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Increased Loss of Chromosome 9p21 but not *p16* Inactivation in Primary Non-Small Cell Lung Cancer from Smokers[1]

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ABSTRACT

Epidemiological studies have demonstrated a causal association between tobacco use and carcinoma of the lung, and some genetic targets of the carcinogens in cigarette smoke have been defined recently. We further examined the effect of cigarette smoking on the frequency of allelic losses on chromosome 9p21 and the incidence of *p16* inactivation. Chromosomal loss at 9p21–24 was determined by microsatellite analysis using 14 markers in 47 patients with non-small cell lung cancer. In addition, *p16* gene inactivation was determined by DNA sequence analysis, methylation-specific PCR, and immunohistochemistry. Tumors from a group of nonsmokers ($n = 14$) were compared with tumors from a group of smokers ($n = 33$) matched for cell type, tumor stage, and gender. Allelic loss encompassing the *p16* locus was present significantly ($P = 0.01$) more often in smokers (23 of 33 smokers, 70%) than in nonsmokers (4 of 14 nonsmokers, 28%). No significant differences in the frequency of *p16* inactivation were observed between smokers and nonsmokers (45% *versus* 36%). However, homozygous deletion of the *p16* gene locus and point mutation of *p16* gene were only observed in tumors from smokers, whereas the *p16* gene was inactivated in tumors from nonsmokers only through promoter hypermethylation. Thus, inactivation of the *p16* gene is a common event in all non-small cell lung cancer, but the mechanism of gene alteration differs between smokers and nonsmokers. The significant link between tobacco and loss of the *p16* locus identifies additional genetic targets of smoking in the pathogenesis of lung cancer.

INTRODUCTION

NSCLC[4] is the leading cause of cancer death in the United States among both men and women (1). Tobacco smoking is strongly associated with lung cancer, and only 5–10% of cases develop in individuals who have never smoked (2, 3). Molecular epidemiological studies have begun to link specific environmental carcinogens, including those in tobacco smoke, with specific gene mutations in cancer progression (4, 5). Mutation of the *p53* gene has been correlated with heavy smoking in patients with NSCLC (6–8). In addition, BPDE, a potent carcinogen in tobacco smoke, has been shown to selectively bind codons 157, 248, and 273 of the *p53* gene (9, 10). Allelic loss at the *FHIT* gene on chromosome 3p14 and K-*ras* oncogene mutations are also more frequent in smokers than nonsmokers (11, 12).

Chromosomal arm 9p contains the *p16* tumor suppressor gene and is frequently deleted in NSCLC (13, 14). The *p16* gene is inactivated in primary tumors by homozygous deletion, methylation of the promoter region of the gene, or, less commonly, by point mutation (13–17). Inactivation of the *p16* gene has been reported in up to 63% of NSCLCs, representing one of the most frequently altered genes in this disease (18). However, the influence of cigarette smoking on the frequency of allelic loss at this region and the mechanism of *p16* inactivation in NSCLC remains unknown. The aim of the present study was to determine the effects of cigarette smoking on chromosome 9p21 loss and inactivation of the *p16* gene. We found that tumors from nonsmokers arise exclusively through inactivation of *p16* by promoter hypermethylation, whereas most tumors from smokers demonstrate deletion or point mutation of the *p16* gene. Because *p16* inactivation (including *p16* promoter methylation) occurs early in NSCLC progression, our work provides insight into the underlying mechanisms that lead to lung cancer in nonsmokers (19).

PATIENTS AND METHODS

Patients. Primary tumor and blood or normal lung tissue were collected from 47 patients undergoing a potentially curative resection of NSCLC at The Johns Hopkins Hospital, The Johns Hopkins Bayview Medical Center, or the Medical College of Wisconsin. Demographic data were collected from patient interviews, review of hospital charts, and review of the tumor registry. Pathological stage was determined using the revised International System for Staging Lung Cancer (20). A history of cigarette smoking was carefully documented. Nonsmokers ($n = 14$) were defined as patients who had smoked fewer than 100 cigarettes during their lifetime (21). Thirty-three patients were selected from a larger group of smokers with NSCLC to closely match the gender, tumor type, and pathological stage of the 14 nonsmokers.

