DNA Methyltransferases Messenger RNA Expression and Aberrant Methylation of CpG Islands in Non–Small-Cell Lung Cancer: Association and Prognostic Value

Daniel Vallböhmer,[1,2*] Jan Brabender,[1,2*] Dongyun Yang,[1] Paul M. Schneider,[2] Ralf Metzger,[2] Kathleen D. Danenberg,[3] Arnulf H. Hölscher,[2] Peter V. Danenberg[1]

Abstract

BACKGROUND: A significant association between aberrant methylation in regulatory regions of tumor suppressor genes and clinical outcome in various different cancer types has been described. The molecular events for this epigenetic alteration still remain unknown. Evidence suggests that overexpression of DNA methyltransferases (DNMTs) is one potential mechanism for hypermethylation. PATIENTS AND METHODS: Therefore, we investigated the influence of gene expression levels of the 3 DNMT isoforms (DNMT1, DNMT3a, and DNMT3b) and the hypermethylation of adenomatous polyposis coli (*APC*), the death-associated protein kinase (*DAPK*), glutathione S-transferase π (*GSTPI*), and the DNA repair gene O6-methylguanine DNA transferase (*MGMT*) in the pathogenesis and prognosis of patients with non–small cell lung cancer and determined their association to each other. Using a quantitative real-time reverse-transcriptase polymerase chain reaction, we measured messenger RNA expression of DNMT1, DNMT3a, and DNMT3b and DNA hypermethylation of *APC*, *DAPK*, *GSTPI*, and *MGMT* in 91 matching tumor and nonmalignant lung tissue samples from patients with curatively resected non–small-cell lung cancer. RESULTS: In tumor tissue, the expression of all 3 DNMT isoforms was significantly higher compared with matched normal-appearing tissue ($P < 0.001$). Hypermethylation in tumor tissue was found in 95% for *APC*, in 92% for *DAPK*, in 18% for *GSTPI*, and in 38% for *MGMT*. CONCLUSION: No correlation was found between the DNMT messenger RNA expression and DNA hypermethylation status in tumor tissues. Multivariate analysis revealed DNA hypermethylation status and TNM stage as independent prognostic factors.

Clinical Lung Cancer, Vol. 8, No. 1, 39-44, 2006

Key words: Methylation index, Molecular prognostic factors, Polymerase chain reaction

Introduction

Lung cancer is the leading cause of cancer-related mortality in the United States. In 2006, this malignant disease is estimated to cause 174,470 deaths.[1] Studies investigating the potential of targeted therapy in combination with traditional chemotherapeutic drugs revealed no improvement in survival of patients with lung cancer, and the current 5-year survival remains 15%.[1-3] Therefore, the discovery of further molecular alterations in non–small-cell lung cancer (NSCLC) should be pursued to provide the basis for future surgical and pharmacologic improvement in lung cancer treatment.

Aberrant methylation of normally unmethylated 5′CpG islands in gene regulatory regions of tumor suppressor genes has become established as an epigenetic mechanism for transcriptional silencing in human cancers.[4-7] Currently, this molecular event has been recognized as a crucial component in cancer initiation/progression and has been intensively studied in connection with the prevention, early detection, treatment, and prognosis of cancer.[6] In lung cancer, several genes involved in essential cellular pathways have been described that are inactivated by promoter hypermethylation, including adenomatous polyposis coli (*APC*), cyclin-dependent kinase inhibitor 2A (*CDKN2A*), the death-associated protein kinase (*DAPK*), E-cadherin, glutathione S-transferase π

[1]Norris Comprehensive Cancer Center, University of Southern California, Los Angeles
[2]Department of Visceral and Vascular Surgery, University of Cologne, Germany
[3]Response Genetics, Inc, Los Angeles, CA
*These authors contributed equally to this article.

