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Imaging, Diagnosis, Prognosis

Clinical Cancer Research

# Molecular Analysis of Plasma DNA for the Early Detection of Lung Cancer by Quantitative Methylation-Specific PCR

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## Abstract

**Purpose:** Aberrant promoter hypermethylation of tumor suppressor genes is a promising marker for lung cancer detection. We investigated the likelihood of detecting aberrant DNA methylation of tumor suppressor genes in plasma samples of patients with abnormalities of the lung detected upon computed tomography (CT) scan.

**Experimental Design:** In a small evaluation cohort, four gene promoters (*DCC*, *Kif1a*, *NISCH*, and *Rarb*) were found to be methylated with increased frequency in samples from cancer patients specifically. We then examined DNA from 93 plasma samples from patients with abnormal findings in the lung detected upon CT scan for aberrant methylation of these four gene promoters by quantitative fluorogenic real-time PCR. The patients were divided into two groups, ground glass opacity ($n$ = 23) and cancerous tumors ($n$ = 70). Plasma DNA from age-matched nodule-free individuals were used as controls ($n$ = 80).

**Results:** In plasma, 73% of patients with cancerous tumors showed methylation of at least one gene with a specificity of 71% ($P$ = 0.0001). Only 22% patients with ground glass opacity exhibited methylation of at least one gene. When smoking history was taken into account, 72% of cancer patients with no smoking history or those who smoked <20 pack-years showed methylation of at least one gene with 100% specificity ($P$ = 0.05) when compared with matched controls. Among heavy smokers with 20+ pack-years of smoking history, 30% of the control group and 73% of the patients with cancerous tumors showed methylation ($P$ = 0.0001).

**Conclusions:** These biomarkers can distinguish between cancerous and noncancerous abnormal CT findings. *Clin Cancer Res; 16(13); 3463–72. ©2010 AACR.*

Lung cancer is the leading cause of cancer related–death in the United States and other developed countries (1). A major factor in the high mortality of lung cancer patients is the presence of metastatic tumors in approximately two-third of patients at the time of diagnosis (2). Detection of lung cancer at earlier stages could potentially increase survival rates by 10- to 50-fold (2). Using chest X-ray and sputum cytology as screening techniques has proven ineffective in increasing patient survival (3, 4). The search for more sensitive and specific tests is ongoing. One approach is the identification of lung cancer–specific biomarkers and noninvasive methods for the detection of these biomarkers at an early stage.

Epigenetic changes, such as DNA methylation, are one of the most common molecular alterations in human neoplasia (5, 6). DNA hypermethylation refers to the addition of a methyl group to the cytosine ring of those cytosines that precede a guanosine (called 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" (7). Several tumor suppressor genes contain CpG island in their promoters, and many of them show evidence of methylation silencing (5, 6). Aberrant promoter methylation may affect genes involved in cell cycle control (*p16INK4Ap15*, *Rb*, and p14; refs. 8–10), DNA repair (*MGMT* and *hMLH1*; refs. 11, 12), cell adhesion (*H-cadherin* and *CDH-1*; refs. 13, 14), signal transduction (*RASSF1A*; refs. 15, 16), apoptosis (*DAPK* and *TMS1*; ref. 17) and cell differentiation (*RAR*$\beta$*2*; ref. 18). 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 (19), lung (20), colon (21), and

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

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## Translational Relevance

The development of a blood-based test for lung cancer based on gene promoter methylation could augment current early detection approaches such as computed tomography scan and sputum cytology. The methylation markers of lung cancer used in this study show great potential in molecular detection approaches. These markers can be detected in the plasma of stage I lung cancer patients and therefore show promise in the early detection of lung cancer. Moreover, methylated targets also have real therapeutic potential with the continuing development of demethylating agents that can be used in the clinic.

endometrium (22). Thus far, genes such as *APC*, *p16*, *CDH1*, *RARβ-2*, and *RASSF1A* have been found to have hypermethylated promoters in 30% of lung tumors (23, 24).

