Sacrococcygeal Chordomas in Patients with Tuberous Sclerosis Complex Show Somatic Loss of *TSC1* or *TSC2*

Lisa Lee-Jones,[1*] Irene Aligianis,[2] Peter A. Davies,[1] Ana Puga,[3] Peter A. Farndon,[2] Anat Stemmer-Rachamimov,[4] Vijaya Ramesh,[3] and Julian R. Sampson[1]

[1]Tumour Molecular Genetics Group, Institute of Medical Genetics, University of Wales College of Medicine, Cardiff, United Kingdom
[2]West Midlands Regional Clinical Genetics Service, Birmingham Women's Hospital, Birmingham, United Kingdom
[3]Molecular Neurogenetics Unit, Massachusetts General Hospital, Charlestown, Massachusetts
[4]Molecular Neuro-Oncology Laboratory, Massachusetts General Hospital, Charlestown, Massachusetts

Chordomas are rare sacrococcygeal/sacral, sphenooccipital/clivus, and spinal tumors whose molecular etiology remains relatively understudied. As several anecdotal reports had described chordomas in individuals with tuberous sclerosis complex (TSC), a multisystem hamartoma syndrome, we hypothesized that the genes that cause TSC may have an etiological role in chordomas. In two cases of sacrococcygeal chordomas in individuals with TSC, one with a germ-line *TSC2* mutation and the other with a germ-line *TSC1* mutation, we confirmed somatic inactivation of the corresponding wild-type allele by loss of heterozygosity analysis and immunohistochemistry. These data provide the first evidence of a pathogenic role by TSC genes in sacrococcygeal chordomas.

Chordomas [MIM 215400] are rare tumors that originate from notochordal remnants along the axial skeleton in the sacrococcygeal/sacral (45%), sphenooccipital/clivus (40%), and spinal (15%) regions (Stepanek et al., 1998; Börgel et al., 2001). They are characterized by slow growth, local destruction of bone, extension into adjacent soft tissue, and, rarely, distant metastatic spread (Stepanek et al., 1998). The incidence of sacrococcygeal chordomas is only 1 in 40,000 births (Harbon and Pheline, 1989). However, they are the most prevalent type of chordoma in children (Sassin and Chutorian, 1967). Chordomas are usually sporadic. However, 8 families have been reported to have multiple family members affected by chordomas (Foote et al., 1958; Enin, 1963; Kerr et al., 1975; Chetty et al., 1991; Korczak et al., 1997; Stepanek et al., 1998; Dalprà et al., 1999), suggesting that inherited variants may predispose to these tumors. Three of these families demonstrated autosomal dominant transmission (Korczak et al., 1997; Stepanek et al., 1998; Dalprà et al., 1999). To date, knowledge of the molecular pathology of chordomas is rudimentary. Of the limited cytogenetic data available on approximately 40 cases of chordoma worldwide (Dalprà et al., 1999; Miozzo et al., 2000; Sawyer et al., 2001; Scheil et al., 2001), no recurrent aberrations have been found (Scheil et al., 2001). A previous molecular cytogenetic study using comparative genomic hybridization identified chromosomal imbalances in 16 of 16 chordomas analyzed (5 clivus, 10 sacrococcygeal, and 1 spinal). However, none showed losses of 9q34 or 16p13, to which *TSC1* and *TSC2* map (Scheil et al., 2001). The only previous loss of heterozygosity (LOH) study of chordomas was of *RB1* (13q14), at which LOH was identified in 2 of 7 sphenooccipital/clivus chordomas (Eisenberg et al., 1997). The tumors demonstrating 13q14 LOH were noted to be particularly aggressive, and consequently the LOH may have been a late genetic event in these tumors. Two further loci have been implicated in the pathogenesis of chordomas. A susceptibility allele at 7q33 was suggested by linkage analysis of one family with apparent autosomal dominant transmission (Kelley et al., 2001). A putative tumor-suppressor gene that may play a role in both familial and sporadic chordomas also was reported (Miozzo et al., 2000) after linkage studies and cytogenetic studies that identified somatic losses of 1p in chor-

domas (Mertens et al., 1994; Butler et al., 1995; Dalprà et al., 1999).

