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ARTICLE

Characterisation of *TSC1* promoter deletions in tuberous sclerosis complex patients

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Tuberous sclerosis complex (TSC), an autosomal dominant disorder, is a multisystem disease with manifestations in the central nervous system, kidneys, skin and/or heart. Most TSC patients carry a pathogenic mutation in either *TSC1* or *TSC2*. All types of mutations, including large rearrangements, nonsense, missense and frameshift mutations, have been identified in both genes, although large rearrangements in *TSC1* are scarce. In this study, we describe the identification and characterisation of eight large rearrangements in *TSC1* using multiplex ligation-dependent probe amplification (MLPA) in a cohort of 327 patients, in whom no pathogenic mutation was identified after sequence analysis of both *TSC1* and *TSC2* and MLPA analysis of *TSC2*. In four families, deletions only affecting the non-coding exon 1 were identified. In one case, loss of *TSC1* mRNA expression from the affected allele indicated that exon 1 deletions are inactivating mutations. Although the number of TSC patients with large rearrangements of *TSC1* is small, these patients tend to have a somewhat milder phenotype compared with the group of patients with small *TSC1* mutations.
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INTRODUCTION

Tuberous sclerosis complex (TSC, MIM no. 191 100) is an autosomal dominant disorder characterised by seizures, mental retardation and hamartomas in multiple organ systems, including brain, skin, heart, lungs and kidneys.[1] Mutations in either *TSC1* or *TSC2* are the underlying cause of the clinical symptoms in TSC patients. In about 75–85% of the patients meeting the definite clinical criteria, a pathogenic *TSC1* or *TSC2* mutation is identified.[2–7] The genes are categorised as tumour suppressor genes, as loss of heterozygosity has been shown in TSC-associated lesions.[8]

TSC1 consists of 23 exons, of which exon 1 and 2 are non-coding. A core promoter has been defined by functional analysis.[9] This region of 587 bp of size is situated 510 bp upstream of exon 1 and runs into exon 1. No TATA or CAAT boxes are present in this promoter region. Several transcription factor-binding sites are present including SP1, E2F and GATA sites. For the detection of small (point) mutations in *TSC1* and *TSC2*, several screening technologies have been undertaken: denaturing gradient gel electrophoresis (DGGE), single strand conformation polymorphism, protein truncation test, denaturing high-pressure liquid chromatography and direct sequencing.[3,5,10–14] Larger rearrangements have been detected by fluorescence *in situ* hybridisation (FISH), southern blotting, long-range (LR) PCR and multiplex ligation-dependent probe amplification (MLPA) analysis.[15–18] Mutations in *TSC2* are more common than in *TSC1*, particularly in sporadic cases. Interestingly, although large rearrangements account for approximately 10% of all *TSC2* mutations identified to date, they appear to be much less frequent in *TSC1*. To our knowledge, only eight different *TSC1* deletions have been described so far.[18–20]

MLPA analysis of *TSC1* was undertaken in patients suspected of TSC, in whom no pathogenic mutation had been identified in either *TSC1* or *TSC2*. In four cases, a deletion of the non-coding exon 1 was identified and in a further four cases multi-exon deletions were detected. The deletions were characterised and it was demonstrated that deletion of exon 1 prevents *TSC1* expression.

MATERIALS AND METHODS

Patient samples

Samples of patients with either a putative or definite clinical diagnosis of TSC were received for mutation analysis. Details on clinical symptoms were obtained from the referring physician using a standardised clinical evaluation form.[3]

Mutation analysis

Extraction of DNA from peripheral blood cells was performed according to the standard techniques. Mutation analysis of *TSC1* and *TSC2* was performed by DGGE[3] or by direct sequence analysis of all coding exons and exon/intron boundaries (primers available on request). For the detection of large rearrangements in *TSC2*, southern blotting, FISH and/or MLPA were performed. After the introduction of MLPA for *TSC1*, all patients without an identified pathogenic mutation were tested using the SALSA MLPA kit P124 (MRC-Holland, Amsterdam, The Netherlands). MLPA was performed according to the manufacturer's instructions; products were run on an automated sequencer (ABI 3730XL, Applied Biosystems, Foster City, CA, USA) and data were analysed using Genemarker version 1.5 (Softgenetics, State College, PA, USA). If possible, all pathogenic mutations were confirmed on an independent DNA sample.

