Gene Promoter Methylation in Plasma and Sputum Increases with Lung Cancer Risk

Steven A. Belinsky,1 Donna M. Klinge,1 Joseph D. Dekker,1 Mitzi W. Smith,1 Theresa J. Bocklage,2 Frank D. Gilliland,3 Richard E. Crowell,2 Daniel D. Karp,4 Christine A. Stidley,2 and Maria A. Picchi1

Abstract

Purpose: Lung cancer is the leading cause of cancer mortality in the United States, due in part to the lack of a validated and effective screening approach for early detection. The prevalence for methylation of seven and three genes was examined in DNA from sputum and plasma, respectively, from women at different risk for lung cancer.

Experimental Design: Lung cancer survivors (n = 56), clinically cancer-free smokers (n = 121), and never smokers (n = 74) comprised the study population. Plasma was collected from all three groups, whereas sputum was collected from lung cancer survivors and smokers.

Results: Methylation was detected in plasma DNA from 10 of 74 women who never smoked. Prevalence for methylation of the p16 gene in plasma was highest in lung cancer survivors. Lung cancer survivors showed a significant increase in the odds of having at least one or more genes methylated in plasma (odds ratio, 3.6; 95% confidence interval, 1.9-9.1) than never smokers. The prevalence for methylation of the O6-methylguanine-DNA methyltransferase, ras effector homologue 1, death associated protein kinase, and PAX5 genes in sputum was significantly higher in lung cancer survivors compared with smokers. Lung cancer survivors had 6.2-fold greater odds (95% confidence interval, 2.1-18.5) for methylation of three or more genes in sputum compared with smokers. Methylation was more commonly detected in sputum than plasma for O6-methylguanine-DNA methyltransferase and ras effector homologue 1, but not p16, in lung cancer survivors.

Conclusion: Concomitant methylation of multiple gene promoters in sputum is strongly associated with lung cancer risk.

Lung cancer is the leading cause of cancer mortality in the United States, due in part to the lack of a validated or effective screening approach for early detection. The significance of this public health burden is evident worldwide, with 1.5 million deaths from lung cancer projected by 2010 (1). Five-year survival rates for cancers of the breast, colon, and prostate for deaths from lung cancer projected by 2010 (1). Five-year survival rates for cancers of the breast, colon, and prostate for 5 years is <50% (5–7). This is contrasted by a median survival of 13 months for patients for whom surgery is not an option (2). Although cessation of smoking is associated with a leveling off 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 for an individual who has never smoked (3). The impact of the continued risk of smoking is seen in the cancer clinics, where more than half of lung cancers diagnosed are in former smokers (4). The benefit of early detection is seen in patients who present with stage I tumors (<3 cm), where surgical resection is commonly the preferred treatment option, and the rate of recurrence within 5 years is <50% (5–7). This is contrasted by a median survival of 13 months for patients for whom surgery is not an option (2).

Studies in our laboratory have shown that detection of aberrant promoter methylation of tumor suppressor genes in sputum has the potential to become a viable screening approach for the early detection of lung cancer. In our initial studies, the detection of methylation of either the CDKN2A (p16) or the O6-methylguanine-DNA methyltransferase (MGMT) gene promoter predicted the development of squamous cell carcinoma up to 3 years before clinical diagnosis (8). The exposure of the entire respiratory tract to inhaled carcinogens within cigarette smoke leads to field cancerization that is characterized by the generation of multiple, independently initiated sites throughout the lungs of people with a long history of smoking (9). The presence of field cancerization presents an obstacle to early detection of lung cancer. Genes,
such as p16, which is inactivated by methylation early in lung cancer development, can be detected in sputum from some cancer-free subjects who are either current or former smokers and as a single marker could have limited specificity for predicting lung cancer (8, 10, 11). Lung cancer is a disease that develops over ≥40 years and involves the acquisition of multiple genetic and epigenetic changes. Therefore, screening for a panel of genes inactivated by methylation at different stages of neoplastic development could help identify subjects with early-stage lung cancer or those who might benefit from chemopreventive agents.