Submitted: Apr 25, 2006; Revised: Jun 26, 2006; Accepted: Jun 26, 2006

Address for correspondence: Daniel Vallböhmer, MD, Department of Visceral and Vascular Surgery, University of Cologne, Joseph-Stelzmannstraße 9, 50924 Cologne, Germany
Fax: 49-221-4786333; e-mail: daniel.vallboehmer@freenet.de

Table 1 Polymerase Chain Reaction Primers and Probes for the Analysis of Messenger RNA Levels

Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	TaqMan® Probe (5′-3′)
β-*actin*	TGAGCGCGGCTACAGCTT	CCTTAATGTCACACACGATT	ACCACCACGGCCGAGCGG
DNMT1	GGTTCTTCCTCCTGGAGAATGTC	GGGCCACGCCGTACTG	CCTTCAAGCGCTCCATGGTCCTGAA
DNMT3a	CAATGACCTCTCCATCGTCAAC	CATGCAGGAGGCGGTAGAA	AGCCGGCCAGTGCCCTC
DNMT3b	CCATGAAGGTTGGCGACAA	TGGCATCAATCATCACTGGATT	CACTCCAGGAACCGTGAGATGTCCCT

(*GSTPI*), and DNA repair gene O6-methylguanine DNA transferase (*MGMT*).[7,8] Recently, aberrant methylation of several genes, including *MGMT, APC,* and *DAPK,* have been reported to be potential prognostic biomarkers in patients with NSCLC.[9-11]

The molecular events leading to aberrant DNA methylation remain unclear. Evidence suggests that the overexpression of the 3 known DNA methyltransferases (DNMTs), ie, DNMT1, DNMT3a, and DNMT3b, is one potential mechanism for DNA hypermethylation.[12-16] In contrast, other investigations indicate that the increase of DNMT isoforms are not responsible for DNA methylation in cancer.[17-19]

To determine whether DNMT gene expression is associated with DNA methylation and whether methylation of *APC, DAPK, GSTPI,* or *MGMT* is associated with clinical outcome in terms of patient survival, we analyzed (1) the messenger RNA (mRNA) expression of all 3 DNMT isoforms and (2) the quantitative methylation levels of these genes in matching tumor and normal lung tissue from 91 patients with curatively resected NSCLC.

Patients and Methods

Ninety-one patients with histopathologically confirmed NSCLC were included in this study. There were 22 women (24%) and 69 men (76%) with a median age of 63 years (range, 34-83 years). Forty-three patients (47%) had squamous cell carcinomas, 33 (36%) had adenocarcinomas, and 15 (17%) had large-cell carcinomas. Twenty patients (22%) had a well/moderately differentiated tumor, and 71 patients (78%) had a poorly differentiated tumor. According to the International Union Against Cancer (UICC), 45 patients (49%) had stage I tumors, 19 (21%) had stage II tumors, and 27 (30%) had stage IIIA tumors. All tumors were completely resected (R0 category) by at least a lobectomy as quality control. Patients with histopathologic stage IIIA tumors received postoperative radiation therapy. With a median follow-up period of 85.9 months (range, 63-105 months) for all 91 patients included in this study, the median survival time was 55.8 months, and no patient was lost to follow-up. Approval for this study was obtained from the local ethics committee. Written informed consent was obtained from participating patients.

Tissue Acquisition

Tumor and adjacent normal tissue samples were obtained immediately after lung resection before starting mediastinal lymphadenectomy and were frozen in liquid nitrogen and stored at –80°C. Tissues were analyzed from the following 2 locations: tumor and uninvolved lung tissue taken from the greatest distance to the tumor. Frozen sections of 6 µm were taken from blocks of tumor tissue/adjacent normal tissue, and starting with the first section, every fifth one was routinely stained with hematoxylin and eosin and evaluated by a pathologist. Only areas of estimated 75% cells of interest were pooled for the analysis.

Polymerase Chain Reaction Quantification and Messenger RNA Expression

Total RNA was isolated by a single-step guanidinium isothiocyanate method using QuickPrep™ Micro mRNA purification according to the manufacturer's instructions. After RNA isolation, complementary DNA was prepared from each sample as described previously.[20]

Quantitation of DNMT1, DNMT3a, DNMT3b, and an internal reference gene (β-*actin*) was done using a fluorescence-based real-time detection method (ABI Prism® 7900 Sequence Detection System [TaqMan®]). The polymerase chain reaction (PCR) reaction mixture consisted of 600 nmol/L of each primer; 200 nmol/L probe; 5 U of AmpliTaq® Gold Polymerase; 200 µmol/L each of 2′-deoxyadenosine 5′-triphosphate, 2′-deoxycytidine 5′-triphosphate, 2′-deoxyguanosine 5′-triphosphate, and 2′-deoxythymidine 5′-triphosphate; 400 µmol/L 2′-deoxyuridine-5′-triphosphate; 5.5 mmol/L $MgCl_2$; and 1 × TaqMan® Buffer A containing a reference dye, to a final volume of 25 µL. Cycling conditions were 50°C for 10 minutes, 95°C for 10 minutes, followed by 46 cycles at 95°C for 15 seconds and 60°C for 1 minute. The primers and probes used are listed in Table 1.