The presence of abnormally high DNA concentrations in the sera and plasma of patients with various malignant diseases has been described (25, 26). Recent publications have shown the presence of promoter hypermethylation of various bodily fluids including plasma, sputum, and bronchoalveloar lavage DNA of lung cancer patients (20, 27–30), which may offer an alternative approach to screening. Further, it may also be of interest in providing data for clinical long-term management of cancer patients. Overall, these studies have established an association between the epigenetic alterations found in primary tumor specimens and in plasma, suggesting the potential utility of these alterations as surrogate tumor markers.

We undertook the current study to determine the frequency of promoter methylation of five candidate tumor suppressor genes (*RarB*, *NISCH*, *B4GALT1*, *KIF1a*, and *DCC*) in the plasma of individuals who evidenced abnormal findings upon computed tomography (CT) scans beginning with a small set of patients (evaluation set). These genes have previously shown cancer-specific methylation in the lung tumor tissue (31), with the exception of *DCC*. *DCC* was validated as a tumor marker in head and neck cancer (32) and esophageal squamous cell carcinoma (33). We then examined plasma DNA from a larger population of patients with abnormal findings on CT for the methylation of the top four candidate genes (*Kif1a*, *DCC*, *NISCH*, and *RarB*) from our evaluation set. This independent set examined plasma from patients with abnormalities ($n$ = 93) the in lung by CT scan, as well as 80 plasma DNA samples from age-matched individuals without any pathologic changes as controls. Patients with abnormal CT findings were divided into two groups, histologically confirmed cases of lung cancer ($n$ = 70) and "ground glass opacity" (GGO; $n$ = 23). GGO is a finding on high-resolution CT that is defined as hazy increased attenuation of the lung with preservation of bronchial and vascular margins (34). GGO is an abnormal finding, and biopsied lesions have an unclear propensity for cancer progression (35). The current study identified novel cancer-specific methylation markers in plasma samples. Our long-term goal for this study is to develop a blood-based screening modality of clinical utility in application in individuals at high risk for developing lung cancer.

## Materials and Methods

### Collection of samples and DNA extraction

***Evaluation set.*** Individuals were selected from study populations recruited by the Specialized Program of Research Excellence in Lung Cancer at the University of Pittsburgh. Plasma was collected from 24 disease-free individuals and 13 individuals with histologically confirmed cases of primary lung cancer. A complete smoking history was collected for each individual. Both control and cancer groups were made up of lifetime nonsmokers, current smokers, and ex-smokers.

***Independent set.*** Individuals were recruited from the New York University (NYU) Lung Cancer Biomarker Center. The participants underwent chest CT and pulmonary function and collection of blood samples banked for biomarker studies. Plasma was collected from 23 individuals with small solid or GGO on CT as well as 70 patients with lung cancer and 80 smokers with no nodules on CT scan. A complete smoking history was collected for each individual. The majority of the control group was made up of 20 pack-year + smokers. Patients with lung cancer included lifetime nonsmokers, current smokers, and ex-smokers. Age, gender, and tumor stage, as well as outcome information was also recorded.

All samples (for both evaluation and independent sets) were received by Dr. Sidransky's laboratory in a blinded fashion in a tube marked with a number. No prior knowledge of case versus control was received.

Plasma DNA was extracted by digestion with 50 μg/mL proteinase K (Boehringer Mannheim) in the presence of 1% SDS at 48°C overnight followed by phenol/chloroform extraction and ethanol precipitation.

### Bisulfite treatment

DNA extracted from 1 mL of blood plasma was subjected to bisulfite treatment, using the EpiTect Bisulfite kit from Qiagen according to the manufacturer's conditions, http://www.Qiagen.com. Bisulfite-treated DNA was eluted in 30 μL of elution buffer and stored at −80°C.

### Methylation analysis

Bisulfite-modified DNA was used as template for fluorescence-based real-time PCR, as previously described (36). Amplification reactions were carried out in duplicate in a volume of 20 μL that contained 2 μL bisulfite-modified DNA; 600 nmol/L forward and reverse primers; 200 nmol/L probe; 5 units of Platinum *Taq* polymerase (Invitrogen); 200 μmol/L each of dATP, dCTP, and dGTP; 200 μmol/L dTTP; and 5.5 mmol/L $MgCl_2$. Primers and probes were designed to specifically amplify the promoters



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of the five genes of interest and the promoter of a reference gene, *ACTB* (Supplementary Table S1). Amplifications were carried out using the following profile: one step at 95°C for 3 minutes, 50 cycles at 95°C for 15 seconds, and 60°C to 62°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 SDS 2.2.1 Sequence Detector System (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 assay's range of sensitivity and reproducibility based on amplification of internal reference standard (CT value for ACTB of 40 or less). The relative level of methylated DNA for each gene in each sample was determined as a ratio of methylation-specific PCR-amplified gene to ACTB (reference gene) and then multiplied by 1,000 for easier tabulation (average value of duplicates of gene of interest/average value of duplicates of ACTB × 1,000). Methylation cutoffs were set by receiver characteristic operator curves (ROC).

