Original articles

Mutations in the TSC1 gene account for a minority of patients with tuberous sclerosis

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Abstract

Tuberous sclerosis (TSC) is an autosomal dominant disorder characterised by tumour-like malformations (hamartomas) of the brain, skin, and other organs, often associated with seizures and learning disability. There is genetic heterogeneity with loci for TSC on chromosomes 9q34 (TSC1) and 16p13.3 (TSC2). The recently cloned TSC1 gene has 23 exons spanning some 40 kb of genomic DNA with an 8.6 kb transcript. We now report the results of mutation screening by SSCP and heteroduplex analysis of genomic DNA for all 21 coding exons of TSC1 in 83 unrelated cases of tuberous sclerosis.

TSC1 gene mutations were found in 16 of the 83 cases (19%). These comprised base substitutions, small insertions, or small deletions giving rise to six nonsense mutations, eight frameshifts, and two splice site mutations, all of which would be expected to result in a truncated or absent protein. In the 10 cases predicted to have TSC1 mutations by linkage analysis or loss of heterozygosity studies, the mutation was identified in eight (80%). In the remaining 73 unassigned cases, only eight mutations were found (11%). From these data we estimate that TSC1 mutations accounted for 24% of the cases in this sample (and an estimated 22% of all TSC cases). This contrasts with data from linkage studies suggesting that TSC1 and TSC2 mutations account for approximately equal numbers of families.

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Tuberous sclerosis (TSC) is an autosomal dominant condition characterised by tumour-like malformations (hamartomas) of the skin, brain, heart, kidney, and other organs. Most patients have seizures and a minority have learning disability.[1] The birth incidence is 1 in 10 000 or higher.[2] In two-thirds of cases the parents are unaffected (so called sporadic cases) and the disease is the result of a new dominant mutation. Linkage studies have identified loci for TSC on chromosomes 9q34 (TSC1) and 16p13.3 (TSC2) and suggest that they account for approximately equal numbers of families.[3]

The TSC2 gene was characterised in 1993.[4] Both truncating and missense mutations are common and large deletions of 1-5 kb have also been reported.[4–10] The gene has 41 exons spanning approximately 45 kb of genomic DNA. The 5.5 kb mRNA encodes the protein tuberin, which has a region of sequence homology to the GTPase activating protein rap1GAP.[4] The finding of loss of heterozygosity in TSC hamartomas suggests that tuberin acts as a tumour suppressor.[11]

The TSC1 gene was first mapped to chromosome 9q34 in 1987.[12] Loss of heterozygosity (LOH) in the TSC1 region in hamartomas suggests that the gene product also acts as a tumour suppressor.[13 14] However, LOH for the TSC1 region is significantly less common than for TSC2.[15 16] In a concerted effort by a Consortium of six research groups, the TSC1 gene has recently been isolated by a combination of positional cloning and large scale sequencing.[17] The gene spans 40 kb with 23 exons and an 8.6 kb mRNA. There are 21 coding exons, the largest being exons 15 (559 bp) and 23 (517 bp of coding sequence) and the remainder varying in size from 44 to 188 bp. The TSC1 protein, hamartin, contains 1164 amino acids with a predicted mass of 130 kDa.

In the Consortium publication, eight exons were analysed in a large panel of TSC cases. We now present the results of screening all 21 coding exons of the TSC1 gene for mutations in 83 unrelated UK patients.

Methods

PATIENTS

Patients were selected from a panel of 93 cases of tuberous sclerosis fulfilling the diagnostic criteria proposed by Gomez.[18] Four familial cases and six sporadic cases were excluded because they had previously been shown to have TSC2 mutations[9] or there was linkage or other evidence to assign them to TSC2. The remaining 83 patients comprised 23 familial and 60 sporadic cases. Ten were predicted to have TSC1 mutations on the basis of either (1) exclusion of TSC2 by linkage analysis with affected relatives having different 16p13.3 haplotypes, or (2) evidence of linkage to 9q34

