Acta Neuropathol (2006) 112: 106–111
DOI 10.1007/s00401-006-0084-6

CASE REPORT

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Ganglioglioma arising in a Peutz-Jeghers patient: a case report with molecular implications

Received: 22 March 2006 / Revised: 9 May 2006 / Accepted: 9 May 2006 / Published online: 30 May 2006

Abstract The Peutz-Jeghers syndrome (PJS), an autosomal dominant disorder caused by inactivating germline mutations in the serine–threonine kinase gene LKB1, is characterized by mucocutaneous pigmentation, multiple gastrointestinal hamartomatous polyps, and by an increased risk for developing tumors involving several different organs. To date, no brain tumors have been described in PJS patients. In this report, we describe a case of ganglioglioma in a 22-year-old PJS patient. Single-strand conformation polymorphism-Heteroduplex analysis evidenced an abnormal pattern in exon 6 of the LKB1 gene. Sequencing revealed a 821delTinsAC mutation creating a termination codon 29 nucleotides downstream (p.Asn274fsX11). RNA studies showed an out-of-frame LKB1 isoform derived from the wild type allele and generated by exon 4 skipping. Since the LKB1 gene is expressed in the fetal and adult brain, our data would suggest its likely involvement in the pathogenesis of a subset of gangliogliomas.

Keywords Ganglioglioma · Peutz-Jeghers syndrome · LKB1 · Splicing · Exon skipping

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Introduction

The Peutz-Jeghers syndrome (PJS) is an autosomal dominant disorder characterized by mucocutaneous pigmentation and multiple gastrointestinal hamartomatous polyps [15]. PJS patients have an increased risk for development of gastro-intestinal cancer and extra-intestinal neoplasms involving diverse organs, such as breast, pancreas and kidney: these patients could also be affected by other rare tumors, for example, sex-cord tumors with annular tubules (SCTAT) in the ovary and tumors of sex-cord and Sertoli-cell type in the testis [15].

The PJS is caused by inactivating germline mutations in the serine–threonine kinase gene LKB1 [4, 6]. The cause of the increased tumor risk and the molecular mechanism of carcinogenesis in LKB1-deficient cells are unclear. LKB1 seems to be a master kinase involved in the control of cell cycle arrest, p53-mediated apoptosis, WNT and TGF-β signaling, Ras-induced transformation, energy metabolism and cell polarity. LKB1 has also been molecularly linked to TSC1 and TSC2 [2, 13] and PTEN [5] genes, whose expression is frequently altered in brain tumors.

In this report, we describe for the first time the presence of a brain tumor, ganglioglioma, in a young female carrier of an LKB1 gene mutation.

Case report

A 22-year-old female patient was admitted to the Institute of Neurosurgery of the Catholic University on December 2003 due to a 1-week history of increasing headaches and vomiting. On physical examination, the patient was awake and reactive and no focal neurological deficits or neck stiffness were noted. Magnetic resonance imaging (MRI) (Fig. 1a) revealed the presence of a right frontal juxtaventricular lesion of 4 cm, with cystic and solid components, which appeared iso-hypointense