### Statistical analysis

Fisher Exact tests (two sided) were done to detect significant methylation differences between groups. *P* values of $<0.05$ are significant.

ROCs were calculated using the Web-based calculator for ROC curves from http://www.Medcalc.be. The diagnostic performance of a test or the accuracy of a test to discriminate diseased cases from normal cases is evaluated using ROC analysis. When considering the results of a particular test in two populations, one population with a disease, the other population without the disease, one will rarely observe a perfect separation between the two groups. ROC curves can be used to establish methylation cutoffs or threshold. The cutoff is chosen so that the classifier gives the best trade off between the costs of failing to detect positives against the costs of detecting false positives. MedCalc calculates the cutoff based on the value corresponding with the highest average of sensitivity and specificity. Area under the curve (AUC) indicates the value of the test. A perfect test (one that has zero false positives and zero false negatives) has an area of 1.00. The closer the AUC is to 1.0, the more sensitive and specific the test is.

## Results

### Evaluation cohort analysis

The demographic characteristics of the evaluation set subjects are summarized in Table 1. Patients with lung cancer ranged in age from 44 to 78 years, whereas the controls ranged in age from 34 to 83 years. Of the patients with lung cancer, 2 were lifetime nonsmokers and 10 had a smoking history. The control group was

**Table 1.** Demographics of study participants in the evaluation set

| **Controls** | ***n* = 24** |
|---|---|
| Gender | |
| Male | 5 |
| Female | 19 |
| Age median (range) | 54 (34-83) |
| Smoking | |
| Current | 4 |
| Former | 10 |
| Lifetime nonsmoker | 9 |
| Not available | 1 |
| Pack-years | |
| Lifetime nonsmoker | 9 |
| <20 | 4 |
| 20-34 | 7 |
| 35-49 | 2 |
| 50+ | 1 |
| Not available | 1 |
| **Cancers** | ***n* = 13** |
| Gender | |
| Male | 10 |
| Female | 3 |
| Age median (range) | 64 (44-78) |
| Smoking | |
| Current | 5 |
| Former | 5 |
| Lifetime nonsmoker | 2 |
| Not available | 1 |
| Pack-years | |
| Lifetime nonsmoker | 2 |
| <20 | 1 |
| 20-34 | 3 |
| 35-49 | 2 |
| 50+ | 4 |
| Not available | 1 |
| Tumor type and stage | |
| Adenocarcinoma | 1 |
| Squamous | 6 |
| Not specified | 6 |

NOTE: Demographics of samples tested (evaluation set). Evaluation set was obtained from University of Pittsburgh Medical Center. Patients with lung cancer (*n* = 13) ranged in age from 44 to 78 y, whereas the controls (*n* = 24) ranged in age from 34 to 83 y. Of the patients with lung cancer, 2 were lifetime nonsmokers and 10 had a smoking history. The control group was made up of 9 lifetime nonsmokers and 14 with a smoking history.



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**Table 2.** Methylation of candidate genes in the evaluation set

| | *KIf1a* | *NISCH* | RarB | DCC | B4GALT1 |
|---|---|---|---|---|---|
| Controls | 1/24 (4%) | 2/24 (8%) | 1/24 (4%) | 0/11 (0%) | 2/10 (20%) |
| Cancers | 3/11 (27%) | 4/11 (36%) | 5/13 (38%) | 6/11 (54%) | 1/9 (11%) |
| | $P = 0.08$ | $P = 0.06$ | $P = 0.01$ | $P = 0.01$ | $P = 1$ |

NOTE: The plasma samples from the evaluation set were tested for methylation of five candidate tumor suppressor genes, (*RarB*, *NISCH*, *B4GALT1*, *KIF1a*, and *DCC*). *Kif1a*, *DCC*, *RarB*, and *NISCH* showed differences in methylation status that seemed to be associated with cancer.

made up of 9 lifetime nonsmokers and 14 with a smoking history.

