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Advances in Brief

Mutations in the $p16^{INK4}$/*MTS1*/*CDKN2*, $p15^{INK4B}$/*MTS2*, and *p18* Genes in Primary and Metastatic Lung Cancer[1]

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Abstract

We examined the genomic status of cyclin-dependent kinase-4 and -6 inhibitors, $p16^{INK4}$, $p15^{INK4B}$, and *p18*, in 40 primary lung cancers and 31 metastatic lung cancers. Alterations of the $p16^{INK4}$ gene were detected in 6 (2 insertions and 4 homozygous deletions) of 22 metastatic non-small cell lung cancers (NSCLCs; 27%), but none were detected in 25 primary NSCLCs, 15 primary small cell lung cancers (SCLCs), or 9 metastatic SCLCs, indicating that mutation in the $p16^{INK4}$ gene is a late event in NSCLC carcinogenesis. Although three intragenic mutations of the $p15^{INK4B}$ gene were detected in 25 primary NSCLCs (12%) and five homozygous deletions of the $p15^{INK4B}$ gene were detected in 22 NSCLCs (23%), no genetic alterations of the $p15^{INK4B}$ gene were found in primary and metastatic SCLCs. The *p18* gene was wild type in these 71 lung cancers, except 1 metastatic NSCLC which showed loss of heterozygosity. We also examined alterations of these three genes and expression of $p16^{INK4}$ in 21 human lung cancer cell lines. Alterations of the $p16^{INK4}$ and $p15^{INK4B}$ genes were detected in 71% of the NSCLC cell lines ($n = 14$) and 50% of the NSCLC cell lines ($n = 14$), respectively, but there were none in the 7 SCLC cell lines studied. No *p18* mutations were detected in these 21 cell lines. These results indicate that both $p16^{INK4}$ and $p15^{INK4B}$ gene mutations are associated with tumor progression of a subset of NSCLC, but not of SCLC, and that $p15^{INK4B}$ mutations might also be an early event in the molecular pathogenesis of a subset of NSCLC.

Introduction

Frequent occurrence of homozygous deletions and intragenic mutations of the $p16^{INK4}$ gene, as well as loss of $p16^{INK4}$ protein expression in various tumor cell lines, suggest that $p16^{INK4}$ is a tumor suppressor gene in a variety of human cancer types (1–5). Recently, the $p15^{INK4B}$ and *p18* genes encoding additional inhibitors of cyclin-dependent kinase 4 (CDK4) and CDK6 have been identified (6, 7). Since ectopic expression of $p16^{INK4}$ and *p18* suppresses cell growth in a manner that inversely correlates with the existence of an endogenous wild-type pRb function (5, 7, 8), the $p16^{INK4}$, $p15^{INK4B}$, and *p18* genes may be critical targets in human carcinogenesis. Recent studies have demonstrated that $p16^{INK4}$ mutations are less frequent in primary human tumors *in vivo* (3–5, 9–13), except for esophageal tumors (14), pancreatic tumors (15), and glioblastoma (16). Possible explanations for this variation include: (*a*) the frequency of $p16^{INK4}$ alterations differs among tumors of different histological types; (*b*) different mechanisms are involved in the inactivation of $p16^{INK4}$, such as mutations in the promoter or noncoding regions, altered gene expression by DNA methylation, and degradation of $p16^{INK4}$ protein; (*c*) $p16^{INK4}$ is inactivated in the late stage of tumor progression; (*d*) $p16^{INK4}$ is involved in *in vitro* cultivation or cellular senescence; (*e*) homozygous deletion of the $p16^{INK4}$ gene was masked by the contamination of noncancerous cells in tumor tissues; and (*f*) there are other tumor suppressor genes at chromosome 9p21. To address several of these possibilities, we examined alterations of the $p16^{INK4}$, $p15^{INK4B}$, and *p18* genes in 22 NSCLC[3] and 9 SCLC metastatic tumors and 25 NSCLC and 15 SCLC primary tumors by Southern blot analysis, PCR-SSCP analysis, and DNA sequencing. We also examined alterations of these three genes and expression of $p16^{INK4}$ and pRb in 22 human lung tumor cell lines. In this study, we found that both $p16^{INK4}$ and $p15^{INK4B}$ gene alterations were correlated with the progression of NSCLC but not of SCLC and that the $p15^{INK4B}$ gene also may be involved in the genesis of NSCLC. The *p18* gene was wild type in the tumors examined.

