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p16^{INK4a} and Histology-specific Methylation of CpG Islands by Exposure to Tobacco Smoke in Non-Small Cell Lung Cancer[1]

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ABSTRACT

The p16^{INK4a} protein inhibits cyclin-dependent kinase 4, a key regulator of progression through the G_1 phase of the cell cycle. Methylation of CpG islands in the promoter region is an important avenue for inactivation of p16. The mechanism of methylation of the p16 promoter region, however, has not been elucidated. Recent reports investigating p16 methylation in non-small cell lung cancer (NSCLC) suggest that carcinogens in tobacco smoke induce the DNA methylation process. We investigated the association between methylation of the p16 promoter region and exposure to tobacco smoke in 185 primary NSCLCs. We also studied the relationship of p16 methylation with mutation of the K-*ras* and *p53* genes, as well as with methylation at the *DAP-kinase* and *p14*^{ARF} loci. Finally, we evaluated the prognostic significance of p16 methylation in NSCLC. The prevalence of p16 methylation was greater in squamous cell carcinoma (41%) compared with adenocarcinoma (22%; $P = 0.03$; Fisher's exact test). Methylation of p16 was significantly associated with pack-years smoked ($P = 0.007$; Wilcoxon rank sum test), duration of smoking ($P = 0.0009$; Wilcoxon rank sum test), and negatively with the time since quitting smoking ($P = 0.03$; Wilcoxon rank sum test). No methylation of the nearby *p14*^{ARF} locus was detected, and methylation of the *DAP-kinase* locus was not associated with either p16 methylation or with exposure to tobacco smoke. In patients with stage 1 adenocarcinoma, p16 methylation was an independent risk factor predicting significantly shorter postsurgery survival ($P = 0.03$), controlling for the significant effects of other factors, including K-*ras* mutation. These findings suggest that methylation of CpG islands in tobacco-associated cancers occurs in a gene- and tissue-specific manner and is induced directly or indirectly by exposure to tobacco smoke in NSCLC.

INTRODUCTION

The *INK4a* locus on chromosome 9p21 encodes both the p16 and p14^{ARF}[3] proteins by alternative splicing (1, 2). The first exon (exon 1β) of p14^{ARF} is ~20 kb centromeric to the first exon (exon 1α) of p16. The p16 and p14^{ARF} proteins have homology at the amino acid level (3), but their transcription occurs in a different reading frame. Thus, they have different protein structures and are involved in different cellular control mechanisms. The p16 gene product is an inhibitor of Cdk4, which phosphorylates the serine/threonine residues of the retinoblastoma protein (4, 5). The p16 protein binds to the Cdk4 and Cdk6 proteins and thereby controls cell cycle progression through G_1 into S phase, past the G_1 checkpoint (6, 7). Hence, the p16 protein plays a major role in maintaining the Rb protein in the unphosphorylated state, inhibiting cell cycle progression. In contrast, the p14^{ARF} product binds to MDM2 and promotes its degradation (8). MDM2 can also bind p53, inactivate its transcriptional activity, and promote its rapid degradation (9).

Inactivation of p16 has been detected in >70% of cell lines derived from human NSCLCs (7). In addition, p16 inactivation has been detected in ~50% of primary NSCLCs (10–12). Homozygous deletions (9–25%; Refs. 13–15) and mutations of p16 (0–8%; Refs. 16–19) have been described in NSCLCs with a highly variable frequency. More recently, methylation (15–35%) at the 5′ CpG islands of the *p16* gene has been identified as an alternative to mutation or deletion as a mechanism of p16 inactivation in NSCLCs (20–22). Genetic alteration of p14^{ARF} has been detected in T-cell acute lymphoblastoid leukemia (23). Point mutation of exon 1β of the p14^{ARF} has not been reported in human cancer, but, similar to p16, aberrant methylation of the p14^{ARF} promoter region has been found in colorectal cancer cell lines (24). However, less is known about the relationship of methylation of these coincident genes and the possible therapeutic importance of methylation of both the p16 and p14^{ARF} promoter region.

