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Comprehensive Mutation Analysis of *TSC1* and *TSC2*—and Phenotypic Correlations in 150 Families with Tuberous Sclerosis

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Summary

Tuberous sclerosis (TSC [MIM 191090 and MIM 191100]) is an autosomal dominant disorder characterized by hamartomas in many organs. Two thirds of cases are sporadic and are thought to represent new mutations. TSC is caused by mutations affecting either of the presumed tumor-suppressor genes, *TSC1* and *TSC2*. Both appear to function as tumor suppressors, because somatic loss or intragenic mutation of the corresponding wild-type allele is seen in the associated hamartomas. Here we report the first comprehensive mutation analysis of *TSC1* and *TSC2* in a cohort of 150 unrelated TSC patients and their families, using heteroduplex and SSCP analysis of all coding exons and using pulsed-field gel electrophoresis and conventional Southern blot analysis and long PCR to screen for large rearrangements. Mutations were characterized in 120 (80%) of the 150 cases, affecting *TSC1* in 22 cases and *TSC2* in 98 cases. *TSC1* mutations were significantly underrepresented in sporadic cases ($P = .000185$). Twenty-two patients had *TSC2* missense mutations that were found predominantly in the GAP-related domain (eight cases) and in a small region encoded in exons 16 and 17, between nucleotides 1849 and 1859 (eight cases), consistent with the presence of residues performing key functions at these sites. In contrast, all *TSC1* mutations were predicted to be truncating, consistent with a structural or adapter role for the encoded protein. Intellectual disability was significantly more frequent in *TSC2* sporadic cases than in *TSC1* sporadic cases ($P = .0145$). These data provide the first representative picture of the distribution and spectrum of mutations across the *TSC1* and *TSC2* loci in clinically ascertained TSC and support a difference in severity of *TSC1*- and *TSC2*-associated disease.

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Introduction

Tuberous sclerosis (TSC [MIM 191090 and MIM 191100]) is a disorder characterized by hamartomatous involvement of many organs (Gomez 1988) and is estimated to affect ⩾1/10,000 live births (Osborne et al. 1991). It is inherited as an autosomal dominant trait, but 60%–70% of cases are sporadic and are thought to represent new mutations (Fleury et al. 1980; Sampson et al. 1989). The phenotype is highly variable. The brain, skin, kidneys, and heart are all involved frequently, the lungs, skeleton, endocrine glands, and most other organs occasionally (Gomez 1988). Traditional diagnosis has been based on combinations of clinical, radiological, and histopathological signs; the criteria for definite diagnosis have been revised recently and have become more stringent than before (Roach et al. 1998). Significant and common medical complications include epilepsy (75%), intellectual disability (50%), and behavioral problems (including autism and attention-deficit disorder with hyperactivity, in 40% of children). Penetrance is very high, and the only cases of apparent nonpenetrance reported after rigorous clinical evaluation have been recently disproved by molecular genetic analysis (Young et al. 1998).

Two TSC-determining genes have been identified by positional cloning—*TSC1* at 9q34 (van Slegtenhorst et al. 1997) and *TSC2* at 16p13.3 (European Chromosome 16 Tuberous Sclerosis Consortium 1993). Molecular genetic analysis of hamartomatous and occasional malignant growths from patients with tuberous sclerosis have revealed loss of heterozygosity or truncating intragenic mutations affecting the corresponding wild-type allele (Henske et al. 1996; Sepp et al. 1996; van Slegtenhorst et al. 1997), indicating that *TSC1* and *TSC2* act as tumor suppressors, as defined by Knudson (1971). The tumor-suppressing properties of *TSC2* have now been demonstrated directly by transgenic expression in the Eker rat, a naturally occurring model in which the homologous *Tsc2* gene is mutated (Kobayashi et al. 1997). The mechanisms through which *TSC1* and *TSC2* mediate cellular growth control have been only partially elucidated. Exons 34–38 of *TSC2* encode a GTPase-activat-

ing protein (GAP)–related domain having significant homology to the GAPs rap1GAP and murine Spa1 (Maheshwar et al. 1996). Tuberin, the *TSC2* product, possesses modest GAP activity for rap1 (Wienecke et al. 1995) and rab5 (Xiao et al. 1997). These members of the Ras superfamily of GTPases serve roles in mitogenesis (Altschuler and Ribeiro Neto 1998), neuronal differentiation (York et al. 1998), and early endosome fusion (Gorvel et al. 1991). Tuberin also appears to play a role both in cyclin-dependent kinase (CDK)–dependent regulation of transition from G_0/G_1 to S phase during the cell cycle (Soucek et al. 1997) and in neuronal differentiation (Soucek et al. 1998). Specific cellular roles have not yet been reported for hamartin, the *TSC1* product, but hamartin and tuberin have been shown to coimmunoprecipitate, interact in the yeast two-hybrid system, and colocalize on immunofluorescence (Plank et al. 1998; van Slegtenhorst et al. 1998), suggesting that the proteins participate in at least some common pathways.