The plasma samples from the evaluation set were tested for the methylation of five candidate tumor suppressor genes (*RarB*, *NISCH*, *B4GALT1*, *KIF1a*, and *DCC*). Methylation frequencies for each gene are listed in Table 2. *Kif1a*, *DCC*, *RarB*, and *NISCH* showed differences in methylation status that seemed to be associated with cancer.

**Independent cohort analysis**

Methylation of these four candidate genes, *Kif1a*, *DCC*, *RarB*, and *NISCH*, were then tested for cancer-specific methylation in a larger cohort of plasma samples from lung cancer cases and controls. This independent set was obtained from NYU. The demographic characteristics of the independent set are listed in Table 3.

Methylation of each gene marker was normalized to β-actin and multiplied by 1,000 for easy tabulation (Supplementary Table S2). Scatter plots for quantitative fluorogenic real-time PCR values are shown in Fig. 1. ROC curves were established for each gene to determine the cutoff value. Methylation of the *Kif1a* gene was present in plasma from 1% of the control subjects, 4.3% of GGO patients, and 18% of lung cancer patients, respectively ($P = 0.0003$; Table 4). *DCC* gene methylation was significantly different between plasma from controls, GGO, and lung cancer patients [4 of 80 (5%), 0 of 23 (0%), and 19 of 70 (26%), respectively; $P = 0.0002$]. Methylation of *RARB* showed significant differences between groups in plasma samples. Three of 80 (3.7%) of controls, 2 of 23 (9%) of GGO, and 11 of 70 (16%) of plasma from lung cancer patients ($P = 0.02$) showed methylation in *RARB*. *NISCH* was found to be methylated in 66% of controls, 82% of GGO, and 86% of cancer patients' samples before a ROC curve cutoff was established. Using a cutoff of 334, the *NISCH* gene was methylated in 25% of control patients, 8.6% of GGO, and 41% of patients with lung cancer ($P = 0.037$).

In summary, *Kif1a*, *DCC*, *RARB*, and *NISCH* all showed significant differences in methylation between controls and lung cancer patient samples. These four genes were examined in the independent set to create a combined panel of methylation markers (Table 4). Seventy-three precent (51 of 70) of patients with cancerous tumors showed methylation of at least one gene, whereas 23 of 80 (29%) of control subjects showed methylation ($P = 0.0001$). Only 5 of 23 (22%) of patients with GGO exhibited methylation of at least one gene. There is a significant difference in methylation between patients with GGO and patients with confirmed cases of lung cancer ($P = 0.0001$). Overall, this panel of tumor markers can help discriminate between cancerous and noncancer findings after CT scan with a sensitivity of 73% and specificity of 71% (AUC = 0.643). To increase the specificity of the markers, we considered methylation positivity to be two or more markers methylated. The specificity increased to 93% but sensitivity decreased to 23%. Combinations of different markers were examined to maximize specificity and sensitivity. The best combination remained all four genes (Table 5).

**Patient demographics and smoking status versus methylation**

We also investigated the association of personal demographic variables with methylation status in plasma samples (Supplementary Table S3). There was no correlation between differences in methylation and age, ethnicity, or tumor histologic subtype (Supplementary Table S3A, B, and D). There was no difference in methylation in the control group with respect to gender; however, in the tumor group, there was a slight increase in methylation in male cancer patients compared with female patients (Supplementary Table S3C). With respect to tumor stage, all four markers were able to detect some stage I tumors (Supplementary Table S3E). When methylation of at least one of the four genes was examined as a group, 74% of stage I tumors showed methylation. Follow-up information was collected for 21 controls, 20 GGOs, and 10 lung cancer patients in the independent set. None of the controls were available for follow-up for developed lung cancer. One of the GGO patients did develop lung cancer 5 years after the original CT scan and died 7 years later. This patient showed an increased level of methylation of *NISCH* only. This patient was a 110 pack-year smoker. Of 10 lung cancer patients available for follow-up, 3 have died. The deceased patients all showed methylation of the NISCH marker only. Two were diagnosed with



