Original Paper

European Journal of Human Genetics

Eur J Hum Genet 1997;5:280–287

Received: March 10, 1997
Revision received: June 17, 1997
Accepted: June 20, 1997

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Deletion of 11 Amino Acids in Tuberin Associated with Severe Tuberous Sclerosis Phenotypes: Evidence for a New Essential Domain in the First Third of the Protein

Key Words
Tuberous sclerosis
Bourneville's disease
TSC2
Tuberin
In-frame deletion
Splice site mutation
Tumor suppressor gene
DGGE
SSCP
16p13.3

Abstract
Tuberous sclerosis complex (TSC) is an autosomal dominant disorder displaying a large spectrum of symptoms. Linkage studies have shown two loci, TSC1 in 9q34 and TSC2 in 16p13.3, to be involved in the disease. The TSC2 gene, composed of 41 exons, has been isolated and is shown to encode a protein, tuberin, from a 5.5-kb transcript. Mutation screening for both clinical diagnosis and identification of functional domains within the tuberin is in progress. In this study we identify a 33-bp in-frame deletion (1462del33) in the mRNA which segregates in two unrelated French families with severe TSC phenotypes. The corresponding 11 amino acids deletion (aa 482–492) is shown to result from two different splice site mutations at exon 14 and, when compared with the position of two previously described missense mutations, indicates a novel functionally important region of the protein.

Introduction

Tuberous sclerosis complex (TSC), also known as Bourneville's disease, is an autosomal dominant disorder with a prevalence of at least 1/10,000 in the general population. About 60% of the cases seem to be sporadic [1]. The disease is characterized by the development of benign tumors (hamartomas) in several organs. These various lesions present a wide inter- and intrafamilial phenotypic variability [2–5]. Nevertheless, the criteria defined by Gomez [3] and Roach et al. [6] allow a reliable clinical diagnosis.

Linkage studies have revealed a genetic heterogeneity with at least two loci: TSC1 in 9q34 [7–11] and TSC2 in 16p13.3 [12], with apparently indistinguishable corresponding phenotypes. Virtually all the affected families can be linked to either chromosome 9 or 16 loci, in approximately equal numbers [13, 14]. The TSC1 gene is located between markers D9S149 and A6 in a region spanning 1.5 Mb [15], but has not been cloned. The TSC2 gene was isolated by positional cloning in 1993 [16]. The corresponding transcript is 5.5 kb long, spans nearly 43 kb of genomic DNA and comprises 41 exons [17]. Numerous isoforms resulting from alternative splicing have been

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1018–4813/97/0055–0280$15.00/0

This article is also accessible online at:
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identified in human, rat and mouse tissues [17–20]. Northern blot analysis from human cell cultures has demonstrated that TSC2 mRNA is widely expressed in brain, kidney, skin, liver, adrenal gland, colon and white blood cells [16]. RT-PCR studies with embryonic rodent tissues reveal particularly high levels of the TSC2 mRNA in the developing CNS [21]. Tuberin, the TSC2 gene product, has been immunohistochemically detected in the adult and developing mouse nervous system [22]. The exact function of tuberin has not been elucidated, but in vitro studies indicate that the C-terminal part of the protein specifically stimulates the intrinsic GTPase activity of Rap1a (GAP domain) [23] and that the tuberin is colocalized in the Golgi apparatus with Rap1a [24]. Another study reports the presence of putative transactivation domains in the carboxyl terminus of the protein (AD1 and AD2) [25], although this result needs to be confirmed.

The analysis of various TSC lesions has shown loss of heterozygosity for both loci 9q34 and 16p13.3 [26–31] suggesting a tumor suppressor activity for the TSC1 and TSC2 gene products, in accordance with the two-hit hypothesis proposed by Knudson [32]. This hypothesis is supported by the development in the Eker rat of renal cell carcinoma due to lack of TSC2 activity [33, 34]. Furthermore the neoplastic phenotype of these cancerous cells is suppressed in vitro by transcomplementation with an active TSC2 product [35, 36].

According to the tumor suppressor gene hypothesis, the mutations of the TSC2 gene in affected individuals are generally interpreted as causing loss of function. The observation of huge germline deletions [16, 37–39] and nonsense mutations or frameshift deletions leading to truncated proteins which do not express normal activity [40–44] confirmed this hypothesis. A small number of missense mutations and in-frame deletions have been detected.

In this study we report an in-frame deletion of 33 bp in the coding part of the TSC2 mRNA segregating with the disease in two unrelated families with TSC. This deletion is generated by variant splicing mutations at the acceptor splice site of exon 14.

Materials and Methods

Patients and Nucleic Acids Extraction

Eighty unrelated French patients and 163 unaffected Caucasian controls were studied. Clinical diagnoses were established according to the criteria defined by Gomez [3] and Roach et al. [6]. A lymphoblastoid cell line was established for each individual by transformation with Epstein-Barr virus. From those families in which a mutation was detected, a second blood sample was taken and used for analysis without being immortalized.

Total RNA was isolated either from blood or cell lines using the RNA-B kit from Bioprobe. Genomic DNA was extracted from blood as previously described [45].

