Mutational Analysis in a Cohort of 224 Tuberous Sclerosis Patients Indicates Increased Severity of *TSC2,* Compared with *TSC1,* Disease in Multiple Organs

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Tuberous sclerosis (TSC) is a relatively common hamartoma syndrome caused by mutations in either of two genes, *TSC1* and *TSC2.* Here we report comprehensive mutation analysis in 224 index patients with TSC and correlate mutation findings with clinical features. Denaturing high-performance liquid chromatography, long-range polymerase chain reaction (PCR), and quantitative PCR were used for mutation detection. Mutations were identified in 186 (83%) of 224 of cases, comprising 138 small *TSC2* mutations, 20 large *TSC2* mutations, and 28 small *TSC1* mutations. A standardized clinical assessment instrument covering 16 TSC manifestations was used. Sporadic patients with *TSC1* mutations had, on average, milder disease in comparison with patients with *TSC2* mutations, despite being of similar age. They had a lower frequency of seizures and moderate-to-severe mental retardation, fewer subependymal nodules and cortical tubers, less-severe kidney involvement, no retinal hamartomas, and less-severe facial angiofibroma. Patients in whom no mutation was found also had disease that was milder, on average, than that in patients with *TSC2* mutations and was somewhat distinct from patients with *TSC1* mutations. Although there was overlap in the spectrum of many clinical features of patients with *TSC1* versus *TSC2* mutations, some features (grade 2–4 kidney cysts or angiomyolipomas, forehead plaques, retinal hamartomas, and liver angiomyolipomas) were very rare or not seen at all in *TSC1* patients. Thus both germline and somatic mutations appear to be less common in *TSC1* than in *TSC2.* The reduced severity of disease in patients without defined mutations suggests that many of these patients are mosaic for a *TSC2* mutation and/or have TSC because of mutations in an as-yet-unidentified locus with a relatively mild clinical phenotype.

Introduction

Tuberous sclerosis (TSC [MIM 605284, MIM 191092]) is an autosomal dominant condition characterized by seizures, mental retardation, and development of widespread hamartomatous lesions (Jozwiak et al. 1994; Kwiatkowski and Short 1994; Webb et al. 1996; Ewalt et al. 1998; Gomez et al. 1999). TSC affects 1/6,000–10,000 individuals (Sampson et al. 1989; Osborne et al. 1991). Two-thirds of cases are sporadic, without antecedent family history, reflecting a high spontaneous mutation rate. Inactivating mutations in either *TSC1* or *TSC2* cause this tumor suppressor–gene syndrome (Eu-

ropean Tuberous Sclerosis Consortium 1993; van Slegtenhorst et al. 1997). *TSC1* consists of a 3.4-kb coding region with 21 coding exons, spanning 50-kb genomic extent on 9q34, and *TSC2* consists of a 5.4-kb coding region with 41 exons and spans 40-kb genomic extent on 16p13.

So far, 154 patients with mutations in *TSC1* and 292 patients with mutations in *TSC2* have been reported (Cheadle et al. 2000; Human Genetics Online Review Tables). Fifteen or more of these have been seen recurrently, but none accounts for >5% of all patients (European Tuberous Sclerosis Consortium 1993; Sampson et al. 1997; van Bakel et al. 1997; van Slegtenhorst et al. 1997, 1999; Au et al. 1998; Beauchamp et al. 1998; Kwiatkowska et al. 1998; Young et al. 1998; Jones et al. 1999). Most (88%) identified mutations are small changes—38% small deletions or insertions and 50% nonsense, splice-site, or missense point mutations. Large genomic deletions and rearrangements account for 12% of identified mutations and have been reported exclusively in *TSC2.* A number of methods have been used

to identify mutations in these two genes: heteroduplex analysis using conformation-sensitive gel electrophoresis (van Slegtenhorst et al. 1997; Kwiatkowska et al. 1998), SSCP (Au et al. 1998; Beauchamp et al. 1998; Young et al. 1998; Jones et al. 1999; van Slegtenhorst et al. 1999), denaturing-gradient gel electrophoresis (Dabora et al. 1998), the protein-truncation test (van Bakel et al. 1997; Mayer et al. 1999), pulsed-field gel electrophoresis combined with Southern blotting (European Tuberous Sclerosis Consortium 1993; Sampson et al. 1997), and FISH (Michalet et al. 1997). The most comprehensive analysis performed to date in a large series of patients with TSC used a combination of SSCP and heteroduplex analysis, as well as screening for large deletions by several methods, and yielded mutations in 130 of 163 patients: a detection rate of 80%, which improves to 87% if some patients without defined mutations after large deletion screening are removed (Jones et al. 1999, 2000).

In this same series, it appeared that patients with mutations in *TSC1* (hereafter denoted "*TSC1* disease" and distinguished from "*TSC2* disease") had less mental retardation on average than patients with *TSC2* disease. To avoid bias, the analysis was restricted to sporadic patients only, and 59 (67%) of 88 patients with sporadic *TSC2* had mental retardation, compared with 4 (32%) of 13 patients with *TSC1* disease ($P = .0145$, Fisher's exact test; Jones et al. 1999). However, this has not been seen uniformly (Kwiatkowska et al. 1998; Niida et al. 1999; van Slegtenhorst et al. 1999), and no study has examined the influence of *TSC1* disease versus *TSC2* disease on the myriad other clinical features of TSC.

Here we present an analysis of 224 unselected and sequentially ascertained patients with TSC, derived largely from pediatric neurology practices in three separate geographic locations. We have developed a combination of efficient methods to comprehensively identify mutations in *TSC1* and *TSC2*. A clinical instrument for recording clinical data on patients with TSC was also developed and applied at each center. These methods have yielded both a very high detection rate of mutations in *TSC1* and *TSC2* in these unselected patients (83% overall) and significant new information on the difference in phenotype between patients with *TSC1* and *TSC2* disease.

Subjects and Methods

Patients and Clinical Assessment

The patients with TSC that we studied were a consecutive, unselected series of 224 index patients derived from four sources: 36 were obtained through patient-derived requests, all from the United States; 105 were derived from an academic pediatric neurology practice in Warsaw, Poland; and 83 were derived from academic pediatric neurology practices in the United States (65 from Cincinnati and 18 from Boston). All patients provided informed consent, and this study was approved by institutional review boards at each institution. All patients encountered at each institution with an initial diagnosis of TSC were offered participation. Although initially included in these cohorts, 14 patients were not studied because of either a lack of an adequate DNA sample or failure to meet formal TSC diagnostic criteria (Roach et al. 1998). An additional three patients were dropped after partial mutational analysis, when it was discovered that they failed to meet diagnostic criteria.

Standardized clinical information was collected prospectively on all patients and updated by additional studies and follow-up during the period of mutational analysis. This information consisted of the age and family status of the patient and 16 different measures of both the clinical and radiographic features of TSC (see fig. 1). The information was collected by persons blinded to the mutational status of the patients. General clinical features of the patients from Warsaw, Cincinnati, and Boston were determined by clinicians experienced in the manifestations of TSC (S.J., D.F., and E.T.). Medical records were reviewed for the patients studied through mail solicitation. Specialized examinations or radiographic studies were used to assess the extent of lesions in the brain, kidney, liver, retina, and heart. Most manifestations were graded according to a binary scale (present or absent), but several features were graded in severity at 3–5 levels, including mental retardation, facial angiofibromas, kidney and liver angiomyolipomas (AMLs), kidney cysts, and lung involvement. In addition, for a subset of patients in which high-resolution brain magnetic-resonance imaging (MRI) and/or computed-tomography (CT) scans were available, the number of subependymal nodules (SENs) and cortical tubers were counted. Most of these studies were reviewed by a single radiologist (J.E.).