Mutation analysis of both *TSC1* and *TSC2* has not been reported previously in a series of patients with TSC. Comprehensive analysis of *TSC2* has been frustrated both by the size and structure of the gene (i.e., 41 coding exons and 1 leader exon) and because, until very recently, the full genomic sequence has not been available. There are several reports of single or small numbers of *TSC2* mutations identified during incomplete screening of the gene (Kumar et al. 1995*a,* 1995*b,* 1997; Vrtel et al. 1996; Jobert et al. 1997; Verhoef et al. 1998; Wang et al. 1998). Three studies have attempted more complete analysis of *TSC2*. Wilson et al. (1996) have used single-strand conformation polymorphism (SSCP) analysis of reverse-transcriptase–PCR products to study 30 unrelated probands and have reported nine possible mutations. One mutation, ΔF1509, has subsequently been seen in unaffected controls and is believed to be nonpathogenic (Jones et al. 1997). The pathogenicity of several missense changes identified in that study remains unconfirmed. van Bakel et al. (1997) have used the protein-truncation test to study 18 unrelated patients and have identified truncating mutations in 5 of them. Au et al. (1998) have used SSCP analysis of all coding exons to study 90 unrelated cases and have identified 22 putative mutations. In addition, several studies have been made of patients with TSC and severe polycystic kidney disease, most of whom appear to have a contiguous gene–deletion syndrome involving *TSC2* and the adjacent autosomal dominant polycystic kidney disease type 1 gene, *PKD1* (Brook-Carter et al. 1994; Longa et al. 1997; Sampson et al. 1997). Analysis of the *TSC1* gene has proved more straightforward. The complete genomic sequence of *TSC1* was established when the gene was identified. It is significantly less complex than *TSC2,* comprising 21 coding and 2 leader exons. At least five systematic studies of *TSC1* mutations have been reported (Jones et al. 1997; Ali et al. 1998; Kwiatkowska et al. 1998; Young et al. 1998; van Slegtenhorst et al., in press); all identified *TSC1* mutations in only 10% of sporadic cases of TSC. It is therefore clear that previous studies of TSC have failed to identify most of the causative mutations.

We set out to determine the distribution and nature of *TSC1* and *TSC2* mutations in a large and representative series of patients and families with TSC. Knowledge of these parameters is required both for development of efficient molecular diagnosis and for studies of the genotype-phenotype relationship. In previous studies of this patient cohort, we have screened all coding exons of the *TSC1* gene and exons 1, 25, 31, 34–39, and 41 of the *TSC2* gene, and we have searched for large-scale rearrangements at the *TSC1* and *TSC2* loci (Jones et al. 1997; Maheshwar et al. 1997). To obtain comprehensive data, we have completed SSCP and heteroduplex analysis of all *TSC2* exons and, by means of Southern analysis and long PCR, have supplemented our previous search for large rearrangements at both loci.

Patients and Methods

Patients

The study was approved by the ethics committee of the Division of Medicine, South Glamorgan Health Authority. The index cases comprised 150 consecutive unrelated patients with TSC who were listed with the Institute of Medical Genetics, Cardiff, prior to April 1994 and for whom samples were available for comprehensive analysis of the *TSC1* and *TSC2* genes. Patients specifically ascertained by us for a separate study of renal cystic disease in TSC (Sampson et al. 1997) were not included, but nine cases ascertained for other reasons who were subsequently found to have polycystic kidneys were included. Initially the series comprised 171 apparently unrelated patients, but 21 were excluded, 7 because they failed to satisfy the recently revised criteria for diagnosis of definite TSC (Roach et al. 1998), 13 because further samples required for completion of the analysis were not available, and, in 1 case, because previously unrecognized kinship to another index case who carried the same mutation was identified. Intellectual disability was considered to be present when formal developmental assessment revealed a developmental quotient <70, when unassisted mainstream schooling was impossible because of intellectual disability (not behavioral problems), or when an adult was institutionalized or required supervision in the community.

PCR

Genomic DNA was prepared from peripheral blood samples by standard methods. PCR primers were de-

signed with the aid of the "Oligo Analysis" software package (Wojciech Rychlik). Primer sequences and annealing temperatures for amplification of all *TSC1* and *TSC2* coding exons and for long PCR at the *TSC1* and *TSC2* loci are available at the Cardiff-Rotterdam Tuberous Sclerosis Mutation Database Website. Standard PCR was done in 50-μl reaction volumes containing 100 ng of genomic DNA, 25 pmol of each primer, 0.2 mM dNTP, 5 μl of reaction buffer (100 mM Tris pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin), and 1 U of Ampli*Taq* Gold Polymerase (Cetus). Cycling parameters were 94°C for 10 min, followed by 32 or 33 cycles of 54°C–58°C for 1 min, 72°C for 1 min, and 94°C for 30 s, and a final step of 72°C for 10 min. Long PCR was done in 50-μl reaction volumes containing 100 ng of genomic DNA, 4 pmol of each primer, 0.35 mM dNTP, 5 μl of Boehringer-Mannheim reaction buffer 3 (20 mM Tris -HCl, 100 mM KCl, 22.5 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 0.5% [v/v] Tween 20™, 0.5% [v/v] Nonidet P40™, 50% [v/v] glycerol), and 2 U of Expand Long Template polymerase (Boehringer Mannheim). Cycling parameters were 95°C for 2 min, followed by 28 cycles of 65°C for 30 s, 68°C for 8 min, and 94°C for 30 s. The elongation step was increased by 20 s for each cycle after cycle 10.

