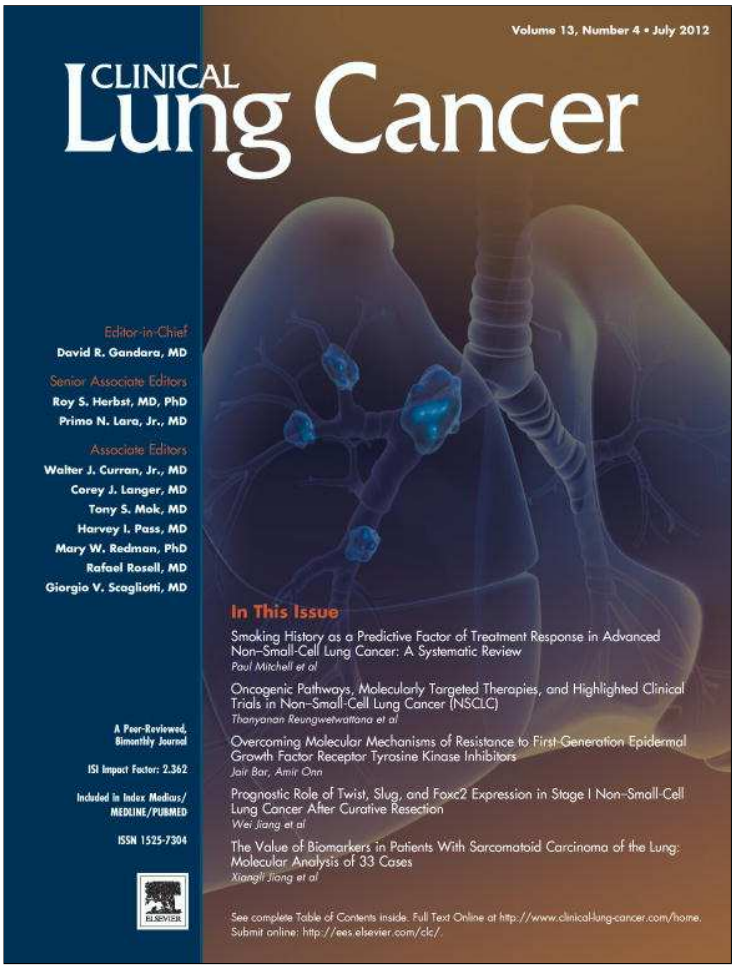

Original Study

Aberrant Promoter Methylation of *CDH13* and *MGMT* Genes is Associated With Clinicopathologic Characteristics of Primary Non–Small-Cell Lung Carcinoma

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Abstract

Non–small-cell lung carcinoma (NSCLC) (n = 65) were analyzed for promoter methylation of *RASSF1A, CDH13, MGMT, ESR1,* and *DAPK* genes in matching lung tumors, normal lung tissue, and blood samples. Aberrant methylation in *CDH13* and *MGMT* was associated with clinicopathologic features of NSCLC. Hypermethylation detected in primary tumors was not observed in corresponding blood samples, which rendered this an unsuitable blood-based test for NSCLC detection.

Introduction: Systemic methylation changes may be a diagnostic marker for tumor development or prognosis. Here, we investigate the relationship between gene methylation in lung tumors relative to normal lung tissue and whether DNA methylation changes can be detected in paired blood samples. **Material and Methods:** Sixty-five patients were enrolled in a surgical case series of non–small-cell lung carcinoma at a single institution. By using bisulfite pyrosequencing, CpG methylation was quantified at 5 genes (*RASSF1A, CDH13, MGMT, ESR1,* and *DAPK*) in lung tumor, pathologically normal lung tissue, and circulating blood from enrolled cases. **Results:** The analyses of methylation in tumors compared with normal lung tissue identified higher methylation of *CDH13, RASSF1A,* and *DAPK* genes, whereas *ESR1* and *MGMT* methylation did not differ significantly between these tissue types. We then examined whether the 3 aberrantly methylated genes could be detected in blood. The difference in methylation observed in tumors was not reflected in methylation status of matching blood samples, which indicated a low feasibility of detecting lung cancer by analyzing these genes in a blood-based test. Lastly, we probed whether tumor methylation was associated with clinical and demographic characteristics. Histology and sex were associated with methylation at the *CDH13* gene, whereas, stage was associated with methylation at *MGMT*. **Conclusion:** Our results showed higher methylation of *RASSF1A*, *CDH13*, and *DAPK* genes in lung tumors compared with normal lung. The lack of reflection of these methylation changes in blood samples from patients with non–small-cell lung carcinoma indicates their poor suitability for a screening test.

