Somatic Mutations in the *STK11/LKB1* Gene Are Uncommon in Rare Gynecological Tumor Types Associated with Peutz-Jegher's Syndrome

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Peutz-Jegher's syndrome (PJS) is a rare autosomal dominant disorder characterized by mucocutaneous pigmentation, hamartomatous polyposis, and predisposition to benign and malignant tumors of the gastrointestinal tract, breast, ovary, uterine cervix, and testis. Germline-inactivating mutations in one allele of the *STK11/LKB1* gene at chromosome 19p13.3 have been found in most PJS patients. Although ovarian sex cord tumors with annular tubules (SCTATs) and minimal deviation adenocarcinomas (MDAs) of the uterine cervix are very rare in the general population, both tumor types occur with increased frequency in women with PJS. An earlier report indicated that the 19p13.3 region containing the *STK11* gene was affected by loss of heterozygosity (LOH) in nearly 50% of MDAs of the uterine cervix. We investigated the role of *STK11* mutations and LOH of the 19p13.3 region in two PJS-associated SCTATs and in five SCTATs and eight MDAs of the uterine cervix, which occurred in patients lacking features of PJS (referred to here as "sporadic" cases). Germline mutations in the *STK11* gene, accompanied by LOH of markers near the wild-type *STK11* allele, were found in the two PJS-associated SCTATs. Somatic mutations in the coding region of *STK11* were not found in any of the sporadic SCTATs or MDAs studied, although LOH of the 19p13.3 region was seen in three of eight MDAs. Our findings indicate that *STK11*, like other tumor suppressor genes, is affected by biallelic inactivation in gynecological tumors of PJS patients. In addition, although LOH of the 19p13.3 region was seen in sporadic MDAs, somatic *STK11* mutations are rare. A yet-to-be-defined tumor suppressor gene in the 19p13.3 region may be the specific target of inactivation in these tumors. (*Am J Pathol* 2000, 156:339–345)

Peutz-Jegher's syndrome (PJS) is a rare, autosomal dominant disorder characterized by benign intestinal hamartomatous polyps and mucocutaneous pigmentation.^{1,2} PJS patients are reported to have a 10- to 18-fold increased risk of developing various tumors of the gastrointestinal tract, breast, testis, ovary, and uterine cervix.^{3,4} This risk appears to be particularly enhanced in women (~20-fold), because of an increased risk for the development of breast and gynecologic malignancies.⁴ A PJS susceptibility locus was recently mapped to chromosomal region 19p13.3.^{5–7} The chromosome 19p13.3 gene responsible for PJS was subsequently found to be a previously identified gene, *LKB1* (GenBank accession number U63333), which encodes a serine threonine kinase, also referred to as *STK11*.^{8,9} A second PJS locus has been proposed because a subset of families with PJS lack mutations in *STK11*.^{10,11}

Women with PJS have an increased incidence of two extremely rare gynecological tumors, SCTAT and MDA, also known as adenoma malignum, of the uterine cervix. SCTAT is an ovarian neoplasm with histological features intermediate between granulosa cell tumor and Sertoli cell tumor.^{12,13} This neoplasm is characterized by sex cord cells growing in the form of a ring, with the nuclei peripherally oriented around a central hyalinized body (Figure 1A). In women with PJS, SCTATs are usually multifocal, bilateral, and benign, whereas the sporadic forms of SCTAT are usually unilateral, often large (palpable), and, in 20% of cases, malignant. Approximately 36% of SCTATs are associated with PJS.¹² MDA is also a very rare malignancy, accounting for only 1% to 3% of all adenocarcinomas of the uterine cervix,¹⁴ and well under 1% of all cervical carcinomas. MDA is a very well-differentiated, mucin-producing adenocarcinoma that, despite its aggressive behavior, has a deceptively bland appearance and lacks cytological features typical of malignancy in all or most of the tumor. The benign-appearing endocervical glands are haphazardly arranged and extend deeply (>5 mm) into the cervical wall (Figure 1C). Occasional mitotic figures are usually present. Approximately

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Figure 1. H&E-stained sections of rare gynecological tumor types associated with Peutz-Jeghers syndrome. **A:** Sex cord tumor with annular tubules (SCTAT) from a patient without PJS (original magnification, $\times 400$). **B:** PJS-associated sex cord tumor with annular tubules showing three SCTAT "tumorlets" (original magnification, $\times 400$). **C:** Minimal deviation adenocarcinoma (MDA) of the uterine cervix showing deep extension of neoplastic glands into the cervical stroma (original magnification, $\times 25$).

