Germline and Somatic Mutations of the STK11/LKB1 Peutz-Jeghers Gene in Pancreatic and Biliary Cancers

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Peutz-Jeghers syndrome (PJS) is an autosomal-dominant disorder characterized by hamartomatous polyps in the gastrointestinal tract and by pigmented macules of the lips, buccal mucosa, and digits. Less appreciated is the fact that PJS also predisposes patients to an increased risk of gastrointestinal cancer, and pancreatic cancer has been reported in many PJS patients. It was recently shown that germline mutations of the STK11/LKB1 gene are responsible for PJS.6–9 The increased risk for cancer among PJS patients would suggest that STK11/LKB1 is a candidate tumor-suppressor gene,10 but the role of STK11/LKB1 gene inactivation in neoplasia has not been conclusively demonstrated.11–14

Pancreatic cancer is an attractive neoplasm to examine for inactivation of STK11/LKB1, because it is one of the more common neoplasms to develop in PJS patients. Of the 53 PJS patients reported in four independent studies, six (11%) were diagnosed with pancreatic adenocarcinoma.2–5 The demonstration that the STK11/LKB1 is inactivated in the pancreatic cancer of a PJS patient and in sporadic pancreatic cancers would strongly support a causal link between these mutations and the development of pancreatic cancers and would help establish the tumor-suppressor role of STK11/LKB1 in neoplasia.

Materials and Methods

PJS Patient and DNA Analysis

Patient PJS1 was an affected family member of a well-followed kindred with PJS.15 She had biopsy-proven Peutz-Jeghers polyps of the duodenum (Figure 1A) and was diagnosed with adenocarcinoma at the age of 35 on biopsy of a peripancreatic lymph node, thought originally and on review to be most consistent with a pancreatic origin on the basis of histological features (Figure 1B).

DNA was prepared from microdissected histological sections of her surgically biopsied cancer and Peutz-Jeghers polyps. Microdissected samples were incubated overnight at 37°C in 0.04% proteinase K, 10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA, and 1% Tween-20. Proteinase K was inactivated at 95°C for 8 minutes before DNA analysis.

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Cancers of the pancreas and distal common bile duct resected at The Johns Hopkins Hospital between 1992 and 1997 were xenografted as described. In addition, at the time of the surgery, resected normal duodenal mucosa was frozen and stored at $-80^\circ$C. The pancreatic cell lines Su86.86, CFPAC1, AsPC1, Capan1, Capan2, Panc1, MiaPaCa2, BxPc3, and Hs766T were purchased from American Type Culture Collection (Manassas, VA) and COLO357 from European Collection of Animal Cell Cultures (Salisbury, Wiltshire, UK). Pancreatic cell line PL45 was established in our laboratory.

Homozygous Deletion Analysis

Genomic DNA samples (40 ng per sample) were screened for homozygous deletions using PCR analysis as previously described. The primers used to amplify exon 1, 4/5, and 9 of $STK11/LKB1$ were as reported previously. Duplex PCR analyses were performed with pairs of internal control primers and $STK11/LKB1$-specific primers. Amplification of integrin-β−4 or MKK4 was used as a positive internal control. Primers are as listed in Table 1.

Loss of Heterozygosity and Sequence Analyses

Loss of heterozygosity (LOH) was determined using three polymorphic markers, D19S886, D19S565, and D19S216 (Research Genetics, Huntsville, AL). LOH was considered to be conclusive only when analysis of the neoplastic DNA showed the complete loss of one of the two alleles present in the patient’s corresponding normal DNA. When a normal DNA sample was unavailable, LOH status was presumptively shown by the unambiguous presence of only a single allele size at all three polymorphic markers evaluated. All samples which displayed conclusive or presumptive LOH were subject to sequencing. Each exon was amplified by PCR from genomic DNA, treated with exonuclease I and shrimp alkaline phosphatase (USB, Cleveland, OH), and subjected to cycle-sequencing (ThermoSequenase, Amersham, Arlington Heights, IL). The majority of the PCR primers have been reported previously. Additional primers are listed in Table 1.

Results

Germline Mutation of $STK11/LKB1$ and Tumorigenesis

To determine the genetic basis for the increased risk of cancer among PJS patients, we examined the status of the $STK11/LKB1$ gene in cancer tissues obtained from a patient diagnosed with PJS. In patient PJS1, the known
germline mutation of this family at the splice donor site of intron 3 of STK11/LKB1\(^{15}\) was demonstrated in nonneoplastic tissue (Table 3) (Figure 2A). DNA from this patient’s microdissected adenocarcinoma and epithelium of a Peutz-Jeghers intestinal polyp were then sequenced and the second allele of STK11/LKB1 was lost (>80% decrease in allele intensity by densitometry) in the pancreatic cancer, but not in the intestinal polyp (Figure 2B). Due to the limited amount of archival material, only limited sequencing was performed. Because LOH is not the only mechanism of gene inactivation, it is possible that the second allele of STK11-LKB1 in the polyp could be inactivated by methylation, small deletions, or point mutation outside of intron 3. The germline mutation is predicted to affect splicing of the STK11/LKB1 transcript.

