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Loss of LKB1 Kinase Activity in Peutz-Jeghers Syndrome, and Evidence for Allelic and Locus Heterogeneity

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Summary

Peutz-Jeghers syndrome (PJS) is an autosomal dominant disease characterized by mucocutaneous pigmentation and hamartomatous polyps. There is an increased risk of benign and malignant tumors in the gastrointestinal tract and in extraintestinal tissues. One PJS locus has been mapped to chromosome 19p13.3; a second locus is suspected on chromosome 19q13.4 in a minority of families. The PJS gene on 19p13.3 has recently been cloned, and it encodes the serine/threonine kinase LKB1. The gene, which is ubiquitously expressed, is composed of 10 exons spanning 23 kb. Several LKB1 mutations have been reported in heterozygosity in PJS patients. In this study, we screened for LKB1 mutations in nine PJS families of American, Spanish, Portuguese, French, Turkish, and Indian origin and detected seven novel mutations. These included two frameshift mutations, one four-amino-acid deletion, two amino-acid substitutions, and two splicing errors. Expression of mutant LKB1 proteins (K78I, D176N, W308C, and L67P) and assessment of their autophosphorylation activity revealed a loss of the kinase activity in all of these mutants. These results provide direct evidence that the elimination of the kinase activity of LKB1 is probably responsible for the development of the PJS phenotypes. In two Indian families, we failed to detect any LKB1 mutation; in one of these families, we previously had detected linkage to markers on 19q13.3–4, which suggests locus heterogeneity of PJS. The elucidation of the molecular etiology of PJS and the positional cloning of the second potential PJS gene will further elucidate the involvement of kinases/phosphatases in the development of cancer-predisposing syndromes.

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Introduction

Peutz-Jeghers syndrome (PJS; MIM 175200) is a rare autosomal dominant disease characterized by mucocutaneous pigmentation and gastrointestinal hamartomatous polyposis (Peutz et al. 1921; Jeghers et al. 1949). Patients with PJS demonstrate a high risk for gastrointestinal and extraintestinal cancers (ovaries, testes, breast, and uterine cervix; Giardiello et al. 1987; Spigelman et al. 1989). In addition, benign ovarian tumors may occur in female PJS patients. One locus for PJS has been mapped to chromosome 19p13.3, in the vicinity of marker D19S886, by comparative genomic hybridization and linkage studies (Hemminki et al. 1997), and subsequently has been confirmed by other investigators (Amos et al. 1997; Mehenni et al. 1997; Olschwang et al. 1998; Nakagawa et al. 1998). In the sample of six PJS families studied by Mehenni et al. (1997), a potential second PJS locus, on 19q13.4, in the vicinity of marker D19S891, was suggested. The presumed locus heterogeneity of PJS has also been observed in the sample studied by Olschwang et al. (1998), in which not all families showed linkage to 19p13.3.

In recent studies, the PJS gene on 19p13.3 has been identified (Hemminki et al. 1998; Jenne et al. 1998). Two groups discovered mutations in the LKB1, or STK11, gene in patients with PJS. This gene encodes a potential serine/threonine kinase, first cloned by J.-i. Nezu (unpublished data; Genbank entry U63333), that shows strong homology with the cytoplasmic serine/threonine kinase XEEK1 of *Xenopus laevis* (Su et al. 1996) and shows weaker similarity to many other protein kinases. The 10 exons of LKB1 span 23 kb of genomic DNA, and the gene is expressed ubiquitously in all human tissues (Jenne et al. 1998). Most of the LKB1 mutations identified in PJS are null alleles (including nonsense codons, deletions, insertions, and rearrangements), and only one missense mutation, L67P, has been observed (Hemminki et al.1998; Jenne et al. 1998). It is likely that LKB1 is a tumor-suppressor gene, since loss of the normal allele was observed in the polyps from a PJS patient

with a germ-line mutation on the other allele (Hemminki et al. 1998). In more-recent studies, loss of heterozygosity and a somatic mutation (G163D) have been observed in some tumor tissues (Avizienyte et al. 1998; Bignell et al. 1998).

To contribute to the study of the molecular pathophysiology of PJS and, thus, to the study of cancer predisposition, we analyzed the occurrence of gene mutations in nine families, using single-strand conformation analysis (SSCA) and sequencing. Mutations were found in heterozygosity in seven of these families, including two missense substitutions. We also show that the mutant LKB1 proteins with these amino acid substitutions lost their kinase activity, as measured by the rate of autophosphorylation. Our data also support the locus heterogeneity of PJS, because one of the two families in which an LKB1 mutation was not found showed strong evidence for linkage to a genomic region on 19q13.4.

Families and Methods

Families with PJS

Samples of genomic DNA from individuals of nine families that met the clinical criteria (Mehenni et al. 1997) of PJS were isolated from peripheral blood lymphocytes by standard methods. The study was approved by the appropriate ethics committee, and the samples from all families that participated in the study were collected after informed consent was obtained from the participants. The pedigrees of six of these families (PJS01 and PJS03 from Spain, PJS05 from Utah, PJS06 from Portugal, and PJS07 and PJS08 from India) have been published elsewhere (Mehenni et al. 1997). The additional three families were from Turkey (PJS02), France (PJS04), and Tahiti (PJS09). All families had multiple affected individuals, except family PJS02, in which there was only one affected member.

