Inactivation of LKB1/STK11 Is a Common Event in Adenocarcinomas of the Lung

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Abstract

Frequent losses of chromosome 19p have recently been observed in sporadic lung adenocarcinomas, targeting the location of a critical tumor suppressor gene. Here we performed fine mapping of the short arm of chromosome 19 and found that the LKB1/STK11 gene mapped in the minimal-deleted region. Because germ-line mutations at LKB1/STK11 result in the Peutz-Jeghers syndrome and an increased risk of cancer, we performed a detailed genetic screen of the LKB1/STK11 gene in lung tumors. We detected a high frequency of somatic alterations (mainly nonsense mutations) in primary lung adenocarcinomas and in lung cancer cell lines. Thus, our findings demonstrate for the first time that LKB1/STK11 inactivation is a very common event and may be integrally involved in the development of sporadic lung adenocarcinoma.

Introduction

A major challenge in cancer genetics is the identification of the complete set of genes that are mutated in sporadic tumors. In the past 10 years, a plethora of chromosomal arms showing a high frequency of loss of heterozygosity have been reported, which are likely targeting inactivation of critical tumor suppressor genes (1, 2). However, for most chromosomes the targeted gene still remains unidentified and, unfortunately, to date, only a few genes harboring functionally disrupting mutations have been described in nonhereditary tumors. In lung cancer, the most relevant genetically inactivated tumor suppressor genes are p53, p16, and Rb (3, 4). Mutations at these genes usually cluster within the different histological types that constitute lung tumors (5). However, data on the patterns of chromosomal abnormalities that characterize lung primary tumors indicate that other important oncogenes/tumor suppressor genes play a critical role in the development of this tumor type (6, 7).

Recently, we and others reported frequent deletions at the short arm of chromosome 19 in LADs (8, 9). Many genes potentially important for cancer development are located on this chromosomal arm such as the LKB1/STK11 gene. Germ-line mutations of the LKB1/STK11 gene cause PJS (10, 11), an autosomal dominantly inherited disorder characterized by the constellation of intestinal hamartoma, oral mucocutaneous hyperpigmentation, and increased risk for gastrointestinal and extraintestinal malignancies (12–14). The LKB1/STK11 protein is a serine-threonine kinase in which its biological function has not been completely elucidated, nor have its in vivo substrates been identified. However, in vitro studies have demonstrated its specific growth-suppressing activity in cells lacking functionally active LKB1/STK11 protein (15). Although LKB1/STK11 is implicated as a tumor suppressor gene in PJS, only a limited number of inactivating mutations has been identified in sporadic tumors (16).

The high frequency of loss of heterozygosity at chromosome 19p that we had observed in lung tumors prompted us to search for the tumor suppressor gene located on this chromosomal arm.

Materials and Methods

Tumor Specimens. Primary lung tumors and matching normal lymphocytes were collected from 42 patients diagnosed with lung cancer and treated at the Johns Hopkins University School of Medicine between 1995 and 1999. None of the patients showed any of the clinical manifestations of PJS. Cell lines were purchased at the American Type Culture Collection (Manassas, VA) and grown under the recommended conditions.

DNA Extraction. Representative sections from tissue used for DNA extraction were stained with H&E. Fresh-frozen tissue was meticulously dissected to ensure that the specimen contained at least 75% tumor cells. Approximately 35 12-μm sections were then collected and placed in 1% SDS/protease K (0.5 mg/ml) at 58°C for 24 h. Digested tissue was then subjected to phenol-chloroform extraction and ethanol precipitation. Normal, control DNA was obtained by venipuncture and isolation of lymphocyte DNA as described previously (17). DNA from cell lines was extracted after standard protocols.

Fine Deletion Mapping. To search for the minimal region of deletion on chromosome 19p, we used 18 highly polymorphic microsatellite markers from Research Genetics (Huntsville, AL): D19S886, D19S883, D19S565, D19S424, D19S894, D19S534, D16S216, D19S427, D19S901, D19S592, D19S391, D19S413, D19S586, D19S355, D19S914, D19S411, D19S410, and D19S460. Before amplification, 200 ng of one primer from each pair was end labeled with [32P]. PCR reactions were carried out in a total volume of 10 μl containing 20 ng of genomic DNA, 2 ng of labeled primer, and 60 ng of each unlabeled primer (17). PCR amplifications of each primer set were performed for 35 cycles, consisting of denaturation at 95°C for 30 s, annealing at 55°C for 60 s, and extension at 72°C for 60 s. One-third of the PCR product was separated on 8% urea-formamide-polyacrylamide gels and exposed to X-ray film from 4 to 48 h. Cases were considered informative if two alleles were present in the normal sample. Allelic loss was scored if a reduction in intensity of ≥50% was documented visually by both experienced observers (D. S., M. S.-C.) in the tumor allele compared with the corresponding allele in the normal DNA.