Mutational Analysis of Exons by Denaturing High-Performance Liquid Chromatography (DHPLC)

DNA was extracted from leukocytes or from Epstein-Barr virus–transformed lymphoblastoid cell lines by use of standard methods. Primers were designed by use of *TSC1* and *TSC2* genomic sequence information (GenBank accession numbers AC002318, AC002096, and AC005600) and the GCG version 8 primer program or the WI Primer 3 program. Amplified fragments were 186–507 bp in length. A detailed description of our method of performing DHPLC is published elsewhere, describing its application to exons 1–20 of *TSC2* (Choy et al. 1999). For analysis of the remaining exons of these genes, similar methods were used, and complete infor-

Clinical features - please circle appropriate term   date of last exam:

Grade
3 | Severe: no speech intelligible to a stranger
2 | Moderate: some speech, no reading
1 | Mild: reading at less than 3rd grade level
0 | None: reading above 3rd grade level

1. seizures   chronic   ever present   never
2. mental retardation   none   mild   moderate   severe   ←
3. hypomelanotic macules (>2)   present   absent
4. facial angiofibroma   grade   0   1   2   3   ←   grade 0: none
5. shagreen patch   present   absent   grade 1: macular (flat) lesions only on cheek
6. ungual fibromas   present   absent   # ____   grade 2: papular lesions, <3 mm diameter
7. forehead plaque   present   absent   grade 3: papular lesions, >3 mm diameter;
                                                                                      and/or extending to below mouth

Radiographic studies or other evaluation - please circle appropriate term or give number; please give dates of all studies

8. SENs   ____ # periventricular lesions seen on CT/MRI, date of exam:
9. SEGA   present   absent
10. cortical tubers   _____# seen on MRI (do not use CT); date of exam:

11. kidney cysts   grade   0   1   2   3   4   ←   grade 0: none   grade 1: 1-2 small (<2 cm) cysts
        date of exam: grade 2: >2 small (<2 cm) cysts   grade 3: >2 and at least one >2 cm
                                                                          grade 4: classic polycystic kidney disease- multiple cysts with renal enlargement

12. kidney angiomyolipoma   grade   0   1   2   3   ←   grade 0: none   grade 1: one or more, all <1 cm diameter
13. liver angiomyolipoma   grade   0   1   2   3   ←   grade 2: multiple, one or more >1 cm, and all <4 cm in diameter
                                                                                      grade 3: multiple, one or more >4 cm

14. retinal hamartomas   present   absent
15. cardiac rhabdomyomas   ever present   never   date of exam
16. lymphangiomatosis   severe (clinical symptoms)   mild (abn. chest X-ray)   absent
17. other

Demographic
18. Ethnic background:
19. Family history- draw a family tree and/or provide a list of all immediate relatives of all members of family with TS.

Name   relationship   age   medical condition potentially related to TS

Figure 1   TSC clinical features grading form

mation on amplicons and run conditions is available (Tuberous Sclerosis [TSC] Project Web site). After completion of analysis of patient DNA samples using a single set of run conditions for DHPLC, we modified the temperature for 20 exons in which analysis by the WAVE-MAKER 3.4 software (Transgenomic) or by the DHPLC MELT program (N. F. Hansen and P. Oefner) suggested that higher or lower temperatures might detect additional sequence variants. All samples remaining without identified mutations were then analyzed under these different conditions. Four additional mutations were detected in this manner.

PCR was performed using AmpliTaq Gold (PE Biosystems) in 20-μl reactions with 10–50 ng of genomic DNA. Amplified fragments showing a DHPLC elution shift were confirmed in a repeat analysis. Sequence var-

iation was identified by bidirectional sequencing using an ABI 377 machine (PE Biosystems) with Big Dye terminator chemistries (PE Biosystems). Sequence traces were analyzed using the GAP4 program of the Staden package, and sequence variation within *TSC1* and *TSC2* was positioned using the VARIATION WIZARD program (Tuberous Sclerosis [TSC] Project). This program permits inspection of genomic and cDNA sequence information for alignment and interpretation of sequence variation. It is freely available from M.P.R. In two amplicons with DHPLC shifts, no sequence variation could be detected, despite repeated sequencing analyses. In those cases, the amplified fragments were cloned and tested by mixing studies and DHPLC analysis, and the variant sequence was identified by sequencing.

Missense mutations were considered to be noncon-

servative amino acid substitutions if they had a negative score in the BLOSUM matrix (Henikoff and Henikoff 1992). A table of polymorphisms discovered during this study is available (Tuberous Sclerosis [TSC] Project Web site).

Mutational Analyses for Large Deletions and Insertions

Two methods were used for identification of larger deletions, insertions, and genomic rearrangements. First, long-range PCR was performed (Dabora et al. 2000). Large amplicons for long-range PCR were chosen to provide overlapping coverage of the entire 40-kb genomic extent of *TSC2* in 19 fragments of size 1.7–11.6 kb. PCR products were analyzed by electrophoresis on 0.8% agarose gels with long run times, to permit visual identification of fragments smaller or larger than normal. Such fragments were gel purified and reamplified with additional PCR primers, to permit isolation and sequencing of the junction site of all apparent deletions and insertions. Multiplex long-range PCR was also performed using a single forward primer and multiple reverse primers (each >15 kb from the forward primer), to enable detection of larger deletion fragments.

Second, quantitative PCR was performed for detection of large deletions and duplications (Yau et al. 1996; Morgan et al. 1999). 6-FAM–labeled forward primers were used with unlabeled reverse primers for multiplex PCR of selected *TSC2* exons. Primer sequences were identical to those used for single *TSC2* exon amplification used for DHPLC (see Tuberous Sclerosis [TSC] Project Web site). Sets of exons of *TSC2* were amplified in multiplex PCR as follows—set 1: exons 4, 7, and 26; set 2: exons 7, 16, and 26; and set 3: exons 25, 26, and 36. In each of these sets, *TSC1* exon 17 was also amplified as a control. PCR conditions were identical to those used for DHPLC analysis, except that multiple primer pairs were present in individual PCR reactions at concentrations from 0.25 to 0.75 μM to normalize the amount of amplicon generated for each exon, and only 18 cycles of PCR were performed, to ensure that amplification was still in the exponential phase. PCR products were analyzed by electrophoresis of 2 μl on 4.25% polyacrylamide gels on the ABI 377. Electropherogram patterns were analyzed and quantitated using GENESCAN version 3.1 software (PE Biosystems). All samples were analyzed in duplicate, and those that indicated a probable deletion were repeated for confirmation. Every PCR reaction set and gel run also included at least one positive control sample with a deletion previously defined by long-range PCR and five control samples.

To determine whether a deletion or duplication of an exon had occurred, we calculated the relative amount of each *TSC2* exon produced in the multiplexed PCR reaction according to the method described elsewhere (Yau et al. 1996). For example, to calculate the dosage quotient (DQ) of *TSC2* exon 4 in an unknown sample (U), in comparison with a control sample (C), the following formula was used:

$$DQ_{TSC2ex4}(U) = \frac{\text{Area } TSC2 \text{ exon 4(U)/Area } TSC1 \text{ exon 17(U)}}{\text{Area } TSC2 \text{ exon 4(C)/Area } TSC1 \text{ exon 17(C)}}.$$

We actually used the average value of five control samples run on the same gel as the denominator in this calculation. Control samples have DQ values in the range of 0.80–1.25 (Yau et al. 1996). DQ mean values <0.61 were scored as exon deletions, and mean values >1.30 were scored as exon duplications. For each deletion patient, the SD of ≥4 DQ measurements was ≤0.11; for each duplication patient, the SD of ≥4 DQ measurements was ≥0.41. One patient DNA sample repeatedly gave intermediate values in the 0.61–0.80 interval, average 0.73 ($n = 4$), which we scored as a mosaic deletion.

Paternity and Maternity Testing

Paternity and maternity testing was performed for all families in which *TSC2* missense mutations were found in sporadic patients and in which parental DNA samples were available. This was performed by analysis of at least three highly informative microsatellite markers (D15S127, D15S131, D16S515, and D16S520), using fluorescently labeled primers and analysis by gel electrophoresis on the ABI 377 and GENESCAN version 3.1 software.

Statistical Analysis

Statistical comparisons were performed using the STATVIEW version 5.0 package for the Macintosh. Fisher's exact test was used for the analysis of categorical variables, and Student's unpaired *t* test was used for the analysis of quantitative variables.

Results

Patient Characteristics

Two hundred twenty-four patients meeting formal diagnostic criteria for TSC were studied. These were derived by sequential ascertainment without selection and were primarily from three academic pediatric neurology settings (tables 1and 2). The index patients were 1–51 years old, with a median age of 10 years and an average age of 11.5 years. Eighty-three percent of index patients

Table 1

Patient Characteristics

	No. (%) of Patients Aged				
Characteristic	1–5 years	6–10 years	11–15 years	16–25 years	>25 years
All	67 (30)	54 (24)	45 (20)	42 (19)	16 (7)
Sporadic TSC	56 (31)	44 (24)	36 (20)	35 (19)	12 (7)
Familial TSC	10 (26)	10 (26)	9 (23)	6 (16)	3 (8)
TSC1 mutation	7 (25)	8 (32)	3 (11)	7 (25)	3 (11)
TSC2 mutation	45 (28)	36 (23)	35 (22)	33 (21)	9 (6)

were sporadic cases, 8% were from families in which there were two affected individuals, and 9% were from families in which there were three or more affected individuals. All of the two-patient families contained an affected parent and child; there were no families with two affected children and unaffected parents.

Detailed clinical data were collected on each patient, using a standardized clinical instrument that covers the classic clinical features of TSC, including both physical and radiographic findings (fig. 1). Several clinical features were graded according to severity. For a subset of patients in which high resolution brain CT and/or MRI scans were available, the number of SENs (CT or MRI) and/or cortical tubers (MRI) were counted. A summary of the clinical findings in these patients is presented in table 3. Adjusting for age, the prevalence of brain, kidney, and skin involvement in this cohort is similar to that in other series (Jozwiak et al. 1992, 1994, 1998; Webb et al. 1996; Ewalt et al. 1998; Gomez et al. 1999), with a slightly higher frequency of neurologic manifestations, consistent with ascertainment of most patients through neurology practices.

