Promoter hypermethylation of the *p16* and *Wif-1* genes as an independent prognostic marker in stage IA non-small cell lung cancers

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Abstract. Hypermethylation of promoter CpG islands is a major inactivation mechanism of tumor suppressor genes, some of which are thought to be related to the prognosis of patients with non-small cell lung cancer (NSCLC). Therefore, hypermethylation of the specific genes may be expected to serve as a prognostic biomarker for NSCLC. In this study, the methylation status of 14 genes was analyzed in 44 stage IA NSCLC cases using methylation-specific PCR. Hypermethylation was detected in *PTGER2* (70% of cases), *DRM/Gremlin* (66%), *sFRP-2* (57%), *IL-12Rß2* (48%), *Reprimo* (41%), *APC* (39%), *CXCL12* (39%), *HPP1* (30%), *SPARC* (30%), *sFRP-5* (30%), *p16* (25%), *RUNX3* (20%), *sFRP-1* (20%) and *Wif-1* (16%). Patients with *p16*, *sFRP-5*, *Wif-1* or *CXCL12* methylation had a significantly shorter duration of relapse-free survival than their counterparts with an unmethylated gene (*p16,* P=0.011; *sFRP-5*, P=0.030, *Wif-1*, P=0.036; *CXCL12*, P=0.026). Also, those with methylated *HPP1*, *p16* or *Wif-1* had a significantly shorter duration of overall survival (*HPP1*, P=0.031; *p16*, P=0.026; Wif-1, P=0.008). Multivariate analysis revealed that *p16* methylation in relapse-free survival and *Wif-1* methylation in overall survival were the strongest independent prognostic factors (*p16*, P=0.036; Wif-1, P=0.035). In conclusion, the hypermethylation of the *p16* and *Wif-1* genes has potential as biomarkers that may be used to predict the prognosis of stage IA NSCLC.

Introduction

Lung cancer is the most frequent cause of cancer-related death in the world (1). Despite advances in the detection and treatment of lung cancer, the overall 5-year survival rate remains approximately 15% (2). The tumor, lymph node, metastasis (TNM) staging system for lung cancer is widely used as a guide for predicting prognoses (3,4). However, this system makes it difficult to accurately determine the prognosis for each patient, since recurrence is not uncommon even in surgically resected early-stage disease.

Alterations in DNA methylation patterns are the earliest and most common events during the process of tumorigenesis. Promoter region methylation of certain genes results in down-regulation of transcriptional activity through local effects on DNA-binding proteins and alterations of chromatin structure. Hypermethylation of tumor suppressor genes (TSGs) has been reported in a wide spectrum of human cancers (5-7), and it may be the most common mechanism of inactivating TSGs in lung cancer. Hypermethylation of several genes has been correlated with malignant potential in non-small cell lung cancer (NSCLC) (8,9): *FHIT* (associated with poor survival) (10), *p16* and/or *RASSF1A* (11), *DH1* (12), co-hypermethylation of *p16* and *FHIT* (2), and *p16* and *CDH13* (correlated with recurrence) (13). These findings suggest that hypermethylation of specific genes may serve as biomarkers to predict prognosis after complete resection of NSCLC. In addition, hypermethylation is a potentially reversible epigenetic change; accordingly, it has recently become a target for gene therapy (14).

Postoperative adjuvant chemotherapy has been established as a standard course of treatment in certain operable cases of NSCLC. However, rather than improving survival in stage IA, there is evidence that adjuvant chemotherapy may be harmful (15,16). On the other hand, there are some patients who suffer from recurrence even if stage IA NSCLC. If patients who are likely to suffer from recurrence could be identified at diagnosis, then a tailor-made strategy may be instituted.

In this study, we determined the methylation status of 14 cancer-related genes (*HPP1*, *DRM/Gremlin*, *RUNX3*, *p16*, *Reprimo*, *IL-12Rß2*, *SPARC*, *sFRP-1*, *sFRP-2*, *sFRP-5*, *Wif-1*, *APC*, *CXCL12* and PTGER2) in patients with stage IA NSCLC. We also investigated the relationship between the methylation status of these genes and the mutation profiles of the EGFR and KRAS mutations.

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Table I. Clinicopathological features of stage IA NSCLC patients.

Variables	No. of patients	Relapse-free 5-year survival (%)	P-value	Overall 5-year survival (%)	P-value
Gender					
M	22	86	0.6	90	0.6
F	22	81		85	
Age[a]					
<63	21	81	0.8	90	0.9
≥63	23	85		85	
Histology					
Adenocarcinoma	30	82	0.8[b]	89	0.4[b]
Squamous cell carcinoma	11	82		81	
Large-cell carcinoma	3	100		100	
p-factor					
p0	28	86	0.4	89	0.3
p1	16	76		84	
Tumor size					
≤2 cm	14	86	0.4	93	0.6
>2 cm	30	82		84	
Smoking					
Never	21	80	>0.9	89	0.5
Smoker	23	87		86	

[a]Divided into 2 groups by median age. [b]Adenocarcinoma vs. squamous cell carcinoma.

