Am. J. Hum. Genet. 64:1632–1637, 1999

High Rate of Mosaicism in Tuberous Sclerosis Complex

Senno Verhoef,[1] Lida Bakker,[1] Anita M. P. Tempelaars,[1] Arjenne L. W. Hesseling-Janssen,[1] Tadeusz Mazurczak,[5] Sergiusz Jozwiak,[4] Alberto Fois,[6] Gabriella Bartalini,[6] Bernard A. Zonnenberg,[2] Anthonie J. van Essen,[3] Dick Lindhout,[1] Dicky J. J. Halley,[1] and Ans M. W. van den Ouweland[1]

[1]M. G. C. Department of Clinical Genetics, Erasmus University and Academic Hospital, Rotterdam, [2]Department of Internal Medicine, Academic Hospital Utrecht, Utrecht, [3]Department of Medical Genetics, State University Groningen, Groningen, The Netherlands; [4]Department of Neurology, Szpital Pomnik-Centrum Zdrowia Dziecka, and [5]Department of Genetics, Institute of Mother and Child, Warsaw; and [6]Università degli Studi di Siena, Istituto di Clinica Pediatrica, Siena, Italy

Summary

Six families with mosaicism are identified in a series of 62 unrelated families with a mutation in one of the two tuberous sclerosis complex (TSC) genes, *TSC1* or *TSC2*. In five families, somatic mosaicism was present in a mildly affected parent of an index patient. In one family with clinically unaffected parents, gonadal mosaicism was detected after TSC was found in three children. The detection of mosaicism has consequences for genetic counseling of the families involved, as changed risks apply to individuals with mosaicism, both siblings and parents. Clinical investigation of parents of patients with seemingly sporadic mutations is essential to determine their residual chance of gonadal and/or somatic mosaicism, unless a mosaic pattern is detected in the index patient, proving a de novo event. In our data set, the exclusion of signs of TSC in the parents of a patient with TSC reduced the chance of one of the parents to be a (mosaic) mutation carrier from 10% to 2%. In the five families with somatic mosaicism, the parent was given the diagnosis after the diagnosis was made in the child.

Introduction

Tuberous sclerosis complex (TSC) is an autosomal dominant, hereditary disease, characterized by the growth of hamartomas and hamartias that can occur in almost all tissues. Most lesions occur in the brain (subependymal nodules or giant cell astrocytomas, or subcortical and cortical tubers), in the skin (facial angiofibromas, white macules, shagreen patches, or ungual fibromas), in the kidneys (cysts and angiomyolipomas), in the heart (rhabdomyomas), and in the eyes (retinal hamartomas). Patients with severe disease suffer from epilepsy (found in 60%–70% of patients with TSC) and mental retardation (found in ~40%–50% of patients with TSC). Mortality due to TSC is mostly from two complications: (1) renal bleeding from an angiomyolipoma or (2) the development of an intracerebral giant cell astrocytoma. The prevalence of TSC is estimated at ~1/10,000 births. More than half the patients with TSC represent sporadic cases, reflecting a high mutation frequency, estimated at 2.5/10,000/gene in each generation (Sampson et al. 1989).

TSC is caused by a mutation in either the *TSC1* gene, located on chromosome 9 (MIM 191100), or the *TSC2* gene, on chromosome 16 (MIM 191092). After both genes were characterized, the identification of mutations became feasible. Mutation analysis for both genes reveals that most families have a unique mutation, although recurrent mutations have been described (van Slegtenhorst et al. 1997, 1999; Au et al. 1998; Verhoef et al. 1998). Mutation detection has provided an opportunity to study possible relationships between genotype and phenotype, to investigate presumed de novo mutations, and, occasionally, to provide diagnostic certainty in individuals with a particularly mild clinical phenotype.