DGGE Analysis on mRNA

MELT 87 and SQHTX programs kindly provided by Dr L.S. Lerman [see 46, 47] and a Sun Sparc station IPX were used for computer analysis. The graphical interface was provided by GNUPLOT software.

cDNAs were synthesized as follows: 600 ng of total RNA was denatured at 65 °C for 2 min with 250 pmol of random hexamers (Pharmacia) in a total volume of 5 µl. Then 15 µl of RT reaction buffer was added and the mixture was incubated for 30 min at 42 °C and then for 30 min at 46 °C. RT reaction buffer contained 1 × PCR buffer, 5 m*M* $MgCl_2$, 1 m*M* DTT, 500 µ*M* dNTP, 8 units of RNasin and 3 units of AMV RT (PCR buffer and enzymes were supplied by Promega Corporation).

PCR amplifications were performed using 5 µl of cDNA mixture with a hot-start procedure in a final volume of 50 µl containing 10 m*M* Tris-HCl (pH = 8.3 at 20 °C), 50 m*M* KCl, 100 µg/ml gelatine, 1.5 m*M* $MgCl_2$, 200 µ*M* dNTP, 15 pM of each primer and 0.4 unit of Taq DNA polymerase (ATGC). Target DNA were amplified in a PTC100 thermal cycler (MJ Research Inc.) with a first denaturation at 93 °C for 5 min followed by 35 cycles of 93 °C for 60 s, 55 °C for 60 s and 72 °C for 90 s with a final 5-min extension at 72 °C. The forward and reverse primer sequences were respectively 5′ gcc cgc cgg ccc gac ccc cgc gcg tcc ggc gcc cgG ATT CTT CAG GAG CGA GT 3′ (lower case letters correspond to the 35 bp GC-clamp previously used by Fanen et al. [48]) and 5′ GGC AGG GTG TAG CTG TGC TTG T 3′.

The size and specificity of PCR products were checked by electrophoresis on a 2% agarose gel (1% standard agarose, Eurobio; 1% metaphore agarose, FMC).

Fifteen microliters of each PCR product were run for 14 h at 80 V on a 6.5% gel containing a linear gradient (30–60%) of denaturing solution in TAE X1 buffer (pH=7.5 at 20 °C) (100% denaturing solution contains 6.5% acrylamide / bisacrylamide 37.5:1, 7 *M* urea and 40% formamide). All the runs were performed at 65 °C with the denaturing gradient gel electrophoresis system from CBS Scientific (DGGE 4000).

Mutation Analysis on Genomic DNA

RT-PCR products were subcloned into the pGEM-T vector (Promega). Insert DNA were prepared with the Miniprep kit (Qiagen) and sequenced with the M13 primers and the Sequenase sequencing kit (USB – Amersham).

Exon 14 was amplified from genomic DNA by PCR under the conditions described above with a first denaturation at 93 °C for 5 min followed by 35 cycles of 93 °C for 40 s, 64 °C for 45 s and 72 °C for 90 s with a final 5-min extension at 72 °C. The forward and reverse primer sequences were respectively 5′ TCG CGC TCA GCG GTG CTG T 3′ and 5′ GAG CAT TGC TGC CCA CGG A 3′ (chosen from the data published by Maheshwar et al. [17]).

The PCR products were then sequenced without any purification using the dsDNA Sequencing Kit (USB – Amersham).

SSCP analysis was performed on exon 14 PCR products according to the procedure of Orita et al. [49] with minor modifications.

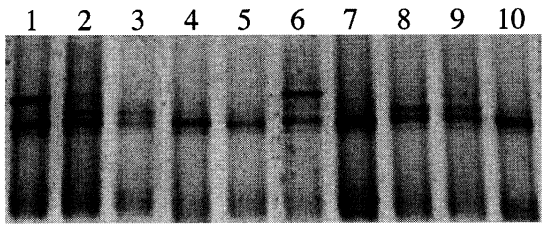


Fig. 1. DGGE analysis of TSC patients (1, 3, 6, 8, 9) and TSC-unaffected controls (2, 4, 5, 7, 10). (1) B-17, (2) control 1, (3) B-13, (4) control 2, (5) control 3, (6) B-95, (7) control 4, (8) B-42, (9) B-55, (10) control 5.

Table 1. Summary of TSC patient clinical findings

	B-17		B-95	
	II-1	III-2	I-1	II-1
Angiofibroma	+	+	+	+
Ungual fibroma	+	+	+	+
Hypomelanic macules	+	+	–	+
Shagreen patch	+	+	–	+
Seizures	+	+	+	+
Mental retardation	–	+	–	+
CT	+	+	+	+
MRI	+		+	+
Eye	–		+	+
Renal abnormalities	+	–	+	+

Amplification was carried out as described above with addition of 0.1 µl α^{33}P-dATP (3,000 Ci/mmol, Amersham). 10 µl of the labelled amplified DNA was diluted in 10 µl of formamide, denatured for 10 min at 95 °C and placed immediately on ice prior to loading. The samples were run for 18 h at 4 W on a 0.5X MDE gel (AT-Biochem) in TBE 0.6X buffer at room temperature. The gels were dried and then exposed to Kodak Biomax MS film overnight at room temperature.