Table 2 Polymerase Chain Reaction Primers and Probes for the Analysis of DNA Methylation

Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	TaqMan® Probe (5′-3′)
MYOD1	CCAACTCCAAATCCCCTCTCTAT	TGATTAATTTAGATTGGGTTTAGAGAAGGA	TCCCTTCCTATTCCTAAATCCAACCTAAATACCTCC
APC	TTATATGTCGGTTACGTGCGGTTTATAT	GAACCAAAACGCTCCCCAT	CCCGTCGAAAACCCGCCGATTA
DAPK	GGATAGTCGGATCGAGTTAACGTC	AACGCAAATCCCTCCCAAAC	TTCGGTAATTCGTAGCGGTAGGGT
GSTPI	AGTTGCGCGGCGATTTC	GCCCCAATACTAAATCACGACG	CGGTCGACGTTCGGGGTGTAGCG-
MGMT	CGAATATACTAAAACAACCCCGCG	GTATTTTTTCGGGAGCGAGGC	AATCCTCGCGATACGCACCGTTTA

Table 3 Association Between Gene Expression Levels of DNMT Isoforms and Clinicopathologic Variables

Factor	N	DNMT1		DNMT3a		DNMT3b	
		Median (Range)	*P* Value	Median (Range)	*P* Value	Median (Range)	*P* Value
All Patients	91	3.32 (0-13.6)		2.89 (0-26.4)		1.13 (0-16.1)	
Sex							
Male	69	3.2 (0-13.3)	NS	2.89 (0.7-26.4)	0.43	1.05 (0-16.1)	NS
Female	22	4.53 (1.1-12.6)		3.71 (0.5-14.7)		1.16 (0-7.6)	
Smoking Status							
Nonsmoker	10	4.03 (1.9-7.1)	NS	2.81 (1.3-12.4)	0.85	1.14 (0-8.4)	NS
Smoker	81	3.25 (0-13.3)		2.93 (0.5-26.4)		1.05 (0-16.1)	
Disease Stage							
I	45	3.42 (1.5-12.6)	NS	2.89 (0.5-21.1)	0.29	0.79 (0-13.2)	NS
II	19	2.82 (0.89-13.3)		2.33 (0.7-26.4)		1.17 (0-16.1)	
IIIA	27	3.36 (0-11.1)		3.2 (0.86-14.7)		1.49 (0.2-8.4)	
Histology							
Squamous cell	43	3.54 (1.2-13.3)	NS	2.89 (0.7-21.1)	0.94	1.16 (0.09-16.1)	NS
Adenocarcinoma	33	3.27 (0-12.6)		2.91 (0.5-14.7)		1.01 (0-8.4)	
Large-cell	15	2.08 (0.8-11.7)		2.89 (0.8-26.4)		0.83 (0-7.6)	
Grade							
Well differentiated	1	1.88 (1.88)	NS	1.17 (1-117)	0.42	0.15	NS
Moderately differentiated	19	2.75 (0.87-7.1)		2.61 (1.1-6.6)		1.01 (0-4.4)	
Poorly differentiated	71	3.83 (0-13.3)		3.05 (0.5-26.4)		1.13 (0-13.1)	

Abbreviation: NS = not significant with Hochberg correction for multiple comparisons

TaqMan® measurements yield cycle threshold (Ct) values that are inversely proportional to the amount of complementary DNA in the tube, ie, a higher Ct value means it requires more PCR cycles to reach a certain level of detection. Gene expression values (relative mRNA levels) are expressed as ratios (differences between the Ct values) between the gene of interest and an internal reference gene (β-*actin*) that provides a normalization factor for the amount of RNA isolated from a specimen. Colon, lung, and liver RNAs were used as a control calibrator on each plate.

DNA Methylation Analysis

Genomic DNA was isolated from frozen tissues by standard methods of proteinase K digestion and phenol-chloroform extraction using the GenomicPrep™ Cells and Tissue Isolation Kit according to the instructions of the manufacturer. Sodium bisulfite conversion of genomic DNA was performed as described previously.[21] Quantitation of bisulfite treated DNA of the genes of interest (*APC, DAPK, GSTPI*, and *MGMT*) and an internal reference gene myogenic differentiation 1 (*MYOD1*) was done using the fluorescence-based real-time detection method as previously described.[9,10,17] The primer and probe sequences are listed in Table 2.

