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# An Epigenetic Marker Panel for Detection of Lung Cancer Using Cell-Free Serum DNA

Shahnaz Begum<sup>3</sup>, Mariana Brait<sup>1</sup>, Santanu Dasgupta<sup>1,5</sup>, Kimberly L. Ostrow<sup>1</sup>, Marianna Zahurak<sup>2</sup>, André L. Carvalho<sup>1,6</sup>, Joseph A. Califano<sup>1,4</sup>, Steven N. Goodman<sup>2</sup>, William H. Westra<sup>1,3</sup>, Mohammad Obaidul Hoque<sup>1</sup>, and David Sidransky<sup>1</sup>

## Abstract

**Purpose:** We investigated the feasibility of detecting aberrant DNA methylation of some novel and known genes in the serum of lung cancer patients.

**Experimental Design:** To determine the analytic sensitivity, we examined the tumor and the matched serum DNA for aberrant methylation of 15 gene promoters from 10 patients with primary lung tumors by using quantitative methylation-specific PCR. We then tested this 15-gene set to identify the more useful DNA methylation changes in the serum of a limited number of lung cancer patients and controls. In an independent set, we tested the six most promising genes (*APC*, *CDH1*, *MGMT*, *DCC*, *RASSF1A*, and *AIM1*) for further elucidation of the diagnostic application of this panel of markers.

**Results:** Promoter hypermethylation of at least one of the genes studied was detected in all 10 lung primary tumors. In majority of cases, aberrant methylation in serum DNA was accompanied by methylation in the matched tumor samples. In the independent set, using a single gene that had 100% specificity (*DCC*), 35.5% (95% CI: 25–47) of the 76 lung cancer patients were correctly identified. For patients without methylated *DCC*, addition of a logistic regression score that was based on the five remaining genes improved sensitivity from 35.5% to 75% (95% CI: 64–84) but decreased the specificity from 100% to 73% (95% CI: 54–88).

**Conclusion:** This approach needs to be evaluated in a larger test set to determine the role of this gene set in early detection and surveillance of lung cancer. *Clin Cancer Res*; 17(13); 4494–503. ©2011 AACR.

## Introduction

Lung cancer kills more people than breast, colon, and prostate cancers combined (1). Lung cancer remains the second most diagnosed cancer in the United States and the most common cause of cancer mortality, with an estimated

161,000 deaths in 2008, with 80% being non-small cell lung cancer (NSCLC; ref. 2). Although the overall prognosis for patients with lung cancer is poor with a 5-year survival rate of less than 15%, patients diagnosed with early-stage disease have a much more favorable prognosis. Patients with pathologic stages I and II disease have 5-year survival rates of 57% to 67% and 38% to 55%, respectively (3, 4). Unfortunately, more than half of patients with NSCLC present only after metastasis to lymph nodes or distant sites because of a lack of symptoms early in the disease (4, 5). Detection of lung cancer at earlier stages could potentially increase survival rates by 10- to 50-fold (6). Using chest X-ray and sputum cytology as screening techniques has proven ineffective in increasing patient survival (7, 8). Recently, low-dose spiral computed tomography (CT) screening in high-risk smokers was proven to find more lung tumors and to reduce mortality (3, 4). Identification of lung cancer-specific biomarkers combined with other noninvasive methods may allow for much needed further refinement of lung cancer screening to reduce mortality.

Methylation plays an important role in normal cells as well as in tumor development. In normal cells, it contributes to chromatin organization, silencing of transposable elements, X chromosome inactivation, tissue-specific expression, and genetic imprinting (9–11). In addition to

**Authors' Affiliations:** <sup>1</sup>Department of Otolaryngology and Head and Neck Surgery and <sup>2</sup>Division of Biostatistics, Department of Oncology, The Johns Hopkins School of Medicine; <sup>3</sup>Department of Pathology, Johns Hopkins Medical Institutions; <sup>4</sup>Milton J. Dance Head and Neck Center, Greater Baltimore Medical Center, Baltimore, Maryland; <sup>5</sup>Department of Human Molecular Genetics, Virginia Commonwealth University, School of Medicine, VCU Institute of Molecular Medicine, Richmond, Virginia; and <sup>6</sup>Department of Head and Neck Surgery, Barretos Cancer Hospital, Barretos, Sao Paulo, Brazil

**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

S. Begum and M. Brait contributed equally to this study.

This article/analysis is based on a web database application provided by Research Information Technology Systems (RITS): <https://www.rits.onc.jhmi.edu/>.

