Multivitamins, Folate, and Green Vegetables Protect against Gene Promoter Methylation in the Aerodigestive Tract of Smokers

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Abstract

One promising approach for early detection of lung cancer is by monitoring gene promoter hypermethylation events in sputum. Epidemiologic studies suggest that dietary fruits and vegetables and the micronutrients they contain may reduce risk of lung cancer. In this study, we evaluated whether diet and multivitamin use influenced the prevalence of gene promoter methylation in cells exfoliated from the aerodigestive tract of current and former smokers. Members (N = 1,101) of the Lovelace Smokers Cohort completed the Harvard Food Frequency Questionnaire and provided a sputum sample that was assessed for promoter methylation of eight genes commonly silenced in lung cancer and associated with risk for this disease. Methylation status was categorized as low (fewer than two genes methylated) or high (two or more genes methylated). Logistic regression models were used to identify associations between methylation status and 21 dietary variables hypothesized to affect the acquisition of gene methylation. Significant protection against methylation was observed for leafy green vegetables [odds ratio (OR) = 0.83 per 12 monthly servings; 95% confidence interval (95% CI), 0.74–0.93] and folate (OR, 0.84 per 750 μg/d; 95% CI, 0.72–0.99). Protection against gene methylation was also seen with current use of multivitamins (OR, 0.57; 95% CI, 0.40–0.83). This is the first cohort-based study to identify dietary factors associated with reduced promoter methylation in cells exfoliated from the airway epithelium of smokers. Novel interventions to prevent lung cancer should be developed based on the ability of diet and dietary supplements to affect reprogramming of the epigenome. Cancer Res; 70(2); 568–74. ©2010 AACR.

Introduction

Lung cancer, the leading cause of cancer-related death in the United States, occurs largely from chronic exposure to tobacco carcinogens (1). The development of this disease over 30 to 40 years involves field cancerization, characterized as the acquisition of genetic and epigenetic changes throughout the respiratory epithelium (2, 3). The silencing of genes through promoter hypermethylation is now recognized as a major and causal epigenetic event that occurs during lung cancer initiation and progression to affect the function of hundreds of genes. Gene silencing involves methylation of cytosines in the gene promoter region, recruitment of transcriptional corepressors, and modification of histone tails that culminate in the establishment of chromatin modifications that block transcription (4, 5). Genes involved in all aspects of normal cell function, such as regulating the cell cycle, differentiation, adhesion, and death, are silenced in lung tumors (3). Importantly, the tumor suppressor gene p16, which plays a critical role in regulating the cell cycle, is not only commonly silenced by methylation in lung cancer but also inactivated early in the development of this disease. Silencing of p16 and other genes is detected in bronchial epithelium of smokers and in precursor lesions to adenocarcinoma and squamous cell carcinoma and increases during disease progression, substantiating a vital role for gene silencing in lung cancer etiology (6–8).

Based on the silencing of key tumor suppressor genes in the lungs of smokers, we hypothesized that the detection of genespecific promoter hypermethylation in exfoliated cells in sputum would provide an assessment of the extent of field cancerization that in turn may predict early lung cancer. This hypothesis has been validated in two studies, the first detecting methylation of the p16 and MGMT genes in sputum up to 3 years before clinical diagnosis of squamous cell carcinoma (9). The second study, a nested, case-control study of incident lung cancer cases from a high-risk cohort, identified six genes associated with >50% increased lung cancer risk. Importantly,
concomitant methylation of three or more of these six genes was associated with a 6.5-fold increased risk of incident lung cancer and sensitivity and specificity both at 64% (10). These studies suggested that gene promoter hypermethylation in sputum could be used as a molecular marker for identifying people at high risk for cancer incidence (11). However, the precise mechanism by which carcinogens disrupt the capacity of cells to maintain the epigenetic code during DNA replication and repair is largely unknown.

