Advances in Brief

Inactivation of *LKB1/STK11* Is a Common Event in Adenocarcinomas of the Lung[1]

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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[4] (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 LKB/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 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/proteinase 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, D19S535, D19S914, D19S411, D19S410, and D19S460. Before amplification, 200 ng of one primer from each pair was end labeled with [^{32}P]. 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 manufacture's instructions (Perkin-Elmer, Roche Molecular Systems, Inc., Brachburg, 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 for 1 min, and 72°C 1 min for 34 cycles. 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′-AGGAAGGACCGCTCACCCG-3′ and PJ1R 5′-AACCATCAGCACCGTGACTG-3′; Exons 2–3, PJ2F 5′-CGTTGGGTCG-GCTGATACAC-3′ and PJ3R 5′-TATCAGGACAAGCAGTGTGG-3′; Exons 4–5, PJ4F 5′-CCTGGACTTCTGTGACTTCC-3′ and PJ5R 5′-AGTGTGCGT-GTGGTGAGTGC-3′; Exons 6–7, PJ6F 5′-GTCAACCACCTTGACTGACC-3′
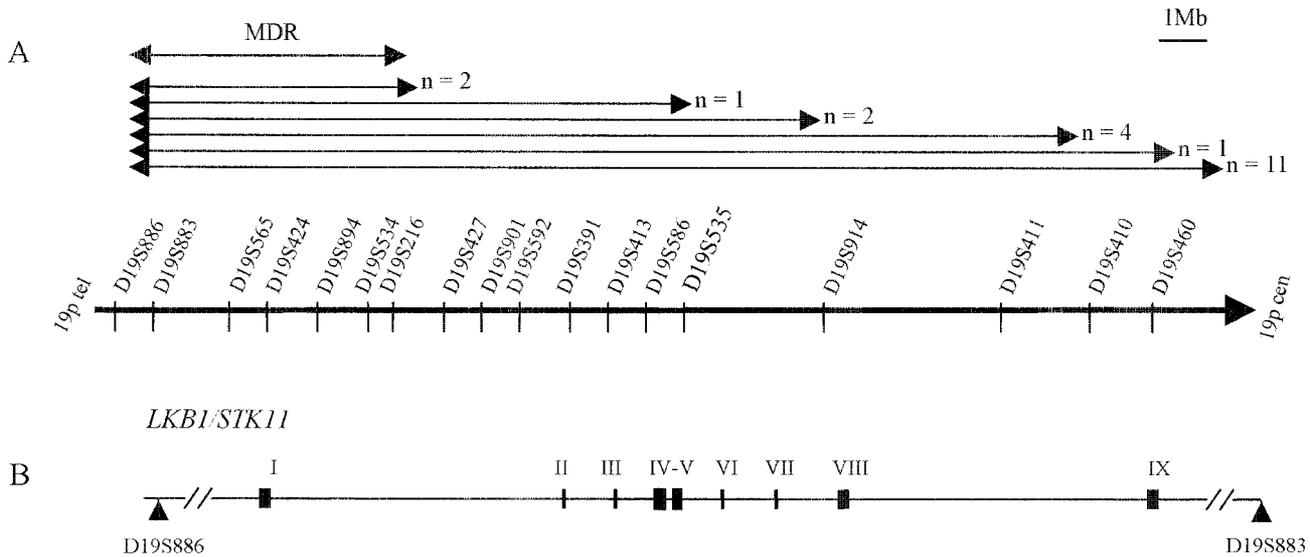
Fig. 1. Identification of the tumor suppressor gene located on chromosome 19p in LADs. *A*, physical map of the microsatellite markers used in fine deletion mapping. The MDR was localized between the telomere and marker *D19S216*. Each *arrow* represents the length of the chromosomal deletions and the number of tumors showing those deletions. *B*, schematic representation of the structure of *LKB1/STK11* gene.

and PJ7R 5′-CTCAACCAGCTGCCCACATG-3′; Exon 8, PJ8F 5′-CCTGAG-TGTGTGGCAGGTAC-3′ and PJ8R 5′-TGTTGCAGACAGGCAGGCAC-3′; Exon 9, PJ9F 5′-GCAGCAGCTGTAAGTGCGTC-3′ and PJ9R 5′-CACCAT-GACTGACTAGCGCG-3′; and primers for cycle sequencing: Exon 1 SPJ1, 5′-CCGCGGACTCAGGGC-3′; Exon 2 SPJ2, 5′-GAGGCCCCGCGGTCC-3′; Exon 3 SPJ3, 5′-CCCTGAGCTGTGTGTCC-3′; Exon 4 SPJ4, 5′-CAGCTG-CAAAGGGGACC-3′; Exon 5 SPJ5, 5′-CCCTCCCGGGCACTC-3′; Exon 6 SPJ6, 5′-CCTGGGGCTGCGGGC-3′; Exon 7 SPJ7, 5′-GGTCTCAGGCCT-GTGC-3′; Exon 8 SPJ8, 5′-GTGCCAGCCTGACAGG-3′; Exon 9 SPJ9, 5′-CCTCAGCTCAGGCCAC-3′.

