DNA Methylation in Tumor and Matched Normal Tissues from Non-Small Cell Lung Cancer Patients

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Abstract

We used MethyLight assays to analyze DNA methylation status of 27 genes on 49 paired cancerous and noncancerous tissue samples from non-small cell lung cancer (NSCLC) patients who underwent surgical resection. Seven genes (*RARB*, *BVES*, *CDKN2A*, *KCNH5*, *RASSF1*, *CDH13*, and *RUNX*) were found to be methylated significantly more frequently in tumor tissues than in noncancerous tissues. Only methylation of *CCND2* and *APC* was frequently detected in both cancerous and noncancerous tissues, supporting the hypothesis that the methylation of these two genes is a preneoplastic change and may be associated with tobacco smoking exposure. Methylation of any one of eight genes (*RASSF1*, *DAPK1*, *BVES*, *CDH13*, *MGMT*, *KCNH5*, *RARB*, or *CDH1*) was present in 80% of NSCLC tissues but only in 14% of noncancerous tissues. Detection of methylation of these genes in blood might have utility in monitoring and detecting tumor recurrence in early-stage NSCLC after curative surgical resection. (Cancer Epidemiol Biomarkers Prev 2008;17(3):645–54)

Introduction

Primary lung cancer remains the leading cause of cancer death in the United States with an estimated 213,380 new patients diagnosed and 160,390 deaths expected in 2007 (1). Non-small cell lung cancer (NSCLC) accounts for 80% of these new cases and includes the following histologic types: adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and mixed histologies.

Epigenetic inheritance refers to changes in gene function that occur independent of changes in nucleotide sequence. The most well-studied epigenetic change is DNA methylation, which is the addition of a methyl group to cytosines preceding guanidines (also called CpG dinucleotides). Methylation of CpG clusters (also called CpG islands) in the promoter region of the gene leads to gene silencing. It has been proposed that DNA methylation of promoter regions of tumor suppressor genes plays as important a role as genetic mutations in tumor development (2, 3). Multiple studies have assessed the epigenetic changes present in NSCLC (4, 5), reporting a wide range of hypermethylation frequencies. Some studies have compared the methylation changes of NSCLC with that of matching noncancerous lung tissue from the same patient (6-17). Most of these studies relied on a qualitative methylation-specific PCR (MSP) methodology that is subjective, relying on the detection of a band after electrophoresis, and does not distinguish between low-level and high-level methylation. Only four studies have used a quantitative approach to investigate gene methylation frequencies in matched tissue pairs (8, 9, 18, 19). Comparing methylation changes in NSCLC with those found in matched noncancerous lung is particularly important for determining potential links between DNA methylation changes and smoking, because, in any given patient, both tissue types have sustained the same exposure to tobacco smoke and other environmental factors. Further, to identify a panel of DNA methylation markers for detection of tumor recurrence, it is necessary to identify genes that are only methylated in tumor tissues but not in matched normal tissues.

In this study, we determined the DNA methylation status of 27 genes using quantitative MethyLight assays in tumor and matching noncancerous lung samples from 49 NSCLC patients who underwent complete surgical resection of their tumors (Table 1). We further identified a panel of genes that differentiates tumor tissues from noncancerous tissues. Such a panel of genes could potentially be used to detect tumor recurrence early after surgery by detecting tumor-associated circulating methylated DNA in plasma.

Materials and Methods

Inclusion and Exclusion Criteria. All subjects in the present methylation study are a subset of patients included in a larger prospective study of fluorodeoxyglucose positron emission tomography imaging in NSCLC conducted under the University of Washington Human Subjects Division approval. All patients were clinically diagnosed with NSCLC and had been referred

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Informed consent was obtained according to procedures approved by the Human Subjects Committee of the University of Washington.

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Table 1. Baseline demographic and tumor characteristics of 49 NSCLC patients, *n* (%)

Age	
<50	3 (6)
50-59	11 (22)
60-69	23 (47)
≥70	12 (24)
Female	23 (47)
Race	
White	42 (86)
African American	3 (6)
Asian	3 (6)
Hispanic	1 (2)
Histologic stage	
I	21 (43)
II	17 (35)
III	11 (22)
IV	0 (0)
Tumor differentiation	
Well	3 (6)
Moderate	16 (33)
Poor	30 (61)
Histologic type	
Adenocarcinoma	20 (41)
Squamous	14 (29)
Large cell	8 (16)
Bronchoalveolar (adenocarcinoma)	3 (6)
NSCLC, not otherwise specified/mixed	4 (8)
Tumor size, cm (mean ± SD)	3.2 ± 1.5
<3	24 (49)
3-5	20 (41)
>5	5 (10)
Pack-years	
0	1 (2)
1-39	14 (29)
40+	32 (65)
Smoker but amount unknown	2 (4)

to the thoracic surgery clinics at the University of Washington Medical Center or the Veterans Affairs Puget Sound Health Care System between February 1998 and August 2004. If histology of the lesion was subsequently confirmed to be other than NSCLC or if histology could not be confirmed, the patient was excluded from the study. Patients with a history of prior cancer were allowed to participate in the study if they had been disease free for at least 5 years. All patients had to be able to tolerate surgical resection if determined to be resectable. Patients who underwent wedge resection or segmentectomy and were later found to have local recurrence were excluded from the study due to the noncurative nature of this resection. Patients were also excluded if they had a history of type I diabetes (for the fluorodeoxyglucose imaging portion of the study), an inability to give informed consent, weighed over 350 pounds, or were either pregnant or breastfeeding. For imaging reasons, the primary NSCLC lesion had to be >1 cm on the mediastinal windows of the chest computed tomographic scan. Based on these criteria, 208 patients were enrolled into the imaging trial, the results of which have been recently reported in the literature (20), and followed the standard NSCLC care algorithm described previously (21). Among the 208 participants, a subgroup of 103 patients, plus 1 additional patient not reported previously, was surgically staged and underwent primary resection without any neoadjuvant or adjuvant therapy. Forty-nine of these resected patients had both NSCLC tissue blocks and matching noncancerous lung tissue collected at the time of surgery and constituted the study population.