**Somatic Inactivation of STK11/LKB1 in Pancreatic and Biliary Cancers**

To further validate STK11/LKB1 as a tumor-suppressor gene, we evaluated the role of somatic mutation in STK11/LKB1 in sporadic pancreatic cancer. Using primers specific for exon 1, 4/5, and 9 of STK11/LKB1, we screened for homozygous deletions among a panel of 100 xenografts of primary pancreatic ductal adenocarcinomas, 16 xenografts of primary distal common bile duct adenocarcinomas, 19 xenografts of other primary carcinomas of the peripancreatic region (predominantly duodenal and ampullary cancer), and 11 pancreatic cancer cell lines (Table 2). One pancreatic (PX30) and one distal common bile duct (PX115) adenocarcinoma exhibited

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**Table 2. Sporadic Neoplasms Analyzed for STK11/LKB1 Mutations**

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Total number of samples</th>
<th>Homozygous deletion screening</th>
<th>LOH study</th>
<th>Sequencing</th>
<th>Number of mutated samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic adenocarcinoma</td>
<td>100</td>
<td>100</td>
<td>92</td>
<td>30</td>
<td>4</td>
</tr>
<tr>
<td>Biliary adenocarcinoma</td>
<td>16</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Pancreatic cell lines</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Other carcinomas(^*)</td>
<td>19</td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^*\)Primary carcinomas of the peripancreatic region, predominantly duodenal and ampullary cancers.
homozygous deletions of STK11/LKB1 (Figure 3). The entire genomic sequence of STK11/LKB1 was deleted from PX30, whereas only exon 1 of STK11/LKB1 was deleted in PX115. Both homozygous deletions were confirmed by duplex PCR (Figure 3) and verified in parallel xenografts derived from the same primary tumor samples (data not shown). In PX115, adequate DNA was available for Southern blot analysis, which confirmed the absence of STK11/LKB1 sequences (data not shown). The homozygously deleted regions in PX30 and PX115 did not extend to the closest available neighboring markers, D19S886 and D19S565. These markers were originally used to define the distal and proximal boundaries in maps of the PJS gene localization.6,7

LOH frequency at the STK11/LKB1 locus in pancreatic cancer was determined with the highly polymorphic markers D19S886, D19S565, and D19S216 (heterozygosity index = 0.61, 0.81, and 0.76, respectively). Conclusive LOH was found in 22 of the 69 pancreatic cancers for which normal DNA was available (32%) and presumptive LOH was inferred in 8 of the 23 pancreatic cancers (35%). Presumptive LOH of 19p at the STK11/LKB1 locus was seen in 9 of the 11 pancreatic cancer cell lines (82%). In addition, four cancers harbored LOH breakpoints between D19S886 and D19S565. The localization of these breakpoints to the STK11/LKB1 locus further suggests that STK11/LKB1 is the target of the allelic loss observed. All coding sequences and splice junctions of the STK11/LKB1 gene amplified from the genomic DNA of the 39 selected pancreatic xenografts and cancer cell lines exhibiting conclusive or presumptive LOH were sequenced (Table 2). One non-sense and two frameshift mutations were detected (3 of 103 (3%) studied for LOH) (Figure 4, A and B, and Table 3) and confirmed in independent PCR products amplified from the samples. One mutation was in exon 1 and one in exon 5, and both of these were within the catalytic kinase domain of STK11/LKB1 (codons 37–314).8 The third mutation was in exon 8, and it potentially would affect the function of the regulatory domain of STK11/LKB1 that comprises the 119 residues at the carboxyl-terminus.8 Two of the three
testicular, and breast cancers. Here, we provide biallelic somatic inactivation of a gene has been cloned, several efforts have failed to show been several reports of LOH on 19p in breast, colorectal, in the general population. The average age at which cancer is diagnosed in patients with PJS ranges from 13- to 30-fold greater than the risk shown that the risk of death from gastrointestinal cancer latency from the time of PJS diagnosis.

In summary, we demonstrated the biallelic inactivation of STK11/LKB1 in a pancreatic cancer of a patient with PJS and in 4–6% of sporadic pancreatic and biliary adenocarcinomas, illustrating the role of this gene in familial and sporadic cancer development.

