Exclusive Mutation in Epidermal Growth Factor Receptor Gene, HER-2, and KRAS, and Synchronous Methylation of Nonsmall Cell Lung Cancer

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Received May 16, 2005; revision received November 17, 2005; accepted December 12, 2005.

BACKGROUND. Both genetic and epigenetic changes in nonsmall cell lung cancer (NSCLC) are known to be a common event.

METHODS. Mutations in the epidermal growth factor receptor gene (EGFR), HER-2, and KRAS and the methylation profile of 9 genes for NSCLC were analyzed and correlated with clinical and histologic data.

RESULTS. Thirty-nine EGFR, 4 HER-2, and 6 KRAS mutations were found in 150 NSCLC cases, with the methylation percentages of the genes ranging from 13% to 54%. Most mutations were present in adenocarcinomas, but mutations of the 3 genes were never found to be present in individual tumors. The frequency of methylation for all the genes was correlated with the Methylation Index, a reflection of the overall methylation pattern (all genes, $P \leq .01$), supporting the presence of the CpG island methylator phenotype (CIMP) in NSCLC. On the basis of the methylation profile, CRBP1 and CDH13 methylation were good indicators of CIMP in NSCLC, and were correlated with a poorer prognosis in adenocarcinomas. Mutations in EGFR, HER-2, and KRAS were found to be present exclusively, whereas methylation tended to be present synchronously. A comparison of mutation and methylation demonstrated that the EGFR mutation had an inverse correlation with methylation of SPARC (secreted protein acidic and rich in cysteine), an extracellular Ca^{2+}-binding matricellular glycoprotein associated with the regulation of cell adhesion and growth, and the $p16^{INK4A}$ gene.

CONCLUSIONS. The findings of the current study suggest that adenocarcinoma cases with CIMP have a poorer prognosis than adenocarcinoma cases without CIMP, and the EGFR mutation was shown to have an inverse correlation with methylation of SPARC and the $p16^{INK4A}$ gene in NSCLC. ***Cancer*** **2006;106:2200–7.**

KEYWORDS: epidermal growth factor receptor gene, mutation, methylation, methylator phenotype, lung cancer.

Nonsmall cell lung cancer (NSCLC), the major form of lung cancer, is divided into 3 types: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. It is characterized by an accumulation of multiple genetic and/or epigenetic alterations, including activation of oncogenes and inactivation of tumor suppressor genes (TSGs).[1] An increased understanding of the molecular mechanisms of NSCLC may provide new and more effective strategies for chemoprevention, early diagnosis, and targeted treatment for lung cancers.[2] Although most lung cancers are smoking-related, there are major somatically acquired genetic differences that exist between adenocarcinomas and squamous cell carcinomas.[3–5] Mutation of epidermal growth factor receptor (EGFR) as a genetic alteration, and aberrant

DOI 10.1002/cncr.21853
Published online 5 April 2006 in Wiley InterScience (www.interscience.wiley.com).