Although there is no direct evidence that smoking induces DNA methylation, recent reports have associated DNA methylation with exposure to tobacco carcinogens (25–28). Lung tumors induced in F344/N rats after exposure to cigarette smoke by inhalation displayed *de novo* methylation of p16 exon 1 (25). Methylation of the p16 promoter region was also induced in 94% of adenocarcinomas of rats treated with tobacco-specific NNK (26). In this same study, the prevalence of p16 methylation increased with disease progression from basal cell hyperplasia (17%) to squamous metaplasia (24%) and then to carcinoma *in situ* (50%). Regional methylation also has been reported in transgenic cell lines treated with nickel, a carcinogenic metal found in cigarette smoke (27). Lee *et al.* (27) demonstrated nickel-induced silencing of *gpt* expression in Chinese hamster ovary cells by DNA methylation, as well as nickel-induced condensed chromatin and heterochromatization of the *gpt* integration site. In humans, Eguchi *et al.* (28) have reported DNA methylation at the *D17S5* locus to be significantly higher in smokers than in nonsmokers in both tumors and nontumorous lung tissues.

The clinical significance of p16 alterations remains unclear. Lack of p16 expression has been negatively associated with the prognosis of patients with early stage (I-II) NSCLC, especially in squamous cell carcinoma (29). It has also been reported that higher expression of p16 correlates with longer patient survival time (30). However, in another study, methylation of the *p16* gene was not associated with patient prognosis (31).

The mechanistic links of p16, p53, and ras alterations in a single lung tumor are not clear, but several studies have suggested that the three proteins may cooperate through different pathways to abrogate growth control. Adenoviral transfection of the *p16* and *p53* genes has shown a synergistic effect in the induction of apoptotic cell death in tumor cells (32). Negative immunostaining for p16 has also been directly correlated with p53 overexpression in NSCLC (12). Finally,

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[3] The abbreviations used are: ARF, alternative reading frame; Cdk, cyclin-dependent kinase; NSCLC, non-small cell lung cancer; DAP-K, death-associated protein kinase; OR, odds ratio; CI, confidence interval; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; MSP, methylation-specific PCR.

primary murine fibroblasts arrested by ras show an accumulation of p53 and p16 proteins (33).

Here we have studied the relationship of methylation of the p16 promoter region with exposure to tobacco smoking and asbestos, as well as with patient clinical and demographic factors. We also examined the relationship of inactivation of p16 by methylation with methylation of the coincident *p14*^{ARF} locus and methylation of another gene important in cell growth control, the *DAP-K* locus. This was done in 185 NSCLCs using MSP, as well as immunohistochemistry and PCR, and PCR-single strand conformational polymorphism for examination of p16, p53, and mutation of K-ras. Finally, we investigated the prognostic significance of p16 methylation, controlling for other known prognostic factors.

MATERIALS AND METHODS

Study Population. A total of 185 primary NSCLC specimens were included in this study. All specimens were obtained from patients who underwent surgical resection at the Massachusetts General Hospital Thoracic Surgery, Oncology, and Pulmonary Services from November 1992 through January 1996. Tumors were snap-frozen in liquid nitrogen and stored at −70°C until genomic DNA preparation. Information regarding smoking habits, occupational exposure to asbestos, and other sociodemographic characteristics was obtained from an interviewer-administered questionnaire. Written informed consent was obtained from all participants. Genomic DNA was isolated using QIA tissue kit according to the manufacturer's instructions (Qiagen). A DyNA Quant 200 fluorometer (Hoefer, San Francisco, CA) was used to measure DNA concentration.