SSCA Analysis, Southern Blot, and Sequencing

Genomic DNA from at least one affected individual per family was PCR-amplified for eight different genomic regions that covered the entire coding sequence of the LKB1 gene and all of the splice junctions. The oligonucleotide primers used and the conditions for PCR amplification were as described by Jenne et al. (1998). For SSCA, the PCR products were denatured with an equal volume of denaturation buffer (95% formamide, 0.05% xylene cyanol, and 0.05% bromophenol blue) for 10 min at 94°C, and 4 μl were then loaded onto a 12.5% GeneGel Excel (Pharmacia Biotech). Electrophoresis was at 600 V at 12°C for 2–3 h, depending on the size of the PCR product. The gels were stained by DNA silver staining (Pharmacia Biotech). SSCA variants were purified with QIAquick spin columns and were directly sequenced, in both directions, with the appropriate primers on an ABI377 sequencer, by use of standard protocols. Primer sequences are available on request (Hamid.Mehenni@medecine.unige.ch). Because of the autosomal dominant nature of the disease and, therefore, the heterozygosity of the mutations in patients' DNA, all mutant alleles were also cloned by the Original TA cloning kit (Invitrogen) and were purified and sequenced as previously described. In order to distinguish between polymorphisms and disease-causing mutations, a panel of 100 normal, unrelated Caucasian individuals was screened for some of the sequence variants described here, by either restriction-endonuclease analysis or SSCA. The numbering of the nucleotide changes described in the present article are in accordance with the LKB1 cDNA sequence (Genbank U63333). Genomic Southern blots were prepared after endonuclease digestion with *Eco*RI, *Hin*dIII, and *Bam*HI/*Pst*I and were probed with a cloned cDNA fragment that comprised the entire coding region of LKB1.

Construction of Expression Vectors

The expression plasmid DNA, pcDNA3/LKB1 myc, which contained the wild-type LKB1 coding sequence and c-myc epitope tag (EQKLISEEDL) on its carboxyl terminal, was constructed by PCR, as follows. A DNA fragment amplified by PCR, by use of an LKE1 primer (*5* gat gaa ttc ggg tcc agc atg gag gtg gtg gac 3′) and an LKE2 primer (5′ gat gaa ttc tta gag gtc ttc ttc tga gat gag ctt ctg ctc ctg ctg ctt gca ggc cga 3′), was cloned into an *Eco*RI site of pcDNA3 vector (Invitrogen). The clones with the correct orientation were selected, and their sequences were verified.

Each of the mutant plasmids (pcDNA3/LKB1-K78I-myc, pcDNA3/LKB1-D176N-myc, pcDNA3/LKB1-W308C-myc, and pcDNA3/LKB1-L67P-myc) were generated from this clone by in vitro mutagenesis, by the GENEEDITOR in vitro site-directed mutagenesis system (Promega), in accordance with the procedure suggested by the manufacturer. The mutagenic oligonucleotides, LK K78I (5′ agg agg gcc gtc atc atc ctc aag aag 3′), LK D176N (5′ att gtg cac aag aac atc aag ccg ggg 3′), LK W308C (5′ cgg cag cac agc tgc ttc cgg aag aaa 3′), and LK L67P (5′ gtg aag gag gtg ccg gac tcg gag acg 3′), were hybridized to single-stranded pcDNA3/LKB1 myc plasmid DNA with the selection oligonucleotide (bottom strand), and mutant strands were synthesized. The plasmid DNAs with the introduced mutations were selected by use of the GENEEDITOR-produced antibiotic-resistant clone, and the mutations were confirmed by sequencing.

cDNA Expression and Kinase Assay

Ten micrograms of DNA from each of the four mutant and normal constructs were transformed into 10^6 COS7 cells by SuperFect Transfection Reagent (Qiagen). The

transfected cells were incubated for 48 h and were extracted in an NP40 kinase lysis buffer (10 mM Tris-Cl [pH 7.8], 1% NP40, 0.15 M NaCl, 1 mM EDTA, 50 mM NaF, 5 mM sodium diphosphate, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride) at 4°C. Cell lysates were precleared by mixing with protein A/G plus agarose (Santa Cruz) for 30 min, and then the transiently expressed proteins were immunoprecipitated with an anti–c-myc antibody, A14 (Santa Cruz). Precipitates were washed six times with an NP40 kinase lysis buffer containing 0.15 or 1 M NaCl, followed by one wash in 50 mM Tris-Cl (pH 7.8). For the kinase assays, samples were resuspended in 50 μl of 50 mM Tris-Cl pH 7.8, 1 mM DTT, 10 mM $MnCl_2$, and $\gamma[^{32}P]$-ATP (10 μCi) and incubated at 37°C for 30 min. The reaction was stopped by the addition of the SDS sampling buffer, and the samples were incubated at 95°C for 5 min. Products of the reaction were subjected to SDS-PAGE and were visualized by autoradiography. Expressed normal and mutant proteins in the lysates were quantified by western blotting using the anti–c-myc antibody A14.