The ratio between the Ct values obtained from the genes of interest (ie, *APC, DAPK, GSTPI,* and *MGMT*) and the reference gene (*MYOD1*) was used as a measure of the degree of methylation at this locus for each particular sample. A methylation-positive human lung cancer cell line (L132) was used as a positive control and for constructing the calibration curve on each plate.

Statistical Analysis

Associations between gene expression levels of DNMT expression in tumor and normal adjacent tissue were tested using the Wilcoxon signed rank test. Associations between continuous DNMT mRNA expression levels and DNA hypermethylation were tested using the Kruskal-Wallis test. To assess the associations between the expression levels of DNMTs as well as CpG island hypermethylation and overall survival, cutoff values were determined for each factor that best segregated patients into poor and good prognosis subgroups (in terms of likelihood of surviving).[22,23] To compare the extent of methylation for the panel of genes examined, we calculated the quantitative methylation index (qMI) for each patient according to the previously described methylation index.[24] In our study, the qMI is defined as a fraction representing the number of methylated genes in the poor prognosis subgroup divided by the number of genes tested. The log-rank test was used to compare patients' survival time between groups. Multivariate analysis was performed with the Cox proportional hazard regression model. The Mann-Whitney *U* and Kruskal-Wallis tests were used to evaluate the associations between the expression of the DNMT isoforms and patients' clinicopathologic variables. The Jonckheere-Terpstra

Table 4 Association Between Quantitative Methylation Index and Clinicopathologic Variables

Factors	0	0.25	0.5	0.75	*P* Value
Sex					
Male	10 (83)	28 (74)	23 (77)	8 (73)	0.76
Female	2 (17)	10 (26)	7 (23)	3 (27)	
Smoking Status					
Smoker	12 (100)	33 (87)	27 (90)	9 (82)	0.4
Nonsmoker	0	5 (13)	3 (10)	2 (18)	
pT Category					
pT1	3 (25)	12 (32)	5 (17)	0	0.27
pT2	6 (50)	21 (55)	22 (73)	9 (82)	
pT3	3 (25)	5 (13)	3 (10)	2 (18)	
pN Category					
pN0	7 (58)	21 (55)	17 (57)	5 (46)	0.39
pN1	5 (42)	11 (29)	5 (17)	4 (36)	
pN2	0	6 (16)	8 (27)	2 (18)	
UICC Stage					
I	7 (58)	18 (47)	15 (50)	5 (46)	0.59
II	2 (17)	10 (26)	4 (13)	3 (27)	
IIIA	3 (25)	10 (26)	11 (37)	3 (27)	
Histology					
Squamous Cell	5 (42)	18 (47)	14 (47)	6 (55)	0.94
Adenocarcinoma	4 (33)	13 (34)	12 (40)	4 (36)	
Large-Cell	3 (25)	7 (18)	4 (13)	1 (9)	
Grade					
Well differentiated	0	0	1 (3)	0	0.53
Moderately differentiated	4 (33)	4 (10)	7 (23)	4 (36)	
Poorly differentiated	8 (67)	34 (90)	22 (73)	7 (64)	

Values in parentheses are percentages.

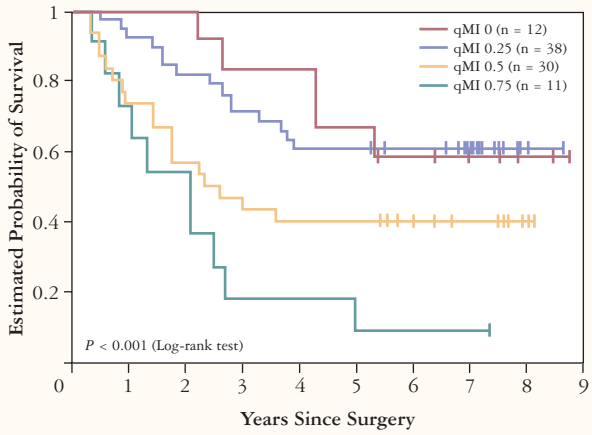
exact test was used to analyze associations between methylation status and categoric clinicopathologic variables. The level of significance was set at $P < 0.05$. All reported P values are based on 2-sided tests.

