STK11/LKB1 germline mutations in the first Peutz-Jeghers syndrome patients identified in Slovakia*

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Peutz-Jeghers syndrome (PJS) is characterized by number of hamartomatous polyps in the gastrointestinal tract and by mucocutaneous hypermelanocytic lesions at different sites. Older patients have an increased risk of the cancers of small intestine, stomach, pancreas, colon, esophagus, ovary, testis, uterus, breast and lung. In majority of PJS cases, the germline mutations in serine/threonine kinase *STK11/LKB1* gene were found to be associated with disease. Here we report the results of a first mutational screen of *STK11/LKB1* in PJS patients characterized in Slovak population. The first patient with unusual carcinoma of duodenum was a sporadic case and carried c.842delC change residing in a mutational C6 repeat hotspot. Neither the polyp nor the tumor of the patient displayed the loss of heterozygosity at the site of mutation suggesting different mechanism involved in the formation of polyp and tumor in this case. The second patient belonged to a three-generation family with typical PJS features but not cancers. Interestingly, the patient displayed concomitant occurrence of adenomatous and hamartomatous polyps. Molecular analysis revealed an IVS2+1A>G mutation that alters the second intron 5' splice site and was shown to lead to aberrant splicing mediated by the U12-dependent spliceosome. The same mutation was present in the 9 affected members of the family but in none of their normal relatives. We also observed novel c. IVS2+61G>A unclassified variant, and recurrent IVS2+24G>T and 3'UTR+129C>T polymorphisms. Based on the achieved results, we could offer predictive genetic testing and counseling to other members of the patients' families.

Key words: STK11/LKB1 gene; pathogenic mutations; Peutz-Jeghers syndrome

During last decade, molecular diagnosis of several hereditary cancer syndromes was established in Slovakia, with the aim of reducing cancer mortality through identification of at-risk individuals and effective prophylaxis [1–9]. Peutz-Jeghers syndrome (PJS; MIM# 175200) is another hereditary predisposition to cancer to which molecular diagnosis is of the urgent need to be established in the Slovak Republic. PJS is autosomal dominant multi-organ cancer syndrome involving the gastrointestinal tract as well as non-gastrointestinal sites such as pancreas, female and male reproductive organs and the lung [10, 11]. The cancer risk is increasing with the age of the PJS patient and recently it was estimated to be approximately 1%, 3%, 19%, 32%, 63%, and 81% at ages 20, 30, 40, 50, 60 and 70 years, respectively [12]. About half of PJS patients were estimated to be likely to die from cancer before age 57 years [13].

The disease is primarily characterized by hamartomatous polyps throughout the gastrointestinal tract which may cause obstruction or intussusception. Additional interesting phenotypical feature of PJS is mucocutaneous hypermelanocytic pigmentation which may be found predominantly on the lips, buccal mucosa, hands and soles of the feet [14– 16]. The main clinical symptoms of the syndrome include abdominal pain, rectal blood loss and anemia.

Linkage studies have mapped the disease to 19p13.3 [17, 18] and germline mutations in the *LKB1* also called *STK11*

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gene (NM_000455.4, MIM# 602216) at this locus have been identified as a major cause of PJS [19–21]. Human *STK11/LKB1* consists of nine coding exons with a 433-amino acid coding sequence and one non-coding exon 10. The STK11/LKB1 protein is serine/threonine kinase 11 and is suggested to act as a tumor suppressor. However the precise mechanism leading to carcinogenesis in PJS is unclear and remains to be elucidated. Inactivating mutations of the wild-type *STK11/LKB1* allele were often but not always found in polyps as well as tumors of PJS patients [22–25]. STK11/LKB1 protein is known to mediate its cellular functions through interactions with a number of proteins [26]. For instance, it is proposed to induce cell cycle arrest through p21, be involved in p53-dependent apoptosis and vascular endothelial growth factor (VEGF) signaling, as well as in chromatin remodeling [27–29]. STK11/LKB1 protein is also known to have effects on metabolism, polarity, and proliferation [30].