Pathology. All biopsy and resection specimens were reviewed by the Department of Pathology of the University of Washington Medical Center or the Veterans Affairs Puget Sound Health Care System to (*a*) verify non-small cell histology of the lung cancer samples and (*b*) verify the noncancerous status of the tissue block selected as a matching noncancerous normal lung for analysis. The noncancerous lung sample was acquired at the time of evaluation of the lobectomy or pneumonectomy specimen by routine pathology procedures and selected from a location away from the primary tumor.

DNA Isolation from Paraffin Blocks. Six 20-μm sections were cut from each block and deparaffined by xylene extraction. The resulting tissue pellets were digested with proteinase K at 48°C overnight. The genomic DNA was isolated by phenol/chloroform extraction and ethanol precipitation. Finally, the DNA was purified using QIAamp DNA mini-column according to the manufacturer's protocol (Qiagen).

Sodium Bisulfite Conversion. Unmethylated human sperm DNA and *in vitro* fully methylated DNA were converted with clinical samples as described before (22). Briefly, ~1 μg DNA was modified by 5 mol/L sodium bisulfite, desulfonated with NaOH, then purified and resuspended in 80 μL EB buffer (10 mmol/L Tris-HCl, pH 8.0).

DNA Methylation (MethyLight) Analysis. For each gene, the primers and probe were designed specifically for bisulfite-converted fully methylated DNA (Appendix 1). Amplification of bisulfite converted *ACTB* was used to normalize for input DNA. Samples that were negative for *ACTB* were excluded in the methylation analysis. A plasmid containing bisulfite converted *ACTB* gene of known concentration was diluted and used as the standard curve for quantification. The assay for a given set of samples was considered valid only if the converted unmethylated human sperm DNA was not amplified, whereas the converted methylated DNA was amplified. The percentage methylated reference (PMR) for each locus was calculated by dividing the GENE/reference ratio of a sample by the GENE/reference ratio of methylated DNA and multiplying by 100 (23). Twenty-seven genes, plus the control gene *ACTB*, were analyzed in this study.

Statistical Methods. In this study, we dichotomized the semiquantitative MethyLight data in two different ways: the first classifying a specific gene positive for any hypermethylation and the second positive for high levels of hypermethylation. Earlier studies have reported that the 4% cutoff best discriminates between normal and premalignant/malignant tissues, and PMR ≥ 4% was associated with loss of expression (24, 25). However, other researchers have evaluated three levels of methylation based on PMR values, assigning PMR = 0 as no methylation, and using two levels of methylation (low and high) based on PMR values above and below the median value for a given gene (26). In our unpublished data, we confirmed the presence of low levels of methylation when PMR values were positive but <4%, but the significance of this level of methylation is not

clear. Because of the uncertainty regarding an appropriate cutoff for PMR values, we have presented our data in two ways: (*a*) a specific gene was considered positive for any hypermethylation if the PMR > 0% and (*b*) a specific gene was considered positive for high levels of hypermethylation if the PMR ≥ 4% in the sample. When multiple cancerous and/or noncancerous tissue samples were available, we randomly chose one of the cancerous and/or noncancerous blocks to be used in this analysis. McNemar's and Exact McNemar's tests for paired data were used to compare the frequency of gene-specific hypermethylation between NSCLC tissues and matched noncancerous tissue. A two-sided 0.05 test level determined statistical significance for all analyses. Gene sensitivity was defined as the proportion of cancerous tissue samples, which were methylated at a given level (PMR > 0% or PMR > 4%), whereas gene specificity was defined as the proportion of noncancerous tissue samples that were not methylated at that level. A gene panel consisting of methylated (at any level) genes that best distinguished cancerous from noncancerous tissue was created by first identifying genes that were 100% specific for cancerous tissue (not detected in the noncancerous tissue). These genes were ranked and added in a stepwise manner by increasing sensitivity, with the most sensitive, 100% specific gene first. Additional genes were subsequently added in a stepwise manner, by decreasing specificity, if the gene increased sensitivity by at least 4% (2 cases) and the increase in sensitivity exceeded the decrease in specificity. This process was continued until all the genes that met the above criteria were included. This same panel was then evaluated in terms of sensitivity and specificity for methylation at high levels (PMR ≥ 4%). Gene panel sensitivity was defined as the proportion of cancerous tissue samples in which gene methylation was detected in one or more of the genes in the panel, whereas gene panel specificity was defined as the proportion of noncancerous tissue samples in which gene methylation was not detected in any of the genes in the panel. Genes were also classified into nonmutually exclusive pathways according to their function, including apoptosis (*DAPK1, RUNX3, TMS1, PTEN*, and *SOCS3*), cell adhesion/invasion/metastasis (*OPCML, CDH13, BVES, APC, CDH1, IGSF4, KCNH5*, and *PCSK6*), cell cycle control (*CCND2, RASSF1, APC, FHIT, CDKN2A, CDKN2B, P14*, and *PTEN*), cell proliferation/differentiation (*RARB, IGFBP3, KCNH5, KCNH8, SOCS3*, and *PTGS2*), and DNA repair/detoxification (*FHIT, MLH1, MGMT, FANCF*, and *GSTP1*). All analyses were conducted using SAS version 9.1 (SAS Institute) and STATA version 8.2 (Stata).

Results

The mean age of the 49 NSCLC patients was 64.3 years. Approximately half were female (47%) and most were Caucasian (86%). All but one patient reported a history of smoking, with 65% reporting at least 40 lifetime pack-years of smoking. The majority of these NSCLC cases had surgical stage I (43%) or II (35%) disease and nearly half (49%) had tumors less than 3 cm. By histology, 41% of tumors were adenocarcinomas, 29% were squamous cell carcinomas, 16% were large cell carcinomas, and 6% were bronchioloalveolar carcinomas.