We compared the clinical features in sporadic patients with the clinical features seen in index members of familial cases (table 3). The familial-case index patients had a somewhat lower frequency of seizures than sporadic patients (82% vs. 93%, $P = .055$) and a significantly lower frequency of hypomelanotic macules (82% vs. 94%, $P = .02$). However, there was no significant difference between those two groups for all other features. When this analysis was repeated considering only those patients with *TSC2* mutations (see below), there were no significant differences.

Mutation Analysis

We used three complementary strategies for mutation detection in these patients: DHPLC analysis of amplified exons, long-range PCR amplification within the *TSC2* gene, and quantitative PCR analysis of *TSC2* amplicons. These were performed in a sequential manner and were chosen on the basis of their sensitivity and the known mutation spectrum of *TSC1* and *TSC2* genes (Cheadle et al. 2000).

When DHPLC was used, 166 small mutations were detected in these 224 patients (table 4 and fig. 2); 138 (83%) of these mutations occurred in *TSC2* and 28 (17%) in *TSC1*. There were 44 (27%, 12 in *TSC1* and 32 in *TSC2*) small deletions, 14 (8%, 3 in *TSC1* and 11 in *TSC2*) small insertions, 48 (29%, 11 in *TSC1* and 37 in *TSC2*) nonsense mutations, 29 (17%, 2 in *TSC1* and 27 in *TSC2*) point mutations affecting splice sites, and 31 (19%, all in *TSC2*) missense mutations.

Missense and in-frame–deletion mutations accounted for 44 (32%) of 138 of all small *TSC2* mutations and were not seen in *TSC1*. Thirty-five missense changes and in-frame deletions in *TSC2* were confirmed as mutations (mis-c, in-f, del-c in table 4) if either the mutation was shown to be present in a sporadic patient with TSC and not present in parental samples or if similar evidence had been obtained elsewhere by others. When directly confirmed in this way, we also confirmed, by analysis using three microsatellite markers each with heterozygosity >80%, that parental samples were authentic. In nine cases in which parental samples were not available, eight missense changes and one in-frame deletion were considered to be probable mutations (mis-pr, in-f, and del-pr in table 4) if the change in amino acid was non-conservative. In addition, of the 23 unique missense changes in *TSC2*, 21 occurred at amino acids identical in rat and mouse *Tsc2*, and 20 occurred at amino acids identical in fugu (Kim et al. 1995; Kobayashi et al. 1995; Maheshwar et al. 1996). The other three residues that had changed in fugu were very conservative substitutions, in contrast to the missense mutations we identified. Four unique missense mutations (mis-spl in table 4) seen in five patients were predicted by computational analysis (Berkeley Drosophila Genome Project, Splice Site Prediction by Neural Network) to cause a change in RNA splicing.

Seventy-nine (48%) of the 166 small mutations are novel mutations never before reported; 130 of the 166 have not been reported by us previously. Twenty-one mutations were seen more than once in this cohort, from two to nine times, in a total of 59 patients. The most frequent mutation seen here, 5238–5255del18 1746delHIKRLR, is an in-frame deletion mutation that

Characteristic	No. (%) of Patients
Patient origin:	
US/mail	36 (16)
Warsaw	105 (47)
Cincinnati	65 (29)
Boston	18 (8)
Family:	
Sporadic	183 (83)
Small family[a]	17 (8)
Family[b]	21 (9)
Unknown	3

[a] Denotes a family in which there are only two affected members.
[b] Denotes a family in which three or more individuals are affected.

was seen in nine patients (4%) in this cohort. This deletion occurs in the sequence context of a direct repeat of 11 of the 18 residues lost in the deletion. Two patients were found to have two distinct mutations within *TSC2*. One patient had both a nonsense mutation and a missense mutation, and the other had both a deletion mutation and a missense mutation, in each case affecting different exons of *TSC2*. The missense mutations were confirmed in one case and were probable in the other.

Seventeen large-deletion and 3 large-duplication mutations were identified in *TSC2*, 6 by long-range PCR and 14 by quantitative PCR (table 5). Long-range PCR was performed using 33 different sets of amplification primers and detected 6 deletions, ranging in size from 440 bp to 39 kb. The deletion junctions have been sequenced in all cases, and four of these have been reported elsewhere (Dabora et al. 2000). The remaining 15 deletions/duplications were detected by quantitative PCR (fig. 3). Because our quantitative PCR assay analyzes only a subset of the *TSC2* exons, the exact size and location of each deletion or duplication has not been determined. One patient was mosaic for a deletion of exons 25, 26, and 36, since the dosage quotients (see Subjects and Methods section) for these exons were consistently between 0.61 and 0.8.

Genotype-Phenotype Comparisons

We examined the clinical manifestations of patients with different types of mutations to see whether there was any correlation between mutation type and specific clinical features. A major consideration in this analysis is the relative ages of patients in the subsets being compared, since many TSC clinical features are age-dependent in their expression (Jozwiak et al. 1994, 1998; Kwiatkowski and Short 1994; Webb et al. 1996; Gomez et al. 1999). For this reason, comparisons involving mental retardation were restricted to age $\geqslant 6$ years; those involving facial angiofibroma were restricted to age $\geqslant 2$ years, and those involving lymphangioleiomyomatosis of the lung were restricted to females age $\geqslant 16$ years. The first major comparison was between patients with *TSC1* disease and those with *TSC2* disease.

Sporadic patients with *TSC1* disease had an age range, average age, and median age that was similar to that of sporadic patients with *TSC2* disease (table 1 and top of table 3). However, the majority of clinical features were seen at a substantially higher frequency and/or severity in the set of patients with sporadic *TSC2* than in the set of patients with sporadic *TSC1* (table 3, 12/16 clinical measures), with statistically significant differences in eight clinical features. We initially excluded familial cases from this analysis, to avoid ascertainment bias, even though the difference between clinical features in familial cases and sporadic cases was relatively minor, as above. Seizures were significantly more common in patients with sporadic *TSC2* than those with *TSC1* (98% vs. 86%, $P = .02$); moderate to severe mental retardation was more frequent in patients with *TSC2* (46% vs. 14%, $P = .04$), and the average level (scale 0–3) of mental retardation was also higher in patients with *TSC2*, compared with those with *TSC1* (1.4 vs. 0.67, $P = .007$). These clinical manifestations were matched by similar differences in brain radiographic findings. Twenty-nine (69%) of 42 patients with *TSC2* had >10 tubers identified by MRI examination, compared with 1 (11%) of 9 patients with *TSC1* ($P = .002$); the average tuber count was significantly higher in patients with *TSC2*, compared with those with *TSC1* (12.9 vs. 4.4, $P = .002$), and patients with *TSC2* had more SENs than patients with *TSC1* (6.7 vs. 1.7, $P = .0002$).

Renal cystic disease occurred at similar rates in patients with sporadic *TSC2*, compared with those with *TSC1* (25% vs. 16%, $P = $ NS), but patients with *TSC1* who had renal cysts were also somewhat older than patients with *TSC2* who had renal cysts (26.3 vs. 13.8 years, $P = .07$). There was also a higher frequency of grade 2–4 renal cystic disease in patients with *TSC2* (19% vs. 0%, $P = .08$). Renal AMLs were seen at higher frequency and had a higher average grade in patients with *TSC2*, compared with those with *TSC1* (60% vs. 31%, $P = .03$; mean grade 0.97 vs. 0.32, $P = .006$; respectively). Several dermatologic manifestations of TSC tended to be more severe in patients with *TSC2* mutations. The mean grade of angiofibromas was higher in patients with *TSC2* than in those with *TSC1* (1.5 vs. 0.9, $P = .02$), despite the older average age of the patients with *TSC1* who had those lesions, and a fibrous forehead plaque was seen more commonly in patients with *TSC2* than in those with *TSC1* (40% vs. 10%, $P = .01$). Although ungual fibroma were seen at similar frequency in the two groups, the average age of patients

Table 3

Clinical Features of the Patients, with Comparisons of Sporadic versus Familial and *TSC1* Versus *TSC2* Mutation Subsets

