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Genetic defects underlying Peutz–Jeghers syndrome (PJS) and exclusion of the polarity-associated *MARK/Par1* gene family as potential PJS candidates

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Abstract

LKB1/STK11 germline inactivations are identified in the majority (66–94%) of Peutz–Jeghers syndrome (PJS) patients. Therefore, defects inother genes or so far unidentified ways of *LKB1* inactivation may cause PJS. The genes encoding the MARK proteins, homologues of the Par1 polarity protein that associates with Par4/Lkb1, were analyzed in this study because of their link to LKB1 and cell polarity. The genetic defect underlying PJS was determined through analysis of both *LKB1* and all four *MARK* genes. *LKB1* point mutations and small deletions were identified in 18 of 23 PJS families using direct sequencing and multiplex ligation-dependent probe amplification analysis identified exon deletions in 3 of 23 families. In total, 91% of the studied families showed *LKB1* inactivation. Furthermore, a *MARK1, MARK2, MARK3* and *MARK4* mutation analysis and an *MARK4* quantitative multiplex polymerase chain reaction analysis to identify exon deletions on another eight PJS families without identified *LKB1* germline mutation did not identify mutations in the *MARK* genes. *LKB1* defects are the major cause of PJS and genes of the *MARK* family do not represent alternative PJS genes. Other mechanisms of inactivation of *LKB1* may cause PJS in the remaining families.

Keywords

LKB1; MARK; MLPA; Peutz; Jeghers

Peutz–Jeghers syndrome (PJS) is an autosomal dominant disorder characterized by mucocutaneous melanin pigmentation, hamartomatous polyps and an increased cancer risk

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(1,2). The discovery of underlying mutations in the tumor suppressor gene *LKB1/STK11*, has provided further insight into this disorder. However, the precise function of LKB1 remains elusive as is the exact molecular mechanism responsible for the phenotypic characteristics of PJS. We recently hypothesized that loss of the polarity function of LKB1 results in mucosal prolapse, ultimately leading to PJS polyp formation, and tumor growth (3).

Although *LKB1* was identified as the PJS gene, germline mutations were found in only 30–70% of patients using conventional mutation analyses (4). *LKB1* might, however, be alternatively inactivated and recently exonic deletions have been described, resulting in 66–94% of PJS patients with *LKB1* inactivation (5–7). A subset of PJS patients remains with seemingly no *LKB1* inactivating mutation and consequently a second PJS gene may exist. Several possible candidates have been studied, including genes encoding LKB1 interacting proteins LIP1, BRG1, STRAD and its co-activator MO25, but to date no second PJS gene has been identified (8–11). In search of a second PJS locus, linkage to chromosome 19q13.4 was found in one Indian PJS family (12) and a 6-day-old patient presenting with a hamartoma with the histology of a PJS polyp had a translocation in the same region (11). Several genes within 0.5 Mb of the breakpoint were sequenced (including *BRSK1/KIAA1811*), but none was mutated in PJS patients without *LKB1* mutation. As the region on chromosome 19q13.4 was implicated in two different PJS families, it may harbor a second PJS gene.

One of the genes located in the 19q13 region is the *MARK4* gene. The MARK proteins are part of the family of AMPK-related kinases of which LKB1 is an upstream activator (13,14). These four microtubule affinity-regulating kinases play a role in microtubule dynamics during polarization of cells (15). *MARK2* knockout and heterozygous mice were also described to present with a phenotype of colorectal prolapse (16). Interestingly, PJS polyps histologically resemble mucosal prolapse (3). However, the *MARK2* knockout mice also develop characteristics not linked to PJS-like immune system dysfunction, overall proportionate dwarfism and a peculiar hypofertility (16,17). The MARK genes have also been implicated in tumorigenesis since in two colorectal tumors a mutation and 1 bp insertion were reported in *MARK3* (18).

The MARK proteins are the human homologues of Par1, which is, like LKB1/Par4, a member of the par family of polarity proteins. This family is conserved during evolution and the six members of the family regulate epithelial polarity in *Drosophila melanogaster*, *Caenorhabditis elegans* and vertebrates, by involvement in cell migration and the establishment of the anterior–posterior axis. Due to their relation to *LKB1* and their role in polarity, the *MARK* genes make interesting PJS candidates.