Results

DGGE Analysis

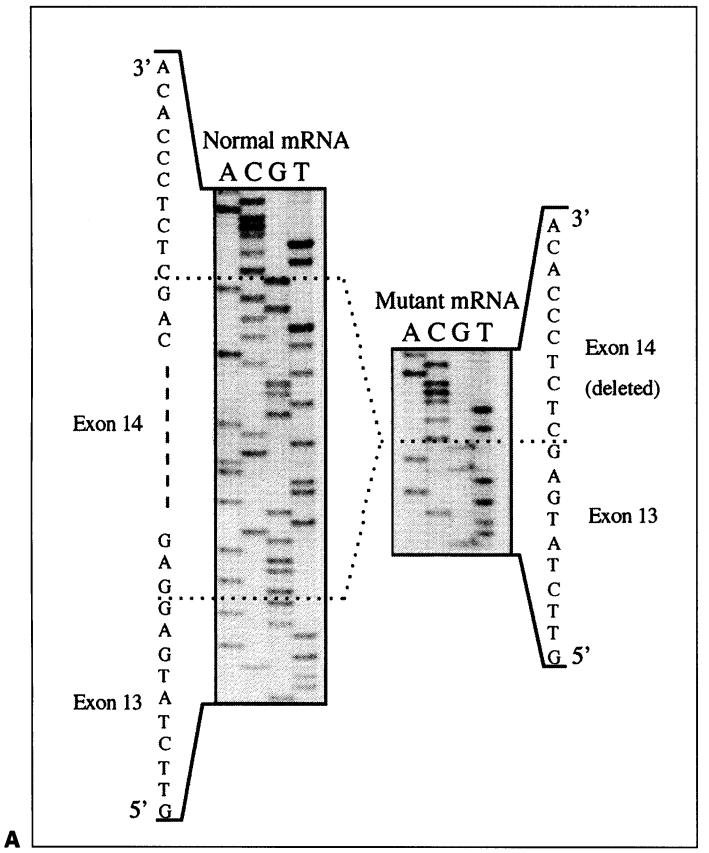
Computer modelling of the TSC2 cDNA led us to divide the sequence into 13 fragments suitable for DGGE analysis (data not shown). In the present study, we focused on domain number 6 (nucleotides 1370–1832). mRNA samples prepared from lymphoblastoid cell lines of 80 independent French TSC patients and 5 normal controls were amplified as described in Materials and Methods. The RT-PCR products were subjected to electrophoresis on denaturing gradient gels, and three different patterns were observed (fig. 1). As expected, 4 of the unaffected controls are characterized by the presence of one band (line 4, 5, 7, 10) while control 1 displays two bands (line 2); this additional band is also present in 3 TSC patients (line 3, 8 and 9). Interestingly a new additional band is observed for 2 TSC patients (line 1 and 6) but not for any of the other 78 patients or the 5 controls.

Analysis of Polymorphism and Mutations in the TSC2 mRNA

Anomalies were identified by direct sequencing of RT-PCR products amplified for the DGGE analysis. TSC patients corresponding to lines 3, 8 and 9 as well as control 1 (line 2) all carried a C→T transition at position 1596 consistent with the polymorphism described by Wilson et al. [43]. Individuals 1 and 6 (respectively individual III-2 of family B-17 and II-1 of family B-95; see fig. 3) were more complex to analyze by direct sequencing but present new transcripts differing from the normal one at the level of nucleotide 1462 for both individuals (data not shown). In order to analyze separately the two mRNA species, RT- PCR products corresponding to these cases were subcloned and several plasmid clones of each product were sequenced. Under this condition it was possible to detect in both patients the normal mRNA (7/12 and 6/11 clones for respectively individual 1 and 6) as well as a new mRNA form with an in-frame deletion of 33 bp ranging from nucleotides 1462 to 1494 (5/12 and 5/11 clones for individuals 1 and 6) (fig. 2A). Going back to the sequence of the previous RT-PCR product, we confirmed that the variants detected on the cloned products correspond to the mutant allele which is expressed at a level comparable with the normal one. Both patients are severely affected and present very similar phenotypes (table 1).

Analysis of Mutations in the Genomic DNA

Southern blotting with 4B2 and s49 probes [16] using three different enzymatic digests (EcoR I, Hind III, Taq I) produced no evidence of genomic rearrangement for individuals 1 and 6 (data not shown). Taking into account that exon 14 starts at nucleotide 1462, we amplified this exon with PCR primers chosen on flanking intronic sequences. As the PCR products for both families were of