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Introduction

Lung cancer is one of the most common causes of cancer-related deaths worldwide.[1] Despite advances in diagnostic methods and treatment, prognosis remains poor. Epigenetic control of gene expression plays an important role in carcinogenesis. Aberrant methylation of CpG dinucleotides is a commonly observed epigenetic modification in human cancer,[2] and it appears to be even more frequent than genetic mutations in cancer. Whereas, tumors are characterized by genome-wide hypomethylation, hypermethylation in the promoter regions of tumor suppressor genes has been a proposed mechanism of transcriptional silencing equivalent to a mutational event.[2]

DNA methylation changes in lung cancer are well established, and translation of this knowledge toward screening has the potential for significant health improvement. With the discovery of new disease screening markers, it may be possible to achieve early diagnosis, to have more informed choices for cancer treatment, and to identify markers associated with prognosis and response to therapy. Researcers in the field have proposed that, in the near future, it may be possible to screen patients for lung cancer by using DNA methylation signatures to measure patient responses to treatment, to identify patients at increased risk, or to monitor interventions designed to reduce cancer incidence.[3,4] To achieve these aims, it will be necessary to demonstrate the specificity of epigenetic change to cancerous cells and to evaluate the sensitivity of detecting aberrations in easily accessible tissue (ie, circulating blood).

A number of genes have been reported as aberrantly methylated in lung tumors. We examined the medical literature to identify a panel of genes most frequently identified as hypermethylated in lung tumors, and favored those with possible association between hypermethylation and clinicopathologic features which led to investigation of a panel of 5 genes (*RASSF1A, CDH13, MGMT, ESR1,* and *DAPK*) by using bisulfite pyrosequencing to determine whether these methylation changes are specific to lung tumors and to test whether these changes are detectable in patients' blood samples. We further analyzed possible associations between DNA methylation and clinicopathologic features.

Material and Methods

Primary tumor samples (n = 65), corresponding nonmalignant lung tissues (n = 65), and matching blood samples (n = 51) were obtained from patients with non–small-cell lung carcinoma (NSCLC) who had been treated in 2009 with curative resectional surgery at the Institute for Lung Diseases, Clinical Center of Serbia, University of Belgrade. This study was approved by the institution's ethics committee, and an informed consent was obtained from all study participants. Five milliliters of blood was collected by using EDTA Vacutainers (Becton Dickinson Vacutainer System, Franklin Lakes, NJ) and was stored at −20°C until processing. DNA was extracted from whole blood samples by using the method described before[5] briefly, blood cells and platelets were lyzed by adding an equal volume of lysis buffer (0.32 M sucrose, 10 mM Tris-HCl pH 7.5, 5 mM $MgCl^2$, and 1% Triton X-100). The lysate was centrifuged at 3000 rpm for 10 minutes. The supernatant was removed, and the pellet again was subjected to lysis and centrifugation. After supernatant removal, the pellet was suspended in 3 mL buffer (10 mM Tris-HCl, 0.4 M NaCl, 2 mM EDTA, and 25 μL proteinase K) followed by the addition of Sodium Dodecyl Sulfate (0.7% final concentration). After overnight incubation at 37°C, 1 mL of 6M NaCl was added, and the proteins were pelleted by centrifugation. Supernatant that contained DNA was transferred into fresh tubes and centrifuged at 4000 rpm for 10 minutes. Supernatant was transferred into new microcentrifuge tubes, and an equal volume of isopropanol was added. DNA became visible and was transferred, washed in 1 mL 70% ethanol, air-dried, then resuspended in distilled water and analyzed for quality by electrophoresis on agarose gel and quantified by using Nanodrop spectrophotometer (ThermoScientific Inc, Wilmington, DE). The isolation of DNA from fresh-frozen tumors was performed as previously described.[3] The subsequent laboratory research was carried out at the Masonic Cancer Center, University of Minnesota, Minneapolis, MN. The detection of DNA methylation is based on a treatment of genomic DNA with sodium bisulfite, which converts unmethylated cytosines to uracil, while methylated cytosines stay unaltered. Bisulfite modification of genomic DNA was performed according to the manufacturer's protocol (Zymo Research, Irvine, CA). A strand-specific polymerase chain reaction (PCR) product was then generated to provide a suitable DNA template for pyrosequencing (Table 1). Primer sequences and PCR conditions for the *RASSF1A, CDH13, MGMT,* and *DAPK* genes have been described previously.[6-9] To facilitate data comparison with other publications, the sequences of the primers as well as the sequences analyzed within each gene are shown in Table 1. Amplicons were resolved by agarose electrophoresis to confirm proper amplification and quality of product. Percentage methylation for each CpG as well as average methylation across CpGs were calculated for each promoter by using PyroMark software (Qiagen, Valencia, CA).