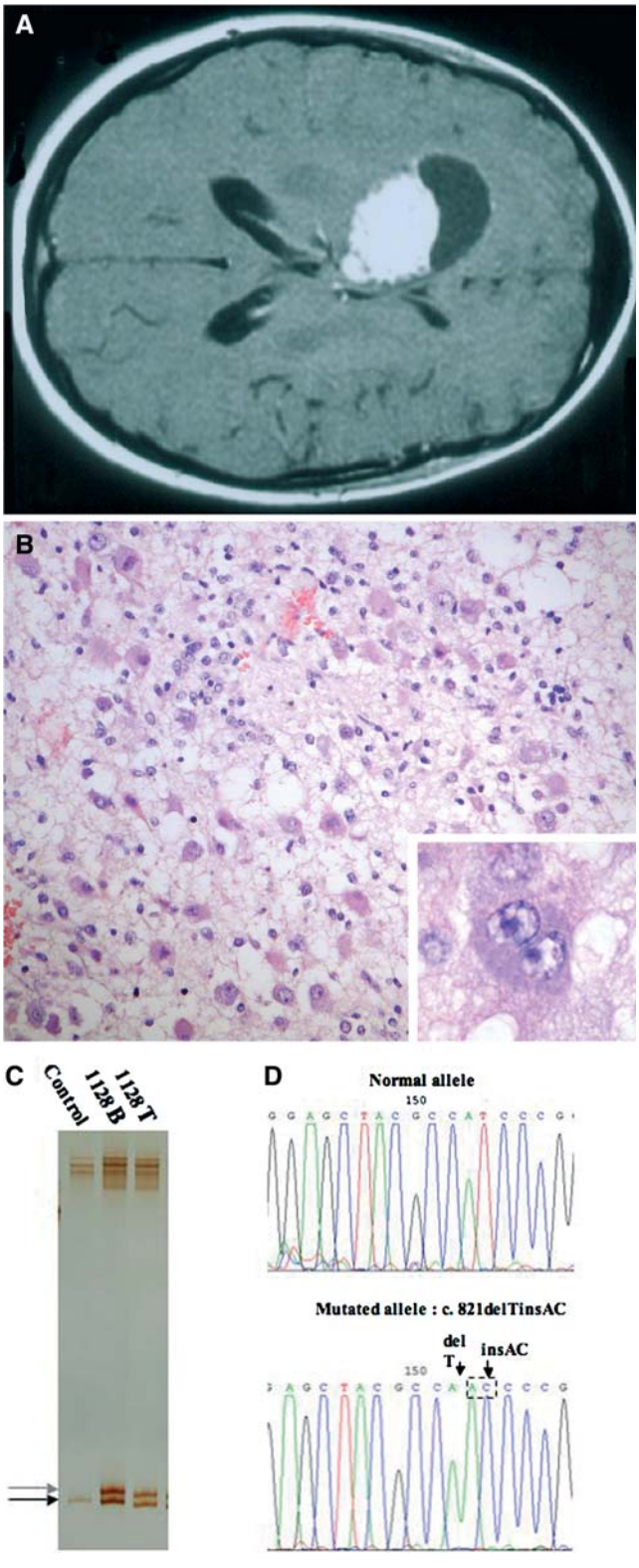

Fig. 1 Neuroradiological (**a**), histopathological (**b**) and genetic (**c**, **d**) features of ganglioglioma in a 22-year-old PJS patient. Axial, gadolinium-enhanced, section of T1-weighted MR image showing a right frontal juxtaventricular lesion with a cystic component; the solid part presented homogeneous contrast enhancement with compression of the right lateral ventricle and midline shift (**a**). Microscopically, the tumor showed a microcystic architecture and was composed of groups of large, multipolar mature neuron-like cells (**b**) that were often binucleated (**b**, *inset*). Single-strand conformation polymorphism (SSCP) heteroduplex analysis of LKB1 exon 6 (**c**). The *grey arrow* indicates the heteroduplexes and the *black arrow* the homoduplexes. The control was a PCR product obtained from normal DNA controls; *1128B* PCR product obtained from DNA obtained from blood of patient #1128; *1128T* PCR product obtained from DNA obtained from ganglioglioma cells of patient #1128). The sequencing analysis (**d**) carried out on cloned PCR products revealed a 821delTinsAC alteration (the two inserted nucleotides are *boxed*)

on T1-weighted images and iso-hyperintense on T2-weighted images revealing a significant and homogeneous enhancement after perfusion with paramagnetic contrast medium (Gd-DTPA). A right frontal craniotomy was performed with a transcortical approach to the lesion; the cystic part was drained and the solid mass, in part hard and in part soft and hemorrhagic, was totally removed. The post-operative course was uneventful. Twenty-four months after surgery, the patient did not present neurological deficits and MRI excluded tumor recurrence.