The fact that gene promoter methylation is a promising marker for lung cancer makes understanding factors that influence the propensity for this epigenetic process throughout the respiratory epithelium a high priority because such knowledge could be used not only for early detection but also to identify persons who would benefit most from chemoprevention. The precise mechanism by which carcinogens disrupt the capacity of cells to maintain the epigenetic code during DNA replication and repair is largely unknown. Carcinogens within tobacco induce single- and double-strand breaks in DNA, and reduced DNA repair capacity (DRC) has been associated with lung cancer (12). Accumulating evidence from our group suggests that extensive DNA damage could be responsible for acquisition of gene promoter hypermethylation during lung carcinogenesis (3, 13). Strong support for this supposition was provided through a recent community-based study in which a highly significant association was seen between DRC and sequence variants within specific DNA repair genes and the propensity for methylation of genes detected in sputum from cancer-free smokers from the Lovelace Smokers Cohort (14). Specifically, smokers with a high methylation index (defined by having three or more genes methylated from an eight-gene panel in sputum) had a 50% reduction in DRC compared with smokers with no genes methylated in sputum. Single nucleotide polymorphisms within five double-strand break DNA repair genes were also highly associated with methylation index. This study suggests that chronic DNA damage coupled with reduced DRC could be an important determinant for inducing gene promoter hypermethylation.

Epidemiologic studies suggest that select dietary nutrients and vitamin supplements might protect against lung cancer (15). Fruits, vegetables, and multivitamins all possess antioxidant activity that should reduce tobacco-induced DNA damage. In addition, folate, a B vitamin, is metabolized to adenosylmethionine (SAM), a universal donor for reactions that include methylation of DNA (16). Low folate has been associated with reduced DRC and an increase in prevalence for gene promoter methylation (17, 18). The purpose of the current investigation was to determine whether diet and multivitamin use influence the presence of methylation in cells exfoliated from the aerodigestive tract of current and former smokers. Composite variables were selected based on our hypotheses that fruits, tomatoes, cruciferous vegetables, leafy vegetables, yellow vegetables, and vitamin intake will be associated with a reduction in number of genes methylated in sputum, whereas animal fat and red and processed meat will be associated with increased methylation.

Materials and Methods

Study population. The Lovelace Smokers Cohort began recruitment of female smokers in 2001 and expanded to include male smokers in 2004 (14). Enrollment, which is still ongoing, is restricted to current and former smokers ages 40 to 75 y with a minimum of 15 pack-years of smoking. Participants primarily are residents of the Albuquerque, New Mexico metropolitan area. Participants complete a standard questionnaire covering demographics, smoking history, personal and family health, and a food frequency questionnaire. Weight and height are measured. Participants provide both blood and sputum samples and undergo standard pulmonary function testing. A total of 1,145 people completed a food frequency questionnaire and were assessed for prevalence for methylation of eight genes in sputum. Those with caloric intake outside of gender-specific bounds (n = 44) were excluded, resulting in a total of 1,101 participants (845 women and 256 men) in this study. All participants signed a consent form, and the Western Institutional Review Board approved this project.

Dietary questionnaire. Participants completed the adult version of the Harvard University Food Frequency Questionnaire Dietary Assessment form, a self-administered instrument that includes ~150 food items (19). The participant indicates consumption frequency for most food items by choosing 1 of 5 to 10 categories that vary depending on the food item and can range from never to six or more servings per day. A food group analysis was conducted to combine food items to obtain estimates of intake of macronutrients and micronutrients. We focused on factors known or suspected of being associated with lung cancer or methylation. Thus, the macronutrients animal fat and total fat and the micronutrients vitamin C, vitamin E, folate (that included supplements and fortified foods), carotene, α-carotene, β-carotene, lycopene, lutein and zeaxanthin, and retinol were examined. Alcohol, multivitamins, and cod liver oil intake were also assessed as categorical variables. In a study of eating patterns, Fung and colleagues (20) created a set of 38 composite variables using the food frequency questionnaire. Six of these composite variables, red and processed meats, fruit, tomatoes, cruciferous vegetables, leafy green vegetables, and yellow vegetables, were related to our hypotheses, so we calculated these variables.

Total caloric intake was assessed. People with either extremely low (n = 8) or extremely high (n = 36) intake were excluded. Cutoffs for extremely low intake were <500 and <800 calories for women and men, respectively. Extremely high intake was defined as >3,500 and >4,200 calories for women and men, respectively. It is standard to exclude participants with at least 70 missing items on the food frequency questionnaire, but none of our participants met this criterion. Participants with missing data on individual food items were excluded from analyses of these items.

Methylation-specific PCR. Eight genes (p16, MGMT, DAPK, RASSF1A, PAX5a, PAX5β, GATA4, and GATA5) were selected for analysis of methylation in sputum based on our previous studies establishing their association with risk for lung
cancer (10, 21). DNA was isolated from sputum and modified with bisulfite as described (10). Nested methylation-specific PCR was used to detect methylated alleles from individual genes in DNA recovered from the sputum samples as described (21). Methylation index, the number of genes methylated in a sputum sample, was also defined. Sputum from males and females was randomly selected and included in batches of 96 samples for assessment of gene methylation. A Hamilton robot was used to assemble PCRs in 96-well plates.