Materials and Methods

Tumor Samples, Cell Lines, and DNA, RNA, and Protein Extraction. Forty lung primary tumors (25 NSCLCs and 15 SCLCs) and 31 metastatic lung tumors (22 NSCLCs, 9 SCLCs, and corresponding primary lung tumors were available in 10 and 7 cases, respectively) were collected at National Cancer Center Research Institute, Tokyo, Japan. Corresponding normal tissues were available in all cases. All of these tumor samples previously showed loss of heterozygosity at more than one locus, indicating a preponderance of tumor cells in the specimens, as we reported previously (17). The cell lines analyzed were A427, A549, A2182, 866MT, HUT292DM, SW1271, SKLU1, CALU1, CALU6, NCI-H358, NCI-H157, NCI-H322, NCI-H596, NCI-H520, NCI-H526, NCI-H446, NCI-H417, NCI-H82, NCI-N231, DMS92, and Lu141. Lu 141 was established in the Pathology Division of the National Cancer Center Research Institute, Japan. All other cell lines were obtained from American Type Culture Collection. DNA, RNA, and protein were extracted as described previously (4).

Southern and Northern Blot Analyses. *Hind*III-, *Bam*HI-, *Eco*RI-, or *Msp*I-digested DNAs were hybridized to human $p16^{INK4}$ cDNA, $p15^{INK4B}$ cDNA including only exon 1, XPB (ERCC3), IFN-β, or L-*myc* probes labeled with [^{32}P]dCTP. Northern blot filters were hybridized with human $p16^{INK4}$ cDNA and GAPDH [^{32}P]dCTP-labeled probes.

Immunoprecipitation and Western Blot Analysis. Protein (300 μg) was immunoprecipitated with the following antibodies: anti-$p16^{INK4}$(18), or anti-Rb (Santa Cruz). Immunoprecipitates were electrophoretically transferred to polyvinylidene difluoride membranes (Millipore), probed with the same antibodies, and detected by chemiluminescence (DuPont).

PCR and PCR-SSCP. PCR intronic primers were identified from the $p16^{INK4}$ genomic sequence. Primers for exon 1 were (5′-3′): 1A, CGGAGAGGGGGAGAGCAG (sense, outer primer); 1A-1, AGAGGGGGAGAG-

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[3] The abbreviations used are: NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; SSCP, single-strand conformational polymorphism; IFN, interferon.

CAGGCA (sense, inner primer); 1B, TCCCCTTTTTCCGGAGAATCG (antisense, outer primer); and 1B-1, AAGCGCTACCTGATTCCAATT (antisense, inner primer). PCR conditions: amplification of exon 1 with outer primers consisted of a 5-min denaturation at 94°C, followed by 40 cycles of 40 s at 94°C, 40 s at 55°C, and 90 s at 72°C. PCR product was then amplified with inner primers consisting of a 5-min denaturation at 94°C, followed by 35 cycles of 40 s at 94°C, 40 s at 55°C, and 90 s at 72°C.

Primers for exon 2 of the $p16^{INK4}$ gene were (5′-3′): 2A, GCTCTACACAAGCTTCCTTTCC (sense, outer primer); 2A-1, AAGCTTCCTTTCCGTCATGCC (sense, inner primer); 2B, GGGCTGAACTTTCTGTGCTGG (antisense, outer primer); and 2B-1, TGGAAAATGAATGCTCTGAG (antisense, inner primer). PCR conditions: amplification of exon 2 with outer primers consisted of a 5-min denaturation at 95°C, followed by 40 cycles of 1 min at 95°C, 1 min at 60°C, and 1 min at 72°C. The PCR product was further amplified with inner primers consisting of a 5-min denaturation at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 60°C, and 1 min at 72°C.

Primers for exon 3 of the $p16^{INK4}$ gene were (5′-3′): 3A, AGGAATTCGGTAGGGACGGCAAGAGAGG (sense); and 3B, GAAGCTTGGGGGAAGGCATATATCTACG (antisense). PCR conditions: amplification of exon 3 with the above primers consisted of a 5-min denaturation at 94°C, followed by 40 cycles of 40 s at 94°C, 40 s at 55°C, and 90 s at 72°C.