**Corresponding Author:** David Sidransky, Division of Head and Neck Cancer Research, The Johns Hopkins School of Medicine, 1550 Orleans Street 5N.03, Baltimore, MD 21231. Phone: 410-502-5155; Fax: 410-614-1411; E-mail: dsidrans@jhmi.edu

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### Translational Relevance

Identification of blood-based noninvasive or minimally invasive detection markers will improve the clinical management of non-small cell lung cancer (NSCLC), the leading cause of cancer-related deaths in the United States and worldwide. From a panel of 15 cancer-specific methylated genes, we derived a 6-gene panel to identify NSCLC in blood by quantitative methylation-specific PCR with high specificity. It would be envisaged that this simple, reliable, and noninvasive blood test could aid the early detection of NSCLC in a high-risk population and could be used most effectively to direct imaging modalities with low specificity such as spiral computed tomography (CT). Such a test could therefore have a significant impact on the long-term survival of these individuals. Moreover, these tests can be developed to monitor the response to therapy and some of the methylated marker can be selected for targeted therapy in future.

general hypomethylation of the genome, hypermethylation (further denoted as "methylation") of CpG islands in gene promoter regions occurs in cancer cells (12). Methylation, one of the most common molecular alterations in human neoplasia, refers to the addition of a methyl group to the cytosine ring of those cytosines that precede a guanine (referred to as CpG dinucleotides) to form methyl cytosine (5-methylcytosine). CpG dinucleotides are found at increased frequency in the promoter region of many genes, and methylation in the promoter region is frequently associated with "gene silencing" (13). Several tumor suppressor genes (TSG) contain CpG island in their promoters, and many of them show evidence of methylation silencing (9, 10). Aberrant promoter methylation may affect genes involved in cell-cycle control (*p16INK4A*, *p15*, *Rb* and *p14*; refs. 14–16), DNA repair (*MGMT* and *hMLH1*; refs. 10, 17), cell adhesion (*H-Cadherin* and *CDH1*; refs. 18, 19), signal transduction (*RASSF1A*; ref. 20), apoptosis (*DAPK* and *TMS1*; ref. 21), and cell differentiation (*RARβ2*; refs. 17, 22). Studies in animals and in humans have shown that these epigenetic changes are an early event in carcinogenesis and are present in the precursor lesions of a variety of cancers including breast (23, 24), lung (25, 26), colon (27), and endometrium (28).

The presence of abnormally high DNA concentrations in the sera and plasma of patients with various malignant diseases has been described (29–31). Recent publications have shown the presence of promoter methylation in various bodily fluids including plasma, sputum, and bronchoalveolar lavage DNA of lung cancer patients (32–36). Using quantitative methylation-specific PCR (QMSP) to test plasma DNA with a panel of 4 genes (*KIF1A*, *DCC*, *RARβ2*, and *NISCH*), we were able to detect 73% of cancer cases with 71% specificity (37). This study evaluated the diagnostic potential of an extended panel of DNA methylation-based markers in pretherapeutic sera of

lung cancer patients. We decided to investigate these markers in patients who had not undergone any form of adjuvant systemic treatment. Of 15 markers tested in an evaluation set, 6 markers (*APC*, *AIM1*, *DCC*, *CDH1*, *MGMT*, and *RASSF1A*) were tested in an independent set of serum samples from lung cancer patients.

### Materials and Methods

#### Patients

The gene evaluation set consisted of patients sera ( $n = 17$ –25) collected before any therapeutic intervention and normal controls ( $n = 15$ –614). The test set consists of sera of 76 patients and sera from 30 age-matched controls. Selected demographics and histopathologic characteristics of these 76 lung cancer patients are shown in Table 1. All patients subsequently underwent a biopsy and were confirmed to have lung cancer.

#### Collection and processing of samples and DNA preparation

We obtained samples of lung tumor tissue and serum from 10 patients with lung cancer who underwent curative surgery at The Johns Hopkins University School of

**Table 1.** Demographic and clinicopathologic characteristics of lung cancer patients ( $n = 76$ )

Characteristics	Number of patients examined
Gender	
Male	40
Female	36
Age, y	
≥65	39
<65	37
Race	
White	52
African American	21
Others	3
Histologic type	
Adenocarcinoma	36
SSC	26
Others	14
Stage	
I	41
II	17
III	11
IV	5
Unknown	2
Tumor size	
≥3 cm	41
<3 cm	29
Unknown	6

Abbreviation: SSC, squamous cell carcinoma.

Medicine. These patients were chosen consecutively on the basis of tissue availability. Tissue specimens were immediately snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Frozen tissue was sectioned (12- $\mu\text{m}$  thick), and every tenth section was stained with hematoxylin and eosin and histologically examined for the presence or absence of tumor cells as well as for tumor density. Only sections that showed more than 70% of neoplastic cells were used for DNA extraction.

Blood samples from all the cancer patients were drawn before any therapeutic intervention at The Johns Hopkins University School of Medicine. The control serum samples consists of subjects enrolled in a community screening study for head and neck cancer approved by the Johns Hopkins Institutional Review Board and through the early detection research networks (EDRN). The experimental protocol was approved by the Johns Hopkins Medical Institutions Institutional Review Board and informed consent was obtained from all enrolled subjects. All subjects were administered a confidential written survey of risk factors for upper aerodigestive tract malignancies, including alcohol and tobacco use as well as the presence of comorbid illnesses. Smoking was defined as use of tobacco, chewable or smoked, for at least 1 year continuously. Heavy alcohol use was defined as intake of more than 2 alcoholic drinks per day. For Head and Neck surveillance study, all individuals were called by phone once a year afterward and interviewed to determine interval changes in tobacco and alcohol consumption and health history including new cancer diagnosis. For the controls used in this study, we excluded those individuals presenting with premalignant or malignant lesions at head and neck area, past history of cancer regardless of site, those who were diagnosed of any cancer regardless of site during follow-up, and those not reachable by phone follow-up.