Protein Modeling

Crystallized proteins homologous to LKB1 were identified by SWISS-MODEL (Peitsch 1996). Three protein kinases sharing enough sequence identity with LKB1 to be used as templates for the construction of a model were retained: PBD entries 1ATP (Zheng et al. 1993), 1KOB (Kobe et al. 1996; Heierhorst et al. 1996), and 1AQ1 (Lawrie et al. 1997). Those kinases share 26%, 22%, and 22% identity and 50%, 43%, and 39% similarity with LKB1, respectively, over residues Y49–I360 of LKB1.

The three crystallized proteins were superimposed in three-dimensional space by use of SWISS-PDBVIEWER (Guex and Peitsch 1997), and the primary sequence of LKB1 was aligned onto the three templates. Residues Y49–I360 of LKB1 were built by use of the average of the backbone position of the three templates, as described by Blundell et al. (1987), and by the addition of the side chains from a rotamer library (Ponder and Richards 1987), so that they matched as closely as possible those present in the three templates. Protein 1ATP was used as the main template, whereas 1KOB and 1AQ1 were used as auxiliary templates. Since LKB1 could not be aligned onto the three templates without gaps, some residues could not be built by homology, and those loops had to be rebuilt de novo in SWISS-PDBVIEWER. The loops that best matched to the corresponding region of the templates while registering a favorable energy reading were selected. Next, one ATP and two Mn^{++} molecules were introduced in the same location as those present in the PDB file 1ATP, and the model was optimized by 2 × 100 cycles of steepest descent, followed by 3 × 200 cycles of conjugate gradients, by use of CHARMM (Brooks et al. 1983). The same procedure was followed to build models with amino acid substitutions D139N and K78I.

Results and Discussion

LKB1 Mutations

The recent discovery that mutations in the LKB1 gene are responsible for most of the cases of PJS (Hemminki et al. 1998; Jenne et al. 1998) prompted us to examine the molecular defects present in our collection of nine families with this syndrome, explore the possibility of locus heterogeneity, and study the importance of the kinase activity of LKB1 in the pathophysiology of the disease. We used a combination of methods, such as SSCA, Southern blot analysis, and nucleotide sequencing, to search for LKB1 mutations.

Seven novel LKB1 mutations were detected in the genomic DNA samples of seven families (table 1 and figs. 1 and 2); in the remaining two families, no mutations were identified. Four of these mutations—three deletions and one mutation in a splice site—are likely to result in a translational frameshift and, thus, premature termination of LKB1. Two mutations cause amino acid substitutions, and one results in the deletion of four amino acids.

The first mutation (fig. 1*A*) is a single-base (A) insertion between nucleotides 574 and 575 of exon 4 of LKB1 and was found in heterozygosity in a sporadic case in a Turkish PJS patient (PJS02). This insertion introduces a frameshift at codon K191, 73 novel amino acids, and the premature termination of a 265-residue protein compared with the normal 433 amino-acid protein. The DNA from both parents of the affected individual did not have this mutation, which is therefore a de novo mutation. In addition, none of the nine siblings carried

Table 1

Mutations in the LKB1 Gene in PJS Families Described in the Present Study

	Mutation in	
Family	DNA	Protein
PJS01	Exon 1, 151–168del18, 150–151ins6	Del LMGD 50–53
PJS02	Exon 4, 574–575insA	Frameshift, 73 new amino acids after K191
PJS03	IVS5+5G→A	Abnormal splicing and truncated protein
PJS04	Exon 8, 924G→T	W308C
PJS05	Exon 4, 526G→A	D176N
PJS06	Exon 7, 903delG	Frameshift, 33 new amino acids after R301
PJS07	No mutation detected	
PJS08	No mutation detected	
PJS09	IVS6 and exon 7, del52 ending at 904	Abnormal splicing and truncated protein