Results

Gene Expression Levels of DNA Methyltransferases and the Correlation to Clinicopathologic Factors

The mRNA levels of the DNMTs in tumor and normal adjacent tissues were detectable by quantitative real-time PCR in the following frequency: DNMT1 normal (96.7%), tumor (98.9%); DNMT3a normal (98.9%), tumor (98.9%); DNMT3b normal (83.5%), tumor (95.6%). The median expression levels of DNMT1 were 3.32 (range, 0-13.6) in tumor tissue and 1.93 (range, 0-9.4) in matching nonmalignant lung tissue. The median expression levels of DNMT3a were 2.89 (range, 0-26.4) in tumor tissue and 2.16 (range, 0-18.4) in matching nonmalignant lung tissue. The median expression levels of DNMT3b were 1.13 (range, 0-16.1) in tumor tissue and 0.44 (range, 0-3.6) in matching nonmalignant lung tissue. For all 3 DNMT isoforms intratumoral expression levels were significantly higher than in matching normal adjacent lung tissue ($P < 0.001$; Wilcoxon test).

Figure 1 Association Between Quantitative Methylation Index and Survival in Patients with Non–Small-Cell Lung Cancer



No significant associations between patients' clinicopathologic data and intratumoral gene expression levels of the DNMT isoforms were detected (Table 3).

Aberrant Methylation of CpG Islands of APC, GSTPI, DAPK, *and* MGMT *and Their Correlation to Clinicopathologic Factors*

Aberrant methylation of CpG islands in 5′ regions in tumor tissues was detectable by quantitative fluorescence-based reverse transcriptase PCR (TaqMan®) at the following frequencies: *APC* (95%), *DAPK* (92%), *MGMT* (38%), and *GSTPI* (18%) and, in matching normal lung tissue, *APC* (88%), *DAPK* (92%), *MGMT* (18%), and *GSTPI* (15%). The median methylation levels relative to the internal reference *MYOD1* were 4.76 (range, 0-240.7) for *APC*, 2.27 (range, 0-144.3) for *DAPK*, 0 (range, 0-134.9) for *MGMT*, and 0 (range, 0-187.9) for *GSTPI* in tumor tissue; and 1.57 (range, 0-73.9) for *APC*, 0.83 (range, 0-116.7) for *DAPK*, 0 (range, 0-82.7) for *GSTPI*, and 0 (range, 0-0.55) for *MGMT* in corresponding normal lung tissue. Methylation levels were significantly higher in tumors compared with normal tissues for *APC*, *DAPK*, and *MGMT* ($P < 0.001$; Wilcoxon test).

The following cutoff values (stratified on methylation levels in tumor tissue) best segregated patients into good and poor prognosis subgroups: 14.1 for *APC*, 0.7 for *DAPK*, positivity versus negativity for *MGMT*, and 0.8×10^{-3} for *GSTPI*. By this criteria, 23 patients (25%) had a high *APC* methylation status, 60 (66%) had a high *DAPK* methylation status, 34 (37%) had a high *MGMT* methylation status, and 14 (15%) had a high *GSTPI* methylation status. Twelve patients (13%) had no high methylated genes (qMI = 0), 38 (42%) had 1 high methylated gene (qMI = 0.25), 30 (33%) had 2 high methylated genes (qMI = 0.5), and 11 (12%) had ≥ 3 high methylated genes (qMI = 0.75). As shown in Table 4, no correlation was found between the qMI and any patient clinicopathologic characteristics.

Table 5 Cox Proportional Hazards Regression Model

Parameter	Hazard Ratio	95% CI	*P* Value
Disease Stage			
I	1	Reference	–
II	2.31	1.01-5.24	0.05
IIIA	6.29	3.13-12.62	< 0.001
qMI Marker			
0	1	Reference	–
0.25	1.11	0.4-3.06	0.84
0.5	2.72	1-7.41	0.05
0.75	5.86	1.93-17.79	0.002

Relationship Between Intratumoral CpG Island Hypermethylation of APC, GSTPI, DAPK, *and* MGMT *and DNA Methyltransferase Expression*

The calculations that were performed to investigate all combinations of the mRNA levels of the 3 DNMT isoforms and CpG island hypermethylation of *APC, GSTPI, DAPK*, and *MGMT* in tumor tissue did not show any significant associations.

Gene Expression Levels of DNMT Isoforms in Tumor Tissue and Clinical Outcome

No cutoff values that best segregated patients into poor and good prognosis subgroups (in terms of likelihood of surviving) could be defined for the DNMT isoforms. No significant correlation between gene expression levels of any DNMT isoforms and clinical outcome was found.