SSCP and Heteroduplex Analysis

SSCP was done on 3 μl of PCR product diluted 1:10 with formaldehyde containing 0.0125% bromophenol blue and 0.75% Ficoll 400. Samples were denatured at 94°C for 5 min, snap-cooled on ice, and triple-loaded (at 2-h intervals) on a mutation detection enhancement (MDE) gel (Flowgen). Electrophoresis was done in 0.6 × Tris borate EDTA at 6 W for 18 h at room temperature. Heteroduplex analysis was performed by mixing of 5-μl aliquots of two PCR products with 0.6 μl of 0.1 M EDTA, denaturing at 94°C for 5 min, and then slow cooling to 37°C. One microliter of electrophoresis dye (Hoefer) was added to each of the samples, which were then quadruple-loaded (at 1-h intervals) on an MDE gel and run at 4.5 W for 15 h at room temperature. Products on SSCP and heteroduplex gels were visualized by silver staining, as described elsewhere (Jones et al. 1997).

DNA Sequencing

PCR products of samples displaying variant banding patterns were sequenced by means of either the Sequenase PCR Product Sequencing kit or the Thermo-Sequenase cycle sequencing kit (Amersham).

Southern Analysis

Genomic DNA for Southern analysis was digested with *Eco*RI, *Hin*dIII, and *Bam*HI, electrophoresed, and blotted, as described elsewhere (Sampson et al. 1997), and were probed with *TSC1* and *TSC2* cDNA clones. Samples yielding variant restriction patterns were subjected to further restriction analysis to characterize the mutations involved. Products generated by long PCR amplification were blotted to Hybond N^+. Amplified exons were used as probes to determine which were deleted and which were preserved on the mutant chromosomes.

Microsatellite Analysis

Evidence for biological paternity and maternity in cases with apparently de novo mutations was assessed by means of highly polymorphic microsatellite repeats on chromosomes 4 (D4S43), 6 (D6S250), 7 (D7S636), 15 (D15S945), and 16 (D16S665).

Mutation Nomenclature

Mutation nomenclature has been inconsistent at the *TSC2* locus, since the transcript initially reported by the European Chromosome 16 Tuberous Sclerosis Consortium (1993) did not contain the nucleotides corresponding to exon 31, which is subject to alternative splicing (Maheshwar et al. 1996). We numbered nucleotides according to the full-length *TSC1* and *TSC2* cDNAs as in GenBank (accession numbers AF013168 and X75621, respectively), and mutations were described as recommended by the Ad Hoc Committee on Mutation Nomenclature (1996).

Results

Sample Characteristics

Of the 150 index cases comprehensively screened for *TSC1* and *TSC2* mutations, 130 were sporadic, 19 were classified as familial (having an affected parent, with or without other affected family members), and 1 patient had been adopted and the clinical status of the biological parents was unknown. At ascertainment, the total number of living affected individuals in the 150 families was 199, and the overall proportions of sporadic and familial cases were 65% and 35%, respectively. Thirteen further cases were screened by pulsed-field gel electrophoresis (PFGE) and by SSCP and heteroduplex analysis of coding exons of *TSC1* but, because of inadequate or degraded DNA samples, were not screened comprehensively for mutations of *TSC2*.

Mutations at the TSC Loci

Likely disease-causing mutations were characterized in 120 (80%) of the 150 index cases; of these 120 mutations, 22 (18%) were at the *TSC1* locus, and 98 (82%) were at the *TSC2* locus. In a previous study, we had reported all but one of the *TSC1* mutations (Jones et al.

1997). In that study, patient 365 with sporadic TSC was shown to have the de novo *TSC1* missense mutation A726E. We have now also demonstrated a de novo *TSC1* nonsense mutation W750X, G→ A at 2471, in this patient. The presence of both de novo mutations was confirmed through repeat samples, and biological parenthood was confirmed through five polymorphic microsatellite markers. The *TSC2* mutations are summarized in tables 1–3. With regard to the *TSC2* locus, 22 patients had whole-exon, multiexon, or whole-gene deletions or other large rearrangements, 20 had nonsense changes, 21 had small insertions or deletions predicted to lead to both a change of reading frame and premature truncation, 8 had changes in splice-site consensus sequences, 5 had in-frame deletions of one to six amino-acid residues, and 22 had missense changes.

Evidence for pathogenicity of the *TSC2* missense changes and small in-frame deletions was obtained by study of additional family members. Two small in-frame deletions—ΔI365 (unique) and 5256del18bp (recurrent)—were shown to arise de novo in sporadic cases, as were 11 of 14 different missense mutations. In all these cases, results of analysis of five polymorphic microsatellite markers were consistent with biological parenthood. A further unique missense change, G294E, occurred in a familial case; its pathogenicity is unconfirmed. Segregation of the missense change with TSC in this family has been demonstrated by others (A. Astrinidis and S. Povey, personal communication). Parental samples were unavailable for two apparently sporadic cases, 050 and 253, who carried the unique missense changes C696Y and R1743P and in whom no other *TSC1* or *TSC2* mutations were identified. The missense mutations were located principally in the GAP-related domain of *TSC2* encoded in exons 34–38 and in a short stretch of nucleotides at positions 1849–1859 (table 2 and fig. 1). Small truncating lesions were distributed across the *TSC1* and *TSC2* genes (fig. 1).

Eight *TSC2* mutations (tables 1 and 2) and one *TSC1* mutation, C→T at 2577, R786X, were seen recurrently. Together, these accounted for 28 (23%) of the 120 mutations identified (i.e., 19% of the 150 index cases).