Tissue Samples. Portions of the primary lung tumor and normal lung tissue were collected from the operative specimen and promptly frozen at −80°C. Lymphocytes were collected from blood and used as a source of normal DNA. Tumor samples of low neoplastic cellularity were microdissected to contain greater than 70% neoplastic cells. Samples were digested overnight at 48°C in 1% SDS/proteinase K (0.5 mg/ml), followed by phenol/chloroform extraction and ethanol precipitation.

Microsatellite Analysis. Allelic loss at chromosome 9p21–24 was determined using 14 highly polymorphic microsatellite markers. Oligonucleotides were synthesized by Operon Technologies Inc. (Alameda, CA; *D9S1749*, *D9S1748*, *D9S1751*, *D9S171*, and *D9S126*) based on sequences in the Genome Database or obtained from Research Genetics (Huntsville, AL; *D9S157*, *D9S265*, *D9S270*, *D9S736*, *D9S144*, *D9S162*, *D9S259*, *D9S942*, and *IFN-α*). One marker from each primer pair was ^{32}P-labeled with T4 polynucleotide kinase (Life Technologies, Inc.). PCR amplification was performed on 60 ng each of nonneoplastic (lymphocyte or normal tissue) and tumor DNA (22). The products were separated by PAGE and visualized using autoradiography. For informative cases, LOH was scored if the intensity of one allele was decreased by 50% in the tumor when compared visually with the same allele in the control sample by two independent observers. Determination of homozygous deletion was based on the presence of one or more markers demonstrating retention flanked by markers showing clear LOH, as described previously (23).

***p16* Sequence Analysis.** One hundred ng were used to individually amplify exons 1 and 2 of the *p16* gene by PCR as described previously (16). PCR products were used as templates for cycle sequencing. Sequencing primers were labeled with [γ-^{33}P]ATP and subjected to PCR amplification using the AmpliCycle sequencing kit (Perkin-Elmer, Roche Molecular Systems Inc., Branchburg, NJ) according to the manufacturer's protocol. Sequenced products were separated electrophoretically through 6% polyacrylamide gels, dried, and finally exposed to film for 24–48 h.

***p16* Methylation-specific PCR.** Methylation-specific PCR was used to determine the methylation status of a CpG island in the promoter region of the

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[4] The abbreviations used are: NSCLC, non-small cell lung cancer; BPDE, benzo-(*a*)pyrene diol epoxide; LOH, loss of heterozygosity.

p16 gene in the tumors not shown to be inactivated by homozygous deletion (24). Two hundred ng of tumor DNA were modified with hydroquinone and sodium bisulfite at 50°C for 16 h. Modified DNA was then purified using the Wizard DNA purification system (Madison, WI), precipitated with ethanol, and resuspended in a Tris-EDTA buffer. The modified DNA was then amplified using both methylated- and unmethylated-specific primers as described previously (24). One primer pair recognizes a sequence in which CpG sites are unmethylated (bisulfite-modified to UpG), and the other pair recognizes a sequence in which CpG sites are methylated (unmodified by bisulfite). The primer sequences are localized to regions in and around the transcription start site of the *p16* gene, a region shown to correlate with loss of gene expression. PCR products were loaded directly onto nondenaturing 6–8% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

P16 Immunohistochemistry. Immunohistochemistry is an accurate and complementary method for evaluating *p16* gene inactivation and was used to confirm the findings of the molecular analysis in 26 of the 47 tumors. Fresh frozen specimens were embedded in Optimum Cold Temperature Medium (Tissue-Tek; Miles, Elkhart, IN), sectioned, and mounted on lysine-coated slides. Immunohistochemistry was performed with the use of C20 rabbit polyclonal antibody (25). Briefly, 5-μm-thick frozen tumor sections were fixed on lysine-coated slides in a 1:1 cold acetone/methanol solution. After blocking sera was applied for 15 min, sections were reacted with primary P16 mAb in a 1:500 dilution overnight at 4°C. Immunolocalization was performed with the use of the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA), 3,3′-diaminobenzidine tetrahydrochloride (Vector Laboratories), chromagen and nickel enhancement. After diaminobenzidine tetrahydrochloride development, tissues were counterstained using Gill's hematoxylin and mounted in an aqueous medium. The slides were evaluated using standard light microscopy by two observers (W. H. W. and M. S-C.). A squamous cell carcinoma of the larynx cell line (JHU-020-SCC-L) with a homozygous deletion of *p16* was used as a negative control. Normal bronchial tissue was used as a positive control. Only nuclear staining was regarded as positive staining. Inflammatory cells and reactive stromal cells served as positive internal controls.

