Loss of Function of p16 Gene and Prognosis of Pulmonary Adenocarcinoma

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BACKGROUND. Stepwise progression of peripheral-type lung adenocarcinoma was characterized morphologically and was related to prognosis. Expression of the tumor suppressor gene p16 in pulmonary adenocarcinoma decreased, mainly as a result of aberrant methylation of the CpG islands of the promoter region.

METHODS. Aberrant methylation status of the p16 promoter region, the expression of its product, and loss of heterozygosity (LOH) on 9p21 were examined in surgically resected lung specimens from 57 patients (28 males and 29 females) with peripheral-type lung adenocarcinoma measuring $\leq$ 2 cm in diameter.

RESULTS. Aberrant methylation of the p16 promoter region, negative p16 protein expression, and LOH of the 9p21 region were detected in 40.4%, 50.9%, and 40.4% of tumor samples, respectively. The alterations of the p16 gene were associated with poor prognosis, and in particular the prognosis of patients with aberrant p16 methylation was significantly worse than that of patients without aberrant methylation. These alterations also were associated with morphologic classification into bronchioloalveolar carcinoma (BAC) and non-BAC adenocarcinoma. Both aberrant methylation and LOH of 9p21 were associated with negative protein expression, but the former was correlated more closely with loss of function than was the latter. Cases with both alterations were completely negative for expression of the p16 gene product.

CONCLUSIONS. Aberrant methylation of the promoter region of the p16 gene and loss of expression of its product were in accord with the multistep progression of peripheral-type lung adenocarcinoma, and these alterations were associated closely with poor prognosis of the disease. ***Cancer* 2005;103:608–15.**

KEYWORDS: nonsmall cell lung carcinoma, pulmonary adenocarcinoma, stepwise progression, p16, CpG island methylation, loss of heterozygosity, immunohistochemistry, tumor suppressor gene, prognostic factor, carcinogenesis.

Lung carcinoma is the leading cause of cancer deaths worldwide, including the U.S. and Japan. Adenocarcinoma of the lung is one of the most common histologic types of lung carcinoma, and its incidence is still increasing in Japan and Western countries.

Histologically, lung adenocarcinoma can be divided into bronchioloalveolar carcinoma (BAC) and non-BAC. According to the recent World Health Organization classification of lung tumors,[1] BAC is a noninvasive carcinoma with an extremely favorable prognosis. However, adenocarcinoma with mixed subtypes, which is believed to be mostly an advanced subtype of BAC, and non-BAC are biologically advanced types of adenocarcinoma. We characterized adenocarcinomas of the lung measuring $\leq$ 2 cm purely on the basis of morphology, and subdivided them into 2 groups.[2] One was replacing-type adenocarcinoma, which includes BAC and advanced-type BAC, and the

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other was nonreplacing-type adenocarcinoma, which is a non-BAC type. The former was subdivided further into three subtypes: type A (localized bronchioloalveolar carcinoma [LBAC]), type B (LBAC with collapse of alveolar structure), and type C (LBAC with foci of fibroblastic proliferation). Types A and B are believed to be biologically in situ adenocarcinoma, because the prognosis of patients is extremely good, but patients with type C have advanced-type adenocarcinoma (advanced BAC).

Inactivation of the tumor suppressor genes is an important factor in lung carcinogenesis. In particular, the p16 gene is associated closely with lung carcinogenesis.[3] The p16 gene encodes a cell cycle protein that is an inhibitor of CDK4 and CDK6 and negatively regulates cyclin D-dependent phosphorylation of the Rb gene product, thereby inhibiting cell cycle progression from G1 to S-phase by sequestration of E2F.[4,5] Loss of function of p16 has been reported to occur mainly by aberrant DNA methylation of the promoter region and/or loss of heterozygosity (LOH) of the chromosomal region on which p16 is located.[4–6]

The aim of our study was to examine the clinicopathologic and biologic significance of loss of function of the p16 gene in the stepwise progression of peripheral-type adenocarcinoma of the lung.

