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Germ-Line Mutational Analysis of the TSC2 Gene in 90 Tuberous-Sclerosis Patients

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Summary

Ninety patients with tuberous-sclerosis complex (TSC) were tested for subtle mutations in the TSC2 gene, by means of single-strand conformational analysis (SSCA) of genomic DNA. Patients included 56 sporadic cases and 34 familial probands. For all patients, SSCA was performed for each of the 41 exons of the TSC2 gene. We identified 32 SSCA changes, 22 disease-causing mutations, and 10 polymorphic variants. Interestingly, we detected mutations at a much higher frequency in the sporadic cases (32%) than in the multiplex families (9%). Among the eight families for which linkage to the TSC2 region had been determined, only one mutation was found. Mutations were distributed equally across the gene; they included 5 deletions, 3 insertions, 10 missense mutations, 2 nonsense mutations, and 2 tandem duplications. We did not detect an increase in mutations either in the GTPase-activating protein (GAP)–related domains of TSC2 or in the activating domains that have been identified in rat tuberin. We did not detect any mutations in the exons (25 and 31) that are spliced out in the isoforms. There was no evidence for correspondence between variability of phenotype and type of mutation (missense versus early termination). Diagnostic testing will be difficult because of the genetic heterogeneity of TSC (which has at least two causative genes: TSC1 and TSC2), the large size of the TSC2 gene, and the variety of mutations. More than half of the mutations that we identified (missense, small in-frame deletion, and tandem duplication) are not amenable to the mutation-detection methods, such as protein-truncation testing, that are commonly employed for genes that encode proteins with tumor-suppressor function.

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Introduction

Tuberous-sclerosis complex (TSC) is an autosomal dominantly inherited disease characterized by hamartomas; the population frequency is 1/10,000–1/6,000 (Wiederholt et al. 1985; Osborne et al. 1991). A hamartoma is defined as "a benign tumor-like nodule composed of an overgrowth of mature cells and tissues that normally occur in the affected part, but with disorganization and often with one element predominating" (*Dorland's Illustrated Medical Dictionary* 1994). In individuals affected with TSC, nearly every organ system has been reported to have hamartomas. These growths rarely progress to malignancy, but they may cause morbidity and mortality because of their size and location (Shepherd et al. 1991). Common clinical findings include hypopigmented macules (HM), facial angiofibromas (FA), renal angiomyolipomas (RA), cardiac rhabdomyomas, and cortical tubers and subependymal glial nodules in the brain (Roach and Delgado 1995). The greatest source of morbidity is the brain tumors, which cause seizures (in 80%–90% of affected individuals), mental retardation (in $\geqslant$50%–60% of affected individuals), and behavioral abnormalities (most prominently, autism; in $\leqslant$50% of affected individuals) (Curatolo et al. 1991; Gomez 1988; Hunt and Dennis 1987, respectively). Approximately two thirds of cases are sporadic and are assumed to result from new mutations (Sampson et al. 1989).

Genetic heterogeneity is characteristic of TSC (Sampson et al. 1989; Janssen et al. 1990; Haines et al. 1991; Northrup et al. 1992). Two loci on different chromosomes have been implicated as causes of the disease. One locus (TSC1) maps to chromosome 9q34.3, and the other (TSC2) maps to chromosome 16p13.3 (Fryer et al. 1987; Kandt et al. 1992). Each locus accounts for ~50% of familial cases (Kwiatkowski et al. 1993). Cloning of the TSC1 gene has recently been reported (van Slegtenhorst et al. 1997). The TSC2 gene, identified in 1993, is encoded by 41 exons, spanning ~50 kb of genomic DNA (European Chromosome 16 TS Consortium 1993). Interestingly, the gene is adjacent, in a tail-to-tail

fashion, to the PKD1 gene, which is the major gene involved in autosomal dominant polycystic kidney disease (European Polycystic Kidney Disease Consortium 1994). The two genes (TSC2 and PKD1) are separated by ~800 bp of intervening sequence (Hughes et al. 1995). The protein encoded by TSC2, tuberin, is hypothesized to function as a tumor-suppressor gene (Green et al. 1994; Wienecke et al. 1995; Henske et al. 1996; Jin et al. 1996). Multiple isotypes of TSC2, identified on the basis of alternative splicing at two different exons, have been reported (Xu et al. 1995). These isotypes of TSC2 are thought to have different developmental and tissue-specific expression patterns.