Materials and methods

Patients and clinical samples. A total of 44 NSCLCs and 32 corresponding normal lung tissue specimens from the same patients were surgically resected and histologically diagnosed as stage IA NSCLC at the Chiba University Hospital, Japan. Institutional Review Board approval and written informed consent from all participants were obtained. Tissue samples were immediately frozen and stored at -80˚C until analysis. The patients had neither undergone any chemotherapy or radiotherapy prior to surgical resection, nor adjuvant chemotherapy or radiotherapy after resection.

The patients included 22 males and 22 females who ranged in age from 44 to 90 years (average, 63.2 years) at the time of diagnosis. TNM staging was based on the TNM classification system of the International Union Against Cancer (UICC) (17). The histological subtypes included 30 adenocarcinomas, 11 squamous cell carcinomas and 3 large-cell carcinomas (18). Twenty-three patients were smokers (including both current and former smokers), and 21 patients had never smoked. Follow-up evaluations, offered to all patients, ranged from 16.0 to 147.2 months after surgery (median, 77.4 months).

DNA extraction and methylation-specific PCR. Genomic DNA was obtained from primary tumors and normal cells by digestion with Proteinase K (Life Technologies, Carlsbad, CA, USA) followed by phenol/chloroform (1:1) extraction. DNA methylation patterns in the CpG island of 14 tumor-related genes were determined using methylation-specific PCR (MSP) as described previously (19-24). These genes were chosen based on reports that their expression is down-regulated by hypermethylation in lung cancer (19-23).

Briefly, 1 μg of genomic DNA was denatured with NaOH and modified with bisulfite. The modified DNA was purified with a Wizard DNA Purification Kit (Promega, Madison, WI, USA), desulfonated with NaOH, precipitated with ethanol, and resuspended in water. PCR amplification was performed with bisulfite-treated DNA as a template using specific primer sequences for the methylated and unmethylated forms of the gene. CpGenome Universal Methylated Control DNA (Chemicon International, Inc., Temecula, CA, USA) and DNA from the blood of healthy individuals were treated with bisulfite as described above and used as methylated and unmethylated controls. Water blanks were included with each assay. PCR products were visualized on 2% agarose gels stained with ethidium bromide. The results were confirmed by repeating the bisulfate treatment and MSP for all samples.

Mutation assay. Sequences of the first 4 exons (18-21) of the EGFR tyrosine kinase domains and exon 2 of KRAS were analyzed as described previously (25). All PCR products were incubated using exonuclease I and shrimp alkaline phosphatase (Amersham Biosciences, Piscataway, NJ, USA) and sequenced using Applied Biosystems PRISM dye-