10% of all cases of MDA of the uterine cervix are found to occur in women with PJS.¹⁵

Several groups of investigators have analyzed sporadic tumors of the gastrointestinal tract, skin, breast, testis, ovary, and cervix for mutations of the *STK11* gene and/or losses of heterozygosity in the region of the *STK11* locus at 19p13.3.^{16–25} Results of these studies indicate that mutations of the *STK11* gene are rare in sporadic forms of common cancer types associated with PJS. Because ovarian SCTAT and MDA of the uterine cervix are strongly associated with PJS, we sought to examine the potential role of somatic mutations in the *STK11* gene and allelic imbalance at chromosome 19p13.3 in sporadic forms of these tumors, and to document bi-allelic inactivation of *STK11* in the tumors arising in PJS patients.

Materials and Methods

Tumor Specimens, Microdissection, and DNA Extraction

Paraffin-embedded tissue specimens from seven ovarian SCTATs were obtained from the pathology archives of the Mayo Clinic (Rochester, MN) and from the consultation files of Dr. Robert Scully (Massachusetts General Hospi-

tal, Boston, MA). Two of the seven SCTATs (SCTAT1 PJS and SCTAT2 PJS) were from patients with known family histories of PJS. Both of these specimens showed multiple small SCTAT "tumorlets" within otherwise normal ovarian tissue (Figure 1B). The remaining five SCTATs were solitary lesions and were presumably sporadic, because they arose in women lacking any history or features of PJS. The sporadic SCTAT specimens contained greater than 90% tumor cells; therefore, microdissection of these specimens was unnecessary. The PJS-associated SCTATs were subjected to laser capture microdissection (Arcturus) to isolate relatively pure populations of tumor and non-neoplastic cells. Archival specimens of eight cases of MDA were obtained from the pathology archives of Kumamoto University Hospital, Kumamoto Red-Cross Hospital, and Kumamoto National Hospital, Kumamoto, Japan. Because the neoplastic endocervical glands were widely dispersed in the cervical stroma, these specimens were also subjected to laser capture microdissection. Genomic DNA was isolated from all laser capture microdissection samples, as recommended by the manufacturer. Briefly, samples were digested overnight at 37°C in 50 μ L of digestion buffer (0.04% proteinase K, 10 mmol/L Tris-HCl, pH 8.0, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), and 1.0% Tween-20) and heated at 95°C for 5 minutes, followed by two

Table 1. PCR Primer Sequences and Annealing Temperatures for Amplification of STK11 Exons 1–9

Primer	Sequence (5'-3')	Fragment size (bp)	Annealing temperature (°C)
1aF	GGAAGTCGGAACACAAGGAA	143	58
1aR	ATACCCACCGACATCAGCTC		
1bF	GAGCTGATGTCGGTGGGTAT	159	58
1bR	GTCCAGCACCTCCTTCACC		
1cF	TCTTACGGCAAGGTGAAGGAGGTG	197	58
1cR	GGGAGGAGAGAAGGAAGGAA		
2F	CGGCTGATACACCCCTGTCC	141	62
2R	GCGGTCCCAACACCGGAAAG		
3F	CCCCCGTGCTCCCTGGGCCTGT	173	65
3R	CCCTGCCCCGCGCACGCA		
4F	CGGCCCCAGGACGGGTGT	226	61
4R	GTGCAGCCCTCAGGGAGT		
4F	CGGCCCCAGGACGGGTGT	181	62
4bR	CCCTAGCACGTGCCTACCAC		
5aF	ACTCCCTGAGGGCTGCAC	217	61
5aR	CCCCTCGGAGTGTGCGTGTGGT		
5bF	CCTGAGGGCTGCACGGCACC	180	64
5bR	GGGGCGGGGCACTTACAGG		
6aF	TCAACCACCTTGACTGACCA	251	64
6R	ACACCCCCAACCCCTACATTT		
6bF	GACCACGCCTTTCTTCCCTCCC	236	64
6R	ACACCCCCAACCCCTACATTT		
7F	TCACCCAGGGCCTGACAACAGAG	193	64
7B	GCAGCCTCGGCCCCACTG		
8F	CCTGACAGGCGCCACTGCTTC	240	64
8R	GGCCCCCGCCAGACTCAC		
9aF	AGCTGTAAGTGCCTCCCGTGG	206	58
9aR	CGCCCTGGATTTGGTGCTC		
9bF	CAGCTGAGCACCAAATCCAG	170	58
9bR	CAGGCGTTGTCCCCACAT		
Sequencing:			
S1aF	CTCAGGGCTGGCGGCGGGACT		
S1bF	ACGTTTCATCCACCGCATCGAC		
S3bF	GCTGTGTGCTCTAGCGCC		
S4bF	GGGTGTGTGCTGCCCGCAG		
S9bR	ACACACGGCCTTGGGGAGG		

extractions in phenol:chloroform (1:1; pH 9.0) and ethanol precipitation. DNA was isolated from manually microdissected deparaffinized tissue sections by overnight digestion in 50 mmol/L Tris-HCl (pH 9.0)–0.02% sodium dodecyl sulfate-proteinase K at 37°C, followed by heating at 95°C for 5 minutes. Digested samples were extracted twice in phenol:chloroform (1:1; pH 9.0) and ethanol precipitated.