MATERIALS AND METHODS

Tissue Specimens and Histologic Typing

Materials used in the current study consisted of surgically resected lung specimens from 57 patients (28 males and 29 females) with peripheral adenocarcinomas measuring $\leq$ 2 cm across the greatest dimension who received mediastinal and pulmonary hilar lymph node dissection. They underwent surgery during the period from December 1996 to October 2003 at the University Hospital of Tsukuba (Ibaraki, Japan), Ibarakihigashi National Hospital (Ibaraki, Japan), and Tsukuba Medical Center Hospital (Ibaraki, Japan). The cases were classified into pathologic stage using the International System for Staging Lung Cancer.[7] Preoperative clinical staging was evaluated mostly by enhanced computed tomography scans (of the chest, head, and abdomen) and bone scintigraphy. All patients underwent curative resections and did not receive adjuvant radiotherapy or chemotherapy. The surgically resected specimens were fixed routinely in 10% formalin and embedded in paraffin for histologic examination. All of the sections (3 μm thick), including the largest cut surface of the tumor, were stained with hematoxylin and eosin and elastica van Gieson and examined by light microscopy. All tumor specimens were classified using the histologic criteria proposed by Noguchi et al.[2] Microscopically, the diagnosis was performed by three pathologists. If two or more opinions coincided, the diagnosis was considered to be firm. Representative parts of the surgically resected samples (57 tumor samples along with 53 samples of nonmalignant lung tissue from the same patients) also were fixed in methanol and embedded in paraffin for DNA analysis. Informed consent was obtained from all patients for specimen collection.

Nucleic Acid Extraction and Methylation-Specific Polymerase Chain Reaction

Approximately six 10-μm sections were cut from the methanol-fixed block. The slices were deparaffinized with xylene and genomic DNA was extracted from the tumor and nontumor samples using the standard technique.[8] A total of 2 μg of genomic DNA obtained from the sample was modified by sodium bisulfite as described previously.[9] The methylation status of the p16 gene promoters was determined by the methylation-specific polymerase chain reaction (PCR) method.[9] A nested (or two-stage) PCR approach, which improved the sensitivity of the detection of methylated alleles, was used in the current study.[10] 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. PCR reactions were performed in a total volume of 25 μL containing 1 $\times$ PCR buffer, 0.25 mM each of the deoxynucleotide triphosphates (dNTP), 5% dimethylsulfoxide, 300 ng of each primer, 1 U of Hot-Start Taq polymerase (Takara, Tokyo, Japan), and the modified DNA (approximately 100 ng) sample. PCR was performed in a Takara PCR thermal cycler MP (Takara). After initial denaturation at 95 °C for 10 minutes, reaction products were subjected to 35 cycles of denaturation at 95 °C for 30 seconds, annealing for 45 seconds, and extension at 72 °C for 30 seconds, with a final extension at 72 °C for 7 minutes. The primer sequences and annealing temperatures, and the sizes of each PCR product, are listed in Table 1. Distilled water without DNA was used as a negative control, and the cell line Colo320 was used as a positive control for p16 analysis.[11] PCR products were analyzed in 2% agarose gels and stained with ethidium bromide. Samples that gave positive methylation products also were analyzed by methylation-sensitive restriction enzyme digestion of the PCR product. For the restriction analysis, the PCR mixture was digested with *Bst*UI under the conditions specified by the manufacturer (New England Biolabs, Beverley, MA).

PCR/LOH Analysis

To obtain relatively pure samples of tumor cells for DNA extraction and LOH analysis, a laser microdissec-

TABLE 1
Summary of Primer Sequences and Annealing Temperatures, and PCR Product Sizes for MSP

Primer	Sequence	Annealing temperature	Product size
1st p16 F	5′-CTACAAACCCTCTACCCACC-3′	60 °C	—
1st p16 R	5′-GAAGAAAGAGGAGGGGTTGG-3′		
2nd p16 MF	5′-TTATTAGAGGGTGGGGCGGATCGC-3′	60 °C	150 bp
2nd p16 MR	5′-GACCCCGAACCGCGACCGTAA-3′		
2nd p16 UF	5′-TTATTAGAGGGTGGGGTGGATTGT-3′	55 °C	151 bp
2nd p16 UR	5′-CAACCCCAAACCACAACCATAA-3′		

F: forward primer; R: reverse primer; MF: methylated/forward; MR: methylated/reverse; UF: unmethylated/forward; UR: unmethylated/reverse; bp: base pair; PCR: polymerase chain reaction; MSP: methylation-specific PCR.