CpG Island Hypermethylation of APC, GSTPI, DAPK, *and* MGMT *in Tumor Tissue and Clinical Outcome*

The qMI was significantly associated with worse survival. The median survival was not reached for the subgroups of patients with a qMI of 0 and a qMI of 0.25, whereas it was reached in 27.7 months (95% confidence interval [CI], 7.6-47.8) for patients with a qMI of 0.5 and 24.7 months (95% CI, 11.6-37.8) for patients with a qMI of ≥ 0.75 (P = 0.0008, log-rank test; Figure 1). The importance of the qMI as a prognostic factor was next determined by the Cox proportional hazards regression model analysis. The Cox model included the following variables: sex, histopathologic type, UICC TNM stage, grade of differentiation of the primary tumor, and qMI marker. Significant independent prognostic factors were shown to be UICC TNM stage (P < 0.001) and qMI marker (P for trend = 0.005; Table 5).

Discussion

In our study, we measured the gene expression levels of all 3 DNMT isoforms and the DNA hypermethylation of *APC, DAPK, GSTPI*, and *MGMT* in matching tumor and histologically normal-appearing lung tissue from 91 patients with curatively resected NSCLC. All 3 DNMT isoforms were found to be significantly upregulated in the tumor compared with normal adjacent tissues. These findings are consistent with recent studies demonstrating a significant upregulation of DNMT isoforms in various tumors, suggesting a role for the methyltransferases in carcinogenesis.[25-28]

To examine whether the expression levels of the DNMT isoforms might contribute to DNA methylation patterns, we measured the methylation of multiple genes, ie, *APC, DAPK, MGMT,* and *GSTPI*, by using a quantitative, methylation-specific, real-time PCR method. Methylation was detected at high frequencies for *APC* (95%), *DAPK* (92%), *MGMT* (37%), and *GSTPI* (18%). Combined analysis of methylation levels of multiple genes showed a significant correlation with survival, suggesting that the quantity of gene methylation in tumor tissues, in addition to its presence or absence, might serve as a novel biomarker of a biologic aggressive disease in NSCLC. Significant associations between methylation and clinical outcome in patients with NSCLC have been described previously but are limited to analysis of single genes or a subgroup of patients with NSCLC.[9,10,29,30] Although there are several studies correlating methylation of multiple genes with clinicopathologic factors (ie, sex, age, histology, and/or tumor stage), to date only 2 studies have assessed the correlation between the methylation of multiple genes and survival in patients with NSCLC.[24,31-34] Toyooka et al analyzed the methylation status of 5 genes ($p16^{INK4a}$, Ras association domain family 1A, *APC,* retinoic acid receptor β, and cadherin 13) in patients with NSCLC and correlated each gene to overall survival, but only increased methylation of $p16^{INK4a}$ was associated with poor survival.[34] Zochbauer-Muller et al assessed the correlation between the methylation index of multiple genes (retinoic acid receptor β, *TIMP-3, MGMT, DAPK,* E-cadherin, $p14^{ARF}$, $p16^{INK4a}$, and *GSTPI*) and survival in NSCLC, but no association between combined methylation patterns and clinical outcome was found.[24] A possible explanation for these discrepant results compared with our study is the use of the conventional methylation-specific PCR, whereas we used a fluorogenic real-time PCR (TaqMan®) method. In addition to its higher sensitivity, this technique allows precise quantitation of methylation levels in relation to a reference gene. As we report here, the methylation levels varied over a range of 240-fold for *APC*, 144-fold for *DAPK*, 187-fold for *GSTPI*, and 134-fold for *MGMT*. This observation of seemingly variable amounts of gene methylation implies heterogeneity of methylation patterns within individual tumors and most likely reflects the biologic behavior of these tumors. Indeed, the most striking finding of the current study is that a high qMI in tumors is correlated with worse clinical outcome in NSCLC. The extent of concordant methylation of multiple loci during tumor transition could be an indicator of the overall methylation propensity of tumors. A greater propensity for methylation might result in a more aggressive tumor because of increased capability for inactivation of undesirable tumor suppressor genes. Regardless of the actual mechanism leading to this alteration, this hypothesis is strengthened by the results of several studies reporting a correlation between promoter methylation and transcriptional silencing of several cancer-related genes in human cancer.[4-8]