To date, more than 140 different mutations in the *STK11/LKB1* gene have been reported [31, 32]. The majority of the mutations (about 80%) leads to a truncated protein product. Some missense or small in-frame deletions have also been reported. Recent studies have shown the presence of large genomic deletions in the *STK11/LKB1* gene in about 30% of PJS patients [33, 34]. The ability to screen for *STK11/LKB1* mutations has important applications in clinical practice.

So far, the disease has not been studied in Slovak population. In the present study, we analyze phenotype and *STK11/LKB1* gene in the first two Slovak families with PJS. Five germline alterations were found, two of which are pathogenic. Molecular characterization of new PJS patients has the potential to reveal new properties of the disease, mechanisms of pathogenicity and links to new biological pathways, which may turn out to be useful in the design of preventive and therapeutic strategies in the future. In addition, the detection of pathogenic germline *STK11/LKB1* gene mutations is one of the key prerequisites for the early identification of at-risk individuals.

Patients and methods

SK-PJ1 patient. The patient was recognized by gastroenterologists at the Department of Internal Clinic of University Hospital in Trnava. A 27 year old male patient suffered severe anemia (Hgb 64 g/l), though he was not reporting blood in the stool. He showed light pigmented macules in the mucosa of the lower lip. In the patient's family there was no history of PJS or cancer. During the colonoscopy multiple sessile polyps of different size and large 8,5–9 cm polyp were found in colon. Further examinations of small intestine showed 12 polyps of different size; six of them were removed surgically while the others were excised by endoscopic polypectomy. After more than one month the patient returned with intestinal obstruction due to the invagination of duodenal tumor that was excised and histologically classified as moderately differentiated adenocarcinoma (T3N0M0).

SK-PJ2 patient. The second patient was referred for a genetic consultation at the Center of Clinical Genetics in Bratislava for differential diagnosis between Peutz-Jeghers syndrome and familial adenomatous polyposis. Extensive personal details, clinical symptoms and medical history of the patient and his relatives were collected during the genetic consultation. The proband is a 25 year old man displaying concomitant adenomatous and hamartomatous polyposis and mucocutaneous pigmentation. He belongs to large kindred with next 13 affected individuals. Two individuals present only pigmentation, the remaining ones both pigmentation and polyposis. Four of the proband's relatives died due to the complications related to polyposis.

The samples and DNA isolation. Peripheral blood samples were collected from the affected and unaffected members of the families after signing informed consent. Several healthy individuals were screened with aim to obtain control-sequencing electrophoretograms. Genomic DNA from peripheral blood samples was extracted using a commercial kit (QIAmp DNA Blood Mini Kit, Qiagen, Hilden, Germany), following the manufacturer's protocol. The paraffin embedded polyp of the colon and duodenal adenocarcinoma samples were sliced and used for DNA isolation by RecoverAll Total Nucleic Acid Isolation Kit according to the manufacturer's instructions (Ambion, Austin, USA).

DNA sequencing. The DNA isolated from peripheral blood leukocytes was used to amplify all nine exons of the *STK11/LKB1* gene with VariantSEQr™ Resequencing System primers (Resequencing Set ID version RSS000015822_02) according to manufacturer's instructions (Applied Biosystems, Foster City, USA). The VariantSeq primers for amplification of exon 6 were not performing well on DNA isolated from paraffin archived tissue presumably due to large size of PCR product and partial degradation of isolated DNA. The successful amplification was achieved by newly designed primer pair 5'-TAGGGCGTCAACCACCTTGACT-3' and 5'-ACCCCCAACCCTACATTTCTG-3' giving a 256 bp long PCR product. Cycling conditions were as follows: 10 min at 95°C, 35 cycles of 94°C/30s, 57°C/30s and 72°C/30s and 7min at 72°C. PCR products were purified by Exonuclease I (Fermentas, Hanover, USA) and SAP (Promega, Madison, USA) and subsequently sequenced in both directions with BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems, Foster City, USA). The sequencing reactions were purified by ethanol-sodium acetate method with PelletPaint NF Co-Precipitant (Novagen, Madison, USA) and electrophoresed on an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, USA). The identified DNA changes were confirmed in two independent PCR products and then compared to reference sequences of *STK11/LKB1* gene at http://www.ensembl.org. The mutation nomenclature used complies with recommendations of Human Genome Variation Society available on http://www.hgvs.org/mutnomen.