To further investigate germline alterations related to PJS, we screened for *LKB1* defects and for mutations in the *MARK1, MARK2, MARK3* and *MARK4* genes. *LKB1* mutation analysis was performed on 23 PJS patients from different families using sequence analysis and multiplex ligation-dependent probe amplification (MLPA) to detect point mutations and exon deletions. Furthermore, a mutation analysis of the four *MARK* genes and an exon deletion screen of *MARK4* on eight PJS patients from families without *LKB1* germline mutations was performed including the Indian PJS family where linkage was found at chromosomal region 19q13.4 (19).

Material and methods

PJS patients study group

This research was carried out in accordance with the ethical guidelines of the research review committee of our institution. All PJS patients fulfilled the clinical criteria for PJS as described by Tomlinson *et al.* (20).

PJS patient material was obtained from pathology archives of several Dutch hospitals including the Academic Medical Center (Amsterdam, The Netherlands), Erasmus University (Rotterdam, The Netherlands) and the Free University (Amsterdam, The Netherlands), and the Johns Hopkins Hospital (Baltimore, MD, USA). Use of anonymous or coded leftover material for scientific purposes is part of the standard treatment contract with patients in our hospital (21).

Formalin-fixed paraffin-embedded tissue was available from 23 PJS patients from different families. Also, genomic DNA isolated from blood and cell lines was available from eight other PJS families without LKB1 germline mutation (22). One of these families was the Indian PJS family PJS07 as described by Mehenni *et al.* (19) kindly provided by Dr S. Antonarakis.

Genomic DNA isolation

Genomic DNA was isolated from paraffin-embedded hamartomatous tissue. Tissue was deparaffinized and DNA was isolated using the Puregene DNA Isolation kit (Gentra Systems, Minneapolis, MN, USA). DNA concentrations were measured using the PicoGreen Double Stranded DNA Quantitation kit (Molecular Probes, Leiden, The Netherlands).

Mutation and sequence analysis

LKB1 coding exons and exon/intron boundaries were amplified by polymerase chain reaction (PCR) as previously described (23) (GenBank accession numbers: exon 1, AF032984; exons 2–8, AF032985; exon 9, AF032986). All coding exons and exon/intron boundaries of *MARK1*, *MARK2*, *MARK3* and *MARK4* were also amplified by PCR (GenBank accession numbers: NM_018650, NM_017490, NM_002376, NM_031417 respectively). Primer sequences and PCR conditions are available upon request.

PCR products were purified using the Qiagen PCR purification kit (QIAGEN Benelux B.V., Venlo, The Netherlands), and the sequencing reaction was performed using the ABI Big Dye Terminator Cycle Sequencing Kit (Applied Bio-systems, Foster City, CA, USA). Samples were run on an ABI 3100 genetic analyzer and analyzed using SEQUENCE NAVIGATOR and CODONCODE ALIGNER.

Multiplex ligation-dependent probe amplification

Deletion of *LKB1* exons was studied using the MLPA kit P101 (MRC-Holland, Amsterdam, The Netherlands). Results were analyzed using the MRC COFFALYSER software (www.MLPA.com). For controls, genomic DNA samples from six normal tissues were used. Results were normalized on all control probes present in the kit and on all six normal tissues. Deletions and duplications were defined as ratios of <.55 and <1.45, respectively and were repeated at least twice.

MARK4 exon deletion screening by quantitative multiplex PCR of short fluorescent fragments

Deletions of *MARK4* were determined by quantitative multiplex PCR of short fluorescent fragments (QMPSF) by a pairwise combination of *MARK4* exons and as an internal reference exon 13 of the household gene *HMBS*. Primer sequences and PCR conditions are available upon request.