TSC1 *and* TSC2 *Mutations in Familial and Sporadic Cases*

Of the 19 familial cases, 9 had *TSC1* mutations and 9 had *TSC2* mutations; no mutation was detected in 1 (5%) of the familial cases. Of the 130 sporadic cases, 13 had *TSC1* mutations and 88 had *TSC2* mutations; no mutation was detected in 29 (22%) of the sporadic cases. *TSC1* mutations were therefore significantly underrepresented among sporadic cases ($\chi^2 = 13.975$, 1 df; $P = .000185$). One adopted case with an unknown family history had a *TSC2* mutation.

Table 1

***TSC2* Nonsense, Frameshift, and Splice Mutations**

Patient	Location	Mutation	Nucleotide Change	Type of Mutation
341	Exon 4	Y155X	C483G	Nonsense
291	Exon 4	485ins5bp	5-bp insertion	Frameshift
046	Exon 5	546delC	1-bp deletion	Frameshift
246	Intron 7	793-1G→A	G793-1A	Splice
024	Intron 11	1275+2T→C	T1275+2C	Splice
255	Intron 13[a]	1462-1G→A	G1462-1A	Splice
366	Exon 14	1506delC	1-bp deletion	Frameshift
100	Exon 14	R505X[b]	C1531T	Nonsense
159	Exon 14	R505X	C1531T	Nonsense
173	Exon 14	R505X	C1531T	Nonsense
017	Exon 16	Y598X	C1812G	Nonsense
289	Exon 16	1813delA	1-bp deletion	Frameshift
222	Exon 18	1993del7bp	7-bp deletion	Frameshift
090	Exon 18	2042del19bp	19-bp deletion	Frameshift
081	Exon 18	2092delG	1-bp deletion	Frameshift
310	Exon 19	W703X	G2127A	Nonsense
086	Exon 19	2160del16bp	16-bp deletion	Frameshift
006	Exon 20	R751X	C2269T	Nonsense
054	Exon 20	R751X	C2269T	Nonsense
245	Exon 20	R751X	C2269T	Nonsense
238	Exon 20	2313insCC	2-bp insertion	Frameshift
142	Intron 20	2373+2T→C	T2373+2C	Splice
134	Intron 20	2373+2del4bp	4-bp deletion	Splice
158	Exon 21	Y790X	C2388G	Nonsense
235	Exon 21	2548delC	1-bp deletion	Frameshift
317	Intron 24	2855+1G→T	G2855+1T	Splice
197	Exon 28	3414delG	1-bp deletion	Frameshift
119	Exon 29	Q1148X	C3460T	Nonsense
166	Exon 29	Q1192X	C3592T	Nonsense
233	Exon 29	Q1192X	C3592T	Nonsense
015	Exon 30	3691ins5bp	5-bp insertion	Frameshift
377	Exon 30	3713delC	1-bp deletion	Frameshift
186	Exon 33	Q1419X	C4273T	Nonsense
001	Exon 33	S1433X	C4316A	Nonsense
027	Exon 33	R1459X	C4393T	Nonsense
040	Exon 33	R1459X	C4393T	Nonsense
240	Exon 33	R1459X	C4393T	Nonsense
373[c]	Exon 34	4512insC	1-bp insertion	Frameshift
147[c]	Exon 34	4559del4bp	4-bp deletion	Frameshift
234[c]	Exon 35	4594del7bp	7-bp deletion	Frameshift
093[c]	Exon 35	4609delG	1-bp deletion	Frameshift
230[c]	Exon 36	Y1571X	C4731G	Nonsense
036[c]	Exon 38	Q1665X	C5011T	Nonsense
248[d]	Intron 38	5087-2A→G	A5087-2G	Splice
351[d]	Exon 39	5178delTG	2-bp deletion	Splice
229	Exon 40	5229del4bp	4-bp deletion	Frameshift
018[d]	Exon 41	5358del32bp	32-bp deletion	Frameshift
357[d]	Exon 41	5406insC	1-bp insertion	Frameshift
172[d]	Exon 41	5425insT	1-bp insertion	Frameshift

[a] G→T has been observed in a patient reported by Jobert et al. (1997).
[b] Also observed in a patient reported by Wilson et al. (1996).
[c] Also reported by Maheshwar et al. (1997).
[d] Also reported by Jones et al. (1997).

Germ-Line Mosaicism

In 1 of the 150 families studied, two affected siblings had parents who were normal on clinical examination. The *TSC2* mutation R905W (C→T at 2731) was iden-

Table 2

***TSC2* Missense Mutations and In-Frame Deletions**

Patient	Exon	Mutation	Nucleotide Change	Type of Mutation	De Novo?
278	9	G294E	G899A	Missense	Familial
060	10	ΔI365	1111del3bp	In-frame deletion	Yes
098	16	R611W[a]	C1849T	Missense	Not tested
208	16	R611W	C1849T	Missense	Not tested
217	16	R611W	C1849T	Missense	Yes
032	16	R611Q[b]	G1850A	Missense	Yes
123	16	R611Q	G1850A	Missense	Not tested
102	16	R611Q	G1850A	Missense	Not tested
353	16	R611Q	G1850A	Missense	Not tested
275	17	A614D	C1859A	Missense	Yes
253	18	C696Y	G2105A	Missense	Not tested
362	23	R905W[b]	C2731T	Missense	Yes
220	33	P1497R	C4508G	Missense	Yes
211	33	S1498N	G4511A	Missense	Yes
306[c]	36	L1594M	C4798A	Missense	Yes
367[c]	37	N1643K	C4947G	Missense	Yes
276[c]	37	N1651S	A4970G	Missense	Yes
084[c]	38	P1675L	C5042T	Missense	Not tested
241[c]	38	P1675L	C5042T	Missense	Not tested
247[c]	38	P1675L	C5042T	Missense	Not tested
302[c]	38	P1675L	C5042T	Missense	Yes
223[c]	38	N1681K	C5061G	Missense	Yes
311	40	5256del18bp	18-bp deletion	In-frame deletion	Not tested
003	40	5256del18bp	18-bp deletion	In-frame deletion	Not tested
044	40	5256del18bp	18-bp deletion	In-frame deletion	Yes
360	40	5256del18bp	18-bp deletion	In-frame deletion	Not tested
050	40	R1743P	G5246C	Missense	Not tested