Mutations in the TSC2 gene have been reported in familial and sporadic cases. Studies suggest that 2%–3% of TSC2 mutations are large DNA deletions, some of which encompass the entire TSC2 gene (European Chromosome 16 Consortium 1993; Brook-Carter et al. 1994; Au et al. 1997). More-subtle mutations are known to cause disease; the literature includes multiple single-mutation reports (Kumar et al. 1995*a,* 1995*b;* Vrtel et al. 1996), as well as a report in which 173 patients were tested for mutations only in exons 34–38 (Maheshwar et al. 1997). There are two reports in which the entire TSC2 gene was surveyed for mutations: Wilson et al. (1996), using an RT-PCR–based system, tested 30 patients, and van Bakel et al. (1997), using the protein-truncation test, tested 18 patients. Using single-strand conformational analysis (SSCA) of genomic DNA, we tested 90 affected individuals (56 sporadic cases and 34 familial probands) for TSC2 mutations. SSCA has the advantage that it screens for mutations at the genomic level; this circumvents potential failure to detect mutations that do not survive either the transcriptional or the translational process. The goals of our study were two-fold: first, to determine the type, number, and distribution of mutations in the TSC2 gene, in an unselected group of tuberous-sclerosis patients, and, second, to assess potential genotype-phenotype correlations at the TSC2 locus.

Subjects and Methods

Family Studies

Ninety patients (56 sporadic cases and 34 familial probands from multiplex TSC families) were tested for variation in the TSC2 gene. TSC diagnoses were reached in accordance with standard diagnostic criteria (Gomez 1991; Roach et al. 1992). Parents of all sporadic patients were examined carefully, and no findings consistent with TSC were detected. Some parents underwent additional clinical testing, including retinal examination, renal ultrasound, and/or computed tomography (CT) or magnetic resonance imaging (MRI) scan of the brain. If possible, blood samples for DNA extraction were obtained from parents of sporadic patients (from both parents, in 38 cases; from the mother only, in 10 cases; from the father only, in 2 cases; or from neither parent, in 6 cases). In multigenerational families, as many family members as possible were enrolled in the study (266 individuals from 34 families). Among the 34 families, 8 showed evidence for linkage to TSC2, and 4 showed evidence for linkage to TSC1. In the remaining 22 families, linkage could not be determined. Linkage in a family was established on the basis of both a posterior probability of >90% for linkage, as calculated by means of HOMOG, to markers in either the TSC1 (markers D9S114, D9S66, DBH, and D9S149) or the TSC2 (markers D16S291, KG8, TSC2 exon 40 *Eco*RV polymorphism, and D16S525) region and exclusion by markers from the other region (Ott 1991). Four of the families included two affected children, but, despite careful examination and internal imaging studies, the parents did not show any findings that were consistent with the diagnosis of TSC. Informed consent was obtained from all individuals enrolled in the study. Patients included 62 (69%) Caucasians, 13 (14%) African Americans, 12 (13%) Hispanics, 2 (2%) Caucasian Hispanics, and 1 (1%) Japanese. The study was approved by the institutional review boards at the University of Texas Medical School, in Houston, and at the University of Texas Southwestern Medical School and the Scottish Rite Hospital, both in Dallas.

SSCA

For all amplifications, primer pairs were designed to flank each exon and to include consensus splice junctions. Primers, therefore, extended ~30–50 bp before and after each exon. Two overlapping sets of primers were designed for exon 33 because of its large size (571 bp). Sufficient sequence information was available from GenBank for all exons except exons 21, 25, and 36. Sequences of the introns that precede and of those that follow exons 21, 25, and 36 were obtained by sequencing PCR fragments amplified from primers that were derived from the exons that flank introns 20, 21, 24, 25, 35, and 36. For all of the exons except 8, 12, 13, 19, 20, and 22, amplification by PCR was accomplished with the initial set of primers. For these remaining six exons, a second set of primers was designed. We were able to obtain a good result by using either the second primer set or a combination of primers from the first and second sets. Information on the sequences of primer pairs, the sizes of PCR products, and the PCR conditions have been submitted to the Genome Data Base (GDB). The 42 GDB accession numbers are the even numbers 6053944–6054026.

PCR reactions were performed in 20-μl reaction

mixtures that contained 40 ng genomic DNA, 1–2 μM primer pairs, 1 μCi [^{32}P]-dCTP, 200 μM dNTPs, and 1 U *Taq* DNA polymerase (Boehringer Mannheim), in standard PCR buffer. Two microliters of the PCR products were denatured at 100°C for 10 min, chilled in ice bath, and loaded into wells of a 6% native Tris-borate EDTA PAGE gel (19 acrylamide:1 bisacrylamide). The samples were electrophoresed, with constant power set at 2 W, at 4°C for 16–22 h, depending on the size of the PCR product. After electrophoresis, the gel was vacuum dried and subjected to autoradiography. When variants were detected by SSCA, we tested either the parents, in the case of sporadic patients, or other family members, in the case of multigenerational families. Changes that were verified either as unique, in sporadic patients, or as segregating with the disease, in the multigenerational families, were tested further.