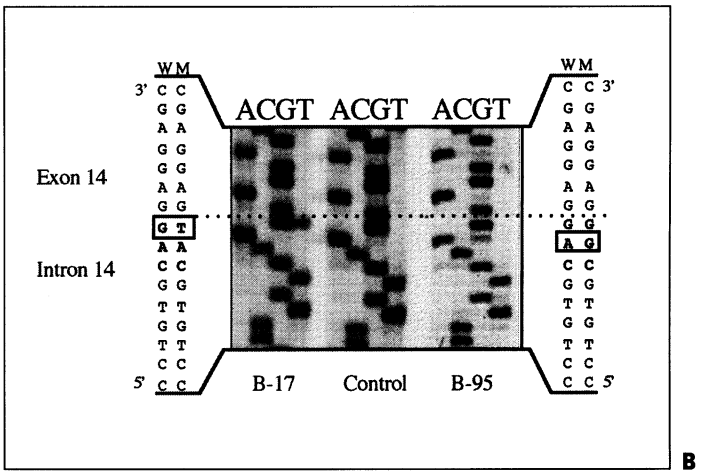


Fig. 2. Sequence analysis of TSC-affected members of families B-17 and B-95. **A** Sequences of cloned RT-PCR products obtained with DGGE primers for domain 6. The 33-bp deletion in the mutant mRNA is indicated by the dotted line. **B** Direct sequencing of PCR products from genomic DNA surrounding exon 14. Mutations for both individuals are shown in the boxes.

the expected length (data not shown), no small deletions of the genomic DNA have been detected. However direct sequencing of the PCR products revealed heteroallelic variation at the acceptor splice site of exon 14 for both families. This result confirms that two alleles are present in the genomic DNA of the patients, and that a large deletion of TSC2 is not germinally transmitted in these families. In the case of family B-17, a G→T transversion abolished the AG consensus sequence required for normal splicing [50]. In family B-95, an A→G transition similarly abolishing the acceptor splice site was identified (fig. 2B).

We then examined whether these changes segregate in the two families. By testing both mRNA and genomic DNA we showed that, for the two families, the mutations segregate with the disease (fig. 3 and table 1). These mutations were not detected by RT- PCR in any of the other 78 patients analyzed or in the 5 unaffected controls.

In order to fully exclude that these mutations were not polymorphisms, we performed SSCP analysis on PCR products of exon 14 from 163 unaffected controls. DNA of members of families B-17 and B-95 were used as controls for the detection of mobility shifts. Three variant forms were detected (fig. 4): the first corresponds to the 1596 C→T polymorphism and was observed in 24 unaffected controls. The second corresponds to the G→T transversion and was only observed in affected patients of

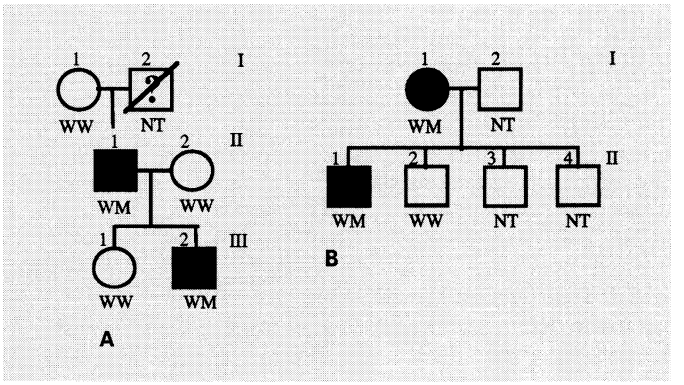


Fig. 3. Pedigree of family B-17 (**A**) and B-95 (**B**). The letters indicate alleles found for each individual: W for wild type, M for mutant and NT for not tested.

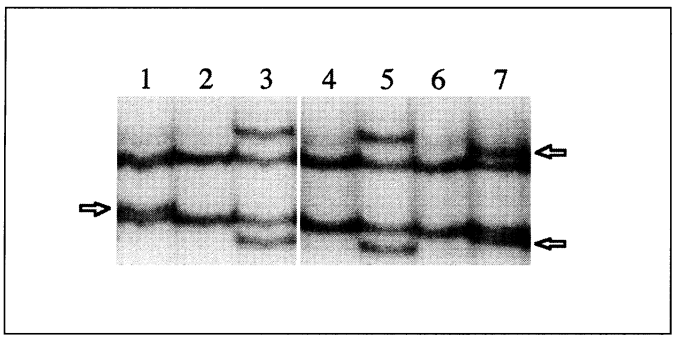


Fig. 4. SSCP analyses of exon 14 on genomic DNA showing different patterns corresponding to the sequence changes. Line 1: B-17, lines 2–6: unaffected controls, line 7: B-95. Control individuals lines 3 and 5 present the 1596 C → T polymorphism. Arrows indicate mutant alleles for individual B-17 and B-95.

family B-17. The third corresponds to the A→G transition and was only observed in affected patients of family B-95.

Discussion

Tuberous sclerosis is an autosomal dominant disease which is clinically and genetically heterogeneous. Analysis of TSC2 gene mutants should lead to a better understanding of the molecular basis of the disorder and facilitate genetic counselling for many affected families.

We used DGGE to screen for abnormalities of the TSC2 mRNA from 80 unrelated French cases with confirmed genetic predisposition to tuberous sclerosis. This method was selected because it is one of the most sensitive techniques for the detection of mutations [46, 47, 51]. Fragments of 500 bp can be analyzed in a single experiment and both small deletions and point mutations can easily be detected. The TSC2 mRNA was divided by computer modelling into 13 domains suitable for DGGE analysis. We report in the present study results obtained for domain number 6 (nucleotides 1370 to 1832). Two unrelated TSC patients (individual III-2 of family B-17 and II-1 of family B-95) showed an additional band which corresponds to new splice site mutations. Another band was also observed in both TSC patients and a normal control confirming after sequencing the 1596 C→T polymorphism previously described by Wilson et al. [43].