The blood was centrifuged at  $2,000 \times g$  for 10 minutes at room temperature, and 1 mL aliquots of serum/plasma samples were stored at  $-80^{\circ}\text{C}$ . DNA was obtained from primary tumor tissues and serum/plasma by digestion with 50  $\mu\text{g}/\text{mL}$  proteinase K (Boehringer) in the presence of 1% SDS at  $48^{\circ}\text{C}$  for 2 days, followed by phenol/chloroform extraction and ethanol precipitation, and finally dissolved in 30 to 60  $\mu\text{L}$  of LoTE (2.5 mmol/L EDTA and 10 mmol/L Tris-HCl).

### Bisulfite treatment

DNA extracted from primary tumors and serum DNA was subjected to bisulfite treatment, which converts unmethylated cytosine residues to uracil residues, as described previously (38), with minor modification. Briefly, 1 to 2  $\mu\text{g}$  of genomic DNA from each sample was denatured with NaOH (final concentration = 0.2 mol/L) in a total volume of 20  $\mu\text{L}$  for 20 minutes at  $50^{\circ}\text{C}$ . The denatured DNA was diluted in 500  $\mu\text{L}$  of a freshly prepared solution of 10 mmol/L hydroquinone and 3 mol/L sodium bisulfite and incubated for 3 hours at  $70^{\circ}\text{C}$ . Bisulfite-modified DNA was purified using a Wizard DNA Clean-Up System (Promega), treated with 0.3 mol/L NaOH for 10 minutes at room temperature, precipitated with ethanol, resuspended in 60 to 120  $\mu\text{L}$  of LoTE

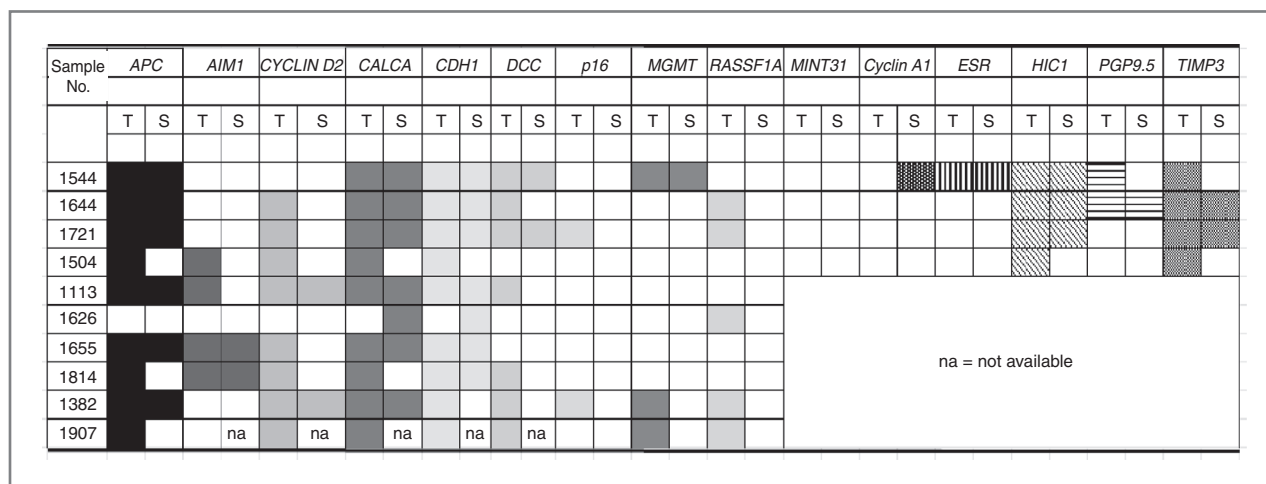
(2.5 mmol/L EDTA, 10 mmol/L Tris-HCl, pH 8), and stored at  $-80^{\circ}\text{C}$ .

