Short Communication

LKB1 Somatic Mutations in Sporadic Tumors

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Germline mutations of *LKB1*/Peutz-Jeghers syndrome gene predispose carriers to hamartomatous polyposis of the gastrointestinal tract as well as to cancer of different organ systems. Although Peutz-Jeghers syndrome patients frequently present with neoplasms of the colon, stomach, small intestine, pancreas, breast, ovaries, and cervix, somatic mutations appear to be rare in the sporadic tumor types thus far studied (colorectal, gastric, testicular, and breast cancers). To evaluate whether somatic mutations of *LKB1* contribute to the tumorigenesis of yet unstudied tumor types, we screened 14 cell lines and 129 tumor specimens from different cancers for a genetic defect in *LKB1*. Six melanoma and eight myeloma cell lines were scrutinized for *LKB1* somatic mutations by genomic sequencing. No changes were found in the coding *LKB1* sequence and exon/intron boundaries. Next, we analyzed 12 pancreatic, 8 gastric, 12 ovarian granulosa cell, 26 cervical, 28 lung, 24 soft tissue, and 19 renal tumors by single-strand conformational polymorphism analysis. Three changes in *LKB1* coding nucleotide sequence were identified. One base pair deletion at A957 and G958 substitution by T occurred in a cervical adenocarcinoma sample, resulting in a frameshift and premature stop codon at position 335. Substitution of A581 by T occurred in a lung adenocarcinoma sample, resulting in the change of aspartic acid at position 194 to valine. A loss of another allele was detected in this sample. One silent change, C1257T, was found in a pancreatic carcinoma sample. The changes were not present in the matched normal tissue DNA samples. Our results suggest that mutational inactivation of *LKB1* is a rare event in most sporadic tumor types. (*Am J Pathol* 1999, 154:677–681)

Peutz-Jeghers syndrome (PJS) is an autosomal dominantly inherited condition characterized by gastrointestinal hamartomatous polyposis and mucocutaneous pigmentation.¹ Although hamartomas are characteristic of the syndrome, hyperplastic and adenomatous polyps are occasionally detected. Mucocutaneous melanin pigmentation tends to be present on the lips, oral area, and buccal mucosa. Melanin spots are typically absent at birth but develop during childhood and may diminish or disappear during aging.^{1,2} PJS patients frequently develop various neoplasms, and an 18-fold increased risk of cancer has been reported in PJS patients.³ The neoplasms in PJS patients are usually diagnosed at a relatively young age, and survival is worse than expected in the general population.⁴ Gastrointestinal tract cancers frequently occur in PJS patients, although an excess of extraintestinal cancer is also present.^{3,4} The PJS intestinal hamartomas occasionally contain an adenomatous component.^{4–6} A hamartoma-adenoma-carcinoma sequence in PJS polyposis has been proposed. A 100-fold excess of pancreatic carcinoma has been reported in 31 PJS patients.³ Interestingly, some rare tumor types can be found relatively frequently in PJS patients. Adenoma malignum, being very rare in the general population, appears quite frequently in PJS patients as up to 10% of all cases are found in patients with PJS.⁷ A significant number of PJS female patients have sex cord tumors with annual tubules (SCTAT) that are typically multifocal, bilateral, and benign.⁸ Sertoli cell tumors are very rare testicular neoplasms; several studies have reported cases of these tumors in the patients with PJS.^{9,10} An approximately fivefold increased risk of early-onset breast cancer appears to be associated with PJS.¹¹ Cervical adenocarcinoma and ovarian (granulosa cell) tumors are not a rare finding in PJS female patients.¹² A small number of cancers affecting lung, gall bladder, bile duct, basal cells, and blood stem cells have been described in PJS patients.^{3,4,13}

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Recently, germline mutations in *LKB1* have been associated with PJS.¹⁴ The gene encodes a serine/threonine kinase that is highly homologous (87%) to *Xenopus* serine/threonine kinase *XEEK1*.¹⁵ A high degree of homology was detected between *LKB1* and a mouse EST (http://www.ncbi.nlm.nih.gov/irx/cgi-bin/birx_doc?genbank+431188), suggesting the existence of a mouse homologue. *LKB1* is a candidate tumor suppressor gene. Loss of heterozygosity analysis on PJS polyps, derived from one patient, showed that a deletion had occurred in the chromosome inherited from the healthy parent;¹⁶ subsequently it was shown that the other allele was mutated in the germline. *LKB1* mutations are typically of inactivating nature, often causing truncation of the protein product.^{14,17}