[a] Also reported by Wilson et al. (1996)
[b] Also reported by Au et al. (1998)
[c] Also reported by Maheshwar et al. (1997).

tified in the index case, patient 362, and in his affected sibling but was not detected in DNA extracted from leukocytes from each of the parents. Result of analysis of five microsatellite markers were consistent with biological parenthood, suggesting the presence, in one of the parents, of mosaicism involving the germ line for this mutation.

Polymorphisms

Thirty-nine sequence variants likely to represent nonpathogenic polymorphisms were observed at the *TSC2* locus, both in TSC patients and in either normal controls or TSC-family members without clinical or radiographic evidence of TSC. Thirty of these have not been reported previously unreported and are listed in table 4; they include nine missense variants within the coding region. *TSC1* polymorphisms have been reported elsewhere (Jones et al. 1997).

Intellectual Disability

Intellectual disability was present in 86 (57%) of the 150 index cases and in 97 (49%) of the 199 affected members in the 150 different families. Intellectual disability was significantly more common in sporadic cases carrying *TSC2* mutations (59 [67%] of 88 cases) than sporadic cases carrying *TSC1* mutations (4 [31%] of 13 cases) ($P = .0145$, Fisher's exact test; odds ratio 4.5). The frequency of intellectual disability in sporadic cases with *TSC2* missense or small in-frame deletion mutations (15 [68%] of 22 cases) was not significantly different from that in sporadic cases with other classes of *TSC2* mutations (44 [67%] of 66 cases) ($P = .506$, Fisher's exact test).

Unusual Somatic Features

Gross polycystic kidney disease was detected on ultrasound scan (but was not the presenting feature) in nine cases. All of these cases had *TSC2* mutations, and, in six cases, these mutations were contiguous deletions also involving the *PKD1* gene. Four of the six cases with *TSC2/PKD1* deletions were somatic mosaics. Two cases had large intragenic deletions affecting *TSC2,* and one case had a 600-kb inversion disrupting *TSC2* (table 3). All cases have been reported elsewhere (European Chromosome 16 Tuberous Sclerosis Consortium 1993; Brook-Carter et al. 1994; Sampson et al. 1997). Fifteen of 22 index cases with *TSC1* mutations had no evidence

Table 3

Large Rearrangements at the *TSC2* Locus

Patient	Mutation	Method of Detection	Reference
002	Multigene deletion *TSC2/OCTS3/OCTS2*	PFGE	Jones et al. (1997)
019	Intragenic deletion ≈5 kb	Southern	European Chromosome 16 Tuberous Sclerosis Consortium (1993)
022	Mosaic multigene deletion *TSC2/PKD1*	PFGE	Sampson et al. (1997)
076	Multigene deletion *TSC2/OCTS3/OCTS2*	PFGE	European Chromosome 16 Tuberous Sclerosis Consortium (1993)
0786	Multigene deletion *TSC2/OCTS3/OCTS2*	PFGE	Brook-Carter et al. (1994)
080	Multigene deletion *TSC2/OCTS3/OCTS2*	PFGE	European Chromosome 16 Tuberous Sclerosis Consortium (1993)
088	Intragenic deletion ≈10 kb	PFGE	Jones et al. (1997)
111	Mosaic multigene deletion *TSC2/PKD1*	PFGE	Sampson et al. (1997)
136	Intragenic deletion ≈4 kb (exons 17–20)	Long PCR	Present study
148	Multigene deletion *TSC2/OCTS3*	Southern	European Chromosome 16 Tuberous Sclerosis Consortium (1993)
179	Multigene deletion *TSC2/PKD1*	PFGE	European Chromosome 16 Tuberous Sclerosis Consortium (1993)
180	Intragenic deletion ≈3 kb	Southern	European Chromosome 16 Tuberous Sclerosis Consortium (1993)
181	Multigene deletion *TSC2/OCTS2/OCTS3*	PFGE	European Chromosome 16 Tuberous Sclerosis Consortium (1993)
206	Intragenic deletion ≈5 kb	Southern	Jones et al. (1997)
216	Intragenic deletion ≈1 kb (exons 37–38)	Southern	Jones et al. (1997)
270	Mosaic multigene deletion *TSC2/PKD1*	PFGE	Sampson et al. (1997)
277	Intragenic deletion ≈3 kb (exon 11, part of exon 12)	Long PCR	Present study
283	Mosaic multigene deletion *TSC2/PKD1*	PFGE	Sampson et al. (1997)
284	Multigene deletion *TSC2/PKD1*	PFGE	Sampson et al. (1997)
308	Deletion of *TSC2* and 3′ UTR of *PKD1*	Southern	Sampson et al. (1997)
346	Intragenic deletion ≈8 kb (exons 3–8)	Southern	Present study
431	Inversion ≈600 kb	PFGE	Sampson et al. (1997)

of renal cysts on ultrasound scan. Three had unilateral solitary cysts (at age 35, 50, and 64 years). No renal ultrasound scan was performed in four cases with *TSC1* mutations.