Results and discussion

The clinical diagnosis of PJS is based on the occurrence of positive family history, mucocutaneous pigmentation and typical PJS hamartomatous polyps. However, there are substantional differences in severity and age at onset of clinical manifestations among the patients and relatively high frequency of PJS cases (about 35%) is non-familial. It follows that some patients are diagnosed as children, where others might not be diagnosed until they reach their teen or adult years.

The differences in clinical manifestations of disease were observed also between the patients in our study. In the case of SK-PJ1 patient the initial symptom was anemia occurring at age 27 and only very light pigmented macules in the mucosa of the lower lip were observed (Fig. 1). In the patient's family there was no history of PJS or cancer. The second patient (SK-PJ2) belonged to a three-generation PJS family presenting with strong polyposis and mucocutaneous pigmentation typical for PJS (Fig. 2). He suffered from complications due to polyposis from the age of five and underwent several endoscopic polypectomies and resections of large bowel. Patient's brother and aunt affected by severe polyposis died at early age (3 years).

Gastrointestinal examinations of SK-PJ1 patient revealed multiple hamartomatous and atypical polyps of different size in colon and small intestine from which the largest were subsequently excised (Fig. 3). Although, the hamartomatous polyps are indicative of PJS, they do not confirm this diagnosis since there are six other autosomal dominant inherited hamartomatous polyposis syndromes, which may be considered. Histopathological sub-classification of hamartomatous polyps is sometimes difficult and needs an evaluation by an expert pathologist. The microscopic examination of several polyps of patient SK-PJ1 confirmed typical Peutz-Jeghers polyp histology with a branched framework and a tree-like stromal core containing smooth muscle fibers of the lamina muscularis mucosae (Fig. 4). Polyps with such histological pattern usually cast strong suspicion on PJS that can then be ascertained by mutational analysis of the *STK11/LKB1* gene.

Figure 1. Facial photograph of SK-PJ1 patient with subtle PJS mucocutaneous melanocytic spots on lips at age 27 (with the patient's permission).

In order to verify PJS diagnosis of the patients with above mentioned clinico-pathological features, we performed the molecular analysis of the *STK11/LKB1* gene. The exons of the gene together with exon-intron regions were scanned by DNA sequencing. The changes identified in the study are shown at the Figure 5. In addition to

