

Pathogenesis of Adenocarcinoma in Peutz-Jeghers Syndrome¹

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Abstract

Peutz-Jeghers syndrome (PJS) is an autosomal dominant condition characterized by intestinal hamartomatous polyps, mucocutaneous melanin deposition, and increased risk of cancer. Families with PJS from the Johns Hopkins Polyposis Registry were studied to identify the molecular basis of this syndrome and to characterize the pathogenesis of gastrointestinal hamartomas and adenocarcinomas in PJS patients. Linkage analysis in the family originally described by Jeghers in 1949 and five other families confirmed linkage to 19p13.3 near a recently identified gene responsible for PJS. Germ-line mutations in this gene, *STK11*, were identified in all six families by sequencing genomic DNA. Analysis of hamartomas and adenocarcinomas from patients with PJS identified loss of heterozygosity (LOH) of 19p markers near *STK11* in 70% of tumors. Haplotype analysis indicated that the retained allele carried a germ-line mutation, confirming that *STK11* is a tumor suppressor gene. LOH of 17p and 18q was identified in an adenocarcinoma but not in hamartomas, implying that allelic loss of these two regions corresponds to late molecular events in the pathogenesis of cancer in PJS. The adenocarcinomas showing 17p LOH also demonstrated altered p53 by immunohistochemistry. None of the 18 PJS tumors showed microsatellite instability, LOH on 5q near APC, or mutations in codons 12 or 13 of the *K-ras* proto-oncogene. These data provide evidence that *STK11* is a tumor suppressor gene that acts as an early gatekeeper regulating the development of hamartomas in PJS and suggest that hamartomas may be pathogenetic precursors of adenocarcinoma. Additional somatic mutational events underlie the progression of hamartomas to adenocarcinomas, and some of these somatic mutations are common to the later stages of tumor progression seen in the majority of colorectal carcinomas.

Introduction

In several polyposis syndromes, patients have increased risk of gastrointestinal cancer. Familial adenomatous polyposis carries a nearly 100% life-time risk of colorectal cancer, and cancer arises through a series of well-characterized genetic events (1). In contrast, the molecular pathogenesis of cancer in PJS³ is poorly defined. PJS is recognized by the presence of benign hamartomatous polyps of the gastrointestinal tract, often in conjunction with macular light brown pigment deposition on the lips, buccal mucosa, eyelids, and fingertips (2–4). The most common complication of PJS is small bowel obstruction from enlarging hamartomas and intussusception, but these pa-

tients are also at increased risk of gastrointestinal and nongastrointestinal cancer (4–6). Histopathological studies have shown that hamartomas are occasionally identified in contiguity with adenocarcinoma in PJS, and hamartomas with dysplasia may represent early lesions in the pathogenesis of cancer (5, 7–9).

Hemminki *et al.* (10) identified a susceptibility locus for PJS on 19p13.3 using comparative genomic hybridization to conduct a targeted linkage analysis. These investigators demonstrated LOH in this region of 19p for hamartomas and found strong evidence of linkage closest to marker *D19S886*. They postulated that inactivation of this putative tumor suppressor gene is a critical early event in the development of hamartomas and adenocarcinomas, although adenocarcinomas were not analyzed. Recently, two groups simultaneously reported the positional cloning of a candidate gene for PJS, *STK11* (11, 12). This gene is a serine-threonine kinase; the contribution of this gene to the pathogenesis of cancer has not been fully defined.

We studied six PJS families to identify the molecular basis of the phenotype and to characterize the pathogenesis of the gastrointestinal hamartomas and adenocarcinomas that arise in those with this syndrome. Linkage analysis confirmed the mapping of PJS and provided haplotype data for examining LOH. Sequencing analysis of the entire coding region of *STK11* identified germ-line heterozygous mutations that segregated with the PJS phenotype in all six pedigrees. LOH and mutational analyses of hamartomas and adenocarcinomas helped characterize tumorigenesis in this syndrome.

Materials and Methods

Six unrelated families with PJS from the Johns Hopkins Polyposis Registry, including the original family described by Jeghers *et al.* (Fig. 1, Family 1; Ref. 3), were selected for this study based on availability of DNA and tumor specimens. One individual from a seventh family was also included because tumor blocks from multiple hamartomas and an adenocarcinoma arising within a hamartomatous polyp were available. Informed consent was obtained from each family member participating in the study through a protocol approved by the Joint Committee on Clinical Investigation at The Johns Hopkins University School of Medicine. DNA was available from 29 affected and 35 unaffected family members. The diagnosis of PJS was confirmed by histopathology, physical exam, and/or medical records for each family member (4).