Statistical Analysis. Data are expressed as the mean ± SE. Clinical characteristics and frequency of allelic loss were compared using Student's *t* test, Fisher's exact test, or χ^2 test, as appropriate.

RESULTS

Patient Characteristics. Clinicopathological characteristics from all 47 patients are shown in Table 1. Eleven of the 14 nonsmokers were women. Twelve of 14 (86%) lung tumors in the nonsmokers were primary lung adenocarcinoma, and the mean tumor size in the nonsmokers was 3.8 ± 0.6 cm. Tumors from 33 cigarette smokers with NSCLC were selected from a larger group of cancers to closely match the nonsmokers with respect to gender, tumor cell type, and stage. No statistically significant difference in mean age, gender, cell type, stage, histological grade, or tumor size was observed between the nonsmokers and smokers (Table 1).

Table 1 *Clinical and pathological characteristics of nonsmokers and smokers*

	Nonsmokers (n = 14)	Smokers (n = 33)	P
Age (yrs)[a]	67 ± 3	65 ± 3	0.51
Gender			
Male	3	12	0.34
Female	11	21	
Tumor size (cm)[a]	3.8 ± 0.6	3.0 ± 0.2	0.15
Cell type			
Adenocarcinoma	12	29	0.87
Squamous cell cancer	1	3	
Large cell	1	0	
Stage			
I	9	26	0.33
II–IV	5	7	
Grade[b]			
Poorly differentiated	3	7	0.20
Moderately differentiated	4	17	
Well differentiated	6	9	

[a] Data are expressed as mean ± SEM.
[b] Histological grade was available only in 13 nonsmokers.

Microsatellite Analysis. Allelic loss at chromosome 9p21 was determined in all 47 patients using microsatellite analysis. LOH anywhere in the 9p21 region was observed with at least one microsatellite marker in 23 of 33 (70%) smokers and in 5 of 14 (36%) nonsmokers (P = 0.05**)**. LOH including the *p16* locus (*D9S1748*, *D9S942*, or *D9S1749*) was significantly (P = 0.02) more common in smokers (23 of 33 smokers, 70%) than in nonsmokers (4 of 14 nonsmokers, 28%). The different patterns of allelic loss at chromosome 9p21 observed in both the smokers and nonsmokers are shown in Figs. 1 and 2. Among smokers, LOH with all informative cases tested was present in 15 of the 33 (45%) tumors. Four of these 33 (12%) tumors showed partial LOH of chromosomal region 9p21–24, which extended into the *p16* locus in all cases, and another 4 tumors contained a homozygous deletion including the *p16* gene locus. P16 immunohistochemistry was negative in three of these tumors (one sample was not available) with a homozygous deletion. Retention of chromosome 9p21 was present in the remaining 10 of 33 (30%) tumors. Among the nonsmokers, the 9p21 losses were partial in four of the cases (80%). Only one tumor showed LOH in all of the markers analyzed, consistent with monosomy of chromosome 9. LOH included the *p16* locus in four of these five tumors and was restricted to *D9S157* in the remaining tumor with partial LOH.

***p16* Sequencing.** Sequencing of exons 1 and 2 of the *p16* gene detected mutations in 2 of the 33 (7%) smokers and in none of the nonsmokers. One mutation was a T insertion at codon 71 (AAC to TAAC; Fig. 3), and the other was a G to T change at codon 108 (D108Y). P16 immunohistochemistry revealed negative staining in the tumor with the insertion but positive P16 staining in the tumor with the missense mutation. Positive immunohistochemical staining may occur if the epitope recognized by the antibody is not altered by the missense mutation. In addition, two polymorphisms [R144C (n = 1) and A148T (n = 2)] were also detected in three of the patients with a history of smoking.