	All (*n* = 224)	*P*, Sporadic (*n* = 38) vs. Familial (*n* = 183)	*TSC1* Sporadic Cases (*n* = 22)	*TSC2* Sporadic Cases (*n* = 129)[a]	*P*, Sporadic *TSC1* vs. *TSC2*
Age range	1–51 (11.5)	NS	2–51 (13.4)	1–44 (11.2)	NS
Median age (years)	10		9	10	
Neurologic features:					
Seizures:	202/223 (93%)	NS (.055)	19/22 (86%)	127/128 (99%)	.02
Age range	1–50 (11.5)		2–50 (12.3)	1–44 (11.4)	NS
Mental handicap (age ≥6):					
Mild + moderate + severe	102/155 (66%)	NS	7/14 (50%)	66/90 (73%)	NS
Moderate + severe	65/155 (42%)	NS	2/14 (14%)	41/90 (46%)	.04
Age range	6–44 (12.0)		9–24 (13.1)	6–44 (11.7)	NS
Mean grade (scale 0–3)	1.25		.67	1.4	.007
Subependymal nodules:	177/193 (92%)	NS	15/20 (75%)	127/136 (93%)	.02
Age range	1–51 (11.6)		2–51 (13.4)	1–44 (11.6)	NS (.055)
Mean SEN number	4.7		1.7	6.7	.0002
SEGA:	23/207 (11%)	NS	2/21 (9%)	13/118 (12%)	NS
Age range	3–44 (14.7)		9–11 (10)	4–44 (15.9)	
Tubers (any):	100/113 (88%)	NS	13/15 (87%)	55/60 (92%)	NS
>10 tubers	40/78 (51%)	NS	1/9 (11%)	29/42 (69%)	.002
Age range	1–50 (11.4)		2–50 (11.7)	1–44 (11)	NS
Mean tuber number	10.2		4.4	12.9	.002
Renal features:					
Kidney cysts:					
Grades 1–4	52/207 (25%)	NS	3/19 (16%)	30/122 (25%)	NS
Grades 2–4	29/207 (14%)		0/19 (0%)	19/122 (16%)	NS (.08)
Grade 4	6/207 (3%)		0/19 (0%)	5/122 (4%)	NS
Age range	1–50 (13.6)		9–50 (26.3)	1–44 (13.8)	NS (.07)
Mean grade (scale 0–4)	.46		.16	.52	NS (.14)
Kidney AMLs:	113/207 (55%)	NS	6/19 (31%)	72/121 (60%)	.03
Age range	1.5–44 (13.2)		9–21 (11.8)	1.5–44 (13.8)	NS
Mean grade (scale 0–3)	.83		.32	.97	.006
Skin features:					
Hypomelanotic macules:	202/219 (92%)	.02	20/21 (95%)	124/128 (97%)	NS
Age range	1–51 (11.2)				
Facial angiofibromas (age ≥2):	155/208 (75%)	NS	13/22 (59%)	95/121 (78%)	NS
Age range	2–51 (14.3)		4–51 (18.5)	2–44 (13.6)	NS (.09)
Mean grade (scale 0–3)	1.4		.9	1.5	.02
Shagreen patch:	100/210 (48%)	NS	7/20 (35%)	68/130 (52%)	NS
Age range	2–51 (13.1)		4–51 (18.9)	2–42 (12.7)	NS (.06)
Ungual fibroma:	39/215 (18%)	NS	5/20 (25%)	26/128 (20%)	NS
Age range	4–51 (21)		6–51 (29.2)	4–42 (18.7)	NS (.07)
Forehead plaque:	74/217 (34%)	NS	2/20 (10%)	51/128 (40%)	.01
Age range	2–42 (13.3)		6–20 (13)	2–42 (13.5)	NS
Other features:					
Liver AMLs:	12/195 (6%)	NS	0/15	9/117 (8%)	NS
Age range	3–38 (16.7)			10–38 (17.4)	
Retinal hamartomas:	46/192 (24%)	NS	0/16	32/117 (27%)	.01
Age range	1–44 (12.8)			1.5–44 (13.2)	
Cardiac rhabdomyoma:	100/195 (51%)	NS	8/17 (47%)	58/117 (50%)	NS
Age range	1–33 (9.6)		3–20 (7.6)	1–23 (8.5)	NS
LAM (females, age ≥16):	5/48 (11%)	NS	0/5	3/17 (18%)	NS
Age range	18–37 (27.4)			18–37 (25.3)	

NOTE.—Age range (average age [in years]) considers all patients with positive findings. SEGA = subependymal giant cell astrocytoma.

[a] Mosaic case with large *TSC2* deletion not included in this analysis.

with *TSC1* was significantly higher than that for patients with *TSC2* with this finding (29.2 vs. 18.7 years, *P* = .07). Retinal hamartomas were also seen with increased frequency in the patients with *TSC2*, compared with those with *TSC1* (27% vs. 0%, *P* = .01). There was a suggestion of an increased frequency of lymphangioleiomyomatosis (LAM) of the lung in patients with *TSC2*, compared with those with *TSC1*, but this did not achieve

Table 4

Small Mutations in *TSC1* and *TSC2* Identified by DHPLC

Patient	Gene/Exon[a]	Mutation[b]	Mutation Type[c]	Family Type[d]	De novo[e]	Reported[f]	First Author (year)
LR115	TSC1E07	868–869delTT	del	S		u, o	van Slegtenhorst (1997)
BG1601	TSC1E08	954C→T 245R→X	non	F		u, o	
BG401	TSC1E08	954C→T 245R→X	non	S		u, o	
BHM3901	TSC1E08	954C→T 245R→X	non	S		u, o	
BHM4201	TSC1E09	970insT	ins	S		n	
BHM1001	TSC1E09	1055–1056delTC	del	S		n	
LR821	TSC1E09	1122–1134+1del14	del	S		u	Kwiatkowska (1998)
LR721	TSC1E10	1209–1210delCT	del	S		o	van Slegtenhorst (1997)
BHM7001	TSC1E15	1746C→T 509R→X	non	smF		u, o	
ONK431	TSC1E15	1746C→T 509R→X	non	S		u, o	van Slegtenhorst (1997)
BG101	TSC1E15	1918delC	del	S		n	
ONK381	TSC1E15	1929–1930delAG	del	S		u, o	van Slegtenhorst (1997)
ONK661	TSC1E15	1950G→T 577E→X	non	F		u	
ONK421	TSC1E15	2109–2112delAAAG	del	S		u, o	van Slegtenhorst (1997)
ONK741	TSC1E15	2109–2112delAAAG	del	smF		u, o	Kwiatkowska (1998)
LR911	TSC1E15	2124–2125delAC	del	S		u, o	van Slegtenhorst (1997)
LR251	TSC1E15	2181C→T 654Q→X	non	S		u	Kwiatkowska (1998)
BG1001	TSC1E17	2295C→T 692R→X	non	S		o	
BHM3301	TSC1E17	2355–2359delCATCG	del	S		n	
ONK711	TSC1E17	2394–2397delAAAG	del	S		u	Kwiatkowska (1998)
ONK641	TSC1E18	2448C→T 743Q→X	non	U		u, o	van Slegtenhorst (1997)
BHM4601	TSC1E18	2520C→T 767Q→X	non	S		n	
ONK781	TSC1E18	2577C→T 786R→X	non	S		u, o	van Slegtenhorst (1997)
BHM5101	TSC1E19	2722insA	ins	S		n	
LR100	TSC1E20	2729–2108delAAAC	del	S		u, o	Kwiatkowska (1998)
BG801	TSC1E20	2790insG	ins	F		n	
BHM2501	TSC1I04	432–1G→A	spl	S		o	
BHM7101	TSC1I04	432–1G→A	spl	S		o	
ONK121	TSC2E03	268C→T 90Q→X	non	S		u	Choy (1999)
ONK1071	TSC2E04	357delC	del	S		u	Choy (1999)
ONK841	TSC2E04	415delA	del	S		u	Choy (1999)
LR811	TSC2E05	569insA	ins	S		u	Choy (1999)
ONK221	TSC2E06	618C→A 206C→X	non	S		n	
ONK451	TSC2E07	731G→A 244C→Y	mis-c	S	Yes	n	
LR841	TSC2E07	759C→A 253C→X	non	S		n	
ONK861	TSC2E07	774G→C 258K→N	mis-spl-c	S	Yes	u	Choy (1999)
ONK311	TSC2E08	782G→C 261R→P	mis-pr	S		u	Choy (1999)
ONK831	TSC2E08	826–827delAT	del	S		o	
BHM602	TSC2E09	871insC	ins	S		u	Choy (1999)
BHM3601	TSC2E09	911G→A 304W→X	non	S		n	
BHM1103	TSC2E09	972C→G 324Y→X	non	S		u	Choy (1999)
ONK251	TSC2E09	972C→G 324Y→X	non	F		u	
LR951	TSC2E10	1082T→C 361L→P	mis-c	S	Yes	u	Choy (1999)
BHM5301	TSC2E10	1093–1095delATC 365delI	in-f del-c	S		o	
ONK341	TSC2E10	1096G→T 366E→X	non	S		n	
BHM1401	TSC2E11	1219T→G 407Y→D	mis-pr	S		u, o	Choy (1999)
ONK1031	TSC2E11	1255C→T 419P→S	mis-spl-c	S	Yes	n	
Z61	TSC2E12	1336C→T 446Q→X	non	S		u	Choy (1999)
BHM4701	TSC2E13	1372C→T 458R→X	non	S		o	
BHM5701	TSC2E13	1372C→T 458R→X	non	S		o	
ONK351	TSC2E13	1372C→T 458R→X	non	S		o	
ONK881	TSC2E13	1372C→T 458R→X	non	S		o	
ONK1001	TSC2E13	1407delG	del	S		n	
BHM4401	TSC2E14	1472C→A 491S→X	non	S		n	
ONK1091	TSC2E14	1513C→T 505R→X	non	S		o	
BHM1601	TSC2E16	1783C→T 595Q→X	non	S		n	
LR601	TSC2E16	1831C→T 611R→W	mis-c	S		u, o	Choy (1999)
ONK571	TSC2E16	1831C→T 611R→W	mis-c	S		u, o	Choy (1999)