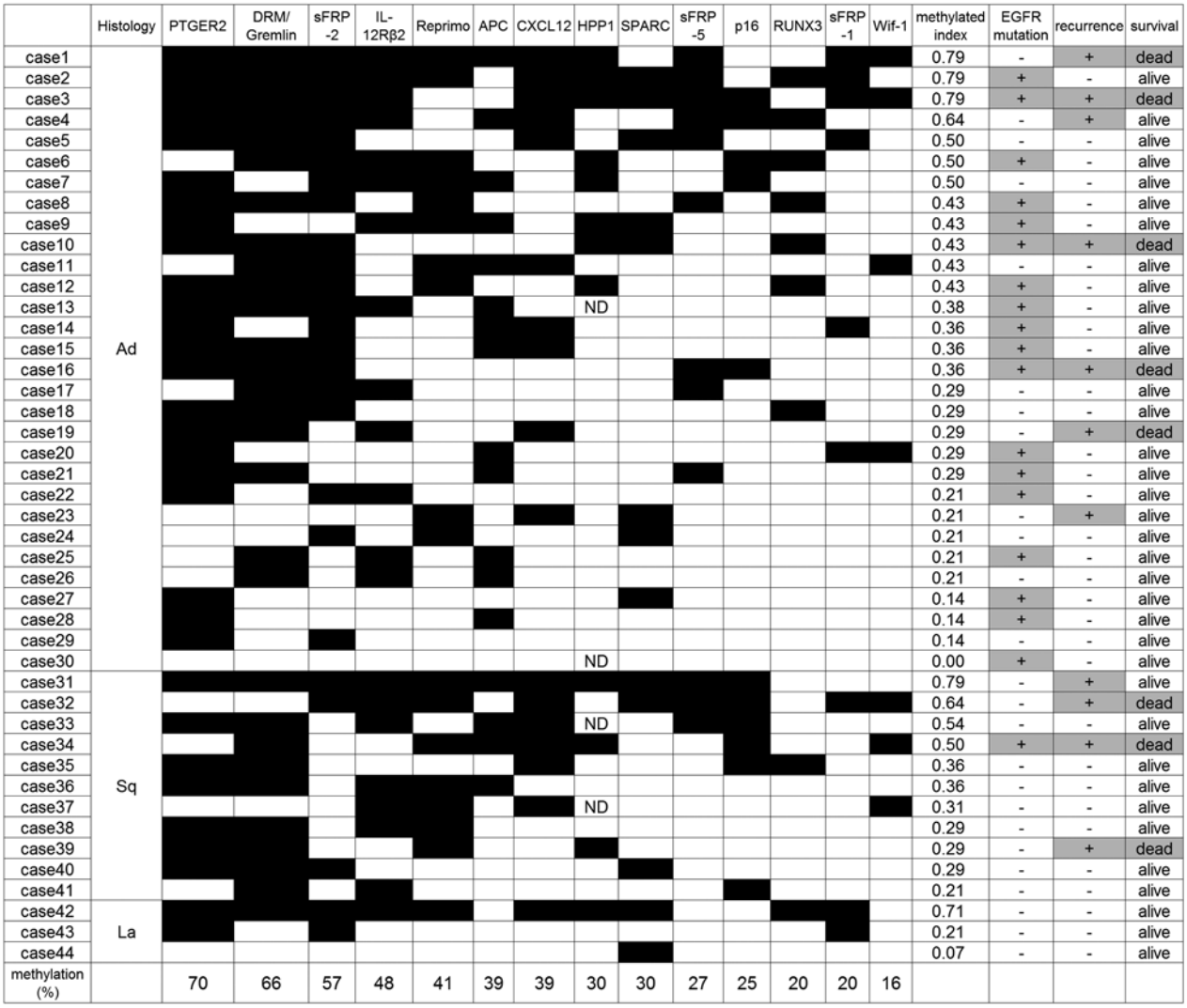

Figure 1. Clustering analysis indicates a correlation between methylation status, methylation index, EGFR mutations, recurrence, and survival for all cases. At least one of the 14 evaluated genes was hypermethylated in 43/44 cases (97.7%). The numerical value of the lower line expresses a positive rate for each genetic methylation. ND, not done; solid box, methylated band detected; open box, unmethylated band detected; Ad, adenocarcinoma; Sq, squamous cell carcinoma; La, large-cell carcinoma.

terminator cycle sequencing (Applied Biosystems, Foster City, CA, USA). All sequence variants were confirmed by independent PCR amplifications and sequencing in both directions.

Statistical analysis. Statistical differences between groups were examined using the Fisher's exact test, Chi-square test, and Mann-Whitney test. Relapse-free and overall survival times were calculated from the date of surgery until recurrence and death, or from the date of the last follow-up (censored). Survival was analyzed using Kaplan-Meier survival analysis, and comparisons between two groups were performed using the log-rank test. For multivariate analysis, independent prognostic factors were assessed using the Cox proportional hazards model. A P-value <0.05 was considered statistically significant.

The methylation index for each case was calculated as Total number of genes methylated/Total number of genes analyzed.

Results

Patient profiles. The clinicopathological features of the patients are described in Table I. Relapse-free and overall 5-year survival rates of all patients were 83.2 and 87.5%, respectively. There was no significant association between survival and clinical features such as gender, age, histology, p-factor, tumor size and smoking.

Correlation between methylation status and clinicopathological factors. Profiles of the methylation status and the EGFR mutation are shown in Fig. 1. Hypermethylation of each gene was observed in 16-70% of cancerous tissues, but in <6% (n=2) of non-neoplastic lung tissue. Thus, each gene methylation was a tumor-specific event (P<0.001).

The relationship between gene methylation status and clinicopathological features such as age, gender, histology, p-factor (p0 vs. p1), tumor size (≤2 vs. >2 cm), smoking history (never vs. smoker) was investigated (Table II). Elderly

Table II. The relationship of the gene methylation status with clinicopathological features in NSCLC patients.