Direct Sequencing

SSCA variants that were unique to the sporadic and the familial patients were followed up by direct sequencing of the variant bands, reamplified in a subsequent PCR, with the excised variant PAGE-gel band as template (Calvert et al. 1995). The center section of the variant band was excised and transferred into a 0.5-ml Eppendorf tube that contained 20 μl 1 × PCR buffer. The gel was rehydrated in the 1 × buffer at 65°C for 10 min, and reamplification PCR was performed in a final volume of 50 μl, under the same conditions. The PCR products were then fractionated on a 3% Nusieve or Metaphor agarose gel to remove excess primers and products of spurious amplifications. Bands near the expected size range were excised and processed in accordance with the β-agarose (New England Biolabs) treatment protocol. Supernatant was sequenced by the Molecular Genetics Core Facility, Department of Microbiology and Molecular Genetics, University of Texas Medical School, Houston. This strategy was successful ~80% of the time. In the remaining cases, it was not possible to excise the pure variant bands. For these variants, the PCR products were subcloned into pGEM-T vector for further analyses.

Additional verification of mutations was accomplished in one of two ways: If a change resulted in creation or loss of a restriction-enzyme site, we tested the affected individual, the individual's parents, and a panel of control individuals, to confirm that the change was present only in the TSC patient. In sporadic cases with a change that did not involve a restriction-enzyme site, we sequenced the same exons from both parents and a control. In all cases except exon 16, the changes determined to be disease-causing mutations were unique among the 90 affected individuals.

Results

Mutations and Polymorphisms

A total of 32 variants were detected by SSCA. Of these, 22 variants, found in 21 patients, were unique changes that appear to be disease related (table 1). Changes comprised 5 deletions, 3 insertions, 10 missense mutations, 2 nonsense mutations, and 2 tandem duplications. All the missense mutations involved changes of amino acids that are conserved between the mouse, rat, and human sequence of TSC2. Mutations were relatively evenly distributed over the exons of the gene (fig. 1). Mutations were found in 18 (32%) of the 56 sporadic patients, compared with only 3 (9%) of the 34 multigenerational families. Thirteen of the 21 mutations were detected in 62 Caucasian patients, 6 in 13 African American patients, and 2 in 12 Hispanic patients. The finding of a greater percentage of mutations among the African American patients was not statistically significant (P = .057).

For several exons (9, 14, 16, and 23), we identified a mutation in two different individuals. One patient was found to have a five-amino-acid tandem duplication, in exon 9, and a missense mutation, Leu1750Phe, in exon 40; neither of these mutations was observed in any of the other 89 patients tested. The mother of the patient did not have either of the changes, and the father was unavailable. The other mutation identified in exon 9 was a missense mutation, Leu292Pro. We identified two mutations in exon 14: a 4-bp deletion (526SerFS→533X) and a nonsense mutation (Arg505X). The nonsense mutation has been reported elsewhere (Wilson et al. 1996). In exon 16, we found the same missense mutation, Arg611Gln, in two unrelated sporadic cases. Wilson et al. (1996) reported a missense mutation, Arg611Trp, in the same codon of exon 16. In exon 23, we identified two mutations—a nonsense mutation (Cys877X) and a missense mutation (Arg905Trp)—in African American sporadic cases. In both cases, the mother and father were tested, and the change was found to arise de novo. Of the three mutations that we identified in multigenerational families, one was a missense mutation in exon 29, Arg1200Trp, which we found in a large African American family. Wilson et al. (1996) reported the same mutation in a Caucasian family. There was no clustering of mutations in the exons that comprise the putative GAP-related domain (exons 34–38) in our population. We found four mutations in the region, in exons 34–37. We did not identify any mutations in either exon 25 or exon 31, which are the spliced exons in the different isoforms of TSC2. Figure 2 provides both representative examples of the SSCA changes and follow-up sequencing that identifies the respective mutations.

Ten of the changes represent polymorphic variation.