Table 2. Promoter hypermethylation in NSCLC tissue and matched noncancerous tissue (*n* = 49)

Gene	Any methylation (PMR > 0%)			High methylation (PMR ≥ 4%)		
	Cancerous tissue, *n* (%)	Matched noncancerous tissue, *n* (%)	*P**	Cancerous tissue, *n* (%)	Matched noncancerous tissue, *n* (%)	*P**
APC	26 (53)	21 (43)	0.30	20 (41)	13 (27)	0.09
RARB	23 (47)	4 (8)	<0.001	20 (41)	2 (4)	<0.001
CCND2	21 (43)	12 (24)	0.07	10 (20)	4 (8)	0.11
BVES	17 (35)	1 (2)	<0.001	12 (24)	0 (0)	0.001 †
CDH1	15 (31)	4 (8)	0.005	4 (8)	1 (2)	0.18
CDKN2A	14 (29)	2 (4)	0.001	14 (29)	1 (2)	<0.001
KCNH5	14 (29)	2 (4)	0.003	9 (18)	0 (0)	0.004 †
RASSF1	12 (24)	0 (0)	0.001 †	12 (24)	0 (0)	0.001 †
CDH13	11 (22)	1 (2)	0.004	8 (16)	0 (0)	0.008 †
KCNH8	9 (18)	3 (6)	0.058	5 (10)	0 (0)	0.06 †
IGSF4	9 (18)	2 (4)	0.035	2 (4)	0 (0)	0.50 †
DAPK1	8 (16)	0 (0)	0.008 †	5 (10)	0 (0)	0.06 †
RUNX	8 (16)	0 (0)	0.008 †	8 (16)	0 (0)	0.008 †
OPCML	7 (14)	3 (6)	0.16	6 (12)	2 (4)	0.10
PCSK6	5 (10)	1 (2)	0.10	3 (6)	0 (0)	0.25 †
MGMT	4 (8)	1 (2)	0.18	2 (4)	0 (0)	0.50 †
TMS1	4 (8)	0 (0)	0.13 †	2 (4)	0 (0)	0.50 †
IGFBP3	3 (6)	0 (0)	0.25 †	1 (2)	0 (0)	1.00 †
CDKN2B	1 (2)	0 (0)	1.00 †	0 (0)	0 (0)	—
MLH1	1 (2)	0 (0)	1.00 †	1 (2)	0 (0)	1.00 †
PTEN	1 (2)	0 (0)	1.00 †	1 (2)	0 (0)	1.00 †
FANCF	0 (0)	1 (2)	1.00 †	0 (0)	0 (0)	—
FHIT	0 (0)	0 (0)	—	0 (0)	0 (0)	—
P14	0 (0)	0 (0)	—	0 (0)	0 (0)	—
SOCS3	0 (0)	0 (0)	—	0 (0)	0 (0)	—
PTGS2	0 (0)	0 (0)	—	0 (0)	0 (0)	—
GSTP1	0 (0)	0 (0)	—	0 (0)	0 (0)	—

*McNemar's test.
†Exact McNemar's test.

This subset of patients ($n = 49$) was similar with regards to age, gender, tumor size, race, lifetime pack-years, differentiation, and histology when compared with the entire series ($n = 208$; data not shown). However, this study has a somewhat larger proportion of lower surgical stages ($P = 0.004$) compared with the larger group. The absence of late-stage patients in the present study is explained by the fact that most stage III and IV NSCLCs do not undergo a primary surgical resection but are treated with chemotherapy. Hence, matching noncancerous lung samples would not be available for these patients.

Of the 27 genes tested, five genes (*FHIT, P14, SOCS3, PTGS2*, and *GSTP1*) were never methylated and four genes (*MLH1, FANCF, CDKN2B*, and *PTEN*) were rarely methylated (≤3%) in cancerous or noncancerous tissue (Table 2). Of the four genes that were rarely methylated in patients with NSCLC, *MLH1, CDKN2B*, and *PTEN* were each detected in the cancerous tissue of a single patient, whereas *FANCF* was detected in the noncancerous tissue of a single patient. The remaining 18 genes had higher frequencies of methylation in NSCLC tissues compared with their matched noncancerous tissues, and methylation of 10 of these genes (*RARB, BVES, CDH1, CDKN2A, KCNH5, RASSF1, CDH13, IGSF4, DAPK1*, and *RUNX*) was detected significantly more often in NSCLC tissues. Similarly, seven genes were significantly more often methylated at high levels (PMR ≥ 4%) in cancerous compared with noncancerous tissue, including *BVES, KCNH5, RASSF1, CDH13*, and *RUNX*, which had high levels of methylation in cancerous but never noncancerous tissue, and *RARB* and *CDKN2A*, which were often methylated at high levels in cancerous tissue but infrequently methylated at high levels in noncancerous tissue.

Methylation of at least one of the 27 genes tested was present in 45 (92%) of 49 cancerous tissues and 26 (53%) of 49 adjacent normal tissues ($P < 0.001$; data not shown). Similarly, high levels of methylation (PMR > 4%) of at least one gene were present in 76% of cancerous tissues but only 39% of normal tissues ($P < 0.001$). Methylation of multiple genes was common, especially in cancerous tissue; methylation of two or more genes was detected in 76% of lung cancer tissue compared with 24% of normal tissue ($P < 0.001$). High-level methylation of multiple genes was detected in 55% of cancerous lung tissues but only 4% of adjacent normal tissues ($P < 0.001$).

We constructed a panel of genes, which was able to sensitively and specifically distinguish cancerous from noncancerous tissue in NSCLC patients. Two genes (*RASSF1* and *DAPK1*) were 100% specific and together identified 39% of NSCLC tissues (Table 3). The presence of methylation of at least one of the eight genes (*RASSF1, DAPK1, BVES, CDH13, MGMT, KCNH5, RARB*, and *CDH1*) identified 80% of NSCLC tissues and only occurred in 14% of the surrounding histologically noncancerous lung tissues of the same individuals. High levels of methylation of six genes (*RASSF1, DAPK1, BVES, CDH13, MGMT*, or *KCNH5*) identified 53% of cancerous tissues and were 100% specific. High levels of methylation of at least one of eight genes (adding *RARB* and *CDH1*) occurred in 63% of NSCLC cancerous tissues and only occurred in 6% of the surrounding histologically noncancerous lung tissues of the same individuals.