Analysis was carried out using an automated ABI 3100 sequencer (Applied Biosystems) with a GENESCAN™ 500 ROX size standard (Applied Biosystems) and the manufacturer's GENESCAN® 5.1 software. The intensity of the genescan peak for a specific exon for a patient sample was normalized for HMBS in the same reaction. The same was calculated for the normal

control. The normalized value of the patient sample was divided by the normalized control sample. Loss of an exon was assumed if the ratio between these two values was less than 0.6.

Results

LKB1 germline mutations and deletions

All coding exons and adjacent exon–intron boundaries of *LKB1* were amplified from genomic DNA of all 23 PJS patients in our study group and used for direct sequencing. *LKB1* germline mutations were detected in 18 of the 23 (78%) patients; 12-point mutations (of which 2 in intron–exon transition), 2 deletions, 4 insertions of a few basepairs and one silent mutation (Table 1). Of these, 13 mutations have been described previously (19,22,24–28). Five novel mutations are reported here: c.551T>C, c.712A>T, c.762delC, c.829–830insGGGCG and c. 547C>T leading to two missense (p.Leu184Pro, p.Ile238Phe) mutations, two frameshift mutations (p.Pro245Pro fsX33, p.Asp277Gly fs X12) and one silent mutation (p.L183L) respectively. For some patients, non-affected family members were used as controls. Furthermore, the described mutations were not identified in a control group of over 250 tumor samples (18,29,30).

To determine if *LKB1* was inactivated in the remaining five PJS patients, an MLPA analysis was performed to identify whole exon deletions. For one patient, MLPA analysis was not possible due to poor quality of the DNA. In total, three patients showed heterozygous exon losses: one patient showed loss of the entire gene, one loss of exon 2 and the last loss of exons 2–7. In total, *LKB1* inactivation is observed in 21 of the 23 (91%) patients analyzed.

MARK1, MARK2, MARK3 and *MARK4* analyses

Similarly, all exons and intron–exon boundaries from the *MARK1, MARK2, MARK3* and *MARK4* genes were amplified and used for direct sequencing. DNA from eight other PJS patients without *LKB1* mutation (22) including the PJS07 family with linkage to 19q14.3 (19) was analyzed. No mutations were found in any of these genes. Several known polymorphisms were found (*MARK1*: rs3737296, rs3737297; *MARK2*: rs224174; *MARK3*: rs2273702, rs2273700, rs2273699, rs4281653, rs1951393, rs10137161, rs1058546; *MARK4*: rs2240672, rs173179) as well as an alteration in exon 14 of *MARK4* in one patient (c.1553C>T, p.518Pro>Leu). This alteration was also detected in one of 50 healthy controls, and, therefore, was considered a (novel) polymorphism.

Since in one specific family the chromosomal region 19q13 might be associated with PJS, we reasoned that if *MARK4* located at 19q13 was involved, it could also be inactivated by loss of complete exons as was the case for *LKB1* in the three PJS families described above. We, therefore, performed an *MARK4* exon deletion analysis based on QMPSF, in which several exons, including an internal control, were co-amplified. However, no *MARK4* exon deletions were identified using this method in any of the 8 PJS families without *LKB1* germline mutation.

Discussion

In the present study, *LKB1* was inactivated in 21 of the 23 (91%) PJS patients in our study group. This included 78% point mutations and 13% exon deletions or even whole gene deletions. Five novel mutations were identified, two frameshift mutations, two missense and one silent mutation. The silent mutation has been included since it has been shown that silent mutations can be pathogenic (31) and, furthermore, this mutation was not identified in the control group. These results are consistent with previous reports where the total percentage of *LKB1* inactivation ranged from 66% to 94% (5–7). Therefore, the question remains whether a second PJS gene exists to explain the percentage of PJS patients without an *LKB1* germline

mutation. Although affected in a minority of cases, inactivation of such a gene could result in the same phenotype if it affected the same pathways as *LKB1*. In sporadic colorectal cancer, a majority of the patients have mutations in the primary CRC gene *APC*. However, in about 10% of cases, *APC* is unaffected, but its target β-*catenin* is mutated in the domain that binds to APC (32). This may also be the case for PJS. Presently, however, no mutations in genes other than *LKB1* have been discovered.