Because of the limited molecular data available on chordomas, we searched the published literature for unusual associations of chordomas with tumor predisposition syndromes, as genes involved in such syndromes often play a role in the pathogenesis of sporadic tumors. We identified three reports of chordomas found in patients with tuberous sclerosis complex (TSC; Dutton and Singleton, 1975; Schroeder et al., 1987; Börgel et al., 2001). TSC [MIM 191100] is an autosomal dominant syndrome characterized by hamartomas in multiple organs, epilepsy, mental retardation, and behavioral problems, with a prevalence of 1 in 6,800 (Osborne et al., 1991). TSC is caused by germ-line mutations in either of two genes, *TSC1* [MIM 605284] and *TSC2* [MIM 191092]. These genes behave like tumor-suppressor genes, as inactivation of the wild-type allele has been demonstrated in hamartomas, consistent with Knudson's two-hit hypothesis. The involvement of *TSC1* and *TSC2* in other unusual tumors in individuals with TSC was reported previously, including identification of biallelic inactivation of *TSC2* in a malignant islet cell tumor in a TSC patient (Verhoef et al., 1999). Furthermore, somatic biallelic inactivation of *TSC* genes also has been established in lymphangioleiomyomatosis (Sato et al., 2002) and some primary bladder cancers (Hornigold et al., 1999). We hypothesized that *TSC1* and *TSC2* are candidate genes in the genesis of chordomas.

We identified two previously unreported cases of TSC with coexisting sacrococcygeal chordoma and have undertaken molecular genetic analysis to investigate somatic involvement of the *TSC* genes in these tumors. Appropriate informed consent was obtained from both families. The first case was a 33-week female fetus from a family with no history of TSC that was terminated because of prenatal ultrasound detection of multiple cardiac rhabdomyomas, suggesting a diagnosis of TSC. Histological examination confirmed the cardiac tumors to be rhabdomyomas, but the brain was not examined because of cephalocentesis and severe disruption of the cranium. A sacrococcygeal chordoma was present. The tumor comprised lobules separated by fibrous septae. Within the lobules, small round cells were arranged in a cordlike fashion in a myxoid stroma. The majority of cells were physaliphorous, with marked vacuolization of the cytoplasm (Fig. 1A).

In view of the clinical findings, mutation analysis of all coding exons of *TSC1* and *TSC2* was undertaken by single-strand conformational polymorphism (SSCP) analysis, as described in Beauchamp et al. (1998), using constitutional DNA (obtained from paraffin-embedded pancreas and left lung by standard proteinase K digestion and phenol chloroform extraction following deparaffinization in xylene). SSCP shifts were characterized by direct sequencing and revealed the *TSC2* nonsense mutation, CAG (glutamine, Q)→TAG (stop, X) at amino acid residue 1010, Q1010X (arrowed), in exon 26 (Fig. 1B), and a 4-bp deletion polymorphism in intron 39 of *TSC2*, 5179-30_5179-27delAGTG (data not shown). Both parents were clinically normal and were not tested for the mutation. This mutation was used for LOH studies by a seminested PCR of *TSC2* exon 26, using the primers LLJ1 (reverse 5′-GCCGTGAAGTTG-GAGAAGACG-3′) and LLJ2 (reverse 5′-GAGC-CATCATGTCCAGACAGG-3′) in the first and second rounds of amplification, respectively, in conjunction with the *TSC2* exon 26 forward primer detailed in Jones et al. (1999, 2000), generating a product of 206 bp in the second step. A seminested PCR was used, as the DNA was extracted from paraffin sections and insufficient product was detectable by denaturing high-performance liquid chromatography (DHPLC) following 32 cycles of amplification. DHPLC analysis of the PCR product from the second round of amplification demonstrated a marked reduction of one of the peaks, indicating LOH (data not shown). Sequencing confirmed that this profile change was attributable to loss of the wild-type allele (Fig. 1B). Assay of the 4-bp deletion polymorphism using the same approach confirmed LOH at the *TSC2* locus (data not shown). Immunohistochemistry was performed using polyclonal antibodies, HF6 for hamartin and TSDF for tuberin, as described in Murthy et al. (2001), and showed consistent immunopositivity for hamartin (Fig. 1C) and focal, very weak staining for tuberin (Fig. 1D). Antihamartin antibody, HF6, was generated in rabbits against the GST fusion protein derived from a GST fusion construct of exon 15 of human hamartin (amino acids 480–666; Murthy et al., 2000). Antituberin antibody, TSDF, was generated in rabbits against amino acids 1165–1393, as detailed in Han et al. (2004).

