Advances in Brief

Mutations in the p16<sup>INK4</sup>/MTS1/CDKN2, p15<sup>INK4B</sup>/MTS2, and p18 Genes in Primary and Metastatic Lung Cancer<sup>1</sup>

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Abstract

We examined the genomic status of cyclin-dependent kinase-4 and -6 inhibitors, p16<sup>INK4</sup>, p15<sup>INK4B</sup>, and p18, in 40 primary lung cancers and 31 metastatic lung cancers. Alterations of the p16<sup>INK4</sup> 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. In 15 primary small cell lung cancers (SCLCs), or 9 primary SCLCs, indicating that mutation in the p16<sup>INK4</sup> gene is a late event in NSCLC carcinogenesis. Although three intragenic mutations of the p15<sup>INK4B</sup> gene were detected in 25 primary NSCLCs (12%) and five homozygous deletions of the p15<sup>INK4B</sup> gene were detected in 22 NSCLCs (23%), no genetic alterations of the p15<sup>INK4B</sup> 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<sup>INK4</sup> in 21 human lung cancer cell lines. Alterations of the p16<sup>INK4</sup> and p15<sup>INK4B</sup> genes were detected in 71% of the NSCLC cell lines (n=14) and 50% of the SCLC 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<sup>INK4</sup> and p15<sup>INK4B</sup> gene mutations are associated with tumor progression of a subset of NSCLC, but not of SCLC, and that p15<sup>INK4B</sup> 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<sup>INK4</sup> gene, as well as loss of p16<sup>INK4</sup> protein expression in various tumor cell lines, suggest that p16<sup>INK4</sup> is a tumor suppressor gene in a variety of human cancer types (1–5). Recently, the p15<sup>INK4B</sup> and p18 genes encoding additional inhibitors of cyclin-dependent kinase 4 (CDK4) and CDK6 have been identified (6, 7). Since ectopic expression of p16<sup>INK4</sup> and p18 suppresses cell growth in human tumors in vivo (3–5, 9–13), except for esophageal tumors (14), but not of SCLC, and that p15<sup>INK4B</sup> gene also may be involved in the genesis of NSCLC.

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).

Southwestern and Northern Blot Analyses. HindIII, BamHI, EcoRI, or MspI-digested DNAs were hybridized to human p16<sup>INK4</sup> cDNA, p15<sup>INK4B</sup> cDNA including only exon 1, XBP (ERCC3), IFN-β, or L-myc probes labeled with [32P]dCTP. Northern blot filters were hybridized with human p16<sup>INK4</sup> cDNA and GAPDH [32P]dCTP-labeled probes.

Immunoprecipitation and Western Blot Analysis. Protein (300 μg) was immunoprecipitated with the following antibodies: anti-p16<sup>INK4</sup> (anti-p16<sup>INK4</sup>), 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 intrinsic primers were identified from the p16<sup>INK4</sup> genomic sequence. Primers for exon 1 were 5′-3′: 1A, CAGGAGGCGGAGACGAG (sense, outer primer); 1A-1, AGAGGCGGAGAG-

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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, TCCCTTTTTCGGGAAATCGC (antisense, outer primer); and 1B-1, AAGGCTACCTGGATCTCATT (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 p16INK4 gene were (5'-3'): 2A, GCTCTACA-CAAGCTTTCTTCC (sense, outer primer); 2A-1, AAGGCTACTTCTGCA-CTG (sense, inner primer); 2B, GGCTGAATTTCTTGGCAGG (antisense, outer primer); and 2B-1, TGAAAGAGTATCTGTTAG (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 p16INK4 gene were (5'-3'): 3A, AGGAAATTCG- GTAGGGCAAGGGAAGAG (sense); and 3B, GAAGCTTGAGGAAAG- GCATATATCTACG (antisense). PCR conditions: amplification of exon 3 with the above primers consisted of a 5-mm 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 p15INK4B gene were (5'-3'): 1C, TAAAGCTT- TACGCGCAAGGTTG (sense, inner primer); 1C-1, AAGGCTTGAT- TATCCGGGAGCCTG (antisense, inner primer); 1D, TGTACAAATCG- CATCGAAT (antisense, outer primer); and 1D-1, AATACCATCATG- GCGATCTAGGT (antisense, inner primer). PCR conditions are the same as those of exon 1 of p16INK4 gene.

Primers for exon 2 of the p15INK4B gene were (5'-3'): 2C, TCTTTAAAT- GCCCTACCGCT (sense, outer primer); 2C-1, TAAAGCTTGACCAT- CTGCGCC (sense, inner primer); 2D, TCCCTGGTGAGCCTTCATCGA (antisense, outer primer); and 2D-1, GTGGCAGGCTTCATAGAAT (antisense, inner primer). PCR conditions are the same as those of exon 1 of the p15INK4B gene.