tion system (Leica Microsystems, Tokyo, Japan) was used according to the methods described previously.[12] To evaluate LOH on 9p21, we used 3 microsatellite markers (D9S162, D9S171, and D9S1747). All primers were obtained from Research Genetics (Huntsville, AL). PCR reactions were performed in a total volume of 50 μL with 50–200 ng of the template DNA, 1.25 mM of each primer, 1.25 mM of each dNTP, 1 $\times$ PCR buffer containing 2 mM $MgCl_2$ and 3 mM $MgCl_2$, and 1 U of Hot-Start Taq polymerase (Takara). After initial denaturation at 96 °C for 5 minutes, reaction products were subjected to 35 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 10 minutes. Ten microliters of each PCR product were used for denaturating high-performance liquid chromatography on the WAVE DNA fragment analysis system (Transgenomic, Santa Clara, CA). Cases were defined as showing LOH when an allele peak signal from tumor DNA was reduced by 50% compared with its normal counterpart.

Immunohistochemistry

For the immunohistochemical analysis of the p16 product, 4-μm sections were cut from methanol-fixed and paraffin-embedded specimens (57 tumor specimens). The sections were deparaffinized in xylene, rehydrated in decreasing concentrations of ethanol, and immunostained by an automated method (Ventana Medical Systems, Tucson, AZ) using an anti-p16 monoclonal antibody (clone G175-405; Pharmingen, San Diego, CA) at a dilution of 1:20. The sections were counterstained with hematoxylin. The slides were evaluated by standard light microscopy. Only nuclear staining was scored, and was considered to be positive when it was more intense than the background cytoplasmic staining. Any cytoplasmic staining was regarded as nonspecific. Tissue specimens of human osteosarcoma were used as positive controls for immunostaining.[13] Inflammatory cells, reactive stromal cells, and bronchial epithelial cells on the same slide served as positive internal controls for immunostaining. The following scale was used: score 0, no immunoreactivity of tumor cells; score 1, $<$ 10 % of the tumor cells displayed strong p16 protein staining; score 2, $>$ 10 % of the tumor cells were strongly positive.[14] In the current study, we judged a score of 0 or 1 as being negative for p16 product expression and a score of 2 as being positive.

TABLE 2
p16 Gene Alteration and Clinicopathologic Features in Patients with Small-Sized Adenocarcinoma of the Lung[a]

		p16 gene alteration		
Clinicopathologic features	All patients	Methylation	LOH on 9p21	Loss of p16 expression
Total no. of patients	57	23	23	29
Mean age (yrs)	64.7	64.3	64.0	65.2
Smoking index				
$\geq$ 600	17	11[b]	9	12[b]
$<$ 600	40	12	14	17
Gender				
Male	28	15	16[b]	17
Female	29	8	7	12
Pathologic stage				
I	50	18[b]	19	22[b]
II	2	0	1	2
III	5	5	3	5
N factor				
N0	50	18	20	22[b]
N1 and N2	7	5	3	7
Ly factor				
Negative	40	11[b]	14	17
Positive	17	12	9	12
V factor				
Negative	48	17	18	22
Positive	9	6	5	7
Pleural invasion				
Negative	45	16	18	22
Positive	12	7	5	7
MI				
1	25	4[b]	5[b]	9
2	19	10	10	10
3	13	9	8	10
Differentiation				
Well	29	8	8[b]	10[b]
Moderately	22	12	9	15
Poorly	6	3	6	4

[a] Smoking index: (the number of cigarettes smoked/day) $\times$ years; pathologic stage: I (IA and IB), II (IIA and IIB), III (IIIA and IIIB); N factor: Lymph node involvement; Ly factor: lymphatic vessel invasion; V factor: vascular vessel invasion; MI: mitotic index (1, $<$ 1 per 10 HPF (high power fields), 2 (1–5 per 10 HPF), 3 ($>$ 5 per 10 HPF), LOH: loss of heterozygosity.
[b] There is a statistically significant difference ($P < 0.05$).