The second case was born at term following an uneventful pregnancy and normal delivery. Antenatal ultrasound scans were normal. At birth, she was noted to have a 3.5-cm sacrococcygeal lesion. The tumor was excised at 5 days of age. Histology revealed this to be a sacrococcygeal chordoma. Microscopic analysis demonstrated a skin-covered

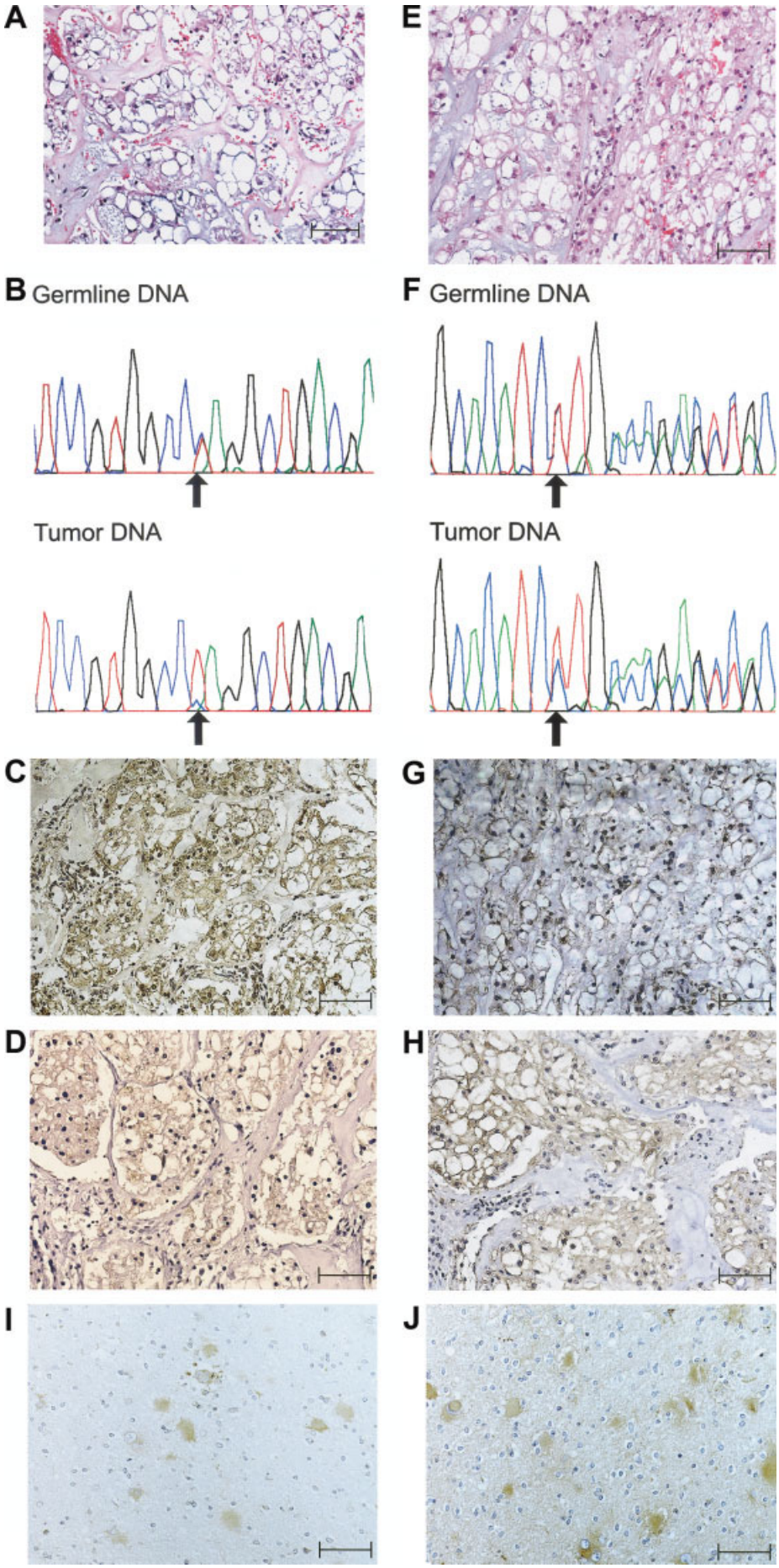

Figure 1.

nodule composed of compact cords of clear cells arranged in a lobular pattern. Thick strands of fibrous tissue intersected the nodule. There were typical physaliphorous cells present, with a clear, bubblelike cytoplasm and vesicular nucleolus (Fig. 1E). Periodic acid Schiff was weakly positive, whereas Alcian-blue stained the mucinous material strongly. Immunohistochemistry was positive with cytokeratin, S100, and epithelial membrane antigen. The cytological features were benign, and no mitotic figures were seen. The findings were consistent with chordoma and were confirmed by electron microscopy. The patient did not receive any subsequent treatment for the chordoma. Ultrasonography of renal and pelvic regions were normal on follow-up. From 11 months, hypomelanotic macules were noted on her skin, which increased in number with age. A formal diagnosis of TSC was made when the patient was 6 years of age, following detection of subependymal nodules on a brain MRI scan. Two lipomas were found on her back, and an early shagreen patch was found on her left hip. No spinal abnormalities were found by MRI, and no further cutaneous signs of TSC were present on reassessment at 8 years of age. Renal ultrasound and fundoscopy were normal. The patient has no history of seizures and is intellectually normal. Both parents were clinically normal.

