Lung cancer is the leading cause of cancer deaths in the United States, accounting for 28% of all cancer deaths and ~157,200 deaths every year (1). Unfortunately, 36% of non–small cell lung cancer cases are detected at an advanced stage often after micrometastasis has developed, leading to an alarmingly low 5-year survival rate of only 14.9% (1). Because nearly 87% of lung cancer cases are due to smoking, yet only 10% of smokers develop lung cancer, it may be worthwhile to screen for susceptible individuals to administer effective preventive measures (1, 2). Thus, the establishment of a combination of early diagnostic markers that could be analyzed in clinical samples obtained using relatively noninvasive procedures could become an asset to efficient detection of changes in preneoplastic tissue before tumor formation and metastasis can occur.

Differential DNA methylation at CpG islands has been associated with regulation of gene expression and is essential for normal development, X-chromosome inactivation, imprinting, suppression of parasitic DNA sequences, and cancer (3–5). Aberrant differential methylation of CpG islands in the promoter region of genes that are implicated in different roles including carcinogen activation or detoxification (CYP1A1 and GSTP1), tumor suppression (p14, p15, p16, p73, APC, and BRCA1), DNA repair (hMLH1 and MGMT), and metastasis and invasion (CDH1, ECAD, TIMP1, and DAPK) occurs in several cancers, including lung cancer (3–10). Thus, the DNA methylation status of critical genes is not only ideal for use as diagnostic markers but also as therapeutic targets for lung cancer. In this study, we chose to analyze ECAD, p16, DAPK, MGMT, GSTP1, and SMAD8 based on their likely involvement in the genesis of lung cancer. The loss of expression of E-cadherin (ECAD), a Ca2+-dependent adhesion molecule responsible for mediating intercellular contacts in morphogenesis and tissue structure maintenance, has been implicated in a number of cancers, including lung cancer (11). Death-associated protein kinase (DAPK) is involved in DNA damage–induced apoptosis and has been shown to be inactivated via hypermethylation in a number of studies involving non–small cell lung cancer (12, 13). Glutathione S-transferase pi (GSTP1), a gene involved in the detoxification of xenobiotics and oxygen radicals, has been shown to be frequently hypermethylated in...
glandular cancers such as prostate, breast, and liver cancers but rarely in lung cancer (14).

One of the extensively studied examples for promoter methylation associated decrease in transcription is the tumor suppressor gene p16 (15–19). Alterations in p16 occur frequently in lung and in most common forms of human cancers including gastric, head and neck, and breast cancers and in leukemia. In general, point mutations in the p16 gene are rare and the loss of p16 gene function occurs frequently via transcriptional silencing associated with abnormal DNA methylation of the transcription start site region. O6-Methylguanine-DNA methyltransferase (MGMT) is a DNA repair gene that removes adducts from the O6 position of guanine, hence providing protection from alkylating agents. It has recently been shown to be hypermethylated in lung cancers including gastric, head and neck, and breast cancers and frequently in lung and in most common forms of human glandular cancers such as prostate, breast, and liver cancers but rarely in lung cancer (14).

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The final study was performed to verify and validate the observations made in the initial study. In summary, we investigated the methylation status of a select group of genes to determine whether we could find unique combinations of methylated genes that could identify distinct stages of non–small cell lung cancer progression providing early diagnostic and therapeutic markers.

### Materials and Methods

**Subject enrollment and tissue collection.** This study, approved by the Boston University Medical Center Institutional Review Board, recruited volunteers who were nonsmokers, former and current smokers, and those suspected of having lung cancer. Subjects (n = 27) provided information on smoking history and family cancer incidence. Subjects were asked to undergo a bronchoscopy in order to collect bronchial brushings in addition to providing 15 mL of blood.

**Tumor specimens.** Primary lung carcinoma tissues and pretreatment blood were collected from participants who consented and enrolled with institutional review board approval from the Johns Hopkins Hospital. Primary tumors were snap frozen immediately after resection until further analysis.