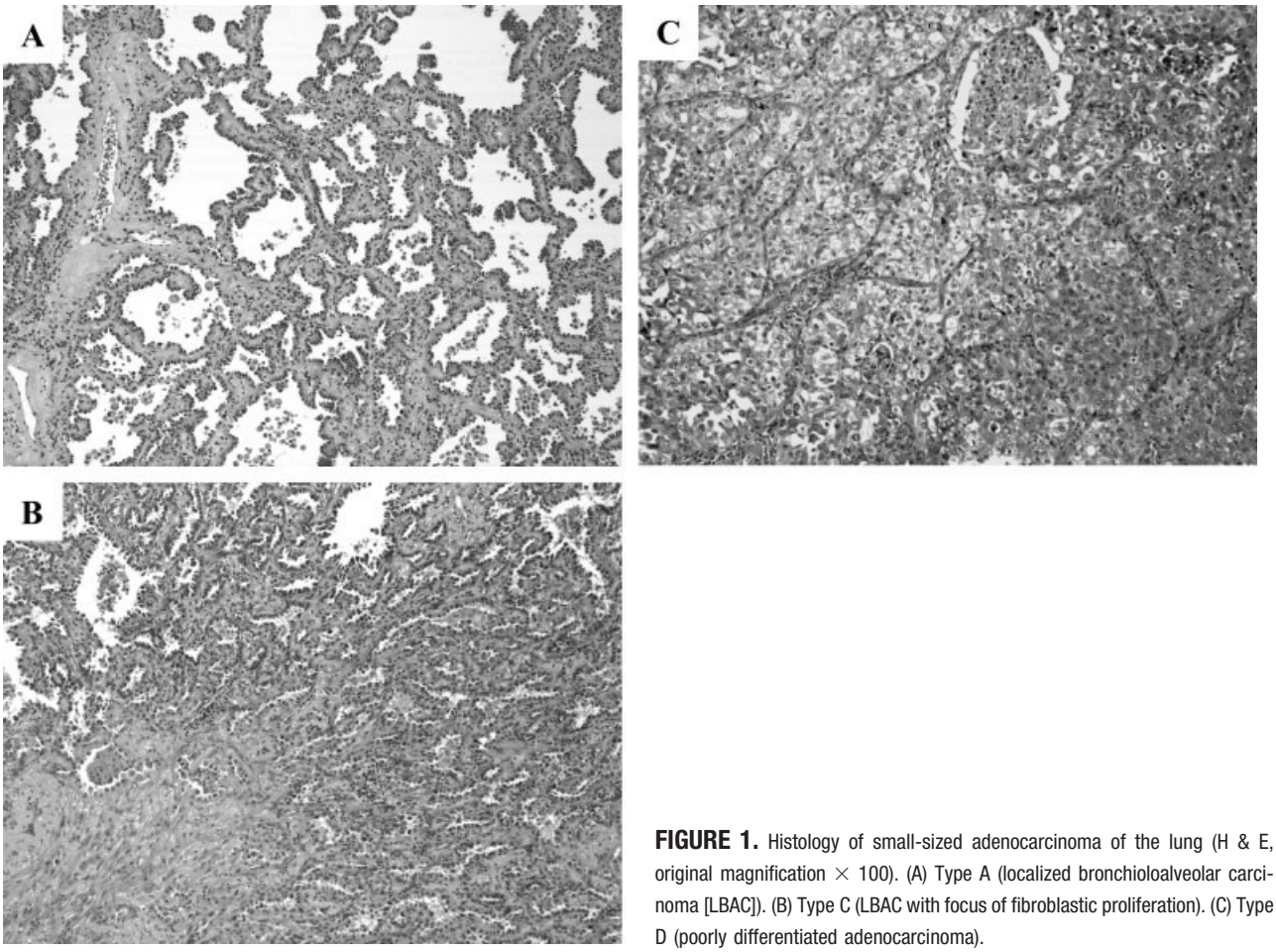

FIGURE 1. Histology of small-sized adenocarcinoma of the lung (H & E, original magnification × 100). (A) Type A (localized bronchioloalveolar carcinoma [LBAC]). (B) Type C (LBAC with focus of fibroblastic proliferation). (C) Type D (poorly differentiated adenocarcinoma).

Statistical Analysis

Associations of categoric variables were evaluated with the Fisher exact test or the chi-square test. Age was compared by the Student *t* test. Survival curves were calculated by the Kaplan–Meier method and then compared with the log-rank test. All statistical calculations were performed with StatView for Windows version 4.54 (Abacus Concepts, Berkeley, CA). Differences were statistically significant when $P < 0.05$ was reached.