Primers for exon 1 of the $p15^{INK4B}$ gene were (5′-3′): 1C, TTAAGTTTACGGCCAACGGTGGAT (sense, outer primer); 1C-1, AACGGTGGATTATCCGGGCCGCT (sense, inner primer); 1D, TGTACAAATCTACATCGGCGATCTA (antisense, outer primer); and 1D-1, AAATCTACATCG GCGATCTAGGTT (antisense, inner primer). PCR conditions are the same as those of exon 1 of $p16^{INK4}$.

Primers for exon 2 of the $p15^{INK4B}$ gene were (5′-3′): 2C, TCTTTAAATGGCTCCACCTGCCTT (sense, outer primer); 2C-1, TAAATGGCTCCACCTGCCTT (sense, inner primer); 2D, TCCCCGTTGGCAGCCTTCATCGA (antisense, outer primer); and 2D-1, GTTGGCAGCCTTCATCGAATTA (antisense, inner primer). PCR conditions are the same as those of exon 1 of the $p15^{INK4B}$ gene.

Primers for exon 1 of the *p18* gene were (5′-3′): 1E, ATGGCCGAGCCTTGGGGGAACGAGTT (sense); and 1F, CAACATTATTGACTTGTTTTCCCCAC (antisense).

Primers for exon 2 of the *p18* gene were (5′-3′): 2E, AGGATTCTACCATTTCTACTTCTTT (sense); and 2F, TTATTGAAGATTTGTGGCTCCCCCA (antisense). PCR conditions consisted of a 5-min denaturation at 94°C, followed by 40 cycles of 40 s at 94°C, 40 s at 55°C, and 90 s at 72°C. For SSCP analysis, ^{32}P-labeled PCR products were heat denatured and applied to a 6% neutral polyacrylamide gel containing 2, 5, or 10% glycerol. PCR products of exon 2 of the $p16^{INK4}$ and $p15^{INK4B}$ genes were digested with *Sma*I, and PCR products of exon 2 of *p18* were digested with *Alu*I before loading.

Sequencing. PCR product was purified, subcloned into the PCR script (Strategene), and DNA pooled from 50 to 100 plasmid clones; individual single clones were purified and used for sequencing by the dideoxy chain termination method using a 7-DEAZA Sequencing kit (U.S. Biochemical). All mutations were confirmed by the direct sequencing of the amplified tumor DNA and DNA from nontumor tissue to identify germline mutations and polymorphisms (19).

Results and Discussion

Forty lung primary tumors and 31 metastases of lung tumors were examined for alterations of the $p16^{INK4}$ gene by Southern blot, PCR-SSCP, and DNA sequencing analyses. Southern blot analysis detected homozygous deletions of the $p16^{INK4}$ gene in 4 of 22 metastatic NSCLCs but none of 25 primary NSCLCs, 15 primary SCLCs, or 9 metastatic SCLCs (Tables 1 and 2; Fig. 1, *A* and *B*). PCR-SSCP and sequencing analyses detected intragenic mutations in two metastatic NSCLCs (Tables 1 and 2; Fig. 1*C*). Both of these insertions cause premature stop codons in exon 2 of the $p16^{INK4}$ gene. The DNA from either the corresponding normal tissue or the primary NSCLC of these metastases with one-base pair insertions contained wild-type $p16^{INK4}$ (Fig. 1*C*), indicating that $p16^{INK4}$ alteration had occurred in the late stage of NSCLC carcinogenesis.

Table 1 *Somatic mutations of the $p15^{INK4B}$, $p16^{INK4}$, and p18 genes in primary and metastatic NSCLC and SCLC lung cancers and lung cancer cell lines*

	$p15^{INK4B}$	$p16^{INK4}$	*p18*
Primary			
NSCLC	3/25 (12%)	0/25	0/25
SCLC	0/15	0/15	0/15
Metastatic			
NSCLC	5/22 (23%)	6/22 (27%)	0/22
SCLC	0/9	0/9	0/9
Cell Lines[a]			
NSCLC	7/14 (50%)	10/14 (71%)	0/14
SCLC	0/7	0/7	0/7

[a] See Table 3 for more detailed information.