Statistical Analysis

Descriptive statistics were calculated for each CpG site as well as the average over multiple CpGs at each gene. Medians and interquartile ranges were calculated for continuous variables because the data were not normally distributed. Box plots were created to visually assess the distribution of values at each CpG site. Due to the nonnormality of methylation values, differences in methylation between tissue types were assessed by using the paired Wilcoxon signed rank test on the average methylation values over all CpG sites within a gene region. Using individual CpG sites or the average CpG site produced similar results.

Tumor hypermethylation was assigned for those samples that had methylation values more than 3 SDs away from the mean of the normal tissue values for average methylation over CpG sites within the gene. We compared methylation in blood samples between patients with and without hypermethylation in the tumor sample by using the Wilcoxon rank sum test. The association between tumor hypermethylation and patient and tumor characteristics was assessed by using the Fisher exact test. Variables assessed included estimated patient age in years (<55, 55-59, 60-64, >65), sex (men, women), smoking (current, former/never), pack-years (<40, 40-70, >70), histology (squamous cell carcinoma, adenocarcinoma, other), grade (<2, 2, >2) and stage (I, II, III, IV). Patient age was estimated

Table 1 Promoter Methylation Analysis in Genes *ESR1, RASSF1A, CDH13, MGMT,* and *DAPK* by Pyrosequencing: Shown Are the Amplification Primers (Forward and Reverse), Sequencing Primer(s) Used, and the Sequence That Was Analyzed

	ESR1	RASSF1A	CDH13	MGMT	DAPK
Forward Primer	TTTTTGGGTTATTTTTAGTAGATT	Biotin-ATAGGTTTTTGTTTAATGAGT	TTGGGAAGTTGGTTGGTTG	TTGGTAAATTAAGGTATAGAGTTTT	GAGGGTAGTTTAGTAAGTTGTTATAG
Reverse Primer	Biotin-CAAAAAACAACTTCCCTAAACT	CTACACCCAAATTTCCATTAC	Biotin-ACAACCCCTCTTCCCTACCT	Biotin-AAACAATCTACRCATCCT	Biotin-CCTCCAACTACCCTACCAAA
Sequencing Primer	GGTTATTTTTAGTAGATTTT	ATCTAAATCCTAAAAAAAAC	GGAAAATATGTTTAGTGTAG	GGAAGTTGGGAAGG	TTTAGTAATGTGTTATAGGT
Analyzed Sequence	YGTGYGTTTTYGTTTTTGGTYGTG	RCTAAAATCRAAACCCRCCCTATAACCCCRCCCRACCCRCRCTTACTAACRCCCAAAACCAACRAAACACRAACCCAACCRAACC	TYGYGTGTATGAATGAAAAYGTYGTYGGGYGTTTTTA	YGTYGTTYGGTTTGTATYGGTYGAAGGGTTA	GGGGYGTTYGYGTTTYGGGYGGAYGTATTGGTTTTTYGGTYGGYGTGGGTGTGGGGYGAGTGGGTGTGTGYGGGGTGTGYGYGGT
Sequencing Primer 2	AATTTAGTTTTTATTTAGTA				
Analyzed Sequence 2	GYGAYGATAAGTAA				

Table 2 Subject Demographic, Tumor Histology, Grade, Stage, and Smoking Status Data