Genotyping was performed on DNA derived from lymphocytes for 237 dinucleotide markers spanning the genome at a marker distance of 10–20 cM. PCR and electrophoresis conditions were performed as described previously (13). Two-point linkage analysis using FASTLINK (14) assumed autosomal dominant inheritance for PJS with a penetrance of 100% and a gene frequency of 0.0001. Marker allele frequencies were estimated as $1/n$ for the whole genome scan, where n is the number of alleles at a given locus. Allele frequencies from Center Etudes Polymorphisme Humaine were used for the detailed linkage analysis of chromosome 19p markers. All analyses were repeated using a gene frequency of 0.002, and no important differences were observed. Linkage heterogeneity tests were conducted using the HOMOG program (<http://linkage.rockefeller.edu/soft/homog.html>; Ref. 15).

Genomic DNA from lymphoblastoid lines of affected individuals was

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³ The abbreviations used are: PJS, Peutz-Jeghers syndrome; LOH, loss of heterozygosity; APC, adenomatous polyposis coli; *STK11*, human serine threonine kinase 11; GDB, GenBank database.

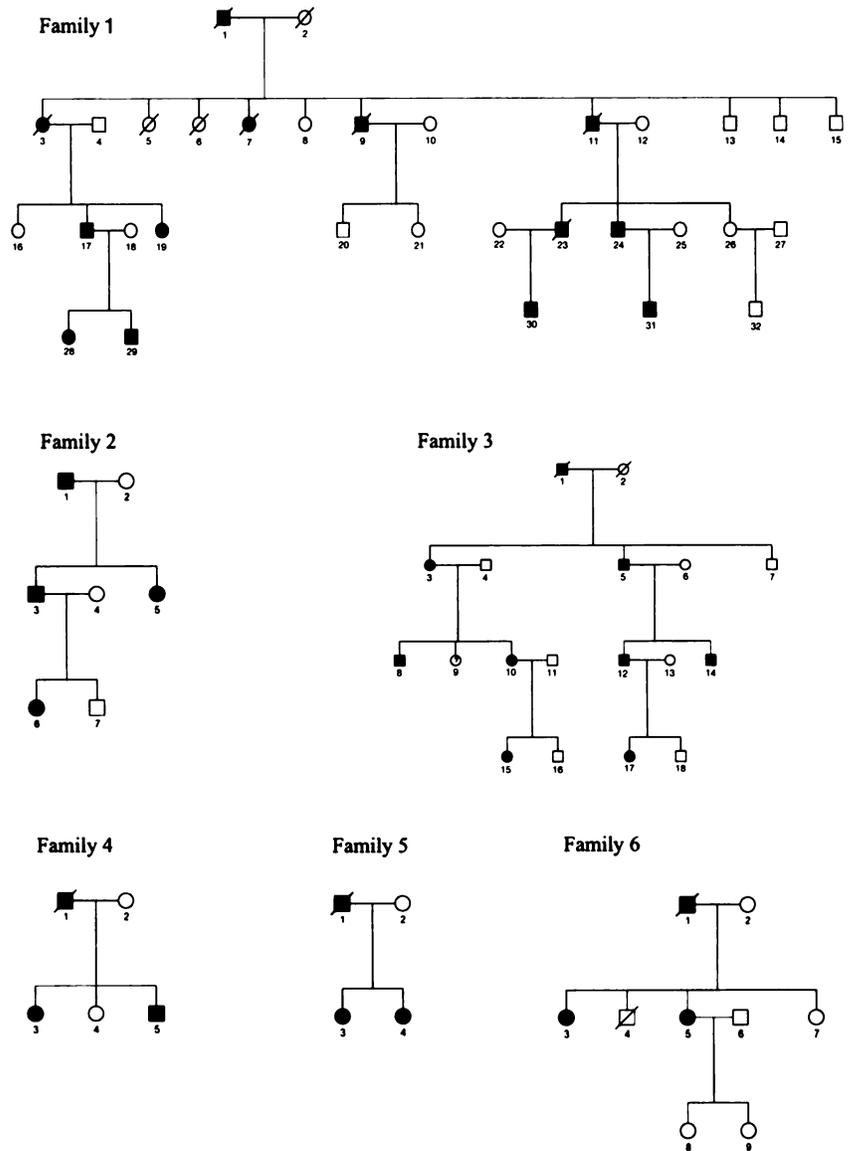


Fig. 1. Pedigrees of six families with PJS. *Solid symbols*, affected individuals; *open symbols*, unaffected individuals. *?*, family members without knowledge of affection status. Family 1 was originally described by Jeghers *et al.* (3).

amplified to sequence the complete coding region of *STK11* (described previously as LKB1). Primer sequences were generously provided by L. Aaltonen prior to publication (11). Fluorescent sequencing reactions were analyzed on an ABI automated sequencer. Sequence homology was facilitated using a BLAST search to help identify mutations in comparison with the published sequences of LKB1 in GenBank (<http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/>).