Three female patients had a history of pneumothorax and either cystic or honeycomb changes in the lungs on radiographic investigation, consistent with a diagnosis of lymphangioleiomyomatosis (LAM). One case, patient 080, had a large deletion mutation involving both the 5′ end of *TSC2* and the *OCTS3* and *OCTS2* genes, which lie immediately 5′ of *TSC2*. Another case, patient 208, had the recurrent *TSC2* missense change R611W. The third case, patient 256, had bony sclerosis, with expansion of the right clavicle, and asymmetry affecting the right side of the face, in addition to cystic changes in the right lung. This patient carried the *TSC1* nonsense change S334X. All of these cases were sporadic.

Localized overgrowth affecting the limbs was observed in three female patients, all of whom otherwise had typical multiorgan involvement. *TSC2* mutations were identified in DNA extracted from venous blood in two of these patients. Patient 211, carrying the de novo missense change S1498N, had massive overgrowth of the right leg, involving the bones and soft tissues, that progressed during infancy and childhood and that, at age 30 years, led to amputation of the leg. Patient 046 carried the single-base deletion 546delC and had overgrowth localized to the left little finger. No mutation was identified in the third case, who had overgrowth affecting the bones and soft tissues of the left forearm and of the middle and ring fingers.

Discussion

We studied 150 unrelated individuals with TSC, as well as their family members. The proportions of familial and sporadic cases among all 199 affected members of their families were 35% and 65%, respectively, and the overall frequency of intellectual disability was 49%. These figures are similar to those reported in large epidemiological studies (Sampson et al. 1989; Osborne et al. 1991), suggesting that the present series is representative of clinically ascertained TSC. We identified mutations in a total of 120 (80%) of these patients; 98 (65.4%) were shown to have mutations at the *TSC2* locus, and 22 (14.6%) had mutations at the *TSC1* locus. One hundred one different mutations were characterized (21 affecting *TSC1* and 80 affecting *TSC2*), of which 42 had not been described previously. Mutations were not identified in 1 of 19 familial cases or in 29 of 130 sporadic cases. Recent studies have shown that somatic mosaicism may be a frequent finding among sporadic cases of TSC (Sampson et al. 1997). The SSCP and heteroduplex analyses used for exon screening in this study are unlikely to have had sufficient sensitivity to detect low-level mosaicism, and they also have limited sensitivity in the detection of some nonmosaic mutations. These factors, as well as our incomplete assessment of