Screening for Point Mutation. One hundred ng of genomic DNA were used for exon amplification. Cycle sequencing reaction was performed according to the manufacturer’s instructions (Perkin-Elmer, Roche Molecular Systems, Inc., Branchburg, NJ). Sequencing conditions were: 95°C 30 s, 56°C 1 min, and 72°C 1 min for 1 cycle; and 95°C 30 s, 53°C 1 min, and 72°C 1 min for 34 cycles. Sequencing products were separated by electrophoresis through 6% polyacrylamide gels and exposed to X-ray film from 4 to 48 h. Sequencing products were separated by electrophoresis through 6% polyacrylamide gels, dried, and finally exposed to a film for 24–48 h (18). The primers used for the screening of point mutations of the LKB1/STK11 gene were as follows: primers for PCR: Exon 1, PJ1F 5′-AGAAGGGCCCTACCCCG-3′ and PJ1R 5′-ACCACGACACCCGTAC-3′; Exons 2–3, PJ2F 5′-CTGTTGGTGC-GCTGATACAC-3′ and PJ2R 5′-TATCAGGACAAGCAGTGTGG-3′; Exons 4–5, PJ4F 5′-CCCTGACCTTTGTGACCC-3′ and PJ5R 5′-AGTTGGCCGT-GTGGTGAGTGC-3′; Exons 6–7, PJ6F 5′-GCTAACACCTTGACTGACC-3′.
and PJ7R 5'-CTAACCAGCTGCCACATG-3' and PJ9R 5'-GACAGCGCAGCTGACAGG-3'; Exon 9, PJ9F 5'-GTCGAGTGCTGAAGTCGTC-3' and PJ9R 5'-CAGGAGGTGCGCTGACAGG-3'; and primers for cycle sequencing: Exon 1 SPJ1, 5'-CCGGCGACTCAGGGC-3'; Exon 2 SPJ2, 5'-GAGGCCCCCGGGCTCC-3'; Exon 3 SPJ3, 5'-CCCTGAGCCTGGGTCTCC-3'; Exon 4 SPJ4, 5'-CAGCTGCAAAAGGGGACCTC-3'; Exon 5 SPJ5, 5'-CCTCCCCGGGCACTC-3'; Exon 6 SPJ6, 5'-CCTGGGGCTGCGGGC-3'; Exon 7 SPJ7, 5'-GTTCTCAGGGGTCCCTGCC-3'; Exon 8 SPJ8, 5'-GTGCCAGCCTGACAGG-3'; Exon 9 SPJ9, 5'-CTTGAGCTGACAGG-3'.

Promoter Hypermethylation Analysis. To screen for promoter hypermethylation, we initially analyzed primary LADs as described in some PJS families (12) and previously described in the germline of families with PJS (10, 11). In addition to the somatic point mutations, we observed three new examples of some mutations are depicted in Fig. 2A. The frameshift mutation found in a primary tumor was identical to another mutation, previously described in the germline of families with PJS (10, 11). Lung carcinomas do not appear frequently in this syndrome, but a few cases of LAD have been described in some PJS families (12–14). Given this background, we decided to examine the LKB1/STK11 gene first.

To elucidate the role of LKB1/STK11 in lung cancer, we performed complete molecular analysis of the gene to search for (a) point mutations in all exons and intron-exon boundaries, (b) partial and complete deletions and insertions, and (c) gene promoter hypermethylation using MSP. We initially analyzed primary LADs selected for loss of heterozygosity at chromosome 19p from 20 patients and nine lung cancer cell lines. None of the patients or their families had the clinical characteristic of PJS. The LKB1/STK11 gene consists of nine coding exons separated by eight introns (Fig. 1B). Sequence analysis of each individual exon revealed mutations in five of the primary tumors analyzed and in two lung cancer cell lines (A549 and H23). The mutations found in the primary tumors were absent in DNA from the matched blood specimens. We identified the putative candidate tumor suppressor gene located within our MDR, we examined the mapviewer database from the Lawrence Livermore National Laboratory web site. Among several interesting candidates was the LKB1/STK11 gene, responsible for PJS (10, 11), that predisposes to gastrointestinal, pancreatic, ovarian, testicular, breast, and uterine tumors (14). Lung carcinomas do not appear frequently in this syndrome, but a few cases of LAD have been described in some PJS families (12–14). Given this background, we decided to examine the LKB1/STK11 gene first.