Table 1

TSC2 Mutations in Sporadic and Familial Patients

Patient	Exon	DNA Sequence Change[a]	Codon Change	Consequence
TS94-35	5	del2bp (T_{487}–T_{488})	163PheFS→187X	Frameshift (early termination)
TS92-05	8	ins1bp (C_{831}–C–C_{832})	277CysFS→337X	Frameshift (early termination)
TS91-03	9	15-bp tandem dup (C_{900}–C_{914})	Amino acids 300–304 tandemly repeated (GMALW)	5-Amino-acid tandem duplication
TS94-02	9	875T→C	Leu292Pro	Missense
TS87-144	14	del4bp (G_{1577}–CCT_{1580})	526Ser FS→533X	Frameshift (early termination)
TS94-96	14	1513C→T[b]	505Arg→stop	Nonsense (early termination)
TS94-31	16	1832G→A[c]	Arg611Gln	Missense
TS93-29	16	1832G→A[c]	Arg611Gln	Missense
TS94-104	18	del1bp ($C_{2070/2071}$)	690Phe FS→697X	Frameshift (early termination)
TS94-82	21	ins2bp (C_{2490}–AT–A_{2491})	830Leu FS→948X	Frameshift (early termination)
TS94-86	23	2661T→A	887Cys→stop	Nonsense (early termination)
TS95-12	23	2713C→T	Arg905Trp	Missense
HOU23	24	ins1bp (C_{2779}–C_{2784})	928ProFS→939X	Frameshift (early termination)
TS87-117	27	3252C→G	Asp1084Glu	Missense
HOU11	29	3598C→T[b]	Arg1200Trp	Missense
TS93-41	30	17-bp tandem dup (G_{3611}–G_{3627})	1209Leu FS→1215X	Frameshift (early termination)
TS93-44	32	3887A→T	Asp1295Val	Missense
TS93-20	34	del4bp (A_{4475}–A_{4478})	1492Ser FS→1574X	Frameshift (early termination)
TS92-08	35	4577A→G	Tyr1526Cys	Missense
TS93-14	36	del (4770–4772)	dl Ile 1591	In-frame deletion
TS94-53	37	4859A→T	Asn1620Ile	Missense
TS91-03	40	5179C→T	Leu1727Phe	Missense (see exon 9)

[a] Nucleotide numbering is based on the published sequence X75621 (GenBank); nucleotide A of the initiation codon ATG is the reference nucleotide number 1.

[b] An identical change was reported by Wilson et al. (1996).

[c] A change in the same codon was reported by Wilson et al. (1996).

Two of the polymorphic variants caused loss or gain of a restriction-enzyme site (Au et al. 1997).

Clinical Findings

Clinical data for each of the patients in whom a mutation was identified are displayed in table 2. Every patient in whom a mutation was identified has HM and positive brain-imaging (CT or MRI) findings. Other clinical findings varied between patients.

Discussion

In an unselected TSC population, our rate of identification of mutations by means of a combination of Southern blotting, for large intragenic deletions, and SSCA of genomic DNA, for more subtle changes, is 25%. Our first phase of testing by Southern blotting detected two mutations in 92 individuals; one individual was the proband in a multiplex family, and the other was a sporadic case (Au et al. 1997). These two individuals were eliminated from our second phase of testing. Disease-causing mutations were identified in 21 of the remaining 90 affecteds. Using estimates derived from family studies, we would predict that 45 patients in our sample have mutations in the TSC2 gene (Kwiatkowski et al. 1993). Therefore, our overall rate of mutation detection by SSCA is 47%. In general, the SSCA method is predicted to detect 76% and 63% of mutations in DNA fragments of sizes 212 bp and 250 bp, respectively (Sheffield et al. 1993). In our population, which included 34 families with more than one affected, we identified mutations in only 3 families, whereas we found mutations in 18 of the 56 sporadic cases. If the denominators are decreased to account for the TSC1 mutations present in half of the individuals, our detection rates are 18% (3/17) for familial cases and 64% (18/28) for sporadic cases. The detection rate for sporadic cases is approximately the same as the rate expected for the SSCA method; the remaining 36% of cases required other detection methods (i.e., heteroduplexes, dideoxy-fingerprinting, or CFLP cleavase) (Keen et al. 1991; Liu and Sommer 1994; Brow and Fors [in press]). It is important to determine which mutation-screening method is best, for the sake of future diagnostic testing. Our strategy (SSCA of genomic DNA) detected more mutations among sporadic cases (32%) than did the reverse transcriptase–PCR strategy of Wilson et al. (1996) (19%). The majority of TSC cases, approximately two thirds, are sporadic in nature. Our method of mutation screening excluded attempts at detection of mutations in the regulatory elements at the 5′ and 3′ UTRs. It is difficult to prove that variants in the regulatory elements are