***p16* Methylation.** Methylation of the *p16* promoter was present in 12 of the 47 (26%) tumors (Fig. 3). *p16* promoter hypermethylation was more common in nonsmokers (5 of 14 nonsmokers, 36%) than in smokers (7 of 33 smokers, 21%), although this difference did not reach statistical significance (P = 0.33). Immunohistochemistry demonstrated an absence of nuclear staining in six of the seven methylated tumors examined. The presence of positive but heterogeneous staining in the remaining tumor is consistent with the emergence of a methylated clone.

***p16* Inactivation.** P16 immunohistochemistry was positive (nuclear staining) in 12 of 14 tumors without evidence of an inactivating event by molecular analysis of the *p16* gene (Fig. 3). Ten of the 12 tumors with a homozygous deletion including *p16*, methylation of the *p16* promoter, or a hemizygous loss coupled with a mutation demonstrated absent P16 staining. Overall, *p16* gene inactivation based on both genetic analysis and immunohistochemistry was present in 5 of 14 tumors (36%) from the nonsmokers and in 15 of 33 tumors (45%) from smokers (Table 2).

DISCUSSION

Allelic loss at chromosome 9p21 is a common event in NSCLC, and is associated with inactivation of the *p16* tumor suppressor gene in the majority of cases. Cigarette smoking has been associated with the mutation or loss of several genes and chromosomal regions in lung cancer. In the present study, allelic loss at the *p16* locus occurred significantly more often in NSCLC from smokers than in tumors from nonsmokers. Although the frequency of LOH in the 9p21 chromosomal region among nonsmokers was 36%, we observed that the losses included only a few markers compared to the larger chromo-

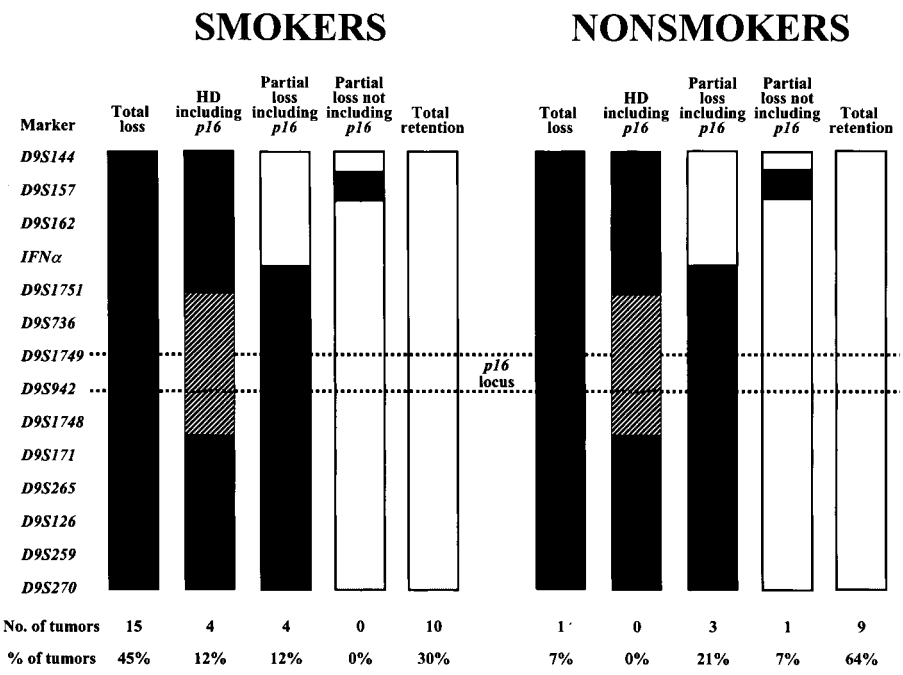

Fig. 1. Patterns of allelic loss at chromosome 9p21 in NSCLC from smokers and nonsmokers. All markers are listed from telomere (*top*) to centromere. Loss of all informative markers tested at chromosome 9p21 (total loss) occurred significantly ($P = 0.02$) more often in NSCLC from smokers (45%) than in NSCLC from nonsmokers (7%). Retention of all informative markers tested was more common ($P = 0.05$) among nonsmokers than smokers. Homozygous deletions were only observed in NSCLC from smokers.

somal deletions (LOH at every marker) observed in most of the smokers. The frequency of *p16* inactivation was similar in both groups of patients, although the mechanisms of inactivation differed. In nonsmokers, *p16* inactivation occurred mainly via gene promoter hypermethylation, whereas in the smoking-related tumors, homozygous deletions and point mutations were more frequent.