### Methylation analysis

Bisulfite-modified DNA was used as a template for fluorescence-based real-time PCR, as previously described (39). Amplification reactions were carried out in duplicate or triplicate in a volume of 20  $\mu\text{L}$  that contained 3  $\mu\text{L}$  of bisulfite-modified DNA; 600 nmol/L concentrations of forward and reverse primers; 200 nmol/L probe; 5 U of platinum Taq polymerase (Invitrogen); 200  $\mu\text{mol}/\text{L}$  concentrations each of dATP, dCTP, and dGTP; 200  $\mu\text{mol}/\text{L}$  dTTP; and 5.5 mmol/L  $\text{MgCl}_2$ . Primers and probes were designed to specifically amplify the promoters of the 15 genes of interest and the promoter of a reference gene, *ACTB*; primer and probe sequences and annealing temperatures are provided in Supplementary Table S1. Amplifications were carried out using the following profile:  $95^{\circ}\text{C}$  for 3 minutes, followed by 50 cycles at  $95^{\circ}\text{C}$  for 15 seconds and  $60^{\circ}\text{C}$  to  $62^{\circ}\text{C}$  for 1 minute. Amplification reactions were carried out in 384-well plates in a 7900 sequence detector (Perkin-Elmer Applied Biosystems) and were analyzed by a sequence detector system (SDS 2.2.1; Applied Biosystems). Each plate included patient DNA samples, positive (*in vitro* methylated leukocyte DNA) and negative (normal leukocyte DNA or DNA from a known unmethylated cell line) controls, and multiple water blanks. Leukocyte DNA from a healthy individual was methylated *in vitro* with excess SssI methyltransferase (New England Biolabs Inc.) to generate completely methylated DNA, and serial dilutions (90–0.009 ng) of this DNA were used to construct a calibration curve for each plate. All samples were within the range of the assay of sensitivity and reproducibility based on amplification of internal reference standard [threshold cycle ( $C_t$ ) value for *ACTB* of  $\leq 40$ ]. The relative level of methylated DNA for each gene in each sample was determined as a ratio of MSP-amplified gene to *ACTB* (reference gene) and then multiplied by 1,000 for easier tabulation (average value of triplicates of gene of interest divided by the average value of triplicates of *ACTB*  $\times 1,000$ ). The samples were categorized as unmethylated or methylated on the basis of the sensitivity of the assay.

### Statistical analysis

The major statistical endpoints in this study involved comparing normal and cancer methylation levels of 6 genes thought to be associated with lung cancer. The presence or absence of methylation was evaluated for an association with cancer using cross-tabulations and  $\chi^2$  or Fisher's exact tests as appropriate. Continuous methylation levels were evaluated using logistic regression and receiver operating characteristic (ROC) curves. One gene with 100% specificity was identified in this group of patients. Among those patients without methylation of this gene, logistic regressions utilizing the remaining genes were conducted. ROC curves were produced by combining the point of 100% specificity from the first step with the logistic results from the second step. Internal validation of the logistic



**Figure 1.** Summary of methylation of pattern of 15 genes in corresponding tumor tissue (left) and serum DNA samples (right). Filled boxes represent samples that are methylated; white boxes represent samples devoid of methylation.

regression models was done using an approximation to the leave one out jackknife procedure provided by the SAS classification table option.

Methylation values were visually compared using box plots of the log transformed values. In these plots, the length of the box is the interquartile range (IQR) of the data and depicts the spread of the middle 50 percent of the observations. The median is displayed with a horizontal line inside of this box. The lines extending out from the box extend from the upper and lower quartiles to values defined as adjacent values. The adjacent values are the upper quartile plus  $1.5 \times \text{IQR}$  and the lower quartile minus  $1.5 \times \text{IQR}$ . Any value lying outside of this range is displayed with an open circle and can be considered an outlier.

Correlations of the methylation levels of genes were calculated with Spearman correlation coefficients. All *P* values are 2-sided. Computations were conducted using the Statistical Analysis System.

## Results

### Methylation in primary tumor and matched serum

We initially measured the levels of promoter methylation for all the 15 genes (*APC*, *AIM1*, *Cyclin D2*, *CALCA*, *CDH1*, *DCC*, *p16*, *MGMT*, *RASSF1A*, *MINT31*, *Cyclin A1*, *ESR1*, *HIC1*, *PGP9.5*, and *TIMP3*) in paired primary lung tumor and serum DNA samples from 10 lung cancer patients. Figure 1 summarizes the methylation profiles of each of the 15 genes for the 10 paired samples. In paired samples, the majority of genes methylated in serum DNA were always accompanied by methylation of tumor DNA, whereas methylation of tumor DNA was not always accompanied by methylation of serum DNA. No aberrant methylation (i.e., hypermethylation) was detected in the serum of lung cancer patients who did not also have aberrant methylation of the same promoter in the corresponding tumor sample with one exception (sample 1626). In general, relative methylation levels, which reflect the number of methylated

alleles, were higher in tumor DNA than in serum DNA (Supplementary Fig. S1). The frequency of methylation of all 15 genes in primary tumors and the analytic sensitivity of the QMSP assay are summarized in Table 2. Among the 10 primary tumor samples, the frequency of promoter methylation at each of the 15 loci ranged from 0% (for *MINT31* and *Cyclin A1*) to 100% (for *HIC1* and *TIMP3*), and the analytic sensitivity of the individual genes ranged from 0% to 75% (Table 2).

### Determination of the best performing genes for inclusion in a gene evaluation set for serum DNA

In addition to determine the analytic sensitivity and clinical sensitivity as described above, to further characterize the best performers, we investigated 15 genes in the sera of 15 to 25 patients with lung cancer and 15 to 614 controls with no known neoplastic disease for the presence of aberrant methylation. The controls and cases used here for preselection of genes and were based on the availability of DNA from the samples. Because of limited amount of DNA, we were not able to evaluate all the 15 genes in the same cohort of samples and we used different set of samples for preselection of genes. An overview of the frequency of methylation in this test set in serum samples is given in Table 3. The most appropriate genes for our additional analyses were determined to be those that show high analytic sensitivity (unmethylated or very low frequency of methylation in serum samples from healthy controls but comparatively high frequency of methylation in serum samples from primary lung cancer patients) and a reported high frequency of lung cancer specific methylation. On the basis of the latter criteria, a total of 6 genes, namely *APC*, *AIM1*, *CDH1*, *DCC*, *MGMT*, and *RASSF1A*, were selected for further analysis in an independent set of samples. Although we have more promising genes (*HIC1* and *PGP9.5*) in our evaluation set, we were not able to include all the interested genes in the same cohort of samples due to lack of required DNA from this cohort.