TSC-affected members of families B-17 and B-95 carry mutations at the acceptor splice site of exon 14. The normal CAG trinucleotide consensus sequence at the acceptor junction is changed to a CAT in family B-17 (IVS13-1G→T), and to a CGG in family B-95 (IVS13-2A→G). The normal acceptor splice site is thus abolished in affected members in both families leading to the use of a cryptic site present 30 bp downstream in exon 14. By comparing the 14-bp upstream of the two acceptors splice sites (normal and cryptic one), it appears that they both contain the necessary AG consensus with the only difference being one less pyrimidine for the cryptic splice site [50]. Consequently a new transcript characterized by an in-frame deletion of 33 bp at the 5′ end of exon 14 is generated (fig. 5). Our findings are consistent with data described in a survey by Krawczak et al. [52] which showed that 87% of mutations in acceptor splice sites affect the invariant AG dinucleotide sequence and can lead to the use of a cryptic acceptor splice site. Moreover, the genomic mutations do not substantially affect the transcription and the stability of the corresponding messenger since the intensity of the DGGE RT-PCR bands for both normal and mutant alleles are very similar (fig. 1).

This in-frame deletion cosegregates with the disease in these two unrelated families, and no mutation at the acceptor splice site of exon 14 was detected within the 163 controls tested in this study. Thus it is reasonable to exclude the possibility of a polymorphic variant generating an alternative spliced form encountered in some individuals of the general population. Clinical data show that all the affected members in these two families present severe TSC phenotypes, regarding the detection in each case of most of the major symptoms encountered in TSC. This in-frame deletion of 33 bp, corresponding to the deletion of amino acids 482–492 from the protein, is therefore likely to be responsible for the genetic predisposition to the disease in these families and for the severity of its expression. Supporting these data, it has to be pointed out that these amino acids have always been present in the variant spliced forms described to date. Furthermore, all the evidence we have is in favor of the production of a normal and mutant protein in comparable levels. However, to affirm that the 11 amino acids deletion could give a dominant negative effect to the mutant protein requires further experimentation.

Mutations on the TSC2 mRNA which do not lead to a truncated protein include one mutation in the AD1 domain, two mutations near the AD2 domain and two missense mutations close to the deletion we report in the present study (aa 482–492) (fig. 6). The presence of several mutations in this region, located in the first third of the tuberin, suggests that this domain of the protein may play

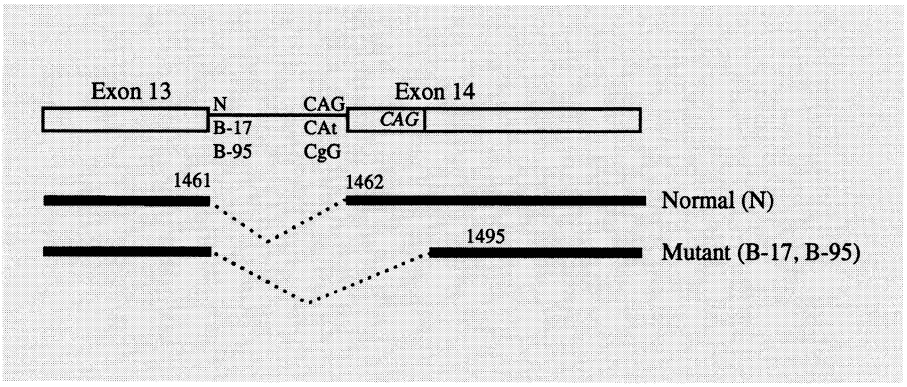
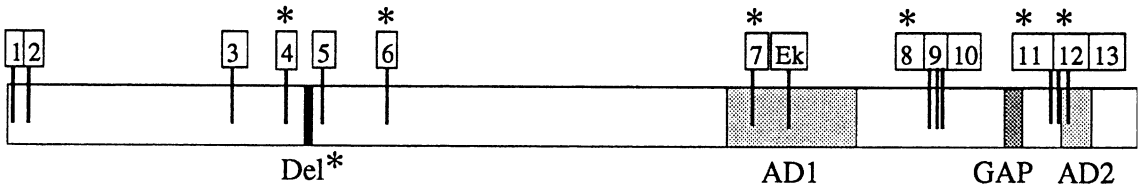


Fig. 5. Normal and mutant form of the TSC2 transcript due to mutations in the acceptor splice site of exon 14 in families B-17 and B-95. The cryptic acceptor splice site is shown in italics.