Variable	Levels	Number (%)
Grade	<2	21 (33.3)
	2	23 (36.5)
	>2	19 (30.2)
	Missing	2
Histology	Squamous cell carcinoma	35 (53.8)
	Adenocarcinoma	20 (30.8)
	Other	10 (15.4)
Stage	1	16 (25.0)
	2	25 (39.1)
	3	19 (29.7)
	4	4 (6.3)
	Missing	1
Sex	Women	21 (32.3)
	Men	44 (67.7)
Smoking Status	Current	53 (81.3)
	Former	10 (15.6)
	None	2 (3.1)
	Missing	1
Pack-Years	<40	18 (28.6)
	40-70	33 (52.4)
	>70	12 (19.0)
Estimated Age	<55 y	14 (21.5)
	55-59 y	17 (26.2)
	60-64 y	15 (23.1)
	≥65 y	19 (29.2)
	Mean (SD)	60.00 (6.86)

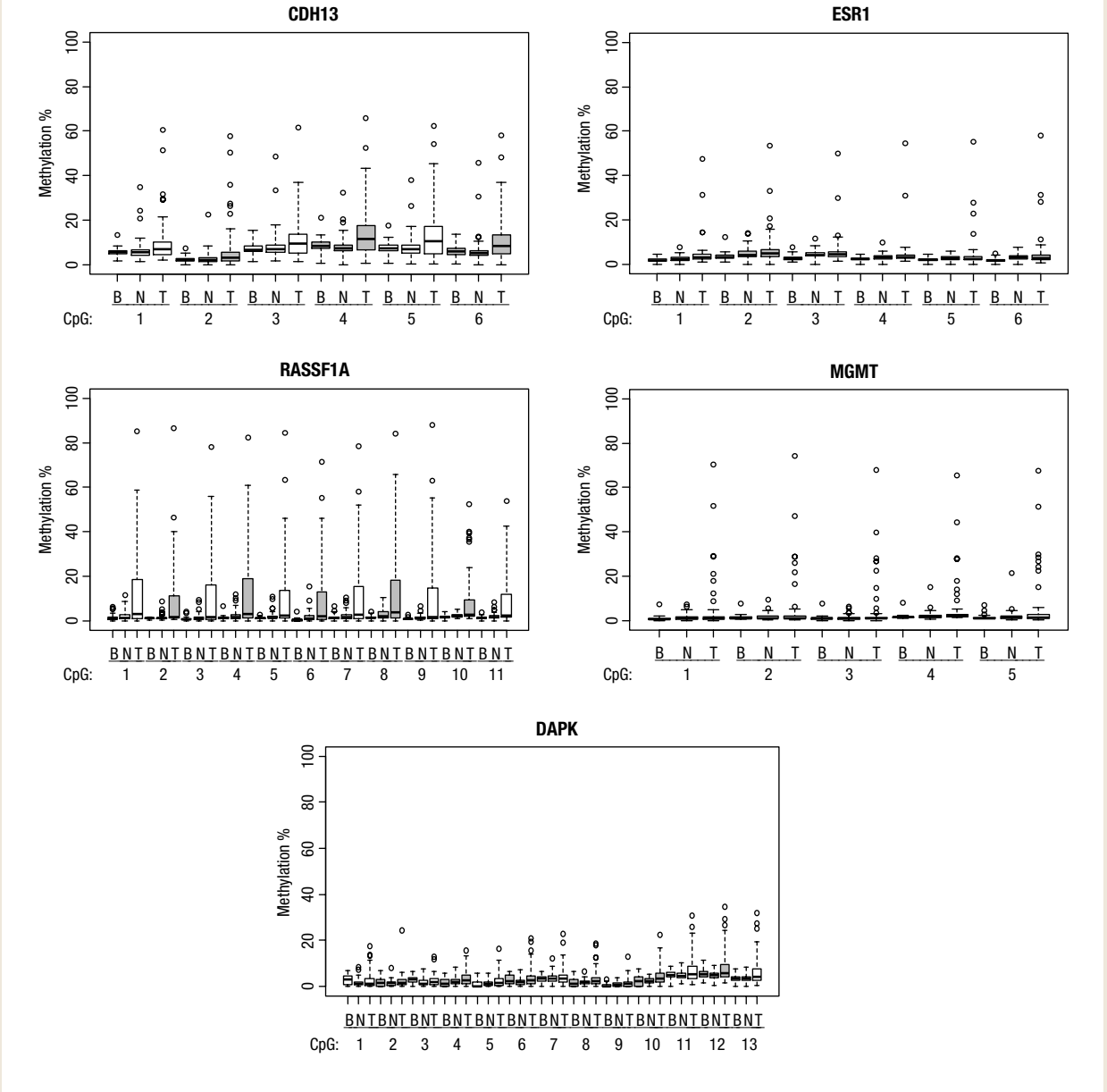
because only year of birth was available; July 1 of the corresponding year was used to estimate age. All analysis was performed by using SAS 9.2 (SAS Institute Inc, Cary, NC).