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Quantitative (Q)-PCR, LR-PCR and sequence analysis of breakpoints

All apparent deletions detected by MLPA were confirmed by further delineation of the breakpoint regions using Q-PCR, followed by LR-PCR and sequence analysis.

Real-time Q-PCR was performed using Fam-labelled Taqman assays.[21] Primers were designed with Primer Express 2.0.0 (Applied Biosystems) in the vicinity of single nucleotide polymorphisms (SNPs) mapping to the *TSC1* locus (Table 1). Primer specificity was checked by performing BLAST analysis. Taqman probes were synthesised with a melting temperature (Tm) 8–10 °C higher than the primers by incorporating locked nucleic acid (LNA) monomers in the probe. Tm values for the LNA probes were calculated using the Exiqon website (http://lna-tm.com/). The LNA-based Taqman assays were manufactured by Eurogentec (Maastricht, The Netherlands).

Table 1 Oligonucleotides used in this study

SNP for Q-PCR analysis	*Sequence 5′–3′*	*Reference sequence. NM_000368.3 5′–3′*
rs36021960	ttcctgtcttgctcggttactca	134.838.750
	ttttcggtgagactggg	134.838.712
	catgacgttcctgcccttaag	134.838.670
rs2905078	ggctggaagtcggaaatcaa	134.831.005
	tgtcggcaggattg	134.830.983
	cctcggagactccagaaggaa	134.830.948
rs3011289	cctgccaaagtacagcagtttg	134.821.751
	ctccaagtgcaccctt	134.821.773
	tccagcaacaggtggtacattt	134.821.812
rs7040593	acccttgacagtggaggacatt	134.816.600
	cgagaactcttcatcgac	134.816.626
	ttgcagttccaagagaggtttga	134.816.670
rs869116	ctccctccgcaccagatg	134.814.011
	agcctgtgctggtca	134.814.037
	cccagacagagaagggcaaa	134.814.078
rs12380834	agttccaccaatctgcaaactacttt	134.811.775
	aggaacagactcttccct	134.811.803
	gaatcccttccctggtgtgaa	134.811.846
rs11796704	ccagccttcttgtttccataaaat	134.807.844
	agtgcttcaggtcctg	134.807.817
	ccagtgaggaagaggacaactga	134.807.775
rs700797	tggactcagttgccctctgaa	134.805.668
	ctgtgggaagctatgg	134.805.644
	tgggacataaagggtagaagagaaa	134.805.598
rs13300390	gctctctcacagctcataatgcat	134.803.943
	ctagggcagagatatgtgaa	134.803.914
	caggacccgtacgccattt	134.803.874
rs5203101	ttaggaggagccaaaggtagactct	134.801.009
	taggctcaggaatggg	134.800.982
	ccttggctaagccacatgct	134.800.946
rs12337302	ctggttctcgtctgtgcctagtac	134.784.975
	cagctgtcatcctagtct	134.784.950
	cacaggagagaggcgaagga	134.784.903
rs2809244	tcactggctccttcctaccaa	134.760.050
	ccctgcctcagctg	134.760.075
	gcctaagaactgtggtctggtgtt	134.760.113
rs2073869	tcctgcagataccctcatgatg	134.753.491
	tcagctgtgacgaggc	134.753.524
	acgccgccgtagtggtt	134.753.560
rs2231405	tgcttggcatcccacagtt	134.749.724
	ctcaacccccagtgga	134.749.704
	ggatcctgtccttcccatca	134.749.666
rs2519759	agagggaaaatggcacagtca	134.744.564
	tgcagacagcccc	134.744.586
	aagcggagccagaacttgaa	134.744.621
rs2072058	tgaaacccaacaggcgacat	134.739.974
	agggcaggcgaaag	134.739.999
	cagaatgacccatggaatcaatta	134.740.042
rs12555164	cacccaaacacaaagagtgtaacg	134.693.461
	tgggacagagcacg	134.693.486
	caggcagaactttccgtcatg	134.693.523
rs35602700	tggcaactaaggctgcatga	134.673.378
	ttaagctcgaccctgttt	134.673.399
	caggagtttgctctgcatacgа	134.673.439
rs4962216	tgaattcatttcttctcacattttcc	134.654.970
	cccccatctttgagc	134.655.000
	ttctaagcatgacacagcattggt	134.655.039
Deletion-specific PCR		
c.[–24893_–15354 del9540; –24902_ –24847inv56]	agctccttgggaaacaggat	134.810.337
	gggctctgcaaatagctgtc	134.809.122
	aaacagggggcaggaaatag	134.819.205
c.[–21656_–14846 del6811; –18011_–17856inv156]	ccccatacaaacagagaaagc	134.816.026
	actgccatcccaaacaaaag	134.808.562
	ttcgagaaggaggggaggt	134.809.913
c.[–18348_–18313 del36;–18267_–11107del7161ins7]	tggactcagttgccctctgaa	134.805.668
	gggcagtggttctcaaaatg	134.805.005
	ctggggctggtactgtgatt	134.812.518
c.–16116_–15364del753	gggatccctaccaagcaagt	134.809.625
	gggctctgcaaatagctgtc	134.809.122
	agctccttgggaaacaggat	134.810.337
c.–12499_*67438 del112575		
	tgggacataaagggtagaagagaaa	134.805.598
	cacccaaacacaaagagtgtaacg	134.693.461
	ggtgaattttgggctctgaa	134.807.378
c.738–1380_*101485 del119088	tttgtgtctcttcccccacttct	134.779.656
	tggaaggctctatggcagat	134.660.735
	ccatttttccctgcctagaa	134.659.847
c.738–1292_*4009 del21524	accctcctgtccaaacactg	134.757.047
	taaaaatattcttggccgggtaca	134.779.420
	gcagggaaaaatgtcctttg	134.777.973
c.–38403_*17484 del88525	cacttcacccatactggagc	134.832.923
	ctggttctgtcagtgctccc	134.743.808
	aagcggagccagaacttgaa	134.744.621