Sputum has both advantages and disadvantages as a biological fluid for screening of lung cancer. The main advantage is that the cells recovered are from the lungs and often include the lower respiratory tract. The main disadvantages of sputum are the variability in composition of the specimen (i.e., epithelial versus inflammatory component) and the fact that up to 30% of current or former smokers do not have increased bronchial secretions and will not produce a sputum specimen even after induction with nebulized saline (12). An alternative to using sputum could be to examine DNA recovered from plasma or serum for gene methylation. Nanogram per milliliter quantities of DNA circulating in blood are present in healthy people (13). Tumor DNA is also released in plasma and serum, and some studies report an enrichment of free circulating DNA compared with controls (14, 15). This accumulation of DNA is speculated to arise from cell lysis by necrosis or apoptosis or local angiogenesis. Several studies have addressed whether gene-promoter methylation could be used to detect prevalent lung cancer in DNA recovered from serum or plasma. These studies report mixed success with detection rates ranging from 45% to 75% in samples where the primary tumor was positive for the methylated gene independent of tumor stage (16–20). Interestingly, only one study reports methylation in serum from people without cancer, suggesting that blood may not be a good biological fluid for early detection (20). Details concerning the amount of DNA recovered from plasma of cancer-free subjects is generally not provided in these studies—circulating free DNA in these people is generally <50 ng/mL plasma. However, some studies do report obtaining <5 mL of blood that would yield ~1.5 mL of plasma. Therefore, the low amount of DNA present in the samples and consequent diminished sensitivity of the assay could be one explanation for the failure to detect methylation.

The purpose of the current study is to assess the hypothesis that the prevalence for methylation of three genes [p16, MGMT, and ras effector homologue 1 (RASSF1A)] in DNA from sputum and plasma varies in three groups of subjects at different risk for lung cancer. Never smokers (minimal risk, \(<1 \times 10^{-4}\) lifetime risk), current, and former chronic smokers (0.3% yearly risk), and persons presumed cured after resection of stage I lung cancer (6.0% risk per patient year; 5-year survival of 60%) comprised the three groups. We examined this hypothesis in a cohort of women at risk for lung cancer. The selection of genes examined for methylation was based on their common prevalence for inactivation in lung cancer (25-50%) and their difference in timing for inactivation—early, intermediate, and late for the p16, MGMT, and RASSF1A genes, respectively (10, 11, 21, 22). The prevalence of methylation of additional early detection candidate genes that included PAX5, PAX5f1, death associated protein kinase (DAPK), and H-cadherin was examined in sputum. These genes are also inactivated in lung cancer at prevalences ranging from 20% to 50% (23–25).

Materials and Methods

Subject enrollment. Never smokers (n = 74, defined as <100 cigarettes smoked during their lifetime) and current and former smokers (n = 121) were from the Albuquerque, New Mexico, metropolitan area. Subjects were recruited through advertisement in a local newspaper and are part of a developing cohort of women at risk for lung cancer. The criteria for participation were females between the ages of 40 and 80, smokers with a minimum smoking history of 15 pack-years, the ability to understand and sign the informed consent, and the ability to perform the requested study procedures. In addition, subjects could not have prior diagnosis of cancer of the aerodigestive tract. Lung cancer survivors were enrolled through a national phase III chemoprevention trial being conducted by the Eastern Cooperative Oncology Group that is investigating the activity of l-selenomethionine to decrease the rate of secondary primary tumors in patients resected for a stage I–IIib cell lung cancer. Patients recruited through the prevention trial (n = 56) must have undergone complete resection of a histologically proven stage IA (pT1N0) or IB (pT1N1) non– small cell lung cancer and be currently disease free. In addition to being pathologically stage N0, at least one mediastinal lymph node must have been sampled at resection. Eligible participants are ≥18 years of age, between 6 and 36 months from the date of surgical resection, have not received chemotherapy or radiation therapy, and have a negative chest X-ray or computed tomography scan ≤8 weeks before randomization to the prevention trial. The Lovelace Respiratory Research Institute Institutional Review Board approved this study and all participants gave written informed consent.

All smokers completed a standardized respiratory questionnaire based on an adult respiratory 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.), general health, family history, and occupational exposures. Never smokers completed the same questionnaire. Subjects enrolled on the prevention trial completed a baseline questionnaire that documented tobacco history, including pack-years, duration, and smoking status.