Sodium Bisulfite Modification. Genomic DNA was modified by treatment with sodium bisulfite. In this reaction, all unmethylated cytosines are converted to uracil, but methylated cytosines are resistant to this modification. DNA was modified using a method described previously (34). Briefly, 1-μg aliquots of DNA in a volume of 36 μl were denatured by adding freshly prepared sodium hydroxide to a final concentration of 0.3 M and incubating for 15 min at 37°C. Twenty-four μl of 10 mM hydroquinone and 416 μl of 3.6 M sodium bisulfite (pH 5.0), both freshly prepared, were added to the denatured DNA. The sample was mixed gently, overlayed with enough mineral oil to cover the surface of the aqueous phase, and incubated at 55°C for 16 h. The DNA was recovered from under the mineral oil layer after snap freezing the reaction and removing the unfrozen oil. Bisulfite-modified DNA was purified using a desalting column (Promega Wizard DNA clean-up system and Vacuum Manifold), according to the manufacturer's instructions, and eluted in 50 μl of autoclaved distilled water. Freshly prepared sodium hydroxide (final concentration, 0.3 M) was added for desulfonation, and the mixture was incubated at 37°C for 15 min. The solution was neutralized by adding 17 μl of 10 M ammonium acetate. The neutralized DNA was ethanol precipitated at −20°C overnight and resuspended in 20 μl of autoclaved distilled water. DNA was quantitated by DyNA Quant 200 fluorometer (Hoefer) and stored at −70°C until use.

MSP–the *p16* Locus, *p14*^{ARF4}, and *DAP-K* Locus. Methylation status of the promoter region of the *p16*, *p14*^{ARF4}, and *DAP-K* locus was determined by MSP (Fig. 1) described by Herman *et al.* (34). Two sets of primers were designed, one specific for DNA methylated at the promoter region of each gene and the other specific for unmethylated DNA. Approximately 50 ng of sodium bisulfite-modified DNA was used as a template for MSP. MSP amplification for the *p16* gene was carried out in a final volume of 50 μl, containing sodium bisulfite-modified DNA, primers (300 ng each), deoxynucleotide triphosphates (each at 1.25 mM), $MgCl_2$ (1.5 mM), 2-mercaptoethanol (1 mM), ammonium sulfate (16.6 mM), Tris (6.7 mM, pH 8.8), and 1.25 units of Taq polymerase under the following conditions: 4 min at 94°C (hot start), 40 cycles (45 s at 94°C, 30 s at annealing temperature, and 45 s at 72°C), followed by a final 10-min extension at 72°C. Annealing temperature was 65°C for the methylated reaction and 60°C for unmethylated reaction. The primer sequences for detecting methylated *p16* gene were 5′-TTA TTA GAG GGT GGG GCG GAT CGC-3′ (sense) and 5′-GAC CCC GAA CCG CGA CCG TAA-3′ (antisense). The size of the PCR amplification for the methylated reaction was 234 bp. The primer sequences for the unmethylated promoter were 5′-TTA TTA GAG GGT GGG GTG GAT TGT-3′ (sense) and 5′-CAA CCC CAA ACC ACA ACC ATA A-3′ (antisense). The size of the PCR product for the unmethylated reaction was 151 bp. The SW 480 colon cancer cell line and NCI-H 209 lung cancer cell line were used as a positive control for the methylated and unmethylated p16 reactions, respectively.

A multiplex PCR was used to detect the presence or absence of the methylated p14^{ARF}. Primer sequences for the methylated region of p14^{ARF} were 5′-GGA AGG CGG GTG CGC GTT-3′ (sense) and 5′-CTC GAC AAC CGC TAC GCC G-3′ (antisense). Primer sequences for the unmethylated region of p14^{ARF} were 5′-AGG GAA GGT GGG TGT GTG TT-3′ (sense) and 5′-ACC ACA CAC ACA CCA AAT CCA-3′ (antisense). PCR conditions consisted of a 1-min denaturation at 94°C, followed by 35 cycles of 30 s at 94°C, 10 s at 65°C, and 30 s at 72°C. Four % of DMSO, 100 ng of modified DNA, primers (300 ng for primers for methylated region and 150 ng for primers for unmethylated region), and Taqstart antibody were used in a 50-μl reaction.