Reports of "apparent nonpenetrance" in TSC, with multiple offspring of clinically unaffected parents, suggested that parental mosaicism could be expected, as had been suggested by Hall and Byers (1987). Several examples of somatic (Verhoef et al. 1995; Sampson et al. 1997) and gonadal (Yates et al. 1997) mosaicism in TSC have been reported.

We present six families in which mosaicism was detected in a group of 62 unrelated families with TSC with a known mutation (from our study population of 225

Received October 16, 1998; accepted for publication March 16, 1999; electronically published May 3, 1999.

Address for correspondence and reprints: Dr. S. Verhoef, MGC Department of Clinical Genetics, Erasmus University and Academic Hospital Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands. E-mail: Verhoef@kgen.fgg.eur.nl

0002-9297/99/6406-0015$02.00

families). All five individuals with somatic mosaicism had mild phenotypic features of TSC; in one family the mosaicism was proved to be gonadal and, possibly, somatic as well. Clinical data on the affected individuals in the families are presented.

Subjects and Methods

The present study was approved by the Medical Ethics Committee of the Academic Hospital Rotterdam Dijkzigt.

Subjects

Clinical data and blood samples from patients with TSC have been collected since 1987. Data from a total of 350 individuals with TSC from 225 unrelated families have been entered in a clinical registry. Mutation analysis is generally performed with the use of DNA from the index patients of each family. Details of the affected family members presented in this paper are discussed in the Results section.

Methods

Mutation analysis was undertaken in 225 unrelated patients with TSC. The DNA of affected individuals (and parents, when available) was isolated from peripheral leukocytes obtained by venipuncture. Mutation analysis of the *TSC1* gene consisted of SSCP analysis of all coding exons. Primer sequences used for the amplification reactions are those described by van Slegtenhorst et al. (1999). Direct-sequence analysis was performed according to standard methods. For the identification of mutations in the *TSC2* gene, Southern blotting was used to detect large-size abnormalities, followed by SSCP analysis for all coding exons (still in progress). Relevant primer sequences are given in table 1; the other exon primers are used as published elsewhere (Au et al. 1998).

Southern blotting was done according to standard procedures by means of genomic DNA digested with the restriction enzymes *Hin*dIII and *Eco*RI. Probes used for analysis of large deletions in the *TSC2* gene were 4B2, 4.9E0.7, and 1A1 (The European Chromosome 16 Tuberous Sclerosis Consortium 1993).

Oligonucleotides were designed for the normal and the mutated sequences (table 1). Allele-specific oligonucleotide (ASO) hybridizations were performed on duplicate filter sets at 37°C for 30 min. Filters were washed in 0.3 × SSC for 10 min at 37°C.

For haplotype analysis in family 2, flanking markers were used on chromosome 9: D9S2126 and D9S1830, proximal to *TSC1;* D9S1199 and D9S1198, distal to the gene (Van Slegtenhorst et al. 1997). Reverse-transcription (RT)-PCR analysis for family 4 was done on total RNA from fibroblast cells of the index patient according to standard procedures. The primers used in the RT-PCR reaction were forward 5′-GCAAAGATTCAGGCTTGAAGG-3′ (exon 1) and 5′-CCACATTCCATGCTCAGT-3′ (exon 2). Direct-sequence analysis was performed on the isolated aberrant RT-PCR product with the same primers. Families were tested to confirm family structure with the Profiler Plus Kit (Perkin-Elmer), which contains nine highly polymorphic markers from nine different chromosomes.

Table 1

Oligonucleotides for Amplification of Exons and Introns for SSCP Analysis and for ASO Hybridization