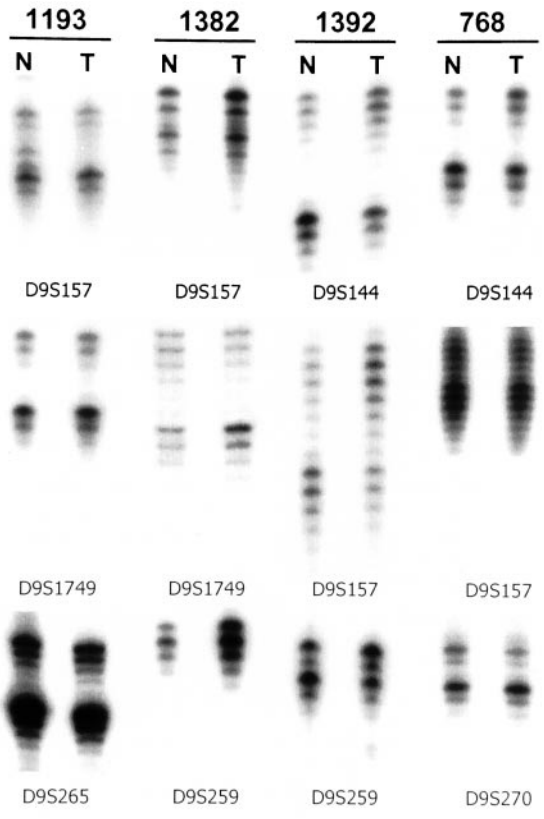

Fig. 2. Representative examples of microsatellite analysis at chromosome 9p21 in nonsmokers and smokers. Case numbers are shown at the *top*, and microsatellite marker numbers are identified at the *bottom*. Cases 1193 and 1382 (nonsmokers) demonstrate total retention and partial loss, respectively. Cases 1392 and 768 (smokers) demonstrate total loss and homozygous deletion of 9p21–24, respectively. *N*, normal; *T*, tumor.