MSP amplification for the promoter region of *DAP-K* gene was performed in a final volume of 50 μl containing bisulfite-modified DNA (50 ng), primers (20 pmol each), deoxynucleotide triphosphate (each at 1.25 mM), $MgCl_2$ (1.5 mM), Tris-HCl (6.7 mM, pH 8.4), and 1.25 units of Taq polymerase under following conditions: 4 min at 94°C (hot start), 40 cycles (30 s at 94°C, 30 s at annealing temperature, and 30 s at 72°C), followed by a final 10-min extension at 72°C. Annealing temperature was 63°C for the methylated reaction and 68°C for the unmethylated reaction. The primer sequences for methylated DAP-K were 5′-GGA TAG TCG GAT CGA GTT AAC GTC-3′ (sense) and 5′-CCC TCC CAA ACG CCG A-3′ (antisense). The size of the PCR amplification for the methylated reaction was 98 bp. The primer sequences for the unmethylated region were 5′-GGA GGA TAG TTG GAT TGA GTT AAT GTT-3′ (sense) and 5′-CAA ATC CCT CCC AAA CAC CAA-3′ (antisense). The size of the PCR product for the unmethylated reaction was 106 bp. All products were analyzed by electrophoresis (3% metaphore agarose) and ethidium bromide staining.

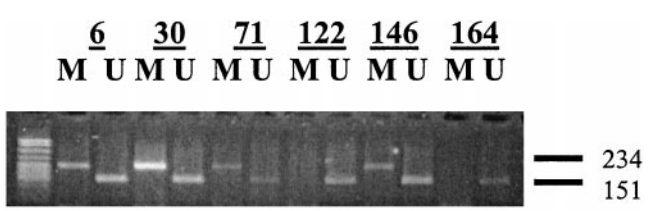

Fig. 1. MSP of *p16* gene. PBR322/MSP1 digests are shown at *left* as molecular markers. Primer sets used for amplification are designated as unmethylated (*U*) or methylated (*M*). Twenty μl of PCR product were run on 3% metaphore agarose gel, stained with ethidium bromide, and visualized under UV illumination. The numbers indicate the identification numbers of samples.

Immunohistochemistry of p16. Tissue sections were deparaffinized in EZ-DeWax solution (Biogenex Laboratories, San Ramon, CA) and washed in PBS. The sections were incubated in Antigen Retrieval Citra (Biogenex Laboratories) in a microoven, and treated with peroxidase for 10 min. The sections were then incubated for 60 min at room temperature with mouse monoclonal anti-p16 antibody (Neomark, Fremont, CA) diluted to a final concentration of 4 μg/ml and washed in PBS. Mouse IgG monoclonal antibody at the same concentration as the primary antibody was used as a negative control. Detection of immunoreactivity was carried out by the Biotin-Streptavidin amplified method according to the manufacturer's instructions (Biogenex Laboratories). 3,3′-diaminobenzidine solution was used as a chromogen, and Mayer's hematoxylin was used as a nuclear counterstain. Nuclear reactivity for p16 protein was considered as positive or negative as described previously (35). Briefly, sections were examined for evidence of nuclear staining above any cytoplasmic background. If there was no nuclear reactivity in the nonneoplastic tissue, the sample was considered to be uninterpretable. If the proportion of stained nuclei was >10% of all nuclei in the tumor, it was considered to be positive. If the proportion of stained nuclei was <10% of all nuclei in the tumor and the admixed nonneoplastic tissue also showed nuclear reactivity, it was then considered to be negative.

Mutation of K-*ras* Codon 12 and p53 Exons 5–9. The K-*ras* alterations in codon 12 were detected by PCR-RFLP as described previously (36). Mutation screening of exons 5–9 of p53 was done with the use of PCR-single strand conformational polymorphism and DNA sequencing (37).

Statistical Analysis. SAS software was used for statistical analysis. Wilcoxon rank sum test and Fisher exact test (or χ^2 test) were used for continuous and categorical variables in univariate analysis, respectively. Multivariate logistic regression was conducted to estimate the relationship between methylation of p16 and covariates that were statistically significant in univariate analysis and that were important biologically.

Kaplan-Meier analysis was performed to estimate a survival function over time for individual covariates. Survival estimates between tumors with methylation of p16 and without methylation of p16 were compared by log-rank test. Cox proportional hazards model was used to explore the relationship between survival and explanatory variables that were identified as important in Kaplan-Meier analysis.