Primers for exon 2 of the p18 gene were (5'-3'): 1E, ATGGCCGAGCCT- TGGGGAAAGGTT (sense); and 1F, CAACATGATGATGGTCTTCCC- ACCAC (antisense).

Primers for exon 2 of the p18 gene were (5'-3'): 2E, AGGATTCAC- CATTCTACCTTCTT (sense); and 2F, TTGATGACTGTTCTGCT- CCCCC (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, 32P-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 p16INK4 and p15INK4B genes were digested with Smal, and PCR products of exon 2 of p18 were digested with Alul 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 dyeoxy 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 p16INK4 gene by Southern blot, PCR-SSCP, and DNA sequencing analyses. Southern blot analysis detected homozygous deletions of the p16INK4 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. 1C). Both of these insertions cause premature stop codons in exon 2 of the p16INK4 gene. The DNA from either the corresponding normal tissue or the primary NSCLC of these metastases with one-base-pair insertions contained wild-type p16INK4 gene (Fig. 1C), indicating that p16INK4 alteration had occurred in the late stage of NSCLC carcinogenesis.

We found three intragenic mutations of the p15INK4B gene in 25 primary NSCLCs (12%; Tables 1 and 2). All of the tumors with intragenic mutations of the p15INK4B gene were stage I and were wild type for the p16INK4 gene. Five homozygous deletions of p15INK4B were detected in 22 metastatic NSCLCs (23%), and four of these cases showed homozygous deletion of the p16INK4 gene (Tables 1 and 2). One case with homozygous deletion of p15INK4B (N2191T) contained a p16INK4 gene mutation. Seven NSCLC cell lines showed concordant homozygous deletions of the p15INK4B gene and seven homozygous deletions of the p18 gene were detected in NSCLC cell lines (71 and 50%, respectively), and four of these cases showed homozygous deletion of the p16INK4 gene (Tables 1 and 2). Case 191 contained a p16INK4 gene mutation. Seven NSCLC cell lines showed concordant homozygous deletions of the p15INK4B and p18 genes. These results indicate that p16INK4 gene was altered in the late stage of NSCLC carcinogenesis.

<table>
<thead>
<tr>
<th>Case</th>
<th>Tumor</th>
<th>Histology</th>
<th>Codon</th>
<th>Mutation Type of mutation</th>
<th>p16INK4</th>
</tr>
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<tr>
<td>N211T</td>
<td>M</td>
<td>Adeno</td>
<td>58 &amp; 59</td>
<td>CAGGTCG -&gt; CAGGTCG</td>
<td>Frameshift</td>
</tr>
<tr>
<td>N251T</td>
<td>M</td>
<td>Large</td>
<td>86</td>
<td>GCC -&gt; GCC</td>
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</tr>
<tr>
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<td>M</td>
<td>Adeno</td>
<td>Homozygous deletion</td>
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p18

<table>
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<th>Codon</th>
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<td>M</td>
<td>Large</td>
<td>Loss of heterozygosity</td>
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<td></td>
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</table>

a P, primary tumor; M, metastatic tumor; Adeno, adenocarcinoma; Large, large cell carcinoma; Squamous, squamous cell carcinoma.
and p16INK4A gene alterations are much more common in NSCLC cell lines (20, 21). Moreover, p16INK4A mRNA or p16INK4A 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 p16INK4A and Rb protein indicates that a mutation in either Rb or p16INK4A is sufficient to disrupt the G1 checkpoint pathway as we and others reported previously (4, 21—24).

A genetic polymorphism of the p16INK4A gene (codon 135; GGG→GGA) 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 p16INK4A 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* 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 p16INK4A gene were detected in 30% of the Japanese primary NSCLCs. Although we examined 25 Japanese primary NSCLCs, no p16INK4A 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 p16INK4A 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 p16INK4A 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

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* Unpublished results.
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 p16INK4A gene alterations only in metastatic NSCLC (27%) and not in primary NSCLC, primary SCLC, or metastatic SCLC. Alterations of the p16INK4A gene were detected in 10 of the 14 NSCLC cell lines (71%) but in none of 7 SCLC cell lines (23%). These results taken together indicate that both p16INK4B and p16INK4A gene alterations preferentially in non-small cell lung cancers. Cancer Res., 55: 20—23, 1995.

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References


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Mutations in the $p_{16}^{\text{INK4}/MTS1/CDKN2}$, $p_{15}^{\text{INK4B}/MTS2}$, and $p_{18}$ Genes in Primary and Metastatic Lung Cancer

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