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Frequent Epigenetic Silencing of the *p16* Gene in Non-small Cell Lung Cancers of Tobacco Smokers

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Epidemiological studies have demonstrated a causal link between tobacco smoking and lung cancer. We investigated the association between inactivation of the *p16* gene and tobacco smoking in 51 non-small cell lung cancers (NSCLCs). Aberrations of the *p16* gene were studied by PCR single-strand conformation polymorphism analysis, followed by direct sequencing, microsatellite analysis, methylation-specific PCR, and immunohistochemistry. Mutations were detected in 3.9% (2/51) of the tumors; the tumors carrying mutations were from smokers. The incidences of loss of heterozygosity, homozygous deletion, and promoter methylation in 37 smokers vs. 14 non-smokers were; 45.9% vs. 28.6%, 16.2% vs. 7.1%, and 35.1% vs. 7.1%, respectively. Among these, only the association between promoter methylation and tobacco smoking was statistically significant (P<0.05). Therefore, epigenetic aberration is considered to be a major causative event in *p16* silencing by tobacco smoking. Loss of p16 protein expression was apparent in 49% (25/51) of the tumors, and was associated with tobacco smoking (P<0.05) and with histological type (P<0.05). These findings suggest that tobacco smoking leads to inactivation of the *p16* gene mainly through the epigenetic mechanism, ultimately increasing the risk of NSCLC, especially the squamous cell histological type.

Key words: *p16* gene — Non-small cell lung cancer — Tobacco smoking — Promoter methylation

Tobacco smoking is strongly associated with lung cancer. Tobacco smoke contains many carcinogens including 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK), polyaromatic hydrocarbons, chromium, cadmium, and nickel.[1)] In addition, tobacco smoke is a direct mucosal irritant and induces inflammation that results in significant endogenous oxygen free radical generation.[2, 3)] Molecular epidemiological studies have begun to link specific environmental carcinogens, including those in tobacco smoke, with specific gene mutations in cancer progression. Mutation of the *p53* gene has been correlated with heavy smoking in patients with non-small cell lung cancers (NSCLCs).[4–7)] Allelic loss at the *FHIT* gene on chromosome 3p14 and *K-ras* oncogene mutations are also more frequent in smokers than in non-smokers.[8–10)] The *p16/INK4A* tumor suppressor gene on chromosome 9p21 encodes the p16 protein, an inhibitor of cyclin-dependent kinase 4 (Cdk4),[11, 12)] which normally phosphorylates the serine/threonine residues of the retinoblastoma protein, a step necessary for cell cycle progression past the G1 checkpoint into the S phase.[13, 14)] Hence, the p16 protein plays a major role in maintaining the Rb protein at the unphosphorylated state, thereby inhibiting cell cycle progression. In NSCLCs, inactivation of the *p16* gene has been detected in more than 70% of cell lines[14)] and in nearly 50% of primary tumors.[15–17)] Homozygous deletions and mutations of *p16* have been described in NSCLCs at variable frequencies.[10, 18–21)] More recently, methylation at the 5′ CpG islands of the *p16* gene has been identified as an alternative mechanism to mutation or deletion in *p16* inactivation in NSCLCs.[3, 10, 22, 23)] To investigate the mechanism of *p16* inactivation associated with tobacco smoking in primary NSCLCs, we studied *p16* mutation, loss of heterozygosity (LOH) and homozygous deletion (HD) on chromosome 9p21, as well as *p16* promoter methylation, and expression of the p16 protein.

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MATERIALS AND METHODS

Tissue samples and DNA extraction A total of 51 pairs of NSCLCs and corresponding normal lung tissues were obtained surgically at the Yamagata University Hospital. Written informed consent was obtained from all participants. Tissue samples were immediately frozen and stored at −80°C until analysis. The patients had not undergone any chemo- or radiotherapy prior to surgical resection, thus avoiding up- or down-regulation of cell-cycle proteins due to DNA damage. DNA was extracted from 51 primary lung cancer samples and corresponding normal lung tissue samples using SepaGene (Sanko Junyaku Co., Tokyo).