RESULTS

Clinical and Demographic Characteristics and Methylation of p16. There was no difference in clinicopathological characteristics between patients with and without fresh frozen tissue available for the study (Table 1). Fresh frozen tissue was available in 185 of 360 patients. Table 2 summarizes the clinical and pathological characteristics of the 185 participants included in this study. Fifty-one (28%) of 185 tumors showed methylation of the p16 promoter region. The mean age of patients with methylation of p16 was 69 ± 9 years, slightly but not significantly older than those without methylation of the *p16* gene ($P = 0.19$; Wilcoxon-rank sum test). Methylation of p16 was also slightly more frequent in males (32%; 32 of 101) than females (23%; 19 of 84; $P = 0.19$). Methylation of p16 occurred more frequently in squamous cell carcinoma (41%) than adenocarcinoma (22%; Fig. 2).

Those with methylation at p16 were more likely to be current smokers than those without p16 promoter methylation ($P < 0.05$). Methylation at p16 was significantly associated with pack-years smoked ($P = 0.007$), duration of smoking ($P = 0.0009$), and negatively associated with duration since quitting smoking ($P = 0.03$; Table 2; Fig. 3). Asbestos exposure was not associated with methylation of the p16 promoter region. Because the *p16* and *p14*^{ARF} genes are physically linked on chromosome 9p21, we also looked for methylation inactivation at p14^{ARF}. None of the 126 tumors that we could evaluate showed methylation (data not shown). Methylation of DAP-K occurred in 25% of patients and was not associated with exposure to smoking and asbestos (38). No association was found between methylation of p16 and disease stage or methylation of the *DAP-K* locus.

Relationships among p16, p53, and K-ras. There was no evidence of any relationship of methylation of p16, mutation of p53 exons 5–9, and mutation of K-*ras* codon 12 (Table 2). Because there is evidence (39, 40) that overexpression of K-*ras* can affect the activity of methyltransferase and because mutation of K-*ras* might be

Table 1 *Characteristics of patients with and without fresh frozen tissue available for study*

	Fresh tissue (n = 185)	No fresh tissue (n = 175)	Total group (n = 360)
Age[a]	67 ± 11	65 ± 10	66 ± 10
Sex			
Female	84 (45%)	85 (49%)	169 (47%)
Male	101 (55%)	90 (51%)	191 (53%)
Pack-years[a]	56 ± 40	56 ± 36	56 ± 38
Years smoked[a]	38 ± 16	37 ± 15	37 ± 15
Years quit[a]	7 ± 10	8 ± 10	7 ± 10
Smoking status			
Never	13 (7%)	9 (5%)	22 (6%)
Ex-smoker	97 (52%)	97 (55%)	194 (54%)
Current	75 (41%)	69 (39%)	144 (40%)
Asbestos exposure[b]			
No	159 (88%)	148 (85%)	307 (86%)
Yes	22 (12%)	26 (15%)	48 (14%)
Stage[b]			
I	102 (56%)	100 (58%)	202 (57%)
II	35 (19%)	18 (11%)	53 (15%)
III	40 (22%)	45 (26%)	85 (24%)
IV	5 (3%)	9 (5%)	14 (4%)
Histology[b]			
Adenocarcinoma	96 (53%)	101 (57%)	195 (54%)
Squamous cell carcinoma	56 (31%)	49 (28%)	108 (30%)
Other	31 (17%)	25 (15%)	56 (16%)

[a] Mean ± SD.
[b] Asbestos exposure, stage, and histology data are missing for 5, 6, and 2 patients, respectively.