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**Table 3.** Demographics of study participants in the independent set

| Controls | *n* = 80 |
|---|---|
| Gender | |
| Male | 39 |
| Female | 41 |
| Age median (range) | 53 (34-83) |
| Smoking | |
| Current | 42 |
| Former | 36 |
| Lifetime nonsmoker | 2 |
| Pack-years | |
| Lifetime nonsmoker | 2 |
| <20 | 1 |
| 20-34 | 32 |
| 35-49 | 32 |
| 50+ | 13 |
| Follow-up | |
| Patients available | |
| For follow-up | 21 |
| Cancer on follow-up | 0 |
| **Ground glass** | ***n* = 23** |
| Gender | |
| Male | 4 |
| Female | 19 |
| Age median (range) | 64 (44-75) |
| Smoking | |
| Current | 8 |
| Former | 15 |
| Lifetime nonsmoker | 0 |
| Pack-years | |
| Lifetime nonsmoker | 0 |
| <20 | 1 |
| 20-34 | 6 |
| 35-49 | 9 |
| 50+ | 7 |
| Follow-up | |
| Patients available | |
| For follow-up | 20 |
| Cancer on follow-up | 1 |
| Deaths | 1 |
| **Cancers** | ***n* = 70** |
| Gender | |
| Male | 37 |
| Female | 33 |
| Age median (range) | 69 (31-85) |
| Smoking | |
| Current | 14 |
| Former | 54 |
| Lifetime nonsmoker | 2 |
| Pack-years | |
| Lifetime nonsmoker | 2 |
| <20 | 9 |
| 20-34 | 14 |
| 35-49 | 12 |
| 50+ | 33 |
| Follow-up | |
| Patients available | |
| For follow-up | 10 |
| Cancer recurrance | 0 |
| Deaths | 3 |
| Tumor type and stage | |
| Adenocarcinoma | |
| Stage 1a | 26 |
| 1b | 8 |
| 2a | 0 |
| 2b | 1 |
| 3a | 7 |
| 3b | 1 |
| 4 | 4 |
| Squamous | |
| Not specified | 1 |
| 1a | 3 |
| 1b | 2 |
| 2b | 1 |
| Adenosquamous 1a | 1 |
| Mucinous 1a | 1 |
| Non–small cell lung carcinoma | |
| 1a | 1 |
| 3a | 1 |
| BAC 1a | 4 |
| Large cell | |
| 1a | 2 |
| 1b | 1 |
| 3 | 1 |
| 4 | |
| Spindle cell | 1 |
| No stage | 3 |

NOTE: Demographics of samples tested (independent set). The independent set was collected by NYU. Patients underwent chest CT scan and grouped into three groups based on CT findings. Controls (*n* = 80) had no findings on CT scan. GGO (*n* = 23) had ground glass abnormalities on CT scan. Cancers (*n* = 70) had confirmed solid cancerous tumors. Cancers detected were squamous cell carcinomas and adenocarcinomas.

stage IIIa adenocarcinomas. One had a smoking history of 20 pack-years and the other had 60 pack-years. The third deceased patient was diagnosed with stage Ia adenocarcinoma with BAC features and had a 51 pack-year smoking history.