Variables (cases) (n=44)	Methylated patients (%)														Mutated patients (%)
	HPP1[b]	DRM/ Gremlin	RUNX3	p16	Reprimo	IL-12Rß2	SPARC	sFRP-1	sFRP-2	sFRP-5	Wif-1	APC	CXCL12	PTGER2	EGFR[d]
Gender															
M (n=22)	4 (21)	14 (64)	2 (9)	8 (36)	9 (41)	12 (55)	6 (27)	4 (18)	10 (46)	5 (23)	4 (18)	11 (50)	12 (55)	14 (64)	5 (23)
F (n=22)	8 (38)	15 (68)	7 (32)	3 (14)	9 (41)	9 (41)	7 (32)	5 (23)	15 (68)	7 (32)	3 (14)	6 (27)	5 (23)	17 (77)	14 (64)[c]
Age[a]															
<63 (n=21)	3 (15)	13 (62)	3 (14)	5 (24)	7 (33)	8 (38)	8 (38)	3 (14)	9 (43)	6 (29)	3 (14)	9 (43)	9 (43)	11 (52)	10 (48)
≥63 (n=23)	9 (45)	16 (70)	6 (26)	6 (26)	11 (48)	13 (57)	5 (22)	6 (26)	16 (70)	6 (26)	4 (17)	8 (35)	8 (35)	20 (87)[c]	9 (39)
Histology															
Ad (n=30)	8 (29)	19 (63)	7 (23)	5 (17)	10 (33)	13 (43)	8 (27)	6 (20)	20 (67)[c]	9 (30)	4 (13)	13 (43)	10 (33)	22 (73)	18 (60)[c]
Sq (n=11)	3 (33)	9 (82)	1 (9)	6 (55)[c]	7 (64)	7 (64)	3 (27)	1 (9)	3 (27)	3 (27)	3 (27)	4 (36)	6 (55)	7 (64)	1 (9)
La (n=3)	1 (33)	1 (33)	1 (33)	0 (0)	1 (33)	1 (33)	2 (67)	2 (67)	2 (67)	0 (0)	0 (0)	0 (0)	1 (33)	2 (67)	0 (0)
p-factor															
p0 (n=28)	7 (28)	19 (68)	6 (21)	6 (21)	12 (43)	14 (50)	4 (14)	5 (18)	15 (54)	7 (25)	4 (14)	15 (54)[c]	10 (36)	21 (75)	15 (54)
p1 (n=16)	5 (33)	10 (63)	3 (19)	5 (31)	6 (38)	7 (44)	9 (56)[c]	4 (25)	10 (63)	5 (31)	3 (19)	2 (13)	7 (44)	7 (44)	4 (25)
Tumor size															
≤2 cm (n=14)	2 (18)	9 (64)	3 (21)	2 (14)	3 (21)	4 (29)	3 (21)	4 (29)	8 (57)	5 (36)	1 (7)	5 (36)	4 (29)	12 (86)	8 (57)
>2 cm (n=30)	10 (34)	20 (67)	6 (20)	9 (30)	15 (50)	17 (57)	10 (33)	5 (17)	17 (57)	7 (23)	6 (20)	12 (40)	13 (43)	19 (63)	11 (37)
Smoking															
Never (n=21)	6 (30)	14 (67)	7 (33)	4 (19)	6 (29)	9 (43)	7 (33)	6 (29)	15 (71)	8 (38)	2 (10)	7 (33)	7 (33)	17 (81)	15 (71)[c]
Smoker (n=23)	6 (30)	15 (65)	2 (9)	7 (30)	12 (52)	12 (52)	6 (26)	3 (13)	10 (43)	4 (17)	5 (22)	10 (43)	10 (43)	14 (61)	4 (17)
Methylation frequency (%)	30	66	20	25	41	48	30	20	57	27	16	39	39	70	43

Analyzed by Fisher's exact test. [a]Divided into 2 groups by median age. [b]n=40. [c]The frequency of the group is significantly higher ($p<0.05$) than the other group. [d]EGFR column indicates mutation states. Ad, adenocarcinoma; Sq, squamous cell carcinoma; La, large-cell carcinoma.

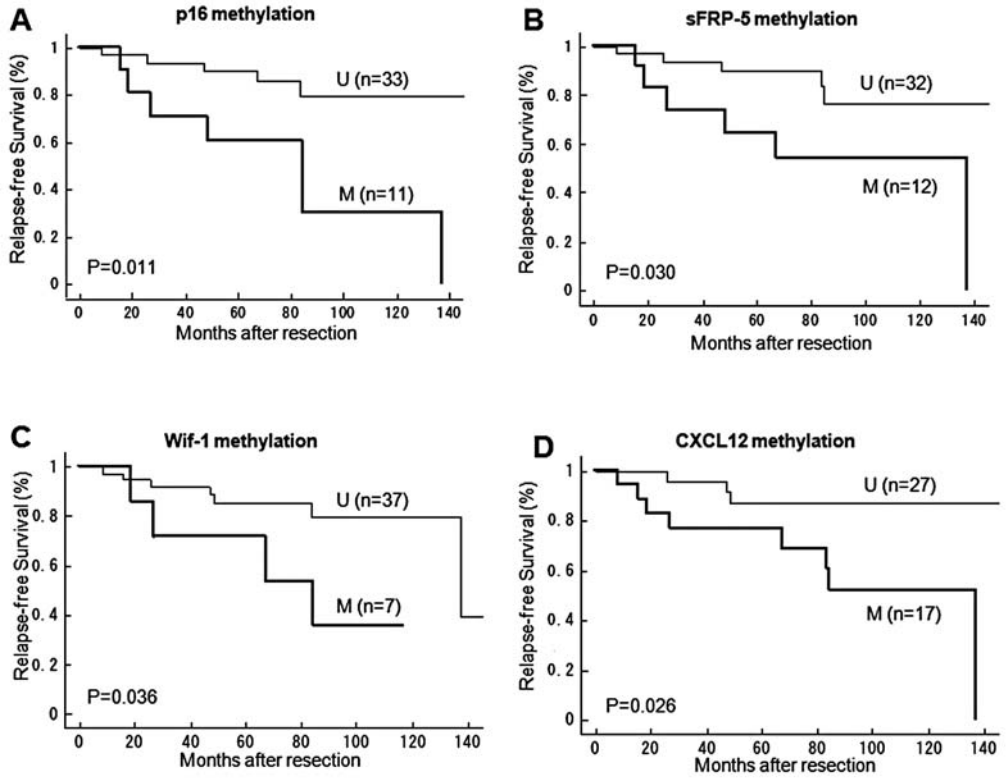

Figure 2. Correlation between the methylation status of *p16* (A), *sFRP-5* (B), *Wif-1* (C), *CXCL12* (D) and relapse-free survival of 44 NSCLC patients using the Kaplan-Meier method to generate survival curves. U, unmethylated cases; M, methylated cases. P=0.011 (A), P=0.030 (B), P=0.036 (C) and P=0.026 (D); log-rank test.