The actual pathways, inducing the initiation and maintenance of tumor methylation, are still controversial. Several previous studies suggested that abnormal methylation patterns

could arise as a result of an overactivity of methylation-inducing factors, such as the overexpression of DNMT isoforms.[12-16] However, we did not find any apparent correlation between the mRNA levels of the 3 DNMT isoforms and the methylation status of *APC*, *DAPK*, *GSTPI*, and *MGMT* in patients with curatively resected NSCLC, which is in concordance with other recent reports failing to show a significant correlation between expression levels of DNMTs and DNA methylation.[17-19] A possible reason for the noncorrelation between DNMT isoform expression and methylation might be that the methyltransferases contribute to carcinogenesis through alternative pathways, besides the possible direct induction of DNA methylation. Milutinovic et al showed that treatment with a DNMT inhibitor in lung cancer cells induces p21 expression without the involvement of p21 demethylation.[35] Moreover, in vitro studies by Soejima et al suggested that DNMT3b might contribute to gene silencing through the recruitment of chromatin remodeling enzymes rather than the direct methylation target loci.[36]

Conclusion

This study shows that upregulation of DNMT isoforms and a high frequency of aberrant methylation of several genes (*APC, DAPK, GSPTI*, and *MGMT*) occurs in patients with curatively resected NSCLCs. Promoter methylation status and mRNA expression levels of DNMTs do not correlate with each other. Combined quantitative methylation analysis of multiple genes appears to define good and bad prognosis subgroups in patients with curatively resected lung cancer, thus showing promise as a novel biomarker for biologic aggressiveness in this disease.