TABLE 1
Genes Tested and Primer Sequences for Mutation and Methylation Assays

Gene		Forward primer	Reverse primer
EGFR TK domains	Exon 18	AGC ATG GTG AGG GCT GAG GTG AC	ATA TAC AGCTTG CAA GGA CTC TGG
	Exon 19	CCA GAT CAC TGG GCA GCA TGT GGCACC	AGC AGG GTC TAG AGC AGA GCA GCT GCC
	Exon 20	GAT CGC ATTCAT GCG TCT TCA CC	TTG CTA TCC CAG GAG CGC AGA CC
	Exon 21	TCAGAG CCT GGC ATG AAC ATG ACC CTG	GGT CCC TGG TGT CAG GAA AATGCT GG
KRAS	Exon 2	GTA TTA ACC TTA TGT GTG ACA	GTC CTG CAC CAG TAA TAT GC
HER-2 TK domains	Exon 19	TGG AGG ACA AGT AAT GAT CTC CTG G	AAG AGA GAC CAG AGC CCA GAC CTG
	Exon 20	GCC ATG GCT GTG GTT TGT GAT GG	ATC CTA GCC CCT TGT GGA CAT AGG
RASSF1A	M	GGG TTT TGC GAG AGC GCG	GCT AAC AAA CGC GAA CCG
	U	GGT TTT GTG AGA GTG TGT TTA G	CAC TAA CAA ACA CAA ACC AAA C
RARβ	M	TCG AGA ACG CGA GCG ATT CG	GAC CAA TCC AAC CGA AAC GA
	U	TTG AGA ATG TGA GTG ATT TGA	AAC CAA TCC AAC CAA AAC AA
APC	M	TAT TGC GGA GTG CGG GTC	TCG ACG AAC TCC CGA CGA
	U	GTG TTT TAT TGT GGA GTG TGG GTT	CCA ATC AAC AAA CTC CCA ACA A
$p16^{INK4A}$	M	TTA TTA GAG GGT GGG GCG GAT CGC	GAC CCC GAA CCG CGA CCG TAA
	U	TTA TTA GAG GGT GGG GTG GAT TGT	CAA CCC CAA ACC ACA ACC ATA A
CDH13	M	TCG CGG GGT TCG TTT TTC GC	GAC GTT TTC ATT CAT ACA CGC G
	U	CGC GGG GCT CGC TCC TCG C	GGC GTT TTC ATT CAT GCA CGC G
HPP1	M	GTT ATC GTC GTC GTT TTT GTT GTC	GAC TTC CGA AAA ACA CAA AAT CG
	U	GTT ATT GTT GTT GTT TTT GTT GTT GT	AAC AAC TTC CAA AAA ACA CAA AAT CA
CRBP1	M	TTG GGA ATT TAG TTG TCG TCG TTT C	AAA CAA CGA CTA CCG ATA CTA CGC G
	U	GTG TTG GGA ATT TAG TTG TTG TTG TTT T	ACT ACC AAA ACA ACA ACT ACC AAT ACT ACA
Reprimo	M	GCG AGT GAG CGT TTA GTT C	TAC CTA AAA CCG AAT TCA TCG
	U	TTG TGA GTG AGT GTT TAG TTT	TAA TTA CCT AAA ACC AAA TTC ATC
SPARC	M	GAG AGC GCG TTT TGT TTG TC	AAC GAC GTA AAC GAA AAT ATC G
	U	TTT TTT AGA TTG TTT GGA GAG TG	AAC TAA CAA CAT AAA CAA AAA TAT C

EGFR: epidermal growth factor receptor gene; TK: tyrosine kinase, M: methylated-specific primers; U: unmethylated-specific primers, SPARC: secreted protein acidic and rich in cysteine.

methylation of the promoter as an epigenetic alteration, are now the focus of study in NSCLC.

EGFR (alternatively called ErbB1) is the prototype member of a structurally homologous family of transmembrane receptor tyrosine kinases that also includes ErbB2 (HER-2), ErbB3, and ErbB4. EGFR and HER-2, which are all expressed in the bronchial epithelium,[6] are highly expressed in many epithelial cancers.[7] The clinical relevance of the EGFR has been heightened in light of the development of the EGFR tyrosine kinase (TK) inhibitor gefitinib (Iressa, ZD1839, AstraZeneca, Wilmington, DE), which has been shown to have antitumor activity in NSCLC.[8,9] Moreover, EGFR mutations in the TK domain are predictors for sensitivity to gefitinib therapy,[10,11] although among patients receiving erlotnib, the presence of an EGFR mutation does not appear to confer a survival benefit.[12] Conversely, trastuzumab (Herceptin; Genentech, South San Francisco, CA), a humanized monoclonal antibody that binds to the extracellular domain of HER-2, has shown less of an effect in NSCLC,[13,14] although it has been effective in breast cancers that overexpress HER-2.[15] Mutations in the TK domain of HER-2 also have recently been reported in lung adenocarcinomas.[16,17]

Aberrant methylation of the promoter region of TSGs and the resultant gene silencing play an important role in the pathogenesis of human cancer, and individual tumor types frequently have a characteristic pattern of acquired aberrant methylation.[18] Simultaneous, multiple-gene hypermethylation, which has been referred to as a CpG island methylator phenotype (CIMP), has been documented in colorectal and gastric cancers, pancreatic adenocarcinomas, and hepatocellular carcinoma.[19–22] Aberrant methylation of TSGs is a frequent and common occurrence in NSCLC[23,24]; however, to our knowledge, the existence of CIMP in NSCLC is unclear.

