Identification of a nonsense mutation at the 5' end of the TSC2 gene in a family with a presumptive diagnosis of tuberous sclerosis complex

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Abstract

Tuberous sclerosis complex (TSC) is an autosomal dominantly inherited disease with a high mutation rate. It is clinically a very variable disorder and hamartomas can occur in many different organs. TSC shows genetic heterogeneity; one gene, TSC1, is on chromosome 9q34, and the second gene, TSC2, on chromosome 16p13.3.

Clinical criteria for diagnosis have been established, but diagnosis of patients with minimal expression of the disease can be very difficult. In children the phenotype is often incomplete or not fully assessable. Hence mildly affected subjects, at risk for severely affected offspring, may remain undiagnosed.

The detection of (small) mutations in the tuberous sclerosis gene located on chromosome 16 (TSC2) has recently become possible and may be helpful in the diagnosis of ambiguous cases. To our knowledge, this is the first report of a point mutation in the TSC2 gene in a familial case of tuberous sclerosis. A nonsense mutation was detected in a family in which the father had only minor signs hinting at tuberous sclerosis. The son had multiple cardiac tumours and white patches, but full clinical investigation was impossible in this child.

This case illustrates that mutation analysis can contribute to a diagnosis of tuberous sclerosis in families with an incomplete phenotype.

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Key words: tuberous sclerosis; TSC2 gene; nonsense mutation.

Tuberous sclerosis complex (TSC) is an autosomal dominant neurocutaneous disorder characterised by hamartomas affecting multiple organ systems, including skin, kidney, brain, and heart.¹ The most prominent clinical signs of brain dysfunction are mental retardation and seizures. TSC is a very variable disease with clinical manifestations ranging from very severe illness to absence of symptoms. Also, considerable variation is observed within families.² The prevalence of tuberous sclerosis has been

reported to be 1/6000 to 1/10 000.³ However, considering the variability of the disease, it has been suggested that the prevalence is underestimated.⁴

Locus heterogeneity has been shown by linkage analysis.⁵⁻⁸ One locus for tuberous sclerosis, TSC1, has been assigned to chromosome 9q34,⁹ while the second locus, TSC2, is on chromosome 16p13.3.¹⁰ Approximately half of the families can be linked to either locus. Sporadic patients represent about half of the total tuberous sclerosis population. Mutations are expected in equal proportion in either the TSC1 or TSC2 gene. No evidence exists for clinical difference in severity, nor in range of symptoms, between chromosome 9 and chromosome 16 linked families.¹¹ In one report there is a suggestion of a mild physical phenotype with behavioural problems in a chromosome 16 linked family.²

Recently, the TSC2 gene has been cloned.¹² Taking into account the loss of heterozygosity of the TSC2 containing region observed in renal angiomyolipoma, cardiac rhabdomyoma, and giant cell astrocytoma of tuberous sclerosis patients, it has been suggested that the TSC2 gene acts as a tumour suppressor gene.¹³

In tuberous sclerosis, rearrangements have been identified in 4% of the patients using pulsed field gel electrophoresis and Southern blotting.^{4,12,14} No point mutations, detectable by single strand conformation polymorphism (SSCP) and related techniques, have been reported. Mutation analysis for TSC might be compared to that of neurofibromatosis type 1 (NF1), another neurocutaneous disorder caused by mutations in a tumour suppressor gene.^{15,16} To date, no hot spot for mutations in the NF1 gene has been published.

In this article we report a point mutation in the TSC2 gene, present in two family members, a father and his son. Familial tuberous sclerosis was suspected but could not be proven on clinical grounds.

Case reports

At 20 weeks of her first pregnancy the mother of the index case was referred to a specialised unit for prenatal diagnosis because of fetal bradycardia and arrhythmia. At 24 weeks' gestation an intracardiac mass suspected of being a rhabdomyoma was detected by fetal ultrasound

Figure 1 Echocardiographic subcostal four chamber view showing the large septal tumour (arrow) and a smaller tumour (arrowhead) in the atrioventricular region. LA = left atrium, LV = left ventricle, RA = right atrium.

evaluation. The likely diagnosis of tuberous sclerosis was made.