Due to the close association with LKB1, the MARK proteins may be involved in LKB1 signaling and therefore, be of importance in the etiology of PJS. In this study, no mutations or deletions were identified in the *MARK* genes in PJS patients without *LKB1* germline mutation. This indicates that the *MARK* genes are unlikely to be second PJS genes. Also, protein expression of the MARK proteins was investigated by performing immunohistochemistry on paraffin material of PJS patients, but, unfortunately, the staining was not specific and provided no further information.

The MARK proteins are members of the AMPK family of kinases, all involved in energy metabolism. Since LKB1 functions upstream of these kinases, a role for the AMPK kinases has been suggested in PJS. AMPK itself is a multi-subunit protein; knockouts of both the α2 and the γ2 subunits have been published but neither have a phenotype comparable with PJS. The phenotype of the α2 knockout is glucose intolerance and that of the γ2 knockout electrocardiographic failure. In humans, a germline AMPK subunit γ2 mutation results in the Wolff–Parkinson–White syndrome (33). Although inactivation of AMPK has significant effects on energy metabolism, these are not similar to PJS symptoms, suggesting that the effect on energy metabolism is not the main cause of PJS symptoms.

If no candidate genes that are associated or related to *LKB1* can be found with germline defects in patients without *LKB1* mutations or deletions, *LKB1* may be the only gene affected in PJS. Presently, germline mutations and exonic losses have been described for 66–94% of PJS patients. Here, we report 9% of the patients without detectable alterations in *LKB1*. The question remains whether the syndrome in the remaining patients can be genetically explained by the existence of a second PJS gene or that *LKB1* might also be inactivated via intronic mutations or deletions that have not been studied thus far.

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Table 1

Identified *LKB1* mutations and clinical information of PJS patients

	Mutation	Effect of mutation	Reference	Family history	Pigmentation	Polyp location	Tumors in family
1	c.526G>A	p.D176N	Mutation described before (19)	?	?	sb	Yes
2	c.551T>C	p.L184P	New	?	?	st, sb, c	No
3	c.580G>A	p.D194N	Patient described before (22)	?	?	st, sb, c	Yes
4	c.712A>T	p.I238F	New	?	?	r	?
5	c.889G>A	p.R297K	Patient described before (22)	Yes	Yes	st, sb, c, n	No
6	c.924G>T	p.W308C	Mutation described before (19)	?	?	sb	?
7	c.367C>T	p.Q123X	Mutation described before (23)	?	?	sb	?
8	c.468C>G	p.Y156X	Patient described before (27)	Yes	?	sb	Yes
9	c.719C>A	p.S240X	Patient described before (27)	Yes	Yes	sb, c	No
10	c.197-198insT	p. V66V fsX104	Patient described before (24)	Yes	Yes	st, sb, c, n	Yes
11	c.418delC	p.L140W fsX21	Patient described before (25)	Yes	?	sb	Yes
12	c.464-465insG	p.G155G fsX8	Patient described before (22)	Yes	Yes	st, sb, c	Yes
13	c.762delC	p.P245P fsX33	New	?	?	st	Yes
14	c.829-830insGGGCG	p.D277G fsX12	New	?	?	sb, c	?
15	c.989-990insC	p.D331D fsX30	Patient described before (22)	Yes	Yes	sb	Yes
16	IVS1-2A>G	Alternative splicing	Patient described before (22)	Sporadic	Yes	st, sb, c	No
17	IVS5-1G>A	Alternative splicing	Mutation described before (26)	Yes	Yes	st, sb, c	No
18	c.547C>T	p.L183L	New	?	?	sb, appendix	?
19	Loss of exon 2 allele		New	Sporadic	Yes	st, sb, c	No
20	Loss of exon 2–7		New	?	?	sb, c	?
21	Loss of the entire gene		New	?	?	?	?
22	—		New	?	?	st	?
23	—		New	?	?	c	?

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Mutation	Effect of mutation	Reference	Family history	Pigmentation	Polyp location	Tumors in family

st, stomach; sb, small bowel; c, colon; n, nose.