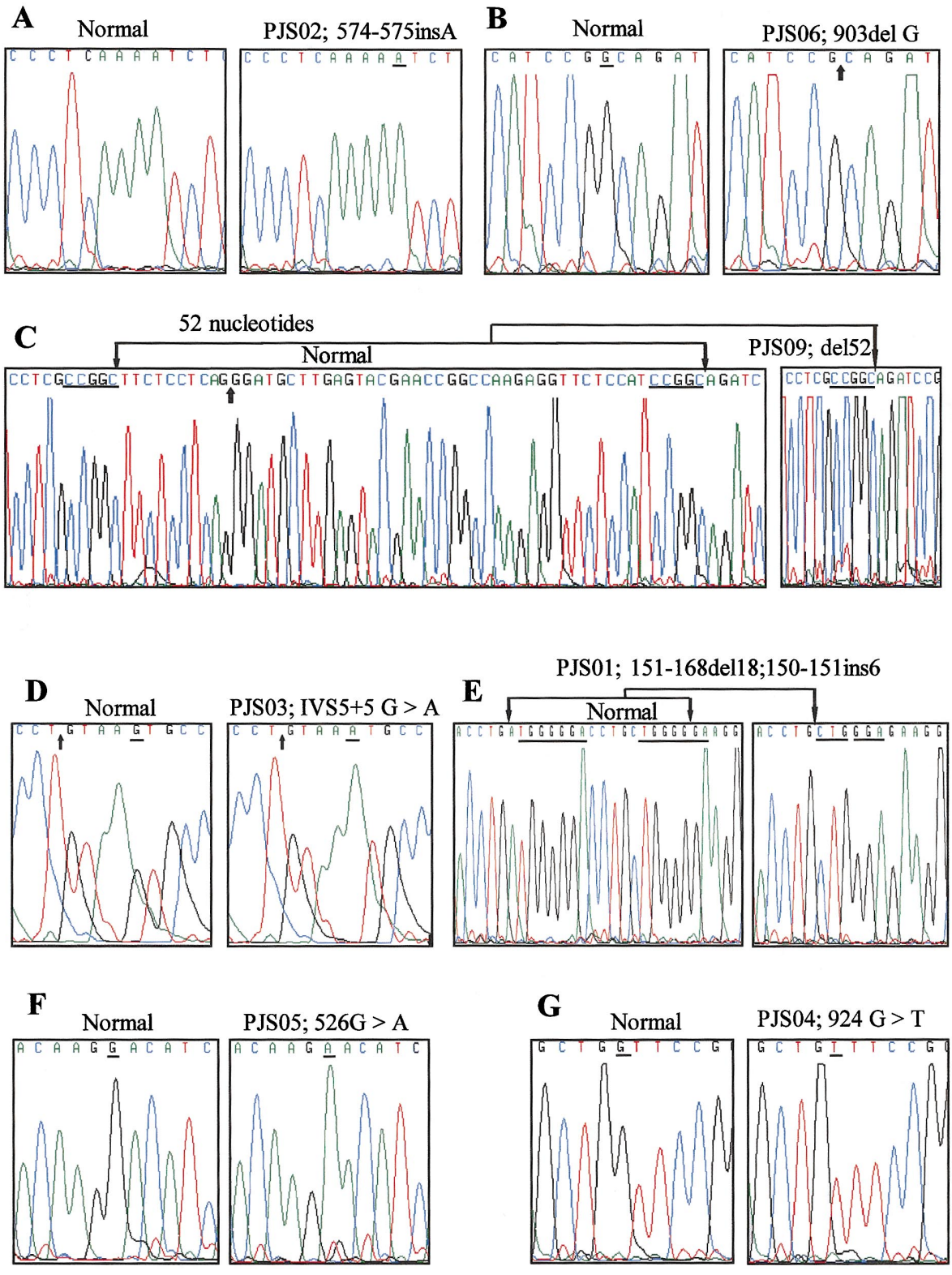

Figure 1 Nucleotide sequences of the LKB1 mutations identified in the present study. Sequences are in pairs, which include the normal sequence (*left*) and the mutant sequence (*right*). The identity of the PJS family in which each mutation was found is denoted above each mutant sequence. The sequences of the mutant alleles were obtained after cloning of the PCR products (see Families and Methods).

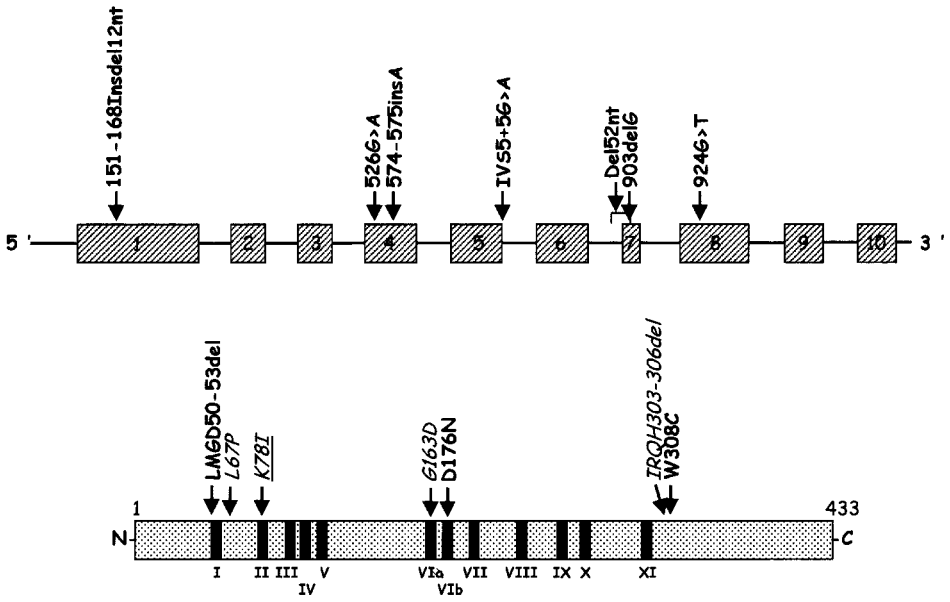

Figure 2 Schematic representation of the LKB1 gene (*top*) and protein (*bottom*). The exons are depicted as striped boxes; the intron sizes are not to scale. The different subdomains (I–IX) of the kinase domain of LKB1, as described by Hanks and Hunter 1995, are depicted (*bottom*). The mutations described in the present study are shown above the genomic structure. The amino acid substitutions or deletions are also shown in the schematic of the predicted protein. The amino acid lesions shown in italics have been described by Avizienyte et al. (1998) and Hemminki et al. (1998). The mutation in italics and underlined has been described in the *Xenopus* XEEK1 (Su et al. 1996).