patients exhibited significantly more hypermethylation of *PTGER2* (<63 years, 52% (11/21) vs. ≥63 years, 87% (20/23); P=0.02). *p16* hypermethylation was more frequently observed in squamous cell carcinoma (Sq) (Sq, 55% (6/11) vs. Ad, 17% (5/30); P=0.041). In contrast, hypermethylation of *sFRP-2* was more frequently observed in adenocarcinoma (Ad) (Ad, 67% (20/30) vs. Sq, 27% (3/11); P=0.036). In pleural invasion, *SPARC* methylation was more prevalent in p1 cases (p0, 14% (4/28) vs. p1, 56% (9/16); P=0.006), and *APC* methylation was found in more p0 cases (p0, 54% (15/28) vs. p1, 13% (2/16); P=0.010).

Mutation state of NSCLC and its correlation with methylation and prognosis. We examined the sequences of KRAS and EGFR tyrosine kinase domains of 44 stage IA NSCLCs (Table II). Nineteen patients (43%) had EGFR mutations, but the KRAS mutation could not be found in all cases. The frequency of the EGFR mutation was significantly higher among women (female, 64% (14/22) vs. male, 23% (5/22); P=0.014) and never-smokers (never-smoker, 71% (15/21) vs. smoker, 17% (4/23); P=0.001). The EGFR mutation was noted in 18 adenocarcinomas and one squamous cell carcinoma. There was no significant correlation between the presence of the EGFR mutation and hypermethylation of the 14 cancer-related genes (Fig. 1), and the methylation index and EGFR mutation were not connected in adenocarcinoma.

Association of methylation status and prognosis. We examined the correlation between methylation and relapse-free or overall survival using Kaplan-Meier survival curves and the Cox proportional hazards model. Fig. 2 shows the curves, and the results of the log-rank tests are listed in Table III. Patients who had *p16*, *sFRP-5*, *Wif-1* or *CXCL12* methylation in their tissues were found to have a significantly shorter duration of relapse-free survival than patients with a negative methylation status for each gene (*p16*, P=0.011; *sFRP-5*, P=0.030; *Wif-1*, P=0.036; *CXCL12*, P=0.026). Methylation status of the remaining genes had no correlation with relapse-free survival. In addition, patients with methylated *HPP1*, *p16* or *Wif-1* had a significantly shorter duration of overall survival compared to patients with negative methylation status (*HPP1*, P=0.031; *p16*, P=0.026; *Wif-1*, P=0.008) (Table III, Fig. 3). Methylation status of the remaining genes did not correlate with overall survival.

Although the number of cases was small, we analyzed the survival of each case with adenocarcinoma or squamous cell carcinoma. Cases with *p16* methylation had a shorter duration of relapse-free and overall survival in adenocarcinoma (P=0.003, P=0.004 respectively), while those with *sFRP-1* methylation had a shorter overall survival in squamous cell carcinoma (P=0.002). There were no significant survival differences noted between the remaining 11 genes in the comparison of methylation-positive and methylation-negative tumors tested in histology (Fig. 4).

The Cox proportional hazards model was used to determine if the association between gene methylation and relapse-free or overall survival remained after adjusting for covariates of age, gender, tumor size and histology (Tables IV and V). A multivariate analysis revealed that the presence of hypermethylated *p16*, *sFRP-5*, *Wif-1* or *CXCL12* was an

Table III. Correlation between gene methylation and relapse-free and overall survival by log-rank test.

Gene	Relapse-free 5-year survival rate (%)			Overall 5-year survival rate (%)		
	Methylated	Unmethylated	P-value	Methylated	Unmethylated	P-value
HPP1[a]	72.7	85.1	0.069	72.7	92.3	0.031[b]
DRM/Gremlin	78.6	92.9	0.,233	84.9	92.9	0.181
RUNX3	77.8	84.7	0.917	87.5	87.7	0.583
p16	60.6	90.4	0.011[b]	77.8	90.1	0.026[b]
Reprimo	88.2	80.0	0.447	87.8	87.3	0.687
IL-12Rß2	80.1	86.5	0.323	83.3	90.9	0.463
SPARC	76.2	86.3	0.464	76.2	92.7	0.633
sFRP-1	77.8	84.5	0.523	77.8	90.1	0.257
sFRP-2	78.7	89.5	0.591	86.4	88.8	0.664
sFRP-5	64.8	90.0	0.030[b]	80.0	89.7	0.077
Wif-1	71.4	85.6	0.036[b]	71.4	90.8	0.008[b]
APC	94.1	75.9	0.552	100.0	79.8	0.261
CXCL12	76.5	87.3	0.026[b]	81.3	91.3	0.172
PTGER2	79.6	91.7	0.709	85.7	91.7	0.735

CI, confidence interval. [a]n=40. [b]Statistically significant.