Method, Gene, and Primer Type	Primer Sequence
SSCP:	
TSC1:	
Exon 8:	
Forward	5′-attcagccctttataatttgtcaac-3′
Reverse	5′-ctcctagatcacattttcaatctctc-3′
Exon 12:	
Forward	5′-tgttctgcccttgtctctaag-3′
Reverse	5′-agtgagtcactgtgcctgg-3′
TSC2:	
Intron 1:	
Forward	5′-cagaggtgttgctcagatgtccc-3′
Reverse	5′-atttccctctagcctagcaaaga-3′
Intron 20:	
Forward	5′-ggcctgaggtgtcctgtct-3′
Reverse	5′-agtgctgcagggcggggac-3′
ASO:	
TSC1:	
942insA:	
Normal	5′-ggaccatgaactgg-3′
Mutant	5′-ggaccatgaaactg-3′
1473delC:	
Normal	5′-tcacacccccag-3′
Mutant	5′-tcacacccccagg-3′
TSC2:	
156+1G→A:	
Normal	5′-atactgagagtgagtga-3′
Mutant	5′-aatactgagaatgagtga-3′
2374−2A→C:	
Normal	5′-tcctgcagcgcga-3′
Mutant	5′-tcctgccgcgcga-3′

Results

After mutation analysis of the complete coding region of the *TSC1* gene, Southern blotting for the *TSC2* gene, and SSCP analysis of ~25% of the coding region of the *TSC2* gene, 29 mutations were detected in *TSC1* and 33 mutations were detected in *TSC2*. In five of the 62 families, parental somatic mosaicism could be proved. In the sixth family, occurrence of TSC in three sibs with clinically unaffected parents was consistent with germline mosaicism, as no somatic copies could be detected in the parents (fig. 1).

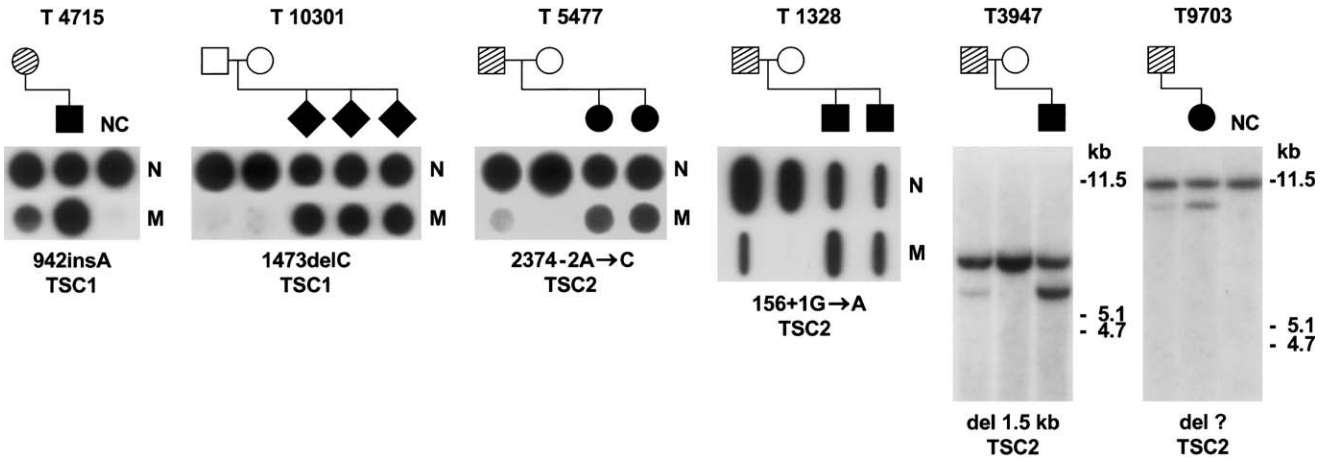

Figure 1 Six families with TSC showing mosaicism. From left to right are *TSC1* mutations 942insA and 1473delC and TSC2 mutations 2374-2A→C (all are dot blots of ASO hybridization analysis), 156+1G→A (slot blot of ASO hybridization analysis), a deletion of 1.5 kb (probe 4.9E0.7), and a deletion of unknown size (probe 4B2), on Southern blot analysis. In each pedigree, squares denotes males, circles denote females, and diamonds denote individuals whose sex is not indicated (for purposes of confidentiality); in each pedigree, the person with mosaicism is denoted by the hatched symbols. NC = negative control sample.