**Table 2.** Sensitivity of the quantitative methylation-specific PCR assay in lung cancer detection in serum/plasma DNA

Marker	Number of tumors with methylation/total number of tumors (%, 95% CI)	No. of patients with methylation in serum or plasma/no. of patients with methylation in primary tumor (analytic sensitivity <sup>a</sup> , 95% CI)
<i>APC</i>	9/10 (90, 56–100)	6/9 (67, 30–93)
<i>AIM1</i>	4/10 (40, 12–74)	2/4 (50, 7–93)
<i>Cyclin D2</i>	8/10 (80, 44–97)	2/8 (25, 3–65)
<i>CALCA</i>	9/10 (90, 56–100)	7/9 (78, 40–97)
<i>CDH1</i>	9/10 (90, 56–100)	6/7 (86, 42–100)
<i>DCC</i>	7/10 (70, 35–93)	2/6 (33, 4–78)
<i>P<sup>16</sup></i>	2/10 (20, 3–56)	0/2 (0, 0–84)
<i>MGMT</i>	3/10 (30, 7–65)	1/3 (33, 1–91)
<i>RASSF1A</i>	5/10 (50, 19–81)	0/5 (0, 0–52)
<i>MINT31</i>	0/4 (0, 0–60)	0/0 (0, 0–100)
<i>Cyclin A1</i>	0/4 (0, 0–60)	Not applicable
<i>ESR</i>	1/4 (25, 1–81)	1/1 (100, 3–100)
<i>HIC1</i>	4/4 (100, 40–100)	3/4 (75, 19–99)
<i>PGP9.5</i>	2/4 (50, 7–93)	1/2 (50, 1–99)
<i>TIMP3</i>	4/4 (100, 40–100)	2/4 (50, 7–93)

<sup>a</sup>The fraction of cases in which methylation of a marker was found in serum DNA for case patients who had confirmed methylation of the same marker in the tumor DNA. Analytic sensitivity were calculated only when tumor and paired DNA were available.

### Detection of lung cancer in an independent set of samples using the six-gene panel

We tested quantitative analysis of promoter methylation in the serum DNA samples from 76 lung cancer patients and 30 age-matched control subjects. The demographic and clinical characteristics of the 76 lung cancer patients included in this study are summarized in Table 1. The study population was almost equal with males and females and had a median age of 65 years (IQR = 42–85 years). All lung cancer cases were eventually confirmed by standard pathology. Among the serum samples from the 76 lung cancer patients, we detected aberrant methylation of the *APC* promoter in 12 samples (15.8%, 95% CI: 8.4–26), of the *AIM1* promoter in 14 samples (18.4%, 95% CI: 10.5–29.0), of the *CDH1* promoter in 47 samples (61.8%, 95% CI: 50–73), of the *DCC* promoter in 27 samples (35.5%, 95% CI: 25–47), of the *MGMT* promoter in 13 samples (17%, 95% CI: 9.4–27.4) and of the *RASSF1A* promoter in 6 samples (7.9%, 95% CI: 3.0–16.4). The box plots in Figure 2 show the distribution of relative methylation values for each of the 6 genes of interest versus *ACTB* obtained by QMSP using serum DNA from cancer patients and control subjects. These 6 genes were examined in the independent set to create a combined panel of methylation markers (Table 3). Eighty-four percent (64 of 76) of patients with cancerous tumors showed methylation of at least 1 gene, whereas 13 of 30 (43%) of control subjects showed methylation ( $P \leq 0.0001$ ;  $\chi^2$ ).

Figure 3A depicts the combined 2-stage algorithm that we used for disease classification. The ROC curves obtained by using the 2-stage approach, which was based on 1

marker (*DCC*) with 100% specificity followed by logistic regression analysis on the remaining 5 markers, are shown in Figure 3B. Both curves have been corrected for overfitting via internal validation. The *DCC* gene that had 100% specificity correctly identified 35.5% (95% CI: 25–47) of the 76 lung cancer patients (Fig. 3B). For patients without methylated *DCC*, addition of a logistic regression score that was based on the 5 remaining genes improved sensitivity from 35.5% to 75% (95% CI: 64–84) but decreased the specificity from 100% to 73% (95% CI: 54%–88%; Fig. 3B). The detailed regression coefficients for the additional genes were also conducted (data not shown). We then compared this overall ROC curve to those obtained by adding each of the 5 genes individually to the model (data not shown). As expected, we found that the individual genes performed less well as predictors than did the multivariable logistic score using the entire group.