The medical history of the patient began in 1997 with alteration of the menstrual cycle. A pelvic ultrasonography evidenced a left ovarian cyst, which was successively removed. On histological examination, a SCTAT of the ovary was revealed. In August 2002, endoscopic examination including biopsies demonstrated multiple hyperplastic polyps in the gastro-duodenal tract, distal ileum and rectum, and a voluminous hyperplastic polyp of the ascending colon was successively removed. Ultrasonography and CT scans of the abdomen excluded other intra-abdominal lesions. Based on the clinical history of the patient at the moment of the neurosurgical treatment, blood samples and tumor specimens were collected with informed consent to perform genetic investigations. The family history for other cases of PJS was negative.

Materials and methods

Histopathological studies

Tissue specimens were formalin-fixed, paraffin-embedded and stained with H&E according to standard procedures. Immunohistochemistry was performed using a DAKO Autostainer (DAKO, Milan, Italy) with the following monoclonal antibodies: anti-GFAP (clone 6F2 DAKO), anti CD34 (clone QBend10, DAKO), anti-Synaptophysin (clone SY38, Ylem, Avezzano, Italy), anti-MAP-2 (clone HM-2, SIGMA, St. Louis, USA) and anti-Neurofilaments (clone NR4/RT97, Ylem). The proliferation index was evaluated by using antibodies against the Ki-67 antigen (monoclonal antibody, clone MIB-1, DAKO). Indirect immunostaining was performed using the ABC-peroxidase technique (Vector, Burlingame, USA). The peroxidase was developed with the DAB substrate kit (Vector).

Culture and chromosomal analysis of the tumor biopsy

For the primary cell culture and chromosomal analysis of the tumor biopsy, tumor specimens minced into small pieces were transferred to a test tube and washed by settling twice with HBSS and were then treated with 1 mg/ml collagenase II (Sigma) in RPMI without calf serum for 2 h at 37°C in a CO_2 incubator. Dispersed cells and cell clusters were collected by centrifugation. The pellet was suspended in 5 ml of Amniomed plus medium (Euroclone, West York, UK) and seeded in a 25 cm^2 flask. Subcultures were obtained by trypsinization of the confluent primary cell cultures. Chromosome preparations were performed by a standard procedure on cells from early passage semi-confluent cultures harvested after 3 h with colcemid (0.1 μg/ml) exposure. A normal female karyotype was observed in 100 metaphases obtained from different cultures. An elevated rate (35%) of tetraploidy was observed.

LKB1 mutational analysis

Genomic DNA was extracted from tumor biopsy, blood sample and paraffin-embedded samples of three sporadic gangliogliomas and one desmoplastic infantile ganglioglioma using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). For mutational analysis, genomic DNA was PCR-amplified for each of the nine exons of the LKB1 gene using intronic primers and PCR conditions as described previously [6]. Single-strand conformation polymorphism (SSCP) analysis was performed as previously reported [11]. The PCR products showing an abnormal pattern in the SSCP analysis were purified with Nucleo Spin columns (Macherey-Nagel, Duren, Germany) and directly sequenced, in both directions, using appropriate primers on an ABI310 sequencer (Applied Biosystem, Foster City, USA).

Methylation-specific PCR (MSP) assay

Analysis of the methylation patterns within the 5′-CpG island of the LKB1 gene was performed on ganglioglioma cells of patient #1128 and DLD-1 colon cancer cell line using a chemical modification of 1 μg of genomic DNA from specimens with sodium bisulphite- and methylation-specific polymerase chain reaction. Primer sequences and PCR conditions were those reported by Trojan et al. [14].

