or col-

![Fig. 1. Methylation of the p16 gene in BECs and corresponding sputum. Representative scenario for detection of methylation in BECs and sputum from 5 different controls are depicted. The sample number corresponds to an individual and the fraction (e.g., 1.1 and 1.2) of BECs collected from different lung lobes from that individual, e.g., in the first control, only the second bronchial epithelial site was positive for p16 methylation. CaLu6 and H209 are cell lines known to contain a methylated and unmethylated p16 gene, respectively.](image)
selected during bronchoscopy of never-smokers. LOH for 9p23 and 9p21 was detected in 1.3 ± 0.3% and 1.7 ± 0.1% BECs, respectively, of never-smokers. These values are similar to percentages seen with other FISH probes (trisomy of chromosomes 7 and 2) used by our laboratory with BECs. Therefore, LOH frequencies >3 SD from those seen in never-smokers (>2.2 and 2% for LOH at 9p23 and 9p21, respectively) were scored as positive for LOH within the BECs evaluated. The use of 3 SDs from the background value combined with scoring of 400 cells ensures a confidence limit of >95% for assessing positivity (36).

Because of the labor involved in assessing LOH by FISH, analyses were conducted on a subset of cases (n = 35) and controls (n = 26). Sites (210) were examined for LOH at 9p23 with positive values ranging from 2.5 to 4.8%. Twenty-five of 35 cases (71%) and 19 of 26 controls (73%) had LOH at 9p23 in ≥1 BEC site (Table 2). There was considerable multiplicity for LOH at this locus throughout the lungs of cases and controls. LOH was present in ≥2 or 3 sites from 51 and 30% of cases and in 31 and 23% of controls (Table 2). Finally, in 22% of cases and 8% of controls (of those for whom 4 sites were available), all 4 BEC sites were positive for LOH at 9p23. The percentage of cases with LOH did not differ by tumor histology. Smoking status had no association with the detection of LOH at 9p23 in the bronchial epithelium. In addition, none of the smoking variables (duration, pack-years, and time quit) was associated with whether allelic loss was detected in the bronchial epithelium. Independent of case control status, 13 sites were positive for 9p23 LOH and p16 methylation; however, methylation of p16 was also detected in 23 sites that were negative for LOH (data not shown).

Approximately 50% of the sites examined for LOH at 9p23 were also evaluated for LOH at 9p21. Positive values for LOH at this locus ranged from 2.3 to 3.8%. There was an 85% concordance between detecting LOH at 9p21 versus 9p23 in BECs from the cases and controls (data not shown). Therefore, additional sites were not analyzed at this locus. Homozygous deletion was not observed at 9p21 or 9p23, nor was aneuploidy of chromosome 9 observed in any of the BECs (data not shown).

Although the percentage of cells with LOH at 9p21 and 9p23 was rather low (2- to 4-fold greater than baseline values), these values were consistent with those seen for trisomy 7 and 20 (8, 9). Our sampling procedure that collects cells over an area of ~1 cm, combined with expansion of the BECs in culture, would disrupt any small clones of cells containing this chromosome abnormality. This conclusion is supported by recent studies (37, 38) that demonstrated multiple small clonal or subclonal patches containing multiple chromosome abnormalities within biopsies of normal bronchial epithelium adjacent to tumors.

Gene Promoter Hypermethylation in Sputum from Controls. Recent studies by our laboratory have indicated that aberrant promoter hypermethylation in sputum could be a valuable biomarker for predicting lung cancer. Furthermore, our initial studies of p16 and MGMT methylation in a limited number of cancer-free smokers suggest that the prevalence for these changes approximate lifetime risk for lung cancer (10). Thus, during the recruitment of subjects for this case control study, the protocol was amended to include the collection of sputum from controls to gather additional information on the prevalence of the p16, MGMT, DAP kinase, and RASSF1A genes in this population of veterans. Sputum was obtained from 66 controls; 18 underwent bronchoscopy.