Results

Patient characteristics are presented in Table 2. There were more male than female patients. Most patients (81.3%) were current smokers, and 15.6% were former smokers. Specific histologic subtypes included 20 (30.8%) adenocarcinomas, 35 (53.8%) squamous cell carcinomas, and 10 (15.4%) other subtypes of NSCLC (4 large-cell carcinomas, 3 giant cell carcinomas, 1 atypical carcinoid, 1 mucinous carcinoma, and 1 carcinoma sarcomatoid). The distribution of methylation across CpG sites for each gene is presented in Figure 1. Methylation percentages were fairly similar across CpG sites for each gene studied. Tumor samples showed more variation in methylation, with a substantial number of outliers for each CpG site. Blood samples tended to have consistently low methylation levels for all CpG sties.

Methylation at 3 of the 5 genes (*DAPK, RASSF1A,* and *CDH13*) was significantly higher in tumor compared with normal lung tissue (Table 3). We next investigated whether methylation changes at these 3 genes could be detected in circulating blood samples. All tumors were classified as either positive or negative for methylation at each gene. There was no significant association between average promoter methylation of *DAPK, RASSF1A, MGMT,* and *CDH13* in DNA isolated from blood and tumor methylation status (Table 4). However, there was a significant association between methylation in blood for those with hypermethylation in tumors for the ESR1 gene. Finally, we evaluated the clinical and demographic correlates of gene methylation (Table 5). Histology and sex were both associated with hypermethylation at *CDH13*, which was observed more frequently in women and patients with adenocarcinoma. In addition, stage was associated with hypermethylation at *MGMT*; patients with stage IV disease were more likely to have hypermethylation at *MGMT*.

Figure 1 Graphical Representation (boxplots), Comparing the DNA Methylation Levels in Tumors (T), Normal Lung Tissue (N), and Blood Samples (B); Results Are Obtained By the Analysis of Individual Genes and CpG Sites



Discussion

In this European-based case series of NSCLCs, we evaluated the specificity of DNA methylation changes for lung tumors and investigated whether these changes are detectable in patients' blood. In our 5-gene panel, we observed that 3 genes were significantly hypermethylated in lung tumors compared with matched normal tissue, however, these changes could not be detected in patients' blood samples. In contrast, one gene not found to be statistically significantly different in tumor compared with normal samples did have higher methylation levels in blood from individuals with hypermethylation in their tumor sample. This association should be confirmed in a larger study.

Several studies investigated the clinical correlates of DNA methylation in selected genes in lung cancer.[2,3,6,10] Interestingly, the bulk of this literature used methylation-specific PCR as the method for quantification of promoter methylation levels.[2,3,6,7,11-14] We are not aware of any study that directly compared this method with the bisulfite pyrosequencing method that we used to analyze the same methylation sites, thus, it is not possible to address whether any

Table 3 Difference in CpG Methylation at 5 Tumor Suppressor Genes in Tumor Tissue and Pathologically Normal Lung Samples Removed at Surgical Resection

Gene	Methylation Change ($Methylation_{tumor} - Methylation_{normal\ lung}$)		*P* Value[a]
	n	Median (IQR)	
CDH13	64	2.43 (−1.41 to 6.9)	<.001
RASSF1A	62	0.91 (−0.15 to 11.7)	<.001
ESR1	65	0.28 (−0.67 to 1.46)	.07
MGMT	64	0.12 (−0.27 to 0.95)	.08
DAPK	47	0.87 (−0.97 to 2.26)	.03

Abbreviation: IQR = interquartile range.
[a] Wilcoxon signed rank test of within-person difference in methylation (tumor vs. normal lung).