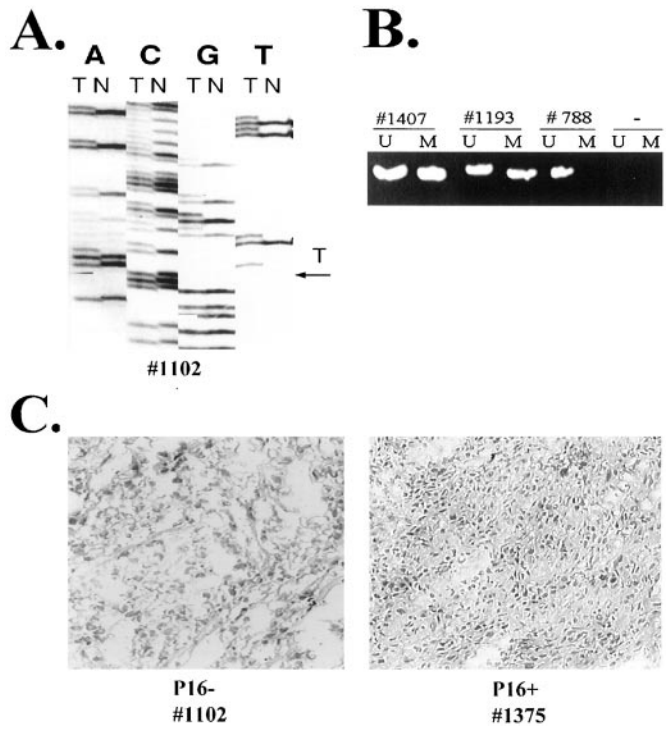

Fig. 3. Representative examples of *p16* gene mutation analysis, methylation-specific PCR, and immunohistochemistry. *A*, insertion of a single bp in exon 2 of the *p16* gene. *N* and *T* represent normal and tumor DNA from the same patient. *B*, examples of *p16* promoter hypermethylation. *Lanes U* and *M* correspond to the unmethylated and methylated reactions, respectively; − is the water as a negative control. *C*, P16 immunohistochemistry. *P16−*, tumor (case 1102) with *p16* LOH and *p16* gene mutation showing absence of P16 immunostaining. *P16+*, tumor showing positive nuclear staining for the P16 protein in a lung tumor from a nonsmoker without *p16* gene alterations.

Chromosome 9p21 is a critical region of loss in NSCLC (13). Previous studies using fine mapping at chromosome 9p21 with closely spaced microsatellite markers have reported allelic loss in 63–100%

Table 2 *Chromosomal loss at 9p21 and p16 inactivation among nonsmokers and smokers*

	Nonsmokers ($n = 14$)	Smokers ($n = 33$)
LOH at *p16* locus	4 (28%)	23 (70%)[a]
Homozygous deletion at the *p16* locus	0 (0%)	4 (12%)
p16 promoter hypermethylation	5 (36%)	7 (21%)
p16 mutation	0 (0%)	2 (6%)
p16 gene inactivation (including immunohistochemistry results)	5 (36%)[b]	15 (45%)[c]

[a] $P = 0.02$ *versus* nonsmokers.

[b] One tumor with promoter hypermethylation showed a heterogeneous pattern in P16 immunostaining.

[c] One tumor with a missense mutation showed positive immunostaining. Three tumors with LOH but without homozygous deletions, point mutation, or promoter hypermethylation demonstrated coherent staining.

of NSCLCs (13–15). Several minimal regions of loss at chromosome 9p21 have been described including the *p16* gene locus and a second region at *D9S126* (13, 14). Okami *et al.* (13) recently performed detailed deletion mapping of 82 NSCLCs using microsatellite analysis and fluorescence *in situ* hybridization, but they were unable to identify any tumors with deletions limited to the region at *D9S126*. Twenty-one percent of the tumors had small homozygous deletions, and all of these deletions included the *p16* gene locus, suggesting that *p16* is the primary target of deletion in NSCLC. Furthermore, immunohistochemistry and genetic analysis of the *p16* gene uncovered inactivation by homozygous deletion, promoter methylation, or point mutation in the majority of tumors from patients with LOH at chromosome 9p21 (26). Thus, allelic loss at chromosome 9p21 is a crucial step leading to loss of function of the *p16* gene. Furthermore, chromosome 9p21 loss is an early event in the neoplastic progression of lung cancer in smokers with the frequency of allelic loss ranging from 28% in histologically normal bronchial epithelium to >75% in epithelium with *in situ* carcinoma (27). The frequency of allelic loss is higher in squamous cell lung cancer than in adenocarcinoma of the lung, and the percentage of allelic loss (70%) in our matched population of smokers may reflect the high frequency of adenocarcinoma in this group (28). The patterns of allelic loss observed in the group of smokers with NSCLC in our study are similar to those reported previously (13).

As demonstrated previously, immunohistochemistry correlates well with genetic analysis in determining inactivation of the *p16* gene, and these two methods yielded similar results in the vast majority of tumors in this study (23, 26). Positive staining was observed in one tumor with a missense mutation in the ankyrin III repeat. These observations indicate that the point mutation does not result in a structural modification of the P16 protein, thus retaining binding to the antibody. Nevertheless, *p16* missense mutations within ankyrin repeats II and III are critical to P16 function (29). Heterogeneous staining was observed in a second tumor with promoter methylation, suggesting the emergence of a methylated clone. Although methylation-specific PCR cannot distinguish between methylation of one or both *p16* alleles, the presence of *p16* promoter methylation using this assay has strongly correlated with absent P16 protein in the setting of both hemizygous loss or retention of both *p16* alleles (17, 23). Two tumors did not stain with the P16 antibody despite the absence of an inactivating event on genetic analysis, suggesting the presence of a mutation outside of the region sequenced or another alteration affecting transcription or translation of the *p16* gene.