RESULTS

The mean age of the 57 patients was 64.9 years (range, 46–82 years; Table 2). Fifty patients had pathologic Stage I disease, 2 patients had Stage II disease, and 5 patients had Stage III disease. Four of the five patients with Stage III disease died of the disease. The resected adenocarcinoma specimens were classified using the histologic criteria for adenocarcinoma ≤ 2 cm proposed by Noguchi et al.[2]. Of 57 tumor specimens, 3 (5.2%) were type A, 12 (21.1%) were type B, 24 (42.1%) were type C, and 18 (31.6%) were nonreplacing-type adenocarcinomas (types D, E, and F; Fig. 1). The 5-year survival rates of patients with type A and B tumors were both 100%, but the survival rates of patients with type C and nonreplacing-type adenocarcinomas were 94.1% and 73.7%, respectively.

Methylation Status of the p16 Gene Promoter Region

Aberrant methylation of the p16 promoter region was detected in 23 of 57 tumor samples (40.4%) and in 9 of 53 nontumorous samples (17%) from the corresponding 53 nontumorous lungs. Aberrant methylation of tumor tissue specimens was detected in all of the nine cases in which aberrant methylation of the nontumorous region was detected. Figure 2A shows representative results of methylation-specific PCR analysis, and Table 2 summarizes the association between p16 methylation status and various clinicopathologic features of the examined patients. Positivity for aberrant p16 methylation was significantly higher in heavy smokers (smoking index > 600) and in patients with