We also examined how the multivariable logistic score model performed for detecting stage I or II tumors. The sensitivity of the model increased with more advanced tumor stage, ranging from 73% for stage I to 76.5% of stage II tumors detectable by QMSP with a specificity of 73% and 77%, respectively. Sample size did not permit this analysis for the stage III and IV subset.

Logistic regression was used to examine associations between clinicopathologic and demographic parameters (age  $\geq 65$  at diagnosis, tumor stage  $\geq$  III, adeno cell type, and tumor size  $\geq 3$  cm) and the methylation status of the 6 genes chosen for testing in the final data set. *CDH1* methylation was the only gene significantly associated with any of these factors. The presence of any *CDH1* methylation

**Table 3.** Frequency of methylation**A. 15 genes in gene evaluation set**

Markers	Number of methylation positive/number of total cases (methylation positive)		
	Serum or plasma (cancer)	Serum (control)	Cutoff values
<i>APC</i>	9/25 (36%)	2/30 (7%)	0
<i>AIM1</i>	4/17 (24%)	1/15 (7%)	0
<i>Cyclin D2</i>	4/17 (24%)	3/35 (9%)	0
<i>CALCA</i>	12/17 (71%)	23/35 (66%)	0
<i>CDH1</i>	10/17 (59%)	1/15 (7%)	0.3
<i>DCC</i>	3/17 (18%)	0/136 (0%)	0
<i>p16</i>	0/25 (0%)	0/30 (0%)	0
<i>MGMT</i>	5/25 (20%)	1/30 (3%)	0
<i>RASSF1A</i>	2/25 (8%)	1/30 (3%)	0.1
<i>MINT31</i>	0/20 (0%)	0/30 (0%)	0
<i>Cyclin A1</i>	2/20 (10%)	0/155 (0%)	0
<i>ESR</i>	1/20 (5%)	0/35 (0%)	0
<i>HIC1</i>	10/20 (50%)	42/614 (7%)	0
<i>PGP9.5</i>	3/20 (15%)	9/318 (3%)	0
<i>TIMP3</i>	5/20 (25%)	0/30 (0%)	1

**B. 6 genes in gene independent set**

Markers	Number of methylation positive/number of total cases (methylation positive)				
	Serum or plasma (cancer)	Serum (control)	<i>P</i>	Sensitivity, %	Specificity, %
<i>APC</i>	12/76 (16%)	3/30 (10%)	0.55	15.8	90
<i>AIM1</i>	14/76 (18%)	1/30 (3%)	0.06	18.4	96.7
<i>CDH1</i>	47/76 (62%)	9/30 (30%)	0.003	61.8	70
<i>DCC</i>	27/76 (36%)	0/30 (0%)	0.0002	35.5	100
<i>MGMT</i>	13/76 (17%)	1/30 (3%)	0.11	17.1	96.7
<i>RASSF1A</i>	6/76 (8%)	1/30 (3%)	0.67	7.9	96.7
1 of 6 markers	64/76 (84%)	13/30 (43%)	<0.0001	84.2	56.7

decreased the probability of tumor size of 3 cm or greater, OR = 0.22 (95% CI: 0.06–0.74),  $P = 0.01$ .

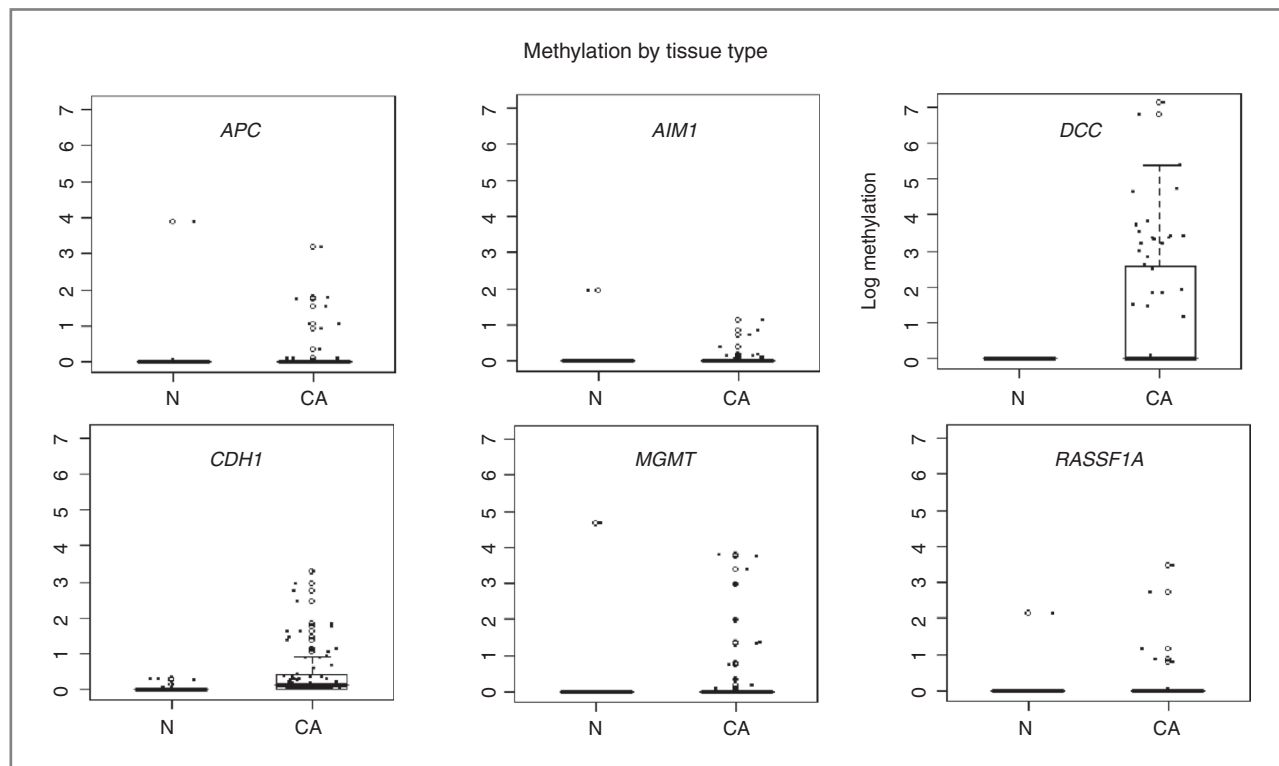
An indicator for the aberrant methylation of any 1 of the 6 genes investigated in serum DNA of lung cancer patients was also not associated with other clinical or demographic characteristics (data not shown). Methylation frequencies of 6 genes for different histologic subtypes are shown in Supplementary Table S2.