Mosaicism for Mutations in the TSC1 *Gene*

Family 1 (*T4715*).—A boy had epilepsy, mental retardation, facial angiofibroma, ungual fibroma, white macules, a shagreen patch, and gingival fibromas. Cortical tubers and subependymal nodules were visible on the CT scan. His mother had normal intelligence and no epilepsy, but, when examined, was found to have facial angiofibroma, white macules, and a shagreen patch on the skin. A CT scan of the mother's brain showed subependymal nodules. SSCP analysis showed an abnormal pattern of exon 8 of the *TSC1* gene. Direct-sequence analysis resulted in the identification of a frameshift mutation 942insA (Van Slegtenhorst et al. 1999). ASO hybridization analysis showed that the ratio of the mutant-to-normal allele was less in the DNA of the mother than in that of her son.

Family 2 (*T10301*).—The index patient was 9 years old, the sixth child in a large family. His medical history revealed congenital cardiac tumors, detected antenatally with ultrasound after analysis of irregular fetal heart rhythm. The tumors had regressed with age. At age 8 years, myoclonic epilepsy occurred. White macules were then noted on the skin. On the basis of these findings, together with the congenital heart tumors (most probably rhabdomyomas), a clinical diagnosis of TSC was made. A CT scan of the brain showed subependymal nodules and confirmed the diagnosis. The youngest sibling developed epilepsy at age 2 years and had three hypopigmented macules on the skin. A CT scan showed subependymal nodules and an irregular cortical gyration pattern, demonstrative of TSC. Both parents were presumed to be unaffected (by skin examination only; they had no history of epilepsy or learning difficulties). SSCP analysis of the *TSC1* gene in the index patient in this family showed an abnormal pattern of exon 12. Direct sequencing followed by ASO hybridization analysis showed a frameshift mutation 1473delC (Van Slegtenhorst et al. 1999). Subsequent analysis of DNA from the complete family with ASO hybridization analysis identified the mutation in both clinically affected children and in a third sib, aged 10 years, suspected of having TSC because of developmental delay and possibly the beginning of a facial angiofibroma. The mutation could not be detected in DNA from blood cells of the parents, suggesting mosaicism. Using flanking markers to the *TSC1* gene, we performed haplotyping of the parents and all children. In addition to the three affected children, two more children were shown to carry the affected allele without the mutation, proving gonadal mosaicism in one of the parents (results not shown). Because the parents do not wish to know their carrier status, the identity of the parent of origin cannot be disclosed, and further clinical evaluation of the parents has been postponed.

Mosaicism for Mutations in the TSC2 *Gene*

Family 3 (*T5477*).—The elder of two sisters had epilepsy between the ages of 6 mo and 1 year. Subsequently she was diagnosed with moderate mental retardation, facial angiofibroma, white macules, a shagreen patch, and an intracardiac tumor. A CT scan of the brain confirmed the diagnosis of TSC. Her younger sister had epilepsy with severe mental retardation, facial angiofibroma, and ungual fibromas. She also had a cardiac

tumor and renal angiomyolipoma. A CT scan of the brain confirmed TSC in her as well. Their father appeared to have facial angiofibroma, white macules, and a shagreen patch on the skin, depigmentations of the retina, angiomyolipoma of the kidney, and gingival fibroma of the mouth. He had no epilepsy or mental retardation. In this family, SSCP analysis followed by direct sequencing of intron–exon boundaries led to the detection of a splice-site mutation 2374-2A→C in intron 20 of the *TSC2* gene, present at a mosaic level in the father. The effect of this splice-site mutation could not be studied, because no RNA was available.

