Lack of $STK11$ Gene Expression in Homozygous Twins with Peutz-Jeghers Syndrome

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Abstract. Clinical features of Peutz-Jeghers syndrome (PJS), an autosomal dominant disorder, include clusters of melanotic spots on the lips and limbs, polyposis of the gastrointestinal (GI) tract, and propensity to develop neoplasms of the GI tract, ovaries, testes, and other sites. We report twin sisters with PJS who were found to be homozygous, based on analyses of 9 DNA markers containing short tandem repeats (STR). Aberrant expression of a putative tumor suppressor gene, $STK11$, which encodes a serine threonine kinase, has been suggested as the etiologic factor in PJS. In both of the twin sisters with PJS, mRNA analyses by RT-PCR demonstrated a complete lack of $STK11$ gene expression. These results provide direct evidence that $STK11$ gene expression is abnormal in PJS. Detecting abnormal expression of the $STK11$ gene may serve as a molecular approach to the diagnosis of PJS and may facilitate genotype-phenotype correlations in PJS patients. (received 28 January 2004; accepted 8 March 2004)

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Introduction

Peutz-Jeghers syndrome (PJS, MIM #175200), an autosomal dominant genetic disorder, is clinically characterized by melanotic macules on the lips, perioral skin, and extremities and by polyposis of the gastrointestinal (GI) tract. This disorder was reported by Hutchinson [1] in 1896 and delineated by Peutz [2] in 1921 and Jeghers et al [3] in 1949. Intussusception and GI bleeding are frequent in PJS patients. Although the polyposis was not initially considered premalignant, metastatic carcinoma from a malignant polyp was reported in an early PJS case [4]. Malignancies involving the GI tract, bladder, bronchus, nose, esophagus, pancreas, mesenteric lymph nodes, breast, ovary, and testis have been observed in PJS patients [5-9]. About half of PJS patients were estimated to be likely to die from cancer before age 57 yr [10].

The gene responsible for PJS is believed to be located on the short arm of human chromosome 19. Hemminki et al [11] used comparative genomic hybridization (CGH), targeted linkage analysis, and loss of heterozygosity (LOH) to localize the susceptibility locus of PJS. A DNA marker, D19S886, was shown to be closely linked to the susceptible gene (multipoint lod score = 7.00) [11]. Mutation of a serine threonine kinase encoded by the $STK11$ gene, a putative tumor suppressor gene (also designated $LKB1$), is considered likely to be the etiologic factor in PJS. The $STK11$ gene is located between 19p13.3 and 19p13.4 [12].

In this investigation, we studied homozygous twin sisters with a familial history of PJS. By investigating the $STK11$ gene expression in these sisters, we obtained direct genetic evidence that links PJS with abnormal $STK11$ gene expression.
Materials and Methods

**Patients.** A 20-yr-old woman came to our hospital complaining of post-coital bleeding and abnormal spotting for 2 mo. She was recognized as a PJS patient with a PJS father and a twin sister. She had multiple typical brown spots on her perioral skin and lips (Fig. 1). A cervical tumor (size 2x1x1 cm) was identified. Pathological examination of the cervical biopsy showed adenocarcinoma. Proctoscopic examination for polyposis was negative. No ovarian abnormalities were evident. Radical hysterectomy and retroperitoneal lymphadenectomy were performed.

The twin sister, also a PJS patient, had brown spots present mainly on the extremities (Fig. 2). Her cervical Papaniculau smear was normal. A diagnosis of benign intestine polyposis had been made 4 yr previously, and she had laparoscopic enucleation of a benign ovarian mucinous tumor 2 yr previously. The family inheritance of PJS is shown in Fig 3.

**DNA extraction.** Genomic DNA was extracted from both PJS patients’ peripheral blood samples using a commercial kit (QIAmp blood kit, Qiagen GmbH, Hilden, Germany), following the manufacturer’s protocol. Recovered DNA was rehydrated in a total volume of 50 µl. The genomic DNA was stored at -20°C until assay.

**Zygosity test.** A commercial kit (Profiler Plus, ABI Corp., Foster City, CA, USA) that contained nine DNA markers composed of tetranucleotide repeats (D3S1358, von Willibrand factor gene, fibrinogen A gene, TH01, TPOX, CSF1PO, D5S818, D13S317, and D7S820), was used to test the homozygosity of the twin sisters [13].

**LOH analysis.** A DNA marker, D19S886, has been reported to be closely linked to STK11 gene [12,14]. Therefore, PCR amplification with a fluorescent dye-labeled primer, followed by 6% denaturing polyacrylamide gel electrophoresis, was used to test the heterozygosity of the STK 11 gene of the sisters.