Selected demographics by group status are summarized in Table 1. The significant differences seen were greater age and lower amount of free DNA recovered from plasma for lung cancer survivors compared with smokers and never smokers.

Sputum and blood collection and processing. On enrollment, current and former smokers were asked to provide sputum by induction. Our attempts to collect sputum from never smokers without lung cancer have had limited success most likely because they lack a productive cough and have limited damage to their lungs. Therefore, sputum was not collected from the never smokers. A variation of the ultrasonic nebulization technique described by Saccomanno (27) was used for sputum induction. Subjects used water or saline to gently brush tongue, buccal surfaces, teeth, and gingiva to remove superficial epithelial cells and bacteria, followed by gargling and rinsing with tap water. Participants then inhaled a nebulized 3% saline solution from an ultrasonic nebulizer for 20 to 30 minutes. Sputum was collected in a sterile specimen cup and an equal volume of Saccomanno solution was added immediately. Subjects enrolled in the prevention trial used the early morning spontaneous cough technique to collect sputum. The baseline sputum collected before randomization on the prevention trial was used to assess gene-specific promoter hypermethylation. Participants were provided with two sterile specimen cups containing Saccomanno’s fixative in a self-addressed return mailer. To increase
the probability that material of deep lung origin was obtained, subjects received detailed verbal instructions by study personnel at the participating institution and written instructions on how to perform the technique. Basically, for three consecutive mornings, they coughed deeply and the resulting mucus is expectorated into the first cup. Then, they repeated this process. Participants then placed the sputum cups in the postage-paid mailer that has been addressed to the study coordinator at Lovelace. The collection of two sputum samples at each time point was based on the finding by Kennedy et al. (12) that the second sample seems to have a higher success rate (80%) in producing an adequate sputum sample based on established cytologic standards (12). This result is attributed to the fact that there may be a “learning effect” in adequate sputum collection. Three lung cancer survivors and three smokers were unable to provide a sputum specimen. Cytology and DNA isolation for methylation assays were conducted on the second sputum sample. All sputum samples, irrespective of adequacy (see Results), 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 Papanicolaou staining for morphologic examination by a certified cytopathologist.

Blood (32 mL) was collected by phlebotomy from all participants into Citrate Vacutainer cell preparation tubes (Becton Dickinson, Franklin Lakes, NJ). These tubes are designed to separate plasma and mononuclear cells from whole blood. Following separation, the plasma fraction was centrifuged at 1,500 rpm to remove any contaminating mononuclear cells. The plasma fraction was then frozen at –80°C until processing for DNA isolation. Blood was not collected from 12 of the lung cancer survivors.

Nucleic acid isolation and methylation-specific PCR. DNA was isolated from sputum by protease digestion followed by phenol-chloroform extraction and ethanol precipitation. Plasma DNA (10 mL) was isolated using the Qiagen blood maxi kit (Invitrogen, Carlsbad, CA). DNA was quantitated by spectrophotometer at absorbance of 260 nm. In addition, a subset of DNA recovered from plasma samples was quantitated by the DNA dip stick test (Invitrogen). Quantitation by these two techniques differed by <5%. The total amount of DNA (median) recovered from sputum and plasma was 24 and 0.3 μg for lung cancer survivors and 14 and 0.45 μg for cancer-free smokers.

Nested methylation-specific PCR was used to detect methylated alleles in DNA recovered from sputum or plasma. We used our nested methylation-specific PCR assay described in detail previously (8) because of its increased sensitivity for the detection of promoter hypermethylation in biological fluids and because of the ability to perform stage I multiplex PCR. The amplification of three genes in a stage I PCR was needed due to the low amount of DNA recovered from the plasma of some subjects. To accurately compare the prevalence for methylation in specimens across all groups with a sensitivity of 1 in 10 to 20,000, 50 to 120 ng of DNA were used for stage I PCR following modification with bisulfite. PCR primers for stage I and II have been described (8, 11, 23). A subset of samples (20%) that gave positive methylation products was also analyzed by methylation-sensitive restriction enzyme digestion of the resulting PCR product. The restriction digestion allows one to examine the methylation state of CpGs within the amplified PCR product and serves as a control for false priming. Digestion within at least one of the restriction sites was seen for all samples positively confirming methylation.