To assess the importance of inactivation of biological pathways through methylation in NSCLC, genes were categorized into nonmutually exclusive functional pathways, including apoptosis, cell adhesion/invasion/metastasis, cell cycle control, cell proliferation/differentiation, and DNA repair/detoxification (see methods for specific genes). In four of the five pathways assessed, any methylation and high levels of methylation (PMR > 4) were significantly more frequent ($P < 0.05$) in cancerous lung compared with normal lung tissue (Table 4), whereas for DNA repair/detoxification, methylation was infrequent (≤10%) and not significantly different in cancerous and normal lung. Methylation of genes associated with apoptosis was observed in 29% of cancerous lung tissue but was not observed in normal tissue. Similarly, methylation of genes associated with cell proliferation/differentiation was common (57%) in cancerous lung tissue but rare (12%) in normal tissue, especially at high levels (4%). Methylation of genes associated with cell adhesion/invasion/metastasis and cell cycle control was present in ~50% of normal tissue and the majority (78%) of cancer tissues. Because *APC*, associated with cell adhesion/invasion/metastasis and

Table 3. Panel of methylated genes sensitive and specific for cancerous NSCLC tissue in paired NSCLC cancerous and noncancerous tissue ($n = 49$)

Panel	Any methylation (PMR > 0%)		High methylation (PMR ≥ 4%)	
	Cancerous tissue, *n* (%)	Matched noncancerous tissue, *n* (%)	Cancerous tissue, *n* (%)	Matched noncancerous tissue, *n* (%)
RASSF1	12 (24)	0 (0)	12 (24)	0 (0)
+*DAPK1*	19 (39)	0 (0)	16 (33)	0 (0)
+*BVES*	25 (51)	1 (2)	20 (41)	0 (0)
+*CDH13*	28 (57)	1 (2)	23 (47)	0 (0)
+*MGMT*	30 (61)	1 (2)	24 (49)	0 (0)
+*KCNH5*	32 (65)	2 (4)	26 (53)	0 (0)
+*RARB*	36 (73)	5 (10)	31 (63)	2 (4)
+*CDH1*	39 (80)	7 (14)	31 (63)	3 (6)

NOTE: Panel of hypermethylated genes that best distinguish cancerous and noncancerous tissues from NSCLC patients. Genes are shown in the order in which they were added to the panel, starting with the most sensitive gene (*RASSF1*), which was 100% specific for NSCLC tissue. A two-gene panel of hypermethylation of *RASSF1* and/or *DAPK1* identified 39% of cancerous tissues but did not identify any noncancerous tissues. The three-gene panel (*RASSF1, DAPK1*, and/or *BVES*) identified 51% of cancerous tissues and only 2% of noncancerous tissues. This process was continued as long as adding genes resulted in at least a 4% gain in sensitivity with a corresponding loss of specificity that was less than the gain in sensitivity and resulted in a final eight-gene panel.

Table 4. Hypermethylation, by gene function, in paired NSCLC cancerous and noncancerous tissue (n = 49)

Pathway	Any methylation (PMR > 0%)			High methylation (PMR ≥ 4%)		
	Cancerous tissue, n (%)	Matched noncancerous tissue, n (%)	P	Cancerous tissue, n (%)	Matched noncancerous tissue, n (%)	P
Apoptosis*	14 (29)	0 (0)	<0.001	11 (22)	0 (0)	0.001
Cell adhesion/invasion/metastasis †	35 (71)	25 (51)	0.03	25 (51)	15 (31)	0.03
Cell adhesion/invasion/metastasis ‡	28 (57)	8 (16)	<0.001	17 (35)	3 (6)	<0.001
Cell cycle control§	41 (84)	24 (49)	<0.001	33 (67)	16 (33)	<0.001
Cell cycle control‖	23 (47)	2 (4)	<0.001	23 (47)	1 (2)	<0.001
Cell proliferation/differentiation¶	28 (57)	6 (12)	<0.001	21 (43)	2 (4)	<0.001
DNA repair/detoxification**	5 (10)	2 (4)	0.26	3 (6)	0 (0)	0.25

*Apoptosis genes: *DAPK1, RUNX3, TMS1, PTEN*, and *SOCS3*.
† Cell adhesion/invasion/metastasis genes: *OPCML, CDH13, BVES, APC, CDH1, IGSF4, KCNH5*, and *PCSK6*.
‡ Cell adhesion/invasion/metastasis genes, excluding *APC*.
§ Cell cycle control genes: *CCND2, RASSF1, APC, FHIT, CDKN2A, CDKN2B, P14*, and *PTEN*.
‖ Cell cycle control genes, excluding *APC* and *CCND2*.
¶ Cell proliferation/differentiation genes: *RARB, IGFBP3, KCNH5, KCNH8, SOCS3*, and *PTGS2*.
**DNA repair/detoxification genes: *FHIT, MLH1, MGMT, FANCF*, and *GSTP1*.

cell cycle control, and *CCND2*, important in cell cycle control, were frequently methylated in both cancerous and noncancerous tissue, we reassessed these pathways excluding these two genes. In this analysis, methylation of cell adhesion/invasion/metastasis genes was present in the majority (57%) of lung tumor tissue but was less commonly detected (16%) in normal tissue. Likewise, methylation of cell cycle control genes (excluding *APC* and *CCND2*) was present in nearly half (47%) of cancerous tissue and always at high levels but was almost never present (4%) in normal tissue.

Methylation of multiple genes in cancerous tissue was common, occurring in 28 (72%) of 39 cases that were identified by the gene panel (Fig. 1). For example, case 4 had high levels of methylation of *RASSF1, BVES, KCHN5*, and *RARB* and lower levels of methylation of *CDH13* in the same cancerous tissue sample. Similarly, case 7 had high levels of methylation of the same five genes. In contrast, some genes, such as *RASSF1* and *DAPK1*, were rarely methylated in the same cancerous tissue sample. Methylation of multiple genes was present in noncancerous tissue samples in only two samples (cases 3 and 48), and in one case (case 48), low-level methylation of a number of genes in the panel was present in noncancerous tissue but was not detected in cancerous tissue.