Microsatellite analysis DNA from tumors and normal lung tissues were analyzed for LOH and HD by amplification of repeat sequences using PCR. Five polymorphic

microsatellite markers spanning chromosomal bands 9p21-23 were used. All primers (D9S161, D9S126, D9S942, D9S1749, D9S162) were obtained from MapPairs (Research Genetics, Huntsville, AL). The PCR mix contained 1× PCR buffer [15 m*M* Tris-HCl (pH 8.0), 50 m*M* KCl], 1.5 m*M* $MgCl_2$, primers (1 μM each per reaction), 0.2 m*M* dNTPs, 0.5 unit of *Taq* polymerase (AmpliTaq Gold DNA Polymerase, PE Applied Biosystems, Foster City, CA), 5% dimethyl sulfoxide and 2.5 μCi of [α-^{32}P] dCTP (Amersham, Buckinghamshire, England) and genomic DNA (100 ng) in a final volume of 10 μl. Amplification was carried out in a GeneAmp PCR System 9700 (PE Applied Biosystems) for 35 cycles, each cycle consisting of denaturation at 95°C for 30 s, annealing at between 55°C and 60°C for 30 s, and extension at 72°C for 60 s, followed by a final 7 min extension at 72°C. PCR products were diluted 1:10 in denaturing loading buffer (95% formamide, 10 m*M* EDTA (pH 8.0), 0.02% xylene cyanol FF, and 0.02% bromophenol blue), heated at 95°C for 5 min, and placed on ice, then 2.0 μl aliquots were subjected to electrophoresis. Gels for microsatellite analysis consisted of 6% polyacrylamide and 7 *M* urea. Criteria for the definition of LOH and HD were described previously.[24, 25]

Methylation-specific PCR (MSP) Promoter methylation status of the *p16* gene was determined by MSP, as described previously.[26] MSP distinguishes unmethylated from methylated alleles of a given gene based on sequence changes that are produced following bisulfite treatment of DNA, which converts unmethylated cytosines to uracils while leaving methylated cytosines unaffected. Subsequent PCR using primers specific to sequences corresponding to either methylated or unmethylated *p16* promoter was performed. The primer sequences for detecting the methylated *p16* gene promoter were 5′-GGGTCGGAGGGGGTTTTTTC-3′ (sense) and 5′-CAACCGCCGAACGCACTCGA-3′ (antisense). The size of the PCR amplification product for the methylated reaction was 97 bp. The primer sequences for detecting the unmethylated *p16* gene promoter were 5′-TTATTAGACGGTGGGGTGGATTGT-3′ (sense) and 5′-CAACCCCAAACCACAACCATAA-3′ (antisense). The size of the PCR amplification product for the unmethylated reaction was 151 bp. Briefly, 2 μg of genomic DNA was denatured by treatment with NaOH and modified with sodium bisulfite. DNA samples were then purified using a Wizard DNA purification resin (Promega, Madison, WI), treated with NaOH, precipitated with ethanol, and resuspended in 30 μl. Modified DNA was amplified in a total volume of 20 μl using GeneAmp PCR Gold Buffer (PE Applied Biosystems) containing 1.0 m*M* $MgCl_2$, 20 μM of each primer, 0.2 m*M* dNTPs, and 1 unit of *Taq* polymerase (AmpliTaq Gold DNA Polymerase, PE Applied Biosystems). After activation of the *Taq* polymerase at 95°C for 10 min, PCR was performed in a thermal cycler (GeneAmp 2400, PE Applied Biosystems) for 35 cycles, each cycle consisting of denaturation at 95°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 30 s, followed by a final 7 min extension at 72°C. The PCR products were then loaded onto a non-denaturing 6% polyacrylamide gel, stained with ethidium bromide, and visualized under UV illumination.