Abnormal sputum cytology was seen in 25% of controls. Metaplasia was the most common change, being present in 19% of sputum samples, whereas mild dysplasia was observed in 6% of sputum samples. Methylation of the p16 gene was detected in sputum from 23 of 66 controls (35%; Fig. 2). DAP kinase methylation was also seen in sputum from 16 controls, and 8 of these subjects were positive for methylation of p16 (Fig. 2). MGMT methylation was seen in 9 controls; 4 of these subjects were also positive for either p16 (1 subject) or DAP kinase (3 subjects). In addition, 3 subjects positive for MGMT methylation also had methylation of both p16 and DAP kinase in their sputum (data not shown). Methylation of RASSF1A was only detected in two sputum samples; however, 1 person positive for this gene contained p16, DAP kinase, and MGMT methylation in the sputum sample. The other person’s sputum was positive for DAP kinase and MGMT methylation. Thus, 3 (5%) and 1 (2%) of the control subjects were positive for three or four of the methylation markers, respectively, in their sputum. As seen in our previous study of methylation changes in sputum (3), sputum cytology was not associated with positivity for aberrant promoter methylation. Smoking status had no association with the detection of gene promoter methylation in sputum. In addition, none of the smoking variables (duration, pack-years, and time quit) was associated with whether methylation was detected in the sputum.

The concordance between finding p16 methylation in sputum and bronchial epithelium from the 18 controls was examined. In this subset, p16 methylation was seen in BECs and sputum of 10 and 7 controls, respectively (Fig. 3). Of the 7 subjects whose sputum was positive for this marker, 5 displayed the methylation change in their bronchial epithelium. However, there were 5 controls whose bronchial epithelium was positive and sputum negative for p16 methylation (Fig. 3).

**DISCUSSION**

The results of this study indicate that aberrant promoter hypermethylation of the p16 gene and to a lesser extent, DAP kinase, occurs...
frequently in the bronchial epithelium of lung cancer cases and cancer-free controls, who smoked. These methylation changes persist after smoking cessation. They can also be detected within exfoliated cells, supporting their potential use as markers in assessing lung cancer risk reduction in response to cancer chemopreventive agents. Prevalence and tissue multiplicity for the five genetic and epigenetic markers examined in this study did not differentiate cases from controls. This finding has been corroborated by investigators examining other genetic markers and is likely because of the overwhelming damage to the bronchial epithelium through years of exposure to tobacco carcinogens (13). Furthermore, the lack of any association between these markers and smoking dose or duration may be because 92% of cases and 73% of controls had >30 pack-years of smoking.

The fact that promoter methylation was seen at similar prevalence in cases and controls irrespective of smoking status (current versus former) does not diminish our previous findings that suggest these methylation changes confer increased risk for lung cancer (3). Approximately half of the lung cancers is diagnosed in former smokers, a statistic quite evident in our own study. Although cessation of smoking is associated with a decrease in lung cancer risk, the cumulative risk for lung cancer by age 75 for a person who quits smoking at age 50 is still six times greater than a never-smoker (39). Furthermore, the strong association seen between p16 methylation in the bronchial epithelium and corresponding primary tumor reinforces our hypothesis that inactivation of genes such as p16, although not transforming by themselves, are most likely permissive for the acquisition of additional genetic and epigenetic changes that ultimately lead to malignant cancer. Supporting this concept are the findings from our previous (3) and current study where methylation of both p16 and MGMT was seen in sputum from 43% of persons with confirmed SCC but only in 3–6% of cancer-free controls. Therefore, the time to acquire the additional genetic and epigenetic changes that promote tumor progression is likely a critical determinant for lung cancer risk.

Inactivation of the p16 gene by promoter hypermethylation was seen more frequently in bronchial epithelium than DAP kinase, whereas RASSF1A methylation was never detected. This finding may reflect the timing and role of these genes in the development of lung cancer. Our previous studies have demonstrated that p16 methylation is detected at the earliest cytological stages of SCC and adenocarcinoma (2). Inactivation of p16 has been proposed as an early step to immortalization by allowing cells to escape the immortality checkpoint “MO” (40, 41). The importance of p16 in the development of NSCLC is evident by the fact that it is inactivated by promoter methylation in 60–70% of SCCs (2) and ≤48% of adenocarcinomas (34).

The timing for inactivation of the DAP kinase gene in NSCLC has not been defined. However, several functional studies have revealed a role for this gene in γ-IFN, tumor necrosis-α, and Fas-induced apoptosis (42, 43). Most recently, expression of DAP kinase suppressed oncogenic transformation of primary embryonic fibroblasts by activating p53 in a p19ARF-dependent manner (44). Thus, it appears that DAP kinase could play an important role in an early checkpoint to eliminate premalignant cells during cancer development. This finding could place inactivation of DAP kinase as a subsequent event to inactivating p16.

The RASSF1A gene was found recently to be inactivated by aberrant promoter hypermethylation (25). The encoded protein interacts with the human DNA repair gene XPA, and the COOH terminus of the translated nucleotide sequence shows 55% homology to the mouse Ras effector protein Nore1. Thus, as concluded by Dammann et al. (25), this protein could function in a RAS signaling pathway as a negative regulator of cell growth. The facts that promoter methylation was not seen in bronchial epithelium and that only two sputum samples were positive for RASSF1A methylation suggest that inactivation of this gene could be a later event in malignant transformation. This supposition is supported by our findings that both control subjects with RASSF1A methylation in their sputum also had methylation of at least two other genes.