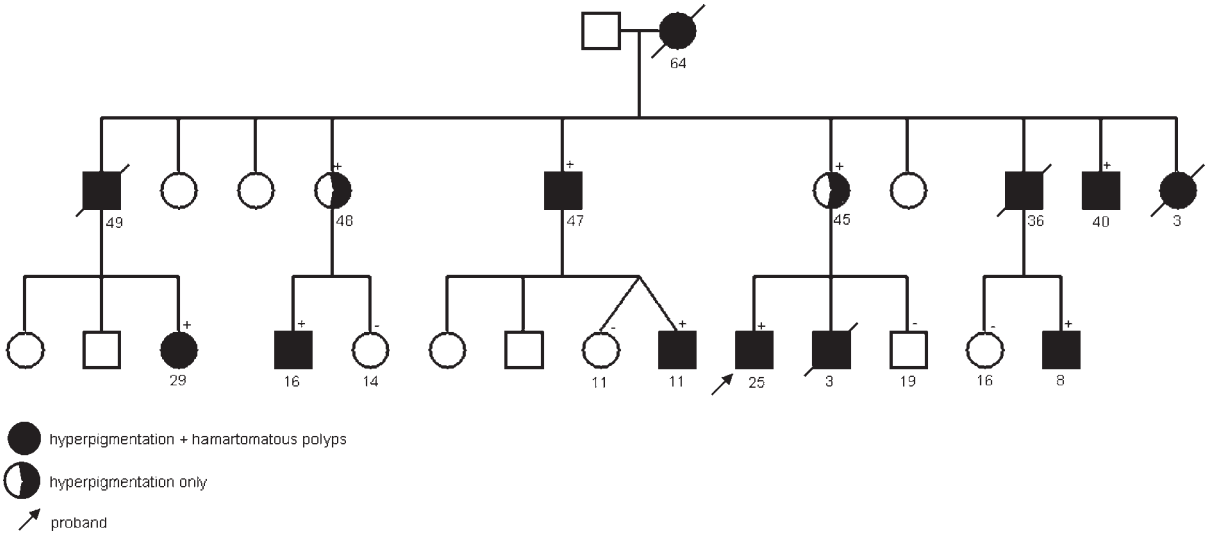

Figure 2. The pedigree of the SK-PJ2 patient suffering from PJS. The numbers under the symbols are the subjects' ages at the time of publication (yr). The numbers under the succumbed individuals are the ages at death (yr); IVS2+1A>G mutation carrier (+); IVS2+1A>G mutation negative (-).

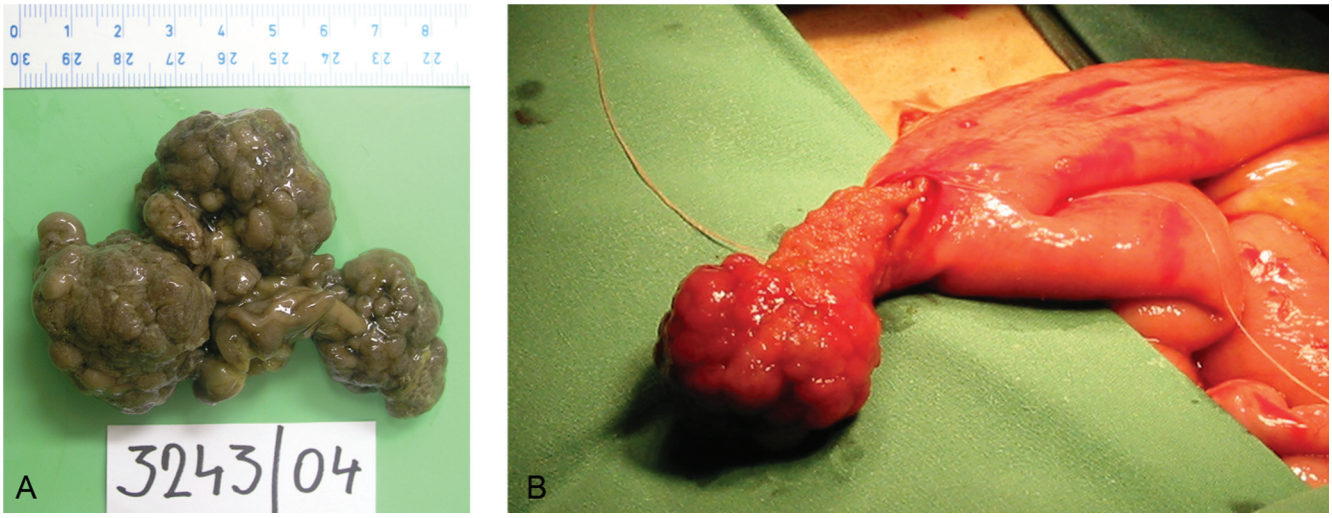

Figure 3. Polyps of SK-PJ1 patient. (A) a large atypical polyp excised from colon; (B) intraoperative picture of small intestine showing a large lobulated and pedunculated polyp with a long stalk typical for PJS.

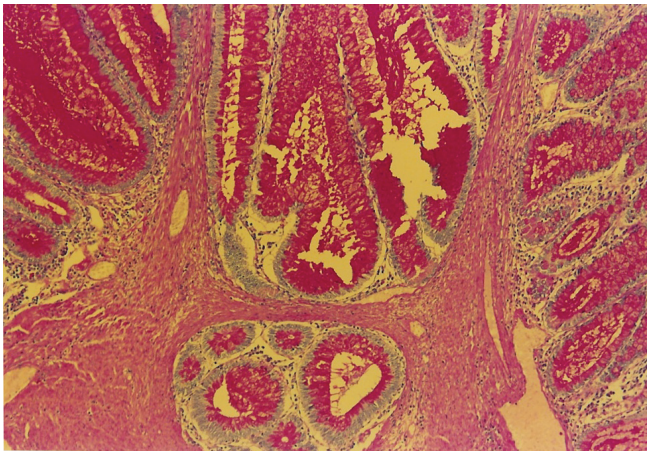

Figure 4. Histological view of PJS hamartomatous polyp with characteristic arborization of nonstriated muscles and branching pattern.

pathogenic changes (Fig. 5 A, B) we found two polymorphisms IVS2+24G>T (Fig. 5C) and 3'UTR+129C>T (Fig. 5D) described previously [35], and one unclassified intronic variant IVS2+61G>A (Fig. 5E). According to our knowledge the last alteration has not been described in the literature yet.