Several previous studies have examined the role of smoking in the molecular pathogenesis of NSCLC. *p53* mutations are more common in heavy smokers than in light smokers, and the *p53* mutational spectrum differs between cancers from smokers and nonsmokers (5–10). BPDE, the active form of benzopyrene, has been shown to bind preferentially to select regions of the *p53* gene (10). Formation of these benzopyrene adducts likely reflects the high frequency of certain *p53* mutations in smoking-associated tumors and correlates with several known mutational hot spots in the *p53* gene (9). The G:C to A:T transitions predominate in NSCLC from nonsmokers, whereas G:C to T:A transversions are more common in smokers (5). Similarly, one of the *p16* gene mutations detected in the smokers in the present study was a G to T transversion in exon 2 of the *p16* gene. K-*ras* mutations are also significantly more common in NSCLCs from smokers than in those from nonsmokers (12).

Allelic loss at the *FHIT* gene locus on chromosome 3p14.2 and at the *HRAS* locus on chromosome 11p are both more common in cancers from smokers than in cancers from nonsmokers (11, 30). Sozzi *et al.* (11) noted a dramatic difference in the LOH rate at the *FHIT* gene locus between smokers and nonsmokers (80% *versus* 22%) but observed no difference in the LOH rate at a control locus (*D10S197*). Furthermore, allelic losses at chromosomal regions 3p14.2, 3p14.3, 3p21, and 3p22–24.2 or chromosomal arms 5q, 9p, 17p, and 13q occur much more frequently in precancerous bronchial epithelium from smokers than in epithelium from nonsmokers (27, 31). Clearly, the most frequently observed genetic alterations in NSCLC occur more commonly in smokers than in nonsmokers. The large difference in the frequency of allelic loss at chromosome 9p21 in our study appears to be specifically related to cigarette smoking.

A small minority (<10%) of lung cancer cases occur in nonsmoking individuals (2, 3). Nevertheless, with the high prevalence of lung cancer in this country, the number of deaths from lung cancer in nonsmokers (11,000) is similar to the number of deaths from cancer of the esophagus, bladder, and kidney (1). NSCLC in nonsmokers has clinical, pathological, and genetic features distinct from those seen in patients with a history of cigarette smoking. We have noted a higher incidence of other cancers in nonsmokers with lung cancer, suggesting that these patients may have an inherited predisposition to develop cancer (32).

Genes or areas of chromosomal loss commonly mutated or deleted in smokers are often less frequently involved in nonsmokers. In the present series, promoter hypermethylation was the sole mechanism for *p16* inactivation in nonsmokers. In contrast, *p16* inactivation through loss of one chromosomal arm followed by point mutation or loss of the second copy (homozygous deletion) was observed only in smokers. The higher frequency of chromosomal loss among smokers suggests that prolonged tobacco exposure leads to chromosome instability. Previous studies have demonstrated that specific tobacco carcinogens like BPDE can form stable covalent DNA adducts and induce DNA single-strand breaks (33).

Epidemiological data have strongly linked lung cancer with cigarette smoking. Allelic loss at chromosome 9p21 has been documented frequently in preneoplastic bronchial lesions, and *p16* inactivation may be an important initial step in the progression of NSCLC. Cell culture studies suggest that loss of *p16* is critical for immortalization of keratinocytes (19, 34). Most of these immortalized cells escape from crisis by methylation of the *p16* promoter. In the absence of cigarette smoke, endogenous methylation appears to be the preferred pathway for *p16* inactivation. The low rates of *p16* LOH in nonsmokers, together with the high frequency of *p16* promoter hypermethylation, suggest that *p16* inactivation is a critical event in lung carcinogenesis. However, the mechanisms of this inactivation may be different in the patients with and without tobacco exposure. The strong link between tobacco exposure and 9p21 loss further strengthens the role of smoking in the pathogenesis of lung cancer, whereas the high rate of *p16* methylation in nonsmokers now provides an important clue as to how these cancers may also occur in nonsmokers.

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