The results of this study indicate that LOHs within the 9p21 and 9p23 loci are frequent within bronchial epithelium. The high concordance seen for LOH at both loci suggests that the breaks detected are quite large, although we are sampling areas with minimal or no cytological changes. The lack of any abnormal cytology in the presence of chromosome damage at this locus has been confirmed (19, 20) and seen with other chromosome aberrations (21, 22). Our results using FISH to detect LOH also corroborate findings by Mao et al. (11), who reported that 57% of cancer-free persons show microsatellite loss at this locus and that 24% of the total sites examined exhibit this change. The lack of aneuploidy in the BECs compared with that commonly seen in lung tumors (33) supports the finding of LOH at 9p as a very early change before chromosome duplication during lung carcinogenesis. Allelic loss at 9p21 also precedes the development of aneuploidy in esophageal cancer (45), and aneuploidy during the genesis of SCC of the lung is generally not observed until bronchial squamous metaplasia with high-grade atypia (46). Additional studies of LOH at chromosome 3p by FISH also support the dogma of LOH occurring before chromosomal duplication (47). Our studies did not reveal a strong association between p16 methylation and LOH. This finding parallels that of Zöchbauer-Müller et al. (24), who reported no association between LOH at 9p21 and p16 methylation.

One critical issue confronting the chemoprevention field is the identification of intermediate markers whose modulation would be indicative of a reduction in lung cancer risk. Current efforts have focused on assessing changes in histopathology and LOH in the bronchial epithelium. Because some cytological atypia and metaplasia are reversible after smoking cessation (48), these histological changes may not be modulated by intervention because their presence may better reflect the constant remodeling of the airway epithelium in response to wounding and damage by carcinogens within tobacco smoke. In addition, assays for LOH are limited by sensitivity and a lack of informativeness at loci in some subjects. Our studies have demonstrated that p16 and DAP kinase are inactivated by promoter hypermethylation in nonmalignant bronchial epithelium of current and former smokers, which is also detected in exfoliated cells within sputum. Methylation biomarkers detected in sputum could be used to monitor the efficacy of selenium, a promising chemopreventive agent for lung cancer that has been shown to inhibit cytosine DNA methyltransferases (49, 50). This inhibition could lead to demethylation and subsequent re-expression of genes, such as p16 and DAP kinase, in premalignant bronchial epithelium.

Sputum samples contain cells from the large bronchi and cells to a lesser extent from the smaller airways that include the small bronchi, bronchioles, and alveoli. Although our comparison of p16 methylation in sputum-BEC pairs was limited by sample size, it is interesting that a positive sputum sample reflected methylation in the bronchial epithelium 70% of the time (five of seven p16-positive sputum). It is unlikely that a single bronchial site positive for p16 methylation would in turn give rise to the methylation detected in sputum in persons who are clinically cancer-free. Thus, it is attractive to speculate that the lack of p16 methylation in sputum from the five controls whose bronchial epithelium was methylated is because of an overall lower amount of field cancerization than seen in the subjects who

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4 K. Divine and S. Belinsky, unpublished data.
showed methylation in both sputum and BECs. The confirmation of this hypothesis must await larger studies to examine this relationship.

In summary, our data demonstrate that BECs that harbor p16 or DAP kinase genes inactivated through promoter hypermethylation persist after cessation of smoking. The impact that silencing of these and other critical regulatory genes has on absolute risk for lung cancer will be better addressed through the analysis of exfoliated cells within a biological fluid, such as sputum, that represents a composite of the genetic damage throughout the aerodigestive tract. Although the current study and our previous work (3) imply that the presence of promoter methylation in sputum from cancer-free smokers conveys a higher risk for lung cancer, the necessity for obtaining additional genetic and epigenetic changes that promote tumor progression will ultimately impact on the time to clinical disease. Through gene discovery and evaluation of additional genes already identified that are inactivated in lung cancer by promoter hypermethylation, we will develop a panel of markers whose inactivation occurs at different stages of malignant transformation. Conducting longitudinal studies with this panel of hypermethylation markers will determine whether more accurate risk models can be developed that incorporate time to tumor and the relationship to multiplicity of these biomarkers in sputum.

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Aberrant Promoter Methylation in Bronchial Epithelium and Sputum from Current and Former Smokers

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