Statistical methods. Data were summarized using frequencies and percents for categorical variables. Differences in distribution between groups were tested using either the χ² test or Fisher's exact test. The distribution of continuous variables that had skewed distribution was summarized with medians and ranges and differences between groups were tested using the nonparametric Kruskal-Wallis test. McNemar's test was used to assess differences in frequencies of gene methylation between sputum and plasma among smokers and lung cancer survivors, respectively. Sputum samples were obtained from the two groups—lung cancer survivors and smokers. The association between methylation and group status was assessed using logistic regression with group status as the outcome variable. Each gene or the cumulative effect of methylation of multiple genes in sputum was included as independent variables in separate models. To address the effect of one, two, and three or more methylated genes, indicators for each of these were included in the model. The likelihood ratio test was computed to assess the overall significance of these multiple gene effects. Age and smoking (duration) were entered as continuous variables. Never smokers were assigned zero years duration. Smoking duration rather than pack-years was used because duration and pack-years had similar strengths as predictors, but more participants had data available on duration. Plasma samples were obtained from participants in all three groups.

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

<table>
<thead>
<tr>
<th>Variable</th>
<th>Lung cancer survivors (n = 56)</th>
<th>Smokers (n = 121)</th>
<th>Never smokers (n = 74)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)†</td>
<td>66 (42-80) *</td>
<td>58 (41-76)</td>
<td>54 (40-73)</td>
</tr>
<tr>
<td>Ethnicity (%)</td>
<td>Non-Hispanic White 54 (96)</td>
<td>91 (75)</td>
<td>59 (80)</td>
</tr>
<tr>
<td></td>
<td>Hispanic</td>
<td>0</td>
<td>24 (20)</td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>2 (4)</td>
<td>6 (5)</td>
</tr>
<tr>
<td>Smoking status (%)</td>
<td>Never</td>
<td>6 (11)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>Former</td>
<td>37 (77)</td>
<td>53 (44)</td>
</tr>
<tr>
<td></td>
<td>Current</td>
<td>11 (23)</td>
<td>68 (56)</td>
</tr>
<tr>
<td>Smoking history‡</td>
<td>Duration (y)§</td>
<td>40 (13-61)</td>
<td>36 (14-59)</td>
</tr>
<tr>
<td></td>
<td>Pack-years‡</td>
<td>40 (8-94)</td>
<td>40 (15-166)</td>
</tr>
<tr>
<td>Plasma DNA (ng/mL)†</td>
<td>30 (6-794)</td>
<td>45 (6-369)</td>
<td>37 (11-386)</td>
</tr>
</tbody>
</table>

*Numbers may vary because of missing data for some variables.
† Median (minimum and maximum).
‡ P < 0.05 compared with never smokers and smokers.
§ Includes only current and former smokers.
The association between methylation in plasma and group was assessed with multinomial logistic regression with the three groups as unordered categories. Individual genes or the cumulative effect of one or more methylated genes were included in separate models. Models were adjusted for age and ethnicity. Exact multinomial regression methods were also fitted to assess the effects of low prevalence for methylation on model fit. Because the exact results were similar to the large-sample methods that allowed the inclusion of age as a continuous variable, only the large-sample results are given. All analyses were conducted in SAS version 8.02 (SAS Institute, Inc., Cary, NC) or LogXact version 6.0 (Cytel Software Corporation, Cambridge, MA).

Results

Sputum cytology and DNA recovery from plasma. Sputum adequacy, defined as the presence of deep lung macrophages or Curschmann’s spiral (27), was observed for 77% and 97% of specimens collected from lung cancer survivors and cancer-free smokers, respectively. Persons with sputum inadequacy were mostly former smokers (85%). Mild atypia was seen in sputum from three current smokers, whereas no cytologic abnormalities were evident in sputum from lung cancer survivors. The median amount of DNA recovered from blood ranged from 30 to 45 ng/mL (Table 1). The amount of DNA in plasma was lower in lung cancer survivors than never smokers and smokers. The range of DNA varied by three orders of magnitude within each study group.