Delivery of a boy, weighing 2500 g, was uncomplicated at 39 weeks. The postnatal ECG showed an intermittent second and third degree atrioventricular block. The echocardiogram showed a large multilobulated cardiac tumour and two additional smaller tumours, one of which was situated in the region of the atrioventricular node (fig 1). Echography of the brain was normal at 1 month of age. Investigation of the retina and echography of liver and kidneys showed no abnormalities. At 3 months of age a hypomelanotic macule, 25 × 15 mm, was noted on the buttock using Woods light. Recent evaluation at the age of 18 months showed the intracardiac tumours to be smaller. Although cardiac function was still impaired, atrioventricular conduction had normalised. Another hypomelanotic patch was seen on the leg, 15 × 15 mm. Psychomotor development was apparently normal. CT scan was postponed in the index case because of the risk of complications of complete anaesthesia. The features elicited in the child would allow a presumptive by not a definite diagnosis of TSC,

according to the criteria of Gomez (table), though the diagnostic criteria of Webb and Osborne would be fulfilled.¹⁷

The proband's father was evaluated at 30 years of age. CT scan of the brain, echography of the heart, and investigation of the skin (Woods light) and retina were normal. Echography of the kidney showed slightly increased echodensity with unilateral multiple echodensities 1 mm in diameter, located either in the capsula or in the peripheral cortex. These echodensities were non-specific, unlike renal angiomyolipoma, the characteristic renal lesion in TSC, which is generally located in the parenchyma in combination with renal cysts. On all tooth surfaces pit shaped enamel defects were seen, corresponding to the dental pits described in patients with tuberous sclerosis.¹⁸ In addition two gingival fibromas were found (fig 2). Thus, the father shows two "suspect" oral symptoms (table).

In summary, from the combination of signs in the father and son it is very likely that they both have TSC. All first degree relatives of the father and son were screened for signs of tuberous sclerosis, but no abnormalities were detected on full clinical evaluation.

Methods

CLINICAL EXAMINATION

All first degree relatives of III-1 and II-3 were screened for signs of tuberous sclerosis. Physical examination, dermatological examination with Woods lamp, a CT scan of the brain, x rays of both hands and feet, and echographical examination of the heart and kidneys were performed in all family members, including the father of the proband, and were normal.

MUTATION ANALYSIS

In principle all patients included in the mutation analysis fulfilled the diagnostic criteria of Gomez,¹⁹ unless circumstantial evidence, like in this case, was highly suggestive of tuberous sclerosis or related disease. Genomic DNA was extracted from peripheral blood leucocytes by the salting out procedure.²⁰

Structure of the TSC2 gene

A genomic phage library of the TSC2 region was prepared. Exon-intron boundaries were determined by sequencing with vector primers (Maheshwar *et al*, manuscript submitted).

Exon amplification

SSCP analysis was performed according to the method described by Orita *et al*.²¹ Primers for amplification of exon 1 of the TSC2 gene were as follows: forward 5'-cagaggtgtgtcagatgtccc-3', reverse 5'-attccctctagcctagcaaaga-3'. The length of the normal PCR product is 256 base pairs. PCR conditions for 10 µl volume were 1 mmol MgCl₂, 0.5 mmol spermidine, 1 pmol of each primer, 200 µmol mix of dATP, dGTP, dTTP, 2.5 µmol dCTP, 0.06 µl ³²P dCTP (10mCi/ml) (ICN), and 0.2 U of *Taq* poly-

Figure 2 The tooth surfaces of the father (II-3) with pit shaped enamel defects and two gingival fibromas.