Often the genes involved in hereditary cancer syndromes are also targets of somatic mutations. Several studies, with an exception of one study on colorectal cancer,¹⁸ have reported a low frequency of *LKB1* somatic mutations in colorectal, testicular, breast, and gastric cancers.^{19–23} Deletion mapping data in sporadic adenoma malignum samples revealed 67% loss of heterozygosity at marker *D19S886* where *LKB1* resides.²⁴ No results on *LKB1* mutation analysis in these samples have been reported so far. The aim of our study was to investigate the frequency of *LKB1* somatic mutations in a wider range of sporadic tumors.

Materials and Methods

Cell Lines and Tumor Specimens

Six melanoma cell lines were used in the *LKB1* mutation analysis: Bowes 2159, SK-MEL-28, SK-MEL-2, A-375, G-361, and MALME-3M. Light myeloma cell lines were also scrutinized. A series of 12 pancreatic, 8 gastric, 12 ovarian, 26 cervical, 28 lung, 24 soft tissue, and 19 renal tumors was studied next. All pancreatic and renal tumors were adenocarcinomas. The gastric cancers were classified as intestinal type (five specimens) and diffuse type (three specimens). All ovarian tumors were of granulosa cell type. Among the cervical tumors, 18 were epidermoid carcinomas and 8 adenocarcinomas. Of the lung tumors, 12 were squamous cell, 3 were large cell, and 1 was a small cell cancer, and 12 were adenocarcinomas. Among the sarcomas, 6 were liposarcomas, 10 were malignant fibrous histiocytomas, 3 were synovial sarcomas, 2 were leiomyosarcomas, 1 was a fibrosarcoma, 1 was an extrasosseal Ewing's sarcoma, and 1 was a malignant schwannoma.

DNA Extraction

DNA samples were prepared from cell lines and fresh-frozen histologically verified tumor specimens according to standard methods. DNA extraction from paraffin-embedded tissue (pancreatic, gastric, and cervical cancers) was performed as described elsewhere.²⁵

Table 1. Oligonucleotide Primers Used for *LKB1* Mutation SSCP Analysis

Exon	Sequence of oligonucleotide primer	Product size (bp)
1a	GTCCAGCATGGAGGTGGT TACTTGCCGATGAGCTTGG	153
1b	CAAGCTCATCGGCAAGTACC ACTTCTTCACGTTGGCCTCC	164
2	GAGGTACGCCACTTCCACAG CTTCAAGGAGACGGGAAGAG	288
3	TGAGCTGTGTGTCCTTAGCG AGTGTGGCCTCACGGAAA	196
4	GTGTGCCCTGGACTTCTGTGA GTGCAGCCCTCAGGGAGT	324
5	ACAGGCACTGCACCCGTT GAGTGTGCGTGTGGTGAGTG	184
6	TCAACCACCTTGACTGACCA ACACCCCAACCCTACATT	251
7	CCAGCTGACAGGCTCCTG CTCTAGCGCCCGCTCAAC	172
8	ACTGCTTCTGGGCGTTTG CACCGTGAAGTCTGAGTGT	205
9	CAGGACAGGTCCCAGAGAG CCAGCCTCACTGCTGCTT	212

Sequencing

LKB1 mutations were analyzed in melanoma and myeloma cell lines by direct genomic sequencing. PCRs were performed using the primer pairs and conditions described in a previous study,¹⁹ and 5 μ l of PCR products was run in 3% agarose (NuSieve, Bioproducts, Rockland, ME) gel to verify the specificity of the PCR. The rest of the reaction product was purified using QIAquick PCR purification kit (QIAGEN, Valencia, CA). Direct sequencing of the PCR products was performed using the ABI PRISM Dye Terminator cycle sequencing kit (Perkin-Elmer Corp., Foster City, CA). Cycle sequencing products were electrophoresed on 6% Long Ranger gels (FMC Bioproducts, Rockland, ME) and analyzed on an Applied Biosystems model 373A automated DNA sequencer (Perkin-Elmer Corp.).