the mutation, which makes it unlikely that there was a parental mosaicism for this molecular defect. The insertion of A occurred in a run of four A's, which is commonplace in insertional mutagenesis, and is potentially a result of polymerase pause and of frameshifting (Cooper et al. 1995). No cancer has been reported in this patient.

The second mutation (fig. 1*B*) is a deletion of one nucleotide (903G) in exon 7 of LKB1, found in heterozygosity in affected individuals of a Portuguese family (PJS06). This results in a frameshift after codon R301, the introduction of 33 novel amino acids, and the premature termination of a 335-amino-acid protein. All eight affected individuals in this family were heterozygotes for 903delG, and none of their 17 normal relatives who were examined showed the mutation in their DNA samples taken from blood lymphocytes. No cancers were reported in the affected members of this family.

The third mutation (fig. 1C) is a deletion of 52 bp at the end of intron 6, which includes the majority of exon 7, that was found in a Tahitian family (PJS09). This deletion ends at nucleotide 904 of the LKB1 cDNA in exon 7 and probably results in abnormal splicing and truncated protein. The deleted DNA is flanked by a pentanucleotide direct repeat, CCGGC, and therefore the mechanism of this deletion is compatible with the slipped-mispairing model (Cooper et al. 1995). Only one affected individual from this family was available for DNA analysis; this patient had developed colon carcinoma. In addition, two of his siblings with PJS had died from gastrointestinal carcinomas. The mutation was not found in the DNAs of four available normal relatives.

The fourth mutation (fig. 1*D*) was a G→A substitution at nucleotide +5 of the consensus donor splice site of

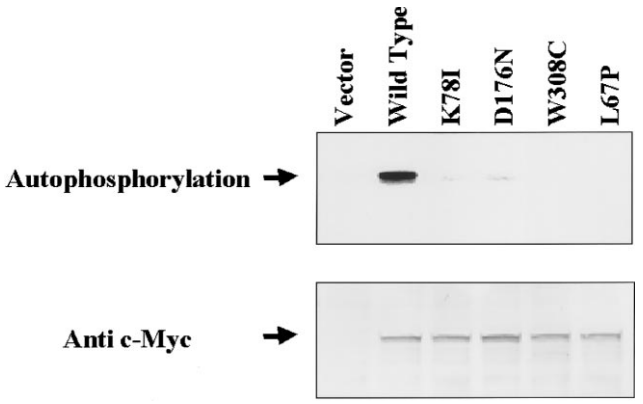

Figure 3 Autophosphorylation assays of the wild-type and mutant LKB1 recombinant proteins, as described in Families and Methods. The lower panel shows the results of western blotting by use of the anti-myc antibody. The top panel reveals that the wild-type LKB1 is autophosphorylated, whereas the same amount of each of the mutants tested, including the K78I, D176N, W308C, and L67P, shows either no or dramatically reduced autophosphorylation.