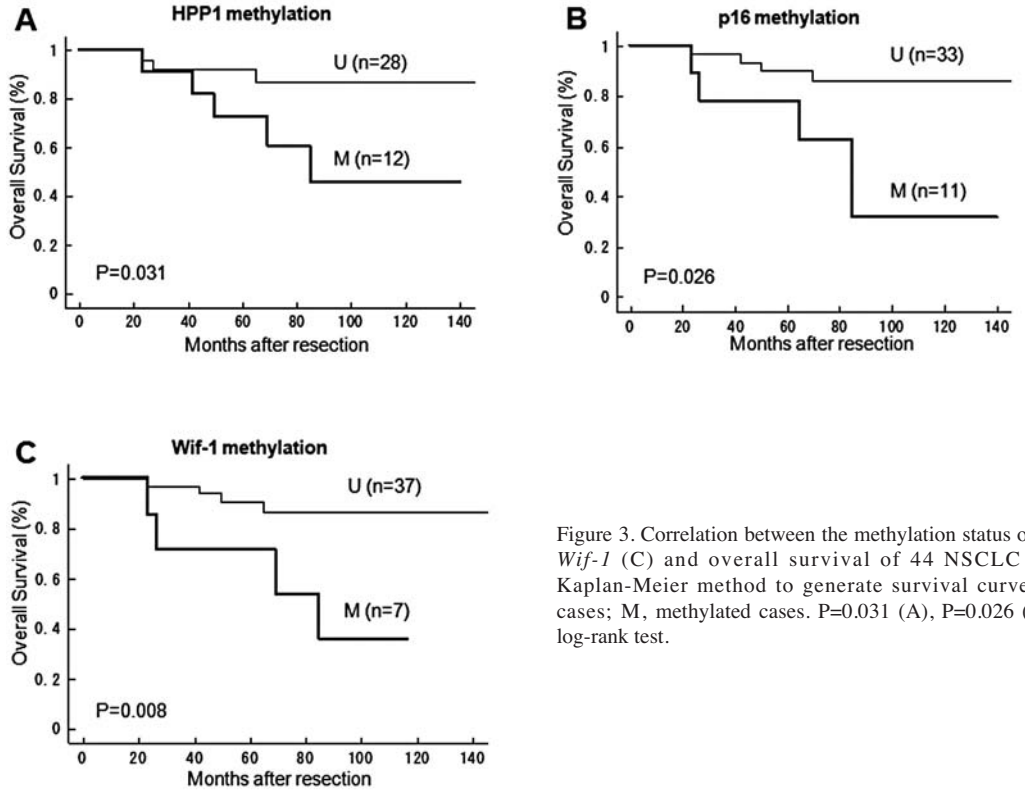

Figure 3. Correlation between the methylation status of *HPP1* (A), *p16* (B), *Wif-1* (C) and overall survival of 44 NSCLC patients using the Kaplan-Meier method to generate survival curves. U, unmethylated cases; M, methylated cases. P=0.031 (A), P=0.026 (B) and P=0.008 (C); log-rank test.

independent prognostic factor in relapse-free survival (*p16*, P=0.004; *sFRP-5*, P=0.014; *Wif-1*, P=0.045; *CXCL12*, P=0.025) (Table IVA). Multivariate analysis of these four genes was performed to assess which methylation is strongly related to convalescence, and *p16* methylation was revealed as the strongest prognostic factor for recurrence (Table VA). Likewise, an examination of overall survival showed that methylation of *p16* or *Wif-1* is an independent prognostic factor (*p16*, P=0.021; *Wif-1*, P=0.014) (Table IVB). Further multivariate analysis of these two genes revealed that *Wif-1* methylation was the only strong prognostic factor in overall survival (Table VB), and that the methylation index was significantly related in both relapse-free survival and overall survival.