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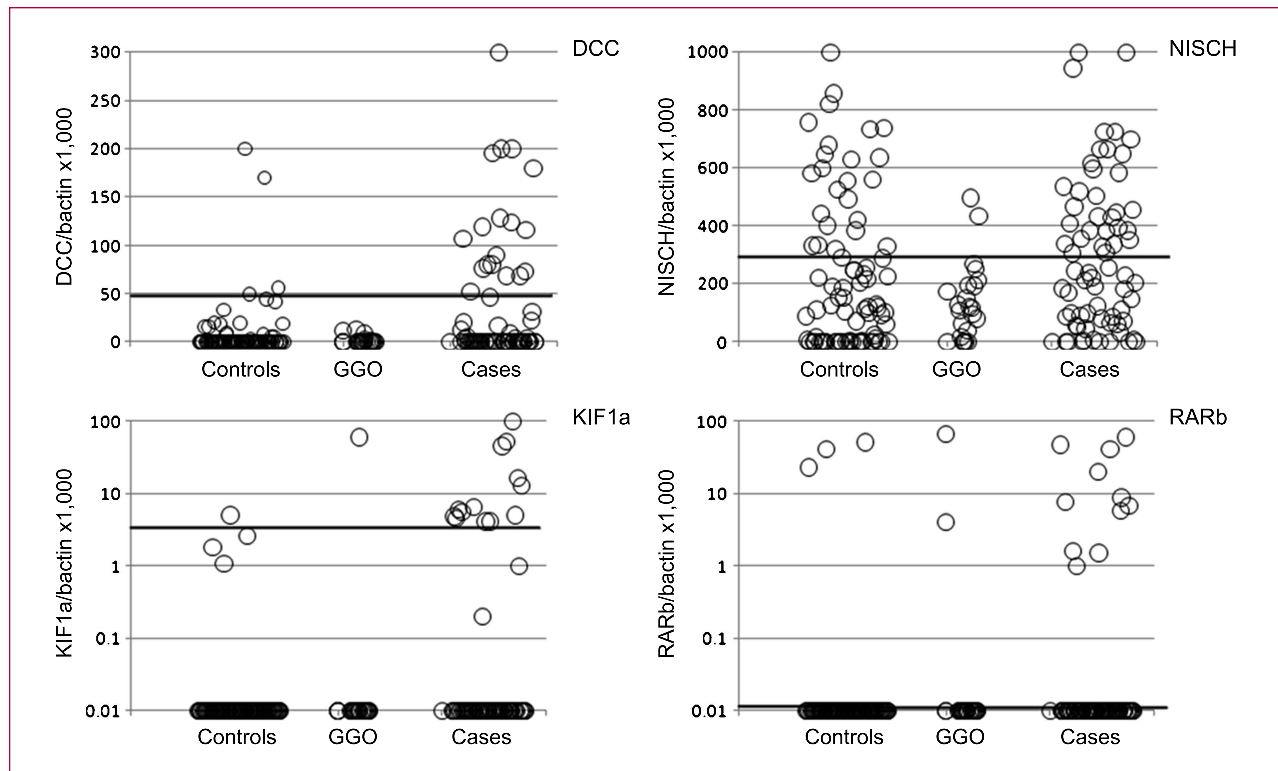


**Fig. 1.** Scatter plots of quantitative fluorogenic real-time PCR analysis of candidate gene promoters Kif1a, DCC, RARb, and NISCH. The samples were categorized as unmethylated or methylated based on detection of methylation above a threshold set for each gene (horizontal bar).

All of the samples in the NYU cohort (independent set) were at high risk for lung cancer based on their smoking history. The smokers were divided into four groups: 0 to 20, 20 to 34, 35 to 49, and 50+ pack-years, respectively. The methylation of *Kif1a*, *DCC*, *RarB*, and *NISCH* were examined in these groups (Supplementary Table S3F). When methylation of at least one of the four genes was considered, 72% of lung cancer patients with no smoking history or who had a smoking history of <20 pack-years showed methylation of at least one gene with 100% specificity (Table 6). No never-smoking or light-smoking controls showed methylation ($P$ = 0.05; Table 6). In the 20 to 34 pack-year group, 44% of controls, 33% of patients with GGOs, and 64% of lung cancer patients exhibited methylation. In the 35 to 49 pack-year group, there was an increase in methylation of lung cancer patient samples (75%) versus controls (19%) and GGOs (22%; $P$ = 0.0009 cancer versus controls). The controls who smoked 50+ pack-years showed 23% methylation,

**Table 4.** Methylation of genes in independent set

| Gene | Frequency of positivity | | | *P* | Cutoff | AUC (95% confidence interval) | Sensitivity % | Specificity % |
|---|---|---|---|---|---|---|---|---|
| | Controls | GGO | Cancers | | | | | |
| KIF1A | 1 of 80 | 1 of 23 | 13 of 70 | *P* = 0.0003 | 2.6 | 0.583 (0.5-0.662) | 18 | 98.8 |
| DCC | 4 of 80 | 0 of 23 | 19 of 70 | *P* = 0.0002 | 44 | 0.603 (0.521-0.681) | 26.4 | 95 |
| RARB | 3 of 80 | 2 of 23 | 11 of 70 | *P* = 0.02 | 0 | 0.556 (0.47-0.636) | 16 | 96.3 |
| NISCH | 20 of 80 | 2 of 23 | 29 of 70 | *P* = 0.037 | 334 | 0.596 (0.518-0.670) | 41 | 75 |
| 1 of 4 genes | 23 of 80 | 5 of 23 | 51 of 70 | *P* = 0.0001 | | 0.64 | 73 | 71 |