Abbreviations: Q-PCR, quantitative PCR; SNP, single nucleotide polymorphism.
Overview of oligonucleotides used for Q-PCR analysis and deletion-specific breakpoint PCR.

Gene dosage alterations were detected on an ABI7500 real-time PCR system (Applied Biosystems) by performing a relative quantification run. Real-time PCR reactions were performed in a total volume of 25 μl, containing 20 ng

DNA, 1× qPCR mastermix Plus-low ROX (Eurogentec: RT-QP2x-03-WOULR), 1 × RNAse P (endogenous control) (Applied Biosystems), 30 μM forward and reverse primers and 10 μM probe. PCR conditions were as follows: an initial 2 min incubation at 50 °C, followed by 95 °C for 10 min and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All samples were analysed in triplicate and compared with a normal control sample.[22]

LR-PCR was performed with the Expand Long Template PCR System (Roche Applied Science, Indianapolis, IN, USA). LR-PCR products were sequenced using an automated sequencer (ABI 3730XL). Nomenclature of the deletions is according to the recommendations of the Human Genome Variation Society, using reference sequence NM_000368 (17 December 2004; build 36, NCBI).

RNA analysis

Fibroblasts were cultured according to the standard procedures. To increase the probability of recovering (truncating) mutant *TSC1* RNA, nonsense-mediated decay of RNA was prevented by adding cycloheximide to the cells 4.5 h before harvesting. RNA was isolated using the RNeasy Mini kit (Qiagen Inc, Valencia, CA, USA). Reverse transcriptase (RT)-PCR (oligo-dT primed) was performed using the Omniscript ReverseTranscription kit (Qiagen). The primers used for RNA analysis were as follows:

Exon 20, forward: 5′-TGTAAAACGACGGCCAGTACAGGCAGCTGTTGGTTCTT-3′

Exon 23, reverse: 5′-CAGGAAACAGCTATGACCGCCAGATGCCTCTTCATTGT-3′

Exon 20/21, forward: 5′-TGTAAAACGACGGCCAGTGCACTCAGATACCACAAAGGAA-3′

Exon 23, reverse: 5′-CAGGAAACAGCTATGACCTCTGAGCACCCGTCATTACA-3′

A first round PCR was performed, followed by a nested PCR using 1 μl of the first round PCR product. The PCR conditions were: 10 s at 94 °C, followed by 10 cycles of 30 s 94 °C, 30 s 68 °C, 1 min 72 °C with a decrease of 1 °C in the annealing temperature per cycle, an additional 25 cycles with an annealing temperature of 58 °C and finally 5 min at 72 °C and 5 min at 20 °C. PCR products were directly sequenced using an automated sequencer (ABI 3730XL). Data were analysed using the SeqScape software (version 2.6; Applied Biosystems).

RESULTS

Mutation analysis

Mutation analysis was performed in a diagnostic cohort of 986 TSC cases. This cohort includes the group (n=362) previously described.[3] Of those 362 patients, 276 (76%) had a definitive clinical diagnosis of TSC. In the total group of 986 patients, it was not possible to give a percentage of patients meeting the clinical diagnosis TSC, as no clinical information was available of the new patients. In 172 cases (17.4%), a pathogenic mutation in *TSC1* was identified, whereas *TSC2* mutations were present in 487 cases (49.3%; data not shown). In 327 cases (33.2%), no pathogenic mutation was identified in *TSC1* (by direct sequence or DGGE analysis of all coding exons) or *TSC2* (by direct sequence, DGGE, southern, FISH and MLPA analysis). MLPA analysis of *TSC1* in these 327 patients showed abnormal patterns in 8 unrelated patients: in 4 cases (patient numbers 30 628, 21 722, 21 899 and 1264; Figures 1b–e), a deletion of the non-coding exon 1 was detected, 1 patient (31 457; Figure 1f) had a deletion of exons 2–23, 2 patients (29 445 and 28 121; Figure 1g–h) had a deletion