Table 2 *Clinicopathological characteristics of methylation of p16 in non-small cell lung cancer*

	Methylation		
	Absent (n = 134)	Present (n = 51)	P[a]
Age[b]	66 ± 11	69 ± 9	0.19
Sex			
Female	65	19	
Male	69	32	0.19
Pack-years[b]	51 ± 38	69 ± 43	0.007
Years smoked[b]	36 ± 16	44 ± 16	0.0009
Years quit[b]	8 ± 10	4 ± 7	0.03
Smoking status			
Never	11	2	
Ex-smoker	76	21	
Current	47	28	0.05
Asbestos exposure[c]			
No	116	43	
Yes	16	6	1.00
DAP-K methylation			
No	97	41	
Yes	37	10	0.35
Stage[c]			
1	79	23	
2	21	14	
3	27	13	
4	5	0	0.11
Histology[c]			
Adenocarcinoma	73	21	
Squamous cell carcinoma	35	24	
Other	25	5	0.07
K-ras mutation			
No	110	43	
Yes	24	8	0.83
p53 exons 5–10[c]			
Wild-type	96	33	
Mutant	34	17	0.36

[a] Wilcoxon rank sum test and Fisher's exact (or χ^2) test was used for continuous and categorical variables, respectively.
[b] Mean ± SD.
[c] Asbestos exposure, stage, histology, and p53 exon 5–10 data are missing for 4, 3, 2, and 5 patients, respectively.

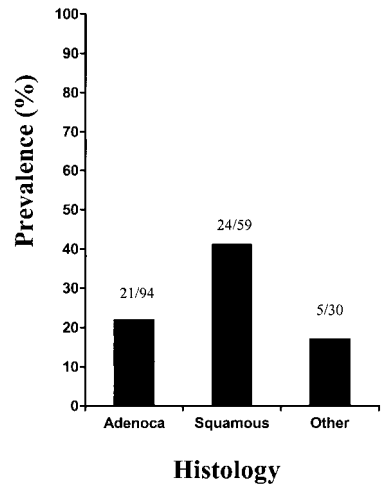

Fig. 2. Frequency of methylation of p16 promoter region by histology in NSCLC. *Columns*, proportion of methylation of the p16 promoter region in each histology.

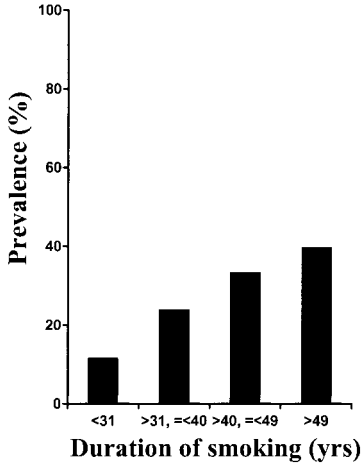

Fig. 3. Frequency of p16 methylation by quartile, according to duration of smoking in all of the NSCLC patients studied. The association between methylation of p16 promoter region and duration of smoking was calculated by Wilcoxon rank sum test. The unit of duration of smoking is years. *Columns*, proportion of methylation of p16 promoter region in each quartile of smoking.

Table 3 *Association of abnormal expression of p16, p53, and K-ras codon 12 mutation in 106 NSCLCs*

	p53[a]			K-*ras* codon 12		
	Normal	Abnormal	P^b	Wild-type	Mutant	P^b
p16[a] Normal	11	22		26	7	
p16[a] Abnormal	26	47	0.82	62	11	0.44

[a] p16 was scored as positive nuclear staining (normal) or no nuclear staining (abnormal). p53 was scored as normal (no staining) or abnormal (nuclear staining).
[b] *P* was calculated by χ^2 test.

Table 4 *Multivariate logistic regression analysis[a] of the association between p16 methylation and clinicopathological features in NSCLC (n = 185)*

	OR	95% CI	*P*
Duration of smoking			
<31 yr	1.00		
≥31 yr, <40 yr	2.07	0.67–6.39	0.21
≥40 yr, <49 yr	3.21	1.11–9.33	0.03
≥49 yr	4.27	1.48–12.30	0.007
Histology			
Adenocarcinoma	1.00		
Squamous cell carcinoma	2.36	1.10–5.08	0.03
Other	1.03	0.37–2.88	0.95
Age	1.00	0.96–1.04	0.97

[a] Sex and stage were adjusted as potential confounders.

