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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 CT-scan.

Experimental design—In a small evaluation cohort, 4 gene promoters (DCC, Kif1a, NISCH, 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 4 gene promoters by quantitative fluorogenic real-time PCR (QMSP). The patients were divided into 2 groups, ground glass opacity (GGO 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 GGO 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 to 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 non-cancerous abnormal CT findings.

Statement of Clinical Relevance

The development of a blood based test for lung cancer based on gene promoter methylation could augment current early detection approaches such as CT scan and sputum cytology. The methylation markers of lung cancer utilized in this study show great potential in

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molecular detection approaches. These markers can be detected in 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.

Introduction

Lung Cancer is the leading cause of cancer related death in the United States and other developed countries¹. 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 diagnosis². Detection of lung cancer at earlier stages could potentially increase survival rates by 10–50 fold². 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 non-invasive 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 (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”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 (p16INK4A, p15, Rb and p14)^{8–10}, DNA repair (MGMT and hMLH1)^{11, 12}, cell adhesion (H-cadherin and CDH-1)^{13, 14}, signal transduction (RASSF1A)^{15, 16}, apoptosis (DAPK and TMS1)¹⁷ and cell differentiation (RAR β 2)¹⁸. Studies in animals and in humans have demonstrated that these epigenetic changes are an early event in carcinogenesis and are present in the precursor lesions of a variety of cancers including breast¹⁹, lung²⁰, colon²¹, and endometrium²². Thus far, genes such as APC, p16, CDH1, RAR β -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 demonstrated the presence of promoter hypermethylation of various bodily fluids including plasma, sputum and bronchoalveolar 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 5 candidate tumor suppressor genes (RarB, NISCH, B4GALT1, KIF1a, DCC) in plasma of individuals who evidenced abnormal findings upon CT scans beginning with a small set of patients (evaluation set). These genes have previously demonstrated cancer – specific methylation in lung tumor tissue³¹, with the exception of DCC. DCC was validated as a tumor marker in head and neck cancer³² and esophageal squamous cell carcinoma (ESCC)³³. We then examined plasma DNA from a larger population of patients with abnormal findings on CT for methylation of the top 4 candidate genes (Kif1a, DCC, NISCH and RarB) from our evaluation set. This independent set examined plasma from patients with abnormalities (n=93) in lung by CT scan, as well as 80 plasma DNA samples from age matched individuals without any pathological 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). Ground Glass Opacity is a finding on high-resolution CT that is defined as hazy increased attenuation of the lung with preservation of bronchial and vascular margins³⁴. GGO is an abnormal finding and biopsied lesions have an unclear propensity for cancer progression³⁵. The current study identified novel cancer specific methylation markers in plasma samples. Our long term goal of 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 (SPORE) 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 non-smokers, current smokers and ex- smokers.

Independent set—Individuals were recruited from the New York University 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 ground-glass opacity (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 non-smokers, 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 lab 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, Germany) in the presence of 1% SDS at 48°C overnight followed by phenol/chloroform extraction and ethanol precipitation.

Bisulfite treatment

DNA extracted from 1mL of blood plasma was subjected to bisulfite treatment, using the EpiTect Bisulfite kit from Qiagen (Valencia, Ca) according to manufacturer’s conditions, www.Qiagen.com. Bisulfite treated DNA was eluted in 30µl 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 described³⁶. Amplification reactions were carried out in duplicate in a volume of 20 µL that contained 2 µL bisulfite-modified DNA, 600 nM forward and reverse primers, 200 nM probe, 5 U of Platinum *Taq* polymerase (Invitrogen), 200 µM each of dATP, dCTP, dGTP, 200µM dTTP and 5.5 mM MgCl₂. Primers and probes were designed to specifically amplify the promoters of the 5 genes of interest and the promoter of a reference gene, ACTB (Supplementary Table 1). 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., Beverly, MA) to generate completely methylated DNA, and serial dilutions (90–.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 1000 for easier tabulation (average value of duplicates of gene of interest/average value of duplicates of ACTB $\times$ 1000). Methylation cutoffs were set by Receiver Characteristic Operator Curves.

Statistical analysis

Fisher Exact tests (2 sided) were performed to detect significant methylation differences between groups. *p* values of <0.05 are significant.

Receiver Characteristic Operator Curves (ROC) were calculated using the web based calculator for ROC curves from 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 Receiver Operating Characteristic (ROC) curve 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.

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 44–78 years while the controls ranged in age from 34–83 years. Of the patients with lung cancer, 2 were lifetime non-smokers, and 10 had a smoking history. The control group was made up of 9 lifetime non-smokers and 14 with a smoking history.

The plasma samples from the evaluation set were tested for methylation of 5 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 appeared to be associated with cancer.

Independent cohort analysis

Methylation of these 4 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 beta-actin and multiplied by 1000 for easy tabulation (Supplementary Table 2). Scatter plots for QMSP values are shown in Figure 1.