**Covariates.** The questionnaire included questions on gender, age, ethnicity, and smoking. Cigarette smoking history included current status (former or current), pack-years, and duration of smoking. Body mass index (BMI) was calculated from measured height and weight and categorized as normal (<25 kg/m²), overweight (25–29.9 kg/m²), and obese (≥30 kg/m²). Age was categorized as 40 to 54, 55 to 64, and ≥65 y. Pack-years of smoking were categorized as light (<29), moderate (30–49), and heavy (≥50).

**Statistical methods.** Demographic, dietary, and methylation variables were summarized overall and by gender. Proportions were used for categorical variables and medians with the interquartile range (IQR) for continuous variables. Differences between men and women in clinical covariates and categorical dietary variables were assessed with Fisher’s exact test. For continuous dietary variables, a two-step linear regression analysis was used to account for differences in total caloric intake between genders. In the first step, the dietary variable was regressed on the total caloric intake. In the second step, the residuals from the first analysis were regressed on gender, which resulted in an estimate of the difference in the mean dietary variable after adjustment for total caloric intake, along with 95% confidence intervals (95% CI).

The total number of methylated genes in the eight-gene panel was dichotomized into low (fewer than two genes methylated) and high (two or more genes methylated). This binary outcome, methylation status, was modeled with logistic regression. Initially, only the clinical covariates gender, age, BMI, and three smoking variables (status, pack-years, and duration) were assessed. Interactions among the covariates, including interactions with gender, were evaluated. After the development of a model with only clinical covariates, individual dietary variables were included, along with adjustment for total caloric intake. Continuous variables, such as total fat intake, were included as a continuous variable or were categorized into quartiles, with the quartiles defined by gender and the quartile medians used as the predictor values. Interactions between dietary variables and clinical covariates were assessed. Only the 21 dietary variables specific to our hypothesis were examined to reduce the potential for false-negative results. In addition, no formal adjustment for multiple comparisons was made to reduce the chance of false-negative results because this is one of the first studies to examine the association between dietary factors and methylation. However, the issue of examining multiple predictor variables is considered in the interpretation of the results. Methylation index was also used as the outcome variable to further assess the association between methylation and dietary factors. Because the methylation index could theoretically take on nine values, but actually took on only seven, it was unclear that linear regression would be appropriate. Thus, results obtained from linear and ordinal logistic regression were compared. The association between significant dietary factors and each of the individual genes was explored using logistic regression models but viewed as secondary analyses to reduce the issue of multiple comparisons. All statistical analyses were conducted in Statistical Analysis System 9.2.

**Results**

**Population characteristics.** The demographics and smoking history of the 1,101 participants are described in Table 1. The Lovelace Smokers Cohort is largely composed of females (76.7%) and non-Hispanic whites (77.8%). Median age was 56 years, with males slightly older than females. More than half of the participants currently smoked, and median duration of smoking was 33 years. There was no difference between men and women with regard to smoking status or duration, but men had significantly higher pack-years of smoking (median = 39 pack-years for men versus 34 pack-years for women).

**Summary of dietary intake.** The completion rate of the food frequency questionnaire was excellent, as only 2.7% of...
There was no difference in vitamin usage, but men had higher consumption of total calories, but after adjustment for caloric intake, women generally had higher consumption of red and processed meat. There were more likely to report having at least one alcoholic drink per day.

Prevalence for gene promoter hypermethylation in sputum. Methylation of an eight-gene panel that included p16, MGMT, DAPK, RASSF1A, GATA4, GATA5, PAX5α, and PAX5β was evaluated. Methylation of these genes is associated with increased risk for lung cancer (10, 21). The prevalence of methylation ranged from 1.1% and 0.0% for RASSF1A to 33.8% and 51.2% for GATA4 for women and men, respectively (Table 3). Three genes, MGMT, GATA4, and PAX5α, were more frequently methylated among men than women (P < 0.001). Methylation index, the number of methylated genes in each sputum sample, was higher in men than women (median = 2 and 1, respectively; P < 0.001). Our previous study was
Association among women. Age and duration of smoking were increased with pack-years among men, but there was no association was less consistent. The odds of high methylation weight and overweight individuals, whereas for men the association among obese participants than among both normal 0.05). There were relatively more women with high methylation status. Meaningful interactions, including interactions between gender and all of the other covariates, were assessed. There were significant interactions between gender and both BMI and pack-years. 