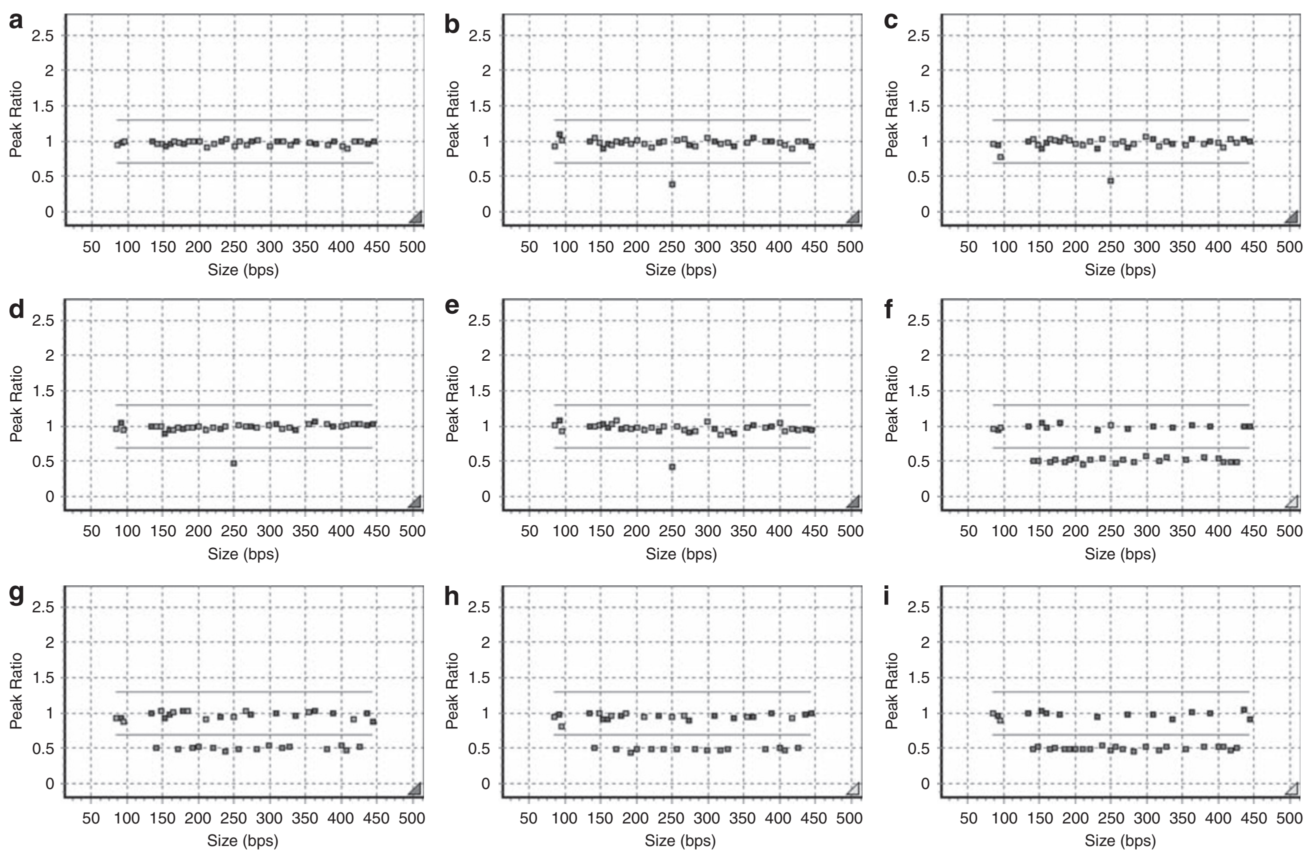

Figure 1 MLPA results. Shown are the graphs after analysis with the Genemarker software. A value of 0.7 or lower is an indication of a deletion of that probe region. (**a**) A sample with a normal pattern (negative control), (**b**) patient 30 628, (**c**) patient 21 722, (**d**) patient 21 899, (**e**) patient 1264, (**f**) patient 31 457, (**g**) patient 29 445, (**h**) patient 28 121 and (**i**) patient 14 249.

of exons 9–23 and 1 patient (14 249; Figure 1i) was identified with a total gene deletion (Figure 1).

Characterisation of the breakpoints

Direct sequence analysis of exon 1 of the four patients with an aberrant MLPA pattern for exon 1 was undertaken to exclude the presence of a SNP interfering with the MLPA probes. No abnormality was identified, indicating that the MLPA results were very likely because of the deletions of this region. To delineate all deleted regions, Q-PCR analyses were performed at several points upstream and downstream of the exon(s) involved in the deletions, followed by LR-PCR using the Q-PCR primers mapping just outside the deleted regions. The breakpoints were identified by sequencing the aberrant LR-PCR products. All four exon 1 deletions had different breakpoints, all resulting in a complete loss of exon 1 (Figure 2). Three of the four deletions did not show a deletion only. In patients 30 628 and 21 722 also an inversion of 56 and 156 nucleotides, respectively, was present. In patient 21 899, an even more complex rearrangement of two deleted regions and an insertion of seven nucleotides was identified. The exon 1 deletion observed in patient 1264 and the multi-exon deletions in the other families (31 457, 29 445, 28 121 and 14 249) did not contain inserted or inverted nucleotides. Only the deletion in patient 28 121 was entirely intragenic. The 5′ breakpoint was located in intron 8 and the 3′ breakpoint in the 3′ UTR of exon 23. The three other deletions started either in the *TSC1* upstream region, in intron 1 or in intron 8 and extended into the *TSC1* downstream region. None of the eight breakpoint junctions showed a sequence that could be an obvious trigger for the rearrangements (Table 2). The *TSC1* promoter region is located between nucleotide positions 16 271 and 15 683 upstream of the ATG codon in exon 3,[9] indicating that three out of the four patients with a complete exon 1 deletion also lack the promoter region. Patient 1264 had a partial deletion of the promoter region. The 155 nucleotides of the 5′ end of the promoter region were still present in this patient.