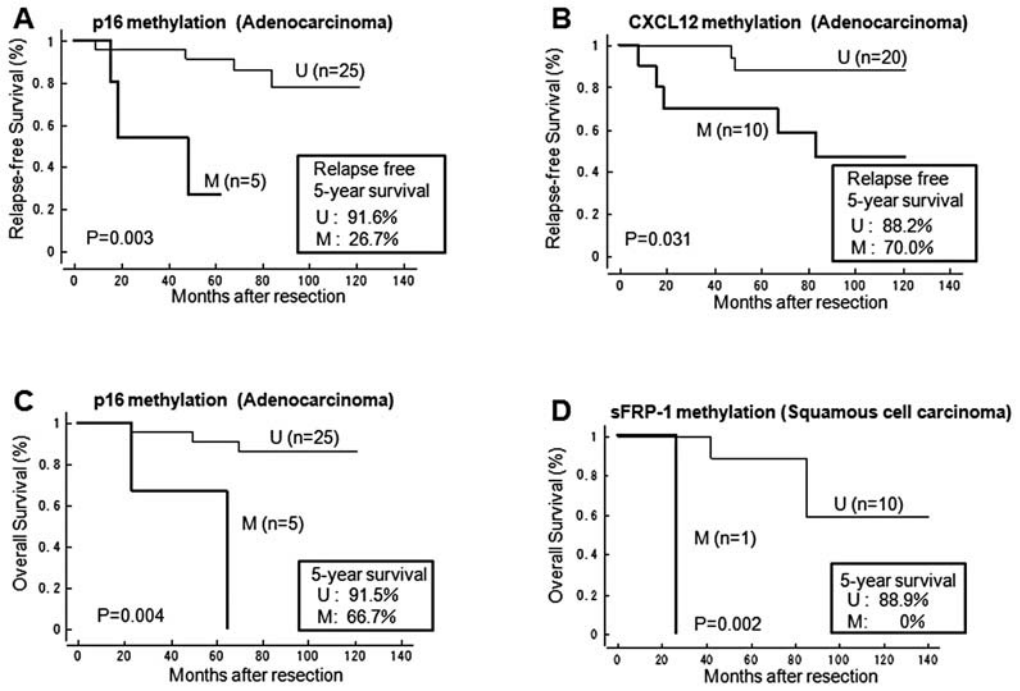

Figure 4. Kaplan-Meier curves of relapse-free survival for 30 patients with adenocarcinoma (A and B), overall survival for 30 patients with adenocarcinoma (C) and 11 patients with squamous cell carcinoma (D). A, Correlation between the methylation status of *p16* and relapse-free survival of 30 adenocarcinoma patients (P=0.001, log-rank test). B, Correlation between the methylation status of *CXCL12* and relapse-free survival of 30 adenocarcinoma patients (P=0.03). C, Correlation between the methylation status of *p16* and overall survival of 30 adenocarcinoma patients (P=0.004). D, Correlation between the methylation status of *sFRP-1* and overall survival of 11 squamous cell carcinoma patients (P=0.002). U, unmethylated cases; M, methylated cases.

Discussion

The prognosis of patients with NSCLC remains poor because of early recurrence and metastasis after complete surgical resection. Therefore, it is necessary to examine the groups with poor prognoses in order to perform effective post-surgical treatment. We examined the methylation frequency of 14 genes to identify biomarkers to predict the prognosis of patients with stage IA NSCLC after surgery, and found possible predictive genes.

The *p16* gene is a TSG located on 9p21 chromosome that encodes a cyclin-dependent kinase, a key protein regulator of progression through the G1 phase of the cell cycle. The *p16* protein plays an important role in the binding and inhibition of cyclin D kinase activity and in regulating phosphorylation of the retinoblastoma protein (p105Rb) (26). Epigenetic alterations, such as methylation of CpG islands in the promoter regions of TSGs, are reportedly frequent events in lung cancer development (27). Frequent inactivation of *p16* by methylation in diverse cancers was reported in 1995. This methylation imposed a loss of *p16* transcriptional expression that was reversible after treatment with 5-deoxyazacytidine (28,29). After these findings, other studies showed that the *p16* promoter region was methylated in lung cancer at frequencies between 20 to 70% (30,31). In this study, we found *p16* methylation in 25% of cases. In addition, when stratified by histological type, *p16* promoter methylation was significantly higher in squamous cell carcinoma (55%) than in adenocarcinoma (15%) (Fisher's exact test, P=0.016) (32). This high prevalence of *p16* promoter methylation in squamous cell carcinoma is probably related to a smoking habit, which is considered a risk factor for squamous cell carcinoma development. Several authors have described a significant association between smoking and the methylation of some genes (33,34), but in our study we found that *p16* promoter methylation was independent of a smoking habit, maybe due to the small number of samples. In addition, *p16* methylation was thought to be correlated with pulmonary metastasis in this study (data not shown).

Members of the Wnt pathway play a critical role in human carcinogenesis. Wnt antagonists were recently identified, and their role in carcinogenesis is gradually being unveiled. Wnt antagonists can be divided into two groups according to their functional mechanisms. The first group includes the secreted frizzled-related protein (*sFRP*) family, Wnt inhibitory factor-1 (*Wif-1*) and Cerberus. They inhibit Wnt signaling by direct binding to Wnt molecules. The second group, including the Dickkof (*DKK*) family, inhibits Wnt signaling by binding to the LRP5/LRP6 component of the Wnt receptor complex (35). *Wif-1* is a highly conserved gene that was first identified in the human retina. *Wif-1* is a secreted antagonist that can bind Wnt in the extracellular space and inhibit Wnt signaling. Recently, down-regulation of *Wif-1* has been reported in several types of human cancers and has been confirmed by immunohistochemistry in 60% of breast cancers and 75% of lung cancers. Mazieres *et al* (36) reported that *Wif-1* silencing correlates with hypermethylation of its promoter in both cancer cell lines and human NSCLC primary tissues. In our study, *Wif-1* hypermethylation was strongly correlated with poor prognosis. Thus, silencing of *Wif-1* may increase the malignant potential of NSCLC.

Hypermethylation of the *SPARC*, *PTGER2* and *p16* genes has been related to the EGFR mutation (24,25). However, we could not find a correlation between the EGFR mutation and

Table IV. Results of multivariate analysis using the Cox proportional hazards model of prognostic factors for relapse-free and overall survival.

