**In Vivo Occurrence of p16 (MTS1) and p15 (MTS2) Alterations Preferentially in Non-Small Cell Lung Cancers**

Osuke Washimi, Masaaki Nagatake, Hirotaka Osada, Ryuzo Ueda, Takashi Koshikawa, Tsuneo Seki, Toshitada Takahashi, and Takashi Takahashi

**Abstract**

Frequent homozygous deletions of the p16 (MTS1) gene encoding a cyclin-dependent kinase inhibitor were recently reported in various tumor cell lines including examples derived from lung cancers, but direct evidence for their occurrence in lung cancer patients has not been reported thus far. In the present study, alterations of p16 and/or p15, a p16-related cyclin-dependent kinase, were observed not only in lung cancer cell lines but also in the corresponding tumor specimens in vivo, excluding the possibility of in vitro artifacts. Interestingly, a clear specificity was also noted in terms of the affected histological subtype; i.e., only non-small cell lung cancers carried alterations (6 of 20 as compared to 0 of 20 small cell lung cancer cell lines).

**Introduction**

Allelic losses are hallmarks of chromosomal regions harboring tumor suppressor genes and cytogenetic and molecular analyses have revealed frequent occurrence of multiple chromosomal deletions involving 3p, 5q, 6q, 9p, 11p, 13q, and 17p. These findings provided the foundation for discovery of inactivation of the retinoblastoma gene on 13q and the p53 gene on 17p (1, 2) and the search has now intensified for tumor suppressor genes in other chromosomal regions.

Recently, two highly related members of the cyclin-dependent kinase inhibitor family, termed p16 (MTS1) and p15 (MTS2), were isolated from the chromosomal region 9p21 (3–5). While p16 appears to be a good candidate for a tumor suppressor gene involved in the oncogenesis of familial and sporadic melanomas (4–7), a high frequency of homozygous deletions of the p16 gene in various tumor cell lines in culture suggests possible wider involvement in multiple types of human tumors. Although such alterations were also reported in lung cancer cell lines in vitro, neither subtle mutations nor homozygous deletions of the p16 gene have been reported in lung cancer specimens in vivo thus far. To date, no extensive search for p15 alterations in lung cancer has been documented. It therefore remains uncertain whether or not alterations of these cyclin-dependent kinase inhibitors occur in lung cancer in vivo as a somatic mutation and whether they may play a role in the oncogenesis at this organ site.

Technical difficulties with the use of in vivo lung cancer specimens can confound detection of homozygous deletions, because samples usually contain an admixture of contaminating inflammatory and stromal cells. Although in vitro-cultured cell lines are free from such contaminants, they might have acquired genetic alterations during a prolonged period of time in culture. We therefore took advantage of our possession of lung cancer cell lines for most of which corresponding tumor samples were available for confirmation of in vivo occurrence by the microdissection technique. We here report on the occurrence of both p16 and p15 alterations in lung cancers in vivo.

**Materials and Methods**

Cell Lines and Tumor Specimens. Twenty SCLC and 11 NSCLC cell lines which had been established in our laboratories at Aichi Cancer Center in Japan (8). These included ACC-LC-5, -35, -36, -48, -52, -60, -61, -67, -80, -87, -96, -170, -171, -172, -173, -174, and -321 SCLC cell lines as well as ACC-LC-33, -71, -73, -91, -93, -174, -176, -314, -319, and -323 NSCLC cell lines. Six sets (4 SCLC and 2 NSCLC) of cell lines which had been established consecutively at different periods of time from different sites of six independent patients were included in this panel (from patient SL, ACC-LC-177 and -178; from patient OT, ACC-LC-48 and -52; from patient SK, ACC-LC-5, -35, and -172; from patient NT, ACC-LC-87 and -173; from patient TS, ACC-LC-94 and -174; from patient MS, ACC-LC-314 and -319). In vivo tumor specimens from the same patients from whom these tumor cell lines derived were available for all cell lines except for ACC-LC-71, -170, and -171. Microdissection and DNA extraction were carried out essentially as described previously. In addition to our cell lines, we also analyzed 9 NSCLC cell lines (SK-LC-10, SK-MES-1, Calu1, Calu6, as well as RERF-LC-MK, -MS, -OK, -MT, and -AI) which were generous gifts of Drs. L. Old and M. Akiyama.