Finally, we conducted a correlation analysis for all pairs of markers (Supplementary Table S3). Promoter methylation of *APC* and *CDH1* were often observed together and this was statistically significant:  $\rho = 0.27$ ,  $P = 0.01$ .

## Discussion

This study confirms and extends previous observations that identification of serum DNA methylation in specific set of genes is a potentially useful approach to detect lung cancer patients. Serum DNA methylation was more fre-

quently observed in patients with lung cancer than those with age-matched controls. Although the sensitivity for the diagnosis of lung cancer was only 35.5% when analyzed by a single gene (*DCC*), the high specificity (100%) indicates the usefulness of QMSP assay for lung cancer detection. For patients without methylated *DCC*, addition of a logistic regression score that was based on the 5 remaining genes improved sensitivity from 35.5% to 75% but decreased the specificity from 100% to 73%. Addition of gene/genomes with reasonable sensitivity and very high specificity with *DCC* may allow higher diagnostic coverage while still proving 100% specificity. Of note, serum DNA methylation could be identified even in patients in the early stages of lung cancer, whereas conventional serum protein tumor markers were rarely elevated, indicating that this DNA-based method is more sensitive than protein-based method for diagnosis of lung cancer in early stage. In our previous study (37), *DCC* methylation was detected in 54% of cases with 100% specificity in evaluation set and fell to 26% in



**Figure 2.** Promoter methylation levels for the 6 markers in serum DNA from lung cancer patients (CA) and age-matched control subjects (N). The quantity of methylated alleles of each gene was expressed as the ratio of the amount of PCR products amplified from the methylated gene to the amount amplified from the reference gene  $\beta$ -actin multiplied by 1,000. Box plots show the middle 50% of the data, the line is the median, and the bars extend the median by 1.5 times the IQR.

the independent set. In the present cohort, *DCC* methylation was detected in 36% of cases, which compares favorably and suggests that about a third of lung cancer cases could be detected by *DCC* alone with high specificity.

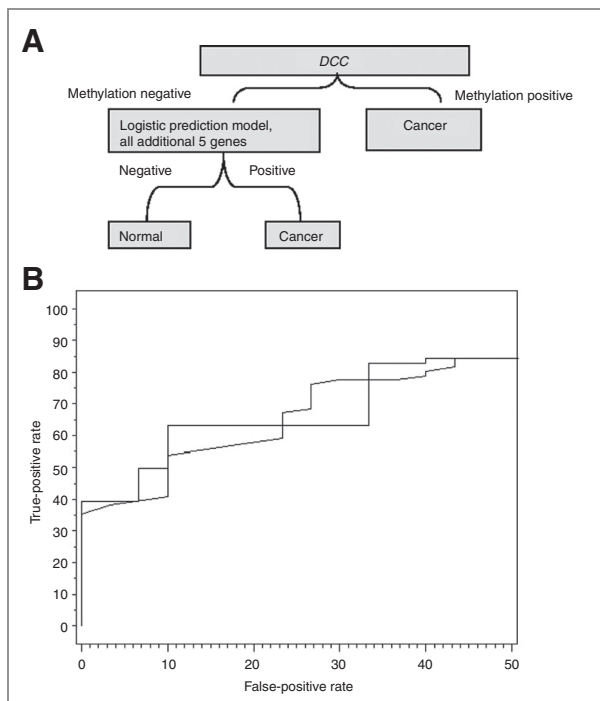
In former studies, methylation in tumor tissues was detected in 25% to 41% for *p16<sup>INK4a</sup>*, 30% to 40% for *RASSF1A*, and 16% to 27% for *MGMT* (26). These results were consistent with our data in 10 tumor tissue samples. The frequency of detecting methylated genes in serum was about half to two thirds compared with that in tumor tissues. However, when we consider that tumor-derived DNA in blood is generally detectable in less than half of cancer patients (32), the frequency of methylation in serum DNA in our study may be quite reasonable. Laird reviewed the studies examining the methylation status of serum/plasma DNA in patients with various neoplasms and indicated that clinical sensitivity of DNA methylation was ~50% (40). Esteller and colleagues did methylation analysis in serum DNA from patients with NSCLC for multiple genes and showed 33% to 80% clinical sensitivity by combination analysis of several genes (41).