Table 4 Continued

Patient	Gene/Exon[a]	Mutation[b]	Mutation Type[c]	Family Type[d]	De novo[e]	Reported[f]	First Author (year)
BHM3201	TSC2E16	1832G→A 611R→Q	mis-c	U	Yes	o	
BHM4301	TSC2E16	1832G→A 611R→Q	mis-c	S	Yes	o	
KS31	TSC2E18	1965insT	ins	S		n	Choy (1999)
BHM802	TSC2E18	2081–2083delTGC 694delL	in-f del-c	S	Yes	u	Choy (1999)
ONK1151	TSC2E19	2108G→A 703W→X	non	S		o	
LR941	TSC2E19	2158–2171insGTCCCTGCGCTATA	ins	S		u	Choy (1999)
ONK551	TSC2E19	2182delT	del	S		n	
BHM2301	TSC2E19	2210–2214insGCTCT	ins	S		n	
LR901	TSC2E20	2225C→G 742S→X	non	S		u	Choy (1999)
ONK111	TSC2E20	2240–2241delTG	del	S		n	
KS41	TSC2E20	2251C→T 751R→X	non	S		u, o	Choy (1999)
ONK531	TSC2E20	2251C→T 751R→X	non	S		u, o	Choy (1999)
BHM7301	TSC2E20	2328C→A 776Y→X	non*	S		n	
ONK731	TSC2E21	2440G→A 814E→K	mis-c	F		n	
ONK501	TSC2E23	2687G→A 896W→X	non	S		n	
ONK921	TSC2E23	2714G→A 905R→Q	mis-pr	S		n	
ONK291	TSC2E24	2785G→T 929E→X	non	S		n	
ONK161	TSC2E26	3094C→T 1032R→X	non	F		n	
ONK761	TSC2E26	3094C→T 1032R→X	non	smF		n	
LR921	TSC2E26	3098insA	ins	S		n	
ONK191	TSC2E26	3098insA	ins	S		n	
ONK23	TSC2E26	3098insA	ins	S		n	
ONK391	TSC2E26	3099C→A 1033Y→X	non	S		n	
ONK981	TSC2E27	3178T→C 1060W→R	mis-c	S	Yes	n	
ONK1081	TSC2E27	3212C→G 1071T→R	mis-c	S	Yes	n	
BHM1901	TSC2E27	3214delA	del	F		n	
ONK701	TSC2E27	3220–3221delGG; 3221 insC	del	F		n	
BHM3401	TSC2E29	3412C→T 1138R→X	non	S		o	
BHM401	TSC2E29	3412C→T 1138R→X	non	S		o	
BHM5801	TSC2E29	3412C→T 1138R→X	non	S		o	
LR831	TSC2E29	3412C→T 1138R→X	non	S		o	
ONK51	TSC2E29	3574delC	del	S		n	
ONK181	TSC2E30	3685C→T 1229Q→X	non	S		n	
Z51	TSC2E30	3685C→T 1229Q→X	non	S		n	
BHM6701	TSC2E30	3750C→G 1250Y→X	non	S		n	
BHM6501	TSC2E32	3984delG	del	smF		n	
BG1701	TSC2E33	4096G→T 1366E→X	non	S		n	
ONK401	TSC2E33	4255delC	del	S		n	
ONK41	TSC2E33	4375C→T 1459R→X	non	S		o	
Z81	TSC2E33	4375C→T 1459R→X	non	S		o	
BG1402	TSC2E33	4422–4423delAG	del	F		o	
ONK371	TSC2E33	4427–4430insTAGA	ins	S		n	
ONK961	TSC2E33	4439T→A 1480L→X	non	S		n	
BG1901	TSC2E34	4544–4547delACAA	del	S		o	
ONK271	TSC2E35	4598–4626ins29bp	ins	smF		n	
ONK1051	TSC2E35	4606C→T 1536Q→X	non	S		n	
ONK1181	TSC2E35	4645T→A 1549Y→N	mis-pr	S		n	
BHM1801	TSC2E35	4655–4657delAAG 1552delE	in-f del-c	S	Yes	n	
ONK1121	TSC2E36	4672G→A 1558E→K	mis-c	S	Yes	n	
ONK11	TSC2E36	4790T→C 1597L→P	mis-c	S	Yes	n	
ONK891	TSC2E36	4823–4825delACT 1608delY	in-f del-pr	S		n	
BHM503	TSC2E36	4825–4831delTGCTGGC	del	S		n	
BHM3801	TSC2E37	4854delC	del	S		n	
ONK751	TSC2E37	4927A→C 1643N→H	mis-pr	S		n	
ONK561	TSC2E37	4934–4935delTT	del	smF		o	
BHM3001	TSC2E37	4943T→C 1648I→T	mis-pr	S		n	
ONK91	TSC2E37	4952A→G 1651N→S	mis-spl-c	F		o	
ONK581	TSC2E37	4989insG	ins	S		n	

(*continued*)

Table 4 Continued

Patient	Gene/Exon[a]	Mutation[b]	Mutation Type[c]	Family Type[d]	De novo[e]	Reported[f]	First Author (year)
BHM103	TSC2E38	5024C→T 1675P→L	mis-c	F		o	
BHM2601	TSC2E38	5024C→T 1675P→L	mis-c	S		o	
LR851	TSC2E38	5024C→T 1675P→L	mis-c	S	Yes	o	
ONK61	TSC2E38	5024C→T 1675P→L	mis-c	S		o	
ONK71	TSC2E38	5024C→T 1675P→L	mis-c	S		o	
ONK991	TSC2E38	5024C→T 1675P→L	mis-c	S		o	
BHM6101	TSC2E38	5051–5068+16del34	del*	S		o	
ONK681	TSC2E39	5108T→G 1703V→G	mis-pr	S		n	
ONK871	TSC2E39	5126C→G 1709P→R	mis-c	S	Yes	n	
BHM6301	TSC2E39	5126C→T 1709P→L	mis-spl-c	S	Yes	o	
ONK591	TSC2E39	5126C→T 1709P→L	mis-spl-c	S	Yes	o	
ONK81	TSC2E40	5167T→C 1723S→P	mis-pr	S		n	
BG301	TSC2E40	5238–5255del18 1746delHIKRLR	in-f del-c	smF		o	
LR61	TSC2E40	5238–5255del18 1746delHIKRLR	in-f del-c	S		o	
LR801	TSC2E40	5238–5255del18 1746delHIKRLR	in-f del-c	S		o	
LR871	TSC2E40	5238–5255del18 1746delHIKRLR	in-f del-c	S	Yes	o	
ONK1101	TSC2E40	5238–5255del18 1746delHIKRLR	in-f del-c	S	Yes	o	
ONK1141	TSC2E40	5238–5255del18 1746delHIKRLR	in-f del-c	S	Yes	o	
ONK231	TSC2E40	5238–5255del18 1746delHIKRLR	in-f del-c	S		o	
ONK261	TSC2E40	5238–5255del18 1746delHIKRLR	in-f del-c	S		o	
ONK281	TSC2E40	5238–5255del18 1746delHIKRLR	in-f del-c	S		o	
BHM302	TSC2E40	5252–5259+19del27	del	F		n	
ONK522	TSC2E40	5252–5259+19del27	del	smF		n	
ONK362	TSC2I01	138+1G→A	spl	F		o	
ONK901	TSC2I01	138+5G→A	spl	S	Yes	u	Choy (1999)
BG1501	TSC2I03	336+2T→G	spl	S		n	
Z21	TSC2I03	337–1G→T	spl	S		u	Choy (1999)
BG1802	TSC2I05	600–2A→G	spl	S		n	
BHM1301	TSC2I07	774+2T→A	spl	S		u	Choy (1999)
ONK211	TSC2I08	849–1G→A	spl	S		u	Choy (1999)
BG1301	TSC2I09	976–15G→A	spl	S		o	
ONK171	TSC2I09	976–15G→A	spl	smF		o	
ONK1061	TSC2I11	1258–2A→C	spl	S		n	
ONK631	TSC2I11	1258–3C→G	spl	S		n	
BG701	TSC2I13	1444–1G→A	spl	S		o	
BHM2001	TSC2I18	2098–1G→A	spl	F		n	
ONK821	TSC2I19	2221–2A→C	spl	S		n	
BHM2701	TSC2I22	2639+1G→C	spl	S		n	
LR931	TSC2I23	2743–2A→C	spl	S		n	
BG601	TSC2I23	2743–2A→G	spl	U		n	
ONK144	TSC2I23	2743–1insGCCAG	spl	F		n	
ONK601	TSC2I26	3131+11–3131+28del18	spl-pr	smF		n	
ONK851	TSC2I29	3610+1G→A	spl	S		n	
BG201	TSC2I32	4005+1G→T	spl	S		n	
BHM902	TSC2I35	4663–1G→A	spl	S		n	
ONK131	TSC2I35	4663–1G→A	spl	S		n	
LR961	TSC2I38	5069–1G→A	spl	S		n	
ONK811	TSC2I39	5160+1G→A	spl	smF		n	
ONK331	TSC2I39	5161–1G→A	spl	S		n	
ONK514	TSC2I39	5161–1G→A	spl	smF		n	

[a] Mutations are listed in the order *TSC1* exon mutations, *TSC1* intron mutations, *TSC2* exon mutations, *TSC2* intron mutations.