RNA analysis

Because exon 1 is a non-coding exon, it was not clear whether deletion of this exon would be pathogenic, nor whether it would have an effect on the expression of *TSC1*. Only one patient (21 899) was heterozygous for a coding SNP in *TSC1* (c.2829C>T in *TSC1* exon 22 (rs4962081; Figure 3a), allowing to assess which alleles of *TSC1* were expressed in this patient. To demonstrate equal expression of both alleles of *TSC1* in cultured fibroblast cells, control RNA from another individual heterozygous for this SNP, was analysed by RT-PCR. Expression of both alleles could be demonstrated in the control RNA, as the RNA showed a heterozygous pattern for the SNP (Figure 3b). In contrast, RNA from patient 21 899 showed only the T nucleotide of SNP rs4962081, indicating monoallelic expression of *TSC1* (Figure 3c). We concluded that the deletion of exon 1 in this patient prevented *TSC1* expression and deletions affecting this non-coding exon are therefore pathogenic mutations.

Deletion-specific PCR analysis

Because the MLPA tests are sensitive to the quality of the DNA[18] (AvdO, unpublished observations), deletion-specific PCRs were developed for diagnostic application of mutation analysis within the family,

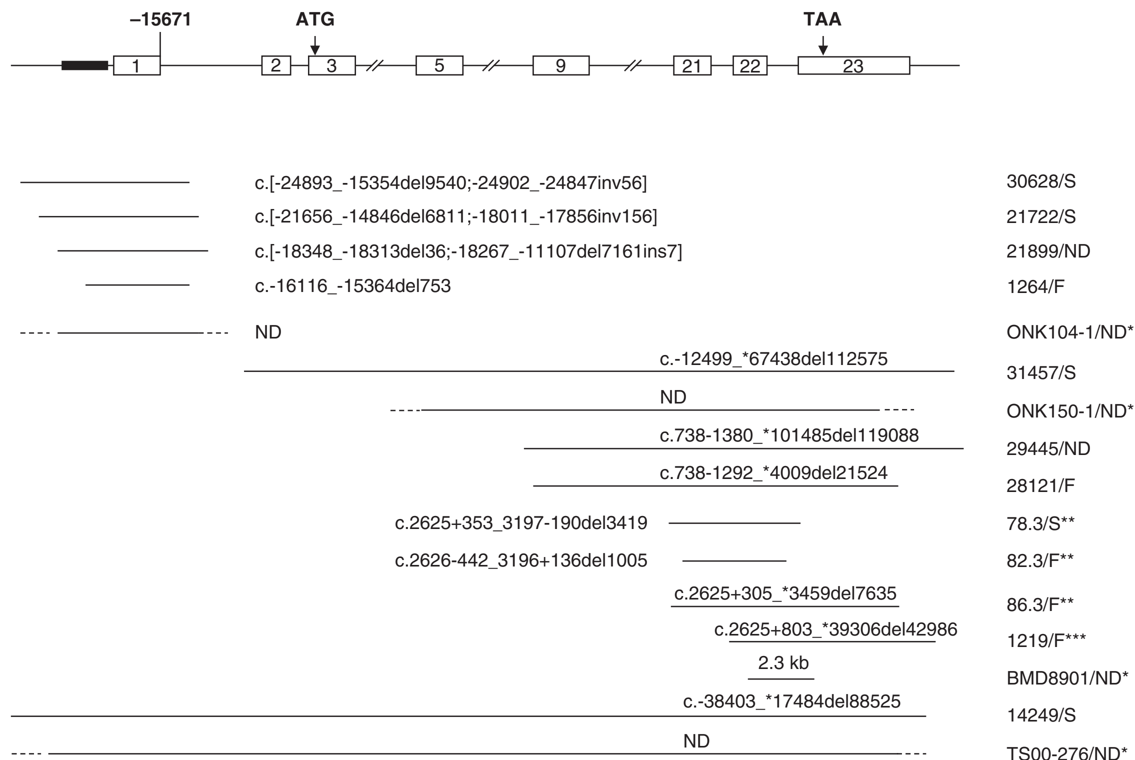

Figure 2 Overview of the *TSC1* deletions identified during this study and described previously. The upper part of the figure represents the genomic region extending from exon 1 to exon 23 of *TSC1* (not drawn on scale). The closed box represents the promoter region and the open boxes represent *TSC1* exons. If the breakpoints of the deletion have been determined, this is given by the HGVS nomenclature (reference sequence NM_000368 (17 December 2004; build 36, NCBI)); ND: breakpoint is not determined; 2.3 kb: in the article of Kozlowski *et al*[18] only the length of the deletion is given. On the right side of each deletion the identification number of the patient is given, followed by the indication S (sporadic), F (familial), ND (not determined) and, if previously published, the references: *Kozlowski *et al*,[18] **Longa *et al*,[19] ***Nellist *et al*.[20]