Table 2 *Genetic alterations of $p16^{INK4}$, $p15^{INK4B}$, and p18 genes in human lung cancers*

Case	Tumor	Histology	Codon	Mutation	Type of mutation
			$p16^{INK4}$		
N211T	M[a]	Adeno	58&59	CGAGTG→CGAGGTG	Frameshift
N521T	M	Large	86	GCC→GGCC	Frameshift
N2131T	M	Adeno		Homozygous deletion	
N181T	M	Adeno		Homozygous deletion	
N571T	M	Adeno		Homozygous deletion	
N2151T	M	Adeno		Homozygous deletion	
			$p15^{INK4B}$		
2201T	P	Adeno	47	GGG→GAG	Gly→Glu
QO51	P	Squamous	64	CTG→CTA	Leu→Leu
N2561P	P	Adeno	50	GCG→GTG	Ala→Val
N2131T	M	Adeno		Homozygous deletion	
N181T	M	Adeno		Homozygous deletion	
N571T	M	Adeno		Homozygous deletion	
N2151T	M	Adeno		Homozygous deletion	
N2191T	M	Adeno		Homozygous deletion	
			p18		
N521T	M	Large		Loss of heterozygosity	

[a] P, primary tumor; M, metastatic tumor; Adeno, adenocarcinoma; Large, large cell carcinoma; Squamous, squamous cell carcinoma.

We found three intragenic mutations of the $p15^{INK4B}$ gene in 25 primary NSCLCs (12%; Tables 1 and 2). All of the tumors with intragenic mutations of the $p15^{INK4B}$ gene were stage I and were wild type for the $p16^{INK4}$ gene. Five homozygous deletions of $p15^{INK4B}$ were detected in 22 metastatic NSCLCs (23%), and four of these cases showed homozygous deletion of the $p16^{INK4}$ gene (Tables 1 and 2). One case with homozygous deletion of $p15^{INK4B}$ (N2191T) contained wild-type $p16^{INK4}$ and retention of the IFN-β locus. The two cases with intragenic mutations of the $p16^{INK4}$ gene showed wild type of coding region of the $p15^{INK4B}$ gene. These data are not consistent with the hypothesis that both the $p15^{INK4B}$ and $p16^{INK4}$ genes must be mutationally inactivated during carcinogenesis (16).

No alterations in the *p18* gene were detected in these 71 lung cancers in total, except one metastatic NSCLC which showed loss of heterozygosity (Table 2). A polymorphic site in exon 2 is located at codon 114 ($GGC^{Gly} \rightarrow GGT^{Gly}$), and this polymorphism was detected in 12 of the 71 cases (17%).

All of the lung tumors with alterations of either the $p16^{INK4}$ gene or $p15^{INK4B}$ gene were NSCLC. Therefore, we examined the genomic status of the $p16^{INK4}$, $p15^{INK4B}$, and *p18* genes and expression of $p16^{INK4}$ in 14 NSCLC cell lines and 8 SCLC cell lines by Southern blot, Northern blot, Western blot, PCR-SSCP, and DNA sequencing analyses (Table 3). Seven homozygous deletions and three intragenic mutations of the $p16^{INK4}$ gene and seven homozygous deletions of the $p15^{INK4B}$ gene were detected in NSCLC cell lines (71 and 50%, respectively), whereas no alterations were detected in SCLC cell lines. Seven NSCLC cell lines showed concordant homozygous deletions of the $p16^{INK4}$ and $p15^{INK4B}$ genes. These results indicate that $p16^{INK4}$