Association of clinical covariates with gene methylation. Gender, age, BMI, and cigarette smoking history were assessed in a multivariate model for association with methylation status. Meaningful interactions, including interactions between gender and all of the other covariates, were assessed. There were significant interactions between gender and both BMI (P = 0.04) and pack-years of smoking (P = 0.05). There were relatively more women with high methylation among obese participants than among both normal weight and overweight individuals, whereas for men the association was less consistent. The odds of high methylation increased with pack-years among men, but there was no association among women. Age and duration of smoking were not associated with methylation, after adjustment for the other clinical covariates, but age was retained in the model. With two other smoking variables included in the model (pack-years and current status), duration was excluded. Thus, the clinical covariates that were included in the modeling with the nutritional variables were gender, pack-years of smoking, current smoking status, BMI, age, and interactions between gender and both pack-years and BMI.

Association of dietary factors with gene methylation. Each dietary factor was assessed for association with methylation status (two or more genes methylated versus fewer than two genes) using logistic regression. A total of 21 variables were examined: three macronutrients, nine micronutrients, six serving variables, and three categorical nutrients, six serving variables, and three categorical variables (consumption of alcohol, vitamins, and cod liver oil; Table 4; Supplementary Table S1). Leafy green vegetable consumption was significantly associated with reduced risk for high methylation status [odds ratio (OR), 0.83; 95% CI, 0.74–0.93], as was higher folate (OR, 0.84; 95% CI, 0.72–0.99; Table 4). The most striking effect seen was the association between current multivitamin use and methylation status (OR, 0.57; 95% CI, 0.40–0.83; Table 4). The duration of vitamin use was not associated with methylation (data not shown). Moreover, because folate levels were higher for participants taking multivitamins, stratification by vitamin use was also conducted and folate remained significantly associated with methylation. There was a marginal increase in odds for methylation associated with total fat and animal fat that did not reach statistical significance (Supplementary Table S1). None of the other dietary or nutrient predictor variables analyzed were associated with methylation (Supplementary Table S1). Interactions between each dietary variable and gender were assessed, but none was significant. The association between these dietary variables and methylation of the individual genes also was examined, but because of the number of tests (8 genes × 3 dietary variables = 24 tests), these results were viewed as exploratory. Associations with at least one of the three significant dietary variables were observed for DAPK, GATA4, PAX5α, and PAX5β (Supplementary Table S2).

A further analysis was conducted to assess whether any extreme outliers influenced the observed results for

### Table 3. Prevalence of gene methylation in sputum

<table>
<thead>
<tr>
<th>Gene</th>
<th>Overall (N = 1,101)</th>
<th>Females (n = 845)</th>
<th>Males (n = 256)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(% positive)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P16</td>
<td>16.6</td>
<td>16.2</td>
<td>18.0</td>
<td>0.50</td>
</tr>
<tr>
<td>MGMT</td>
<td>26.3</td>
<td>24.4</td>
<td>32.8</td>
<td>0.009</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>0.8</td>
<td>1.1</td>
<td>0.0</td>
<td>0.13</td>
</tr>
<tr>
<td>DAPK</td>
<td>17.8</td>
<td>16.7</td>
<td>21.5</td>
<td>0.09</td>
</tr>
<tr>
<td>GATA4</td>
<td>37.9</td>
<td>33.8</td>
<td>51.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GATA5</td>
<td>18.0</td>
<td>17.0</td>
<td>21.1</td>
<td>0.14</td>
</tr>
<tr>
<td>PAX5α</td>
<td>15.3</td>
<td>13.1</td>
<td>22.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PAX5β</td>
<td>9.6</td>
<td>9.5</td>
<td>10.2</td>
<td>0.72</td>
</tr>
<tr>
<td>≥2 genes methylated</td>
<td>39.8</td>
<td>36.6</td>
<td>50.4</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Comparison of females and males, from Fisher’s exact test.