Table 2 Overview of the deletion junction sites and inserted sequences identified in individuals 30 628, 21 722 and 21 899

ID 30 628		
134818993	TTAGTAGAGACAGGGTTTCAccatgttagccaggatggtc	
134809453	ggggcgaggtgggcccccttcGTCAGTCCCTCACTTGGTGG	
134818982	inversion: AGGGTTTCACCATGTTAGCCAGGATGGTC TCAATCTCCTGACCTCGTGATCTGCCC	
ID 21 722		
134815756	TTGGGATTACAGGCGTGAGCcaccsagcctggcctgttcc	
134808945	taacgttaatggagtgctctCTGTGTGCCAGGCACTGTGA	
134812091	inversion: TGTAGAAACAGTGACAATGTGGTTATGGC CCCCTGGAAAGTGAGCAGTTGATGAGCTCTGCTTATGGCT GCTCTCCTCCATGGCAGAATGCATTCCTGCAGGATGAGGT CATGGAGCCAAGGAAGGTCAGGGTGTGGGGGGGCTTCCTG GGTCAGA	
ID 21 899		
134812448	AGAAGGCGAGGGGGAGATGTcacacagccaaatgcagcag	First breakpoint
134812412	gcagatgtcctccaccacgtGCATCCTGTCAGCACGCCCG	Second breakpoint
134812367	GGAGGGCTCAGCCCTTTCATgctcagacaggccggtcagg	Third breakpoint
134805206	gaatgaatgtttttaccttCATCCTTCTTTTTCCTAGAC insertion:AATACAT	
ID 1264		
134810216	CAGGCGCCGCCAGCTTGTTTacgcctctccgccgcgtccc	
134809463	ccctcgggcaggggcgaggtGGGCCCCTTCGTCAGTCCCT	
ID 31 457		
134806599	TTTCTCTGGTTTACTTTTTAagactgtagttgctagaaat	
134694024	tttctataaagctattatatTCCTTTTAAAATAATTCCAA	
ID 29 445		
134779065	CATTGGCTCACTTTTTTTTTttttttttttgagacgga	
134659977	tggcttccccaaagtctggtCAAGTTGATTTTAACAGAGT	
ID 28 121		
134778977	ACTGCAAATTCCGCCTCCTGggttcacgccattctcctgc	
134757453	ttaggtcgaggactggccccTAGGCTGCTGCTGTGACCCT	
ID 14 249		
134832503	TAACAGAATGCCACAGACTGggtcacttataaagaaaaga	
134743978	csactctaggagttgggctgAGGATTTCCACCCGATCCTC	

Retained sequences are shown in capitals; deleted nucleotides are in lower case. On the left, the number of the first nucleotide in each row in the genomic reference sequence NM_000368 (17 December 2004; build 36, NCBI) is given.