Analysis of all coding exons of *TSC1* and *TSC2* in constitutional DNA extracted from EDTA-treated blood was performed using the conditions detailed in Jones et al. (1999, 2000). DHPLC analysis with a WAVE DNA Fragment Analysis System (3500HT, Transgenomic, Crewe, UK), using the methods described on http://www.uwcm.ac.uk/uwcm/mg/tsc_db/dhplc2.html, demonstrated a profile variant in exon 4 of *TSC1* (data not shown) that on sequencing revealed a 9-bp in-frame deletion, 402_410delCTGACCACC (Fig. 1F). Parental DNA samples were analyzed, which confirmed that this mutation had occurred de novo. Biological paternity and maternity were also confirmed by genotyping microsatellite repeats using the Powerplex 1.2 kit (Promega, Madison, WI). Although missense and in-frame deletions are common in *TSC2*, they are exceptionally rare in *TSC1* (Kwiatkowski, 2003). To our knowledge, only one other patient with TSC and an in-frame deletion of *TSC1* has been reported; this patient had a 3-bp deletion of exon 7, N198_F199delinsI; 814_816delACT (van Slegtenhorst et al., 1999). We also identified in-frame deletion V127del; 600_602delGTT (exon 6) in one case (our unpublished data).

LOH analysis of the chordoma from the second case was undertaken using two-step PCR to generate detectable PCR product by DHPLC, as poor-quality DNA had been obtained from the paraffin sections. The template in the secondary PCR of *TSC1* exon 4 from chordoma DNA (data not shown) was 1 μl of a 1:1000 dilution of the primary PCR product. A 4-bp deletion polymorphism in intron 39 of *TSC2*, 5179-30_5179-27delAGTG (the same polymorphism as identified in Case 1), was also assayed (data not shown). In addition, primers were designed to surround the in-frame deletion of *TSC1* exon 4, using an annealing temperature of 61°C and 40 cycles, to generate a small PCR fragment of 53 bp in the wild-type allele. The primer sequences were 5′-CTCTCAGCCGGCATTG-CAC-3′ (forward) and 5′-CCTTGTCATGTG-GCTCTTGC-3′ (reverse). The PCR products were analyzed on a 10% midi polyacrylamide gel and silver-stained according to standard procedures. LOH was not evident by either DHPLC analysis (data not shown) or silver staining of the smaller PCR fragment (data not shown). Sequencing across the region of the germ-line mutation in blood and tumor DNA revealed apparently reduced nucleotide peak heights corresponding to the wild-type allele (Fig. 1F). Further analysis with external primers (forward 5′-TGACAGGAAGCT-GTGTAAGG-3′; reverse 5′-GCACAGAAGCT-

Figure 1. Molecular and histopathological analysis of *TSC1* and *TSC2* in chordomas. Case 1: (A) Chordoma, hematoxylin, and eosin staining (original magnification ×250). The tumor displays the characteristic features of chordoma: lobular and composed of cells with bubbly cytoplasm in a myxoid stroma, high power. (B) Partial sequences of exon 26 of *TSC2* from germ-line and chordoma in forward direction. The germ-line shows a CAG (glutamine, Q)→TAG (stop, X) nonsense mutation at amino acid residue 1010, Q1010X (arrowed). The signal from the wild-type allele is markedly reduced in the chordoma, indicating LOH. Data representative of sequencing undertaken in triplicate (at least). (C) Chordoma immunostaining with HF6 antihamartin antibody showing strong immunopositivity in tumor cells (original magnification ×250). (D) Chordoma immunostaining with antituberin TSDF antibody (original magnification ×250). Some cells demonstrate immunopositivity, whereas other demonstrate the total absence or very low levels of tuberin. Case 2: (E) Chordoma, hematoxylin and eosin, also displaying the characteristic features (original magnification ×250). (F) Partial sequences of exon 4 of *TSC1* in forward direction of germ-line and chordoma DNA. The 9-bp deletion, 402_410delCTGACCACC, was present in both the constitutional and chordoma tissue (start of deletion arrowed), but peak height was reduced for the wild-type allele, suggestive of LOH. Data representative of sequencing undertaken in triplicate (at least). (G) Chordoma, HF6 immunostaining (original magnification ×250). Tumor cells are immunonegative for hamartin. (H) Chordoma, TSDF immunostaining (original magnification ×250). Tumor cells are immunopositive for tuberin. (I) Positive control for HF6, cortical tuber from a TSC patient, giant cells demonstrating HF6 immunopositivity. For further information, see Kerfoot et al. (1996) and Johnson et al. (1999). (J) Positive control for TSDF, cortical tuber from a TSC patient, giant cells demonstrating TSDF immunopositivity. For further information, see Kerfoot et al. (1996) and Johnson et al. (1999).