Gene promoter methylation in plasma. Methylation of p16 was seen in plasma DNA from 7 of 74 women who never smoked, whereas only 2 women were positive for methylation of either MGMT or RASSF1A (Table 2). The plasma DNA from one never smoker contained methylated alleles for p16 and MGMT. The prevalence for methylation of p16, MGMT, and RASSF1A generally increased as a function of increasing risk for lung cancer (Table 2). The largest difference in prevalence across risk groups was seen for the MGMT and p16 genes, where lung cancer survivors had a 5.0- and 3.2-fold increased odds for methylation of these genes in their plasma DNA compared with never smokers. Overall, lung cancer survivors also showed a significant increase in risk for having at least one or more genes methylated [odds ratio (OR), 3.6; 95% confidence interval (95% CI), 1.9-9.1] than never smokers. No association was seen between the detection of gene promoter methylation and ethnicity or the amount of freely circulating DNA in plasma (P > 0.4 for each group).

Gene promoter methylation in sputum. The availability of DNA recovered from sputum allowed us to extend our methylation studies to also examine the promoters of the DAPK, H-cadherin, PAX5α, and PAX5β genes in smokers and lung cancer survivors. The prevalence for methylation of the MGMT, RASSF1A, DAPK, and PAX5α genes in sputum were increased significantly in lung cancer survivors compared with smokers (Table 3). A 2.6- to 5.0-fold increase in odds for methylation of these gene promoters was observed after adjustment for age and smoking duration. Examining multiplicity for gene promoter methylation revealed even greater differences between these two risk groups. Lung cancer survivors had a 6.2-fold greater odds (95% CI, 2.1-18.5) for methylation of three or more genes in their sputum compared with smokers after adjustment for age and smoking duration (Table 3). Restricting the comparison of multiplicity to the five genes with OR >2.0 revealed that lung cancer survivors had a 21-fold greater odds (95% CI, 5.5-80.1) for methylation of three or more genes in their sputum compared with smokers after adjustment for age and smoking duration. The detection of methylation in lung cancer survivors was not associated with ethnicity and was independent of sputum adequacy (not shown).

Comparison of methylation in sputum versus plasma. Methylation of the p16, MGMT, and RASSF1A genes was generally more common in sputum than plasma (Fig. 1). The prevalence for RASSF1A and MGMT methylation was significantly greater in sputum than plasma from lung cancer survivors, whereas p16 methylation occurred more frequently in sputum than in plasma from smokers. The odds for methylation of at least one gene in sputum or plasma in lung cancer survivors relative to smokers was similar (OR, 2.3; 95% CI 1.2-4.5; P = 0.02 for sputum versus OR, 2.0; 95% CI, 0.9-4.2; P = 0.07 for plasma). In addition, methylation of these genes was independent with respect to their detection in sputum versus plasma. For example, it was uncommon to see methylation of p16 in both sputum and plasma from the same person.

Discussion

The results of this study show that concomitant methylation of multiple gene promoters in sputum is strongly associated with lung cancer risk. In this study, lung cancer survivors whose risk for new or recurrent lung cancer is ~6% per patient year

<table>
<thead>
<tr>
<th>Table 2. Prevalence and odds for gene promoter hypermethylation in plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gene</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>p16</td>
</tr>
<tr>
<td>MGMT</td>
</tr>
<tr>
<td>RASSF1A</td>
</tr>
<tr>
<td>≥1 gene</td>
</tr>
</tbody>
</table>

NOTE: Data are adjusted for age.  
Abbreviation: Pos, positive.  
*Reference group.  
†Methylated for one or more genes in the same plasma sample; reference group is zero genes methylated.
showed a 6.2-fold greater odds of having three or more genes methylated in their sputum compared with cancer-free smokers. Interestingly, the percentage of lung cancer survivors and smokers with three or more methylated genes in their sputum (38% and 11%, respectively) approximates the proportion of people who will have a recurrent lung cancer or initial lung cancer diagnosed, respectively (1, 2, 5–7).