In the current study, we searched for mutations in EGFR and HER-2 and determined the frequency of aberrant methylation of 9 TSGs in NSCLC. We also examined the KRAS mutation because KRAS is downstream of EGFR signaling and because the KRAS mutation is the most frequently reported alteration in EGFR signaling pathways.[25]

MATERIALS AND METHODS

Surgically resected samples were obtained from 150 unselected patients with lung cancer who had not received any treatment before resection between 1995 and 1998

TABLE 2
Mutations and Methylation Profile of Nonsmall Cell Lung Cancers

		KRAS	EGFR	HER-2	HPP1	SPARC	Reprimo	CRBP1	RAR-β	RASSF1A	APC	CDH13	p16^{INK4A}
	Cases (n = 150)	6 (4)*	38 (25)	4 (3)	56 (37)	81 (54)	49 (33)	19 (13)	48 (32)	48 (32)	53 (35)	40 (27)	44 (29)
Gender	Male (101)	3 (3)	15 (15)	0 (0)	32 (31)	59 (58)	34 (34)	12 (12)	28 (28)	32 (32)	37 (37)	27 (27)	33 (33)
	Female (49)	3 (6)	23 (47)†	4 (8)†	24 (49)†	22 (45)	15 (31)	7 (14)	20 (41)	16 (33)	16 (33)	13 (27)	11 (22)
Age, y‡	<65 (73)	2 (3)	21 (29)	3 (4)	25 (34)	42 (58)	24 (33)	7 (10)	25 (34)	21 (29)	29 (40)	17 (23)	19 (26)
	≥65 (77)	4 (5)	17 (22)	1 (1)	31 (40)	39 (51)	25 (32)	12 (16)	23 (30)	27 (35)	24 (31)	23 (30)	25 (32)
Smoking	Never (41)	2 (5)	24 (59)†	2 (5)	18 (44)	22 (54)	12 (29)	6 (15)	16 (39)	11 (27)	13 (32)	8 (20)	7 (17)
	Smoker (109)	4 (4)	14 (13)	2 (2)	38 (35)	59 (54)	37 (34)	13 (12)	32 (29)	37 (34)	40 (37)	32 (29)	37 (34)†
Histology	Adenocarcinoma (79)	5 (6)	36 (46)†	4 (5)	30 (38)	38 (48)	27 (34)	7 (9)	31 (39)	23 (29)	32 (41)	23 (29)	16 (20)
	Others§ (71)	1 (1)	2 (3)	0 (0)	26 (37)	43 (61)	22 (31)	12 (17)	17 (24)	25 (35)	21 (30)	17 (24)	28 (39)†
T classification	T1 (38)	1 (3)	12 (32)	3 (8)†	12 (32)	18 (47)	16 (42)	6 (16)	12 (32)	11 (29)	13 (34)	7 (18)	5 (13)
	T2, 3, and 4 (112)	5 (4)	26 (23)	1 (1)	44 (39)	63 (56)	33 (29)	13 (12)	36 (32)	37 (33)	40 (36)	33 (29)	39 (35)
N classification	N0 (78)	4 (5)	18 (23)	1 (1)	28 (36)	41 (53)	30 (38)	13 (17)	13 (17)	27 (35)	26 (33)	16 (21)	19 (24)
	N1, 2, and 3 (72)	2 (3)	20 (28)	3 (4)	28 (39)	40 (56)	19 (26)	6 (8)	35 (49)†	21 (29)	27 (38)	24 (33)	25 (35)
Stage	I (51)	1 (2)	14 (27)	1 (2)	19 (37)	25 (49)	24 (47)†	10 (20)	10 (20)	18 (35)	16 (31)	11 (22)	13 (25)
	II, III, and IV (99)	5 (5)	24 (24)	3 (3)	37 (37)	56 (57)	25 (25)	9 (9)	38 (38)†	30 (30)	37 (37)	29 (29)	31 (31)

EGFR: epidermal growth factor receptor; SPARC: secreted protein acidic and rich in cysteine.