GTTGTACTC-3′) also revealed apparently reduced signal from the wild-type allele, suggestive of LOH. Tumor DNA quality precluded systematic assays for an intragenic somatic mutation, but we performed immunohistochemistry in order to address expression levels of hamartin and tuberin. Immunostaining with the HF6 antibody showed absence of hamartin in the chordoma (Fig. 1G), consistent with somatic inactivation of *TSC1*, whereas the tumor cells were strongly immunopositive for tuberin (Fig. 1H).

Our data are consistent with biallelic inactivation of each gene that causes TSC in the cases studied. Case 1, with a germ-line *TSC2* mutation, demonstrated LOH of *TSC2*, which was supported by the focal loss of tuberin staining in the chordoma on immunohistochemistry. The tumor from Case 2, with a germ-line *TSC1* mutation, did not unequivocally demonstrate LOH of *TSC1* but showed absence of hamartin, suggesting that a more subtle intragenic mutation or hypermethylation caused loss of function of the second allele in this tumor. Analysis of all the coding exons of *TSC1* would have enabled us to address this but was precluded by the poor-quality DNA obtained from the paraffin-embedded tissue.

This report provides the first evidence for the role of somatic mutations of *TSC1* and *TSC2* in the development of chordomas. There have been three previous reports of chordomas in TSC patients. One was a sacrococcygeal chordoma (Dutton and Singleton, 1975), and two were clivus chordomas (Schroeder et al., 1987; Börgel et al., 2001), but no examination of somatic changes of the *TSC* genes was undertaken in those studies. Chordomas are clearly associated with TSC only rarely. Although MRI of the spine is not part of the routine workup of patients with TSC, it is unlikely that spinal or sacrococcygeal chordomas would escape clinical detection. Perhaps the rarity of this tumor type in individuals with TSC is more likely to reflect the small number of target cells (i.e., notochordal remnants) susceptible to somatic second-hit mutations or a requirement for further somatic events. All chordomas associated with TSC have been single lesions, a situation also seen with subependymal giant-cell astrocytoma but not with most other TSC-associated lesions, which are usually multiple.

Case 2 reported here, with a *TSC1* mutation, had minimal clinical manifestations of TSC. The case reported by Börgel et al. (2001), presented at less than 4 years of age with chordoma and had no recognized clinical signs of TSC at the time of his death, at age 6. However, he did have a nonsense mutation, E609X; 2046G>T, in exon 15 of the *TSC1* gene that was also present in his father, who had a definitive clinical diagnosis of TSC. The manifestations of TSC can be so subtle that unless specific investigations are done, the diagnosis can be missed. It is possible that the association between chordoma and TSC has therefore been overlooked in some cases. However, it would be premature to suggest additional investigations in patients with apparently sporadic chordoma. Careful clinical and family history assessment should suffice.

The average age of onset of chordomas not associated with TSC is 38 years for sphenooccipital (Heffelfinger et al., 1973) and 56 years for sacrococcygeal chordomas (Sundaresan et al., 1979), although sacrococcygeal chordomas are the most frequent of these tumors in children (Sassin and Chutorian, 1967) and may be congenital. The TSC-associated cases of sphenooccipital chordomas reported by Schroeder et al. (1987) and Börgel et al. (2001) were in children less than 5 years of age, and the TSC-associated sacrococcygeal chordoma reported by Dutton and Singleton (1975) was discovered during the first few days of life. The early onset of these tumors is also consistent with genetic predisposition to chordomas mediated via germ-line mutation in a *TSC* gene.

Although our data support a role for the *TSC* genes in the etiology of chordomas, this may be true only for a subset of these tumors. Future studies of this rare tumor should investigate involvement of *TSC1* or *TSC2* in cases arising in patients who have been carefully assessed to exclude any manifestations of TSC.

ACKNOWLEDGMENTS

The authors thank the families who provided consent and biological specimens for this study. Roberta Beauchamp is thanked for her assistance with DNA extraction from archival material.

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