Clinical or imaging features for the diagnosis of TSC (adapted from Gomez¹⁹)

Diagnostic features*

Organ	Definitive signs	Presumptive signs	Suspect signs
CNS	Cortical tubers Subependymal nodules		Infantile spasms Seizures
Retina	Hamartomas	Harmartoma	
Skin	Facial angiofibromas Ungual fibroma Fibrous forehead plaque Multiple angiomyolipomas	Confetti-like spots	Hypomelanotic maculas
Kidneys		Angiomyolipoma	Cysts
Heart		Multiple rhabdomyomas	Rhabdomyoma
Teeth			Enamel pits
Gingiva			Fibromas
Bones			Cysts Osteoma thickening

Symptoms at the time of clinical assessment in the K12X family

Person	Definitive signs	Presumptive signs	Suspect signs
Grandmother	—†	—	—
Father	—	—	Enamel pits Gingival fibromas Hypomelanotic maculas
Son	—‡	Multiple rhabdomyomas	

* Only the more frequent signs that are normally screened for in relatives of TSC patients are mentioned.

† Diagnostic signs mentioned in top part of table excluded by adequate clinical examination.

‡ CT scan postponed in the index case.

merase (Gibco/BRL). Thermal cycling conditions were six minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C, 90 seconds at 72°C, with a final elongation of six minutes at 72°C. Electrophoresis was performed on a 6% polyacrylamide gel with 10% glycerol. Running time was 14 hours, 5 Watts, both at 4°C and at room temperature.

Sequence analysis

For sequencing of PCR products, 100 µl of PCR product was purified and concentrated on microcon 30 (Amicon). The double stranded DNA cycle sequencing system (Gibco/BRL) was used to sequence PCR fragments directly: six minutes at 94°C followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C, 60 seconds at 72°C, and six minutes at 72°C in the last cycle. Sequencing of both strands was performed with the primers used for the SSCP analysis.

ASO hybridisation

For the ASO hybridisation the sequence of the "normal" oligonucleotide was 5'-CTTGAAG-GAGAAGTTTA-3' and for the "mutant" oligonucleotide 5'-CTTGTAGGAGAAGTTT-A-3'. Hybridisation was performed at 37.5°C for 30 minutes. Filters were washed to 0.3 SSC for 10 minutes at 37.5°C.

Marker analysis

Markers 3'HVR, KG8, and 16AC2.5 (D16S291) were analysed according to standard procedures.²²

Results

Mutation analysis of the TSC2 gene in TSC patients has been performed using pulsed field gel electrophoresis (PFGE) and Southern blot-

Figure 3 Allele specific oligonucleotide hybridisation for the detection of the K12X mutation. N = normal allele. M = the mutated allele. Note that the normal alleles are of about equally reduced intensity in the affected family members II-3 and III-1.

ting analysis, resulting in the detection of a number of large deletions.^{4,12,14} However, the majority of the TSC2 gene defects are expected to be small mutations. To facilitate the detection of such mutations, the genomic organisation of the TSC2 gene was determined (Maheshwar *et al*, manuscript submitted) and exon specific primers were designed for single strand confirmation polymorphism (SSCP) analysis.

DNA from 116 unrelated TSC patients was analysed to detect mutations in the first exon (nucleotides 1–156 of the published TSC2 cDNA sequence).¹² In one case an additional band was observed. Subsequent sequencing showed an A→T transition at nucleotide position 52, resulting in a change of lysine (AAG) to a stop codon (TAG) at amino acid position 12 (K12X). Allele specific oligonucleotide hybridisation (ASO) was performed on DNA from all family members (fig 3). The K12X mutation was also present in the proband's father. The other family members, including the twin sisters of the father and their parents, were homozygous for the normal allele. To trace the origin of the mutated allele, linkage analysis with the VNTR probe 3'HVR, located distally to the TSC2 gene, and polymorphic CA repeat markers KG8 and 16AC2.5 (D16S291) was undertaken. KG8, the closest marker just proximal to the TSC2 gene, was not informative. 16AC2.5 is positioned about 200 kb proximal to the TSC2 gene.²³ Both informative flanking markers proved the mutated chromosome to be of grandmaternal origin. Both twin sisters had received the other grandmaternal chromosome 16 (data not shown).