Family 4 (*T1328*).—The oldest son in this family had facial angiofibroma, white macules, subependymal nodules, and cortical tubers. Although he had suffered from infantile spasms, he had normal intelligence. His brother, 14 mo younger, had facial angiofibroma, white macules, subependymal nodules, and cortical tubers, with epilepsy and mild mental retardation. He showed cardiac rhabdomyoma and a single gingival fibroma. Facial angiofibroma and white macules were subsequently seen in the father. A CT scan of his brain revealed subependymal nodules. He had no retardation or epilepsy. Mutation analysis resulted in the identification of a splice-site mutation 156+1G→A in intron 1 of the *TSC2* gene. RT-PCR analysis, with primers located in exon 1 and exon 2, resulted in the identification of an abnormal fragment (fig. 2). Direct-sequence analysis of the aberrant fragment with the same primers showed the use of a cryptic splice site in exon 1 at nucleotide position 105–106 (numbering according to The European Chromosome 16 Tuberous Sclerosis Consortium 1993), causing a frameshift resulting in a stop codon at amino acid position 31. This mutation was previously described in another family (Kumar et al. 1997). The signal of the mutant allele was clearly reduced in the father, shown by ASO hybridization analysis, indicating somatic mosaicism.

Family 5 (*T3947*).—This family was originally reported as the first family with TSC showing somatic mosaicism, proved by DNA analysis (Verhoef et al. 1995). A 4-year-old boy had epilepsy for 2 years and multiple white macules. Periventricular subependymal calcifications and cortical hypodensities were seen on his CT scan, proving the diagnosis of TSC. The father showed mild signs of TSC, with subtle facial angiofibroma, dental pits, and, on a CT scan, one paraventricular calcified nodule, but no epilepsy or mental retardation. A 1.5-kb deletion in the *TSC2* gene was detected with Southern blot analysis with marker 4.9E0.7 on *Pvu*II-digested DNA. A consistent difference in signal intensities between normal and aberrant fragments demonstrated somatic mosaicism in the father. This result was confirmed from a newly collected blood sample.

Family 6 (*T9703*).—The index patient in this family was a 2-year-old girl, given a diagnosis of TSC when analyzed for her epilepsy. The parents were then screened. The father was found to have ungual fibromas and facial angiofibroma. A CT scan of the brain revealed several subependymal nodules, and a renal CT scan showed multiple small angiomyolipomas. The father had no history of epilepsy and had normal intelligence. Southern blot analysis, with probes 4.9E0.7 and 4B2, on DNA of the index patient indicated a deletion in the *TSC2* gene. Despite the use of a combination of different restriction enzymes, the exact length of the deletion could not be determined. Her father has a changed ratio of normal to abnormal signals and carries somatic mosaicism. The clinical data on all individuals carrying the mosaicism are summarized in table 2.

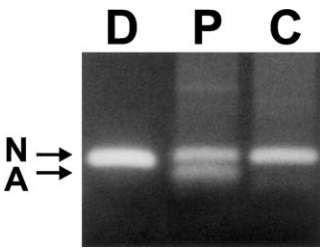

Figure 2 RT-PCR products of cDNA clone containing the complete coding region of the *TSC2* gene (lane D), from fibroblasts of one of the patients with TSC in family 4 (lane P), and from fibroblasts of a negative control (lane C). N = normal RT-PCR fragment; A = aberrant RT-PCR fragment.

Discussion

In general, a high rate of mosaicism has been predicted for conditions with a high percentage of new mutations (Hall 1988) and has recently been demonstrated for another tumor suppressor gene syndrome, neurofibromatosis type 2 (Kluwe and Mautner 1998). Our results indicate a level of gonadal and/or somatic mosaicism of ~10% (6/62) in the group with TSC with a known mutation. The true prevalence of mosaicism in our study population of patients with TSC is probably higher than the 10% (6/62) detected in the present study, because in 17 of the 62 families the parents were not investigated. The proportion of sporadic patients with TSC with a severe phenotype who are mosaic is unknown, as most mutations in TSC are unique mutations, and a distinction between high-grade mosaicism and nonmosaicism can be impossible. Conversely, a low level of mosaicism in blood cells can be undetectable; thus, the mutation will remain unidentified unless other tissues from the patient are tested.