Critical to the development of a marker panel for early detection of lung cancer is the selection of genes whose methylation is common but occurs during different stages of lung cancer development. Inactivation of the $p16$ gene is likely one of the earliest methylation events in lung cancer development, occurring in the bronchial epithelium of some current and former smokers (11). This may, in fact, account for the similarity in prevalence for methylation of this gene in sputum from lung cancer survivors and smokers. Prior studies restricted to examining methylation of $p16$ alone or in conjunction with MGMT have shown an association with methylation of this gene in sputum and the development of lung cancer (8, 28). It is not surprising that the prevalence for methylation in sputum from lung cancer survivors was substantially lower than the 80% prevalence seen in our previous study of incident and prevalent squamous cell carcinoma (8). In that study, 13 of 21 cases had a history of smoking and exposure through uranium mining to radon that deposits in the upper airways of the lung. The very high prevalence for methylation of $p16$ in that study was likely in part, to the presence of tumor DNA in sputum from prevalent cases and the recent association between forms of high linear energy radiation and targeting of $p16$ for inactivation by methylation (29). In contrast, only 6 of 56 (11%) lung cancer survivors had a diagnosis of squamous cell carcinoma and the sputum collection occurred 6 to 36 months after surgery. The 19% prevalence in this group is lower than predicted by some of our other work (11), but this could be due to the smaller sample size examined compared with the cancer-free smokers.

Inactivation of MGMT, DAPK, and RASSF1A all seem to be later events in lung cancer than $p16$ inactivation. Inactivation of MGMT is rare (8%) in hyperplasia, metaplasia, and dysplasia within the central airways, but increases in prevalence between stage I adenocarcinomas and stages I to IV (20). Similarly, methylation of the DAPK and RASSF1A genes also is uncommon (3% and 0%) in bronchial epithelium from smokers (11). Methylation of DAPK has been detected in alveolar hyperplasias in a murine model of lung adenocarcinoma, supporting a role for this gene in the progression of carcinogenesis (30). The odds for methylation of all three of these genes increased significantly in sputum from lung cancer survivors compared with smokers. A previous study suggested that methylation of RASSF1A may be a good marker for lung cancer because its presence predicted disease in three of five people positive for methylation of this gene, 12 to 14 months after diagnosis.

### Table 3. Prevalence and odds for gene promoter hypermethylation in sputum

<table>
<thead>
<tr>
<th>Gene</th>
<th>Lung cancer survivors (n = 53)</th>
<th>Smokers (n = 118)</th>
<th>OR (95% CI) *</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. Pos (%)</td>
<td>No. Pos (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$p16$</td>
<td>10 (19)</td>
<td>30 (25)</td>
<td>0.5 (0.2-1.3)</td>
<td>0.18</td>
</tr>
<tr>
<td>MGMT</td>
<td>19 (36)</td>
<td>17 (14)</td>
<td>2.6 (1.2-5.9)</td>
<td>0.02</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>13 (25)</td>
<td>8 (7)</td>
<td>3.8 (1.4-10.8)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>DAPK</td>
<td>25 (47)</td>
<td>21 (18)</td>
<td>4.1 (1.9-8.7)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>H-cadherin</td>
<td>7 (13)</td>
<td>34 (29)</td>
<td>0.4 (0.2-1.1)</td>
<td>0.08</td>
</tr>
<tr>
<td>PAX5α</td>
<td>21 (40)</td>
<td>14 (12)</td>
<td>5.0 (2.1-11.8)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>PAX5i</td>
<td>13 (25)</td>
<td>11 (9)</td>
<td>2.5 (1.0-6.4)</td>
<td>0.06</td>
</tr>
<tr>
<td>Multiplicity †</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 genes</td>
<td>8 (15)</td>
<td>41 (35)</td>
<td>1.0 (reference)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>1 gene</td>
<td>12 (23)</td>
<td>41 (35)</td>
<td>1.4 (0.5-4.0)</td>
<td></td>
</tr>
<tr>
<td>2 genes</td>
<td>13 (25)</td>
<td>23 (19)</td>
<td>3.0 (1.0-8.9)</td>
<td></td>
</tr>
<tr>
<td>≥3 genes</td>
<td>20 (38)</td>
<td>13 (11)</td>
<td>6.2 (2.1-18.5)</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Data are adjusted for age and smoking duration.