* Parentheses in each column indicate percentage of mutation or methylation.

† The frequency of the group is significantly higher ($P < .05$) than the other group.

‡ Divided by the median age.

§ "Others" include squamous cell carcinoma, adenosquamous cell carcinoma, and large-cell carcinoma.

at the Chiba University Hospital in Chiba, Japan. This study was approved by the Institutional Review Board and written informed consent was obtained from all participants. All patients received curative intent surgery. Seventy-nine patients (53%) were diagnosed as having adenocarcinoma, 58 patients (39%) as having squamous cell carcinoma, 11 patients (7%) as having large cell carcinoma, and 2 patients (1%) as having adenosquamous carcinoma. Resected samples were immediately frozen and stored at −80°C until use.

Genomic DNA was obtained from whole primary tumors after overnight digestion with sodium dodecyl sulfate and proteinase K (Life Technologies, Rockville, MD) at 37°C, followed by standard phenol-chloroform (1:1) extraction and ethanol precipitation.

Mutation Assay

According to our previous study, sequencing of the first 4 exons (exons 18-21) of the EGFR TK domains and exons 19-20 of the HER-2 TK domains were analyzed. The intron-based polymerase chain reaction (PCR) primer sequences for the EGFR and HER-2 TK domain exons examined, as well as exon 2 of KRAS, are summarized in Table 1. All PCR products were incubated using exonuclease I and shrimp alkaline phosphatase (Amersham Biosciences, Piscataway, NJ) and sequenced directly using the Applied Biosystems PRISM dye terminator, cycle-sequencing method (Perkin-Elmer, Foster City, CA). All sequence variants were confirmed by independent PCR amplifications and sequenced in both directions.

Methylation Assay

DNA was treated with sodium bisulfite as described previously.[26] Treated DNA was purified with the Wizard DNA Purification System (Promega, Madison, WI), desulfurated with 0.3 M NaOH, precipitated with ethanol, and resuspended in water. The methylation status of 9 genes was determined by methylation-specific PCR (MSP), using primers specific for the methylated and unmethylated alleles of each gene (Table 1).[27] From our previous observations, all the genes tested were expressed in normal human bronchial epithelial cells and were primarily down-regulated through aberrant methylation of their promoters.[28–32] DNAs from 14 lymphocytes of healthy nonsmoking volunteers were used as negative controls for methylation-specific assays. DNA from lymphocytes of a healthy volunteer treated with Sss1 methyltransferase (New England BioLabs, Beverly, MA) and then subjected to bisulfite treatment was used as a positive control for methylated alleles. Negative control samples without DNA were included for each set of PCR. PCR products were analyzed on 2% agarose gels containing ethidium bromide.

Statistical Analysis

The Fisher exact test and the Mann-Whitney U-test were used to assess the association between categor-