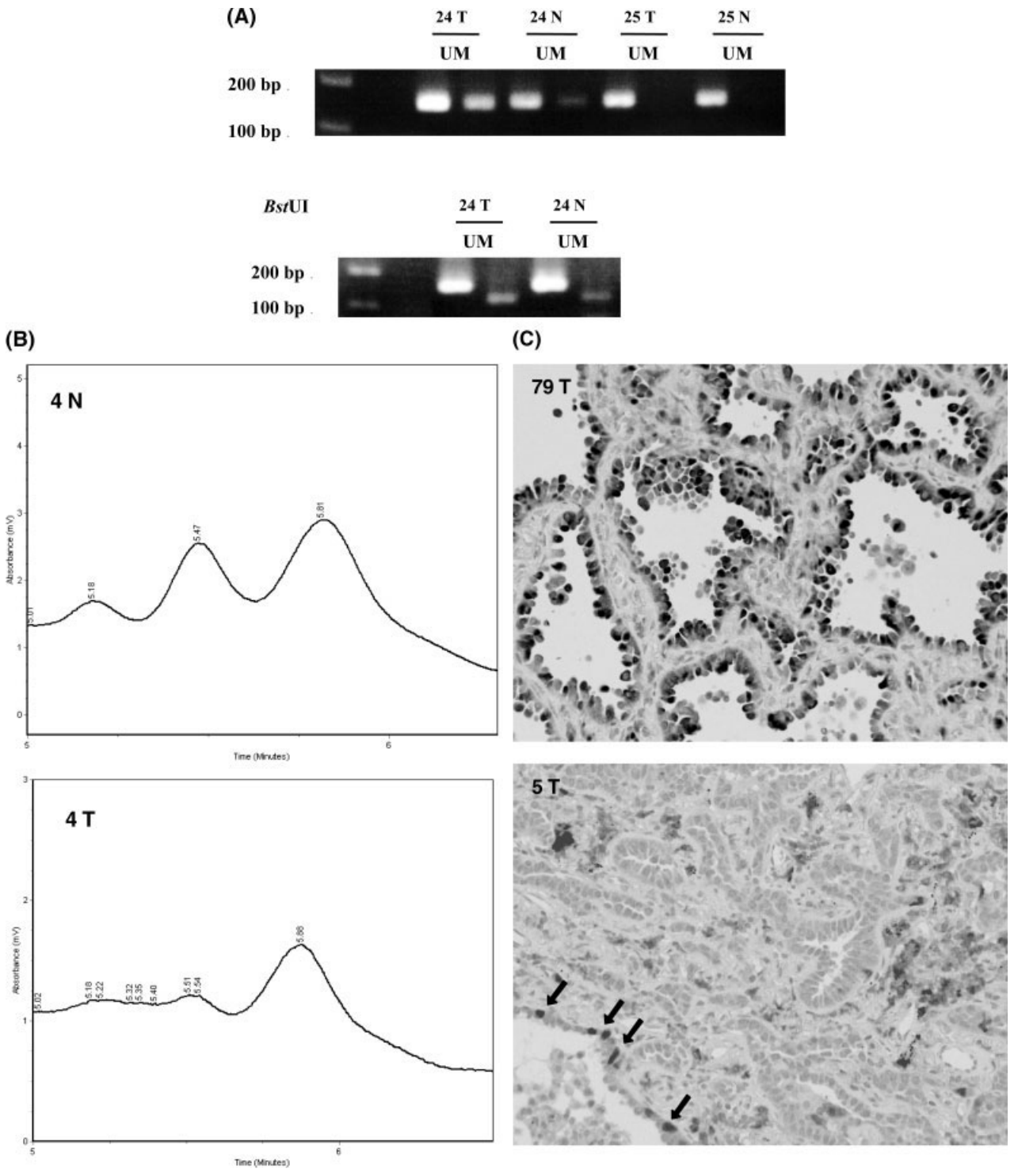

FIGURE 2. (A) Results of methylation-specific polymerase chain reaction (PCR) of p16 gene promoter regions with unmethylated (U) and methylated (M) primers. The asterisk denotes the 100-base pair ladder size marker, 24 and 25 are case numbers, T denotes tumor sample, and N denotes nontumor sample. Both tumor and nontumor samples were methylated in Case 24 (upper panel). Only methylated/modified p16 products were cleaved by *Bst*UI in Case 24 (lower panel). (B) Analysis of PCR products (D9S162) by WAVE DNA fragment analysis showed that 4 N (Case 4, nontumor sample) displayed two distinct high peaks, whereas 4 T (Case 4, tumor sample) showed only a single peak, indicating loss of one allele and confirming that loss of heterozygosity is present in the tumor cells at 9p21. The X-axis is retention time (minutes) and the Y-axis is absorbance (mV). (C) Immunohistochemical staining of p16 protein in small-sized adenocarcinoma of the lung. Positive staining of tumor cells with replacing of bronchioloalveolar structure (upper panel). Although the nuclei of normal bronchial epithelial cells are positive for staining of p16 protein (arrows), there is negative staining of tumor cells with tubular growth in the central scar (lower panel). T denotes tumor sample and 79 and 5 are case numbers.

advanced-stage tumors, lymphatic vessel invasion, and high mitotic index. As Figure 3A shows, the prognosis of patients without aberrant p16 methylation was significantly better than that of patients with aberrant methylation.