CCND2 and *APC* were frequently methylated, often at high levels, in both cancerous and noncancerous lung tissues of smokers with NSCLC, suggesting that hypermethylation of these genes may be associated with some environmental factors, such as chronic smoking (Fig. 2). Methylation of neither *CCND2* (P = 0.07) nor *APC* (P = 0.3) was strongly correlated in paired cancerous and noncancerous tissues. Only one other gene (*OPCML*) had a high level of methylation detected in noncancerous but not cancerous tissue from any NSCLC patient (case 14). Most genes were never or rarely methylated in noncancerous tissue in the absence of methylation in the paired cancerous tissue.

Discussion

In this study, we compared methylation changes in paired tumor and nonmalignant lung tissue blocks using quantitative MethyLight assays from 49 NSCLC patients who underwent surgical resection. We found that methylation of 10 genes was significantly more frequent in tumor than in matched noncancerous tissues. As few genes, except for *CCND2* and *APC*, were methylated in noncancerous tissues of NSCLC patients, methylation of most genes studied is associated with the molecular pathogenesis of lung cancer but is not linked to smoking exposure. We classified genes according to known function and pathway and noted that genes associated with apoptosis and cell proliferation/differentiation as well as cell adhesion/invasion/metastasis and cell cycle control genes, with the exception of *CCND2* and *APC*, were all commonly methylated, and at higher levels, in cancerous lung tissue but were infrequently methylated in normal lung tissue. Finally, we derived a panel of eight genes that identified 80% of NSCLC tissues but only 14% noncancerous lung tissues.

Our study is unique in several aspects: we analyzed more genes on paired samples than most other studies; we showed feasibility of using paraffin tissue blocks for methylation analysis, whereas most previous studies have used frozen tissues; we used quantitative MethyLight assays to detect DNA methylation, whereas most other studies used qualitative MSP; and our study is the first one to show that methylation of *BVES* is a frequent event in NSCLC.

We analyzed the methylation of the largest number (27) of genes on paired tumor and noncancerous tissues. Only a handful of other studies (8, 13-15, 17) have assessed five or more genes with 30 or more paired cases of NSCLC. Several studies have reported findings similar to ours. Toyooka et al. analyzed methylation of seven genes using MSP on 84 cancerous and noncancerous tissue pairs from NSCLC patients and reported methylation of five genes (*APC, RASSF1, CDH13, p16*, and *RARB*) was significantly more frequent in cancerous compared with noncancerous tissues. When methylation was detected in noncancerous tissues, methylation was also present in the corresponding tumor tissues (14). Yanagawa et al. analyzed eight genes using MSP on 75 pairs of tissue from NSCLC patients and found that methylation of five genes (*DAPK1, CDH1, CDKN2A, RASSF1A*, and *RUNX3*) was significantly more frequent in cancerous compared with noncancerous lung tissue (13). As in our study, methylation of *APC* was detected in both noncancerous (48%) and cancerous (37%) tissue.

Zochbauer-Muller et al. analyzed methylation of eight genes using MSP on 104 pairs of NSCLC tissues. Methylation of at least one gene was detected in 82% of tumors and in 29% of the noncancerous tissues. Most genes were rarely methylated in the noncancerous tissues (15). *RARB* was methylated in 14% of the noncancerous tissues, a rate somewhat higher than we found (8%); however, methylation of this gene occurred only at low levels. Further, they showed that most of the methylation detected in the noncancerous tissues was not