In the SK-PJ1 patient, we found a 1-bp deletion in exon 6 at codon 281 and nucleotide position 842 (Fig. 5A) leading to a reading frame shift starting at codon 281 and premature termination at codon 286. The deleted base resides in a mutational hotspot, a six-cytosine repeat (c.837–c.842) between the codons 279–281 which is frequently undergoing a 1-bp deletion or 1-bp insertion, probably due to the predisposition to DNA slippage. The c.842delC mutation was observed previously in 6 families [36–39] and in a sporadic PJS case [40]. It may be predicted that the alteration leads to truncated protein with incomplete catalytic domain that does not exhibit kinase activity.

In the patient SK-PJ2 a mutation at IVS2+1 A>G was identified. This mutation is replacing a non-canonical AT/AC splice junction sequence with a GT/AC sequence of intron 2, which has sequence features of a U12-type AT-AC intron. The alteration was found previously only in one PJS family and since no obvious effect on splicing of intron 2 judged by RT-PCR analyses was detected its pathogenicity was unclear [41]. Recently, the consequences of this unusual mutation were further investigated in details by different assays and it was shown that splicing occurs from the mutated 5' splice site to several cryptic, non-canonical 3'splice sites immediately adjacent to the normal 3' splice site [42]. This aberrant splicing is generating slightly shorter mRNAs which are unstable, and presumably undergo nonsense-mediated decay (NMD). It is notable, that IVS2+1A>G mutation in *STK11/LKB1* gene is the first confirmed case of a disease-associated mutation in a genuine U12-dependent AT-AC intron.

Although more than 140 pathogenic mutations of *STK11/LKB1* gene have been identified so far, it is still unclear how these mutations contribute to the variety of symptoms which occur with PJS. For instance, it seems that some PJS families have higher cancer risk than others. In our study, the SK-PJ1 patient with c.842delC germline mutation developed cancer of duodenum at age 27. This type of cancer is sought to be rather unusual for PJS and it was reported only in one case [43]. In the family of patient SK-PJ2 the cancer risk seems not to be elevated (Fig. 2). The grandmother of proband died

at age 64 without cancer. The mother of the proband as well as her sister at ages 45 and 48, respectively, display mucocutaneous pigmentation without any gastrointestinal symptoms or cancers at any sites. Two living uncles carrying mutation are cancer-free at ages 40 and 47, respectively and one uncle with PJS died by bowel obstruction at age 36. Only one person in this family, another uncle of the proband, died by cancer, however this lung cancer at age 49 could be due to his smoking. Although the contribution of inherited *STK11/LKB1* mutation to cancer can not be excluded in this last case, the overall cancer risk in this family at current stage of collected data seems to be low or none.

Since both identified germline mutations, the c. 842delC as well as IVS2+1A>G lead to similar consequences, i.e. frameshifts in reading frame and probably unstable mRNA products, it is likely that other genetic and/or non-genetic factors influence at least partially different phenotypic manifestation of disease. So far, controversial data were collected on whether for the clinical manifestations such as polyp or tumor formation, the haploinsufficiency of *STK11/LKB1* is sufficient or the loss of the wild-type allele is necessary [44–46]. We have analyzed the colonic polyp and duodenal tumor tissue of the SK-PJ1 patient for loss of heterozygosity (LOH) at the site of germline mutation by sequencing the sixth exon of *STK11/LKB1* and found that wild-type allele was present in equal ratio in both samples. Thus our results support a different mechanism from LOH in the formation of polyp or tumor in this case. In regard to carcinogenesis in PJS it is not clear how the adenocarcinomas develop. It is speculated that direct hamartoma-adenoma-carcinoma sequence as well as concomitant occurrence of adenomatous and hamartomatous polyps may exist within the same patients [47]. In our study we have indeed observed such concomitant occurrence of both types of polyps in the SK-PJ2 patient which is in support of previous hypothesis.