Table 4 DNA Methylation in Circulating Blood Samples Stratified by Tumor Methylation Status

Gene	Cutoff Value for Hypermethylation	Average CpG Methylation in Circulating Blood				*P* Value[a]
		Hypermethylation in Tumor		No Hypermethylation in Tumor		
		n	Median (IQR)	n	Median (IQR)	
CDH13	18.52	9	6.56 (5.42-7.67)	41	6.22 (5.30-6.89)	.26
RASSF1A	6.93	17	1.23 (1.18-1.45)	31	1.26 (1.16-1.45)	.78
ESR1	6.92	4	3.37 (2.88-3.57)	46	2.36 (1.99-2.67)	.02
MGMT	6.68	5	1.17 (1.15-1.24)	45	1.22 (1.09-1.47)	.74
DAPK	5.20	8	2.74 (2.33-3.23)	25	2.52 (2.07-3.11)	.95

Abbreviation: IQR = interquartile range.
[a] Wilcoxon rank sum test.

discrepancies of our study compared with previous studies can be attributed to the differences in the methods used. However, the bisulfite pyrosequencing is regarded as a sensitive method that allows the detection of low copy numbers (ie, the presence of circulating tumor DNA in blood).

These 5 genes were selected because they have previously been reported to be hypermethylated in lung tumors.[6,7,11,12,15,16] Our results were not entirely consistent with those findings, because we failed to observe hypermethylation of *ESR1* and *MGMT* in NSCLC compared with adjacent normal lung tissue. The discrepancies may be attributed to differences in analysis methods used and interpretation of hypermethylation status, as well as different CpG sites investigated, which emphasizes the importance of reporting which methylation sites are being analyzed in defining methylation status, and it has been suggested that such reports would facilitate the comparison of gene-specific methylation data obtained in different studies.[10] Another possible explanation for the inconsistencies in these finding may be the very low levels of methylation observed in the promoters of these 2 genes in our tumor series, obviating the detection of differences in methylation in adjacent normal lung tissues.

It is well established that double-stranded DNA fragments can be detected in considerable quantities in blood (serum or plasma) of cancer patients due to the lysis of tumor cells.[17] Thus, it is possible to detect the tumor-specific DNA alterations in patients' peripheral blood, and it has been demonstrated that they can serve as biomarkers for NSCLC detection in a Chinese population.[18] Specifically, Zhang et al[18] identified a panel of 5 genes as markers for early diagnosis of NSCLC, these included *RASS1A* and *CDH13*. We, therefore, investigated whether hypermethylation of 3 genes observed in tumors would also be present in blood but were unable to detect elevated DNA methylation in blood. This may be due to consistently low methylation levels across all CpG sites characteristic for our blood samples. It remains plausible that the selection of different methylation sites in promoter regions of the 3 genes would yield stronger methylation signals in blood samples, but given that we used average methylation values over all CpG sites within the gene, this is unlikely. Alternatively, the level of tumor DNA present in circulation may have been low, which resulted in low gene-specific methylation signal. The limitation of our study is that it did not include healthy controls. Thus, it remains plausible that differences in methylation in blood samples from healthy controls compared with blood samples from the patients with NSCLC would reveal a valuable target for NSCLC screening. Withstanding this limitation, our results, therefore, suggest that these are not appropriate targets for early screening of NSCLC in our population.