Detection of Homozygous Deletion in the p16 and p15 Genes. The primer pairs and PCR conditions used to amplify exons 1 and 2 of the p16 gene were

**Exon 1:** S1 (sense), 5'-GAAGAAAAGAGGGGCGTG
AS1 (antisense), 5'-GCCTACTTCTTACTGCT
1 cycle at 94°C for 5 min; 1 cycle at 94°C (0.5 min) with annealing temperatures (T_{	ext{an}}) of 65°C (0.5 min) and extension at 72°C (0.5 min), 1 cycle with T_{	ext{an}} = 63°C (0.5 min), 1 cycle with T_{	ext{an}} = 61°C (0.5 min), 1 cycle with T_{	ext{an}} = 59°C (0.5 min), 1 cycle with T_{	ext{an}} = 57°C (0.5 min), and 20 cycles with T_{	ext{an}} = 55°C (0.5 min) in the presence of 5% DMSO

**Exon 2:** S2 (sense), 5'-TGCTCTGACCACATTCTG
AS2 (antisense), 5'-AGGTGGAAAGCTCCTCAG
30 cycles (94°C for 0.5 min, 55°C for 0.5 min, and 72°C for 0.5 min) after the initial denaturation step (94°C for 5 min) in the presence of 5% DMSO.

The primer pair and PCR conditions used to amplify exon 2 of the p15 gene were

**Exon 2:** S3 (sense), 5'-GGGTCTCGACCACACTG
AS3 (antisense), 5'-AGGAATTCGCTGTTGAGAATTGA
30 cycles (94°C for 0.5 min, 55°C for 0.5 min, and 72°C for 0.5 min) after the initial denaturation step (94°C for 5 min) in the presence of 5% DMSO.

**Note:**

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2 To whom requests for reprints should be addressed.

1 The abbreviations used are: SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; PCR-SSCP, polymerase chain reaction-single strand conformation polymorphism.
30 cycles (94°C for 0.5 min, 55°C for 0.5 min and 72°C for 0.5 min) after the initial denaturation step (94°C for 5 min) in the presence of 5% DMSO. PCR products were electrophoresed in a 1.5% agarose gel. The quality of genomic DNAs was confirmed by PCR amplification of a similar-sized fragment (~400 base pairs) of the β-actin gene using the following PCR primer pair: sense, S4 (5'-GAAACTACCTTCAACTCCATC); and antisense, AS4 (5'-CTAGAAGCATTTCGCTTGAGTGGAGGGCC). The PCR amplification consisted of 35 cycles (94°C for 30 s, 55°C for 2 min, and 72°C for 3 min) after the initial denaturation step (94°C for 5 min).

**PCR-SSCP Analysis for the Detection of Subtle Alterations in the p16 and p15 Genes.** Lung cancer cell lines which did not carry homozygous deletions in the p16 and p15 genes were further analyzed for the presence of subtle mutations by the PCR-SSCP method as described previously (9). PCR-SSCP analysis of exons 1 and 2 of the p16 gene was carried out on genomic DNAs using the same PCR primers and conditions described above. PCR-SSCP analysis of the entire coding region of the p15 gene was also performed using random primed cDNAs and the following PCR primers: S5 (sense), 5'-AGCAAGCTTAGTTTACGGCCAACGGTGGAT; and AS3 (antisense, see above). PCR amplification consisted of 30 cycles (94°C for 0.5 min, 55°C for 0.5 min, and 72°C for 0.5 min) after the initial denaturation step (94°C for 5 min) in the presence of 5% DMSO. PCR products were digested with Smal before electrophoretic separation to yield a higher sensitivity due to their smaller size. PCR products of cell lines with an abnormal electrophoretic mobility were cloned into pGEMT (Promega, Madison, WI), and the plasmid DNAs were prepared from pooled clones and sequenced as described previously (9).