Screening of Homozygous Deletions. To screen for homozygous deletions, we selected two length polymorphisms located in intron 1 (LP1) and in intron 2 (LP2), respectively. Apparent retention of heterozygosity flanked by loss of heterozygosity in closely mapped polymorphic microsatellite markers is indicative of homozygous deletion (19). The sequences of the primers used for the amplification of these polymorphisms were as follows: LP1 LP1F, 5′-CACAGCGAGACTCTGTCTC-3′; LP1R, 5′-CGTCTGTCTTCCCATTGAC-3′; LP2 LP2F, 5′-CCCTGAGCTGTGTGTCC-3′; and LP2R, 5′-TATCAG-GACAAGCAGTGTGG-3′.

Screening of Partial Deletions/Insertions. Deletions in the vicinity of exons 2–8 were tested using a long-range PCR strategy by amplifying a 5-Kb fragment containing exons 2–8. The primers used were PJ2F and PJ8R (see sequences above), and the PCR reaction was performed with Elongase Enzyme Mix (Life Technologies, Inc.) following the manufacture's recommendations.

MSP. For *LKB1/STK11* promoter hypermethylation analysis, 1 μg of DNA was denatured by NaOH and then modified by sodium bisulfite as described previously (20). PCR was performed separately with methylation-specific primers and nonmethylation primers for each tumor sample. Controls without DNA and positive controls for unmethylated and methylated reactions were performed for each set of PCR reactions. PCR products were analyzed on nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination (21).

LKB1/STK11 Statement in Cell Lines. Total proteins (40 μg) were analyzed by 10% SDS/PAGE and blotted according to standard protocols (22). Polyclonal antigoat polyclonal IgG (Santa Cruz Biotechnology) was used to detect LKB1/STK11 protein by enhanced chemiluminescence in six lung cancer cell lines (H1155, H1299, H209, H522, A549, and H23).

Results and Discussion

First, we narrowed the minimal region of deletion on chromosome 19p by screening 30 primary LAD specimens with 18 highly polymorphic microsatellite markers (Fig. 1*A*). Among the 30 tumors analyzed, 21 showed loss of heterozygosity at any of the markers evaluated. Two tumor specimens set the MDR, a 5-Mb interval that maps between the telomeric end and microsatellite marker D19S216 (Fig. 1*A*).

To identify the putative candidate tumor suppressor gene located within our MDR, we examined the mapviewer database from the Lawrence Livermore National Laboratory web site.[5] 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.

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. 1*B*). 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 lymphocytes, indicating that these alterations were somatic events. Among the seven mutations detected, six were nonsense and one was a frameshift, all of them predicting a truncated LKB1/STK11 protein with an incomplete catalytic domain (Table 1). Representative examples of some mutations are depicted in Fig. 2*A*. The frameshift mutation found in a primary tumor was identical to another mutation, previously described in the germline of families with PJS (10, 11). In addition to the somatic point mutations, we observed three new germ-line variants that likely represent polymorphisms because they do not result in amino acid changes (codons 46, 88, and 222).

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[5] Internet address: http://www-bio.lnll.gov.

Table 1 *Somatic mutations of* LKB1/STK11 *in primary LADs and in lung cancer cell lines*[a]

Sample Identification	Codon location	DNA change	Predicted effect	K-ras	p53	p16[b]
1012-LAD	37	cag-tag	Q-Ter	mt[c]	mt	wt
758-LAD	44	aag-tag	K-Ter	wt	mt	wt
898-LAD	210	tgc-tga	C-Ter	wt	wt	wt
946-LAD	223	gag-tag	E-Ter	wt	mt	wt
1122-LAD	279	1C deletion	Stop codon 286	mt	wt	wt
997-LAD	Promoter	PH	No protein	wt	mt	wt
A549[d]	37	cag-tag	Q-Ter	mt	wt	mt
H23	332	tgg-tga	W-Ter	mt	mt	wt

[a] Primary tumors were selected for loss of heterozygosity at chromosome 19p.

[b] 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).

[c] PH, promoter hypermethylation; mt, mutant; wt, wild type.

[d] Mutations in cell lines were homozygous. Lung tumors analyzed for *LKB1/STK11* alterations: 20 primary LADs; 12 primary squamous cell carcinomas; and nine lung cancer cell lines (nonadenocarcinomas, H209, H1155, H1299, H1618, H1770, and adenocarcinomas H522, H23, A549, H358).

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. 2*B*).