Among various techniques used for methylation analysis, we adopted a simple method of QMSP analysis. The specificity of the primers and probes we used in this study has been verified using genomic sequencing and/or MSP. Recently, several studies showed improved detection rates

of methylation status using a nested PCR approach or a quantitative real-time PCR technique (42, 43). The sensitivity of the TaqMan method was reported to be 10-fold higher than conventional qualitative MSP and we feel that more sensitive technologies are very likely to reduce specificity (44).

Although promoter methylation was observed predominantly in lung cancer patients, several controls were methylation positive for 1 of the 5 genes (except *DCC*) in the independent test set. In methylation pattern analysis in primary tumors and paired serum samples, 1 lung cancer patient with serum DNA methylation for *CALCA* and *CDH1* did not show the same alteration in the corresponding tumor tissue. We considered the following as possible explanation of these apparent false-positive results. First, the methylated serum DNA might be derived from undetected precancerous lesions in these cases. According to previous reports, aberrant promoter methylation is clearly detectable in precancerous lesions, such as dysplasia and nonmalignant lung tissues of patients with lung cancer (45, 46). In a prospective study, Belinsky and colleagues reported that 3 genes in a panel of 6 were hypermethylated in the sputum of high-risk individuals, resulting in a greater than 6-fold risk of developing lung cancer within 18 months (47). Long-term follow-up of control subjects with methylated DNA of several genes in serum samples





**Figure 3.** A, 2-stage algorithm for disease classification. Patients who were positive for promoter methylation of *DCC* were classified as having cancer. Those who were negative for promoter methylation of *DCC* were analyzed in a second stage, in which a logistic risk score was calculated on the basis of adding additional markers (see text for details). B, ROC curves based on logistic scores using binary dichotomization of the genes at zero/nonzero methylation levels (lighter line). ROC curve based on logistic scores using the actual log methylation levels (darker line). Both curves were corrected for overfitting via internal validation.

will elucidate the risk of developing lung cancer in these subjects. Second, aberrant methylation might be caused by environmental factors, such as smoking, yet may not necessarily lead to clonal expansion (25, 48). Finally, endogenous factors like the occurrence of other occult malignancies or age-related methylation could be the cause in the control group.

Hypermethylation of the *APC* and *CDH1* promoters in serum DNA from lung cancer patients often occurred together. Given that hypermethylation of these genes is common in lung cancer, these associations could have occurred by chance alone and should be interpreted with caution. However, a possible interaction between these genes in lung cancer deserves further evaluation. *CDH1* methylation occurred more often in patients with tumor sizes less than 3 cm (20 of 24; 83%) compared with patients with tumor sizes of 3 cm or greater (24 of 46; 52%;  $P = 0.01$ ). It is well studied that *CDH1* inactivation is

related to invasion, metastasis, and increased tumor size. So, our finding of presence of any *CDH1* methylation decreased the probability of tumor size of 3 cm or above (OR = 0.22; 95% CI: 0.06–0.74;  $P = 0.01$ ) need to be further evaluated.

To consider the use of serum DNA methylation as a marker in lung cancer active screening, several issues must be considered. Certainly, optimal specificity and sensitivity must be achieved before the approach can be used alone or in combination with imaging. It is tempting to combine methylation with promising methods such as low-dose spiral CT (49, 50). Because one of the serious limitations of low-dose spiral CT is its poor specificity, a combination with serum DNA methylation may overcome this limitation.

Serum DNA methylation is found in early-stage disease (73% and 77% in stages I and II, respectively) and thus could be tested before invasive procedures after testing in a larger cohort and in a prospective setting. Although further evaluation is essential, the results in this study indicate the substantial usefulness of methylation marker for detection of lung cancer. Further studies are warranted to confirm the accuracy of the approach and search for the best combination of genes for methylation analysis. Moreover, it is important to investigate prospectively whether methylation-positive nonclinical cancer cases will ultimately develop malignancies in the near future.

## Disclosure of Potential Conflicts of Interest

The funding agencies had no role in the design of the study, data collection, analysis, interpretation of the results, preparation of the manuscript, or the decision to submit the manuscript for publication. Under a licensing agreement between Oncomethylome Sciences, SA, and the Johns Hopkins University, D. Sidransky is entitled to a share of royalty received by the University upon sales of diagnostic products described in this article. D. Sidransky owns Oncomethylome Sciences, SA stock, which is subject to certain restrictions under University policy. D. Sidransky is a paid consultant to Oncomethylome Sciences, SA, and is a paid member of the company's Scientific Advisory Board. The Johns Hopkins University in accordance with its conflict of interest policies is managing the terms of this agreement.

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