We also investigated the correlation between aberrant methylation and clinicopathologic characteristics. Our results demonstrate that methylation of certain genes may be associated with some clinicopathologic characteristics of patients. A comparison of methylation of multiple CpG sites in the promoter and histopathology revealed

Table 5 Patient and Tumor Characteristics and Tumor Suppressor Gene Methylation

Variable	*CDH13* Hypermethylation		*RASSFA1* Hypermethylation		*MGMT* Hypermethylation		*DAPK* Hypermethylation		*ESR1* Hypermethylation	
	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
Sex										
Men	5 (11.4)	39 (88.6)	15 (34.9)	28 (65.1)	6 (13.6)	38 (86.4)	8 (20.5)	31 (79.5)	3 (6.8)	41 (93.2)
Women	6 (28.6)	15 (71.4)	8 (38.1)	13 (61.9)	2 (9.5)	19 (90.5)	4 (26.7)	11 (73.3)	3 (14.3)	18 (85.7)
P value[a]	.15		.99		.99		.72		.38	
Histology										
Squamous cell carcinoma	1 (2.9)	34 (97.1)	12 (35.3)	22 (64.7)	6 (17.1)	29 (82.9)	7 (24.1)	22 (75.9)	2 (5.7)	33 (94.3)
Adenocarcinoma	8 (40.0)	12 (60.0)	9 (45.0)	11 (55.0)	2 (10.0)	18 (90.0)	4 (22.2)	14 (77.8)	4 (20.0)	16 (80.0)
Other	2 (20.0)	8 (80.0)	2 (20.0)	8 (80.0)	0 (0.0)	10 (100.0)	1 (14.3)	6 (85.7)	0 (0.0)	10 (100.0)
P value[a]	<.001		.40		.40		.99		.18	
Tumor Grade										
<2	4 (19.0)	17 (81.0)	7 (33.3)	14 (66.7)	4 (19.0)	17 (81.0)	6 (35.3)	11 (64.7)	2 (9.5)	19 (90.5)
2	2 (8.7)	21 (91.3)	8 (36.4)	14 (63.6)	2 (8.7)	21 (91.3)	3 (15.8)	16 (84.2)	3 (13.0)	20 (87.0)
>2	4 (21.1)	15 (78.9)	8 (42.1)	11 (57.9)	2 (10.5)	17 (89.5)	3 (17.6)	14 (82.4)	1 (5.3)	18 (94.7)
P value	.47		.90		.64		.36		.87	
Clinical Stage										
I	1 (6.3)	15 (93.8)	6 (37.5)	10 (62.5)	2 (12.5)	14 (87.5)	3 (23.1)	10 (76.9)	1 (6.3)	15 (93.8)
II	6 (24.0)	19 (76.0)	9 (37.5)	15 (62.5)	5 (20.0)	20 (80.0)	7 (31.8)	15 (68.2)	3 (12.0)	22 (88.0)
III	3 (15.8)	16 (84.2)	7 (36.8)	12 (63.2)	0 (0.0)	19 (100.0)	2 (13.3)	13(86.7)	2 (10.5)	17 (89.5)
IV	0 (0.0)	4 (100.0)	1 (25.0)	3 (75.0)	1 (25.0)	3 (75.0)	0 (0.0)	3 (100.0)	0 (0.0)	4 (100.0)
P value[a]	.47		.99		.11		.52		.99	
Smoking Status										
Former/never	2 (16.7)	10 (83.3)	4 (33.3)	8 (66.7)	1 (8.3)	11 (91.7)	1 (9.1)	10 (90.9)	1 (8.3)	11 (91.7)
Current	9 (17.3)	43 (82.7)	19 (37.3)	32 (62.7)	7 (13.5)	45 (86.5)	11 (26.2)	31 (73.8)	5 (9.6)	47 (90.4)
P value[a]	.99		.99		.99		.42		.99	
Pack-Years Smoking										
<40	2 (11.1)	16 (88.9)	8 (44.4)	10 (55.6)	2 (11.1)	16 (88.9)	2 (14.3)	12 (85.7)	0 (0.0)	18 (100.0)
40-70	7 (21.2)	26 (78.8)	13 (39.4)	20 (60.6)	5 (15.2)	28 (84.8)	8 (26.7)	22 (73.3)	5 (15.2)	28 (84.8)
>70	1 (8.3)	11 (91.7)	2 (18.2)	9 (81.8)	1 (8.3)	11 (91.7)	2 (25.0)	6 (75.0)	1 (8.3)	11 (91.7)
P value[a]	.53		.34		.99		.66		.40	
Age										
<55 y	4 (26.7)	11 (73.3)	6 (40.0)	9 (60.0)	3 (20.0)	12 (80.0)	3 (25.0)	9 (75.0)	1 (6.7)	14 (93.3)
55-59 y	4 (18.2)	18 (81.8)	6 (28.6)	15 (71.4)	3 (13.6)	19 (86.4)	4 (20.0)	16 (80.0)	2 (9.1)	20 (90.9)
60-64 y	1 (6.7)	14 (93.3)	5 (33.3)	10 (66.7)	1 (6.7)	14 (93.3)	2 (18.2)	9 (81.8)	2 (13.3)	13 (86.7)
≥65 y	2 (15.4)	11 (84.6)	6 (46.2)	7 (53.8)	1 (7.7)	12 (92.3)	3 (27.3)	8 (72.7)	1 (7.7)	12 (92.3)
P value[a]	.54		.76		.73		.97		.99	