PCR-single strand conformation polymorphism (SSCP) and sequencing analyses Mutations in exons 1 and 2 of the *p16* gene were analyzed in 51 tumors and normal lung tissues by the use of PCR-SSCP. PCR primers used for the analysis of *p16* mutations were as follows: exon 1, 5′-GAAGAAAGAGGAGGGGCTG-3′ and 5′-GCGCTACCTGATTCCAATTC-3′; exon 2, 5′-CTGACCATTCTGTTCTCTCTGG-3′ and 5′-CATGGTTACTGCCTCTGGTGC-3′.[20, 27] PCR conditions and treatment of the final products were the same as described for microsatellite analysis, except that dimethyl sulfoxide was added. Gels for SSCP analysis contained 6% polyacrylamide and 5% glycerol. Gels were dried and exposed to Hyperfilm MP autoradiography film (Amersham) for 4–16 h. Direct sequencing was performed using small pieces of the gel containing the shift band detected by SSCP. The gel was immersed in 50 μl of water, heated at 95°C, and then applied to PCR under the conditions described above for SSCP, except that PCR was carried out in a volume of 50 μl. The PCR products were purified using a QIA quick PCR purification Kit (QIAGEN, Tokyo). The purified PCR products were sequenced with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). Gel electrophoresis, data collection and analysis were done with a Genetic Analyzer (model 310, PE Applied Biosystems).

Immunohistochemistry (IHC) Loss of the p16 protein was determined by IHC, as described previously[16] using F-12 antibody (class: IgG 2a mouse monoclonal antibody, epitope: residues 1–167, representing the full length p16) (Santa Cruz Biotechnology, Santa Cruz, CA). IHC was performed according to the indirect biotin-streptavidin-peroxidase method, as described previously.[28, 29] For evaluation of p16 expression, we used published criteria.[28] Results were judged as either normal (No), when more than 90% of the tumor nuclei were stained, or loss (Lo), when there was an absence of nuclear staining. Admixed non-neoplastic cells showed nuclear reactivity. Type II pneumocytes, suprabasal bronchial cells and bronchioli epithelial cells, which were present in the vicinity of the tumor on the same slide, were used as positive internal controls. Slide examination was performed by 2 independent observers (NY and GT).

Statistical analysis Statistical comparisons were performed using Student's *t* test, Fisher's exact test, or the χ^2 test, as appropriate. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Patient characteristics Clinicopathological characteristics of the 51 patients are shown in Table I. Thirty-five of the 37 smokers were male, while 13 of the 14 non-smokers were female ($P<0.001$). Thirteen of the 14 lung cancers of the non-smokers were histologically adenocarcinomas (AdCs), while 20 of the 37 lung cancers of the smokers were squamous cell carcinomas (SCCs) ($P=0.01$).

LOH and HD on 9p21-23 LOH at the *p16* locus (D9S942 and/or D9S1749) was observed in 21 of the 51 (41.1%) cancers (Fig. 1). There were 6 cases with LOH for all informative markers tested (total loss). HD at the *p16* locus was observed in 7 of the 51 (13.7%) cancers (Fig. 2). Eight cases exhibited partial LOH including the *p16* locus.

Table I. Clinicopathological Characteristics of Smokers and Non-smokers

	Smokers (*n*=37)	Non-smokers (*n*=14)	*P* value
Age (years)	66.5±1.4	68.7±2.6	0.42
Gender			
Male	35	1	<0.001
Female	2	13	
Histology			
Adenocarcinoma	15	13	0.01
Squamous cell carcinoma	20	1	
Large cell carcinoma	1	0	
Adenosquamous cell carcinoma	1	0	
Lymph node metastasis			
Negative	26	7	0.17
Positive	11	7	
Stage			
I	25	6	0.14
II	4	1	
III	8	7	

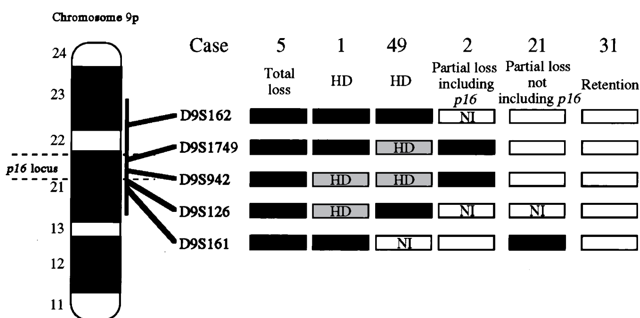

Fig. 1. Patterns of allelic loss on chromosome 9p. Black rectangle, LOH; white rectangle, retention of both alleles; HD in shaded rectangle, homozygous deletion; NI, not informative.