#	Name	Consequence	Reference
1	Lys12X	Nonsense (stop codon at position 12)	Vrtel et al. [42]
2	IVS1+1G→A	Frameshift (assumed stop codon at position 56)	Kumar et al. [44]
3	1112delTC	Frameshift (stop codon at position 385)	Wilson et al. [43]
4	Met449Iso	Missense	Wilson et al. [43]
Del	1462del33	33 bp in-frame deletion (aa 482 to 492)	This study
5	Arg505X	Nonsense (stop codon at position 505)	Wilson et al. [43]
6	Arg611Trp	Missense	Wilson et al. [43]
7	Arg1199Trp	Missense	Wilson et al. [43]
Ek	IVS30ins5kb	Genomic insertion (rat DNA) (stop codon at position 1272)	Yeung et al. [33] Kobayashi et al. [34]
8	Phe1509del	3 pb in-frame deletion (aa 1509)	Wilson et al. [43]
9	4616ins29	Frameshift (stop codon at position 1564)	Wilson et al. [43]
10	4659/4660delC	Frameshift (stop codon at position 1575)	Kumar et al. [41]
11	Pro1709Leu	Missense	Wilson et al. [43]
12	Ala1712Glu	Missense	Wilson et al. [43]
13	5179delA	Frameshift (polypeptide different from aa 1721)	Kumar et al. [40]

Fig. 6. Summary of mutations on the TSC2 cDNA. Del: 482 del 492 deletion described in this study. GAP: GTPase activating protein domain. AD1 and AD2: putative activation domains described by Tsuchiya et al. [24]. Missense mutations or in-frame deletions are marked with a star. Human sequences were numbered according to Maheshwar et al. [17]. Mutations were described according to the nomenclature proposed by Beaudet and Tsui [53] and the Ad Hoc Comittee on Mutation Nomenclature [54].

an important role in its function. However it is not possible to associate the severity of the disease with the mutations found in this area as there is too much phenotypic variation between these affected patients. Moreover it is difficult to establish a correlation between genotype and phenotype in the case of a disease where a second somatic mutation is known to be necessary in most of the cells in order to express the cellular abnormality. Therefore only a large and systematic analysis of mutations in an extended data set could permit a reliable correlation between genotype alterations and TSC phenotypes.

Acknowledgements

We would like to thank Sylvie Dumas for helpful discussions and critical reading of the manuscript. We would also like to thank Sylvie Larget and Jean-François Prud'homme from Genethon for their help with cell cultures. This work was supported in part by grants from the Centre National de la Recherche Scientifique (CNRS), the Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche (MENESR), the Association Française contre les Myopathies (AFM), the Association pour la Recherche contre le Cancer (ARC) and Rhône-Poulenc Rorer (RPR). S.J. held a fellowship from the CNRS and the ARC. G.P. held a fellowship from the MENESR and the University Paris 7. We would also like to thank the patients, their families and clinicians for making this work possible.

References

1 Osborne JP, Fryer A, Webb D: Epidemiology of tuberous sclerosis. Ann NY Acad Sci 1991; 615:125–127.

2 Gomez MR: Tuberous Sclerosis, ed 2. New York, Raven Press, 1988.

3 Gomez MR: Phenotypes of the tuberous sclerosis complex with a revision of diagnostic criteria. Ann NY Acad Sci 1991;615:1–7.

4 Shepherd CW, Gomez MR, Lie JT, Crowson CS: Causes of death in patients with tuberous sclerosis. Mayo Clin Proc 1991;66:792–796.

5 Roach ES, Delgado MR: Tuberous sclerosis. Dermatol Clin 1995;13:151–161.

6 Roach ES, Smith M, Huttenlocher P, Bat M, Alcorn D, Hawley L: Diagnostic criteria: Tuberous sclerosis complex. J Child Neurol 1992;7: 221–224.

7 Fryer AE, Chalmers A, Connor JM, Fraser I, Povey S, Yates AD, Yates JRW, Osborne JP: Evidence that the gene for tuberous sclerosis is on chromosome 9. Lancet 1987;i:659–661.

8 Haines JL, Amos J, Attwood J, Bech-Hansen NT, Burley M, Conneally PM, Connor JM, Fashold R, Flodman P, Fryer A, Halley DJJ, Jewell A, Janssen LAJ, Kandt R, Northrup H, Osborne J, Pericak-Vance M, Povey S, Sampson J, Short MP, Smith M, Speer M, Trofatter JA, Yates JRW: Genetic heterogeneity in tuberous sclerosis: study of a large collaborative data set. Ann NY Acad Sci 1991;615:256–264.

9 Haines JL, Short MP, Kwiatkowsky DJ, Jewell A, Andermann E, Bejjani B, Yang C-H, Gusella JF, Amos JA: Localization of one gene for tuberous sclerosis within 9q32–9q34, and further evidence for heterogeneity. Am J Hum Genet 1991;49:764–772.

10 Northrup H, Kwiatkowski DJ, Roach ES, Dobyns WB, Lewis RA, Herman GE, Rodriguez E, Daiger SO, Blanton SH: Evidence for genetic heterogeneity in tuberous sclerosis: One locus on chromosome 9 and at least one locus elsewhere. Am J Hum Genet 1992;51:709–720.

11 Sampson JR, Janssen LAJ, Sandkuijl LA, and the Tuberous Sclerosis Collaborative Group: Linkage investigation of three putative tuberous sclerosis determining loci on chromosome 9q, 11q and 12q. J Med Genet 1992;29:861–866.

12 Kandt RS, Haines JL, Smith M, Northrup H, Gardner RJ, Short MP, Dumars K, Roach ES, Steingold S, Wall S, Blanton SH, Flodman P, Kwiatkowski DJ, Jewell A, Weber JL, Roses AD, Pericak-Vance MA: Linkage of an important gene locus for tuberous sclerosis to a chromosome 16 marker for polycystic kidney disease. Nat Genet 1992;2:37–41.