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INACTIVATION OF LKB1/STK11 IN LUNG ADENOCARCINOMAS

<table>
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<tr>
<th>Sample Identification</th>
<th>Codon location</th>
<th>DNA change</th>
<th>Predicted effect</th>
<th>K-ras</th>
<th>p53</th>
<th>p16</th>
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<td>W-Ter</td>
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</table>

* Primary tumors were selected for loss of heterozygosity at chromosome 19p.
* Six of 14 primary adenocarcinomas without LKB1/STK11 alterations harbored p16/INK4A inactivation. Kras, p53, and p16 genes mutational status of primary tumors was reported previously (32, 33).

Epigenetic inactivation represents an alternative mechanism of tumor suppressor gene inactivation in human cancer. Previous reports have shown, in tumor cell lines, a direct correlation between LKB1/STK11 promoter hypermethylation and absence of gene transcription (21). We detected gene promoter hypermethylation in one primary tumor, suggesting that this mechanism of LKB1/STK11 gene inactivation is not common in sporadic LADs (Fig. 2B).

Partial deletions and inversions containing some of the exons of the LKB1/STK11 gene have been detected in the germline of individuals with PJS (10, 11). We tested for the presence of large homozgyous deletions and small deletions/insertions using indirect approaches. Homozgyous deletions were analyzed using a previously described method (19). To screen for the presence of small deletions and insertions, we used a long-range PCR approach. No apparent homozygous deletions or small deletions/insertions were detected in our cell lines (nonadenocarcinomas, H209, H1155, H1299, H1618, H1770, and adenocarcinomas H522, H23, A549, H1558).

Interestingly, among the nine lung cancer cell lines, LKB1/STK11 abnormalities were detected only in adenocarcinomas (Table 1). To evaluate whether LKB1/STK11 alterations occur exclusively in this specific lung cancer subtype, 12 primary squamous cell carcinomas of the lung were also screened for genetic and epigenetic abnormalities. No alterations were found in any of those primary tumors. One patient showed a germ-line missense variant of unclear significance in the COOH terminus region (Cys→Ser at codon 418), distant from the kinase domain. Taken together, our results indicate that LKB1/STK11 alterations arise in adenocarcinomas but not in squamous cell carcinomas of the lung. These observations have special significance because they are in agreement with the common finding of adenocarcinomas but not squamous cell carcinomas in malignant tumors from individuals affected with PJS (13, 14).

We further tested LKB1/STK11 protein status in six different lung cancer cell lines (Fig. 2C). Adenocarcinoma cell lines showed either absence of protein because of truncated mutations (A549 and H23) or increased protein stability (H522) compared with nonadenocarcinoma cell lines. Increased amounts of LKB1/STK11 protein in the adenocarcinoma cell line without LKB1/STK11 gene mutations (H522) may reflect the presence of abnormalities at upstream/downstream components of the same biochemical pathway. Similar observations have been reported in other tumor suppressors such as P16. It is common that tumors without p16/INK4A gene alterations tend to up-regulate P16 protein. This usually occurs as a consequence of Rb gene inactivation (3, 24). We could not test primary tumors for protein expression because of technical limitations with currently available antibodies.

LKB1/STK11 is a 436 amino acid serine/threonine kinase widely
expressed in all tissues (11). LKB1/STK11 shows weak sequence similarity with p53 (30). Moreover, LKB1/STK11 induces G1 cell cycle arrest in the G361 melanoma cell line (15), which might be mediated by its interaction with BRG1 protein, an essential component of the chromatin remodeling factor (31). We studied how LKB1/STK11 inactivation correlated with other common genetic alterations in lung cancer. Our samples of p16INK4A inactivation were evaluated using: (a) manual sequencing to detect point mutations in exons 1 and 2; (b) MSP to detect promoter hypermethylation; and (c) microsatellite markers to check for homozygous deletions (32). We compared the presence of p16INK4A and LKB1/STK11 inactivation and found that both rarely occur simultaneously (Table 2), supporting a role for LKB1/STK11 in cell cycle regulation. No association was found between LKB1/STK11 gene inactivation and mutations in Kras and p53.

Somatic mutations at LKB1/STK11 occur in one-third of LADs and, thus, constitute a characteristic genetic signature of LADs. Although it is widely believed that LKB1/STK11 gene mutations are uncommon outside PJS, our data leave little doubt that LKB1/STK11 inactivation may be an important clue to the development of the >50,000 sporadic LADs that occur every year in the United States alone.

References

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