LKB1 gene expression analysis

PolyA+ RNA was obtained from cultured cells before and after 6 h treatment with puromycin (100 μg/ml) (USbiological, Swampscott, USA) which has been shown to inhibit NMD [5]. Poly A+ RNA was extracted from cultured cells derived from ganglioglioma (as tumor biopsy specimen was no more available) and normal lymphoblastoid cell line using QuickPrep Micro mRNA (Amersham Biosciences, Little Chalfont, Buchinghamshire, UK). RNA analysis was carried out on cDNAs synthesized after reverse transcription of polyA+ RNA with M-MLV-RT H (−) point mutant (Promega, Madison, USA) and random hexamers (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. cDNA amplification was performed using the primers localized within the exon 2 and exon 6 of the LKB1 gene and the Expand Long Template System (Roche, Manheim, Germany). The thermocycling profile was as follows: 2 min at 95°C for 1 cycle followed by 35 cycles at 95°C for 10 s, at 55°C for 30 s, and 68°C at 1 min. LKB1 cDNA isoform was sequenced after purification of the PCR products as described above.

Results

On microscopic examination, the cerebral tumor showed a microcystic architecture which was composed of groups of large, multipolar, often binucleated neuron-like cells (Fig. 1b and inset) positively stained for synaptophysin, neurofilaments and MAP-2; neoplastic cells were also positive for CD34. An additional neoplastic GFAP positive glial component was present along with focal calcifications. Mitotic figures were absent as were areas of necrosis. The proliferation index (Ki-67/MIB-1 labeling) ranged from 0.5 to 1.5%. Focal lymphocytic infiltrates were also evident.

The cytological preparation of primary cultures obtained from tumoral specimens showed ganglion-like cells with large nuclei and plump cytoplasms, occasionally binucleated, merging with other medium-sized cells with round or oval nuclei and scarce cytoplasm (not shown). The cells were positive for both glial and neuronal immunohistochemical markers.

Exhaustive LKB1 analyses at the DNA and RNA level to identify either mutations or loss of the wild type LKB1 allele were carried out on tumor-derived cultured cells. The hypothesis that the tumor could be the result of two mutational events (two-hit hypothesis, i.e. germline mutation in one allele and somatic mutation in the other) was examined. Genomic DNA, obtained from peripheral blood lymphocytes, and ganglioglioma specimens were analyzed by PCR-SSCP heteroduplex analysis. SSCP heteroduplex analysis showed an abnormal pattern in exon 6 (Fig. 1c). Sequencing of the corresponding PCR products revealed the presence of c.821delTinsAC in a heterozygous condition in both samples (Fig. 1d). The c.821delTinsAC generated a premature termination codon 29 nucleotides downstream (p.Asn274fsX11) which was located 11 nucleotides upstream of the exon 6–exon 7 junction and thus in a region that was non-sense-mediated decay (NMD) incompetent.

To assess the methylation status of the wild type LKB1 allele, we analyzed the 5′CpG islands of the LKB1 promoter on the DNA extracted from ganglioglioma cells using the DLD-1 colorectal cancer cell line as positive control for the methylated reaction. As shown in

Fig. 2, ganglioglioma cells displayed only the unmethylated promoter island. This result would exclude hypermethylation of the LKB1 promoter as the mechanism of inactivation for the remaining normal allele in the tumor. RNA analysis revealed an out-of-frame LKB1 isoform derived from the wild type allele (Fig. 3a). The sequence analysis demonstrated that the isoform was generated from the skipping of exon 4 (Fig. 3b). Unexpectedly, this isoform was NMD sensitive as shown in Fig. 3 (Fig. 3a, lane 4). In order to identify the mutational event responsible for the exon 4 skipping in the wild type allele, the introns and exons upstream and downstream of exon 4 in tumor DNA were sequenced. Unfortunately, we were unable to identify any LKB1 mutation. To elucidate the eventual etiological role of LKB1 gene in sporadic gangliogliomas, we characterized the sequence of the gene in the DNA extracted from three paraffin-embedded sporadic ganglioglioma and one desmoplastic infantile ganglioglioma samples. The sequencing failed to show the presence of variants.