Polymerase Chain Reaction Amplification and Direct Sequencing

Polymerase chain reaction (PCR) amplifications of *STK11* exons 1–9 were performed using the primer sequences and temperatures listed in Table 1. Multiple primer pairs were used to amplify selected exons because of difficulty amplifying DNA fragments from some of the specimens. PCR reactions containing 1× PCR buffer (20 mmol/L Tris-HCl, pH 8.4, 50 mmol/L KCl), 1.5 mmol/L MgCl₂, 100 μmol/L dNTPs (Pharmacia, Uppsala, Sweden), 0.1 μmol/L forward primer, 0.1 μmol/L reverse primer, and 2.5 U *Taq* polymerase (Life Technologies, Inc., Grand Island, NY) were amplified using an initial denaturation cycle of 5 minutes at 95°C, followed by 42 cycles of 30

seconds at 95°C, 30 seconds at the annealing temperature specified in Table 1, and 30 seconds at 72°C. The resulting PCR products were evaluated on 2% ethidium bromide-stained agarose gels. Next, they were directly sequenced using the ThermoSequenase radiolabeled terminator cycle sequencing kit (Amersham), per the manufacturer's instructions, with either the same primers used for PCR amplification or additional sequencing primers listed in Table 1. Products of the cycle sequencing reactions were resolved on 6% acrylamide–8 mol/L urea denaturing gels. Gels were fixed in a solution of 5% methanol and acetic acid, dried, and subjected to radiography. Results were confirmed by repeating each PCR and direct sequencing reaction.

LOH Analysis

Samples were assessed for loss of heterozygosity by PCR amplification of small segments of DNA, using selected Map pair (Research Genetics, Huntsville, AL) primer sets and annealing temperatures described below. Each 10-μl PCR reaction contained 1× PCR buffer (20 mmol/L Tris-HCl, pH 8.4, 50 mmol/L KCl), 200 μmol/L dATP, 200 μmol/L dGTP, 200 μmol/L dTTP, 25 μmol/L

dCTP, 2 μ Ci (3000Ci/mmol) dCTP, 0.1 μ mol/L of each primer, and 1.0 U *Taq* polymerase. For microsatellite markers D19S886, D19S565, D19S591, D19S216, and D19S395, DNA templates were amplified using an initial denaturation step of 95°C for 5 minutes, followed by 32 cycles of 95°C for 30 seconds, 55°C (D19S886, D19S565, D19S591, D19S216) or 60°C (D19S395) for 1 minute, and 72°C for 30 seconds, and by a final extension step at 72°C for 2 minutes. Touchdown PCR was used for microsatellite marker D19S894 as follows: 1 cycle of 95°C for 5 minutes, followed by 3 three cycles of 95°C for 30 seconds, 59°C for 30 seconds, and 72°C for 30 seconds; 3 cycles of 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds; and 33 cycles of 95°C for 30 seconds, 55° for 30 seconds, and 72°C for 30 seconds, followed by a final extension step at 72°C for 2 minutes. A touchdown PCR program was also used for microsatellite marker D19S549 as follows: 1 cycle of 95°C for 5 minutes, followed by 3 cycles of 95°C for 30 seconds, 64°C for 30 seconds, and 72°C for 30 seconds, 3 cycles of 95°C for 30 seconds, 62°C for 30 seconds, and 72°C for 30 seconds; and 33 cycles of 95°C for 30 seconds, 60° for 30 seconds, and 72°C for 30 seconds, followed by a final extension step at 72°C for 2 minutes. PCR products were resolved by electrophoresis on 6% acrylamide–8 mol/L urea gels. Gels were then fixed in a solution containing 5% acetic acid and methanol, dried, and subjected to autoradiography. Given the limited availability of tumor DNA from the PJS-associated SCTATs, only selected 19p13.3 loci were evaluated in these specimens. In previous studies we have noted erratic results from PCR-based LOH analyses using very small quantities of template DNA. Hence, losses of heterozygosity in the PJS-associated SCTATs were scored only if confirmed by independent PCR reactions. Three sporadic SCTATs were excluded from the 19p13.3 LOH analysis because sufficient quantities of matched normal tissue were not available.