ROC curves were established for each gene to determine 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 4/80 (5%), 0/23 (0%) and 19/70 (26%), respectively ($p=0.0002$). Methylation of RARB showed significant differences between groups in plasma samples, 3/80 (3.7%) of controls showed methylation in RARB, 2/23 (9%) of GGO, and 11/70 (16%) of plasma from lung cancer patients ($p=0.02$). 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 4 genes were examined in the independent set, to create a combined panel of methylation markers (Table 4). (51/70) 73% of patients with cancerous tumors showed methylation of at least one gene while 23/80 (29%) of control subjects showed methylation ($p=0.0001$). Only 5/23 (22%) of patients with ground-glass opacity (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 non-cancer 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 2 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 4 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 3). There was no correlation between differences in methylation and age, ethnicity, or tumor histological subtype (Supplementary Table 3a, 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 to female patients (Supplementary Table 3c). With respect to tumor stage, all 4 markers were able to detect some stage I tumors (Supplementary Table 3e). When methylation of at least one of the 4 genes was examined as a group, 74% of stage I tumors demonstrated methylation. Follow-up information was collected for 21 controls, 20 GGOs, and 10 lung cancer patients in the independent set. Baseline methylation status of these patients is shown in Figure 1. None of the controls were available for follow-up 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 (Figure 1). 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 Stage IIIa adenocarcinomas. One had a smoking history of 20 pack-years and the other 60 pack-years. The third deceased patient was diagnosed with Stage Ia adenocarcinoma with BAC features and had a 51 pack-year smoking history.

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 4 groups; (0–20, 20–34, 35–49, and 50+ pack-years, respectively). The methylation of Kif1a, DCC, RarB, and NISCH were examined in these groups (Supplementary Table 3f). When methylation of at least one of the 4 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 20–34 pack-year group, 44% of controls, 33% of patients with GGOs, and 64% of lung cancer patients exhibited methylation. In the 35–49 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 vs controls). The controls who smoked 50+ pack-years demonstrated 23% methylation, while 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 cytological tests is low³⁷. 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 PCR³⁸ may offer a promising approach for the noninvasive diagnosis of lung cancer. As seen in saliva for the detection of head and neck cancer³⁹, 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 5 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, (RARB²⁹, Kif1a, NISCH, b4GALT1³¹) with the exception of DCC. DCC was validated as a tumor marker in head and neck cancer³² and esophageal squamous cell carcinoma (ESCC)³³. These genes have putative tumor suppressor functions. RARB located on chromosome 3p24, is involved in cell differentiation⁴², 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 and has suppresses the malignant phenotype of epithelial cells⁴⁵ and is involved in apoptosis⁴⁶. Nisch located on chromosome 3p21 inhibits Rac1 oncogenic activity⁴⁷. 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 microtubules⁴⁸ 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 cellular accumulation of beta-catenin which may lead to malignant transformation⁵⁰. Altered expression of Kif1a and other kinesin superfamily genes have been reported in many human cancers including breast⁵¹, glioblastoma⁵² and prostate cancers⁵³.

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 demonstrated 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 4 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, which examined methylation markers in sputum of cancer-free heavy smokers, subjects with one hypermethylated gene develop neoplasia not less than 5 years of follow up⁵⁴. In our study, the GGO patient who harbored NISCH methylation who developed lung cancer within 5 years

and died 6½ 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 demonstrated by looking at methylation in lifetime non-smokers and <20 pack year smokers. The plasma samples from the lifetime non-smokers in the controls group showed no methylation of any of these markers, while lifetime non-smokers and <20 pack years smokers who had confirmed cases of lung cancer were methylated; (72% methylated). This information suggests that 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 demonstrate 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Scatter plots of QMSP 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). Red dots represent subjects with no disease (controls) or stable disease (GGO and lung cancers) upon follow-up (2–6 years). Green dots represent patients who progressed to advanced disease and are deceased. White dots represent subjects with no follow-up.

Table 1
Demographics of study participants in Evaluation set

Evaluation set obtained from UPMC. Patients with lung cancer (n=13) ranged in age from 44–78 years while the controls (n=24) ranged in age from 34–83 years. Of the patients with lung cancer, 2 were lifetime non-smokers, and 10 had a smoking history. The control group was made up of 9 lifetime non-smokers and 14 with a smoking history.