13 Povey S, Burley MW, Attwood J, Benham F, Hunt D, Jeremiah SJ, Franklin D, Gillett G, Malas S, Robson EB, Tippett P, Edwards JH, Kwiatkowski DJ, Super M, Mueller R, Fryer A, Clarke A, Webb D, Osborne J: Two loci for tuberous sclerosis: One on 9q34 and one on 16p13. Ann Hum Genet 1994;58:107–127.

14 Sampson JR, Harris PC: The molecular genetics of tuberous sclerosis. Hum Mol Genet 1994; 3:1477–1480.

15 Kit-Sing A, Murell J, Buckler A, Blanton SH, Northrup H: Report of a critical recombination further narrowing the TSC1 region. J Med Genet 1996;33:559–561.

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

17 Maheshwar MM, Sandford R, Nellist M, Cheadle JP, Sgotto B, Vaudin M, Sampson JR: Comparative analysis and genomic structure of the tuberous sclerosis 2 (TSC2) gene in human and pufferfish. Hum Mol Genet 1996;5:131–137.

18 Kim KK, Pajak L, Wang H, Field LJ: Cloning, developmental expression, and evidence for alternative splicing of the murine tuberous sclerosis (TSC2) gene product. Cell Mol Biol Res 1995;41:515–526.

19 Xiao GH, Jin F, Yeung RS: Identification of tuberous sclerosis 2 messenger RNA splice variants that are conserved and differentially expressed in rat and human tissues. Cell Growth Differ 1995;6:1185–1191.

20 Xu L, Sterner C, Maheshwar MM, Wilson PJ, Nellist M, Short PM, Haines JL, Sampson JR, Ramesh V: Alternative splicing of the tuberous sclerosis 2 (TSC2) gene in human and mouse tissues. Genomics 1995;27:475–480.

21 Geist RT, Gutmann DH: The tuberous sclerosis 2 gene is expressed at high levels in the cerebellum and developing spinal cord. Cell Growth Differ 1995;6:1305–1315.

22 Geist RT, Reddly AJ, Zhang J, Gutmann DH: Expression of the tuberous sclerosis 2 gene product, tuberin, in adult and developing nervous system tissues. Neurobiol Dis 1996;3: 111–120.

23 Wienecke R, König A, DeClue JE: Identification of tuberin, the tuberous sclerosis-2 product: Tuberin possesses specific rap1GAP activity. J Biol Chem 1995;270:16409–16414.

24 Wienecke R, Maize JC Jr, Shoarinejad F, Vass WC, Reed J, Bonifacino JS, Resau JH, de Gunzburg J, Yeung RS, DeClue JE: Co-localization of the TSC2 product tuberin with its target Rap1 in the Golgi apparatus. Oncogene 1996;13:913–923.

25 Tsuchiya H, Orimoto K, Kobayashi T, Hino O: Presence of potent transcriptional activation domains in the predisposing tuberous sclerosis 2 (Tsc2) gene product of the Eker rat model. Cancer Res 1996;56:429–433.

26 Carbonara C, Longa L, Grosso E, Borrone C, Garrè MG, Brisigotti M, Migone N: 9q34 loss of heterozygosity in a tuberous sclerosis astrocytoma suggests a growth suppressor-like activity also for the TSC1 gene. Hum Mol Genet 1994;3:1829–1832.

27 Green AJ, Smith M, Yates JRW: Loss of heterozygosity on chromosome 16p13.3 in hamartomas from tuberous sclerosis patients. Nat Genet 1994;6:193–196.