*Reference group is Smokers.

† Refers to the number of genes methylated in an individual sputum sample.

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Fig. 1. Comparison of methylation of the $p16$, MGMT, and RASSF1A genes in sputum (S) and plasma (P) from former lung cancer patients and smokers. The prevalence for methylation (%) of these three genes was determined by the nested, methylation-specific PCR assay. *P < 0.05 and **P < 0.01 when comparing methylation prevalence in sputum to plasma.
after sputum collection (31). The PAX5x and PAX5β genes function as nuclear transcription factors important for cellular differentiation, migration, and proliferation (32). We have shown previously that inactivation of PAX5β likely contributes to neoplastic development by inhibiting expression of CD19, a gene shown to negatively control cell growth (23). Although the timing for inactivation of PAX5x and PAX5β in lung cancer development is unknown, the fact that the prevalence for methylation of these genes was increased in lung cancer survivors compared with smokers supports a role for these genes in the progression of preinvasive cancer.

This study is the first to detect gene promoter methylation in blood from never smokers. This finding is likely due to the use of more adequate amounts of DNA and the increased sensitivity of the nested methylation-specific PCR assay. The detection of methylation in DNA recovered from never smokers was not surprising given the risk for age-related cancers. The 10 never smokers with positive methylation in their blood reported medical problems that included cervical cancer, ovarian cancer, and peptic ulcer. The one woman with two methylated genes in plasma had a history of hepatic disease. Recently, methylation of p16 has been associated with hepatitis virus infection in people who developed hepatocellular carcinoma (33). Methylation of the p16 gene has also been detected in 40% of ovarian cancer (34). Therefore, it is clear that medical history may confound the use of DNA recovered from plasma for predicting lung cancer. In spite of this obstacle, a significant difference in risk for methylation in blood was seen for lung cancer survivors compared with never smokers. This difference in methylation could reflect the high risk for tumor recurrence seen in lung cancer survivors. Blood may still be a viable fluid for early detection or predicting tumor recurrence if marker panels more specific for lung cancer can be developed. A gene like p16, which is both an early and common alteration in many malignancies, may not be a good marker for early detection in blood.

In this study, little agreement was seen between the detection of methylation of a specific gene in sputum and blood. This, again, is not surprising given the different anatomic compartments providing the DNA source and the contribution of other diseases to the DNA pool recovered in blood. It is presumed that the release of DNA into the circulation requires efficient vascularization of the tumor or premalignant lesion. If this is the case, then, screening of plasma would have a low sensitivity for detecting preinvasive lesions early enough to improve the effects of therapeutic intervention. Recently, a study by Fujiiwara et al. (20) reported the detection of gene methylation in serum from 50% of stage I lung cancer patients. In that study, methylation of more than one gene was uncommon (13%) in lung cancer patients independent of stage; however, 12% of cancer-free smokers showed methylation of one gene in their serum. The higher prevalence for methylation (22%) seen in our smokers is likely due to the greater sensitivity of the nested methylation-specific PCR assay and amount of plasma/serum collected (10 versus 2 mL). A more precise estimate of the sensitivity and specificity for methylation detection in blood will require a nested, case-control study that examines gene methylation in blood collected before cancer diagnosis.

Irrespective of the outcome from that study, methylation detection in blood could be very powerful for monitoring disease recurrence in individuals with a diagnosed cancer. It is also unlikely that a sputum assay would specifically detect a small peripheral adenocarcinoma because such a lesion would only contribute a small number of cells into a heterogeneous sputum sample collected from that individual. Rather, it is our hypothesis that as field carcinization progresses, more methylated genes will be detected in the sputum sample and this will indicate an increased probability for early lung cancer (35). The 6-fold increase seen for methylation of three or more genes in a subset of lung cancer survivors supports this hypothesis and substantiates the potential power of methylation as a biomarker that could impact the lives of millions of people who are at risk for this disease worldwide.

Acknowledgments

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References


Clinical Cancer Research

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