**Discussion**

PJS predisposes affected family members to the development of cancer. Four independent studies have shown that the risk of death from gastrointestinal cancer among PJS patients is 13- to 30-fold greater than the risk in the general population. The average age at which cancer is diagnosed in patients with PJS ranges from 38–50 years, and there is a reported 20–25 years of latency from the time of PJS diagnosis. There have been several reports of LOH on 19p in breast, colorectal, and pancreatic cancers; however, since the STK11/LKB1 gene has been cloned, several efforts have failed to show biallelic somatic inactivation of STK11/LKB1 in colorectal, testicular, and breast cancers. Here, we provide the genetic evidence to support the epidemiological clues that the PJS gene, STK11/LKB1, is a classic tumor-suppressor gene involved in pancreatic and biliary neoplasia. Furthermore, this gene appears to play a role in the development of both sporadic and familial (PJS) pancreatic and biliary cancers. In sporadic cancers, STK11/LKB1 was somatically inactivated in 4% of the pancreatic cancers and in at least 6% of biliary cancers examined. The patient with a familial (PJS) pancreatic cancer inherited a mutated copy of the STK11/LKB1 gene and had somatic loss of the remaining wild-type allele. Indeed, the first kindred described in the seminal report by Jeghers, McKusick, and Katz included a patient who died of pancreatic cancer who could now, with the new understanding of the causal link between PJS and pancreatic cancer, be inferred to be the obligatory mutation carrier. These observations conform to the Knudson model, wherein the same genes are inactivated in both familial and sporadic forms of a cancer.

The xenografted series of pancreatic and biliary cancers, in which we demonstrated the inactivation of STK11/LKB1, have been well characterized genetically, providing additional opportunities to examine the tumor-suppressor role of STK11/LKB1. For example, it would be unusual for two genes in the same pathway to be inactivated in a cancer. We can therefore infer that the STK11/LKB1 suppressive pathway is distinct from the p53, p16, and DPC4 pathways; genetic inactivations of the p53 and p16 genes are known to coexist in tumor PX68, and DPC4 is homozygously deleted from tumors PX30 and PX115. K-ras, which is mutated in 95% of pancreatic cancer cases, is also mutated in tumors PX30, PX68, and PX104.

In summary, we demonstrated the biallelic inactivation of STK11/LKB1 in a pancreatic cancer of a patient with the PJS and in 4–6% of sporadic pancreatic and biliary adenocarcinomas, illustrating the role of this gene in familial and sporadic cancer development.

**Acknowledgments**

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**References**


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**Table 3. Mutations of the STK11/LKB1 Gene Identified in Pancreatic and Biliary Cancers**

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Sample</th>
<th>Allele loss</th>
<th>Position of gene alteration</th>
<th>Gene alteration</th>
<th>Predicted product</th>
<th>Origin of gene alteration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic</td>
<td>PJS1</td>
<td>LOH</td>
<td>Nucleotide +2</td>
<td>CGG gtt to CGG ggtg, Homozygous deletion</td>
<td>Insertion, altered splicing</td>
<td>Germline</td>
</tr>
<tr>
<td>Pancreatic</td>
<td>PX30</td>
<td>LOH</td>
<td>Exons 1 to 9</td>
<td>TAC CAG to TAA CAG</td>
<td>Absence</td>
<td>Somatic</td>
</tr>
<tr>
<td>Pancreatic</td>
<td>PX68</td>
<td>LOH</td>
<td>Codon 36 Exon 1</td>
<td>CGG GCT T to CGG CTT</td>
<td>Tyrosine to Stop</td>
<td>Somatic</td>
</tr>
<tr>
<td>Pancreatic</td>
<td>PX104</td>
<td>LOH</td>
<td>Codon 217 Exon 5</td>
<td>AAA CAT C to AAC ATC</td>
<td>Deletion, frameshift</td>
<td>Somatic</td>
</tr>
<tr>
<td>Pancreatic</td>
<td>PX289</td>
<td>LOH</td>
<td>Codon 312 Exon 8</td>
<td>Homozygous deletion</td>
<td>Absence</td>
<td>Somatic</td>
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<tr>
<td>Biliary</td>
<td>PX115</td>
<td>LOH</td>
<td>Exon 1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Codons, nucleotides, and exons are numbered according to GDB entries AF032984, AF032985, and AF032986.
* The underlined nucleotides are either deleted or inserted. Exonic sequences are in capital letters and intronic sequences are in lower case. The spaces between trinucleotides denote codon structure.