DNA was extracted from tumor specimens after histopathological review and microdissection of nonneoplastic mucosa, epithelium of hamartomas, and adenocarcinomas (16). Persons performing the microdissections of the adenocarcinomas were careful to remove homogeneous areas of epithelial tumor. Areas with different histopathological features were microdissected and analyzed separately. These included discontinuous epithelial regions of hamartomas as well as mucinous and glandular areas of adenocarcinomas. Samples were incubated overnight at 56°C in 1× TK buffer (0.5% Tween 20, 0.2 mg/ml proteinase K, and 0.1× TE9), treated with 2× volume of 5% Chelex in 0.1% TE9, and heated to 100°C for 10 min. Microsatellite markers *D19S886* (GDB: 608640) and *D19S565* (GDB:343597) were amplified with 30 PCR cycles (denatured for 1 min at 95°C, annealed for 1 min at 52°C (*D19S886*) and 50°C (*D19S565*), and elongated for 1 min at 72°C). *D18S55*, *D18S58*, *D18S61*, *D18S64*, and *D18S69* were amplified with 38 PCR cycles (denatured for 30 s at 93°C, annealed for 30 s at 55°C, and elongated for 1 min at 72°C) in 6% DMSO and a PCR buffer composed of 670 mM Tris (pH 8.8), 67 mM magnesium chloride, 166 mM ammonium sulfate, and 100 mM β-mercapto-

ethanol. The primers for the 18q markers were described previously (17). Allelic loss and microsatellite instability were assessed by denaturing gel electrophoresis performed with 6% polyacrylamide gels.

Tumor DNA was amplified for somatic mutational analyses. Amplified DNA from the first exon of *K-ras* was prepared using a PCR Master kit (Boehringer Mannheim, Mannheim, Germany) with 40 amplification cycles (denatured for 1 min at 94°C, annealed for 1 min at 50°C, and elongated for 1 min at 72°C). Primers for amplification were 5'-GAGAATTCATGACTGAATATAAAGTGT-3' and 5'-TCGAATTCCTCTATTGTTGGATCATATTCG-3'. Sequencing reactions were performed with a Sequitherm Excel kit (Epicentre Technologies, Madison, WI) using the sequencing primer 5'-ATTCGTCACAAAATGAT-3' and were run on 6% polyacrylamide gels. A subset of the samples were also evaluated by allele-specific hybridization for mutations of *K-ras* (18). Somatic mutations of p53 were evaluated by immunohistochemical detection of intranuclear p53 gene product and allelic loss of 17p as described previously (19).

Results and Discussion

Linkage to 19p13.3 and *STK11* Germ-Line Mutations in PJS. Each of the six PJS families was consistent with autosomal dominant inheritance with complete penetrance (Fig. 1). A genome-wide linkage analysis in family 1 confirmed linkage to 19p13.3 near *D19S886* with a lod score of $Z = 2.5$ at a recombination fraction of $\theta = 0.0$ and