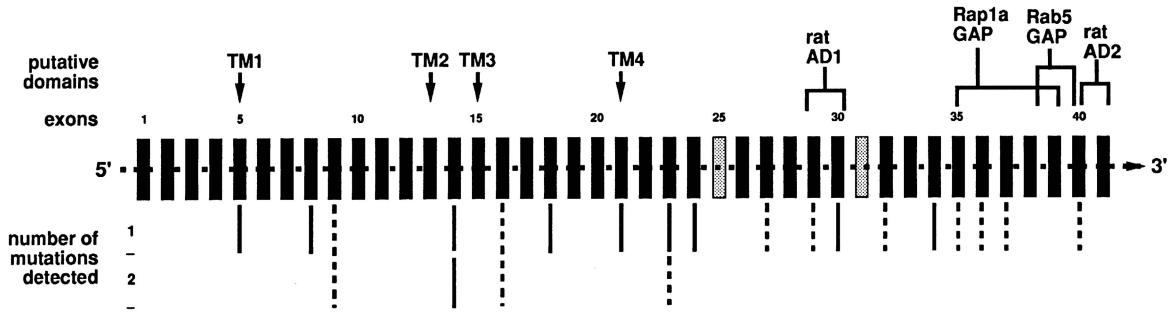

Figure 1 Schematic illustration of the distribution of TSC2 mutations detected in 21 TSC patients. The sizes of exons and introns are not drawn to scale. The locations of the putative transmembrane domains (TM1, TM2, TM3, and TM4) were predicted from the primary amino acid sequence encoded by GenBank sequence X75621, and they reside in exons 5, 13, 15, and 21, respectively. Other functional domains defined experimentally include transcriptional activation domains AD1 and AD2, found in rat tuberin, and GAP-like domains, specific to Rapla (RaplGAP) and to Rab5 (RAB5GAP). The numbers 1, 5, 10, 20, 25, 30, 35, and 40 appear above the corresponding exons, for clarification. Exons denoted by blackened boxes are found in all isoforms of tuberin, and exons denoted by shaded boxes are spliced in some isoforms. Each vertical solid line below an exon represents a single frameshift or nonsense mutation predicted to cause premature termination of tuberin. Each vertical dotted line below an exon represents a single missense mutation predicted to change a single amino acid in that exon. Exons 9, 14, 16, and 23 each harbored two mutations, and the two missense mutations found in exon 16 were identical (Arg611Gln) but occurred in two unrelated patients.

disease-causing mutations; they cannot be distinguished easily from rare polymorphic variants, which are not uncommon in the TSC2 gene (Au et al. 1997; authors' unpublished observation).

There are several possible explanations for our inability to find mutations in the multigenerational families. By chance, our sample may include more families that harbor a mutation in the TSC1 gene. We have no reason to assume that this is the case; in fact, among the families in our sample, only four show definite evidence of linkage to TSC1, whereas eight show evidence of linkage to TSC2. We did not selectively include or eliminate any of our patients, except the two excluded patients in whom we had previously detected large intragenic mutations of TSC2. Another possible explanation is that familial mutations are more difficult to detect by means of the methods that we employed. We did find two unique intronic variants within 40 bp of the splice sites; both variants are in familial cases and have not yet been categorized as mutations or polymorphisms. We are testing whether these intronic mutations would cause incorrect splicing and would thereby lead to production of abnormal or decreased amounts of tuberin. If these changes prove to be mutations, our rate of detection in multiplex families increases to 15% (5/34), which is still less than the predicted rate.

Of the mutations identified, 10 were missense mutations, 2 were in-frame tandem duplications or deletions, and the other 10 were predicted to result in premature termination of the protein. Therefore, >50% of the mutations that we detected would be missed by strategies that rely on production of a smaller protein product. The majority (6/9) of mutations detected by Wilson et al. (1996) were of the former type (missense, in frame, etc.), which led them to conclude that, in terms of mutations, the behavior of the TSC2 gene is similar to the behavior of the *p53* gene (Baker et al. 1990; Chiba et al. 1990; Toguchida et al. 1992). If we combine all of the mutations reported by others, the total in each category (14 missense/in-frame duplications or deletions vs. 17 truncating mutations) is similar to the distribution that we observed (12 missense/in-frame duplications or deletions vs. 10 truncating mutations), with a slight bias toward truncating mutations. This is not surprising, since one group (van Bakel et al. 1997) tested only for truncating mutations (Kumar et al. 1995*a,* 1995*b;* Vrtel et al. 1996; Maheshwar et al. 1997; van Bakel et al. 1997). We predict that, as the database of TSC2 mutations grows, TSC2 will continue to exhibit the trend observed in other phakomatoses—that is, more frameshifts and nonsense mutations than missense mutations (Latif et al. 1993; Legius et al. 1993; Trofatter et al. 1993).