Table 1 Pathogenic mutations identified in the TSC1 gene

Mutn No	Mutation	Location	Mutation type	Family category*
1	585 −1 G>C	Intron 5	Splice acceptor	S
2	875insA	Exon 7	Frameshift	F
3	1122 C>T; Q301X	Exon 9	Nonsense	F (TSC1)
4	1250 +1 G>A	Intron 10	Splice donor	F (TSC1)
5	1424delT	Exon 12	Frameshift	F (TSC1)
6	1552 C>G; S444X	Exon 13	Nonsense	F (TSC1)
7	1746 C>T; R509X	Exon 15	Nonsense	S
8	1938 C>T; Q573X	Exon 15	Nonsense	S
9	1995insGA	Exon 15	Frameshift	S (TSC1)
10	2105delAAAG	Exon 15	Frameshift	S
11	2332delAT	Exon 17	Frameshift	S (TSC1)
12	2395insA	Exon 17	Frameshift	F (TSC1)
13	2519del23bp	Exon 18	Frameshift	S
14	2577 C>T; R786X	Exon 18	Nonsense	F
15	2718 C>T; Q833X	Exon 19	Nonsense	F (TSC1)
16	2887insA	Exon 21	Frameshift	S

*F = familial case; S = sporadic case; TSC1 = predicted to have TSC1 mutation from linkage data or loss of heterozygosity studies. Mutation Nos 7, 9–14 have been reported previously.[17]

markers with a lod score of at least 3, or (3) loss of heterozygosity for 9q34 markers in a TSC hamartoma. In familial cases, samples from affected relatives were used to test whether mutations segregated with the disease. In sporadic cases, the normal parents were tested for mutations identified in their offspring.

PCR AMPLIFICATION

DNA was extracted from peripheral blood lymphocytes using standard methods.[19] Exons 3-23 of the TSC1 gene were individually amplified from genomic DNA by PCR to give products in the size range 150 to 250 bp suitable for SSCP. Exon 15 was amplified in two overlapping segments of approximately 330 bp. Exon 23 was amplified in four overlapping segments of approximately 220 bp. Primer sequences are available from the web site http://expmed.bwh.harvard.edu/projects/tsc/.

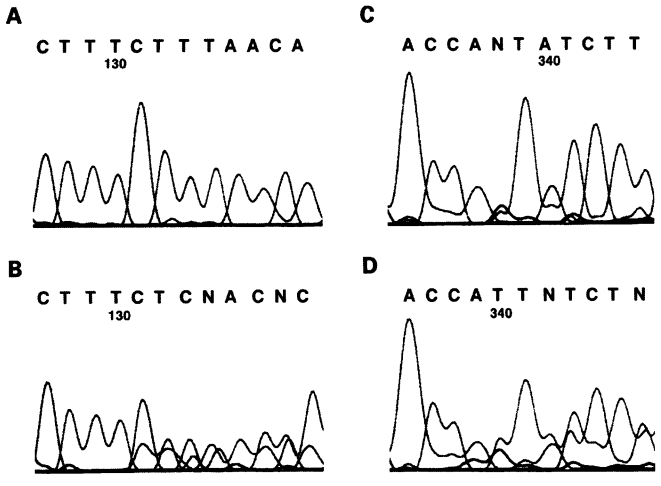

Figure 1 Germline mutation 2105delAAAG and in the same patient the somatic mutation 1957delG in a renal cell carcinoma. Automated sequencing of lymphocyte DNA from (A) a normal control showing reverse sequence CTTTCTTTAACA, (B) patient showing CTTT followed by heterozygous sequence owing to deletion of the CTTT repeat in the mutant allele. At the site of the somatic mutation, automated sequencing showed (C) in lymphocyte DNA the normal sequence ACCAGTATCTT, (D) in renal cell carcinoma DNA the sequence ACCA followed by heterozygous sequence owing to deletion of G in the mutant allele. To confirm that the somatic mutation had occurred on the wild type allele, allele specific amplification of the tumour DNA was carried out using a primer specific for the normal sequence at the site of the 2105delAAAG mutation; automated sequencing of the product confirmed the presence of the 1957delG mutation.

SSCP AND HETERODUPLEX ANALYSIS

PCR products were screened for mutations by single strand conformational polymorphism (SSCP)[20] and heteroduplex analysis.[21] All PCR products showing variant SSCP or heteroduplex patterns were sequenced.

For the SSCP analysis 1 µl of PCR product was mixed with 9 µl of loading buffer (95% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, 10 mmol/l NaOH), heat denatured, snap cooled, and loaded onto a 1 × MDE™ gel (FMC)/10% glycerol (v/v) in 1 × TBE. Electrophoresis was carried out at 10°C for 16-20 hours at 7 W using a Protean II xi Cell (BioRad). Bands were visualised by silver staining.