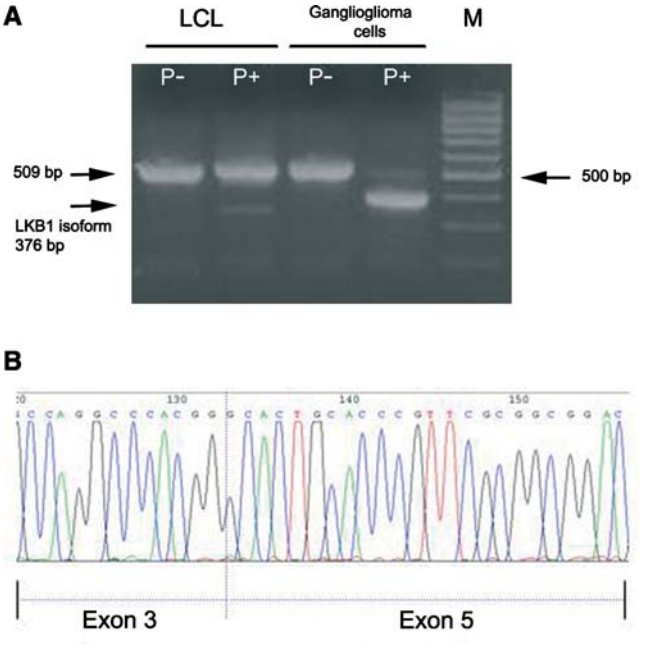

Fig. 3 The PCR analysis (**a**) and sequencing (**b**) of LKB1 cDNA from ganglioglioma cells of patient #1128. LKB1 cDNA analysis (**a**) using the following primers LKB1 ex 2 F 5′-aattcaactactgaggagg-3′ as forward and LKB1 ex 6R 5′-cgatgttctcaaacaact-3′ as reverse on samples treated with puromycin (+P) for 6 h and untreated ones (−P): (*LCLs* normal lymphoblastoid cell line; *M* molecular weight marker). Sequencing after purification of the 376 bp RT-PCR product shows a LKB1 isoform lacking exon 4 (**b**)

Discussion

Ganglioglioma is a well-differentiated, slowly growing, neuro-epithelial tumor composed of neoplastic mature ganglion cells in combination with neoplastic glial cells [9]. Ganglioglioma preferentially affects children or young adults usually arising in the cerebral hemispheres, most frequently in the temporal lobes [9]. Clinically, cerebral gangliogliomas are associated with a variable history of seizures and present the most common tumor associated with temporal lobe epilepsy [9]. In the literature, there are only a few reports of gangliogliomas involving the ventricular system from the adjacent thalamic area as well as those purely intraventricular [9]. Radiologically, ganglioglioma appears as a well-defined cyst with an enhancing mural nodule, forming a T1-weighted hypo-intense/T2-weighted hyperintense circumscribed mass at MRI. Cysts are usually large and can be found in about 50% of cases.

Little is known concerning the molecular pathogenesis of these glioneuronal tumors. Recent findings support a dysontogenic origin from a glioneuronal precursor lesion with neoplastic, clonal proliferation of the glial cell population [1, 9].

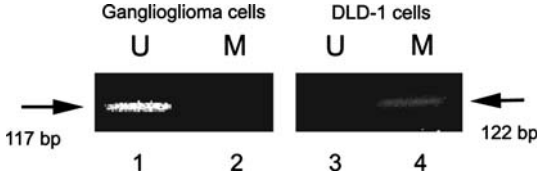

Fig. 2 Analysis of LKB1 methylation by MSP in ganglioglioma cells of patient #1128 and a DLD-1 colon cancer cell line. The amplification was performed using specific primers for unmethylated (*U*) and methylated (*M*) DNA

Mutations of genes implicated in neuro-developmental signaling cascades are presumed to play a role in the pathogenesis of this neoplasm. The TSC1 and TSC2 genes, acting in cortical differentiation and growth control, have been considered potential candidates [1, 9]. Therefore, both genes have been widely studied. LOH (Loss of heterozygosity) has been observed in 5/16 gangliogliomas; however, SSCP screening of all coding exons of the TSC1 or TSC2 genes in the tumors with LOH revealed no intragenic mutations [10]. A comprehensive mutation analysis of TSC1 and TSC2 identified a high incidence of gene polymorphisms in a cohort of patients with gangliogliomas; the frequency of some of these polymorphisms in the ganglioglioma group was significantly increased when compared to controls [1] and, in addition, a somatic mutation, a nucleotide substitution in intron 32 of the TSC2 gene was encountered in one patient [1]. Kam et al. [7] found a significantly lower expression of the *dab1* and *p35* genes, both involved in the reelin pathway, although mutational analysis of the two genes revealed only a *p35* polymorphism in a series of 29 gangliogliomas.