Our findings and those of other investigators indicate a total of 46 mutations, identified in 52 independent patients (Kumar et al. 1995*a,* 1995*b;* Vrtel et al. 1996; Wilson et al. 1996; Maheshwar et al. 1997; van Bakel et al. 1997). Only four mutations (Arg505X, Arg611Gln, Arg1200Trp, and Pro1675Leu) were detected in more than a single individual, and a small number of exons (14, 16, 29, and 38) were more likely to harbor mutations. Of these four exons, only exon 38 is known to be in a functional domain of tuberin, whereas the other three have not yet been identified as having specific functions in the protein. We are targeting these exons in our current functional studies of tuberin. We

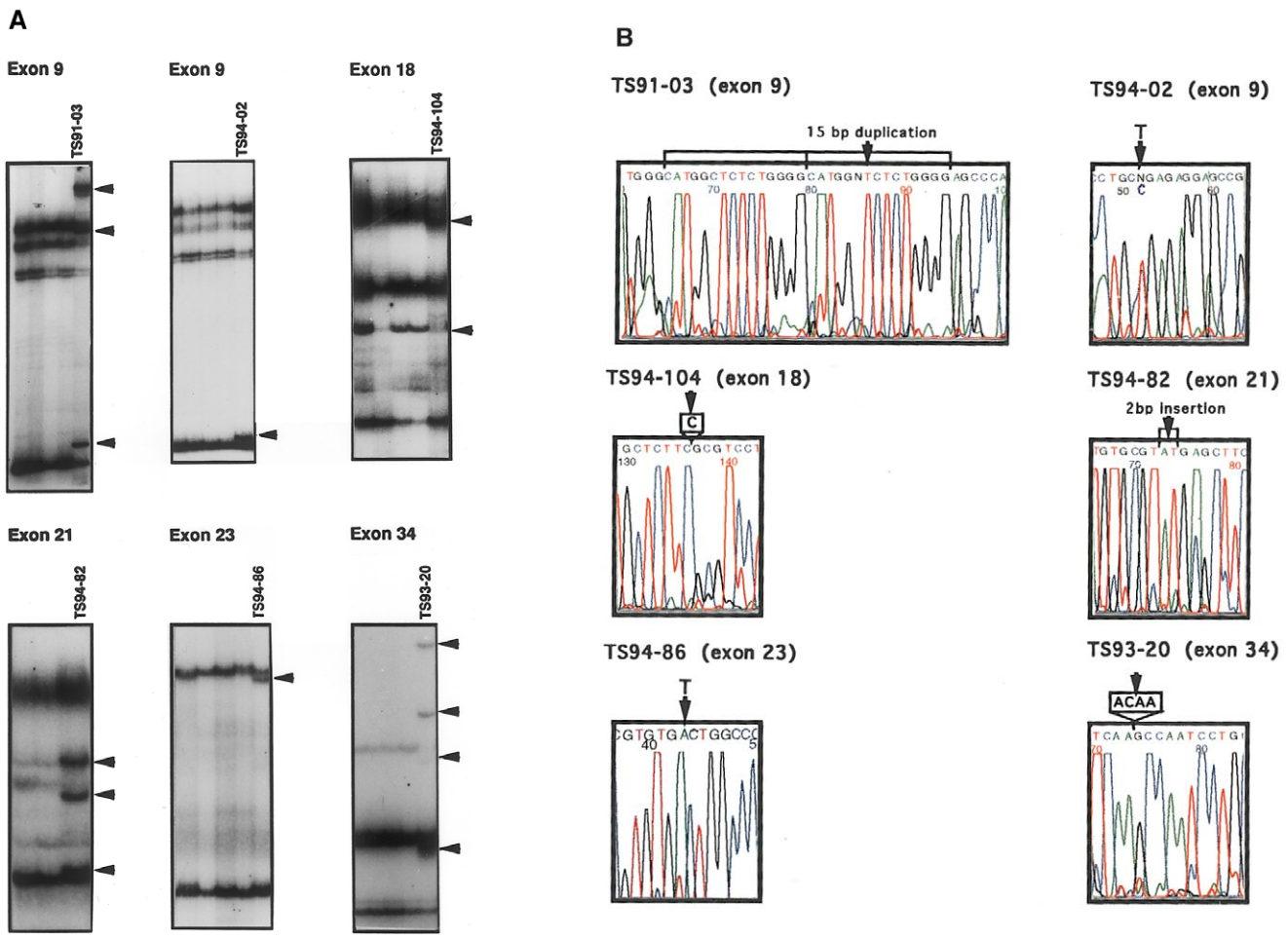

Figure 2 Typical SSCA gel variants and characterization of each mutation by sequencing. *A*, Six typical SSCA variants (*arrowheads*), found in six sporadic TSC probands (TS91-03, TS94-02, TS94-104, TS94-82, TS94-86, and TS93-20 codes above corresponding lanes), in exons 9, 18, 2l, 23, and 34 of the respective patients' TSC genes. These SSCA variants are unique among 90 TSC probands and their parents. The SSCA-variant bands were excised and reamplified for sequencing; results are shown in *B*. The location of each mutation is indicated by an arrow above each variant. Sequences for exons are presented for review of the forward sense-strand sequences, except for exon 21, for which the reverse-complemented sequence is shown. The 15-bp duplication found on exon 9 of patient TS91-03 is bracketed. Both patient TS94-02 and patient TS94-86 have substitution mutations; the sequence of normal TSC2 is shown, with an arrow, above the mutation. Two deletion mutations in patients TS94-104 and TS93-20 are shown; deleted sequences (*boxes*) appear above the deletion sites. In patient TS94-82, the mutation contains a 2-bp (AT) insertion, indicated by an arrowhead.

identified five transition mutations that represent changes in the CpG dinucleotides of the Arg-specific codon, as observed in the NF2 gene (Rouleau et al. 1993); this finding is similar to findings reported by Wilson et al. (1996).