Promoter methylation status of the *p16* gene The *p16* gene promoter was methylated in 14 of the 51 (27.5%) cancers. In contrast, all the corresponding normal lung tissues unmethylated (Fig. 3).

Mutations of the *p16* gene Mobility shifts were found in 2 cases (cases 42 and 44), both in exon 2. DNA sequencing of these abnormal bands revealed a frameshift mutation, a G deletion at codon 60 in case 42, and a missense mutation, G to T substitution at the first letter of codon 100 in case 44 (Fig. 4).

Correlation between IHC and molecular analyses Lo expression was observed in 25 of the 51 (49%) cancers (Fig. 5). Among those 25, 24 harbored either HD, LOH, methylation or mutation of the *p16* gene. In total, 96% of the cancers with loss of p16 protein expression displayed at least one of these alterations.

***p16* abnormalities with reference to tobacco smoking** Lo expression was observed in 22 of the 37 (59.5%)

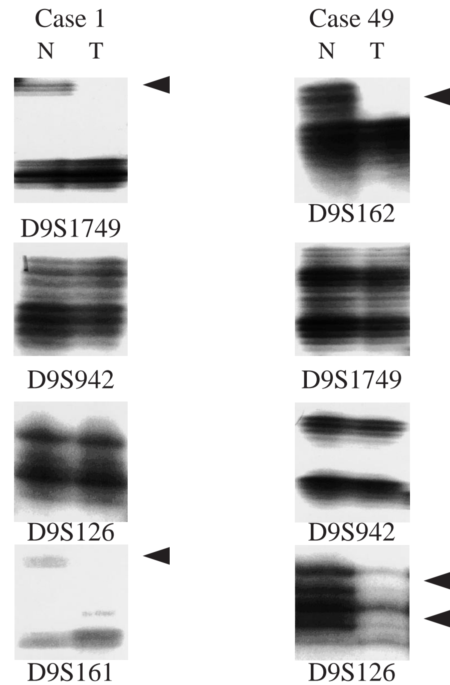

Fig. 2. Representative illustrations of HD on chromosome 9p. Apparent retention (HD) of D9S126 and D9S942 is demonstrated with LOH of the flanking D9S1749 and D9S161 markers in case 1, and apparent retention (HD) of D9S942 and D9S1749 is demonstrated with LOH of the flanking D9S162 and D9S126 markers in case No. 49. Arrows indicate LOH. N, normal lung tissue; T, tumor.

smokers and in 3 of the 14 (21.4%) non-smokers, being more frequent in smokers than in non-smokers (P=0.016). LOH at the *p16* locus was observed in 21 (41.1%) cancers; 17 (45.9%) from smokers and 4 (28.6%) from non-smokers (P=0.34). HD was observed in 7 (13.7%) cancers; 6 (16.2%) from smokers and 1 (7.1%) from a non-smoker (P=0.37). *p16* promoter methylation was more frequent (P=0.043) in tumors from smokers (13 of 37 or 35.1%) than in those from non-smokers (1 of 14 or 7.1%), and was associated with the Brinkman index (data not shown). The two *p16* gene mutations detected were only in tumors from smokers. These results are summarized in Table II. Lo expression of p16 was more frequent in SCCs (14/21; 66.7%) than in AdCs (9/28; 32.1%) (P=0.016).

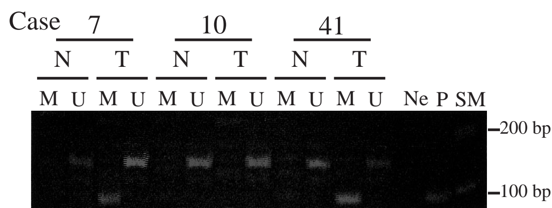

Fig. 3. Methylation analysis of *p16* gene promoter in NSCLCs and their corresponding normal lung tissues by MSP. Primer sets used for amplification are designated as methylated (M) or unmethylated (U). N, normal lung tissue; T, tumor; Ne, negative control (diluted water); P, positive control; SM, size marker.