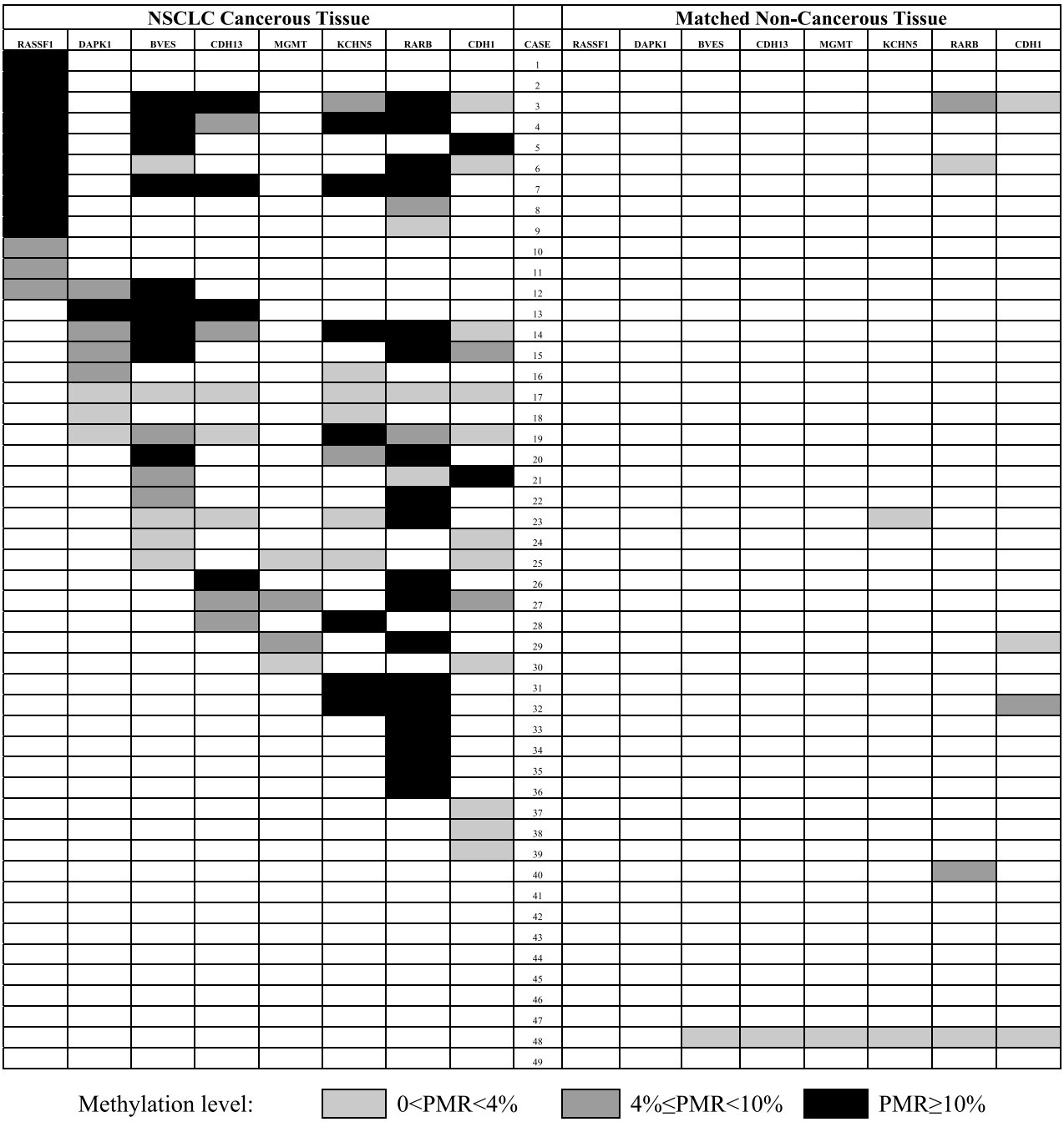

Figure 1. Summary of methylation patterns of paired cancerous (*left*) and noncancerous (*right*) tissues in 49 NSCLC patients for the eight genes included in the panel that best distinguishes cancerous from noncancerous tissue. Each row represents one patient. Genes are shown in the order in which they were added to the panel, starting with the most sensitive gene that was 100% specific for NSCLC tissue, and additional genes were added if the gain in sensitivity was greater than the loss of specificity. *Black shaded boxes,* samples methylated at very high levels (PMR $\geq$ 10%); *dark gray boxes,* samples methylated at high levels (PMR $\geq$ 4%); *light gray boxes,* samples methylated at low levels (PMR < 4%); *white boxes,* samples not methylated.

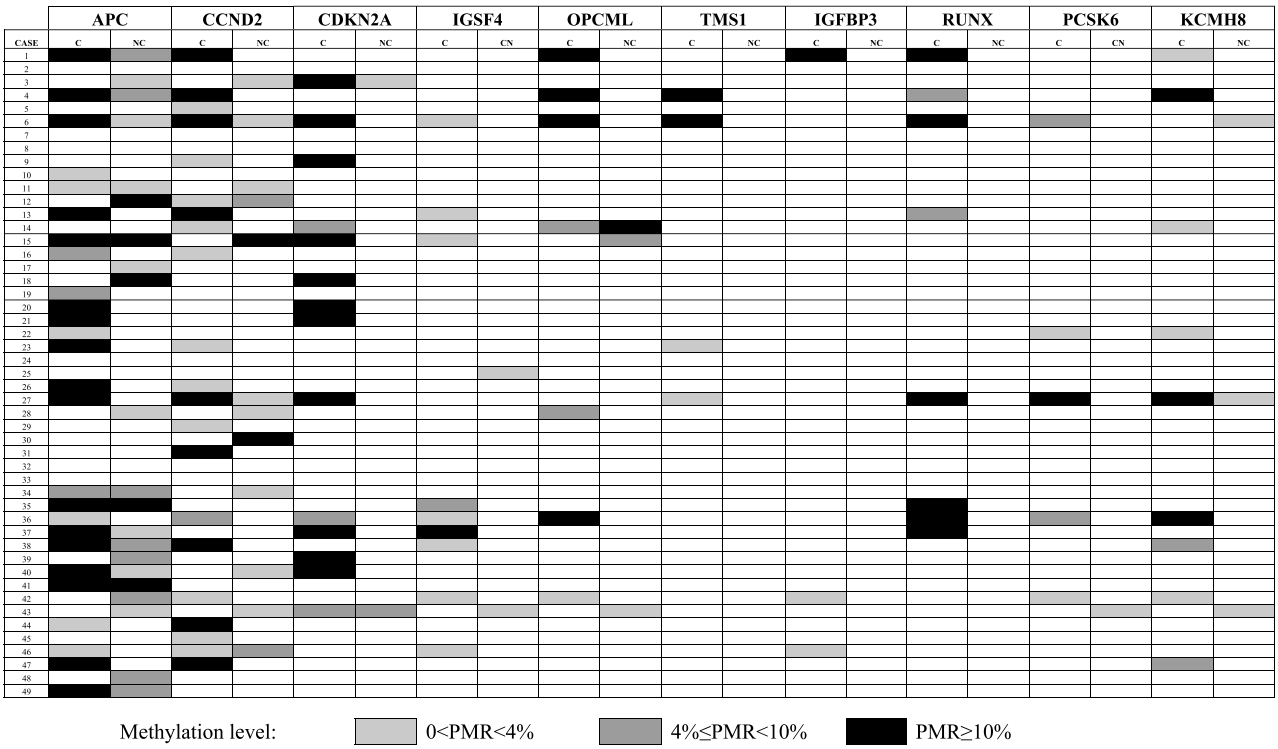

Figure 2. Summary of methylation patterns of paired cancerous (*C*) and noncancerous (*NC*) tissues in 49 NSCLC patients for 10 genes that were not included in the panel that distinguishes cancerous from noncancerous tissue. Genes methylated in fewer than two samples ($n = 9$) are not shown. Each row represents one patient. *Black shaded boxes,* samples methylated at very high levels (PMR $\geq$ 10%); *dark gray boxes,* samples methylated at high levels (PMR $\geq$ 4%); *light gray boxes*, samples methylated at low levels (PMR < 4%); *white boxes,* samples not methylated.

detected in the matched tumor tissues, suggesting that these methylation changes were preneoplastic. Shivapurkar et al. analyzed methylation of 11 genes using quantitative MSP on 40 pairs of NSCLC tissues. Methylation of at least one gene was present in 98% of the tumors. *APC* was frequently methylated in noncancerous lung tissues but never in peripheral blood mononuclear cells from cancer-free patients (8). Methylation of nine genes was significantly more frequent in cancer than in noncancerous tissues. However, in that study, individual gene methylation quantification level cutoffs to differentiate cancerous from noncancerous tissues were optimized *post hoc*, resulting in an overestimate of the importance of specific genes and making comparisons to the present study infeasible.