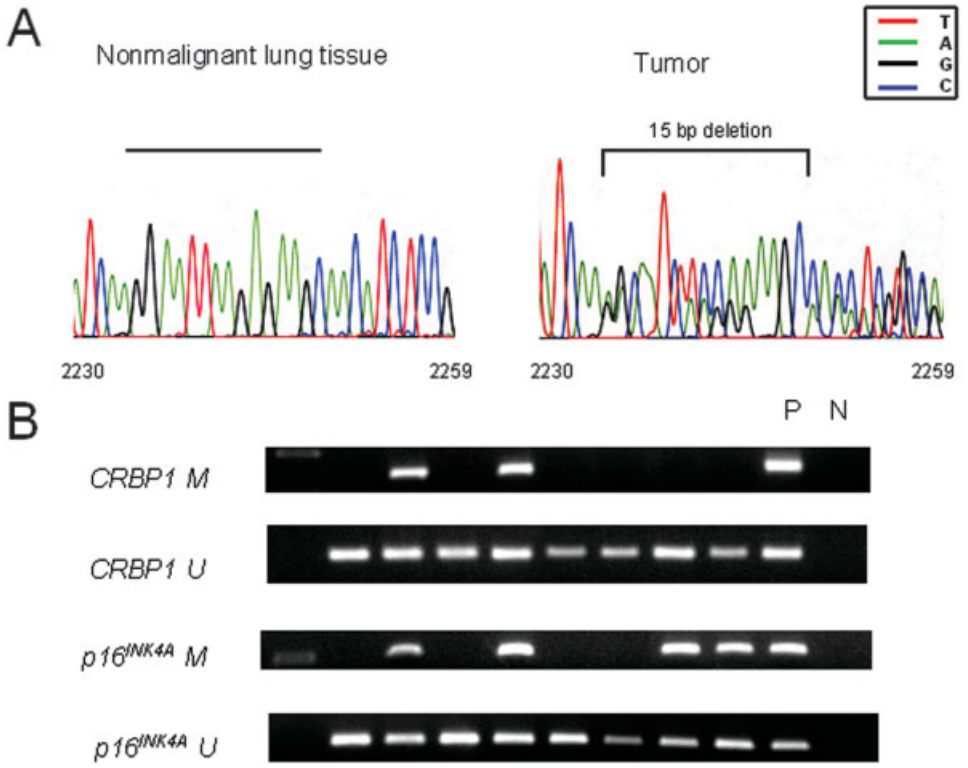

FIGURE 1. (A) Electropherograms demonstrating mutational patterns, highlighting a 15-bp deletion in exon 19 of tumor epidermal growth factor receptor gene. (B) Representative examples of the methylation-specific polymerase chain reaction assay. Results of the methylated forms of CRBP1 and $p16^{INK4A}$ are illustrated. M: methylated form; U: unmethylated form; P: positive control; N: negative control.

ical variables. Overall survival curves were calculated with the Kaplan-Meier method and compared with the log-rank test. The Cox proportional hazards regression model was used for multivariate analyses. Statistical significance was defined as a P value $<.05$. All P values were 2-sided.

To compare the overall extent of methylation for the panel of genes examined, we calculated the methylation index (MI) for each case [MI = (total number of genes methylated)/(total number of genes analyzed)], and determined the mean for the different groups.

RESULTS

Mutation Profile of NSCLC

As shown in Table 2 and in Figures 1 and 2, 38 EGFR mutations, 4 HER-2 mutations, and 6 KRAS mutations were found in all of the 150 NSCLC cases. We examined 60 nonmalignant lung tissues corresponding to the tumors. In 48 nonmalignant lung tissues corresponding to the mutant tumor samples and in 12 nonmalignant lung tissues corresponding to the nonmutant tumor samples, mutations of EGFR, HER-2, and KRAS were absent, indicating that these mutations were somatic in origin. EGFR mutations were found to be higher in females than in males ($P <.0001$), higher in nonsmokers than in smokers ($P <.0001$), and higher in adenocarcinoma cases than in other carcinoma cases ($P<.0001$). KRAS mutations were present in 1 case of squamous cell carcinoma and in 5 cases of adenocarcinoma. HER-2 mutations were found only in females ($P = .01$), and only in adenocarcinoma ($P = .1$). Major mutations included in-frame deletions in exon 19 of EGFR and codon 12 of KRAS, and in-frame duplications/insertions in exon 20 of HER-2 (Fig. 1). Mutations of the 3 genes (EGFR, HER-2, and KRAS) occurred exclusively, not simultaneously.