DISCUSSION

Aberrant methylation of normally unmethylated CpG-rich areas (or islands), in or near the promoter region of many genes, has been associated with transcriptional inactivation of tumor suppressor genes in human cancers.[22, 30] In NSCLCs, such methylation has been reported in the tumor suppressor or tumor-related genes *p16*, *p14*, *DAPK*,

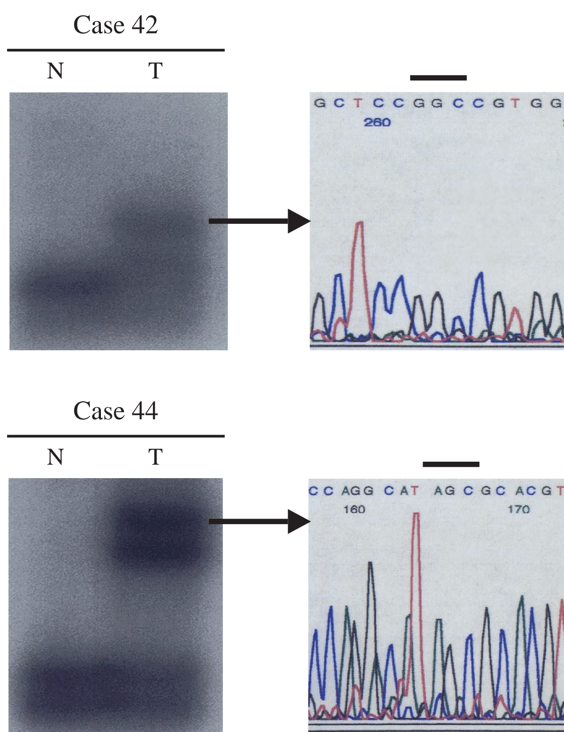

Fig. 4. SSCP analysis of exon 2 of *p16* and the sequencing histograms of exon 2 in *p16* using the antisense primer. Mobility shifts were found in 2 cases (cases 42 and 44) in exon 2. DNA sequencing of these abnormal bands revealed a frameshift mutation, a G deletion at codon 60 in case 42 and a missense mutation, G to T at the first letter of codon 100 in case 44. N, normal lung tissue; T, tumor.

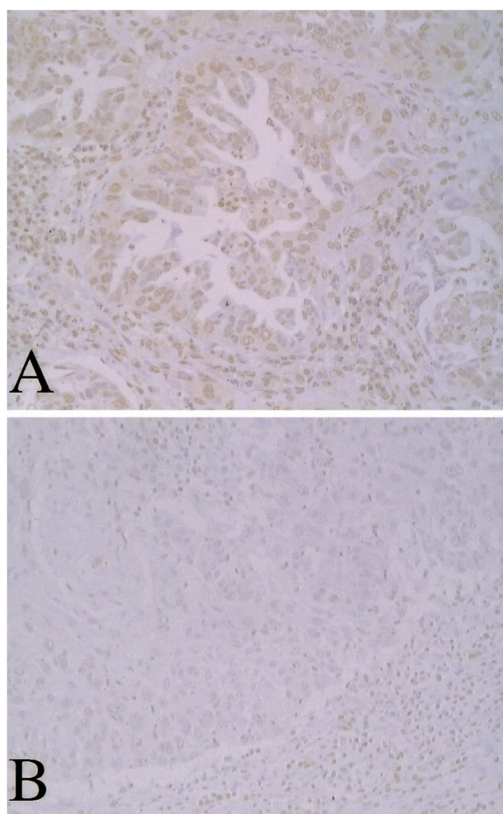

Fig. 5. Immunohistochemistry of the p16 protein. (A) Positive immunostaining in an adenocarcinoma (case 41, ×200). (B) Loss of p16 protein expression in a SCC with positive staining of stromal elements (case 8, ×200).