[b] Nomenclature is per Antonarakis (1998). *TSC1* numbering uses A of initiator ATG as +222; *TSC2* numbering uses A of initiator ATG as +1.

[c] del = deletion; non = nonsense; ins = insertion; spl = splice site; mis = missense; in-fr del = in-frame deletion; mis-spl = missense and probable splice site change; c = confirmed; pr = probable. *, patient with two mutations.

[d] S = sporadic; smF = family with two affected members; F = family with more than two affected members.

[e] "Yes" means shown by parental DNA analysis to be de novo.

[f] o = others; u = us (previous reports); n = novel.

statistical significance because of small numbers of female patients of age >16 years (3/17 patients with *TSC2* had LAM vs. 0/5 patients with *TSC1*).

When this analysis between *TSC1* and *TSC2* mutations was repeated using both sporadic patients and familial index patients, similar results were obtained with generally similar levels of confidence (table 6). The differences in the frequency of grade 2–4 kidney cysts (17% vs. 0%, $P = .03$) and mean grade of kidney cysts (0.55 vs. 0.125, $P = .05$) become significant in this larger population (table 6).

We also compared the clinical features of the 38 patients in whom no mutations could be identified in separate analyses with patients with *TSC1,* patients with *TSC2,* and *TSC1* and *TSC2* patients combined (table 6). We expected that this group was a composite of patients whose mutations may not have been detected for several different reasons. The "no mutation identified" (NMI) group had clinical features that were generally less severe than those in patients with *TSC2* mutations and were generally similar to clinical features of patients with *TSC1* mutations. The NMI group had a lower incidence of seizures, mental retardation, cortical tubers, SENs, hypomelanotic macules, facial angiofibromas, Shagreen patch, and forehead plaque in comparison with patients with *TSC2* mutations. They also had a lower average SEN count, tuber count, and grade of facial angiofibromas. Most of these comparisons were also significant when made between the NMI group and patients with both *TSC1* and *TSC2* combined. This may be partly because *TSC2* cases significantly outnumber *TSC1* cases. When NMI patients were compared with patients with *TSC1,* many clinical features were seen at similar fre-

Table 5

Mutations in *TSC2* Identified by Long-Range and Quantitative PCR

Patient	TSC2 Deletion/Duplication	Detection Method
BHM1501	4.5-kb deletion, introns 2–5	Long PCR[a]
BHM1701	39-kb deletion, introns 1–40	Long PCR[a]
ONK 10-1	34-kb deletion, introns 1–33	Long PCR[a]
ONK 101-1	1.4-kb deletion, exons 37–39	Long PCR[a]
BG501	440-bp deletion, intron 39–3′ UTR	Long PCR
BHM 4001	465-bp deletion, introns 9–10	Long PCR
BHM5401	Duplication, exon 25	Quantitative PCR
BHM5501	Duplication exons 7, 16, and 25	Quantitative PCR
BHM5901	Duplication exons 16 and 25	Quantitative PCR
JR 1-1	Deletion, exons 7, 16, 25, 26, and 36	Quantitative PCR
LR 89-1	Deletion, exons 4, 7, 16, 25, 26, and 36	Quantitative PCR
ONK 15-1	Deletion, exons 25, 26, and 36	Quantitative PCR
ONK 24-1	Deletion, exon 36	Quantitative PCR
ONK 46-1	Deletion, exons 4, 7, 16, 25, 26, and 36	Quantitative PCR
ONK 116-1	Deletion, exons 4 and 7	Quantitative PCR
BHM2901	Deletion, exons 16, 25, and 26	Quantitative PCR
BHM4901	Deletion, exons 4, 7, 16, 25, 26, and 36	Quantitative PCR
BHM6801	Deletion, exon 36	Quantitative PCR
ONK 95-1	Deletion, exon 7	Quantitative PCR
BG1201	Mosaic deletion, exons 25, 26, and 36	Quantitative PCR

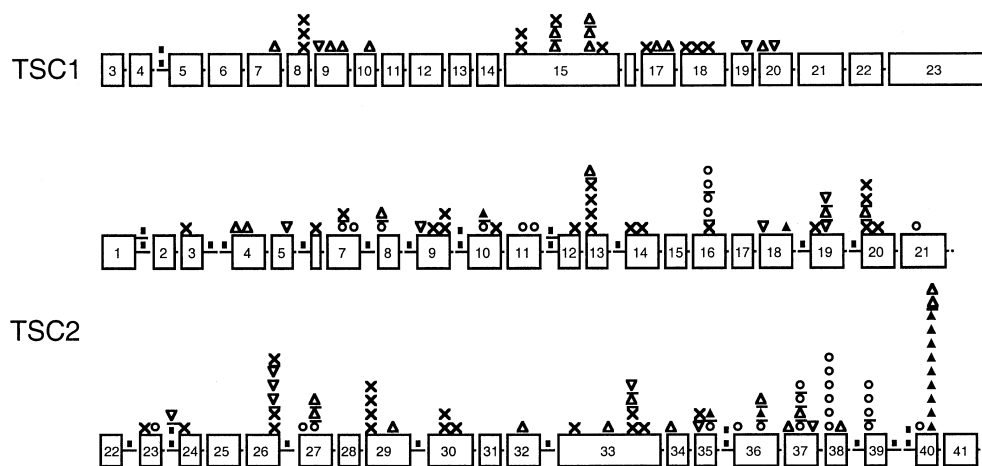[a] Reported elsewhere (Dabora et al. 2000).

Figure 2   Map of the sites of small mutations in *TSC1* and *TSC2*. Proportional drawings (*boxes*) of all of the exons of *TSC1* and *TSC2* are shown. Intron regions are not drawn to scale and are expanded only when a mutation is present. Mutation symbols are x = nonsense; ■ = splice site; △ = deletion; ▽ = insertion; ○ = missense; and ▲ = in-frame deletion. A line separates mutations occurring at nearby but not identical nucleotide positions.
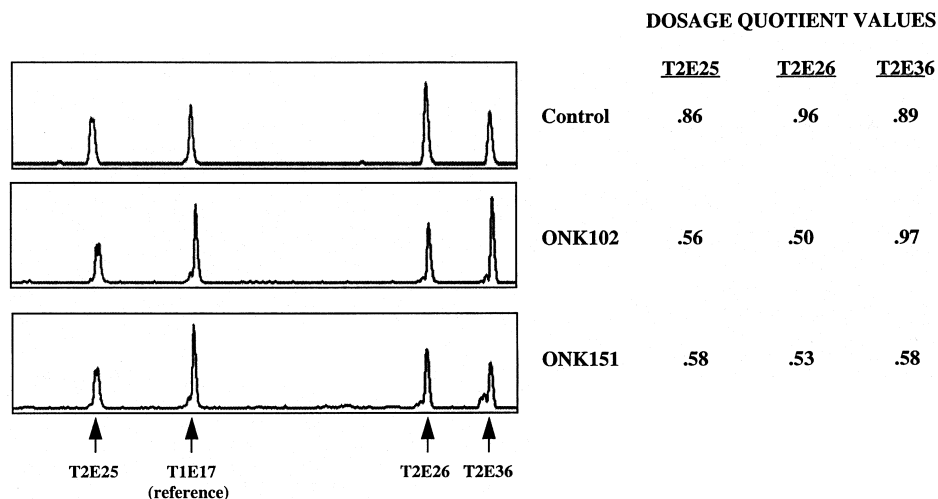
Figure 3 Analysis of deletions and duplication events by quantitative PCR. Representative electropherograms (*left*) and dosage quotient values (*right*) for 3 DNA samples analyzed by quantitative PCR: control sample (*top*); ONK102: 34-kb deletion in *TSC2* identified by long-range PCR (*middle*); ONK151: deletion of *TSC2* exons 25, 26, and 36 identified using quantitative PCR (*bottom*).

quencies (table 6). However, hypomelanotic macules (74% in NMI vs. 96% in *TSC1*, $P = .03$) were seen significantly less frequently in NMI patients, compared with patients with *TSC1*, whereas renal AMLs had greater severity in NMI patients, compared with patients with *TSC1* (average level 0.66 vs. 0.29, $P = .05$).