Methylation Profile of NSCLC

The methylation profile for all the lung cancer patients was determined using the MSP method for 9 genes. Examples of the bands obtained by MSP are illustrated in Figure 1 and detailed data regarding the frequency of aberrant methylation are summarized in Table 2. We examined 60 nonmalignant lung tissues corresponding to the tumors. Aberrant methylation of nonmalignant lung tissue for each gene was between 3%

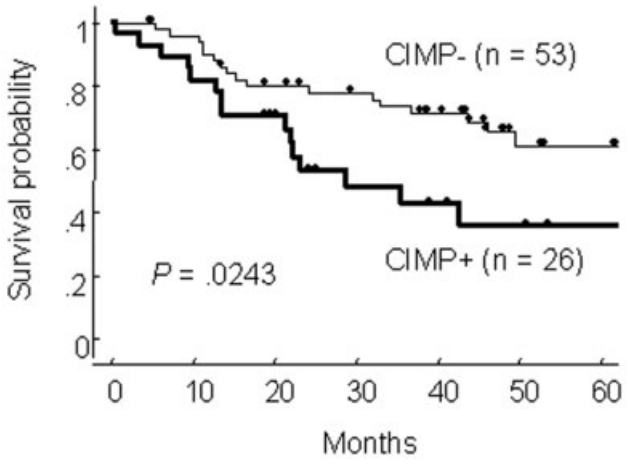

FIGURE 2. Survival of adenocarcinoma patients as a function of CpG island methylator phenotype (CIMP) status. The 5-year overall survival rates of CIMP&positive (CIMP+) and CIMP-negative (CIMP-) cases were 35.2% and 61.3%, respectively.

and 7%. Tumor tissues consist of mixtures of tumor cells (i.e., malignant cells) and nonmalignant cells, and the unmethylated forms of all the genes were present in all of the tumor samples. The methylation frequencies for the genes tested were: 37% for HPP1, 54% for SPARC, 33% for Reprimo, 13% for CRBP1, 32% for RARβ, 33% for RASSF1A, 35% for APC, 27% for CDH13, and 29% for $p16^{INK4A}$. Methylation of all genes was a tumor-specific event ($P<.0001$) when compared with corresponding nonmalignant tissues. The frequency of RARβ methylation was higher in cases with lymph node metastasis ($P<.0001$) and cases with advanced stage disease ($P = .026$). The frequency of $p16^{INK4A}$ methylation was significantly higher in smokers than in nonsmokers ($P = .046$), and was also higher in the other carcinoma cases compared with adenocarcinoma cases ($P = .012$). There was no methylation present in 13 of 79 adenocarcinoma cases (16%) and in only 1 of 58 squamous cell carcinoma cases (2%) ($P = .0041$). The frequency of methylation for all the genes correlated with the MI (all genes, $P \leq .01$). These findings imply that methylation tends to occur frequently and synchronously, and that it supports the presence of CIMP. Also, no significant correlation between age and alteration of the genes was observed. Among the genes tested, methylation of CDH13, CRBP1, and $p16^{INK4A}$ was present only in cases in which the MI was ≥ 0.3 for adenocarcinomas, whereas methylation of CDH13 and CRBP1 was present only in cases in which the MI was ≥ 0.3 for squamous cell carcinomas. In these circumstances, we defined the cases with either CDH13 or CRBP1 methylation as CIMP for NSCLC.

Next, we correlated CIMP with clinical factors, including prognosis of the patients (Table 3). The only significant correlation observed between CIMP and clinical factors was between CIMP and prognosis. No significant association was observed between EGFR mutation and prognosis. On the basis of univariate analysis, the adenocarcinoma patients with CIMP displayed worse prognosis (Fig. 2). To determine whether the association between the presence of CIMP and overall survival remained after adjusting for covariates of gender, age, and stage, we used a Cox proportional hazards model (Table 4). Clinical variables were converted to a binary format. Age was dichotomized by median age of the adenocarcinoma patients. The pathologic stages were classified according to the International System for Staging Lung Cancer. Disease stage was dichotomized into Stage I, then Stages II, III, and IV as advanced disease. The resulting model showed that the presence of CIMP remained significant after adjusting for gender, age, and stage.

Correlation between Mutation and Methylation of NSCLC

We compared mutations with methylation using the Fisher exact test and found SPARC methylation to be significantly higher in cases without EGFR mutation (67 of 112 [60%] cases) compared with cases with EGFR mutation (14 of 38 [37%] cases, $P = .0155$). In addtion, $p16^{INK4A}$ methylation was significantly higher in cases without EGFR mutation (38 of 112 [34%] cases) compared with cases with EGFR mutation (6 of 38 [16%] cases, $P = .039$). No significant correlation was observed between methylation of other genes and CIMP nor between mutation of other genes and mutations.