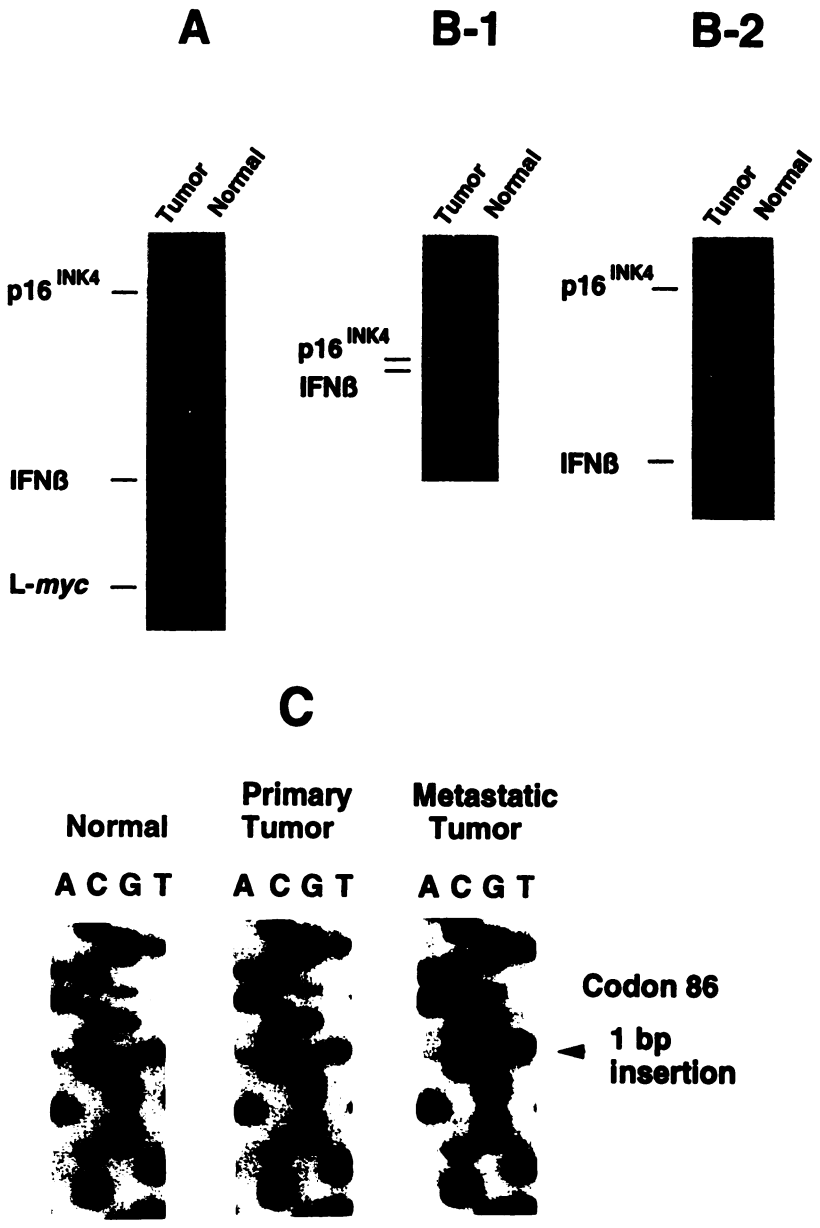

Fig. 1. Homozygous deletion and mutation of the *p16*^{INK4} gene in lung metastases. DNA from two lung metastases and corresponding normal tissues were digested with either *Eco*RI (*A, B-2*) or *Hind*III (*B-1*), respectively, and hybridized to *p16*^{INK4}, IFN-β (*IFNβ*), or L-*myc* probes. Sequencing analysis showed one base insertion in codon 86 in lung metastatic tumor, whereas corresponding normal tissue and primary tumor were wild type (*C*).

and *p15*^{INK4B} gene alterations are much more common in NSCLC cell lines (20, 21). Moreover, *p16*^{INK4} mRNA or *p16*^{INK4} protein was undetected only in NSCLC cell lines. We also examined expression of the Rb protein. Nine of 10 NSCLC cell lines examined expressed Rb protein, and conversely, 5 of 6 SCLC cell lines did not display normal Rb protein expression. This inverse relationship between expression of p16^{INK4} and Rb protein indicates that a mutation in either Rb or *p16*^{INK4} is sufficient to disrupt the G_1 checkpoint pathway as we and others reported previously (4, 21–24).

A genetic polymorphism of the *p16*^{INK4} gene (codon 135; $GGG^{Gly} \rightarrow GGA^{Gly}$) was detected in one primary NSCLC, one primary SCLC, and one metastatic SCLC. This polymorphism may be found in Japanese populations more frequently than in other ethnic groups (4, 5). We also found two unreported polymorphisms in intron 1 of the *p15*^{INK4B} gene (23 nucleotides from exon 2 g→a; 27 nucleotides from exon 2 c→a). Although this latter polymorphism was also detected in American and Polish populations[4] and one SCLC cell line (DMS92), the polymorphism at the twenty-third nucleotide from exon 2 was detected only in the Japanese populations in our experience to date.

Hayashi *et al.* (25) reported that mutations of the *p16*^{INK4} gene were detected in 30% of the Japanese primary NSCLCs. Although we examined 25 Japanese primary NSCLCs, no *p16*^{INK4} mutations were detected. One possibility is that there are some false negative cases by PCR-SSCP. However, we used several conditions for the PCR-SSCP analysis, and PCR products of less than 300 base pairs were analyzed by SSCP, which generally has a false negative rate of less than 10% (26). Moreover, we sequenced the *p16*^{INK4} gene in 14 primary NSCLCs, 22 metastatic NSCLCs, and 27 metastatic colon tumors and compared the result with the PCR-SSCP result to evaluate the reliability of the PCR-SSCP analysis. Sequencing detected 3 mutations, 5 polymorphisms, and 55 wild-type cases of the *p16*^{INK4} gene, while PCR-SSCP detected 7 mobility shifts and 56 normal mobilities. Thus, PCR-SSCP correctly predicted the presence of a mutation in 98% (62 of 63) of the samples, and 88% (7 of 8) of the samples with mutations. Forty NSCLCs showed loss of heterozygosity on at least one locus, as

[4] Unpublished results.