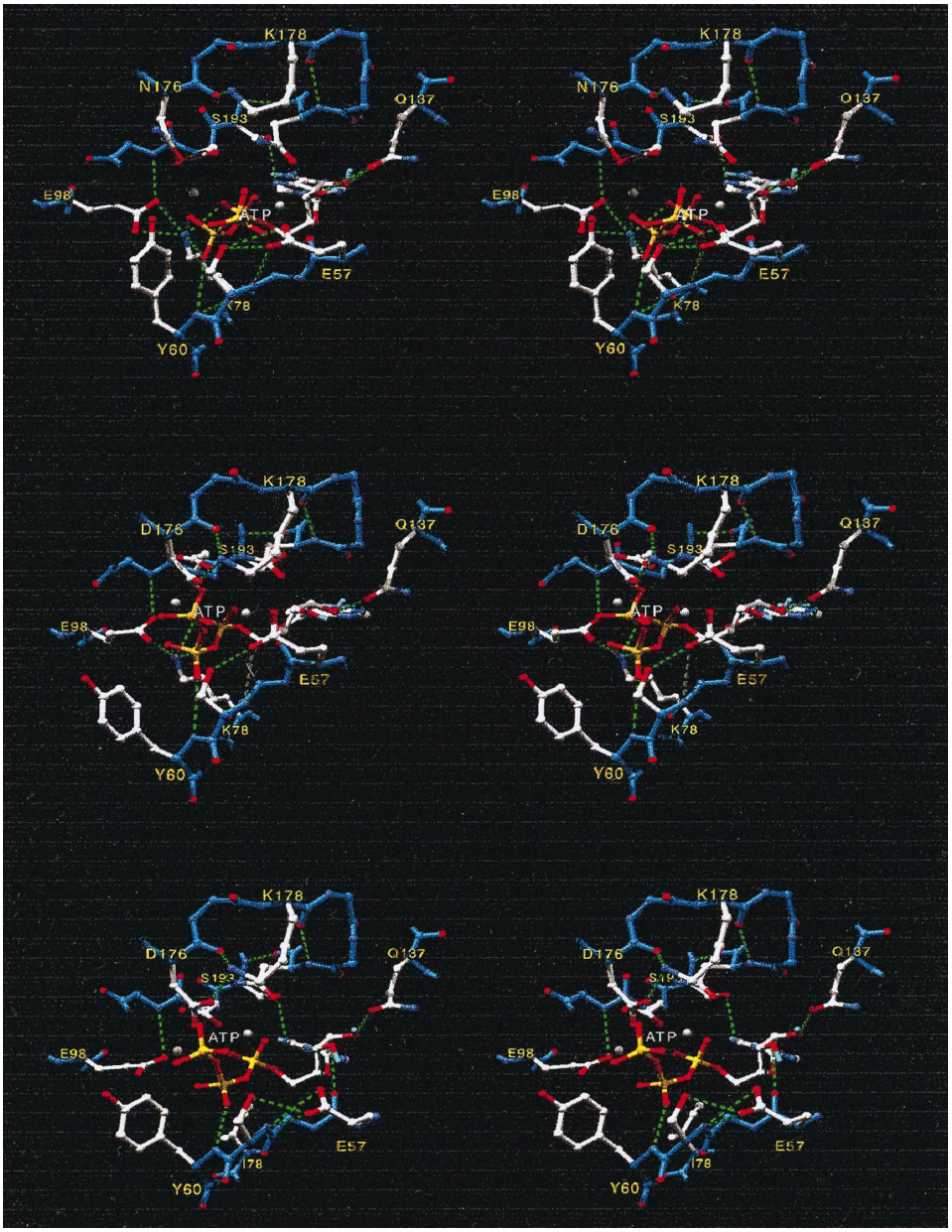

Figure 4 Stereo images of the catalytic core of LKB1 in the presence of ATP after modeling (see Families and Methods). The normal LKB1 is shown in the middle, for easier comparison with the mutants; the D176N mutant is on the top, and the K78I is on the bottom. The protein backbone is shown in blue, whereas side chains and ATP are shown in the following colors: C, white; O, red; N, blue; P, orange; S, yellow; H, cyan. Two Mn^{++} atoms are shown as gray balls. Both mutations greatly disrupt the normal chemical contacts in the catalytic core (see the Protein Modeling subsection).

intron 5 of the LKB1 gene and was found in a Spanish family (PJS03). This substitution was observed in all five affected siblings, and there is no history of carcinomas in this family. The mutation is likely to result in abnormal splicing and a truncated protein, because G is the preferred (85% frequency) nucleotide at position +5 of the splice site (Padgett et al. 1986). The mutation is not a common polymorphism, since it was not detected in 100 normal Caucasians. The Human Gene Mutation database at the Institute of Medical Genetics in Cardiff contains many mutations at nucleotide +5 of the donor splice site that are predicted to reduce significantly the stability of the splice-site base pairing with the complementary region of U1 snRNA (Cooper et al. 1995). No cancers were reported in the affected members of this family.

The fifth mutation (fig. 1*E*) is a combination of a deletion of 18 nucleotides in exon 1 and an insertion of 6 nucleotides at the site of the deletion, which was observed in a Spanish family (PJS01). The mechanism of this complex mutation is unclear; however, it is noteworthy that at the site of the deletion there is a direct repeat of seven nucleotides (TG_5A). The net result is the deletion of four amino acids, LMGD in codons 50–53 of LKB1, because the inserted six nucleotides code for the same amino acids normally present at this position in the protein. The deleted amino acids are within the protein kinase ATP-binding region (subdomain I in Hanks and Hunter 1995; Prosite domain PDOC00100, PS00107), and therefore the mutant protein is likely to be nonfunctional. All 10 affected individuals were heterozygous for this mutation, and none of their 12 normal relatives who were examined carried this molecular defect. No cancers were reported in the affected members of this family.

The sixth mutation (fig. 1*F*) was a nucleotide change, 526G→A in exon 4 of the LKB1 gene, that resulted in the substitution of Asp176 by Asn (D176N), which was found in an American Caucasian family from Utah (PJS05). This substitution occurs within the serine/threonine protein kinase active-site signature (Prosite domain PDOC00100, PS00108; subdomain VIb in Hanks and Hunter 1995). Asp176 is an invariant amino acid of this active site (catalytic loop) that is important for the catalytic activity of the enzyme; it is likely that Asp176 accepts the proton from the attacking substrate hydroxyl group during the phosphotransfer mechanism (Hanks and Hunter 1995). The mutation was not detected in 100 normal Caucasians. All five affected individuals studied were heterozygous for D176N, and none of their eight normal relatives who were examined carried this molecular defect. Affected members of this family had developed cancers (pancreatic and other gastrointestinal neoplasias).