Contrary to the present study, two prior studies have reported similar frequencies of methylation in cancerous and noncancerous tissue samples. Kim et al. analyzed methylation of five genes using MSP on 72 pairs of adenocarcinoma tissues (17). High frequencies of methylation were observed for both tumor and noncancerous tissues, with methylation of at least one gene being found in 88% of cancer tissues and 79% of noncancerous tissues. Similarly, Safar et al. analyzed methylation of eight genes using MSP on 32 pairs of NSCLC tissues, and high frequencies of methylation in 6 genes were observed in cancerous as well as noncancerous tissues (7). The discrepancy is likely due to the shortcomings of the qualitative MSP method these researchers used for methylation detection.

The methylation frequencies of most genes we assessed in NSCLC tissues generally fell within the wide range of frequencies reported in the literature, although most studies used qualitative MSP. However, our findings with regard to methylation of *CCND2, FHIT*, and *TMS1* differed from that reported in other populations. In our study, 43% of the cancerous NSCLC tissues were methylated for *CCND2*, a rate similar to Virmani et al.'s study (40%; ref. 27). However, whereas Virmani et al. reported that *CCND2* was 100% specific for cancerous tissue, we detected *CCND2* in 24% of noncancerous tissues. *TMS1* was not frequently methylated in cancerous tissue in our study (8%), but it is frequently methylated in Virmani et al.'s report (40%). Although methylation of *FHIT* has been shown to be associated with smoking and occurs early during tumor development (28-30), we did not detect any methylation of *FHIT* in our study.

The gene-specific discrepancies we observed are likely due to the different methylation detection assays used in different studies. Although the same CpG island is investigated, the somewhat different primers and probes used in various methylation analyses indicate that different sequence regions are investigated. For example, the primers and probe sequences we used for *FHIT* is located in a different region from other primers and probes used in the literature (30, 31). Even when the

Appendix A. MethyLight Primers and Probes

Gene	Forward primer, probe, and reverse primer
ACTB	Forward: TGGTGATGGAGGAGGTTTAGTAAGT Probe: 6FAM-ACCACCACCCAACACACAATAACAAACACA-TAMRA Reverse: AACCAATAAAACCTACTCCTCCCTTAA
DAPK1	Forward: GGAGAGGGTGGTTACGGTGTT Probe: 6FAM-CAACCTACGACAACGATAA-MGB Reverse: ACCCTCGCCCAAACGATAC
RARB	Forward: TGAGGATTGGGATGTCGAGAA Probe: 6FAM-CTACTCGAATCGCTCG-MGB Reverse: CCCGACGATACCCAAACAAA
CCND2	Forward: CGTGTTAGGGTCGATCGTGTT Probe: 6FAM-ACTACGATAAAATCGCCG-MGB Reverse: CTCGCCAAACTTTCTCCCTAAA
RASSF1	Forward: TAGGTTTTTATTGCGCGGTTTT Probe: 6FAM-CGCGAACCGAACGAA-MGB Reverse: TACTTCGCTAACTTTAAACGCTAACAA
OPCML	Forward: CGGTTAGGGATGGAGTTGTTG Probe: 6FAM-CCGAACCGCCGAAA-MGB Reverse: CGCCTTCCTCCCGAAAAC
CDH13	Forward: GATTTTTGGGTTCGGAATGATTT Probe: 6FAM-TTTTCGTCGTCGCGATC-MGB Reverse: ATCGCCCGACACGAACAA
BVES	Forward: GGACGGAGTGGGCGATATC Probe: 6FAM-CCTACGTACAACCGAACG-MGB Reverse: CCTCGAACCGCGCAAA
APC	Forward: TTATATGTCGGTTACGTGCGTTTATAT Probe: 6FAM-CCCGTCGAAAACCCGCCGATTA-TAMRA Reverse: GAACCAAAACGCTCCCCAT
CDH1	Forward: AATTTTAGGTTAGAGGGTTATCGCGT Probe: 6FAM-CGCCCACCCGACCTCGCAT-TAMRA Reverse: TCCCCAAAACGAAACTAACGAC
FHIT	Forward: CGCGCGTTAGGTTATTATTTCG Probe: 6FAM-TCGGCGTCGTTTAC-MGB Reverse: ACCCTAAAACCCTCGTAAAACGA
MLH1	Forward: CGTTATATATCGTTCGTAGTATTCGTGTT Probe: 6FAM-CAAACGCCACTACGAAA-MGB Reverse: ACCTAATCTATCGCCGCCTCAT
IGSF4	Forward: AGGGAGCGAGGTTTTTCGA Probe: 6FAM-CGAACCCAACCCGAC-MGB Reverse: ACGAAATCCGAACAAACCAATC
MGMT	Forward: GCGTTTCGACGTTCGTAGGT Probe: 6FAM-CGATACGCACCGCGAA-MGB Reverse: ACCCAAACACTCACCAAATCG
CDKN2A	Forward: TGGAGTTTTCGGTTGATTGGTT Probe: 6FAM-ACCCGACCCCGAACCGCG-TAMRA Reverse: AACAACGCCCGCACCTCCT
RUNX	Forward: CGAGGTTTCGTTGGTTCGA Probe: 6FAM-CCGCACGCGAAAC-MGB Reverse: GCCGCGACCCAAACAA
FANCF	Forward: TTTTTATTGGTTGTGTAGTCGTCGTT Probe: 6FAM-CCATTCGCACGACTC-MGB Reverse: CATCCATCGACGCTTTAATCG
TMS1	Forward: CGTTATTTTGGATGCGTTGGA Probe: 6FAM-ACTCCTCGACGATCA-MGB Reverse: ACGACACCGACAACAACTTCAA