In our case the ganglioglioma developed in a patient affected by PJS caused by a germline mutation of the LKB1 gene. The LKB1 gene product is involved in two biological pathways. It has been shown to play a fundamental role in controlling the spatial orientation of structures and in maintaining an ordered, polarized epithelium; on the other hand, it has been suggested to be a regulator of AMP-dependent kinase (AMPK),

which controls the balance of cellular energy [13]. LKB1 directly activates AMPK. Phosphorylation and activation of AMPK redirects cell metabolism towards generation of ATP and away from macromolecular synthesis such as that required for cell division. The control of protein synthesis by AMPK occurs via mammalian target of rapamycin (mTOR), a kinase, which is inhibited following phosphorylation of TCS2 by AMPK [4, 13]. In addition, like TSC2, LKB1 inhibits the phosphorylation of the translational regulators S6 K and 4EBP1. All these data indicate that TSC2 and LKB1 act in the same pathway. To verify the existence of an eventual correlation between the two pathologic conditions, extensive molecular investigations were performed on the tumor. Allelic loss of the wild type LKB1 gene was excluded and sequencing of all LKB1 exons from the tumor DNA revealed several well-known LKB1 polymorphisms in a heterozygous condition. On the other hand, LOH in polyps and benign tumors of PJS patients has been found in only a few instances [3].

Interestingly, the RNA studies carried out on cells from the ganglioglioma culture revealed an out-of-frame LKB1 isoform derived from the wild type allele. Sequence analysis demonstrated that the isoform was generated from an exon 4 skipping. This situation is not unusual considering that we already described two aberrant transcripts in a single PJS patient despite the lack of any apparent genomic alteration [12]. In addition, after comprehensive cloning and sequencing of LKB1 cDNA derived from a normal individual, an LKB1 isoform which lacks exon 4 and retains a cryptic exon in intron 1 was observed in 1/15 clones analyzed [unpublished observations].

At this point, at least two hypotheses can be formulated. If the LKB1 isoform lacking exon 4 even though at its lowest levels is normally expressed representing a sort of background splicing, the imbalance observed among the different isoforms (Fig. 3a, lane 4) could depend on the cell culture conditions. Alternatively, since LKB1 mRNA processing could be tissue specific, and therefore influenced by a differential expression of splicing modulators, such as SR proteins and their antagonistic factors, the imbalance among the different isoforms could be a consequence of an altered expression of the splicing regulators, as demonstrated in several tumors [16]. According to this latter hypothesis, in the presence of a germline mutation, the spliced isoform would reduce the level of expression of the remaining wild type allele.

To demonstrate the involvement of LKB1 in a sporadic ganglioglioma pathogenesis, we searched for LKB1 alterations in three sporadic gangliogliomas and one desmoplastic infantile ganglioglioma by direct sequencing of all the LKB1 gene exons, but our analyses did not reveal any mutations.

Considering that LKB1 gene is expressed in the fetal and adult brain [4, 6], however, our data would still suggest the probable involvement of the LKB1 gene in the molecular pathogenesis of a subset of gangliogliomas. Although the analysis of the LKB1 gene sequence in the DNA extracted from archival samples of three sporadic gangliogliomas and one desmoplastic infantile ganglioglioma failed to reveal variations, further research is needed to unravel its eventual role as novel target in gangliogliomas.

Acknowledgments The authors are grateful to Dr. Rosanna Bagnulo for her skilful technical assistance. This work is partially supported by Fondazione Cassa di Risparmio di Puglia "Progetto integrato per la salvaguardia della salute dell'uomo" and R.S. 2005.

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