PJS is nowadays experiencing a strong upsurge in research and diagnosis options. The precise presymptomatic diagnosis can be provided by characterization of the mutation carried by PJS patient. In such characterized cases, about half of the family members can be released from repeated physical examination and also from concern about an increased risk of tumors, while the individuals who are diagnosed as positive can be provided with special screening program to detect early stages of potentially malignant tumors. We have confirmed that the patient SK-PJ1, which had no family history and his parents were homozygous for the wild-type allele, transmitted the mutation to his child. Although the cancer risk in the second SK-PJ2 family seems to be low so far, the caution should be taken particularly due to concomitant occurrence of adenomatous and hamartomatous polyps in the gastrointestinal tract of examined proband. The mutation carriers identified in our study are encouraged to keep current with all recommended tests and doctor visits and healthcare professionals are aware of importance in keeping abreast of all current developments with PJS. It is hoped that in addition to surveillance options further research of PJS molecular pathogenesis will lead to identification of potential targets for chemoprevention/drugs that will be able to reduce the extent of polyp formation and cancer risk in PJS patients.

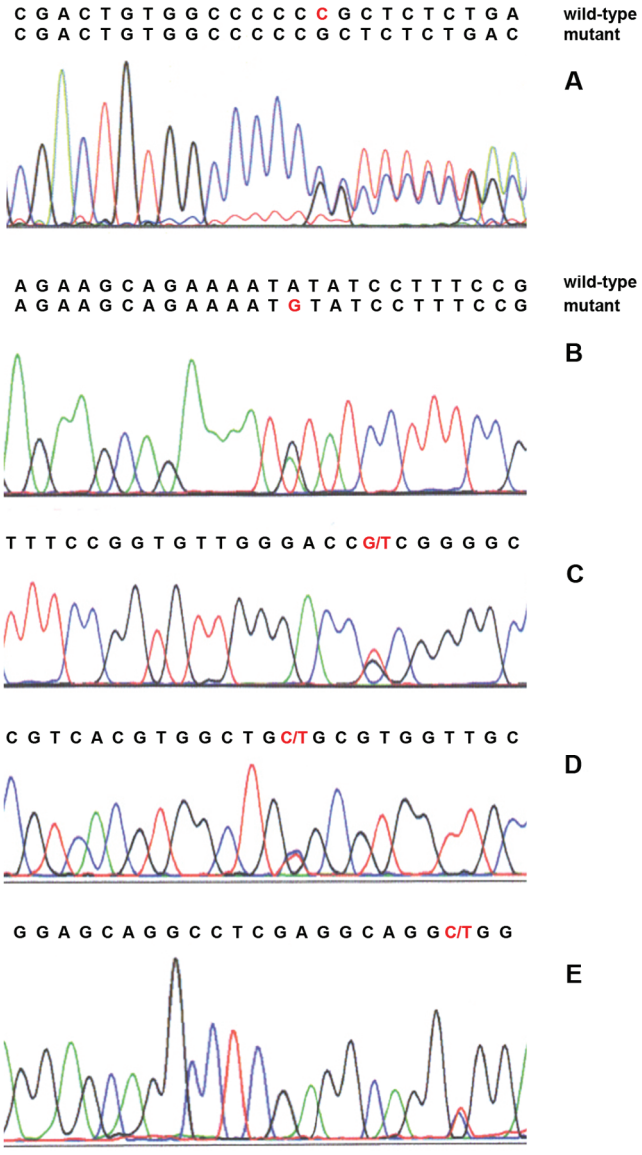

Figure 5. Germline alterations found in *STK11/ LKB1* gene in this study: (A) c.842delC (forward sequence); (B) IVS2+1A>G (forward sequence). (C) IVS2+24G>T polymorphism (forward sequence); (D) 3'UTR+129C>T polymorphism (forward sequence); (E) a novel unclassified variant IVS2+61G>A (reverse sequence).

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