Mutations in the p16INK4/MTS1/CDKN2, p15INK4B/MTS2, and p18 Genes in Primary and Metastatic Lung Cancer

Aikou Okamoto, S. Perwez Hussain, Koichi Hagiwara, Elisa A. Spillare, Marek R. Rusin, Douglas J. Demetrick, Manuel Serrano, Gregory J. Hannon, Masayuki Shiseki, Malmoona Zariwala, Yue Xiong, David H. Beach, Jun Yokota, and Curtis C. Harris


Abstract

We examined the genomic status of cyclin-dependent kinase-4 and -6 inhibitors, p16INK4, p15INK4B, and p18, in 40 primary lung cancers and 31 metastatic lung cancers. Alterations of the p16INK4 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 primary SCLCs, indicating that mutation in the p16INK4 gene is a late event in NSCLC carcinogenesis. Although three intragenic mutations of the p15INK4B gene were detected in 25 primary NSCLCs (12%) and five homozygous deletions of the p15INK4B gene were detected in 22 NSCLCs (23%), no genetic alterations of the p15INK4B 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 p16INK4 in 21 human lung cancer cell lines. Alterations of the p16INK4 and p15INK4B 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 p16INK4 and p15INK4B gene mutations are associated with tumor progression of a subset of NSCLC, but not of SCLC, and that p15INK4B 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 p16INK4 gene, as well as loss of p16INK4 protein expression in various tumor cell lines, suggest that p16INK4 is a tumor suppressor gene in a variety of human cancer types (1–5). Recently, the p15INK4B and p18 genes encoding additional inhibitors of cyclin-dependent kinase 4 (CDK4) and CDK6 have been identified (6, 7). Since ectopic expression of p16INK4 and p15INK4B genes suppresses cell growth in human tumors in vivo (3–5, 9–13), except for esophageal tumors (14), and metastatic SCLCS, indicating that mutation in the p16INK4 gene is a late event in the molecular pathogenesis of a subset 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, A2132, 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).

Immunoprecipitation and Western Blot Analysis. Protein (300 µg) was immunoprecipitated with the following antibodies: anti-p16INK4 (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).

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2 To whom requests for reprints should be addressed, at Laboratory of Human Carcinogenesis, National Cancer Institute, NIH, Bldg. 37, Room 2C01, Bethesda, MD 20892-4255.

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, TCCCCCTTCCGAGAATGCG (antisense, outer primer); and 1A-1B, AAGCCTACCTGATGTTCAATT (antisense, inner primer). PCR conditions: amplification of exon 1 with outer primers consisting 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 p16INK4B gene were (5’-3’): 2A, GCTCTACATGGCCATCCAGTTT (sense, outer primer); 2A-1, AAGCCTACCTGATGTTCAATT (antisense, inner primer); and 2B-1, TGGAAAATGATGTTCAATTG (antisense, inner primer). PCR conditions: amplification of exon 2 with outer primers consisting of a 5-min denaturation at 94°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 94°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 p16INK4B gene were (5’-3’): 3A, AGGAATCGCTACAGGCGAAGGAAGAGG (sense); and 3B, GAAGCTTGGGGGAGAGCATATATCTACG (antisense, outer primer); and 2B-1, TGGAAAATGATGTTCAATTG (antisense, inner primer). PCR conditions: amplification of exon 3 with the above primers consisting 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 p15INK4B gene were (5’-3’): 1C, TTAAGTTTACCGGCAAAGCTGAT (sense, outer primer); 1C-1, AAGCCTACCTGATGTTCAATT (antisense, inner primer); 1D, TGGAAAATGATGTTCAATTG (antisense, outer primer); and 1D-1, AAAGGATGCTTACATCCGAGTCTTTACTCAGTTTTGTTGAGGTG (antisense, inner primer). PCR conditions are the same as those of exon 1 of p16INK4B gene.

Primers for exon 2 of the p15INK4B gene were (5’-3’): 2C, TCTTTAAAACAGCTGACGGCTTACCTG (sense, outer primer); 2C-1, TTAAGTTTACCGGCAAAGCTGAT (antisense, outer primer); 2D, TCCCGTGCTTGAGCCCTTCACTCAGA (antisense, outer primer); and 2D-1, TGGAAAATGATGTTCAATTG (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’): 2E, AGGAATTCTACATGGCCATCCAGTTT (sense); and 2F, TTATGGAATGCTTGTTTACCTCAGTTTTGTTGAGGTG (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 p16INK4B and p15INK4B genes were digested with Smal, and PCR products of exon 2 of p18 were digested with Aul 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 p16INK4B gene by Southern blot, PCR-SSCP, and DNA sequencing analyses. Southern blot analysis detected homozygous deletions of the p16INK4B 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 p16INK4B gene. The DNA from either the corresponding normal tissue or the primary NSCLC of these metastases with one-base pair insertions contained wild-type p16INK4B (Fig. 1C), indicating that p16INK4B 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 p16INK4B gene. Five homozygous deletions of p15INK4B were detected in 22 metastatic NSCLCs (23%), and four of these cases showed homozygous deletion of the p16INK4B gene (Tables 1 and 2). One case with homozygous deletion of p15INK4B (N2191T) contained wild-type p16INK4B and retention of the IFN-β locus. The two cases with intragenic mutations of the p16INK4B gene showed wild type of coding region of the p15INK4B gene. These data are not consistent with the hypothesis that both the p15INK4B and p16INK4B 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 (GGG → GAG), and this polymorphism was detected in 12 of the 71 cases (17%).

All of the lung tumors with alterations of either the p16INK4B gene or p15INK4B gene were NSCLC. Therefore, we examined the genomic status of the p16INK4B, p15INK4B, and p18 genes and expression of p16INK4B 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 p16INK4B gene and seven homozygous deletions of the p15INK4B 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 p16INK4B and p15INK4B genes. These results indicate that p16INK4B...
and p16INK4B gene alterations are much more common in NSCLC cell lines (20, 21). Moreover, p16INK4 mRNA or p16INK4 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 p16INK4 and Rb protein indicates that a mutation in either Rb or p16INK4 is sufficient to disrupt the G1 checkpoint pathway as we and others reported previously (4, 21–24).

A genetic polymorphism of the p16INK4 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 p16INK4B 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 p16INK4 gene were detected in 30% of the Japanese primary NSCLCs. Although we examined 25 Japanese primary NSCLCs, no p16INK4 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 p16INK4 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 p16INK4 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 p16\(^{INK4A}\) gene alterations only in metastatic NSCLC (27%) and not in primary NSCLC, primary SCLC, or metastatic SCLC. Alterations of the p16\(^{INK4A}\) gene were detected in 10 of 12 (83%) lung tumors with \(9p\) loss. Science (Washington DC), 265: 415—416, 1994.

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References


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