Results

Exons 1–9 of the *STK11* gene were PCR amplified, purified, and evaluated by direct sequencing of the PCR products from all 13 sporadic cases. Direct sequencing of exon 6 PCR products amplified from the constitutional DNA of one PJS patient (SCTAT1 PJS) revealed a single-base-pair change from G→T (Figure 2) resulting in a nonsense mutation at codon 256 (GAA [glu]→TAA [stop]). In the constitutional DNA of the second PJS-associated SCTAT (SCTAT2 PJS), a single-base-pair change from A→C (AAC [asn]→ACC [thr]) in codon 181 of exon 4 was identified. These germline mutations were confirmed by independent PCR amplification and sequencing reactions. Sufficient DNA from several pooled SCTAT2 PJS tumorlets was available for sequence analysis. The presence of the missense mutation was confirmed, and no additional *STK11* mutations were identified in the tumorlet DNA. SCTAT1 PJS tumor DNA was not sequenced owing to insufficient DNA availability. All five sporadic SCTATs and eight sporadic MDAs lacked mu-

Figure 2. Nonsense mutation of *STK11/LKB1* (exon 6) in the PJS-associated SCTAT1. The **arrow** indicates a point mutation (GAA [glu] → TAA [stop]) at codon 256. The corresponding sequence in SCTAT5 (sporadic) is wild type.

tations within the *STK11* exons or at the intron/exon boundaries.

All sporadic MDAs, two sporadic SCTATs, and both PJS-associated SCTATs were evaluated for LOH of the chromosome 19p13.3 region. The seven polymorphic microsatellite markers used for this analysis span approximately 6.0 Mb within chromosome 19p13.3 and are depicted schematically in Figure 3A. The schematic diagram for the region was generated based on information from the chromosome-19 mapping project (²⁶; <http://www-bio.llnl.gov/>). The locations for microsatellite markers D19S549 and D19S395 were estimated based on the maps of sex-averaged recombination distances (Genome Database [<http://www.gdb.org>] and Marshfield Center for Medical Genetics [<http://www.marshmed.org/genetics>]). Of particular interest to this study are marker D19S886, which is the marker closest to the *STK11* gene (approximately 190 kb telomeric) and marker D19S216, which is the marker previously shown to be lost with the highest frequency in sporadic cases of MDA of the uterine cervix.²² LOH was scored when there was a relative decrease of greater than 50% in one allele of the tumor compared with the allele signals in matched normal tissue. Cases were scored as not informative when only a single allele size was present in both tumor and matched normal tissue. Cases were scored as not evaluated when there was insufficient material for the assay or when PCR amplification of specific microsatellite markers was unsuccessful.

Figure 3. **A:** Map of chromosomal region 19p13.3. The locations of markers D19S549 and D19S395 are estimates based on maps of sex-averaged recombination distances (Genome Database and Marshfield Center for Medical Genetics). **B:** Summary of chromosome 19p13.3 losses of heterozygosity in SCTATs and MDAs. **C:** LOH analysis in representative tumors. LOH (arrows) at D19S886 is shown in PJS-associated SCTAT1 and in MDA2. MDA1 exhibited LOH at D19S549, but not at proximal markers D19S591 and D19S894.

Results of the LOH analysis are summarized schematically in Figure 3B, and representative examples of LOH assays are depicted in Figure 3C. Allelic loss of 19p13.3 markers was observed in both PJS-associated SCTATs, consistent with biallelic inactivation of *STK11* in these tumors. Two sporadic SCTATs (SCTAT3 and SCTAT7) and five of eight MDAs did not show 19p13.3 LOH at any of the loci tested. *STK11* was likely included in the deleted region in two of the three MDAs demonstrating LOH at 19p13.3 (MDA2 and MDA7). One MDA (MDA1) exhibited LOH at D19S549, but retained heterozygosity of several intervening markers closer to the *STK11* gene.

Discussion

The study of inherited cancer syndromes has proven critical in defining genes that contribute not only to the development of cancers in those carrying germline mutations, but also to cancers arising in the absence of recognized syndromes ("sporadic" forms).²⁷ The recent identification of germline *STK11* gene mutation as the underlying genetic alteration responsible for most cases of PJS^{8,9} has generated substantial interest in evaluating sporadic cancers of the same type as those observed in PJS patients for mutations in this gene.