NOTE: The plasma samples from the independent set were tested for the methylation of RarB, NISCH, KIF1a, and DCC. Significant differences in methylation were detected between cancerous tumors and controls. Differences in methylation were also detected between cancerous tumors and noncancerous abnormal CT findings (GGOs).



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**Table 5.** Methylation sensitivity and specificity for multiple markers

| Gene | Frequency of positivity | | P | AUC | Sensitivity % | Specificity % |
|---|---|---|---|---|---|---|
| | Controls | Cancers | | | | |
| KIF1A, DCC, RARb, NISCH | 23 of 80 | 51 of 70 | 0 | 0.64 | 73 | 71 |
| DCC, RARb, NISCH | 22 of 80 | 47 of 70 | 0 | 0.57 | 67 | 73 |
| Kif1A, NISCH, RARb | 24 of 80 | 43 of 70 | 0 | 0.64 | 62 | 70 |
| KIf1A, DCC, NISCH | 22 of 80 | 46 of 70 | 0 | 0.57 | 68 | 73 |
| KIF1A, DCC, RARb | 6 of 80 | 30 of 70 | 0 | 0.6 | 43 | 93 |
| DCC, NISCH | 20 of 80 | 40 of 70 | 0 | 0.61 | 57 | 75 |
| KIF1a, NISCH | 21 of 80 | 38 of 70 | 0 | 0.61 | 54 | 74 |
| RARB, NISCH | 23 of 80 | 37 of 70 | 0 | 0.6 | 28 | 71 |
| KIF1A, DCC | 7 of 80 | 26 of 70 | 0 | 0.62 | 37 | 91 |
| DCC, RARB | 6 of 80 | 24 of 70 | 0 | 0.59 | 34 | 93 |
| KIF1a, RARB | 4 of 80 | 19 of 70 | 0 | 0.76 | 27 | 95 |

NOTE: ROCs were established for multiple markers. All four markers used in combination had the greatest sensitivity and specificity.

whereas the patients had a higher frequency of methylation (76%; $P = 0.0019$).

## Discussion

Exfoliative biomaterial (present in sputum, bronchoalveolar lavage, and bronchial brushings) offers diagnostic access, but the sensitivity of current cytologic tests is low (37). Diagnostic tools that would provide high specificity and sensitivity would clearly be of enormous benefit to patients, particularly if the specimens could be obtained by noninvasive means. To this end, the detection of aberrant methylation in plasma by quantitative methylation-specific PCR (38) may offer a promising approach for the noninvasive diagnosis of lung cancer. As seen in saliva for the detection of head and neck cancer (39), and urine sediment for the detection of bladder cancer (40, 41), these approaches are highly specific and correlate with tumor methylation status.

Tissue from our cohort of patients was not available and was therefore not tested. The panel of five plasma markers tested in the evaluation set were shown in our previous studies to be cancer-specific markers in lung tumor tissue in previous studies [*RARB* (ref. 29), *Kif1a*, *NISCH*, and *b4GALT1* (ref. 31)] with the exception of *DCC*. *DCC* was validated as a tumor marker in head and neck cancer (32), and esophageal squamous cell carcinoma (33). These genes have putative tumor suppressor functions. *RARB* located on chromosome 3p24 is involved in cell differentiation (42) and has been found to be under expressed in cancers (18, 23, 43, 44). *DCC* or "deleted in colorectal cancer" is located on chromosome 18q, has