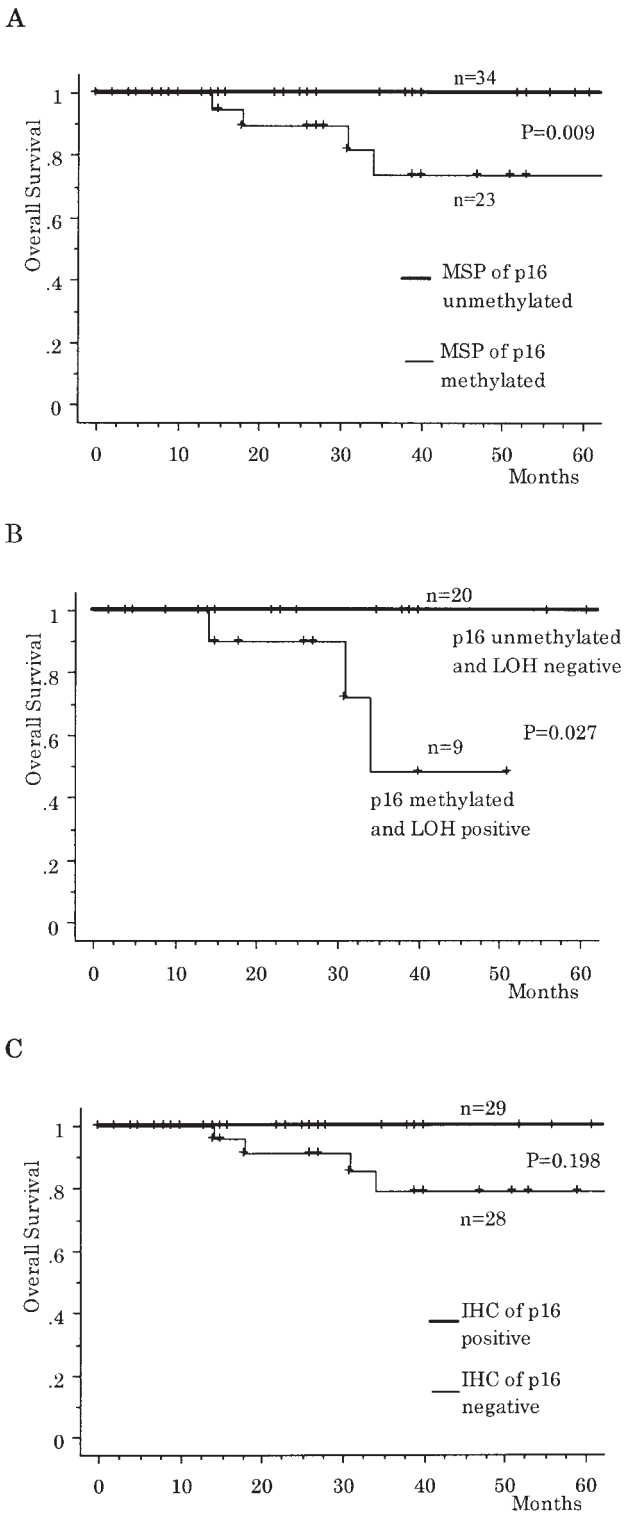

TABLE 3
Immunohistochemistry of p16 Protein and p16 Gene Alteration[a]

	p16 protein expression	
p16 gene alteration	+	–
Methylation positive/LOH positive	0	9
Methylation positive/LOH negative	3	11
Methylation negative/LOH positive	8	6
Methylation negative/LOH negative	17	3

LOH: loss of heterozygosity.
[a] There is a statistically significant correlation between the p16 protein expression and the p16 gene status ($P = 0.0001$, $r = 0.909$).

Loss of Heterozygosity on 9p21

LOH on 9p21 was detected in 23 of 57 tumor samples (40.4%). Figure 2B shows representative results of PCR-LOH analysis. Table 2 summarizes the association of LOH status with the clinicopathologic features of the examined patients. Positivity for LOH on 9p21 was associated significantly with men patients, high mitotic index, and poor differentiation. There was no significant correlation between LOH on 9p21 and the prognosis of patients. However, the 3-year survival rate of patients with both aberrant methylation and LOH on 9p21 was 48.0%, which is worse than the survival rate of patients with only aberrant methylation (Fig. 3B).

Immunohistochemistry of p16 Expression

Twenty-nine of 57 tumor samples (50.9%) showed negative immunoreactivity for p16 protein (Fig. 2C). Table 2 summarizes the association between immunohistochemical results and the clinicopathologic features of the patients examined. The frequency of loss of p16 expression in tumor samples was significantly higher in heavy smokers, in patients with advanced stage tumors, and in patients with lymph node involvement and poor differentiation. As Figure 3C shows, patients with p16 gene expression had a more

FIGURE 3. Survival curves of patients with small-sized adenocarcinoma of the lung. (A) Comparison between the methylated ($n = 23$) and unmethylated p16 ($n = 34$) groups for all patients. The difference in the survival curves was significant ($P = 0.009$). (B) Comparison between patients with both aberrant methylation of the p16 gene and loss of heterozygosity (LOH) on 9p21 ($n = 9$) and those without either alteration ($n = 20$). The difference in the survival curves was significant ($P=0.027$). (C) Comparison between the p16-negative ($n=29$) and the p16-positive ($n=28$) groups of immunostaining in all patients. The difference in the survival curves was not significant ($P=0.198$). MSP: methylation-specific polymerase chain reaction; IHC: immunohistochemistry.

TABLE 4
p16 Gene Alteration and Histologic Subtypes of Small-Sized Adenocarcinoma

	Type of Noguchi's classification (%)			*P* value	
p16 gene alteration	A+B	C	D+E+F	AB : C	AB : DEF
Total no. of patients	15	24	18		
Methylation	2 (13.3)	12 (50)	9 (50)	0.038[a]	0.034[a]
LOH on 9p21	1 (6.7)	9 (37.5)	13 (72.2)	0.057	0.0002[a]
Loss of p16 expression	3 (20)	13 (54.2)	13 (72.2)	0.048[a]	0.005[a]

LOH: loss of heterozygosity.
[a] There is a statistically significant difference ($P < 0.05$).

favorable prognosis than patients without such expression, although the difference did not reach statistical significance ($P = 0.198$).