The seventh mutation (fig. 1*G*), found in a French family (PJS04), was a nucleotide change, 924G→T in exon 8, that resulted in the substitution of Trp308 by Cys (W308C). This substitution does not occur in a recognizable domain of LKB1, but it is likely to be a pathogenic mutation, since it was not detected in 100 normal Caucasians. The introduction of a novel Cys308 may result in a dramatic change of the three-dimensional structure of LKB1 and in the elimination of its normal activity (see the Loss of Protein Kinase Activity of Mutant LKB1 in PJS Patients subsection). All three affected individuals studied were heterozygous for this W308C, and none of their two normal relatives examined carried this molecular defect. No cancers were reported in the affected members of this family.

Loss of Protein Kinase Activity of Mutant LKB1 in PJS Patients

To study the protein kinase activity of certain mutant LKB1 proteins, we produced, by transient expression in COS7 cells, normal and mutant LKB1s, all tagged at their carboxyl-terminal ends with a 10-amino-acid c-myc epitope. The four mutant LKB1 proteins made were those with (1) the D176N mutation (identical to the mutation in family PJS05, described in the LKB1 Mutations subsection), (2) the W308C substitution (identical to the mutation in family PJS04, described in the LKB1 Mutations subsection), (3) the L67P substitution (identical to the mutation in PJS family SL25, reported by Hemminki et al. 1998), and (4) the K78I substitution that corresponds to the mutation in the subdomain II ATP-binding site of the *Xenopus* XEEK1 protein and that was previously shown to abolish the XEEK1 autophosphorylation (Su et al. 1996). The protein kinase activities of the normal and mutant LKB1s were assayed by autophosphorylation, as previously had been per-

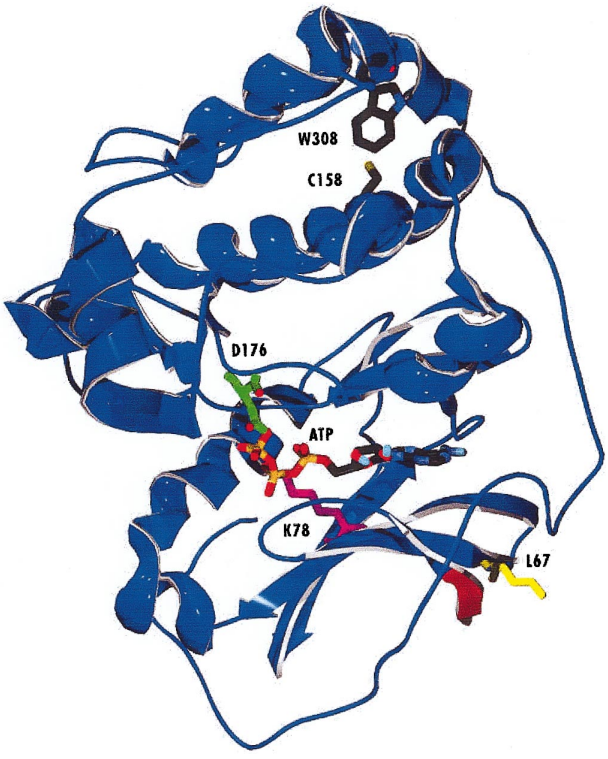

Figure 5 Overall fold of the LKB1 protein modeling. The β-strand that would be removed by the deletion of tetrapeptide LMGD is shown in red. ATP is added in the catalytic core. The D176 side chain that is mutated in family PJS05 is shown in green. The side chain of K78 is shown in purple. The W308 side chain mutated in PJS04 and in C158, which probably forms a disulfide bridge with the mutant W308C, is shown in black. L67, which is mutated to L67P (Hemminki et al. 1998), is shown in yellow.

formed for the LKB1 *Xenopus* homologue, XEEK1. As shown in figure 3, although equal amounts of each protein were assayed (as shown by the anti-myc staining of the hybrid proteins), no or very little autophosphorylation was observed in all four mutant LKB1s compared with normal samples, which clearly shows the loss of kinase activity of LKB1. Thus, this biochemical defect is probably associated with the development of the different phenotypes in neoplasia-susceptibility PJS, but further studies are needed to prove this conclusion. The autophosphorylation defect was expected in LKB1 mutants D176N and K81I, since these amino acid substitutions occur in important residues of the kinase family of proteins (Hanks and Hunter 1995). The other two mutations (i.e., L67P and W308C) do not occur in recognized functional domains of the kinases, but they are likely to disrupt the normal three-dimensional structure and folding of LKB1. Therefore, LKB1 is the first known protein kinase that predisposes to cancer because of loss of the kinase activity, since the germ-line mutations in other protein kinase–encoding genes, such as RET (Eng and Mulligan 1997), MET (Schmidt et al. 1997), and CDK4 (Zuo et al. 1996), are associated with predisposition to neoplasia when mutations result in activation of kinase activity.