Table 5 *Survival outcome by multivariate Cox proportional hazard analysis[a] for stage I adenocarcinoma*

	HR[b]	95% CI	*P*
Methylation of p16	4.72	1.02–21.85	0.04
Mutation of K-*ras* codon 12	7.57	1.60–35.94	0.01

[a] Adjusted for age, sex, duration of smoking.
[b] HR, hazard ratio.

an effect modifier, preceding actual p16 methylation, we further analyzed the relationship of p16 alteration and K-*ras* mutation. When the data were stratified by K-*ras* mutation status, there was no difference in the association of p16 hypermethylation and smoking in the patients with mutation of K-*ras* codon 12 or those with wild-type K-*ras* (data not shown). Immunohistochemical staining for p16 and p53 was conducted to study their relationships at the level of protein. There was no association among overexpression of p53, underexpression of p16, and mutation of K-*ras* codon 12 (Table 3).

Methylation of p16 Is Associated with Exposure to Tobacco Smoke. Logistic regression analysis of the data was performed to control for the potential confounding effects of variables such as age, sex, and histology. Table 4 shows that exposure to tobacco was significantly associated with p16 promoter methylation. When we examined duration of exposure by quartile, the OR for predicting p16 methylation was 2.07 (0.67–6.39), 3.21 (1.11–9.33), and 4.27 (1.48–12.30) for each increasing quartile of exposure. Patients who smoked <31 years were used as a referent group. No significant effect of age was observed (OR, 1.00; Table 4).

Methylation of p16 and Patient's Prognosis. Kaplan-Meier estimates were used to examine the relationship of p16 methylation with survival. Data were stratified by disease stage because stage is an important risk factor in NSCLC. The Kaplan-Meier survival function in stage I of adenocarcinoma is shown in Fig. 4. In our data, there is a significant association of p16 methylation silencing in stage I and a poorer prognosis ($P = 0.04$). In squamous cell carcinoma and more advanced stage disease, no significant association of methylation of p16 and outcome was observed. Because there was limited number of patients available for study of disease survival in stages II, III, and IV, no similar analysis was completed. Cox proportional hazard analysis (Table 5) was conducted to measure the hazard ratio after controlling for potential confounding such as age, sex, the duration of smoking, and mutation of K-*ras* codon 12. Patients with methylation of p16 had a significantly shorter survival time (hazard ratio, 4.72; 95% CI, 1.02–21.85) than patients without methylation of p16 in stage I adenocarcinoma.

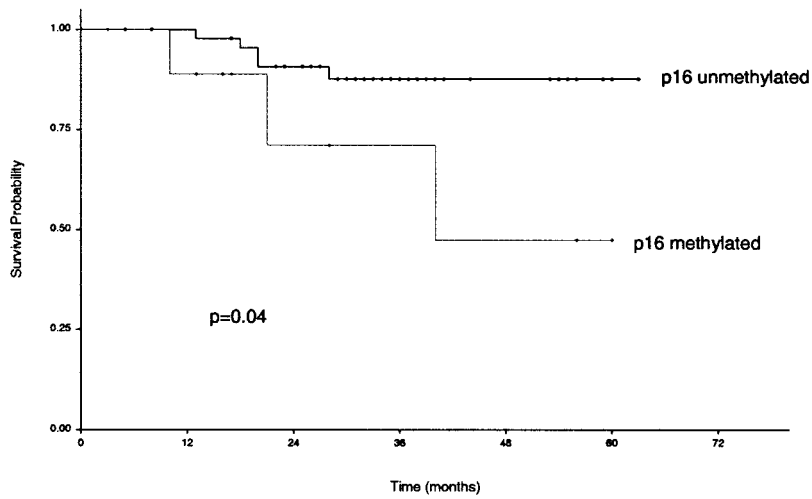

Fig. 4. Overall survival of 58 patients with stage I adenocarcinoma. Patients with methylation of the p16 promoter region had poorer survival compared with those without methylation of p16 promoter region. This difference is statistically significant based on log-rank test ($P = 0.04$).