For the heteroduplex analysis, 1 µl of PCR product was mixed with 5 µl of loading buffer (40% w/v sucrose, 0.1% bromophenol blue, 0.1% xylene cyanol), heat denatured for five minutes, left at 70°C for one hour, allowed to cool to room temperature, and loaded onto a 1 × MDE™ gel (FMC) in 1 × TBE. Electrophoresis was carried out at room temperature for 16-20 hours at 400 V. Bands were visualised by silver staining.

AUTOMATED SEQUENCING

All samples were sequenced using an ABI Prism™ 377 DNA Sequencer (Perkin Elmer). Dye terminator cycle sequencing kits were provided by Perkin Elmer. For amplification of DNA and subsequent sequencing, the same primers were used as for the original PCR. Purification of templates was done using Wizard™ Minicolumns (Promega) or QIAquick™ PCR purification kits (Qiagen). Reaction volumes were halved as compared to ABI protocols. All mutations were confirmed on the opposite strand and from a second PCR product. Mutations were numbered according to the published cDNA sequence.[17]

Results

Pathogenic TSC1 gene mutations were found in 16 of the 83 patients, representing 19% of the samples tested (17% of the total patient sample including TSC2 mutation cases). Seven of these mutations were included in the Consortium paper.[17] The mutations are detailed in table 1. They comprised eight single base substitutions, four small insertions, three small deletions, and one larger deletion of 23 bp, giving rise to six nonsense mutations, eight frameshifts, and two putative splicing mutations. In the patient with the germline mutation 2105delAAAG, analysis of a renal cell carcinoma confirmed the presence of this mutation together with a second mutation, 1957delG, in the other allele which was not present in the germline[17] (fig 1). All the mutations would be predicted to result in a truncated protein.

Several single base substitutions were found which were not considered to be pathogenic (table 2). The coding polymorphisms 1186 T>C (M322T) in exon 10 and 3050 C>T (A943A) in exon 22 were observed in several patients and had heterozygosities of 30% and 18% respectively in normal controls. The rarer polymorphism 2415 C>T (H732Y) in exon 17

Table 2 Polymorphisms identified in the TSC1 gene

Mutation	Location	Heterozygosity (No of controls)	Comments
753 G>A; V178I	Exon 7	0% (100)	Not present in affected daughter
1186 T>C; M322T	Exon 10	30% (20)	Present in 8% of patients
2415 C>T; H732Y	Exon 17	2% (45)	
2646 G>C; E809Q	Exon 19	0% (56)	Present in unaffected father
2867 C>T; A882A	Exon 21	0% (67)	Observed in 2 unrelated patients
3050 C>T; A943A	Exon 22	18% (71)	Present in 20% of patients

Table 3 Mutation detection rate in sporadic and familial cases predicted to have TSC1 mutations compared to cases where locus unassigned. Number of mutations detected and number of patients screened shown in brackets

	Sporadic	Familial	Total
Predicted TSC1 cases	67% (2/3)	86% (6/7)	80% (8/10)
Unassigned cases	11% (6/57)	12% (2/16)	11% (8/73)
All cases	13% (8/60)	35% (8/23)	19% (16/83)

was present in one patient and in one of 90 control chromosomes. It has previously been reported as a rare variant.[22] The silent mutation 2867 C>T (A882A) in exon 21 was observed in two unrelated patients but not in 134 normal chromosomes. This base change would not be expected to have an effect on splicing. Two missense mutations failed to segregate with the disease. One patient had the mutation 753 G>A resulting in the substitution of isoleucine for valine at codon 178. This conservative change was not present in her affected daughter and is likely to be a rare polymorphism of her normal TSC1 allele. Another patient who was a sporadic case had the mutation 2646 G>C resulting in the substitution of glutamine for glutamic acid at codon 809. This mutation was present in his unaffected father.

In the 10 cases predicted to have TSC1 mutations by linkage analysis or loss of heterozygosity studies, eight mutations were identified giving a detection rate of 80% (table 3). In the remaining 73 unassigned cases, eight mutations were found, giving a detection rate of only 11%. From these data we estimate that TSC1 mutations account for 24% of the patients tested and 22% of the whole patient panel including cases with TSC2 mutations. The overall detection rate in familial cases was 35% as compared to 13% in sporadic cases, but the difference did not reach statistical significance (Fisher's exact p value=0.06).