Table 3 *Expression of the $p16^{INK4}$, Rb, and genomic status of $p16^{INK4}$, $p15^{INK4B}$, p18, and p53 in human lung carcinoma cell lines*

Cell line	Histology	$p16^{INK4}$ DNA	$p16^{INK4}$ mRNA	$p16^{INK4}$ Protein	$p15^{INK4B}$ gene	p18 gene	p53 gene[b]	Rb
A427	Ad[a]	HD	–	ND	HD	WT	WT	+
A549	Ad	HD	–	ND	HD	WT	WT	+
A2182	Ad	HD	–	ND	HD	WT	WT	+
866MT	Me	33-bp deletion 2nd base of codon 44-intron 1 13 bp	+	–	WT	WT	M	+
HUT292DM	Me	HD	–	–	HD	WT	WT	+
SW1271	Sq	HD	–	–	HD	WT	M	+
SKLU1	Ad	HD	–	–	HD	WT	M	+
Calu 1	Ep	WT	+	–	WT	WT	HD	+
Calu 6	Ad	WT	+	–	WT	WT	M	+
NCIH358	Ba	WT	+	–	WT	WT	M	+
NCIH157	Sq	$GAG^{Glu}\rightarrow GTG^{Val}$ Codon 69	+	–	WT	WT	M	+
NCIH322	Ba	HD	–	–	HD	WT	M	+
NCIH596	Ad/Sq	WT	+	+	WT	WT	WT	–
NCI-H520	Sq	1-bp deletion Codon 45	ND	–	WT	WT	M	+
NCI-H526	Sc	WT	+	+	WT	WT	M	–
NCI-H446	Sc	WT	+	+	WT	WT	WT	–
NCI-N417	Sc	WT	+	+	WT	WT	M	–
NCI-H82	Sc	WT	ND	+	WT	WT	M	+
NCI-N231	Sc	WT	ND	+	WT	WT	M	±
DMS92	Sc	WT	+	+	WT	WT	M	+
LU141	Sc	WT	ND	+	WT	WT	M	M[c]

[a] Ad, adenocarcinoma; Me, mucoepidermoid carcinoma; Sq, squamous cell carcinoma; Ep, epidermoid; Ba, bronchioalveolar carcinoma; Ad/Sq, adenosquamous cell carcinoma; Sc, small cell lung carcinoma; HD, homozygous deletion; bp, base pair; +, expression; –, not detected; ±, low expression; ND, not done; WT, wild type; M, mutant.
[b] Data from Refs. 23 and 24.
[c] Data from Ref. 25.

we reported previously (16), which indicates a preponderance of tumor cells in the specimens. Another possibility is that there are different clinical pathogenesis such as grading, staging, and prognosis between the two populations of donors.

In this study, we found $p16^{INK4}$ gene alterations only in metastatic NSCLC (27%) and not in primary NSCLC, primary SCLC, or metastatic SCLC. Alterations of the $p16^{INK4}$ gene were detected in 10 of the 14 NSCLC cell lines (71%) but in none of 7 SCLC cell lines studied. These results suggest that inactivation of $p16^{INK4}$ may be a late event in NSCLC carcinogenesis. Homozygous deletions of $p16^{INK4}$ are also found more frequently in the late stages of tumor progression of human astrocytomas (27). We also found intragenic mutations of the $p15^{INK4B}$ gene in primary NSCLCs (12%) and five homozygous deletions of the $p15^{INK4B}$ gene in metastatic NSCLCs (23%). These results taken together indicate that both $p15^{INK4B}$ and $p16^{INK4}$ genes may be targets in tumor progression of NSCLC, and the $p15^{INK4B}$ gene also may be involved in the molecular pathogenesis of NSCLC. A future study should focus on the immunohistochemical and functional examinations of $p16^{INK4}$, $p15^{INK4B}$, and p18 proteins in primary and metastatic lung tumors.

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