### Table 4. Dietary variables significantly associated with methylation status in the Lovelace Smokers Cohort

<table>
<thead>
<tr>
<th>Dietary predictor variable</th>
<th>OR* (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leafy green vegetables (per 12 monthly servings)</td>
<td>0.83 (0.74–0.93)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Folate (per 750 μg/d)</td>
<td>0.84 (0.72–0.99)</td>
<td>0.04</td>
</tr>
<tr>
<td>Multivitamin use versus never</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>0.57 (0.40–0.83)</td>
<td>0.01</td>
</tr>
<tr>
<td>Past</td>
<td>0.68 (0.45–1.03)</td>
<td></td>
</tr>
</tbody>
</table>

*ORs are obtained from models with a single dietary variable but including adjustment for gender, age, BMI, pack-years of smoking, current smoking status, total caloric intake, and interactions between gender and both BMI and pack-years of smoking.
continuous variables by defining quartiles by gender and then using the medians within the quartiles as predictors in logistic regression modeling. Similar results were obtained as in the initial analysis, except that total fat was associated with marginally increased methylation (OR, 1.43 per 40 g/d; 95% CI, 1.03–1.99). The same dietary variables were identified to be significant in analyses that used the continuous methylation index as the outcome. In addition, vitamin E showed a protective effect for methylation per gene (OR, 0.98 per 20 mg/d; 95% CI, 0.96–1.00; \( P = 0.04 \)).

**Discussion**

This is the first cohort-based study to systematically evaluate the association between dietary factors and risk for methylation in cells exfoliated from the aerodigestive tract of smokers and former smokers. Our findings support a significant, biologically plausible role for leafy green vegetables, folate, and multivitamin use in protection against the acquisition of gene promoter methylation.

There has been considerable interest and debate for decades about the effect of diet and vitamins on the risk for cancer. Recent large epidemiologic studies along with functional investigations are beginning to provide a clearer picture as to the dietary variables that may influence risk for cancers such as lung where a clear causative environmental exposure in the form of smoking has been established. Reduced folate intake has been associated with increased risk for lung cancer in current and former smokers (22). A link between folate and gene methylation exists through the role of 5-methyltetrahydrofolate in providing methyl groups for SAM, a key methyl acceptor in the methylation of DNA. Higher folate has been associated with a lower prevalence for methylation of individual and total number of genes in colorectal tumors (17). This finding was validated in a second study of colorectal tumors in which folate was inversely associated with gene-specific promoter hypermethylation (23). Our study shows for the first time that the acquisition of gene promoter methylation throughout the airway epithelium is influenced by folate. The biological mechanisms related to low folate and hypermethylation are still unclear; however, Jhaveri and colleagues (24) suggested that folate deficiency leads to increased levels of SAM and S-adenosylhomocysteine (SAH), an inhibitor of SAM. The increase in free intracellular SAM could contribute to gene-specific hypermethylation if an absolute level of SAH needed to regulate SAM is not maintained. Folate is also known to affect DRC, affect the propensity for methylation. Green leafy vegetables were the only food item in this analysis to exhibit protection against methylation status. Leafy vegetables are rich in phytochemicals such as vitamin C, carotenoids, lutein, and folic acid in addition to vitamins A and K. A comprehensive and systematic review of the literature up to 2007 by the World Cancer Research Fund (WCRF) concluded that probable evidence existed for reduction of lung cancer risk among persons with higher intake of fruits, whereas evidence was inconclusive about green leafy vegetables (15). However, a recent hospital-based case-control study of lung cancer (25) showed a strong protective effect of green leafy vegetables (OR, 0.5; 95% CI, 0.3–0.81). The lack of an effect of cruciferous vegetable on methylation status in our study is not surprising because lung cancer observational studies report only modest effects that may be influenced by genetic variation (26). In addition, the lack of association with red meat and processed meat intake is consistent with the inconclusive evidence, as summarized by the WCRF (15).

In our study, strong protection against gene methylation was also associated with the use of multivitamins that contain some of the same agents as leafy green vegetables. Although a clear connection between vitamin supplements and risk of lung cancer has not been established (28), vitamin supplementation has been associated with reduction in DNA damage by benzo(\( \alpha \))pyrene, a major tobacco carcinogen (29–32). The silencing of genes by promoter hypermethylation is now well established as a major component of lung cancer initiation and progression and has emerged as a potential disease marker for early detection. The ability to affect reprogramming of the epigenome through diet and chemopreventive supplements could significantly affect mortality from lung cancer. This study has identified two dietary variables, leafy green vegetables and folate, along with multivitamin use that could help reduce the incidence of lung cancer by reducing the induction of methylation in the aerodigestive tract of smokers.

**Disclosure of Potential Conflicts of Interest**

S.A. Belinsky is a consultant and has licensed intellectual property with Oncomethylome Sciences. The other authors disclosed no potential conflicts of interest.

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