According to empirical, worldwide data on families with TSC, the incidence of gonadal mosaicism in clin-

Table 2

Clinical Signs of the Person Mosaic for the Mutation

Variable	Designation or Status, by Laboratory (Family) and Relationship						
	4715 (1), mother	10301 (2)[a]		5477 (3), father	1328 (4), father	3947 (5), father	9703 (6), father
		Father	Mother				
No. of affected children	1	3	3	2	2	1	1
Symptom or sign:[b]							
Facial angiofibroma	+	−	−	+	+	+/−	+
White macules	+	−	+/−	+	+	−	−
CT-scan abnormalities	+	Unknown	Unknown	Unknown	+	+/−	+
Intelligence	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Epilepsy	−	−	−	−	−	−	−

[a] Both parents were clinically unaffected—but were incompletely examined.
[b] + = Present; − = absent; +/− = single symptom/minimal sign.

ically unaffected parents of children with TSC is ~1%–2% (Berberich and Hall 1979; Baraitser and Patton 1985; Connor et al. 1986; Rott and Fahsold 1991; Webb and Osborne 1991; Ruggieri et al. 1997). In our study, one such family with mosaicism was detected as a result of multiple affected offspring from healthy parents. Gonadal mosaicism was proven by haplotype analysis, whereas the presence or absence of somatic mosaicism could not be investigated extensively. Gonadal mosaicism was recently reported for TSC (Yates et al. 1997). Both in the family reported by Yates and in the family we studied, it remains uncertain as to whether the mosaicism was present in the gonads only or in other tissues as well.

In all five persons in whom somatic mosaicism was detected, the diagnosis of TSC was made in their children first. Subsequent clinical investigations of the parents showed that the mosaic parents did fulfill the diagnostic criteria of TSC (Roach et al. 1992), although all these parents had normal intelligence and no epilepsy.

For the individual with mosaicism, the risk for future offspring to inherit the mutation is dependent on the proportion of germ cells that contain the mutation. This risk will be $\leqslant 50\%$, as has been shown for other conditions (Zlotogora 1998). An exact estimate of the level of mosaicism in the gonads is difficult to obtain, although sperm analysis is technically possible. Siblings and parents of a patient with proven mosaicism have a population risk of a TSC mutation, because a mosaic mutation is presumably a postzygotic event and thus excludes gonadal mosaicism in one of the parents as a source of the mutation.

Complete clinical screening of parents of seemingly sporadic patients with TSC remains essential to exclude the possibility of a mild phenotype of which parents are not aware, to which somatic mosaicism might contribute. If parents do not show any signs of TSC at full clinical evaluation (including a brain CT scan), the chance of one of them having gonadal mosaicism would be ~2% (in our data set, one family in 45). A substantially higher possible overall mosaicism figure (10% in our data set) might need to be given until parents have completed clinical investigations and have shown no abnormalities.

Since low-degree somatic and gonadal mosaicism can remain undetected in DNA from blood cells, we discuss the low-percentage chance of gonadal mosaicism with parents of a child with TSC seemingly due to a de novo mutation and would be inclined to offer prenatal testing for the mutation in a further pregnancy if requested by the parents. In view of the serious consequences for genetic counseling of families with TSC, a larger series of mosaic and nonmosaic parents would be required, with a more systematic genotype–phenotype analysis, to show whether somatic mosaicism contributes to a milder phenotype in TSC.

Acknowledgments

The authors thank Sarvan Ramlakhan, Yavuz Ariyurek, Qi Wang, and Radek Vrtel, for their technical assistance, and Prof. H. Galjaard, for his continuous support. This work is supported by Zorg Onderzoek Nederland grant 28-1723-1.