Discussion

We have reported the results of mutation screening of the TSC1 gene in 83 unrelated cases of tuberous sclerosis using SSCP and heteroduplex analysis to test all 21 coding exons amplified from genomic DNA. Mutations were detected in 19% of the patients tested (representing 17% of the whole patient panel including cases with TSC2 mutations). This is comparable to the study by Jones *et al*[22] who identified mutations in 13% of their patients using a similar approach. The mutations were either single base substitutions, small deletions, or insertions giving rise to nonsense mutations, frameshifts, or aberrant splicing. This is similar to the mutation spectrum reported by others.[17 22]

Three-quarters of the mutations identified were novel changes only observed in a single family and these data bring to 54 the total number of different mutations reported.[17 22] This illustrates the great diversity of mutations that occur in the TSC1 gene. The recurrent mutations were 2105delAAAG reported in six unrelated families, 2577 C>T in four families, 1746 C>T in three families, and 2887insA in two unrelated patients. Mutations were distributed throughout the gene with a tendency to cluster in exon 15, the largest exon. Mutations have now been reported in the majority of the coding exons, the exceptions being exons 3, 8, 14, 16, 22, and 23.[17 22] During the course of this study several coding polymorphisms were also identified (table 2). Of these, only two occurred at an appreciable frequency, 1186 T>C (M322T) in exon 10 with a heterozygosity of 30% and 3050 C>T (A943A) in exon 22 with a heterozygosity of 18%. Both have been reported previously.[22]

All the mutations described here, in common with the majority of reported mutations, would be predicted to result in a truncated protein with probable loss of protein function. This is consistent with the hypothesis that TSC1 acts as a tumour suppressor gene. Additional support for this comes from the analysis of tumours from two patients in whom we identified the germline mutation and had previously shown loss of heterozygosity for TSC1 markers in the lesions.[13 15] In one case with the germline mutation 1995insGA, we were able to confirm that in the hamartoma (a giant cell astrocytoma) only the mutant allele was present. In another patient with the germline mutation 2105delAAAG, analysis of a renal cell carcinoma confirmed the presence of this mutation together with a separate inactivating somatic mutation 1957delG of the wild type allele[17] (fig 1). This directly implicates the TSC1 gene in the pathogenesis of the tumour.

Only two missense mutations have been reported in the TSC1 gene. This is in contrast to TSC2 where missense mutations are relatively common.[4-10] There are other important differences in the mutation spectrum. No large scale germline rearrangements have been observed in TSC1, whereas large deletions of TSC2 occur in up to 5% of cases.[4] Similarly, in TSC hamartomas, large somatic deletions encompassing TSC1 are rare, whereas somatic TSC2 gene deletions occur in about 30% of lesions.[15 16]

The identification of the TSC1 gene is an important step in providing a molecular genetic diagnostic service for families affected by TSC. However, screening of 21 exons amplified from genomic DNA is labour intensive and analysis of TSC1 mRNA may be a more efficient approach. Since most TSC1 mutations are truncating, the analysis of TSC1 mRNA by PTT might provide an effective mutation screening strategy. We have previously shown that mutations can be detected in the TSC2 gene by analysis of mRNA using the protein truncation test (PTT).[9]

In developing protocols for mutation screening, important considerations are mutation detection rates and the frequencies of TSC1 and TSC2 mutations in familial and sporadic

cases. In the current study, there were 10 cases predicted to have TSC1 mutations by linkage analysis or loss of heterozygosity studies and eight mutations were identified, giving an estimated mutation detection rate for TSC1 mutants of 80%. This would be comparable to the detection rates achieved with SSCP/heteroduplex analysis in studies of other genes. On the assumption that the same detection rate applies to the unassigned cases where another eight mutations were found, we would estimate that two TSC1 mutations were missed. This would give an estimated 20 TSC1 mutants in the sample of 83 patients, representing 24% of the patients tested and 22% of the whole patient panel including cases with TSC2 mutations. This is at odds with the data from linkage studies suggesting that TSC1 and TSC2 contribute equally to familial cases of tuberous sclerosis.[3] In the Consortium paper, the mutation detection rate in patients predicted to have TSC1 mutations by linkage analysis was also substantially higher than in unassigned cases.[17] Jones *et al*[22] reported a significant under-representation of TSC1 mutations in sporadic cases, finding mutations in 37% (9/24) of familial cases as compared to 9% (13/147) of sporadic cases. To account for this, they suggested that the mutation rate in the TSC2 gene is higher than in TSC1, but mutations in TSC2 are associated with a lower reproductive fitness, giving rise to more equal representation in families. In the present study, the comparable figures for TSC1 mutation detection were 35% (8/23) in familial cases and 13% (8/60) in sporadic cases (table 3), showing the same trend but failing to reach statistical significance. More data will be needed to determine whether the under-representation of TSC1 mutations is confined to sporadic cases or also applies to families.

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