A comparison of the clinical features of patients with different types of *TSC2* mutations was also performed, to see whether there was any clinical correlation among those subsets. We divided the *TSC2* mutations into two subsets for this analysis: first, chain-terminating small mutations (insertion, deletion, nonsense, and splice-site mutations) and large genomic deletions and second, non–chain-terminating mutations (missense and in-frame deletion mutations). This analysis was performed in two different ways, once with patients with *TSC2* missense/splice mutations in the first subset and then with those patients in the second subset. In both comparisons, the only significant difference in clinical features was for Shagreen patch, which was seen less often in the missense/in-frame deletion group (38% vs. 59%, $P = .04$). We suspect that this single difference represents random chance. This provides further evidence that *TSC2* missense mutations are pathogenic and inactivating similar to other *TSC2* mutations.

Discussion

We have studied the largest series of patients with TSC yet reported and have identified mutations in 186 (83%) of 224 cases. Twenty-eight (12%) index patients were found to have mutations in *TSC1*, 138 (62%) were found to have small mutations in *TSC2*, 20 (9%) were

found to have large genomic deletions in *TSC2*, and 38 (17%) had no mutation identified. The overall mutation detection rate increases to 190 (88%) of 217 if we include two unconfirmed missense mutations and two samples suspected of being mosaic cases with large *TSC2* deletions, as well as correcting the denominator to exclude seven samples in which there was an inadequate DNA supply to complete the entire analysis. The great majority of these mutations, 148 (78%), have not been reported elsewhere. The cumulative mutation detection rate and distribution of mutations described here are similar to those reported in a British cohort of 150 patients with TSC by Sampson and coworkers over the past several years (Jones et al. 1997, 1999, 2000; Maheshwar et al. 1997). The major difference seen in comparison of these two series is that the British cohort has a somewhat higher frequency of genomic disruptions of *TSC2* (15% vs. 9% in this study, $P = $ NS). This is possibly due to ascertainment and/or selection bias, since there was a higher frequency of patients with TSC in the British cohort who have polycystic kidney disease (6% vs. 3% in this series).

We have developed two relatively novel yet technically simple strategies for detection of large genomic rearrangements within *TSC2*: long-range PCR (Dabora et al. 2000) and quantitative PCR (Yau et al. 1996). Long-range PCR can be performed in any current molecular laboratory, requiring only a PCR machine and agarose gel electrophoresis. Because a complete assay of the *TSC2* gene by this method requires multiple PCR reactions, we perform this analysis only on patient samples in which no small mutation in *TSC1* or *TSC2* has been detected by DHPLC. Quantitative PCR analysis is

Table 6

Clinical Features of Patients with NMI and Comparison with Patients Who Have *TSC1* and *TSC2* Mutations

	NMI (all) ($n = 38$)	TSC1 (all) ($n = 28$)	TSC2 (all) ($n = 158$)	*P*, T1 vs. T2	*P*, T1 vs. NMI	*P*, T2 vs. NMI
Age range (years) (average age)	1–33 (9.8)	2–51 (14.9)	1–44 (11.3)	NS	NS	NS
Neurologic features:						
Seizures	25/37 (68%)	24/28 (86%)	153/158 (97%)	.03	NS (.15)	<.0001
Mental handicap (age ≥6 years):						
Mild + moderate + severe	12/22 (54%)	10/20 (50%)	80/113 (71%)	NS (.08)	NS	NS (.14)
Moderate + severe	9/22 (41%)	4/19 (21%)	52/113 (46%)	.05	NS	NS
Mean MR gr (scale 0–3)	1.1	.79	1.49	.02	NS	NS
SENs:	26/34 (76%)	20/25 (80%)	131/134 (98%)	.003	NS	.0001
Mean SEN number	2.9	2.0	6.3	.0005	NS	.0008
SEGA	2/37 (5%)	4/26 (15%)	17/145 (12%)	NS	NS	NS
Tubers (any):	19/24 (79%)	15/18 (83%)	66/71 (93%)	NS	NS	NS (.12)
>10 tubers	6/20 (30%)	1/11 (9%)	34/47 (72%)	.0002	NS	.002
Mean tuber number	6.9	4.3	12.9	.0004	NS	.002
Renal features:						
Kidney cysts:						
Grades 1–4	6/34 (18%)	3/24 (12.5%)	43/149 (29%)	NS	NS	NS
Grades 2–4	4/34 (12%)	0/24 (0%)	25/149 (17%)	.03	NS	NS
Grade 4	0/34 (0%)	0/24 (0%)	6/149 (4%)	NS	NS	NS
Mean grade	.32	.125	.55	.05	NS	NS
Kidney AMLs:	16/35 (46%)	7/24 (29%)	89/148 (60%)	.007	NS	NS
Mean grade	.66	.29	.96	.001	.05	NS
Skin features:						
Hypomelanotic macules	26/35 (74%)	26/27 (96%)	150/157 (95%)	NS	.03	.0004
Facial angiofibromas (age ≥2 years):	21/36 (58%)	18/28 (64%)	116/155 (75%)	NS	NS	NS (.06)
Mean grade	1.2	1.0	1.5	.01	NS	NS (.06)
Shagreen patch	9/35 (26%)	9/25 (36%)	82/153 (54%)	NS	NS	.004
Ungual fibroma	3/35 (9%)	5/25 (20%)	31/155 (20%)	NS	NS	NS
Forehead plaque	8/35 (23%)	3/25 (12%)	64/155 (42%)	.006	NS	.05
Other features:						
Liver AMLs	1/31 (3%)	0/20 (0%)	11/144 (8%)	NS	NS	NS
Retinal hamartomas	5/28 (18%)	0/20 (0%)	41/144 (28%)	.004	NS (.07)	NS
Cardiac rhabdomyoma	19/34 (56%)	9/21 (43%)	72/140 (51%)	NS	NS	NS
LAM (females, age ≥16 years)	0/7 (0%)	0/6 (0%)	5/36 (14%)	NS	NS	NS

NOTE.—Abbreviations are as in table 3.

also a relatively simple assay, requiring only fluorescently labeled primers, careful PCR technique, and a sequencing machine with fluorescent detection capability, such as the ABI 377 used here. The combination of the two assays should provide detection of all deletion mutations of size >400 bp within the *TSC2* gene. A significant fraction of partial gene duplications and moderate size insertions are also detected by quantitative PCR and/or long-range PCR. Chromosomal translocations involving *TSC2* are not detected by these methods, but these appear to be extremely rare (European Tuberous Sclerosis Consortium 1993; Sampson et al. 1997; Eussen et al. 1999; van Slegtenhorst et al. 1999).

In this analysis, we have found mutations in *TSC1* in only 12% of all index patients, with very similar detection rates in sporadic (12%) and familial (13%) cases. The observation that *TSC1* mutations account for only a small proportion (10%–20%) of all patients with TSC has now been made in several series studied in multiple laboratories (Kwiatkowska et al. 1998; Young et al. 1998; Jones et al. 1999; Niida et al. 1999; van Slegtenhorst et al. 1999). However, our observations of families contrasts somewhat with observations made of limited numbers of extended multigeneration families with TSC in which linkage and mutational studies indicate there is an approximately equal frequency of *TSC1* and *TSC2* mutations (Young et al. 1998; Jones et al. 1999; Niida et al. 1999; van Slegtenhorst et al. 1999). The predominance of *TSC2* mutations in the families studied here appears to be primarily due to their small size, since nearly half of these families consist of just two affected individuals, and there are only six families that extend to three or four (three families each) generations. However, even among these extended families, five of six had mutations in *TSC2*. We conclude

that the estimated frequency of *TSC1* versus *TSC2* mutations in familial cases, based on previous linkage studies, may have been biased because of the small numbers of families studied. Our data suggest that *TSC2* mutations are substantially more frequent in both familial and sporadic cases than *TSC1* mutations.

The greatly lower frequency of de novo mutations in *TSC1* versus *TSC2* (a ratio of 1:6 in this cohort) can be explained somewhat by examination of the genomic structure and types of mutations that occur in these genes. Deletion, insertion, and nonsense mutations occur at lower frequency in *TSC1*, compared with *TSC2* (~1:3 ratio), which is partially explained by the smaller size of the coding region (about two-thirds) and fewer number of exons (about one-half) in *TSC1*, compared with *TSC2* (fig. 2). However, splice-site and missense mutations and genomic rearrangements are all very rare or not seen in *TSC1*, in contrast to *TSC2*. It is the deficiency of these latter mutation types that pushes down the frequency of de novo mutations in *TSC1* to such a low level in comparison with *TSC2*. The differing spectrum and frequency of *TSC1*, compared with *TSC2*, mutations may be due to intrinsic differences in the regional genomic structure of the two genes. The lack of clearly pathogenic missense mutations in *TSC1* suggests the possibility that such a mutation either has no effect or causes a different clinical phenotype, possibly through a dominant negative effect (Cheadle et al. 2000).