Expression of p16 protein was compared with aberrant methylation of the promoter region and LOH on 9p21 (Table 3). Cases with both aberrant methylation and LOH on 9p21 always were completely negative for expression of the p16 protein, but 15% of cases without both alterations were also negative.

p16 Gene Alterations in Small-Sized Adenocarcinomas

As Table 2 and Figure 3 indicate, pathologic stage and prognosis were associated significantly with aberrant promoter methylation and/or p16 expression. We next examined the association of these factors with the histologic subtypes of the tumors (Table 4). Aberrant methylation of the p16 promoter region and negative expression of p16 protein were detected more frequently in patients with advanced BAC (type C) and non-BAC than in BAC (types A and B). Furthermore, LOH on 9p21 was detected more frequently in non-BAC than in BAC (types A, B, and C).

DISCUSSION

Silencing of the important tumor suppressor gene p16 was analyzed in the setting of early-stage adenocarcinoma of the lung. In the current study, we detected aberrant methylation of the p16 promoter region, negative p16 protein expression, and LOH of the 9p21 region in 40.4%, 50.9%, and 40.4% of adenocarcinoma specimens, respectively, and aberrant methylation and negative p16 protein expression were mutually correlated (Tables 2 and 3). Both of these factors were associated with smoking history, staging, and prognosis. These results indicate that silencing of the p16 gene in the early stages of adenocarcinoma of the lung is critical for stepwise progression of the tumor and that smoking is one of the factors that influence progression of adenocarcinoma, as it does for squamous cell carcinoma.[15,16] Nevertheless, 12 of 32 (34.3%) and 17 of 35 (48.6%) nonsmokers showed aberrant methylation and loss of expression, respectively, demonstrating that there are other factors responsible for this epigenetic alteration and change in protein expression. It is interesting to note that when both aberrant methylation of the p16 gene and LOH on 9p21 occurred in the same tumor tissue specimen, expression of the p16 gene was always completely interrupted. Nevertheless, one-seventh of cases showed interruption of gene expression without aberrant methylation and LOH (Table 3). Both aberrant methylation of the promoter site of the p16 gene and LOH on 9p21 seem to be sufficient, but not necessary, to interrupt the expression of the p16 product.

Peripheral-type adenocarcinoma is essentially divided into BAC and non-BAC. Non-BAC tumors are a heterogenous group including advanced BAC (progressed BAC) and pure non-BAC (adenocarcinoma without BAC-type spreading). In the current study, we subclassified the cases according to a histologic classification of small-sized adenocarcinoma that is based on the pattern of cancer architecture. Aberrant methylation and loss of protein expression were detected less frequently in the BAC group, which is believed to represent in situ adenocarcinoma, compared with both advanced BAC and the pure non-BAC group (Table 4). Loss of function of the p16 gene is believed to occur by epigenetic alteration (promoter methylation) and is a very early event in the carcinogenesis of pulmonary carcinoma.[15,16] However, in pulmonary adenocarcinogenesis, it occurs in the course of progression from in situ adenocarcinoma (BAC) to early but advanced adenocarcinoma.

The molecular mechanisms responsible for the aberrant methylation and loss of expression of the p16 gene are still being debated, but their biologic significance is very clear, because they are associated closely with prognosis (Fig. 3A–C). In patients with adenocarcinomas of the lung measuring ≤ 2 cm in diameter, the 5-year survival rate for cases without aberrant methyl-

ation of the promoter region of the p16 gene or showing positive expression of the p16 gene was 100%. In contrast, all the patients with Stage III disease showed aberrant methylation and 80% of them died of the disease. Therefore, aberrant methylation and/or loss of expression of the p16 gene do not appear to be independent prognostic factors but are associated closely with the stepwise progression of pulmonary adenocarcinoma that is indicated by pathologic staging.

We detected nine cases that showed aberrant methylation of the p16 gene in the nontumorous region (Table 2). In all nine cases, aberrant methylation also was detected in the tumor tissue specimen. We can suggest several possible explanations for this. First, the nontumorous tissue specimens were contaminated with small amounts of cancer cells that contained aberrant methylation. Second, the background lung already had been exposed to carcinogens, including cigarette smoke, and aberrant methylation had occurred in nontumorous tissue specimens.[17] Third, the nontumorous tissue specimen contained precancerous lesions, such as squamous metaplasia and/or dysplasia, that carried aberrant methylation of the p16 gene.[15]

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