We found two tandem duplications. This type of mutation has been reported in only two other human disease genes: the NF1 gene and the CO12A1 gene (Tiller et al. 1990; Tassebehji et al. 1993). Wilson et al. (1996) also reported a tandem-duplication mutation in the TSC2 gene. The mutation detected in individual TS91-03 (15-bp tandem duplication) supports the theory that this type of mutation occurs as a result of intrastrand slipped mispairing during meiosis, because it is flanked by two short direct repeats (TGGG) (Roth et al. 1985). No repeat sequences flanking the tandem duplication were observed, either in individual TS93-41 or in the 29-bp duplication reported by Wilson et al. (1996). Interestingly, both duplications in exons 30 and 35 occurred at the 5′ end of the exon; this suggests a separate but perhaps similar mechanism.

Although we did observe mutations in the Rap1GAP-related domain (exons 35–39), the Rab5GAP-related domain (exons 38–40), and the transcriptional activating domains (exons 29, 30, 40, and 41) identified in the rat tuberin, we did not observe a higher number of mutations in these regions than in the rest of the gene (Wienecke et al. 1995; Maheshwar et al. 1997; Tsuchiya et

Table 2

Summary of TSC-Patient Clinical Findings

	Skin				Brain[a]						Kidney		
Patient No. (Age)	HM	FA	UF	SP	CT	MRI	Eye	Seizures	MR/DD	BA/LD	Tumors	Cysts	Heart
TS94-35 (18 years)	+	+	−	+	+	+		+	−	+	+	−	−
TS92-05 (1 year)	+	+	−	−	+	+	+	+	+	−	−	−	+
TS91-03 (9 years)	+	+	−	+	+	+	−	+	+	+			−
TS94-02 (12 years)	+	+	−	+	+	+	−	+	+	−	+	−	+
TS87-144 (20 years)	+	+	+	+	+	+	+	+	+	−			
TS94-96 (19 years)	+	+	+	+	+	+	−	+	+	+			+
TS94-3l (10 years)	+	+	−	−	+	+	+	+	+	+	−	−	+
TS93-29 (30 mo)	+	−	−	−	+	+		+	+		−	−	−
TS94-104 (19 years)	+	+	+	−	+			−	−	+	+	±[b]	
TS94-82 (16 mo)	+	−	−	+	+	+		+	+	−	+	−	−
TS94-86 (12 years)	+	+	+	+	+	+	+	−	−	+	+	−	+
TS95-12 (42 mo)	+	−	−	−	+	+		+	+		−	−	−
HOU23-01 (32 years)	+	+	+	+	+	+		+	−	−	+	+	
TS87-117 (3 mo)	+	−	−	−	+		−	+			−	−	−
HOU11-58 (11 years)	+	−	−	−	+	+		+	−	−			−
TS93-41 (22 years)	+	+	+	+	+		+	+	+	+	+	−	
TS93-44 (7 years)	+	−	−	−		+	−	+	−				
TS93-20 (5 years)	+	+	+	+	+	+	+	+	+	+	−	−	+
TS92-08 (22 years)	+	−	−	−	+	+	−	+	−	+	+	−	+
TS93-14 (5 years)	+	+	−	+	+	+	−	+	+		+	−	+
TS94-53 (5 years)	+	+	−	−		+	−	+	+	+	−	±[b]	−

Note.—MR/DD = mental retardation or developmental delay; BA/LD = behavioral abnormalities or learning disabilities, a plus sign (+) denotes presence; and a minus sign (−) denotes absence.