DISCUSSION

To our knowledge, the current study is the first to date to correlate EGFR, HER-2, and KRAS mutations with aberrant methylation of multiple genes in NSCLC. These findings demonstrate important differences in the mutation and methylation profiles of NSCLC. Mutations of EGFR and HER-2 were frequent in females and in individuals with adenocarcinoma. Mutation of EGFR was frequent in nonsmokers. Simultaneous mutations in EGFR, HER-2, and KRAS were not observed. These findings are consistent with our earlier findings as well as those of other investigators.[17,33] Because EGFR families and KRAS function sequentially, activation of all 3 of these molecules might be functionally redundant: mutation of either EGFR, HER-2, or KRAS is sufficient for lung carcinogenesis. The analysis of EGFR mutations is a useful predictor of clinical outcome with gefitinib treatment in patients with advanced NSCLC.[10,11] Further study is necessary to clarify the usefulness of trastuzumab treatment for patients with the HER-2 mutation.

TABLE 3
Correlation between CIMP and Clinical Characteristics of Nonsmall Cell Lung Cancers

	Cases (n = 150)	CIMP+ 49 (33)*	CIMP- 101 (67)	P
Gender	Male (101)	34 (34)	67 (66)	.9
	Female (49)	15 (31)	34 (69)	
Age in y[†]	64.1 ± 9.6	64.8 ± 8.7	63.8 ± 10.0	.6
Smoking	Never (41)	10 (24)	31 (76)	.2
	Smoker (109)	39 (36)	70 (64)	
Histology	Adenocarcinoma (79)	26 (33)	53 (67)	.9
	Others[‡] (71)	23 (32)	48 (68)	
T classification	T1 (38)	9 (24)	29 (76)	.2
	T2, 3, and 4 (112)	40 (36)	72 (64)	
N classification	N0 (78)	23 (29)	55 (71)	.5
	N1, 2, and 3 (72)	26 (36)	46 (64)	
Stage	I (51)	16 (31)	35 (69)	.9
	II, III, and IV (99)	33 (33)	66 (67)	
KRAS	Mutant (6)	3 (50)	3 (50)	.4
	Wild-type (144)	46 (32)	98 (68)	
EGFR	Mutant (38)	9 (24)	29 (76)	.2
	Wild-type (112)	40 (36)	72 (64)	
HER-2	Mutant (4)	1 (25)	3 (75)	.9
	Wild -type (146)	48 (33)	98 (67)	
HPP1	Methylated (56)	34 (61)	22 (39)	<.0001
	Unmethylated (94)	15 (16)	79 (84)	
SPARC	Methylated (81)	32 (40)	49 (60)	.05
	Unmethylated (69)	17 (25)	52 (75)	
Reprimo	Methylated (49)	28 (57)	21 (43)	<.0001
	Unmethylated (101)	21 (21)	80 (79)	
CRBP1	Methylated (19)	19 (100)	0 (0)	<.0001
	Unmethylated (131)	30 (23)	101 (77)	
RARβ	Methylated (48)	20 (42)	28 (58)	.1
	Unmethylated (102)	29 (28)	73 (72)	
RASSF1A	Methylated (48)	20 (42)	28 (58)	.1
	Unmethylated (102)	29 (28)	73 (72)	
APC	Methylated (53)	23 (43)	30 (57)	.05
	Unmethylated (97)	26 (27)	71 (73)	
CDH13	Methylated (40)	40 (100)	0 (0)	<.0001
	Unmethylated (110)	9 (8)	101 (92)	
$p16^{INK4A}$	Methylated (44)	23 (52)	21 (48)	.002
	Unmethylated (106)	26 (25)	80 (75)	
Methylation	≥0.3 (81)	49 (60)	32 (40)	<.0001
Index	<0.3 (69)	0 (0)	69 (100)	

CIMP: CpG island methylator phenotype; EGFR: epidermal growth factor receptor gene; SPARC: secreted protein acidic and rich in cysteine.

* Parentheses in each column indicate percentage.