Controls	n=24	Cancers	n=13
<i>Gender</i>		<i>Gender</i>	
Male	5	Male	10
Female	19	Female	3
<i>Age median (range)</i>	54 (34–83)	<i>Age median (range)</i>	64 (44–78)
<i>Smoking</i>		<i>Smoking</i>	
current	4	current	5
former	10	former	5
lifetime non-smoker	9	lifetime non-smoker	2
na	1	na	1
<i>Pack years</i>		<i>Pack years</i>	
lifetime non-smoker	9	lifetime non-smoker	2
<20	4	<20	1
20–34	7	20–34	3
35–49	2	35–49	2
50+	1	50+	4
na	1	na	1
		<i>Tumor type and stage</i>	
		Adenocarcinoma	1
		squamous	6
		not specified	6

Table 2

Methylation of candidate genes in evaluation set

The plasma samples from the evaluation set were tested for methylation of 5 candidate tumor suppressor genes, (RarB, NISCH, B4GALT1, KIF1a, and DCC). Kif1a, DCC, RarB and NISCH showed differences in methylation status that appeared to be associated with cancer.

	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%)
	<i>p=0.08</i>	<i>p=0.06</i>	<i>p=0.01</i>	<i>p=0.01</i>	<i>p=1</i>

Table 3
Demographics of study participants in Independent set

The independent set was collected by NYU. Patients underwent chest CT scan and grouped into 3 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.

Controls	n=80	Ground Glass	n=23	Cancers	n=70
<i>Gender</i>		<i>Gender</i>		<i>Gender</i>	
Male	39	Male	4	Male	37
Female	41	Female	19	Female	33
<i>Age median (range)</i>	53 (34–83)	<i>Age median (range)</i>	64 (44–75)	<i>Age median (range)</i>	69 (31–85)
<i>Smoking</i>		<i>Smoking</i>		<i>Smoking</i>	
current	42	current	8	current	14
former	36	former	15	former	54
lifetime non-smoker	2	lifetime non-smoker	0	lifetime non-smoker	2
<i>Pack years</i>		<i>Pack years</i>		<i>Pack years</i>	
lifetime non-smoker	2	lifetime non-smoker	0	lifetime non-smoker	2
<20	1	<20	1	<20	9
20–34	32	20–34	6	20–34	14
35–49	32	35–49	9	35–49	12
50+	13	50+	7	50+	33
<i>Follow up</i>		<i>Follow up</i>		<i>Follow up</i>	
Patients available		Patients available		Patients available	
for follow up	21	for follow up	20	for follow up	10
Cancer on follow up	0	Cancer on follow up	1	Cancer recurrence	0
		deaths	1	deaths	3
				<i>Tumor type and stage</i>	
				<i>Adenocarcinoma</i>	
				stage 1a	26
				1b	8
				2a	0
				2b	1
				3a	7

Controls	n=80	Ground Glass	n=23	Cancers	n=70
				3b	1
				4	4
				squamous	
				not specified	1
				1a	3
				1b	2
				2b	1
				adenosquamous 1a	1
				mucinous 1a	1
				NSCLC	
				1a	1
				3a	1
				BAC 1a	4
				Large cell	
				1a	2
				1b	1
				3	1
				4	
				spindle cell	1
				no stage	3

Methylation of genes in independent set

The plasma samples from the independent set were tested for methylation 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 non-cancerous abnormal CT findings (GGOs).

Table 4

Gene	Frequency of positivity			P	Cutoff	AUC (95% CI)	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 out of 4 genes	23 of 80	5 of 23	51 of 70	p=0.0001		0.643	73	71

Table 5

Methylation sensitivity and specificity for multiple markers

ROC curves were established for multiple markers. All four markers used in combination had the greatest sensitivity and specificity.

Gene	Frequency of positivity		P	AUC	Sensitivity %	Specificity %
	Controls	Cancers				
KIF1A, DCC, RARb, NISCH	23 of 80	51 of 70	0.0001	0.643	73	71
DCC, RARb, NISCH	22 of 80	47 of 70	0.0001	0.569	67	73
Kif1A, NISCH, RARb	24 of 80	43 of 70	0.0001	0.639	62	70
Kif1A, DCC, NISCH	22 of 80	46 of 70	0.0001	0.573	68	73
KIF1A, DCC, RARb	6 of 80	30 of 70	0.0001	0.597	43	93
DCC, NISCH	20 of 80	40 of 70	0.0001	0.607	57	75
KIF1a, NISCH	21 of 80	38 of 70	0.0004	0.608	54	74
RARB, NISCH	23 of 80	37 of 70	0.0027	0.599	28	71
KIF1A, DCC	7 of 80	26 of 70	0.0001	0.619	37	91
DCC, RARB	6 of 80	24 of 70	0.0001	0.586	34	93
KIF1a, RARB	4 of 80	19 of 70	0.0002	0.761	27	95

Methylation of markers vs. pack year smoking

Smokers were divided into 4 groups; (0–20, 20–34, 35–49, and 50+ pack-years). Methylation of Kif1a, DCC, RarB, and NISCH were examined in these groups.

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%)	0.336	64	56
35–49 py	6/32 (19%)	2/9 (22%)	9/12 (75%)	0.0009	75	81
50+ py	3/13 (23%)	1/7 (14%)	25/33 (76%)	0.0019	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%)	0.0001	73	70