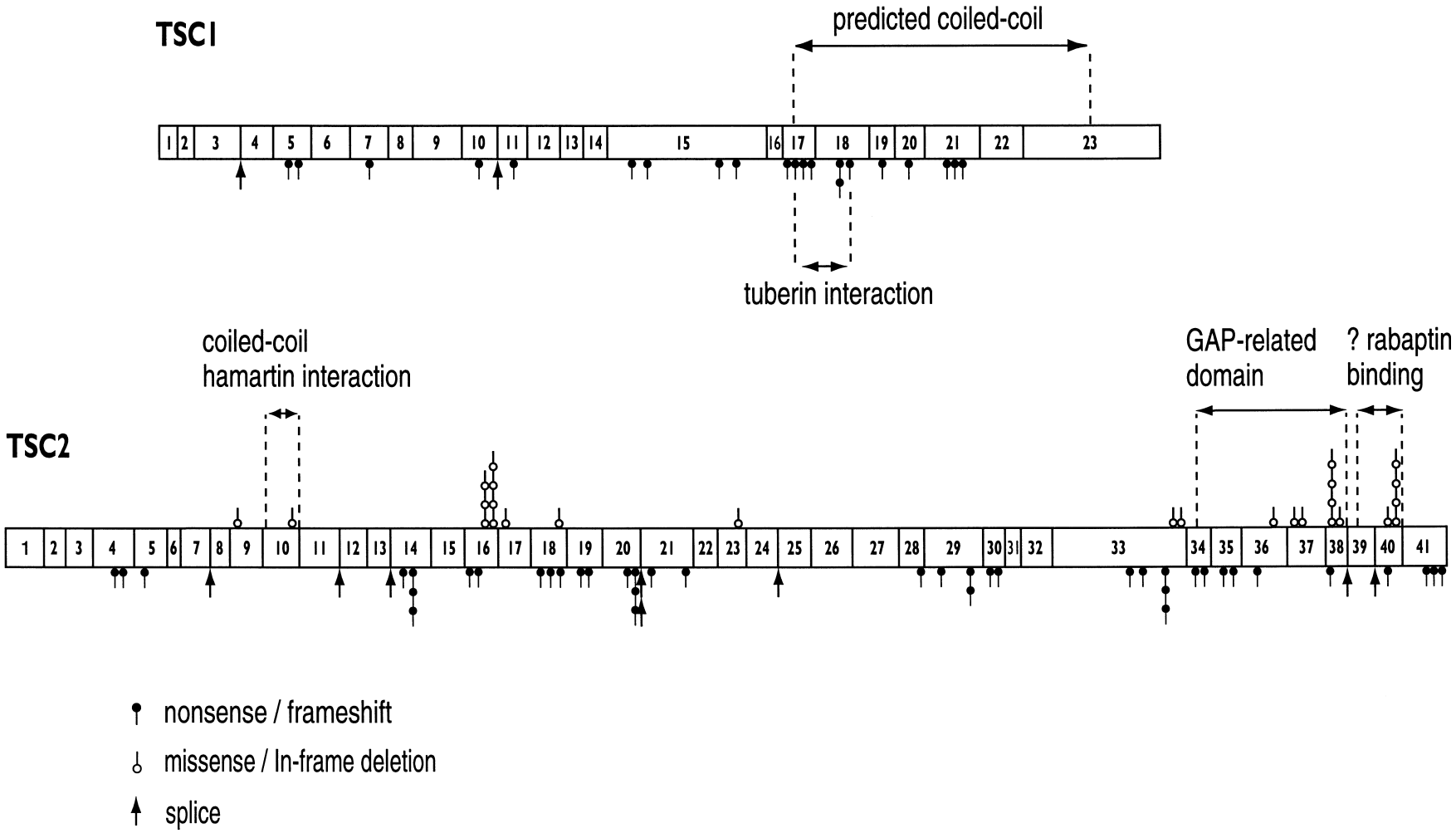

Figure 1 Distribution of point mutations and small deletions and insertions in the *TSC1* and *TSC2* genes. Numbered boxes denote individual exons.

Table 4

Polymorphisms within the *TSC2* Gene

Location	Polymorphism	Nucleotide Change	Frequency
Intron 3	354+31C→T	C354+31T	Unique
Exon 4	L160V	C496G	Unique
Intron 4	500−3C→T	C500−3T	5%–10%
Intron 4	500−7CC→AA	CC500−7AA	Unique
Exon 8	R261W	C799T	Unique
Exon 9	M286V	A874G	<5%
Exon 10	R367Q	G1118A	<5%
Exon 10	Q370	A1128G	<5%
Exon 11	P378L	C1151T	Unique
Exon 12	G440S	G1336A	Unique
Exon 13	G459	C1395T	Unique
Exon 13	I463V	A1405G	Unique
Intron 14	1618−14C→T	C1618−14T	5%–10%
Intron 14	1618−39C→T	C1618−39T	5%–10%
Intron 15	1735−16C→T	C1735−16T	Unique
Exon 16	A583T	G1765A	<5%
Exon 18	P677	C2049T	Unique
Intron 18	2115+35G→A	G2115+35A	Unique
Intron 22	2657+44C→G	C2657+44G	<5%
Intron 31	3901+78G→A	G3901+78A	5%–10%
Intron 31	3902−51C→G	C3902−51G	<5%
Intron 31	3902−55A→T	A3902−55T	Unique
Exon 33	D1436	C4326T	Unique
Intron 35	4681−43G→A	G4681−43A	Unique
Intron 39	5179−9A→C	A5179−9C	5%–10%
Intron 39	5179−26delTGAG	4-bp deletion	<5%
Intron 40	5277+73C→T	C5277+73T	Unique
Intron 40	5277+80C→T	C5277+80T	Unique
Exon 41	G1791S	G5389A	Unique
3′ UTR	5503delAA	2-bp deletion	<5%

intronic and regulatory sequences, likely account for our failure to detect mutations in 20% of the cases. Further locus heterogeneity in TSC also remains a theoretical possibility.

We found that significantly more of the sporadic cases had *TSC2* than had *TSC1* mutations. This would be expected if the germ-line mutation rate at the *TSC2* locus were higher than at the *TSC1* locus. However, additional familial *TSC2* cases would also be expected, and we did not observe this; nor has a significant excess of families without linkage to *TSC1* been observed in linkage studies (Sampson et al. 1991; Povey et al. 1994). A possible explanation for the apparent excess of *TSC2* mutations among sporadic cases—but not among familial cases—would be greater overall severity of *TSC2*-associated disease. We assessed the frequency of intellectual disability in sporadic cases, as a reliable and important aspect of disease severity. Unlike many other components of the phenotype that show age-dependent penetrance, intellectual disability in TSC is almost invariably present from early childhood and rarely escapes detection. We found intellectual disability to be significantly more frequent among sporadic cases with *TSC2* mutations than among those with *TSC1* mutations. If the presence or absence of intellectual disability is, in part, related to the frequency of "second hit" mutations in the developing brain, then our observations could reflect a somatic mutation rate that is higher at the *TSC2* locus than at the *TSC1* locus. Alternatively, tuberin may serve roles in the CNS that are not shared by hamartin.

Elsewhere, we have shown that most patients with TSC and severe polycystic kidney disease have contiguous deletions involving *TSC2* and the adjacent polycystic kidney disease 1 (*PKD1*) gene (Brook-Carter et al. 1994; Sampson et al. 1997). The present series did not include cases specifically ascertained because of polycystic kidney disease, but polycystic kidney disease was identified in 9 of the 150 index cases. Six of these cases had *TSC2-PKD1* deletions, of whom four were mosaics. Deletion of *TSC2* and of only the 3′ UTR of *PKD1* was found in a further case, who did not have polycystic kidneys. Three cases with polycystic kidneys had *TSC2* mutations that did not involve *PKD1*. Solitary cysts—but not polycystic kidneys—were seen in some patients with *TSC1* mutations. Specific study of a larger cohort of patients is required in order to determine whether *TSC1* disease carries a risk of clinically significant renal cystic disease.