[a] All patients with positive CT or MRI findings had subependymal nodules and one or more cortical tubers.

[b] See Discussion.

al. 1996; Xiao et al. 1997). Patients who had mutations in these particular exons did not have a more severe phenotype compared with patients who had mutations in other regions of the gene. We did not identify any mutations in the exons (25 and 31) known to be spliced out in different isoforms (Xu et al. 1995).

Although the significance of frameshift mutations is obvious (production of prematurely terminated tuberin), determination of the significance of missense mutations is more difficult. All the missense mutations that we observed changed amino acids that are conserved among mouse, rat, and human tuberin. Eight of the 10 missense mutations were observed in sporadic cases; 5 of these (Arg611Gln, Arg905Trp, Asp1295Val, Tyr1526Cys, and Asn1635Ile) were verified as de novo, since both parents were available for testing, and neither parent had the change. One of the missense mutations (Arg1200Trp) detected in a family had been observed elsewhere, and the mutation could be traced back to the starting point, for verification (Wilson et al. 1996).

Clinical findings for each patient are summarized in table 2. The mutations can be divided into two groups: those predicted to lead to premature termination of tuberin (frameshifting insertions/deletions and nonsense mutations) and those that do not lead to premature termination of tuberin (missense mutations and in-frame deletions). The 10 patients with truncating mutations have more skin findings (FA, found in 9 patients; ungual fibromas [UF], in 7 patients; and shagreen patch [SP], in 8 patients) compared with the 11 patients in the missense and in-frame–deletion group (FA, found in 5 patients; UF, in 0 patients; and SP, in 3 patients). Similarly, six of eight patients in the premature-termination group have RA, compared with only three of eight in the other group. However, others have noted that skin conditions associated with TSC increase as an affected individual ages (Webb et al. 1996). Examination of the clinical findings as they correspond to age, in our sample of patients with identifiable mutations, reinforces this observation. Our 9 "older" patients ($\geqslant$12 years of age) had a greater number of skin findings (8, 6, and 7 patients had FA, UF, and SP, respectively) and RA (in 7 patients) than did our 12 "younger" patients (<12 years of age), among whom 6, 1, 4, and 2 patients had FA, UF, SP, and RA, respectively. The majority (7/10) of the patients with truncating mutations are in the older group; only 2 of the 11 patients in the missense and in-frame–deletion group fall into the older age bracket. We conclude on the basis of these data that type of germ–line mutation does not influence clinical phenotype.

Of 21 patients in whom a mutation was detected, 16 were evaluated for renal cysts. A definite diagnosis of renal cysts was reached in one case (HOU23-01). Two of our patients (TS94-104 and TS94-53) had MRI scans

of the abdomen, to evaluate the kidneys. In both cases, multiple focal areas of abnormal signal intensity were observed throughout the kidney; these were interpreted as compatible with renal cysts. We have tested these individuals for involvement of the PKD1 gene, by means of loss-of-heterozygosity (LOH) studies of flanking markers (KG8, which is at the 3′ end of PKD1, and D16S665, at the 5′ end of PKD1), and we find no LOH. Our studies do not indicate involvement of PKD1 in these patients. Brook-Carter et al. (1994) described a contiguous-gene syndrome, associated with a severe cystic renal phenotype, that includes both the TSC2 and PKD1 genes. They point out that renal cysts have been observed in chromosome 9–linked TSC patients, and that the number of patients observed with the TSC2-PKD1 contiguous-gene syndrome is small and does not account for the majority of TSC patients reported to have renal cysts. We agree with Brook-Carter et al. (1994) that cysts can be seen as part of the TSC phenotype, on the basis of TSC2 mutations alone.

In summary, diagnostic testing for TSC will be difficult because of the genetic heterogeneity of TSC (which has at least two causative genes: TSC1 and TSC2), the large size of the TSC2 gene, and the variety of mutations. More than half of the mutations that we identified (missense, small in-frame duplication, and tandem duplication) are not amenable to the mutation-detection methods, such as the protein-truncation test, that are commonly employed for genes that encode proteins with tumor-suppressor function. Identification of mutations in the TSC2 gene supplies additional clues toward identification of functionally important regions of the gene. There appears to be no correlation between the type of mutation and the clinical findings. Renal cysts can be seen in TSC patients who have TSC2 mutations, without involvement of the PKD1 gene.

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