**Genomic DNA Isolation.** Blood samples were allowed to sit in the heparinized tube for 45 to 60 minutes at 37°C to allow for separation of serum and dark red blood. The upper layer containing serum and peripheral blood lymphocytes was combined with 1 mL PBS in a 15-mL tube and centrifuged at 1200 rpm for 25 minutes to pellet the lymphocytes. Genomic DNA isolated from the lymphocytes/microdissected tumor was routinely resuspended in genomic DNA sample buffer [100 mmol/L NaCl, 25 mmol/L EDTA (pH 8). 10

### Table 1. Primers used for MSP

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Annealing temperature (°C)</th>
<th>Major product size (bp)</th>
<th>Cycles</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>p16</td>
<td>MF: 5'TTATTAGAGGGTGCCGGATGC-3' MR: 5'GACACCCGAACCGCCAGCTAA-3' UF: 5'TTATTAGAGGGTGCCGGATGC-3' UR: 5'CAACCCCAACCCAAATTTCA-3'</td>
<td>65</td>
<td>150 (M)</td>
<td>36</td>
<td>(19)</td>
</tr>
<tr>
<td>MGMT</td>
<td>MF: 5'TTTCGACGTTGTTAGTTTTCG-3' MR: 5'GACTCTTCGAAACCCAGAAG-3' UF: 5'TTTCGTGTTTTGATGTTTGTAGGTTTTTGT-3' UR: 5'AAATCCACACTCTTTTCCAA-3'</td>
<td>66</td>
<td>81 (M)</td>
<td>31</td>
<td>(19)</td>
</tr>
<tr>
<td>ECAD</td>
<td>MF: 5'TGGGGCGGTGTCGTTAGTCT-3' MR: 5'CTCACAAATACTTTACAAATCCGACG-3' UF: 5'GGGAGATGTTGAGATTTAGTTT-3' UR: 5'AAATCCACCACTCTTTTCCAA-3'</td>
<td>62</td>
<td>173 (M)</td>
<td>33</td>
<td>(19)</td>
</tr>
<tr>
<td>DAPK</td>
<td>MF: 5'GGAGATGCTCGGATCGTGTTAA-3' MR: 5'CCTCTTCCTAAAACCGCC-3' UF: 5'GGGAGATGTTGAGATTTAGTTT-3' UR: 5'AAATCCACACTCTTTTCCAA-3'</td>
<td>62</td>
<td>100 (M)</td>
<td>33</td>
<td>(18)</td>
</tr>
<tr>
<td>GSTPI</td>
<td>MF: 5'TTGGCGGTGTCGTTAGTCT-3' MR: 5'GACCCAATTTAATCTACGAG-3' UF: 5'GGGAGATGTTGAGATTTAGTTT-3' UR: 5'AAATCCACACTCTTTTCCAA-3'</td>
<td>60</td>
<td>91 (M)</td>
<td>30</td>
<td>(14)</td>
</tr>
<tr>
<td>SMAD8</td>
<td>MF: 5'GAGGAGGAGGGATTTACG-3' MR: 5'GACCGACGTACCGGAAAACCTCGG-3' UF: 5'GGGAGATGTTGAGATTTAGTTT-3' UR: 5'CAACCCCAACCCAAATTTCA-3'</td>
<td>64</td>
<td>212 (M)</td>
<td>31</td>
<td>(21)</td>
</tr>
</tbody>
</table>

Abbreviations: M, methylated; U, unmethylated; F, forward; R, reverse.
mmol/L Tris-Cl (pH 8), and 0.5% SDS) and digested with protease K in 0.1% SDS. Overnight incubation at 58°C was followed by standard phenol-chloroform extraction and sodium acetate and ethanol precipitation.

Bronchial DNA samples were isolated from the DNA fraction of the RNA isolation procedure using the Trizol (Invitrogen, Carlsbad, CA) method and further processed by three sodium citrate washes in 10% ethanol. After the final wash, DNA was suspended in 75% ethanol, centrifuged at 2000 × g for 5 minutes, air-dried, and dissolved in 8 mmol/L NaOH.