**Table 6.** Methylation of markers versus pack-year smoking

| At least one of the four genes | Controls | GGO | Cancer | P | Sensitivity | Specificity |
|---|---|---|---|---|---|---|
| 0-20 py | 0/3 (0%) | 0/1 (0%) | 8/11 (72%) | 0.05 | 72 | 100 |
| 20-34 py | 14/32 (44%) | 2/6 (33%) | 9/14 (64%) | 0.34 | 64 | 56 |
| 35-49 py | 6/32 (19%) | 2/9 (22%) | 9/12 (75%) | 0 | 75 | 81 |
| 50+ py | 3/13 (23%) | 1/7 (14%) | 25/33 (76%) | 0 | 76 | 77 |
| **At least one of the four genes** | **Controls** | **GGO** | **Cancer** | **P** | **Sensitivity** | **Specificity** |
| 0-20 py | 0/3 (0%) | 0/1 (0%) | 8/11 (72%) | 0.05 | 72 | 100 |
| 20 + py | 23/77 (30%) | 5/22 (23%) | 43/59 (73%) | 0 | 73 | 70 |

NOTE: Methylation versus smoking history. Smokers were divided into four groups: 0 to 20, 20 to 34, 35 to 49, and 50+ pack-years. Methylation of Kif1a, DCC, RarB, and NISCH were examined in these groups.
Abbreviation: Py, pack-year.



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suppressed the malignant phenotype of epithelial cells (45), and is involved in apoptosis (46). Nisch located on chromosome 3p21 inhibits Rac1 oncogenic activity (47). *Kif1a* located on chromosome 2q37 is a member of the kinesin superfamily of motor proteins. This protein is an anterograde motor protein that transports membranous organelles along axonal microtubules (48) and is highly similar to mouse heavy chain kinesin member 1A (KHC) protein (48, 49). In mouse colon, KHC transports APC protein along microtubules. Suppression of KHC expression abolishes peripheral translocation of APC and induces the cellular accumulation of β-catenin, which may lead to malignant transformation (50). Altered expression of Kif1a and other kinesin superfamily genes have been reported in many human cancers including breast (51), glioblastoma (52), and prostate cancers (53).

In the present study, the plasma methylation markers *Kif1a*, *DCC*, *NISCH*, and *RARB* were evaluated independently and as a combined panel. *NISCH* is the only marker that showed a high level of methylation in the control group (25% of samples in the independent NYU set). The other genes were more lung cancer specific (95-98%) but less sensitive (16-26%). When all four genes were considered as a combined panel, increased sensitivity was achieved (73% of lung cancer plasma samples methylated). The combined specificity of this panel of markers is 71%.

Methylation in control plasma samples may be considered false positives or early events in the initiation of lung cancer. The subject population in the independent set are all heavy smokers, the control group consisting mainly of 20+ pack-year smokers. These methylation events may be disease initiating and predictive of lung cancer, but extensive follow-up of control patients with methylation is not available at this time. Consistent with the study by Baryshnikova et al. (54), which examined methylation markers in the sputum of cancer-free heavy smokers, subjects with one hypermethylated gene develop neoplasia not <5 years of follow-up. In our study, the GGO patient who harbored *NISCH* methylation developed lung cancer within 5 years and died 6.5 years after their initial CT scan. The other subjects in the controls group that evidenced methylation were either not available for follow-up or not contacted after 5 years of their initial scan.

More data suggesting that methylation of these genes plays a role in the development of lung cancer can be shown by looking at methylation in lifetime nonsmokers and <20 pack-year smokers. The plasma samples from the lifetime nonsmokers in the controls group showed no methylation of any of these markers, whereas lifetime nonsmokers and <20 pack-years smokers who had confirmed cases of lung cancer were methylated (72% methylated). This information suggests that the methylation of these genes is associated with the development of lung cancers and not just false positives.

In summary, this study identified a novel set of gene promoter markers that shows an increased level of methylation in plasma of lung cancer patients. There was a progressive increase in methylation from the control group with no abnormalities detected upon CT scan to patients with malignant lung tumors detected by CT scan, but this methylation is also related to smoking. For additional validation, we plan to follow this cohort of patients and collect plasma for follow-up methylation marker analysis. Upon follow-up, we may be able to predict cancer earlier in patients with CT-detected nodules and/or GGOs based on these methylation markers. If our results are confirmed in larger studies, the panel easily could be expanded in the future to simultaneously provide molecular staging and prognostic information in addition to detection.

## Disclosure of Potential Conflicts of Interest

D. Sidransky: share of royalty received by Johns Hopkins University upon sales of diagnostic products described in this article; ownership (stock), consultant, and scientific advisory board member, Oncomethylome Sciences, SA.

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