Discussion

Tuberous sclerosis is a clinically and genetically heterogeneous disease. Half of the patients are expected to have a mutation in the TSC2 gene. From our file of 119 patients, three patients had large deletions in the gene.^{12,14} On the

hypothesis of an equal distribution of mutations across the TSC2 gene, which contains 41 exons (Maheshwar *et al*, manuscript submitted), one or two mutations per exon per 100 patients may be expected. SSCP analysis of the first exon of the TSC2 gene resulted in the identification of the first point mutation. No other mutations were detected in the first exon.

The mutation was found only in the father and in the son. Analysis with flanking polymorphic markers showed that the mutated allele is of grandmaternal origin. After complete clinical examination, the grandmother showed no signs of tuberous sclerosis. Germline mosaicism in the grandmother or a de novo mutation in the father in the early stages of postzygotic cell division has occurred, since the mutation could not be identified in DNA from leucocytes of the grandmother. The possibility of germline mosaicism in the grandmother prompted the analysis of the father's sisters, who were shown to be unaffected, both by mutation analysis and by marker analysis. Recently, we described a case of somatic mosaicism in TSC, based on the identification of a familial mutation.¹⁴ From our analysis there is no suggestion that the father is a somatic mosaic for the mutation although the possibility of a very high grade (>90%) mosaicism cannot be excluded on the basis of the ASO result (fig 3). The father showed signs that make him a presumptive tuberous sclerosis patient if the diagnosis is certain in the child, given the criterion of the affected first degree relative.¹⁹ The detection of the mutation allowed the suspected affected status in the father to be confirmed, thus making an important contribution to the diagnosis of familial TSC and genetic counselling in this family.

The K12X mutation leads to a truncated peptide of 11 amino acids in length. In the TSC2 gene an alternative downstream ATG codon is present at amino acid position 50. The nucleotide sequence of this alternative site is partially consistent with the criteria proposed by Kozak.²⁴ It is feasible that transcription could start at this position resulting in a protein with a lower molecular weight and lacking part of the N-terminus of the protein. None of the affected family members requires medical treatment for epilepsy, has severe mental impairment, or proven renal involvement. These observations may support the hypothesis of the usage of an alternative ATG codon resulting in an altered, partially functional tuberlin. It is probable that the amount of alternative product and its activity would differ from the activity of the normal tuberlin protein. Since no antisera directed against tuberlin are available as yet, this hypothesis has not been tested.

Mosaicism could account for the difference in phenotype in this family, but the father is at the most a high grade mosaic. Another partial explanation for the difference in phenotype between the proband and father may be the different ages at which different signs of tuberous sclerosis are most apparent. Cardiac manifestations have been reported to decrease in both clinical importance and echocardiographic detectability, especially in the

first years of life.²⁵ It is thought that the tumours either regress or are incorporated into the cardiac muscle wall. There is no clinical history of neonatal or infantile heart disease in the father. Dental pits have been reported to be poorly visible in primary teeth. Gingival fibromas often appear only at puberty. The child may still develop oral signs later in life.

With regard to genetic counselling of this family, it has to be noted that the occurrence of the full, possibly severe, phenotype of tuberous sclerosis cannot yet be excluded for the child or for future generations. However, it is also possible that in this family the observed genotype gives a consistent phenotype. More mutations in clinically well delineated families are needed to decide on the extremely important issue of genotype-phenotype correlations. Once a definitive diagnostic test becomes available, and the majority of patients can be detected by DNA analysis, re-evaluation of the diagnostic criteria will be needed.

Our results prove the diagnosis of tuberous sclerosis in this family by means of mutation detection. We recommend molecular analysis in families in which a diagnosis of tuberous sclerosis is considered, even if the diagnosis cannot be made on clinical criteria alone.

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Note

After submission of this article, the first point mutation in the tuberlin gene was reported by Kumar *et al*. A de novo frameshift mutation in the tuberlin gene. *Hum Mol Genet* 1995;4:1471-2.

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