[a] Derived from Wilcoxon rank sum test.

that methylation of *CDH13* ($P < .001$) was higher in adenocarcinoma. This is in contrast with a previous report that the prevalence of hypermethylation was indistinguishable among major histologic subtypes of lung cancer.[16] This discrepancy might be due to differences in exposure and different techniques used for analysis of DNA methylation.[10] However, our study was consistent with the reported differences in the methylation patterns of squamous cell carcinoma and adenocarcinoma.[19] This histology finding is in line with the report of Toyooka et al.[13]

Quantitative profiling in a study by Vaissière et al[16] revealed correlation of *RASSF1A* hypermethylation with sex (men showed higher levels of methylation), as well as Buckingham[10] (women had lower methylation levels in *RASSF1A*). In our study, we found no association of *RASSF1A* hypermethylation and sex. The discrepancies may reflect different methods of analysis and interpretation of hypermethylation status as well as different CpG sites investigated.[10,20] Although aging has been associated with methylation of certain genes,[13] we did not find a correlation between the overall fraction of tumor methylation in *RASSF1A, CDH13, MGMT, ESR1,* and *DAPK* genes and age. The lack of the association may be attributed to the narrow age range of our study participants (the youngest and the oldest age groups were only 10 years apart). Further, we found no

association of hypermethylation at any of our studied genes and smoking status, thus, further studies are needed to elucidate the role of smoking in the epigenetic inactivation of specific genes in smokers.[14,17]

In conclusion, we confirmed that hypermethylation of *RASSF1A, CDH13,* and *DAPK* plays a role in NSCLC pathogenesis but also showed that these genes were not suitable markers for early detection of NSCLC in Central European whites enrolled in our study. Thus, other potential epigenetic biomarkers of NSCLC remain to be examined to identify those biomarkers that might be used as a lung cancer screening tool. Our data indicate that some of the more commonly investigated epigenetic changes in lung cancer may not be well suited for this purpose and that additional studies on larger gene panels are needed. In addition, our study revealed some interesting associations of hypermethylation in specific genes with clinicopathologic features. However, these findings should be considered exploratory due to size limitation and remain to be validated with larger cohorts.

Clinical Practice Points

- DNA methylation changes in lung cancer are well established, and translation of this knowledge toward screening has the potential for significant health improvement. With the discovery of new disease-screening markers, it may be possible to achieve early diagnosis, to have more informed choices for cancer treatment, and to identify markers associated with prognosis and response to therapy.
- A number of genes have been reported as aberrantly methylated in lung tumors. We investigated a panel of 5 genes (*RASSF1A, CDH13, MGMT, ESR1,* and *DAPK*) by using bisulfite pyrosequencing to determine whether these methylation changes are specific to lung tumors and to test whether these changes are detectable in patients' blood samples. We also analyzed possible associations between DNA methylation and clinicopathologic features.
- We observed that 3 genes were significantly hypermethylated in lung tumors compared with matched normal tissue, however, these changes could not be detected in patients' blood samples. We confirmed that hypermethylation of *RASSF1A, CDH13,* and *DAPK* play a role in NSCLC pathogenesis but also showed that these genes are not suitable markers for the early detection of NSCLC in Central European whites enrolled in our study. Thus, other potential epigenetic biomarkers of NSCLC remain to be examined to identify those biomarkers that might be used as a lung cancer screening tool.
- In addition, our study revealed some interesting associations of hypermethylation in specific genes with clinicopathologic features, which remain to be validated with larger cohorts.

Disclosure

The authors have stated that they have no conflicts of interest.

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