† Mean ± the standard deviation.

‡ "Others" included squamous cell carcinoma, adenosquamous cell carcinoma, and large cell carcinoma.

In our methylation study, we noted 2 subpopulations based on the frequency of methylation. One population included cases with an absence of methylation in adenocarcinomas (16%). Although our methylation study was inconclusive, we found the number of cases with absent methylation to be higher in adenocarcinomas (16%) than in squamous cell carcinomas (2%). It appears that mechanisms other than methylation may contribute to the inactivation of TSGs in these adenocarcinomas.

Another population included cases with CIMP, for which to our knowledge there currently is no consensus definition. Although Toyota et al.[34] suggested that investigation of 2 to 4 type "C" (cancer-specific) CpG loci is sufficient for the accurate evaluation of this phenotype, some investigators have insisted on analyzing methylation by a quantitative method,[35] whereas others have not.[34,36,37] We previously established a method to analyze the methylation of the genes tested in this study for many types of cancers,

TABLE 4
Multivariate Analyses of Prognostic Variables in Patients with Adenocarcinomas

Variable*	Hazards ratio	95% CI	*P*
Gender (female)	1.927	0.970-3.831	.0612
Age (≥65 y)	1.368	0.684-2.735	.3750
Stage (≥II)	7.246	2.481-21.28	.0003
CIMP (+)	2.370	1.175-4.785	.0160

95% CI: 95% confidence interval; CIMP: CpG island methylator phenotype.
*Stage and CIMP were found to be significant prognostic variables.

including lung cancer, by MSP and by real-time MSP.[23,24,26,30] In this study, we examined methylation by the MSP method, but not by a quantitative method, because we used whole tumor samples contaminated with stromal cells and normal cells. We found that methylation of either CDH13 or CRBP1 may be a good indicator of CIMP. To the best of our knowledge, the current study is the first report regarding the CIMP concept in NSCLC. However, because the number of the genes ($n = 9$) and the number of the tumors ($n = 150$) tested here were small, and because the samples were contaminated with normal cells, further study with a larger population or methylation analysis by real-time MSP using microdissected tumor cells is needed to clarify the usefulness of these genes as indicators of CIMP. However, we did observe that cases with CIMP were associated with poorer survival for patients with adenocarcinomas, and it is well known that treatment with demethylating agents results in restoration of gene expression.[30,38] Therefore, patients with CIMP are good candidates for a potential method of tumor therapy using demethylating agents.

We found a moderate inverse association between the EGFR mutation and SPARC methylation, as well as a weak inverse association between the EGFR mutation and $p16^{INK4A}$ methylation, in NSCLC. An inverse association between NRAS/BRAF mutations and the frequency of loss of heterozygosity on 9p21, in which the CDKN2A ($p16^{INK4A}$) gene is located, also has been reported in melanomas.[39] SPARC, otherwise known as osteonectin or BM40, is known to be aberrantly expressed in many types of tumors.[40] The functions of SPARC include deadhesion, regulation of extracellular matrix, and antiproliferation.[40,41] SPARC can modulate growth factors such as vascular endothelial growth factor, platelet-derived growth factor, fibroblast growth factor, and tumor growth factor-β.[40,41] Our previous observation demonstrated a down-regulation of SPARC through aberrant methylation in tumor cells with overexpression in stromal cells of NSCLC.[32] Negative EGFR and increased SPARC expression in stromal cells of the tumor also have been reported.[42] Although the exact mechanisms remain to be addressed, our observation in the current study as well as observations from previous studies of the correlation between NRAS/BRAF mutations and $p16^{INK4A}$ suggests that these mutations may to some extent compensate for the requirement of down-regulation of TSGs in the pathogenesis of cancer.

In the current study, we found distinct patterns of mutations and methylation in NSCLC. Three gene mutations appear to occur exclusively, whereas methylation tends to occur synchronously, and the EGFR mutation has an inverse association with the SPARC and $p16^{INK4A}$ genes. We believe that these findings will help to stratify NSCLC patients into clinically important subsets and will lead to improved chemoprevention, diagnosis, and chemotherapy strategies.

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