The detailed analysis of clinical phenotype and its relationship to mutation type performed here permits a delineation of the distinctions in clinical features associated with *TSC1* versus *TSC2* disease. *TSC1* disease is less severe than *TSC2*, by a significant margin, in multiple clinical measures that relate to brain, kidney, dermatologic, and retinal involvement by TSC. The observations have value in the clinical setting in which genetic counseling is offered to patients with known mutations in either *TSC1* or *TSC2*. It is clear, however, that there is a wide degree of variation in clinical phenotype for each of *TSC1* and *TSC2* mutations, so that a precise prediction of the severity of clinical features cannot be made in either case. This clinical variability is nicely demonstrated by the nine independent patients in this series with the *TSC2* in-frame deletion mutation 5238–5255del18 1746delHIKRLR. The age range for eight of these patients was 7–28 years and all had seizures, but mental retardation ranged from none to severe, facial angiofibromas ranged from none to extensive (grade 3), and renal AMLs ranged from minimal to extensive (grades 1–3). Thus, there is extensive clinical variability even for identical mutations.

However, it is possible to derive some clinical guidelines in the assessment of patients with TSC. First, patients that are either sporadic cases or from small families are more likely to be due to *TSC2* than *TSC1* mutation (odds ratio 6:1). Second, as the severity of the disease and number of clinical features increases, so does the chance that the patient bears a *TSC2* mutation. One way to consider this is to group all patients with any of the following clinical features: forehead fibrous plaque, liver AMLs, retinal hamartomas, or significant renal involvement (grade $\geqslant 2$ cysts or AMLs). This group represents the majority of patients with TSC in this series (124 [55%]). In this group, there were 3 (2%) *TSC1* mutations, 104 (84%) *TSC2* mutations, and 17 (14%) without a mutation identified. Thus, any of these clinical features strongly predicts that the patient has a *TSC2* mutation. Conversely, in the group of patients without any of these features, *TSC1* mutations are more common (25%).

There are at least two hypotheses to explain why *TSC1* disease is less severe than *TSC2* disease. First it is possible that second-hit events (following the Knudson two-hit model) occur less often in *TSC1* than in *TSC2*. Second, it is possible that complete loss of hamartin, the *TSC1* gene product, has different effects in cells, compared with the loss of tuberin, the *TSC2* gene product.

The two hit model for hamartoma development in TSC postulates that second somatic mutations occur in the cells that give rise to TSC hamartomas (Green et al. 1994; Henske et al. 1996). This model has been well-proven by loss of heterozygosity analysis for the renal AMLs and cardiac rhabdomyomas of TSC, with more provisional evidence for hamartomas involving the brain and skin (Henske et al. 1996, 1997). A reduced level of somatic mutation in *TSC1* (second-hit events), compared with *TSC2*, is implied by the reduced number of both cortical tubers (4.4 in *TSC1* vs. 12.9 in *TSC2*, $P = .002$) and SENs (1.7 in *TSC1* vs. 6.7 in *TSC2*, $P = .0002$) in patients with *TSC1*, compared with those with *TSC2*. This apparent difference in somatic mutation rates between *TSC1* and *TSC2* is concordant with the difference in germline mutation rates in the two genes. However, it is possible that tuber and SEN counting by MRI/CT scan only detects lesions above a certain size, so this may not provide an accurate count of second-hit events.

In addition, the extreme qualitative difference in kidney disease in patients with *TSC1* versus *TSC2* may be more consistent with the second hypothesis. No significant renal involvement was seen in any index patient with a *TSC1* mutation in this series; all had either no or minimal (grade 1) renal cysts and AML. In contrast, significant renal involvement (grade $\geqslant 2$ renal cysts or AMLs) was seen in 27% of all the patients in this series, and mutations were identified in 53 (87%) of these 61 patients, all with *TSC2*. This distinction is rather striking and may fit better with a differential role of the gene

products tuberin and hamartin in renal cell growth control.

Similar to reports elsewhere, we found that all six patients with polycystic kidney disease had a large deletion involving the 3′ end of the *TSC2* gene and likely involving the adjacent *PKD1* gene (Sampson et al. 1997). One of these patients was mosaic for this deletion.

Although it was not the primary intent of this study, we also examined the correlation between clinical features among this large patient set. Among all patients, 55 (73%) of 75 without mental retardation had seizures, whereas 138 (99%) of 139 with mental retardation had seizures ($P < .0001$), consistent with studies elsewhere that indicate that patients with TSC without seizures do not develop mental retardation at an appreciable rate (Gomez et al. 1999). In contrast, we found a relatively poor correlation between tuber count and mental retardation. Mean tuber counts were 7.5 for patients without mental retardation, 14.7 for mild, 9.7 for moderate, and 12.1 for patients with severe mental retardation. There was a correlation between any degree of mental retardation (tuber count 12.5) versus those without mental retardation (7.5, $P = .0035$). This finding is similar to reports elsewhere in which tuber count has correlated to a modest degree with mental retardation (Goodman et al. 1997). However, a rather striking correlation was seen between the extent of renal AMLs and mental retardation. In index patients of age ≥6 years, 75 (76%) of 99 with kidney AMLs had some degree of mental retardation, whereas only 19 (44%) of 43 without such lesions had any degree of mental retardation ($P = .0004$). In addition, the mean grade of mental retardation in patients with AMLs (1.61) was significantly higher than in those without (0.84, $P = .0004$). Similar results were obtained when only patients with *TSC2* were considered. This correlation between renal angiomyoplipomas and mental retardation in TSC is unexplained, although it has been noted elsewhere (O'Callaghan et al. 2000). A direct causative relationship seems biologically implausible, although it could be viewed as simply a correlation between severity of disease in two distinct organs.

DHPLC has proved to be an efficient tool for mutation detection in TSC. It is ideally suited for genetic disorders like TSC, in which mutations are scattered over multiple exons, in that amplicons may be scanned at high sensitivity and efficiency by the DHPLC technique, followed by directed sequencing. The most frequent mutation in this series was seen in only 9 (4%) of all patients, and most other recurrent mutations were seen in only two patients. In addition, 79 of the 166 small mutations reported here in *TSC1* and *TSC2* have not been previously identified by any group, indicating that the spectrum of mutations in these two genes continues to expand. Therefore, mutation detection approaches directed at identification of specific mutations cannot be comprehensive in this disorder.

At the conclusion of this mutational screening, we were left with a set of 38 (17%) patients in whom no mutation was identified, the NMI set. There are multiple potential factors that could contribute to the lack of identification of a definite mutation in these patients. First, there were two patients in this set in whom missense changes in *TSC2* were identified that could not be classified as mutations because they were only moderately nonconservative and parental samples were not available. Second, there were two cases in which mosaicism for a large deletion in *TSC2* was suspected but results were inconclusive. Third, there were seven patients whose DNA samples were depleted before a complete analysis was performed. Fourth, it is likely that inactivating mutations remote from coding exons and their splice boundaries occur at some rate in *TSC1* and *TSC2*, and these and certain other classes of mutation will go undetected or unconfirmed as mutations by methods that analyze DNA only. Fifth, it is possible that there are additional causative loci that account for a small fraction of TSC cases. Finally, we and others have reported elsewhere that some patients with relatively low level mosaicism for a *TSC1* or *TSC2* mutation may present with diagnostic features of TSC (Sampson et al. 1997; Kwiatkowska et al. 1999; Verhoef et al. 1999). Some of these mutations may be difficult to detect by any conventional method, particularly mosaic single-base substitution mutations. It was of considerable interest to note that the NMI patients as a set had clinical features that were significantly milder than those of patients with *TSC2* mutations and were somewhat different from those with *TSC1* mutations (table 6). Because the numbers of these patients is relatively small, the comparisons are of only modest statistical significance. In addition, the NMI patient set is likely to be a mixed grouping of patients from several of the categories listed above. Nonetheless, the observations indicate that the NMI set of patients has a distinct set of clinical features, which, in our view, suggests the hypothesis that mosaicism for a mutation in *TSC2* and/or additional, as-yet-unidentified TSC loci account for a significant fraction of these patients. Additional study will be required to explore this hypothesis.

Acknowledgments

Electronic-Database Information

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

Berkeley Drosophila Genome Project, Splice Site Prediction by Neural Network, http://www.fruitfly.org/seq_tools/splice .html (for splice-site detection by computation)

GenBank, http://www.ncbi.nlm.nih.gov/Genbank (for *TSC1* and *TSC2* genomic sequence information [accession numbers AC002318, AC002096, and AC005600])

Human Genetics Online Review Tables, http://expmed.bwh .harvard.edu/ts/review/

Online Mendelian Inheritance in Man (OMIM), http://www .ncbi.nlm.nih.gov/Omim (for TSC [MIM 605284 and MIM 191092])

Primer 3, http://www-genome.wi.mit.edu/cgi-bin/primer /primer3_www.cgi

Run the DHPLC MELT Program, http://insertion.stanford.edu /melt.html

Staden Package, http://www.mrc-lmb.cam.ac.uk/pubseq/

Tuberous Sclerosis (TSC) Project, The, http://zk.bwh.harvard .edu/projects/tsc/ (for Primer sequences, PCR and DHPLC conditions, polymorphism tables, and Variation Wizard)

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