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 homozygous deletions and small deletions/insertions using indirect approaches. Homozygous 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 screening. However, our methods did not allow us to discard partial deletions, including exons 1–2 or 8–9 and other complex gene rearrangements.

Overall, we observed that 33% (6 of 20 primary tumors and two of four cell lines) of LADs harbor *LKB1/STK11* gene inactivation (Table 2). Given the intrinsic difficulties of the genetic analysis in primary tumors, necessarily contaminated with normal cells, and the limited amounts of genomic DNA, we expect this frequency to be an underestimate. Thus, it is likely that a substantial proportion of primary LADs contain *LKB1/STK11* alterations. A single previous report on the screening of *LKB1/STK11* mutations in lung tumors identified one somatic missense mutation among 12 primary LADs (23). The use of single-strand conformational polymorphism as a mutational screening method (a lower sensitivity technique compared with manual sequencing), the evaluation of tumors not selected for loss of heterozygosity (although loss of heterozygosity at *LKB1/STK11* locus in LADs

is ~70%), and chance may help explain the differences with our findings.

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. 2*C*). 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

Fig. 2. *LKB1/STK11* molecular alterations. *A,* examples of *LKB1/STK11* gene point mutations in primary tumors (T) and cell line A549, compared with matched normal DNA from lymphocytes (N) or cell lines without *LKB1/STK11* gene mutations (H522). *B,* MSP of lung tumors. The presence of promoter hypermethylation was confirmed in sample no. 997. U, unmethylated reaction; M, methylated reaction; IVD, *in vitro* methylated DNA; NL, normal lymphocytes; C, water as negative control. *C,* Western blot analysis of LKB1/STK11 in several lung cancer cell lines. The *light band* observed in the A549 lane is probably because of the initiation of translation at an internal in-frame methionine (codon 51). Non-AD, nonadenocarcinoma cell lines; AD, adenocarcinoma cell lines.
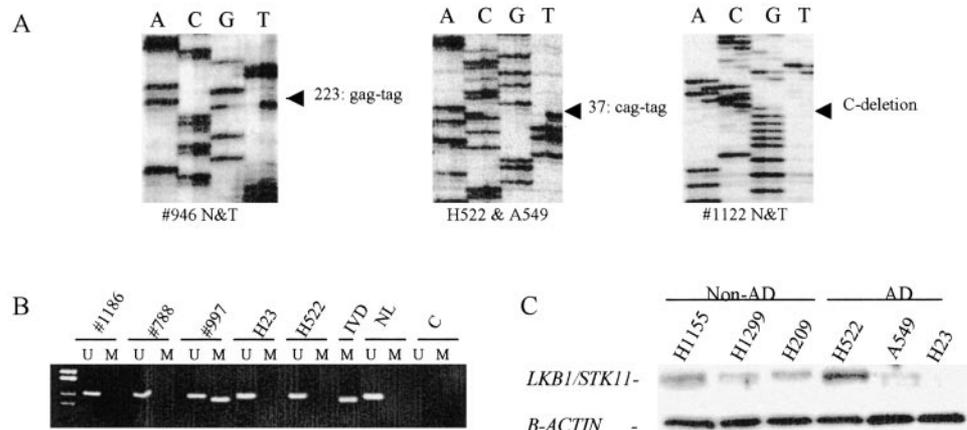
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Table 2 *Correlation between LKB1/STK11 gene inactivation and other genetic alterations in LADs*

	Kras[a]		p53[a]		p16/INK4A[a]	
	+	−	+	−	+	−
LKB1/STK11 +	4	4	5	3	1	7
LKB1/STK11 −	7	9[b]	7	9[b]	10	6[c]

[a] *Kras, p53,* and *p16* genes mutational status of primary tumors was reported previously (32, 33).

[b] Fisher's Exact test. Nonsignificant.

[c] Fisher's Exact test ($P = 0.03$).

expressed in all tissues (11). LKB1/STK11 shows weak sequence homology to many protein kinase over the conserved catalytic core of the kinase domain that is common to both serine/threonine and tyrosine protein kinase family members (25). XEEK1 and Par-4 proteins from *Xenopus lavis* and *Caenorhabditis elegans*, respectively, are LKB1/STK11 orthologs (26, 27). Little is known about the function of any of these proteins, except that they are implicated in the early development of these organisms (26, 27). A mouse homologue has also been identified that shares 88% amino acid identity with human LKB1/STK11 protein (28). Mice deficient in *LKB1/STK11* die at mid-gestation, and embryos show several important defects such as vascular abnormalities with an increase in the expression of vascular endothelial growth factor (29). LKB1/STK11 is widely expressed in all tissues and localizes in the nucleus and cytoplasm (30), although, it translocates to the mitochondria during apoptosis through its physical association with p53 (30). Moreover, LKB1/STK11 induces G_1 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 *p16/INK4A* 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 *p16/INK4A* 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.

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