SSCP Analysis

LKB1 mutation analysis using DNA extracted from lung, renal, pancreatic, gastric, ovarian, and cervical cancers and sarcoma specimens was performed by SSCP. Primers used in SSCP analysis are listed in Table 1. A 93% proportion of the coding *LKB1* sequence was covered by these primers. This set of primers amplifies relatively short fragments and was used to facilitate the amplification of low-quality, especially paraffin-embedded tissue-derived, DNA. Also, the length of these PCR products is optimal for SSCP analysis. The reactions were carried out in a 25- μ l reaction volume containing 100 ng of genomic DNA, 1X PCR reaction buffer (Life Technologies, Frederick, MD), 200 μ mol/L each dNTP (Life Technologies), each primer at 0.6 μ mol/L, 1.5 mmol/L $MgCl_2$, and 1 U of AmpliTaqGOLD polymerase (Life Technologies). The following cycling conditions were used: 10 minutes at 95°C, 35 cycles of 45 seconds at 95°C, 45 seconds at 57°C, and 45 seconds at 72°C with the final extension 10 min-

Figure 1. A: A957 deletion and G958T substitution in a cervical adenocarcinoma sample. B: The same position in the matched normal tissue DNA.

utes at 72°C. After PCR, 5 μ l of each sample was mixed with 5 μ l of denaturing loading buffer (95% formamide, 20 mmol/L EDTA, 0.05% bromphenol blue, 0.05% xylene cyanole FF), denaturated for 5 minutes at 94°C, and loaded into a 0.4-mm $\times$ 30-cm $\times$ 45-cm gel. Electrophoresis was performed using gels containing 0.6 $\times$ MDE solution (AT Biochem, Malvern, PA) and 0.6 $\times$ TBE buffer and that were run at 4 W overnight. The gels were silver stained according to standard procedure. DNA fragments showing aberrant bands in SSCP analysis were sequenced as described above.

Cloning of PCR Products

To better evaluate the deletion in the cervical adenocarcinoma DNA sample, the PCR product of exon 8 was cloned into pGEM vector (Promega, Madison, WI) according to the manufacturer's procedure. DH5 α competent cells were transformed with the ligation product according to standard methods. Plasmid DNA was extracted using QIAprep Spin Miniprep kit (QIAGEN). The insert was sequenced with T7 primer.

Results

We screened six melanoma and eight myeloma cell lines for somatic *LKB1* mutations by genomic sequencing. No mutations in the coding sequences and exon/intron boundaries were found. Next, we performed *LKB1* mutation analysis in a series of 12 pancreatic, 8 gastric, 12 ovarian, 26 cervical, 28 lung, 24 soft tissue, and 19 renal tumor specimens. Three samples showed aberrant bands in the SSCP analysis: one cervical adenocarcinoma, one lung adenocarcinoma, and one pancreatic carcinoma. Subsequent sequencing analysis revealed three aberrations in these tumor DNA samples. A957 deletion and G958 substitution to T resulted in a frameshift and premature stop codon at 335 in a cervical adenocarcinoma sample (Figure 1A). No change was found in the corresponding normal tissue DNA sample (Figure 1B). A581 to T change was detected in a lung adenocarcinoma DNA sample (Figure 2A). Sequencing results demonstrated only T, but no A, at this position, indicating the loss of the wild-type allele. No change was found in a matched normal tissue DNA sample (Figure 2B). The same tumor sample displayed only T at a poly-

morphic site of intron three (Figure 2C), whereas the matched normal tissue DNA sample demonstrated C/T polymorphism at the same position (Figure 2D), confirming loss of an *LKB1* allele. The mutation resulted in valine, which substituted aspartic acid at the position 194. The third aberration occurred in a pancreatic adenocarcinoma but not in a matched normal tissue DNA. C1257T change did not result in a substitution of amino acid at codon 419.