Methylation-specific PCR. Genomic DNA was chemically modified with sodium bisulfite, which converts all unmethylated cytosines to uracil while all methylated cytosines remain unchanged. Briefly, 0.5 to 1.0 μg of genomic DNA was treated with 2 mol/L NaOH. After a 10-minute incubation at room temperature, samples were treated with 10 mmol/L hydroquinone and 3 mol/L sodium bisulfite and incubated for 16 to 20 hours at 50°C. DNA was purified using the Wizard DNA Purification System (Promega, Madison, WI) according to the manufacturer’s protocol. Samples were then desulfonated with 3 mol/L NaOH, precipitated with ammonium acetate, ethanol, and glycogen, and resuspended in distilled H2O.

Primer sequences, designed to amplify specifically methylated or unmethylated CpG islands in the promoter region, are listed in Table 1. PCR amplification was carried out using ~50 ng of treated DNA template, 300 ng forward and reverse primers, 0.4 μmol of 25 mmol/L deoxynucleotide triphosphates, 2.5 μmol/L Taq polymerase (Invitrogen), and 1.5 μL DMSO. PCR conditions were as follows: 94°C for 2 minutes, 30 to 35 cycles (gene dependent) at 94°C for 30 seconds, 60°C to 66°C (see Table 1) for 40 seconds, 70°C for 40 seconds followed by a final extension at 70°C for 10 minutes. The methylation-specific PCR (MSP) products were routinely analyzed by gel electrophoresis in a 6% mini-acrylamide gel, stained with ethidium bromide, and visualized under UV light.

Results and Discussion

Methylation-specific PCR was used to evaluate the promoter DNA methylation status of six genes in samples derived from 27 bronchial epithelial cells and matching blood samples from 22 former/current smokers and five nonsmokers as well as 49 primary non–small cell lung cancer samples with matching normal controls.

We screened likely preneoplastic (smoke-exposed bronchial epithelial cells) and neoplastic (primary lung tumors) lesions with their matching blood samples to evaluate the methylation status of six selected genes (ECAD, p16, DAPK, MGMT, GSTP1, and SMAD8) of interest using MSP analyses. Figure 1A illustrates representative examples of the MSP assays that were used in our studies. We wished to identify methylation patterns in smoke-exposed epithelium before any detectable presence of lung cancer by comparing it to primary lung tumors. In examining these differences, we hoped to find specific genes as targets for inactivation through DNA methylation that could eventually be used as early diagnostic markers and therapeutic targets. In addition, by combining our methylation data with that of genetic alterations, it is our hope to identify a minimal set of markers to provide a highly accurate determination of genetic predisposition to and/or the extent of the progression/spread of lung cancer at the time of diagnosis.

Methylation in preneoplastic bronchial epithelium and blood. We analyzed the bronchial epithelium and blood of the 27 subjects representing the preneoplastic stage, composed of both smokers and nonsmokers for altered promoter DNA methylation patterns (Table 2). Aberrant methylation of ECAD was detected in 27% of smokers’ bronchial epithelium (n = 22) and in 41% of smokers’ blood. Interestingly, no methylation was found for ECAD in either the blood or bronchial epithelium of nonsmokers (n = 5; data not shown). Similarly, 35% of smokers’ bronchial epithelium and 41% of smoker’s blood exhibited hypermethylation for DAPK, whereas the remaining samples from nonsmokers were unaffected. Surprisingly, only one sample of the smokers’ bronchial epithelial cells and one from the smokers’ blood was methylated for p16, whereas one bronchial epithelial cell sample and two blood samples exhibited methylation for MGMT. Methylation of nonsmoker
samples remained consistent in that no methylation was observed. GSTP1 and SMAD8 were rarely or never methylated in both smokers and nonsmokers (data not shown).

In summary, analysis of the four frequently methylated sites (DAPK, ECAD, p16, and MGMT) in this study revealed that smoker blood is methylated in at least one site in 64% of samples, whereas bronchial epithelium is methylated 41% of the time. Alternatively, nonsmoker samples showed methylation in zero of four sites, 100% of the time (n = 5). Although a recent report suggest that one can detect a low level of DAPK methylation in normal lymphocytes using quantitative MSP, in the current study, using standard MSP, we clearly detected methylation only in the blood and bronchial epithelial cell samples from the smokers and not in the blood and bronchial epithelial cell samples of nonsmokers (data not shown), which has also been confirmed in other previous reports (13, 22, 23).