DISCUSSION

Although our data suggest that tobacco smoke can affect the methylation of p16 in NSCLC, the precise mechanism at the DNA level has yet to be defined. Tobacco smoke contains many carcinogens including NNK, polyaromatic hydrocarbons, chromium, cadmium, and nickel. In addition, tobacco smoke is a direct mucosal irritant and induces inflammation that results in significant endogenous oxygen free radical generation. Our data are consistent with recent animal and *in vitro* work, showing that the prevalence of p16 methylation increases with the duration of smoking in a dose-dependent fashion (25–27). After stratifying the data by histology, there was a similar pattern with duration of smoking associated with p16 methylation in both adenocarcinoma and squamous cell carcinoma.

The different degrees of methylation that we observed in adenocarcinoma compared with squamous cell carcinoma might result from a different activity of the *de novo* methyltransferase by cell type (41). *De novo* methylation of unmethylated CpG islands has been reported to increase upon overexpression of DNA methyltransferase in human fibroblasts (42). In addition, the activity of DNA methyltransferase has been measured in different histological cell types of rat lungs prior to and after treatment with NNK (41). Activity of DNA methyltransferase was increased in the alveolar type II cells of the A/J mouse susceptible to lung cancer formation; however, Clara cells from the same A/J mouse did not show the increase of methyltransferase activity. The degree of methylation of DNA also was found to be higher in mouse alveolar cells than Clara cells. Although the activity of *de novo* methyltransferase was not measured in the current study, p16 promoter methylation occurred more often in squamous cell carcinoma than adenocarcinoma (Fig. 3; $P = 0.03$), and our observation of this tissue specificity is clearly consistent with the mouse data (41).

To understand whether the effect of tobacco carcinogen exposure on methylation of promoter sequences is gene and locus specific, we compared p16 methylation with p14^{ARF} and DAP-K methylation. Exposure to tobacco was associated with methylation of the p16 promoter region but not the *DAP-K* gene promoter. Furthermore, the coincident *p14*^{ARF} gene was not methylated in any tumors. It is unclear if the locus specificity for methylation is carcinogen specific (43) or dependent upon other factors that might be acting indirectly, including *trans*-acting factors (44), *cis*-acting elements such as Alu repeats (45, 46), protecting factors against methylation (47, 48), chromosomal location of the gene (49), promoter structure (50), and demethylation by repair systems (51, 52). That is, it is also possible that tobacco carcinogens induce cell clones that become susceptible to methylation events, and hence, gene specificity may be attributable to the prior effects of the genotoxic action of tobacco smoke at other genes. Recently, Huang *et al.* (44) studied the methylation status of >276 CpG island loci in breast cancer cell lines using an array-based method, called differential methylation hybridization. They reported that 5–14% of these loci were methylated in breast cancer cells relative to normal control and that loci with preexisting methylation within a CpG island are more prone to *de novo* methylation. This suggests that CpG islands may have different susceptibility to methylation and that additional *trans*-acting factors may be responsible for aberrant methylation in breast cancer cells.

The prognostic significance of p16 protein in NSCLC has been studied by several groups without consensus. Most reports have examined expression of p16 (10, 29–31, 53) and are not, therefore, strictly comparable with our data. In addition, we have controlled for other risk factors (specifically including K-*ras* mutation) and find that methylation of the p16 promoter is an independent risk factor in stage I adenocarcinoma. In this analysis, mutation of K-*ras* codon 12 remains an independent prognostic factor in the same stage I adenocarcinomas (36). The fact that p16 methylation was not associated with survival in squamous cell lung cancers further emphasizes the important histological differences in the role of p16 inactivation in lung cancer.

In conclusion, the molecular mechanisms underlying *de novo* methylation of CpG islands in cancer are complex. For the *p16* gene, exposure to tobacco-associated carcinogens enhances the likelihood of inactivation by methylation. Methylation of the *DAP-K* locus is not associated with smoking and is independent of p16 methylation. Hence, the mechanism responsible for epigenetic modification of genes responsible for NSCLC is both gene and tissue specific and only sometimes associated with carcinogen exposure. Considerable additional research will be needed to more clearly understand these striking differences in the induction of epigenetic inactivation of critical cell growth control genes.

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