Polymorphic Variability of the LKB1 Gene

During the search for LKB1 mutations, the following polymorphic variants were also identified: a single-nucleotide polymorphism, 1315A→G in IVS2 (nucleotide numbering is that of Genbank entry AF032985), and another single-nucleotide polymorphism, 4053G→C in IVS7 (nucleotide numbering is that of Genbank entry AF032985), both of which were present in normal and affected individuals of different ethnic origins; and a short sequence repeat of the heptamer GGGGGCC (starting at nucleotide 1486 of Genbank entry AF032985) in IVS3, which is polymorphic, with two or three allelic tandem copies in the population. These polymorphisms may be of use in linkage studies of chromosome 19p13.3.

Protein Modeling

Computer models of LKB1 were generated for normal LKB1 and two of the mutants. Although these models are only computer approximations of changes that might occur in the tertiary structure, they aid in the understanding of the role of this kinase in PJS. Figure 4 shows the catalytic core of these three models—one normal, one with the PJS mutation D176N, and one with the K78I mutation that abolishes the autophosphorylation in the *Xenopus* XEEK1 gene (Su et al. 1996). The D176N mutation is likely to prevent a proper catalysis (fig. 4, top). Indeed, the phosphate group transferred during the phosphorylation of the target protein is very close to D176, which is probably crucial to maintain the phosphate group in a proper location for its transfer. In addition, the negative charge seems to be effective to maintain K178 close to the phosphate groups. This lysine is conserved among the kinases and is important for the catalysis. The mutation of the invariant K78I is also likely to prevent proper catalysis (fig. 4, bottom). K78 helps to anchor and orient ATP and also forms an important salt bridge with the carboxyl group of the nearly invariant E98. In the model with I78, this bridge is not formed.

The deletion of tetrapeptide LMGD (codons 50–53 of LKB1) is likely to have a major effect on the folding of the protein. Indeed, proteins can generally tolerate only deletions of a whole domain or deletions of residues located at the surface (in loops), where rearrangements are more likely to be accommodated. In our case, this tetrapeptide is located at the beginning of a β-sheet (fig. 5, *red*). For the protein to keep the same structure, the four residues immediately preceding LMGD (i.e., IGKY) would have to take their place. There is not enough space to accommodate them smoothly, which is likely to disrupt the structure.

The substitution of W308C is located on the top part of the model shown in figure 5. C308 is likely to interact, and to form a disulfide bridge, with C158. This interaction during the folding of LKB1 may result in an aberrant three-dimensional structure with little or no kinase activity, which is demonstrated by the autophosphorylation assay.

Potential Locus Heterogeneity of PJS

After searching for mutations, by means of both a sequencing of all exons and intron-exon junctions of the LKB1 gene and Southern blot analysis by use of the LKB1 cDNA probe, we failed to detect any molecular lesions in the LKB1 genes of affected individuals of Indian families PJS07 and PJS08. The mutations in these two families may be in regions, either deep in the introns or in the regulatory sequences of LKB1, that have not been examined. Since all of the known LKB1 mutant alleles result in either absence of LKB1 protein or elimination of the kinase activity, we expect that, if affected individuals of families PJS07 and PJS08 have mutations in the LKB1 gene, then those mutations will result in severe, "null" LKB1 alleles. Alternatively, and more likely, the PJS in families PJS07 and PJS08 may be a result of mutations in a gene different from LKB1. We have described elsewhere that linkage analysis using DNA samples from family PJS07 resulted in LOD scores of 3.52 at a recombination fraction of 0, for markers D19S877 and D19S891 on chromosome 19q13.4 (Mehenni et al. 1997). That analysis and the absence of LKB1

mutations in family PJS07 strongly suggest that there exists a second locus for PJS syndrome. Linkage analysis of family PJS08 gave inconclusive results, since it was not informative for markers on 19q13.4. It is of interest that, in the initial study by Hemminki et al. (1998), there were PJS patients with no recognizable mutation and that additional linkage studies (Mehenni et al. 1997; Olschwang et al. 1998) detected families without linkage to the LKB1 locus. Linkage analysis and positional-cloning efforts are now justified, to discover potential additional loci of PJS. These genes may uncover molecules in kinase phosphatase–mediated metabolic pathways involved in human tissue overgrowth and neoplasias (Li et al. 1997; Steck et al. 1997; Kinzler and Vogelstein 1998).

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Genbank, http://www.ncbi.nlm.nih.gov/irx/genbank/query_form.html (for serine/threonine kinase [U63333] and nucleotide numbering [AF032985])

The Human Gene Mutation Database at the Institute of Medical Genetics in Cardiff, http://www.uwcm.ac.uk/uwcm/mg/hgmd0.html)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nim.nih.gov/Omim (for PJS)

Prosite, http://www.expasy.ch/sprot/prosite.html (for protein domains PDOC00100, PS00107 and PS00108)

SWISS-MODEL, http://www.expasy.ch/cgi-bin/swmodel-search-de (for protein models)

SWISS-PDBVIEWER, http://www.expasy.ch/spdbv/mainpage.htm (for protein models)

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