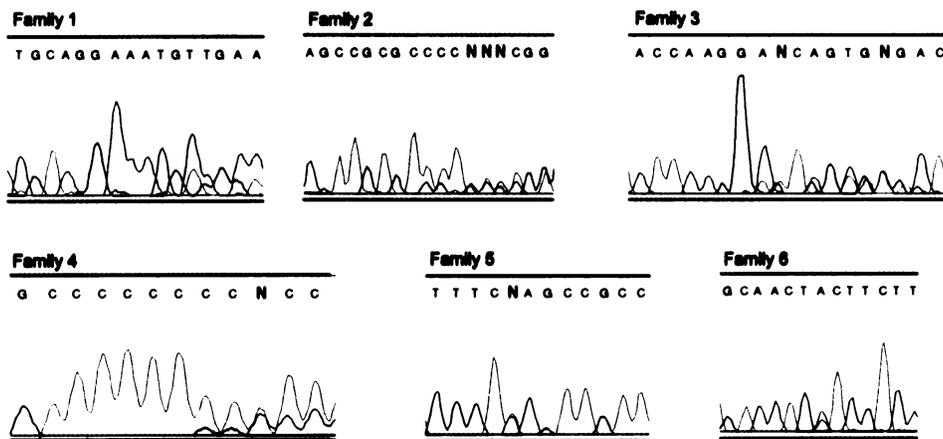


Fig. 2. Germ-line mutations of *STK11* in affected individuals from six families with PJS. All mutations were confirmed by forward and reverse sequencing. Forward sequencing is shown for each family except family 6, because the heterozygous mutation in this family was easier to read on the reverse sequence. Each of these mutations segregated with other affected family members and was not observed in unaffected individuals.

found no evidence of other susceptibility loci. Linkage analysis was less informative for the other five families, although *D19S886* showed the strongest evidence of linkage with a combined lod score of $Z = 3.1$ at $\theta = 0.0$. There was no significant evidence of heterogeneity using the HOMOG analysis program, and the strongest evidence against linkage was shown in family 6 with a $Z = -0.35$ at $\theta = 0.0$.

Mutations in *STK11* were identified in all six families (Table 1; Fig. 2). Families 1–4 had frameshift mutations, and families 5–6 had nonsense mutations. All six mutations were confirmed by sequencing on both strands. In family 1, three affected family members were all heterozygous for the 1407delC mutation, and three unaffected individuals all carried two wild-type alleles. In families 2–6, the familial mutation was confirmed in one other affected individual. The entire coding sequence of *STK11* was examined in each family, and no other truncating mutations were identified.

LOH, Haplotype Analysis, and Somatic Mutations in PJS Tumors. Sixteen hamartomas and three adenocarcinomas arising in four patients with PJS were examined for LOH. One of these patients was

identified from a small family that was not included in the linkage or germ-line analyses. LOH on 19p near *STK11* was identified in 11 of 12 hamartomas or adenocarcinomas studied (Fig. 3). Limited DNA precluded complete evaluations of all tumor specimens. Because markers on 18q are commonly lost in gastrointestinal malignancies (17), DNA from hamartomas and adenocarcinomas were evaluated for LOH in this region. Analysis of 15 hamartomas and 3 adenocarcinomas found LOH in one adenocarcinoma at *D18S55* on the long arm of chromosome 18 near *DPC4* and *DCC*. The LOH on 18q was seen in separately dissected regions of adenocarcinoma but was not seen in the hamartomas from the same patient.

Comparison of the linkage data and with LOH analyses demonstrated that the retained allele carried the germ-line mutation in all cases. These data provide strong evidence that *STK11* is a tumor suppressor gene.

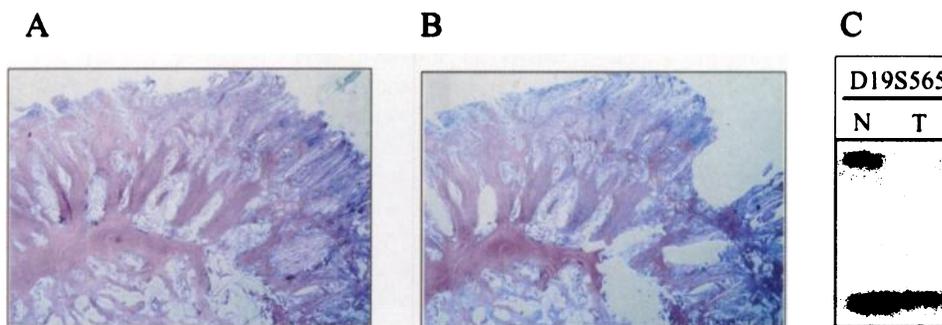
None of the 18 tumor samples had *K-ras* mutations commonly observed in colorectal cancer. Mutations at codon 12 or 13 are found in 40% of colorectal carcinomas and 18–68% of colorectal adenomas depending

Table 1 Germ-line mutations of *STK11* identified in six families with Peutz-Jeghers syndrome

The 5' end of the illustrated sequences are given in parentheses and correspond to the notation in GDB for *STK11* (AF032984 and AF032985). The source of the mutations is shown in boldface.