28 Green AJ, Johnson PH, Yates JRW: The tuberous sclerosis gene on chromosome 9q34 acts as a growth suppressor. Hum Mol Genet 1994;3: 1833–1834.
29 Henske EP, Neuman HPH, Sheithauer BW, Herbst EW, Short MP, Kwiatkowski DJ: Loss of heterozygosity in the tuberous sclerosis (TSC2) region of chromosome band 16p13 occurs in sporadic as well as TSC-associated renal angiomyolipomas. Genes Chrom Cancer 1995; 13:295–298.
30 Carbonara C, Longa L, Grosso E, Mazzucco G, Borrone C, Garrè ML, Brisigotti M, Filippi G, Scabar A, Giannotti A, Falzoni P, Mongua G, Gaini G, Gabrielli M, Riegler P, Danesino C, Ruggieri M, Magro G, Migone N: Apparent preferential loss of heterozygosity at TSC2 over TSC1 chromosomal region in tuberous sclerosis hamartomas. Genes Chrom Cancer 1996; 15:18–25.
31 Green AJ, Sepp T, Yates JRW: Clonality of tuberous sclerosis hamartomas shown by nonrandom X-chromosome inactivation. Hum Genet 1996;97:240–243.
32 Knudson AG: Mutation and cancer: Statistical study of retinoblastoma. Proc Natl Acad Sci USA 1971;68:820–823.
33 Yeung RS, Xiao GH, Jin F, Lee WC, Testa JR, Knudson AG: Predisposition to renal carcinoma in the Eker rat is determined by germ-line mutation of the tuberous sclerosis 2 (TSC2) gene. Proc Natl Acad Sci USA 1994;91:11413–11416.
34 Kobayashi T, Hirayama Y, Kobayashi E, Kubo Y, Hino O: A germline insertion in the tuberous sclerosis (Tsc2) gene gives rise to the Eker rat model of dominantly inherited cancer. Nat Genet 1995:9:70–74.
35 Jin F, Wienecke R, Xiao GH, Maize JC Jr, DeClue JE, Yeung RS: Suppression of neoplasticigenicity by the wild-type tuberous sclerosis 2 (Tsc2) gene and its C-terminal region. Proc Natl Acad Sci USA 1996;93:9154–9159.
36 Orimoto K, Tsuchiya H, Kobayashi T, Matsuda T, Hino O: Suppression of the neoplastic phenotype by replacement of the Tsc2 gene in Eker rat renal carcinoma cells. Biochem Biophys Res Commun 1996;219:70–75.
37 Brook-Carter PT, Peral P, Ward CJ, Thompson P, Hughes J, Maheshwar MM, Nellist M, Gamble V, Harris PC, Sampson JR: Deletion of the TSC2 and PKD1 genes associated with severe infantile polycystic kidney disease – a contiguous gene syndrome. Nat Genet 1994;8: 328–332.
38 Verhoef S, Vrtel R, van Essen T, Bakker L, Sikkens E, Halley DJJ, Lindhout D, van den Ouweland AMW: Somatic mosaicism and clinical variation in tuberous sclerosis complex. Lancet 1995;345:202.
39 Kit-Sing A, Rodriguez JA, Rodriguez E Jr., Dobyns WB, Delgado MR, Northrup H: Mutations and polymorphisms in the tuberous sclerosis complex gene on chromosome 16. Hum Mutat 1997;9:23–29.
40 Kumar A, Wolpert C, Kandt RS, Segal J, Pufky J, Roses AD, Pericak-Vance MA, Gilbert JR: A de novo frame-shift mutation in the tuberin gene. Hum Mol Genet 1995;4:1471–1472.
41 Kumar A, Kandt RS, Wolpert C, Roses AD, Pericak-Vance MA, Gilbert JR: Mutation analysis of the TSC2 gene in an African-American family. Hum Mol Genet 1995;4:2295–2298.
42 Vrtel R, Verhoef S, Bouman K, Maheshwar MM, Nellist M, van Essen AJ, Bakker PLG, Hermans CJ, Bink-Boelkens MThE, van Elburg RM, Hoff M, Lindhoust D, Sampson J, Halley DJJ, van den Ouweland AMW: Identification of a nonsense mutation at the 5′ end of the TSC2 gene in a family with a presumptive diagnosis of tuberous sclerosis complex. J Med Genet 1996;33:47–51.
43 Wilson PJ, Ramesh V, Kristiansen A, Bove C, Jozwiak S, Kwiatkowski DJ, Short MP, Haines JL: Novel mutations detected in the TSC2 gene from both sporadic and familial TSC patients. Hum Mol Genet 1996;5:249–256.
44 Kumar A, Kandt RS, Wolpert C, Roses AD, Pericak-Vance MA, Gilbert JR: A novel splice site mutation (156+1G -> A) in the TSC2 gene. Hum Mutat 1997;9:64–65.
45 Jeanpierre M: A rapid method for the purification of DNA from blood. Nucleic Acids Res 1987;15:9611.
46 Lerman LS, Silverstein K: Computational simulation of DNA melting and its application to denaturing gradient gel eletrophoresis; in Wu R (ed): Methods in Enzymology. New York, Academic Press, 1987, vol 155, pp 482–501.
47 Myers RM, Maniatis T, Lerman LS: Detection and localization of single base changes by denaturing gradient gel electrophoresis; in Wu R (ed): Methods in Enzymology. New York, Academic Press, 1987, vol 155, pp 501–527.
48 Fanen P, Ghanem N, Vidaud M, Besmond C, Martin J, Costes B, Plassa F, Goossens M: Molecular characterization of cystic fibrosis:16 novel mutations identified by analysis of the whole cystic fibrosis conductance transmembrane regulator (CFTR) coding regions and splice site junctions. Genomics 1992;13:770–776.
49 Orita M, Suzuki Y, Sekiya T, Hayashi K. Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. Genomics 1989;5:874–879.
50 Shapiro MB, Senapathy P: RNA splice junctions of different classes of eukaryotes: Sequence statistics and functional implications in gene expression. Nucleic Acids Res 1987;15: 7155–7174.
51 Fodde R, Losekoot M: Mutation detection by denaturing gradient gel electrophoresis (DGGE). Hum Mutat 1994;3:83–94.
52 Krawczak M, Reiss J, Cooper DN: The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: causes and consequences. Hum Genet 1992;90:41–54.
53 Beaudet AL, Tsui LC: A suggested nomenclature for designating mutations. Hum Mutat 1993;2:245–248.
54 Ad Hoc Committee on Mutation Nomenclature: Update on nomenclature for human gene mutations. Hum Mutat 1996;8:197–202.