Symptomatic cystic lung disease presenting as pneumothorax was a feature in three cases in the present series. Two patients with *TSC2* mutations had widespread honeycomb appearances in both lungs that was typical of LAM, and the third, with a *TSC1* mutation, had marked cystic changes in the upper lobe of the right lung. LAM is a disorder seen almost exclusively in females and is characterized by bronchiolar smooth-muscle infiltration and cystic changes in the lung parenchyma. This is usually associated with angiomyolipoma of the kidneys and/or abdominal and hilar lymph nodes. Symptomatic LAM is estimated to occur in ~1/million of the population, without other evidence of TSC, but in several percent of females with TSC, implicating a role for the TSC genes in the etiology of LAM. Loss of heterozygosity for markers in the *TSC2* region has been observed in renal angiomyolipomas and lymph nodes removed from women with LAM but without other signs of TSC (Smolarek et al. 1998), but mutations of the TSC genes had not been reported previously in patients with either TSC-associated LAM or sporadic LAM.

Studies of other components of the TSC phenotype in *TSC1*- and *TSC2*-associated disease are now required, particularly to quantify the frequency and severity of complications. Given our findings in relation to the distribution of *TSC1* and *TSC2* mutations in sporadic and familial cases, as well as the likelihood that familial cases will be less severely affected than are sporadic cases, such studies will have to be based on either sporadic cases or

epidemiologically complete populations, or they will have to employ valid corrections for ascertainment bias.

The large proportion of *TSC2* mutations—as opposed to *TSC1* mutations—that we observed in medically ascertained TSC has implications for the development of molecular diagnostics. Not only is the *TSC2* gene, in its structure, substantially more complex than the *TSC1* gene, but we have also found its mutational spectrum to be more diverse. Several studies have found the great majority of germ-line mutations at the *TSC1* locus to be small truncating lesions (Jones et al. 1997; Ali et al. 1998; Kwiatkowska et al. 1998; Young et al. 1998; van Slegtenhorst et al., in press). In contrast, we have now shown that, at the *TSC2* locus, missense changes, large rearrangements including whole-gene deletions, nonsense mutations, and small insertions and deletions are represented at similar frequencies. The sensitivity of any single currently available approach for mutation detection at the *TSC2* locus is therefore likely to be limited. However, we did observe eight *TSC2* mutations recurrently, at least three of which (R505X, R611W, and R611Q) have also been reported by other investigators (Wilson et al. 1996; van den Ouweland et al. 1997; Au et al. 1998). Seven of the mutations seen recurrently were due to C→T or G→A substitutions at CpG dinucleotides and are likely to result from spontaneous deamination of methylated cytosines in the genomic DNA. The in-frame *TSC2* deletion, 5256del18bp, was identified in four unrelated sporadic cases. It involves a direct repeat of 11 nucleotides and the intervening 7 nucleotides and is likely to be the result of slipped mispairing during replication (Cooper and Krawczak 1993). Several *TSC1* mutations also have been seen recurrently (Jones et al. 1997; van Slegthenhorst et al. 1997, and in press; Ali et al. 1998; Kwiatkowska et al. 1998; Young et al. 1998), suggesting that assay for a small number of *TSC2* and *TSC1* mutations might provide a useful initial screen in the diagnostic setting.

Our characterization of significant numbers of missense mutations and some small in-frame deletions at the *TSC2* locus is likely to be helpful in the investigation of putative functions of the gene product. The effects that the missense changes in the GAP-related domain have on GAP activity toward rap1 and rab5 are now under investigation, as is the effect that the single-residue deletion ΔI365 has on the binding of hamartin by tuberin. Natural mutants of this kind may prove useful for determination of which of tuberin's functions has a requirement for hamartin binding. The recurrent 18-p deletion in exon 40 of *TSC2* lies within the putative rabaptin-binding domain (Xiao et al. 1997). However, demonstration of the binding of rabaptin by tuberin awaits independent confirmation, and the pathogenic mechanism of this mutation is unclear at present. We and others (Wilson et al. 1996; van den Ouweland et al. 1997; Au et al. 1998) have identified recurrent missense mutations within a short stretch of only 11 nucleotides (1849–1859) of *TSC2*. This region does not correspond to any currently recognized functional domain of tuberin, but the effects that these mutations have on recently proposed roles of tuberin, including regulation of G_0/G_1 to S phase transition (Soucek et al. 1997), can now be assessed. In contrast, despite reports of large numbers of single-base changes and small deletions and insertions at the *TSC1* locus, no clearly pathogenic missense mutations have been described, and it appears likely that complete loss of hamartin—rather than more-subtle disruption of specific roles—is usual in *TSC1* associated disease.

Note added in proof.—Since this article was accepted for publication, mutations have been identified in three further sporadic cases, all at the *TSC2* locus. These were (i) an intragenic deletion of ~5 kb in patient 165 (identified by long PCR), (ii) a deletion of 34 bp from a 34-bp tandem repeat spanning the exon 38/intron 38 boundary in patient 112 (the same mutation has been reported elsewhere, in a different patient with TSC [Beauchamp et al. 1998]), and (iii) a deletion of exon 41 and the 3′ UTR of *TSC2* and part of the 3′ UTR of *PKD1* in patient 115 (identified by long PCR).

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Cardiff-Rotterdam Tuberous Sclerosis Mutation Database, http://www.uwcm.ac.uk/uwcm/mg/tsc_db/pcrpub.html (for PCR primers and conditions)

GenBank, http://www.ncbi.nlm.nih.gov/Web/Genbank (for *TSC1* and *TSC2* cDNAs [AF013168 and X75621, respectively] and *TSC2* sequence [AC005600])

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