References

1. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2006. *CA Cancer J Clin* 2006; 56:106-130.
2. Giaccone G, Herbst RS, Manegold C, et al. Gefitinib in combination with gemcitabine and cisplatin in advanced non-small-cell lung cancer: a phase III trial--INTACT 1. *J Clin Oncol* 2004; 22:777-784.
3. Herbst RS, Giaccone G, Schiller JH, et al. Gefitinib in combination with paclitaxel and carboplatin in advanced non-small-cell lung cancer: a phase III trial--INTACT 2. *J Clin Oncol* 2004; 22:785-794.
4. Merlo A, Herman JG, Mao L, et al. 5′CpG island methylation is associated with transcriptional silencing of the tumor suppressor p16/CDKN2/MTS1 in human cancers. *Nat Med* 1995; 1:686-692.
5. Wajed SA, Laird PW, DeMeester TR. DNA methylation: an alternative pathway to cancer. *Ann Surg* 2001; 234:10-20.
6. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med* 2003; 349:2042-2054.
7. Belinsky SA. Gene-promoter hypermethylation as a biomarker in lung cancer. *Nat Rev Cancer* 2004; 4:707-717.
8. Tsou JA, Hagen JA, Carpenter CL, et al. DNA methylation analysis: a powerful new tool for lung cancer diagnosis. *Oncogene* 2002; 21:5450-5461.
9. Brabender J, Usadel H, Danenberg KD, et al. Adenomatous polyposis coli gene promoter hypermethylation in non-small cell lung cancer is associated with survival. *Oncogene* 2001; 20:3528-3532.
10. Brabender J, Usadel H, Metzger, et al. Quantitative O(6)-methylguanine DNA methyltransferase methylation analysis in curatively resected non-small cell lung cancer: associations with clinical outcome. *Clin Cancer Res* 2003; 9:223-227.
11. Tang X, Khuri FR, Lee JJ, et al. Hypermethylation of the death-associated protein (DAP) kinase promoter and aggressiveness in stage I non-small-cell lung cancer. *J Natl Cancer Inst* 2000; 92:1511-1516.
12. Hsu DW, Lin MJ, Lee TL, et al. Two major forms of DNA (cytosine-5) methyltransferase in human somatic tissues. *Proc Natl Acad Sci U S A* 1999; 96:9751-9756.
13. Okano M, Bell DW, Haber DA, et al. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 1999; 99:247-257.
14. Kanai Y, Ushijima S, Kondo Y, et al. DNA methyltransferase expression and DNA methylation of CPG islands and peri-centromeric satellite regions in human colorectal and stomach cancers. *Int J Cancer* 2001; 9:205-212.
15. Rhee I, Bachman KE, Park BH, et al. DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. *Nature* 2002; 416:552-556.
16. Robert MF, Morin S, Beaulieu N, et al. DNMT1 is required to maintain CpG methylation and aberrant gene silencing in human cancer cells. *Nat Genet* 2003; 33:61-65.
17. Eads CA, Danenberg KD, Kawakami K, et al. CpG island hypermethylation in human colorectal tumors is not associated with DNA methyltransferase overexpression. *Cancer Res* 1999; 59:2302-2306.
18. Saito Y, Kanai Y, Sakamoto M, et al. Expression of mRNA for DNA methyltransferases and methyl-CpG-binding proteins and DNA methylation status on CpG islands and pericentromeric satellite regions during human hepatocarcinogenesis. *Hepatology* 2001; 33:561-568.
19. Sato M, Horio Y, Sekido Y, et al. The expression of DNA methyltransferases and methyl-CpG-binding proteins is not associated with the methylation status of p14(ARF), p16(INK4a) and RASSF1A in human lung cancer cell lines. *Oncogene* 2002; 21:4822-4829.
20. Lord RV, Salonga D, Danenberg KD, et al. Telomerase reverse transcriptase expression is increased early in the Barrett`s metaplasia, dysplasia, carcinoma sequence. *J Gastrointest Surg* 2000; 4:135-142.
21. Olek A, Oswald J, Walter JA. A modified and improved method of bisulfite based cytosine methylation analysis. *Nucleic Acids Res* 1996; 24:5064-5066.
22. Miller R, Siegmund D. Maximally selected chi-square statistics. *Biometrics* 1982; 38:1011-1016.
23. Halpern, J. Maximally selected chi-square statistics for small samples. *Biometrics* 1982; 38:1017-1023.
24. Zochbauer-Muller S, Fong KM, Virmani AK, et al. Aberrant promoter methylation of multiple genes in non-small cell lung cancers. *Cancer Res* 2001; 61:249-255.
25. Belinsky SA, Nikula KJ, Baylin SB, et al. Increased cytosine DNA-methyltransferase activity is target-cell-specific and an early event in lung cancer. *Proc Natl Acad Sci U S A* 1996; 93:4045-4050.
26. Girault I, Tozlu S, Lidereau R, et al. Expression analysis of DNA methyltransferases 1, 3A, and 3B in sporadic breast carcinomas. *Clin Cancer Res* 2003; 9:4415-4422.
27. Ramchandani S, MacLeod AR, Pinard M, et al. Inhibition of tumorigenesis by a cytosine-DNA, methyltransferase, antisense oligodeoxynucleotide. *Proc Natl Acad Sci U S A* 1997; 94:684-689.
28. Robertson KD, Uzvolgyi E, Liang G, et al. The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors. *Nucleic Acids Res* 1999; 27:2291-2298.
29. Lu C, Soria JC, Tang X, et al. Prognostic factors in resected stage I non-small-cell lung cancer: a multivariate analysis of six molecular markers. *J Clin Oncol* 2004; 22:4575-4583.
30. Kim DH, Kim JS, Ji YI, et al. Hypermethylation of RASSF1A promoter is associated with the age at starting smoking and a poor prognosis in primary non-small cell lung cancer. *Cancer Res* 2003; 63:3743-3746.
31. Toyooka S, Maruyama R, Toyooka KO, et al. Smoke exposure, histologic type and geography-related differences in the methylation profiles of non-small cell lung cancer. *Int J Cancer* 2003; 103:153-160.
32. Hanabata T, Tsukuda K, Toyooka S, et al. DNA methylation of multiple genes and clinicopathological relationship of non-small cell lung cancers. *Oncol Rep* 2004; 12:177-180.
33. Tomizawa Y, Iijima H, Nomoto T, et al. Clinicopathological significance of aberrant methylation of RARbeta2 at 3p24, RASSF1A at 3p21.3, and FHIT at 3p14.2 in patients with non-small cell lung cancer. *Lung Cancer* 2004; 46:305-312.
34. Toyooka S, Suzuki M, Maruyama R, et al. The relationship between aberrant methylation and survival in non-small-cell lung cancers. *Br J Cancer* 2004; 91:771-774.
35. Milutinovic S, Knox JD, Szyf M. DNA methyltransferase inhibition induces the transcription of the tumor suppressor p21(WAF1/CIP1/sdi1). *J Biol Chem* 2000; 275:6353-6359.
36. Soejima K, Fang W, Rollins BJ. DNA methyltransferase 3b contributes to oncogenic transformation induced by SV40T antigen and activated Ras. *Oncogene* 2003; 22:4723-4733.