**RNA extraction and reverse transcription.** Total RNA was extracted from 200 µl of heparinized whole
blood from both patients and from a healthy control (apparently unrelated to the sisters) using TRIzol reagent (Invitrogen, Groningen, Netherlands) according to the manufacturers’ directions. Total RNA isolated was rehydrated in 50 µl of DEPC water. To convert mRNA to complementary DNA (cDNA) for further analysis and stable storage, reverse transcription was conducted. In brief, in a total volume of 12 µl containing 2 µl of RNA, 1 µl of 20 mM dNTP (ABgene, New York, NY, USA), 1 µl of oligo dT (Invitrogen), and DEPC water were mixed. The mixture was first incubated at 65°C for 5 min and then transferred on ice. Four µl of 5x first strand buffer (Invitrogen), 2 µl of dithiothreitol/DTT (Invitrogen), and 40 U of RNaseOUT (Invitrogen) were added to the mixture. After incubation at 37°C for 2 min, 200 U of M-MLV reverse transcriptase (Invitrogen) was added to convert mRNA to cDNA. The cDNA was stored at -20°C until assay.

**STK11 gene expression analysis.** PCR primers specifically targeted to STK11 cDNA sequence (PJS5L: 5’-GAAGTCGGAACACAAGGAAG-3’, PJS5R: 5’-CCGTAACCTCCTCAGTAGTT-3’) were employed to detect STK11 cDNA in the samples from these two sisters and a healthy individual (apparently unrelated to the sisters) was analyzed likewise as a reaction control. PCR was carried out in a reaction volume of 30 µl containing 1 µM each of the primers, 200 µM of dNTP (ABgene), 1.25 U of Thermo Start DNA polymerase (ABgene), and its reaction buffer. The reaction mixtures were first incubated at 94°C for 15 min to activate the hot start DNA polymerase, then they were cycled 40 times at 94°C, 50 sec, 58°C, 1 min, and 72°C, 1.5 min. An additional 7 min at 72°C was added to the last cycle to complete extension. The size of the PCR amplicon generated from primer set PJS5 was 395 bps. After amplification, PCR products were resolved in a 2% agarose gel.
Results

Zygosity analysis. The twin sisters both had typical clinical features of PJS and suffered from tumors of the reproductive system. Surgical procedures were performed to remove their tumors. To understand if their disorders were related to their genetic composition, samples from the twin sisters were subjected to zygosity analysis with STR polymorphic markers. The nine DNA markers all showed identical repeat numbers in both samples, indicating that the twins were probably homozygous.

D19S886 DNA marker and LOH analysis. Since the PJS locus is believed to be situated on chromosome 19 and closely linked to a DNA marker, D19S886 [12,14], this marker can be used as an indicator of the presence of STK11, a putative tumor suppressor gene. This marker was analyzed to determine if LOH occurred in the patients. The results obtained for the twin sisters both showed a single dominant fluorescent peak with identical electrophoretic mobility (Fig. 4).

RT-PCR test for STK11 gene expression. Using STK11 cDNA-specific primers, the electrophoretic analysis showed no STK11-specific cDNA present in either twin's circulation, whereas a clear band was present in the positive control case. These data show that STK11 gene expression was absent in this pair of homozygous twin sisters (Fig. 5).

Discussion

The neoplasms that are occur in PJS patients may involve the lung [15], colorectum [16], biliary tract [17], breast [18], pancreas [19], cervix [20], ovary [21], brain [22], and testis [23]. Gynecomastia has been noted in some males with PJS [9,10]. The twin sisters that were the subjects of this investigation fit the phenotypic criteria of PJS.

Firmly establishing the genetic susceptibility locus responsible for PJS will greatly facilitate prenatal molecular diagnosis of this disorder. Chromosome 1 [24] and chromosome 6 [25] had been suggested to contain the gene responsible for PJS, but Hemminki et al [11] provided strong evidence that the genetic lesion that leads to PJS is on the short arm of chromosome 19. This finding has been independently confirmed by Amos et al [14] and the STK11 gene has been localized on chromosome 19 [12,26].

Jishage et al [27] established STK11 gene knock-out mice and showed that the STK11 gene is critical for embryogenesis and normal development [27].

Yoon et al [28] investigated 10 PJS cases using the PCR-SSCP method and documented various lesions of the STK11 gene, including missense mutations, frame-shift mutations, a single base mutation leading to a premature stopping codon, and an intronic mutation in the splice-acceptor site. Five of the 10 PJS cases were suspected to be due to germline mutations [28].

For the LOH assays, it would have been ideal to compare the results in blood samples from healthy family members with those from the twin sisters with PJS. Unfortunately, samples from the proband's healthy mother and healthy sister were unavailable for testing. The likelihood that the results (ie, one predominant peak present in the DNA samples from both twin sisters) indicate a loss of heterozygosity is 64%, based upon the reported heterozygosity rate (0.64, Gene Data Bank: D19S886).

Based on RT-PCR analyses of STK11 gene expression using primer set PJ5, mRNA from STK11 gene was undetectable in the specimens from the twin sisters with PJS.

In conclusion, we studied two homozygous twin sisters with family history of PJS. Absence of STK11 gene expression in the patients was demonstrated by RT-PCR analyses. This study provides direct evidence that the STK11 gene is involved in the occurrence of PJS. We plan to test whether the STK11 gene is expressed in amniocytes. If this is shown to be the case, when pregnant women with a family history of PJS seek prenatal diagnosis of PJS, the use of RT-PCR to detect abnormal STK11 gene expression may be a practical diagnostic approach.

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References