Differential DNA methylation patterns could be used to distinguish preneoplastic cells from tumors. ECAD and DAPK exhibited statistically insignificant differences in their levels of methylation among the tumor and bronchial epithelial cell and blood samples from smokers (Table 2). On the contrary, promoter DNA methylation was observed at a relatively higher frequency for the MGMT and p16 genes in tumors when compared with the other test samples (Table 2). Interestingly, similar levels of methylation were observed in bronchial epithelial cells and blood from the smokers for all four genes (ECAD, p16, MGMT, and DAPK), whereas no methylation was detectable for the same genes in nonsmokers’ bronchial epithelial cells and blood (Table 2, Fig. 1B, data not shown). In addition, neither GSTP1 nor SMAD8 exhibited lung cancer-or smoking-related increases in promoter DNA methylation (data not shown).

In summary, our data suggest that ECAD and DAPK are targeted for methylation in the earliest stages of lung cancer, whereas DNA methylation silencing of p16 and hMGMT are likely alterations that occur in the later stages of cancer progression and are often diagnostic of advanced lung cancer (Fig. 2). Interestingly, a recent study reporting the analysis of tobacco smoke–induced murine lung tumors also suggested that promoter DNA methylation of DAPK is an early event in adenocarcinoma development (25). Our data also suggest that when the four genes (ECAD, p16, MGMT, and DAPK) that exhibited differential methylation upon exposure to tobacco smoke or in lung cancer are evaluated, the frequency of methylation in two or more sites could be used as diagnostic of cancer provided that the altered/tumor cells are present in the clinical samples.

Conclusions

We found that DNA methylation changes that affect critical gene expression patterns occur at increments from the initial

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Table 2. The frequency of promoter DNA hypermethylation in primary lung tumors, bronchial brushings, and blood

<table>
<thead>
<tr>
<th>Gene</th>
<th>Tumor (%)</th>
<th>Control blood from tumor subjects (%)</th>
<th>P*</th>
<th>Smokers’ bronchial epithelial cells (%)</th>
<th>P*</th>
<th>Smokers’ blood (%)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAPK</td>
<td>22/49 (45)</td>
<td>2/49 (4)</td>
<td>0.001</td>
<td>7/22 (35)</td>
<td>0.59</td>
<td>9/22 (41)</td>
<td>0.8</td>
</tr>
<tr>
<td>ECAD</td>
<td>18/49 (37)</td>
<td>11/49 (22)</td>
<td>0.18</td>
<td>6/22 (27)</td>
<td>0.59</td>
<td>9/22 (41)</td>
<td>0.79</td>
</tr>
<tr>
<td>MGMT</td>
<td>18/33 (55)</td>
<td>6/33 (18)</td>
<td>0.004</td>
<td>1/9 (11)</td>
<td>0.03</td>
<td>2/12 (17)</td>
<td>0.04</td>
</tr>
<tr>
<td>p16</td>
<td>21/48 (44)</td>
<td>4/48 (8)</td>
<td>0.001</td>
<td>1/9 (5)</td>
<td>0.003</td>
<td>1/12 (5)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

*P values were computed using Fisher’s exact test by comparing with the frequency of DNA methylation observed in tumors.
stages of lung cancer to late stages of lung cancer (Fig. 2). Our studies identify that promoter DNA methylation at DAPK and ECAD are early events, whereas DNA methylation at p16 and MGMT are late events (Fig. 2). Thus, these and other alterations identified from comprehensive studies could aid in predicting the extent of cancer progression or predisposition to subsequent cancer development. Furthermore, our studies also showed that peripheral lymphocytes from the smokers could potentially substitute for test samples such as bronchial epithelial cells that are acquired by using more invasive methods to detect DNA methylation changes as diagnostic/prognostic markers. Thus, in terms of determining susceptibility to lung cancer at the preneoplastic stage, our findings provide strong preliminary data, which upon confirmation in a larger population study could lead to the use of blood from the smokers as a reliable surrogate for detecting similar changes that could be found in bronchial epithelial cells. Therefore, our findings could have implications for the use of alternate clinical sample sources for diagnostic testing for lung cancer risk and susceptibility and could enable one to secure clinical samples with minimum discomfort to the patient in an economically feasible manner and which are within reach of all those who are likely to be affected.

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References

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