Family	Exon	Mutation	Wild-type sequence of <i>STK11</i> (position) mutant sequence	Type
1	Exon 3	1407delC	WT (1400) GGAAATGCTGGA MU (1400) GGAAATGTGGAC	Frameshift
2	Exon 1	455-456insGC	WT (450) CCGCGCCGCAAGCGG MU (450) CCGCGCGCCGCAAGCGG	Frameshift
3	Exon 8	5093-5101delACCGGTGGC	WT (5090) AGGACCGGTGGCCGCA MU (5090) AGGCGCA	Frameshift
4	Exon 6	3360-61insC	WT (3351) GTGGCCCCCGCTCT MU (3351) GTGGCCCCCGCTCT	Frameshift
5	Exon 5	2681C→T	WT (2675) GCTTTCCAG MU (2675) GCTTTCTAG	Nonsense
6	Exon 1	588A→T	WT (582) AAGAAGAAGTTGC MU (582) AAGAAGTAGTTGC	Nonsense

Fig. 3. Histopathology of adenocarcinoma arising in a hamartoma before (A) and after (B) microdissection. Microdissection focused on epithelial components of the tumor. Allelic LOH is shown for adenocarcinoma arising in a PJS hamartoma at a marker on 19p (C). Haplotype analysis demonstrated that the retained allele carried the germ-line mutation in *STK11*. Alleles from microdissected normal DNA (N) tumor DNA (T) are shown.



on morphology, histopathological dysplasia, and history of familial adenomatous polyposis (20, 21). These data suggest that *K-ras* mutations are not essential for tumor progression in this syndrome.

Patients with PJS are at increased risk of gastrointestinal and nongastrointestinal cancer, and our data help elucidate the pathogenesis of a subset of the tumors observed in this syndrome. All of the neoplasms examined in this study were gastrointestinal tumors, and it is important to recognize that not all of the cancers associated with PJS develop in association with hamartomas. Nongastrointestinal cancers that have been associated with PJS include cancers of the breast, ovary, cervix, uterus, thyroid, and lung (4–6), and the molecular basis for these nongastrointestinal tumors in patients with PJS remains unclear. It appears that somatic mutations in *STK11* are rare in sporadic breast (22), colorectal, and testicular cancers (23), but somatic mutations of these tumors arising in patients with PJS have not yet been examined.

The present study offers several observations of the pathogenesis of gastrointestinal neoplasia in PJS. Truncating germ-line mutations in *STK11* appear to be necessary and sufficient to cause PJS, and there is strong evidence that *STK11* is a tumor suppressor gene involved in the earliest step of the pathogenesis of hamartomas and adenocarcinomas in PJS. There does not appear to be any locus heterogeneity by linkage or mutational analysis. We have not yet been able to identify any genotype/phenotype correlations of individual mutations in *STK11* with the phenotypic expression of PJS. All families studied thus far have mutations that interrupt the coding sequence of the gene.

Our data also provide evidence outlining additional steps in the pathogenesis of PJS hamartomas and adenocarcinomas. Mutations of *APC* and *K-ras* that are commonly observed in colorectal cancer do not appear to be required for the development of PJS hamartomas or adenocarcinomas. Identified allelic loss on chromosome 17p and 18q, in combination with immunohistochemistry staining for p53, suggests that p53 and potentially other tumor suppressor genes in these regions are involved at a later stage in the pathogenesis of cancer in PJS.

The histopathological appearance of Peutz-Jeghers hamartomas is quite distinct from other types of gastrointestinal polyps and likely reflects a different pathogenetic sequence for their development. PJS hamartomas show an elongated, frond-like epithelium with cystic dilation of glands overlying an arborizing network of smooth muscle bundles. Hyper mucinous goblet cells are often prominent. In addition, pseudoinvasion by histopathologically benign epithelium is common in PJS hamartomas. These characteristic features are easily distinguished from the cytological atypia and lack of differentiation seen in typical adenomas, and it is not surprising that PJS tumors seem to share few of the earliest genetic events observed in the transition of normal epithelium to dysplastic adenomas. Hamartomatous polyps arising in the juvenile polyposis syndrome arise through yet another mechanism as a consequence of germ-line mutations in *SMAD4/DPC4* (24). The hamartomas of juvenile polyposis are histologically distinct from those in PJS, and the risk of malignancy also differs in these two syndromes. It will be interesting to compare somatic alterations and the regulation of cellular growth in the hamartomas of these two syndromes. Contrasting the pathogenesis of neoplasia in these syndromes should help clarify the roles contributed by stromal and epithelial elements in the development of some cancers (25).

Functional studies of *STK11* are likely to further elucidate the role of the *STK11* serine-threonine kinase in alternative pathways leading to cancer. This highly conserved gene is ubiquitously expressed (12), and additional study of the distinctive phenotype of PJS may contribute to a better understanding of deviations from normal cellular differentiation and growth.

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