Table II. Abnormalities of the *p16* Gene with Reference to Tobacco Smoking

p16 abnormalities	Smokers (n=37) (%)	Non-smokers (n=14) (%)	P value
Loss of p16 protein expression	22 (59.5)	3 (21.4)	0.016
p16 alterations			
LOH at the *p16* locus	17 (45.9)	4 (28.6)	0.34
HD at the *p16* locus	6 (16.2)	1 (7.1)	0.37
p16 promoter methylation	13 (35.1)	1 (7.1)	0.043
p16 mutation	2 (5.4)	0 (0)	0.52

E-cadherin and *MGMT*.[3, 10, 17, 23, 30] In addition, investigators have described an association between genetic/epigenetic alterations of the *p16* gene in NSCLCs and tobacco smoking.[2, 3, 10, 28–32] Kim *et al.*[3] reported that *p16* promoter methylation was more common in tumors from smokers (49/172; 28.5%) than from non-smokers (2/13; 15.4%). Conversely, though, Sanchez-Cespedes *et al.*[10] reported that methylation of the *p16* gene promoter was more common in those from non-smokers (5/14, 36%) than from smokers (7/33, 21%), and that the *p16* gene was inactivated in tumors from non-smokers only through promoter methylation. Eguchi *et al.*[33] reported that DNA methylation at the D17S5 locus was significantly more frequent in smokers than in non-smokers in both tumors (P=0.03) and corresponding non-tumorous lung tissues (P=0.01). In the present study, *p16* promoter methylation was more frequent in tumors from smokers than from non-smokers (P=0.043). Although it is unclear whether smoking induces DNA methylation, recent reports have indicated an association between DNA methylation and tobacco carcinogens in animal models.[1, 32, 34] Lung tumors induced in F344/N rats after exposure to inhaled tobacco smoke displayed *de novo* methylation of *p16*.[32] Promoter methylation of the *p16* gene was induced in 94% of AdCs of rats treated with tobacco-specific NNK.[1] Regional methylation also has been reported in transgenic cell lines treated with nickel, a carcinogenic metal found in cigarette smoke.[34] Similarly, Lee *et al.*[34] 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. These laboratory data, and our present results, support the theory that tobacco smoke affects promoter methylation of the *p16* gene in NSCLC. Apparently conflicting results between studies might be due to differences in histological types examined. Kim *et al.*[3] reported that *p16* promoter methylation occurred more frequently in SCCs than in AdCs, concordant with our present study. In an animal model, activity of DNA methyltransferase was increased in alveolar type II cells of the A/J mouse, which is susceptible to lung cancer formation. However, Clara cells from the same A/J mouse did not show an increase in methyltransferase activity.[35] Therefore, promoter methylation might be cell- and/or tissue-specific.

Allelic loss on chromosome 9p21 is a common event in NSCLC,[10, 17, 18, 28, 29, 36] and occurs more frequently in NSCLCs from smokers than in those from non-smokers, while HD is detected only in smokers. In the present study, although the effects were not statistically significant, increased frequencies of both LOH and HD at the *p16* locus was observed in tumors from smokers. The higher frequency of allelic loss in smokers suggests that prolonged tobacco exposure leads to chromosome instability.[10] Previous studies have demonstrated that specific tobacco carcinogens such as BPDE can form stable covalent DNA adducts and induce DNA single-strand breaks.[37] We detected mutation of the *p16* gene only in smokers, similar to a previous report.[10] Previous studies further examined the role of smoking in the molecular pathogenesis of NSCLC. In general, *p53* mutations are more common in heavy smokers than in light smokers, and the *p53* mutational spectrum differs between cancers from smokers and non-smokers.[4–7] For instance, in the mutation patterns of the *p53* gene, G:C to A:T transitions predominate in NSCLC from non-smokers, whereas G:C to T:A transversions are more common in smokers.[4] One of the *p16* gene mutations actually detected in the present study was G to T transversion in exon 2 of the *p16* gene.

In conclusion, inactivation of the *p16* gene occurs in NSCLCs mainly through promoter methylation induced by tobacco smoking. Alternatively, tobacco smoking leads to inactivation of the *p16* gene mainly through epigenetic alterations, ultimately increasing the risk of NSCLC, especially the squamous cell histological type. Because the number of samples examined in the present study is small, further studies are necessary to confirm this issue.

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