A, Relapse-free survival

Variable	Hazards ratio (95% CI)		P-value
HPP1 methylation	3.743	(0.956-14.656)	0.058
DRM/Gremlin methylation	2.773	(0.557-13.789)	0.213
RUNX3 methylation	0.806	(0.140-4.638)	0.810
p16 methylation	8.158	(1.920-34.650)	0.004[a]
Reprimo methylation	1.470	(0.414-5.219)	0.551
IL-12Rß2 methylation	1.756	(0.478-6.451)	0.396
SPARC methylation	1.423	(0.364-5.559)	0.612
sFRP-1 methylation	2.128	(0.479-9.451)	0.321
sFRP-2 methylation	2.056	(0.433-9.751)	0.364
sFRP-5 methylation	5.945	(1.431-24.705)	0.014[a]
Wif-1 methylation	3.944	(1.033-15.057)	0.045[a]
APC methylation	0.748	(0.187-2.992)	0.681
CXCL12 methylation	5.067	(1.230-20.877)	0.025[a]
PTGER2 methylation	2.959	(0.491-17.834)	0.237
Methylation index	58.886	(2.572-1348.426)	0.011[a]

B, Overall survival

Variable	Hazards ratio (95% CI)		P-value
HPP1 methylation	4.651	(0.947-22.727)	0.058
DRM/Gremlin methylation	3.678	(0.440-30.766)	0.230
RUNX3 methylation	0.355	(0.036-3.476)	0.373
p16 methylation	6.579	(1.326-32.258)	0.021[a]
Reprimo methylation	1.016	(0.223-4.637)	0.983
IL-12Rß2 methylation	1.456	(0.317-6.693)	0.630
SPARC methylation	1.143	(0.245-5.329)	0.865
sFRP-1 methylation	2.767	(0.553-13.837)	0.215
sFRP-2 methylation	1.756	(0.325-9.471)	0.513
sFRP-5 methylation	4.685	(0.988-22.220)	0.052
Wif-1 methylation	6.897	(1.473-32.258)	0.014[a]
APC methylation	0.476	(0.084-2.690)	0.401
CXCL12 methylation	3.062	(0.642-14.598)	0.160
PTGER2 methylation	2.331	(0.264-20.565)	0.446
Methylation index	36.352	(1.118-1168.686)	0.043[a]

Adjusted for age (<63 vs. ≥63), gender, tumor size (≤2 vs. >2 cm) and histology (adenocarcinoma vs. non-adenocarcinoma). CI, confidence interval. [a]Statistically significant.

Table V. Results of multivariate analysis using the Cox proportional hazards model of prognostic factors for relapse-free and overall survival.

A, Relapse-free survival

Variable	Hazards ratio (95% CI)		P-value
Age (<63 vs. ≥63)	2.627	(0.532-12.963)	0.236
Gender	0.201	(0.027-1.504)	0.118
Tumor size (≤2 vs. >2 cm)	2.809	(0.331-23.810)	0.344
Histology (Adeno vs. non-Adeno)	0.473	(0.053-4.237)	0.503
p16 methylation	6.416	(1.128-36.506)	0.036[a]
sFRP-5 methylation	2.013	(0.368-11.004)	0.419
Wif-1 methylation	1.480	(0.307-7.133)	0.625
CXCL12 methylation	3.516	(0.575-21.476)	0.173

B, Overall survival

Variable	Hazards ratio (95% CI)		P-value
Age (<63 vs. ≥63)	1.309	(0.245-7.008)	0.753
Gender	0.157	(0.009-2.782)	0.207
Tumor size (≤2 vs. >2 cm)	1.563	(0.252-9.709)	0.632
Histology (Adeno vs. non-Adeno)	0.696	(0.051-9.526)	0.786
p16 methylation	5.435	(0.961-30.303)	0.056
Wif-1 methylation	5.155	(1.125-23.810)	0.035[a]

CI, confidence interval; adeno, adenocarcinoma. [a]Statistically significant.

genetic methylation in 30 cases of stage IA adenocarcinoma. Again, this finding could have been the result of the small number of cases examined. Another explanation might be that the methylations which show a correlation with the EGFR mutation occur at stage II or higher.

In this study, we confirmed that the methylation status of four genes (*p16*, *sFRP-5*, *Wif-1*, *CXCL12*) is related to a prediction of recurrence, and only two of the four are independent prognostic factors. Therefore, *p16* and *Wif-1* may be added to the list of prognostic markers in stage IA NSCLC. These findings could help improve the survival of stage IA NSCLC patients after complete resection in two ways. First, patients with these paticular methylations may be good candidates for adjuvant chemotherapy. Second, as these are methylation markers, demethylating agents might prove beneficial for such patients. It is well known that both carcinogenesis and tumor progression evolve from the genetic and epigenetic alterations of several genes. 5-Aza-2-deoxycitydine (5-AZA), an inhibitor of DNA methyltransferase can recover such epigenetic changes (31,37). Therefore, if a drug that can selectively reverse epigenetic changes as well as hypermethylation is developed, a unique targeted strategy could emerge. Thus, although further study is needed to clarify the mechanism of these gene methylations, our results may contribute to an improvement in survival rates for specific stage IA NSCLC patients.

In conclusion, the methylation status of *p16* and *Wif-1* was found to be strongly associated with decreased survival in patients with stage IA NSCLC disease, a finding that reveals their potential as novel and unique prognostic factors for NSCLC.

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