Several independent investigations suggest that somatic mutations of the *STK11* gene are infrequent (<10%) in sporadic cancers arising in several different organs, including the gastrointestinal tract, skin (melanoma), breast, testis, and ovary.^{16-21,23,24} A notable exception is a recent report of frequent *STK11* mutations in left-sided colon cancers (53.8%).²⁵ To date, studies that have included gynecological malignancies have focused on the more common types of tumors affecting the ovary

and cervix, specifically ovarian granulosa cell tumors and typical squamous carcinomas and adenocarcinomas of the cervix.^{23,24} *STK11* mutations in these tumor types are reportedly rare.

Although granulosa tumors have been reported to occur with increased frequency in women affected by PJS,^{28,29} a review of several such cases resulted in their reclassification as SCTATs.¹³ Other ovarian tumor types, such as benign cystadenomas and cystadenofibromas, have also been reported in the ovaries of women affected by PJS. However, the frequency of these tumors in the general population raises doubts about the significance of their association with PJS.¹³ In contrast, SCTATs are exceedingly rare in the general population, yet are frequently found in women with PJS. Hence, it appears that SCTATs, rather than typical granulosa tumors or other ovarian tumor types, are the ovarian tumors distinctively associated with PJS. SCTATs are thought to arise from granulosa cells but show characteristic morphological features that warrant their classification as sex cord stromal tumors distinct from granulosa tumors.^{12,13} We are not aware of any reports in the literature that have evaluated the *STK11* gene in sporadic forms of rare tumor types associated with PJS (ovarian SCTATs and MDAs of the uterine cervix). Our interest in assessing the role of *STK11* in sporadic SCTATs and MDAs was further piqued by a recent study demonstrating a high frequency of allelic loss over a large distance (>3.5 Mb) of chromosome region 19p13.3 in nine sporadic cases of MDA of the cervix.²²

Although germline mutations of *STK11* were identified in the two PJS-associated SCTATs, somatic mutations of *STK11* were not found in any of the five sporadic SCTATs or eight sporadic MDAs. Our results are consistent with

previous reports indicating that somatic mutations of *STK11* are infrequent in sporadic cancers of the same type as those observed in PJS patients. Our results are consistent also with biallelic inactivation of *STK11* in the SCTATs arising within the two PJS patients. Our presumption that the wild-type allele was deleted rather than mutated in the PJS-associated SCTATs is supported by the identification of 19p13.3 LOH in both tumors and the *STK11* sequence analysis of SCTAT2 PJS, which demonstrated retention of the allele with the germline mutation and no additional somatic mutations.

Three of eight sporadic MDAs demonstrated allelic loss at chromosome 19p13.3. One of three cases (MDA 1) exhibited allelic loss at marker D19S549, but not at flanking markers D19S565, D19S591, D19S894, and D19S395. Interestingly, D19S549 is located in close proximity to D19S216, which was found to exhibit the highest frequency of LOH (6/6 informative cases) in another study of 19p LOH in sporadic cases of MDA.²² Lee and colleagues observed allelic loss at chromosomal region 19p13.3 in all nine MDAs evaluated, with the highest frequency, over 3 Mb centromeric to *STK11*, at chromosomal marker D19S216. Based on their results, these investigators suggest that there may be a tumor suppressor gene (TSG) distinct from *STK11*, that is involved in the development of sporadic MDA. The pattern of allelic loss we observed in our cases of sporadic MDA is not as striking, but for the cases that do exhibit loss, we cannot exclude the possibility that another TSG exists closer to marker D19S216. The fact that we observed LOH in only three of eight (37.5%) of cases could reflect the different populations from which the cases were obtained (Korea versus Japan) or the small number of cases available for analysis in each study. Other investigators studying relatively large numbers of sporadic colon cancers have observed more frequent (30%) allelic imbalances at the 19p13.3 markers centromeric (approximately 2.0 Mb) to *STK11*, compared with the frequency of losses (16%) closer to the *STK11* locus (D19S886; LA Boardman and SN Thibodeau, personal communication). Moreover, in a recent analysis of pancreatic and biliary cancers, allelic losses at the D19S886, D19S565, and D19S216 loci were observed in 32% to 35% of the tumors evaluated, whereas somatic inactivation of *STK11* was demonstrated in only 4% to 6% of these cases.¹⁷ Taken together, these observations provide further evidence supporting the existence of another TSG located centromeric to *STK11*.

Although the number of cases in our study is small due to difficulty in obtaining specimens of these rare tumors, our results indicate that somatic mutations of the *STK11* gene are infrequent in sporadic cases of SCTAT and MDA. However, our data confirm that allelic losses at chromosome region 19p13.3 occur in at least a subset of sporadic cases of MDA. Furthermore, our results and the results of others suggest that there may be another, yet to be identified, chromosome 19p gene that is important in the development of certain types of sporadic cancers.

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