Appendix A. MethyLight Primers and Probes (Cont'd)

Gene	Forward primer, probe, and reverse primer
IGFBP3	Forward: GGGCGTGTTTTGGGTTATTTC Probe: 6FAM-CGAACGCGCCGACC-MGB Reverse: CGCAATACTCGCATCTAAACGA
CDKN2B	Forward: TCGTTTTTTTGCGGTTTGG Probe: 6FAM-AACCCGAAACTAACGAC-CGA-MGB Reverse: CGCGCCCGCTAATTCTTAA
KCNH5	Forward: ATTGTGTTTCGCGGAGTTTGT Probe: 6FAM-CCGCACACGAAACT-MGB Reverse: GCCGCTACCCAAACTATAACGA
KCNH8	Forward: CGTCGCGCGGTGTTTC Probe: 6FAM-CTCTTTCCGAACGAACG-MGB Reverse: GCCCCGAACCACTACGAA
PCSK6	Forward: TCGGGAGGTTCGAAACGTT Probe: 6FAM-CCAAAAATCGACGAAAAA-MGB Reverse: ACACCGCGTCCCGAAAA
PTEN	Forward: TTAAGCGTTAGTTTCGATAGCGTTT Probe: 6FAM-TCGAACCAACCTCCCGAA-MGB Reverse: CTACGCCGCGACGAAAA
P14ARF	Forward: CGCGGCGGGTCGTA Probe: 6FAM-CGACGAAAACATAATAC-GCA-MGB Reverse: CGACTACCGAACTCGACCCTAA
SOCS3	Forward: TGTGGATTTTACGGTCGTTAATATTT Probe: 6FAM-AACCCGCGCTACGC-MGB Reverse: AACGACGCGACGACGAAA
PTGS2	Forward: CGAAAAGGCGGAAAGAAATAGTT Probe: 6FAM-ATTAATCGCTAACCGAAAAA-MGB Reverse: TCCTAACGCTCACTACAAATCGTATAA
GSTP1	Forward: TCGGAGGTCGCGAGGTT Probe: 6FAM-CTACGACGACGAAACT-MGB Reverse: CGCGCGTACTCACTAATAACGA

same primers are used, the specific PCR conditions may affect the MSP results. For example, several studies analyzed *CDKN2A* methylation using the same primers. However, different methylation frequencies were detected because different annealing temperatures and master mixes were used in these studies (10, 12, 13, 32). Because of the incorporation of sequence-specific probes in quantitative MSP assays, these assays tend to have improved specificities due to elimination of nonspecific products detection and identification of only fully methylated sequences. This emphasizes the importance of standardization of methylation analysis protocols, so that meaningful comparisons can be made among different studies. Finally, because little is known of the origin of cancer-specific DNA methylation changes, the discrepancies observed might be due to the differences in patient population and tumor heterogeneity (14).

Given the central role smoking plays in lung cancer development and that DNA methylation is an early event in tumorigenesis, it is somewhat surprising that we did not detect frequent methylation of most genes we analyzed in the adjacent noncancerous lung tissues in NSCLC patients. Several studies have looked at the effect of smoking on DNA methylation (14, 33-35). Some studies have shown no correlation between methylation

of *CDKN2A, DAPK1, GSTP1*, and *APC* and smoking history (19, 34, 36, 37), whereas Kim et al. showed that increased *CDKN2A* methylation with increased pack-years and duration of smoking (34). Methylation of *RASSF1A* has also been associated with exposure to smoke in lung cancer (14, 38, 39), and methylation of *FHIT* has been shown to be an early event in smoking-caused squamous cell lung cancer (29, 30). It is possible that the DNA methylation of most of the genes we analyzed (except for *APC* and *CCND2*) is a cancer-specific change only present in tumor tissues but not in preneoplastic tissues. However, it is also possible that these genes are only partially methylated in preneoplastic tissues and hence could not be detected by MethyLight assays. Only detailed bisulfite sequencing analysis would be able to detect these changes.

In the current study, we detected methylation of *CCND2* and *APC* at high frequencies in matched noncancerous lung samples, suggesting that methylation of these genes in both cancerous and noncancerous tissue was associated with a common environmental exposure, such as tobacco smoke. Several studies have reported the methylation of *APC* in noncancerous lung tissues. Two studies used conventional MSP to show that *APC* was methylated in 30% to 50% tumors and also methylated in 14% and 48%, respectively, noncancerous tissues (13, 14). Three studies detected *APC* methylation in ~90% tumors using quantitative MSP assays and 58% to 88% in noncancerous tissues (8, 9, 19). Although we detected methylation of *CCND2* in 25% of noncancerous tissues, Virmani et al. did not detect any methylation in adjacent normal lung tissues (27). Unfortunately, in the present study, we did not assess gene hypermethylation in nonsmokers with NSCLC or in smokers without NSCLC, so we were unable to directly assess the relationship between smoking, NSCLC, and gene methylation.

Our data indicate that NSCLC-specific DNA methylation changes of many genes are rare in noncancerous lung tissues from NSCLC patients. Several pathways appear to be important for tumorigenesis of NSCLC, including apoptosis, cell adhesion/invasion/metastasis, cell cycle control, and cell proliferation/differentiation. This has important implications with regards to (*a*) understanding the role of environmental factors (that is, tobacco smoke) in the development of hypermethylation changes in NSCLC and (*b*) using a DNA hypermethylation panel to detect tumor recurrence after surgical resection of NSCLC. The fact that we were able to identify a panel of eight hypermethylated genes that were present in 80% of the NSCLC tissues but only present in 14% of the noncancerous lung tissue suggests that a methylated gene panel from sputum or blood may have potential in detecting occult NSCLC in high-risk individuals, such as smokers, as well in predicting tumor recurrence in postoperative NSCLC patients (40-42).

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