**Results and Discussion**

We first examined genomic DNAs of 20 SCLC cell lines derived from 15 patients as well as 20 NSCLC cell lines originating from 18 patients for the presence of homozygous deletions of the p16 and p15 genes. We observed homozygous deletions of p16 and p15 in 6 NSCLC cell lines (ACC-LC-71, -91, -314, and -319 as well as SK-MES-1 and RERF-LC-OK) established from 5 independent cases, whereas none of the SCLC cell lines carried homozygous deletions, showing a clear specificity in the affected histological types (Fig. 1). All homozygous deletions identified in the present study involved both the p16 and p15 loci and were confirmed by the Southern blot analysis (data not shown). The presence of homozygous deletions in both ACC-LC-314 and -319 was of particular interest, because these two cell lines originated at different time points from distinct metastatic sites (skin metastasis and pleural effusion, respectively) from a single patient, suggesting that the deletions took place prior to metastasis in this case. Careful microdissection of a cryostat section of his in vivo tumor specimen, from which ACC-LC-314 originated, enabled extraction of genomic DNA from a small piece without any contamination of normal stromal cells and infiltrating inflammatory cells and we were able to confirm that the homozygous deletions of p16 and p15 in ACC-LC-314 had indeed occurred in the patient’s corresponding tumor in vivo, excluding the possibility of an in vitro artifact (Fig. 2). Although we could not confirm their presence in the corresponding tumor samples for ACC-LC-71 and -91, because of significant infiltration of nonneoplastic cells, homozygous deletions were found in early passages of ACC-LC-71 (passage 3) and -91 (passages 1 and 3) (data not shown). Primary tumors corresponding to RERF-LC-OK and SK-MES-1 were not available for examination of the nature of the homozygous deletions.

Identification of homozygous deletions in vivo suggests that the p16 and p15 genes may play a role in the oncogenesis of lung cancer. It is, however, still possible that these genes may be fortuitously involved in the homozygous deletions and as yet unidentified target genes for the deletions could reside within the surrounding genomic region. Since intragenic subtle mutations of...
the p16 and p15 genes can lend support for the authenticity of target genes in lung cancer, PCR-SSCP analysis was performed to search for such genetic changes in the p16 and p15 genes (Fig. 3). Abnormal mobility shifts were observed in exon 1 of p16 in two NSCLC cell lines (ACC-LC-94 and -174). Notably, these cell lines had been established from a single patient at the time of diagnosis (cervical lymph node metastasis) and after treatment (pleural effusion), respectively. Since these results strongly suggest that genetic changes occurred in vivo before metastasis and treatment, we performed sequence analysis of microdissected specimens of the corresponding tumor from which ACC-LC-94 originated (Fig. 4). Identical nonsense mutations (TAC to TAA) at codon 36 were observed not only in both ACC-LC-94 and -174 cell lines but also in the corresponding in vivo tumor sample. This nonsense mutation was not present in his peripheral blood lymphocytes, clearly indicating that it occurred as an in vivo somatic mutation in the patient's tumor before metastasis to the cervical lymph node.

In contrast to previously identified tumor suppressor genes such as p53 (2, 10), it appears that homozygous deletion rather than point mutation is the predominant alteration at the p16 and p15 loci. This predominance of homozygous deletion might be favored as a mechanism which causes simultaneous inactivation of both genes with similar sequence structures and functions (3, 11). Another interesting feature of p16 and p15 involvement in lung cancer is that they are preferentially altered in NSCLC but not in SCLC. This apparent specificity in terms of the affected histological type of lung cancer is of interest, because these two histological subtypes have distinct clinicopathological and molecular biological characteristics. Normal progression through G1 is thought to be promoted at least partly by the activity of the cyclin-dependent kinases CDK4 and CDK6, which phosphorylate and inactivate the retinoblastoma gene product, Rb (12, 13). The fact that p16 and p15 are potent inhibitors of CDK4 and CDK6 would provide an explanation for the lack of p16 and p15 alterations in SCLC in which the Rb gene is mutated in virtually all cases (1).
The actual frequency of p16 and/or p15 alterations in vivo could be even lower than that observed in cell lines, since inactivation of p16 and/or p15 may have conferred a selective growth advantage in vitro, leading to successful establishment of cell lines. Similar discordance and in vitro selection have been reported in lung cancer with regard to ras and p53 alterations (16, 17).

In summary, we have shown that p16 and/or p15 alterations can occur in NSCLC in vivo. The present observations lend support to a conclusion of possible involvement in the oncogenesis of NSCLC, although alterations of p16 and/or p15 appear to be less frequent than initially anticipated (12). Our results and others (18, 19) also suggest that their oncogenic contribution may vary with the tumor type, since highly frequent p16 alterations comparable to the incidence of 9p deletions were recently reported in primary esophageal cancers in vivo (20).

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References

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