Discussion

A total of 14 cell lines and 129 solid tumors representing different tumor types were screened for somatic mutations in *LKB1*. We selected the series of tumor samples by considering the sites and histologies of tumors that affect PJS patients. The most frequent cancer sites in PJS patients are colon, small intestine, stomach, and pancreas.^{3,4} In this regard, we have chosen gastric and pancreatic carcinomas, but, unfortunately, no tumors of the small intestine were available. We also investigated sporadic ovarian granulosa cell tumors as this histological type is characteristic of ovarian tumors developing in female PJS patients. Because the classical histological appearance of PJS polyps contains a significant smooth muscle component, we were prompted to choose sporadic soft tissue sarcomas of different histological types. Although the frequency of melanoma is not increased in PJS patients, mucocutaneous melanin pigmentation is often present in PJS patients. In this regard, we aimed at studying whether the gene defect might be associated with sporadic malignant melanoma. Although only two PJS patients with myeloma and one with lung adenocarcinoma were reported in the literature,^{3,17} we wanted to study whether somatic *LKB1* mutations are present in myeloma cell lines and lung cancers. There is also good evidence that genes involved in hereditary cancer syndromes are somatically mutated in tumors that are not associated with the syndrome; eg, *PTEN* somatic mutations are frequent in endometrial carcinomas, which are not characteristic for Cowden's syndrome patients.²⁶ This prompted us to screen a series of renal tumors that was available for this work.

We found one truncating, one missense, and one silent *LKB1* mutation in the series of tumor samples described above, and no changes were found in the melanoma and

Figure 2. A: A581T change in a lung adenocarcinoma DNA sample. B: The same position in the matched normal tissue DNA. C: T at the polymorphic -51 position of intron three in the same lung adenocarcinoma sample. D: C/T polymorphism at the same position in the matched normal tissue DNA.

myeloma cell lines. The mutation that was found in a cervical adenocarcinoma sample is predicted to cause truncation of the Lkb1 protein. No change occurred in the normal tissue sample. The same mutation has been detected earlier in a PJS patient's germline (unpublished results). One missense type mutation was detected in a lung cancer at position 194, which is conserved between *Xenopus* and mouse homologues in the kinase core domain¹⁴ (unpublished data). The experiments with different *LKB1* mutant constructs have showed that even subtle changes, such as missense mutations changing highly conserved amino acids and small in-frame deletions in *LKB1* kinase domain, lead to impairment of kinase function.¹⁷ Loss of heterozygosity was found in the same lung adenocarcinoma sample, indicating a complete inactivation of Lkb1. The mutation proved to be a somatic event as the analysis of matched normal tissue sample showed no changes in the same position. Finally, we found a silent mutation in a pancreatic carcinoma sample. No polymorphism has been reported in this position. The significance of this change remains to be seen.

The mutation screening methods used in our study do not allow detection of all mutation types, thus perhaps underestimating the frequency of *LKB1* mutations in the studied series. We did not perform Southern analysis or RT-PCR. A subset of *LKB1* mutations are likely to be large genomic rearrangements. On the other hand, methylation as an alternative mechanism of *LKB1* inactivation may take place in a subset of sporadic tumors.

Our study shows that the frequency of *LKB1* somatic mutations in a wide range of sporadic tumors is low. Previous studies have reported small numbers of somatic

mutations in colorectal, testicular, breast, and gastric tumors.¹⁹⁻²³ One study, however, reported a relatively high prevalence of *LKB1* mutations in left-sided invasive colon cancers.¹⁸ These tumors were of Korean origin, whereas the series of cancers analyzed in the other three studies^{19,20,23} were from a Western population. The results of the study performed on Korean sporadic gastric cancers, on the other hand, is in agreement with ours, confirming that somatic *LKB1* mutations are not common in sporadic gastric carcinomas.

In summary, our results support the notion that *LKB1* somatic mutations are rare in sporadic tumors. It remains to be seen whether some specific tumor types can be associated with somatic *LKB1* mutations in the future and whether somatic *LKB1* inactivation by epigenetic mechanisms, eg, methylation, contributes to sporadic tumorigenesis.

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