Electronic-Database Information

The URL for data in this article is as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for *TSC1* [MIM 191100] and *TSC2* [191092])

References

Au K-S, Rodriguez JA, Finch JL, Volcik KA, Roach ES, Delgado MR, Rodriguez E Jr, et al (1998) Germ-line mutational analysis of the TSC2 gene in 90 tuberous-sclerosis patients. Am J Hum Genet 62:286–294

Baraitser M, Patton MA (1985) Reduced penetrance in tuberous sclerosis. J Med Genet 22:29–31

Berberich MS, Hall BD (1979) Penetrance and variability in tuberous sclerosis. Birth Defects 15:297–304

Connor JM, Stephenson JB, Hadley MD (1986) Non-penetrance in tuberous sclerosis. Lancet 2:1275

The European Chromosome 16 Tuberous Sclerosis Consortium (1993). Identification and characterization of the tuberous sclerosis gene on chromosome 16. Cell 75: 1305–1315

Hall JG (1988) Somatic mosaicism: observations related to clinical genetics. Am J Hum Genet 43:355–363

Hall JG, Byers PH (1987) Genetics of tuberous sclerosis. Lancet 1:751

Kluwe L, Mautner V-F (1998) Mosaicism in sporadic neurofibromatosis 2 patients. Hum Mol Genet 7:2051–2055

Kumar A, Kandt RS, Wolpert C, Roses AD, Pericak-Vance MA, Gilbert JR (1997). A novel splice site mutation (156+1G→A) in the TSC2 gene. Hum Mutat 9:64–65

Roach E, Smith M, Huttenlocher P, Bhat M, Alcorn D, Hawley L (1992) Diagnostic criteria: tuberous sclerosis complex. J Child Neurol 7:221–224

Rott HD, Fahsold R (1991) Tuberous sclerosis in two sibs of normal parents. Clin Genet 39:306–308

Ruggieri M, Carbonara C, Magro G, Migone N, Grasso S, Tiné A, Pavone L, et al (1997) Tuberous sclerosis complex: neonatal deaths in three of four children of consanguineous, non-expressing parents. J Med Genet 34:256–260

Sampson JR, Maheshwar MM, Aspinwall R, Thompson P, Cheadle JP, Ravine D, Roy S, et al (1997) Renal cystic disease in tuberous sclerosis: role of the polycystic kidney disease 1 gene. Am J Hum Genet 61:843–851

Sampson JR, Scahill SJ, Stephenson JBP, Mann L, Connor JM (1989). Genetic aspects of tuberous sclerosis in the west of Scotland. J Med Genet 26:28–31

van Slegtenhorst M, de Hoogt R, Hermans C, Nellist M, Janssen B, Verhoef S, Lindhout D, et al (1997) Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34. Science 277:805–808

van Slegtenhorst M, Verhoef S, Tempelaars A, Bakker L, Wang Q, Wessels M, Bakker L, et al (1999) Mutational spectrum of the TSC1 gene in a cohort of 225 tuberous sclerosis complex patients: no evidence for a genotype-phenotype correlation. J Med Genet 36:285–289

Verhoef S, Vrtel R, Bakker L, Stolte-Dijkstra I, Nellist M, Begeer JH, Zaremba J, et al (1998) Recurrent mutation 4882delTT in the GAP-related domain of the tuberous sclerosis TSC2 gene. Hum Mutat Suppl 1:S85–S87

Verhoef S, Vrtel R, van Essen T, Bakker L, Sikkens E, Halley D, Lindhout D, et al (1995) Somatic mosaicism and clinical variation in tuberous sclerosis complex. Lancet 1:202

Webb DW, Osborne JP (1991) Non penetrance in tuberous sclerosis. J Med Genet 28:417–419

Yates JRW, Van Bakel I, Sepp T, Payne SJ, Webb DW, Nevin NC, Green AJ (1997) Female germ line mosaicism in tuberous sclerosis confirmed by molecular genetic analysis. Hum Mol Genet 6:2265–2269

Zlotogora J (1998) Germ line mosaicism. Hum Genet 102: 381–386