including prenatal testing. In all cases, three primers were used: one common primer, one primer located in the deleted region and one primer just outside the deletion (Table 1). The primers were chosen so that the fragment specific for the deletion was shorter than the wild-type fragment (Figure 4). Using the deletion-specific PCRs, the unaffected parents of patients 30 628, 21 722 and 14 249 tested negative for the deletion, suggesting that these patients are *de novo* TSC cases. Patients 1264 and 28 121 each had an affected family member. They were available for DNA deletion analysis and tested positive for the respective deletion-specific PCRs (Figure 4). The affected sib of patient 1264 showed mild mental retardation, epilepsy, cortical tubers and subependymal nodules (age of diagnosis 15 years). The mother of patient 28 121 had facial angiofibroma, ungual fibroma, fibrous plaques, hypomelanotic macules and shagreen patches (diagnosed at age 31 years). None of the healthy relatives tested positive for the familial deletions and, thus, non-penetrance was not encountered in these families. The parents of patient 31 457 were tested elsewhere by MLPA analysis. Both parents showed a normal result (data not shown). The parents of individuals 21 899 and 29 445 were not available for testing.

Clinical details

With one exception,[20] no clinical information was available on the other previously reported *TSC1* deletion cases. The clinical features of the nine *TSC1* deletion index patients are summarised in Supplementary Table 3. All patients had a definite clinical diagnosis of TSC. The clinical findings of these nine TSC patients with *TSC1* deletions were compared with other patients with a *TSC1* mutation.[3] Although the number of patients was very small, making comparisons difficult, fewer neurological symptoms and dermatological findings, especially shagreen patches, were found in the *TSC1* deletion patient group (Supplementary Table 3; compare last two columns).

DISCUSSION

In TSC patients, different types of mutations can be identified in *TSC1* or *TSC2*. Approximately one-third are identified in *TSC1*.[3,4] Most of the pathogenic mutations are nonsense, frameshift or splice site mutations and some missense mutations have been described (http://chromium.liacs.nl/LOVD2/TSC/home.php). Recently, functional tests have helped to classify missense changes as pathogenic mutations in *TSC1*.[23–25] So far, only a small number of large rearrangements in *TSC1* have been described.[18–20] In total, 16 large rearrangements, including the deletions described here, have been identified. In our cohort of individuals with a *TSC2* mutation (487), an MLPA abnormality was present in 48 cases (9.9%; data not shown). In one case, an intragenic duplication of several exons was identified, whereas the other cases had (multiple) exon deletions. A *TSC1* pathogenic mutation was present in 181 individuals (data not shown). Of these mutations, nine were large rearrangements (5.0%). The percentage large *TSC1* rearrangements in the patient group of Kozlowski *et al*[18] was lower (0.5% of all *TSC1* mutations) compared with our group. In our cohort, all *TSC1* and *TSC2* rearrangements (n=57) account for 8.5% of all mutations (n=668). This is comparable with the data of Kozlowski *et al* (6.1% of all *TSC* mutations).

Of all 16 patients/families with a large *TSC1* deletion, 5 patients were sporadic, 5 were familial cases and of the remaining 6 cases, the parents were not analysed by molecular techniques. In our previously described cohort of patients with point mutations in *TSC1*, a comparable ratio of sporadic with familial cases was observed (22 sporadic to 20 familial).[3]

As exon 1 is a non-coding exon, RNA analysis was performed to investigate the effect of deleting this exon on *TSC1* mRNA expression. Monoallelic expression was demonstrated, indicating that exon 1 deletions are likely to be null alleles and that there is no alternative promoter present in the *TSC1* region. Regulatory elements necessary for basal transcription of *TSC1* and a region for optimal promoter function have been defined and were localised to the regions between nucleotide positions 16 271–16 003 and 16 002–15 683, respectively, upstream of the start codon in exon 3.[9] In the UCSC Genome Browser only one promoter region is presented. A CpG island containing region and several transcription-binding sites are located in the same region as defined by the functional test and the monoallelic *TSC1* expression in our patient. The promoter region was completely deleted in patients 30 628, 21 722 and 21 899. In patient 1264, a partial deletion of the promoter region was identified; only the most 5′ 155 nucleotides of the 'basal' transcription core were present. Unfortunately, this patient was not heterozygous for a coding SNP and therefore, it was not possible to analyse monoallelic expression of *TSC1*.

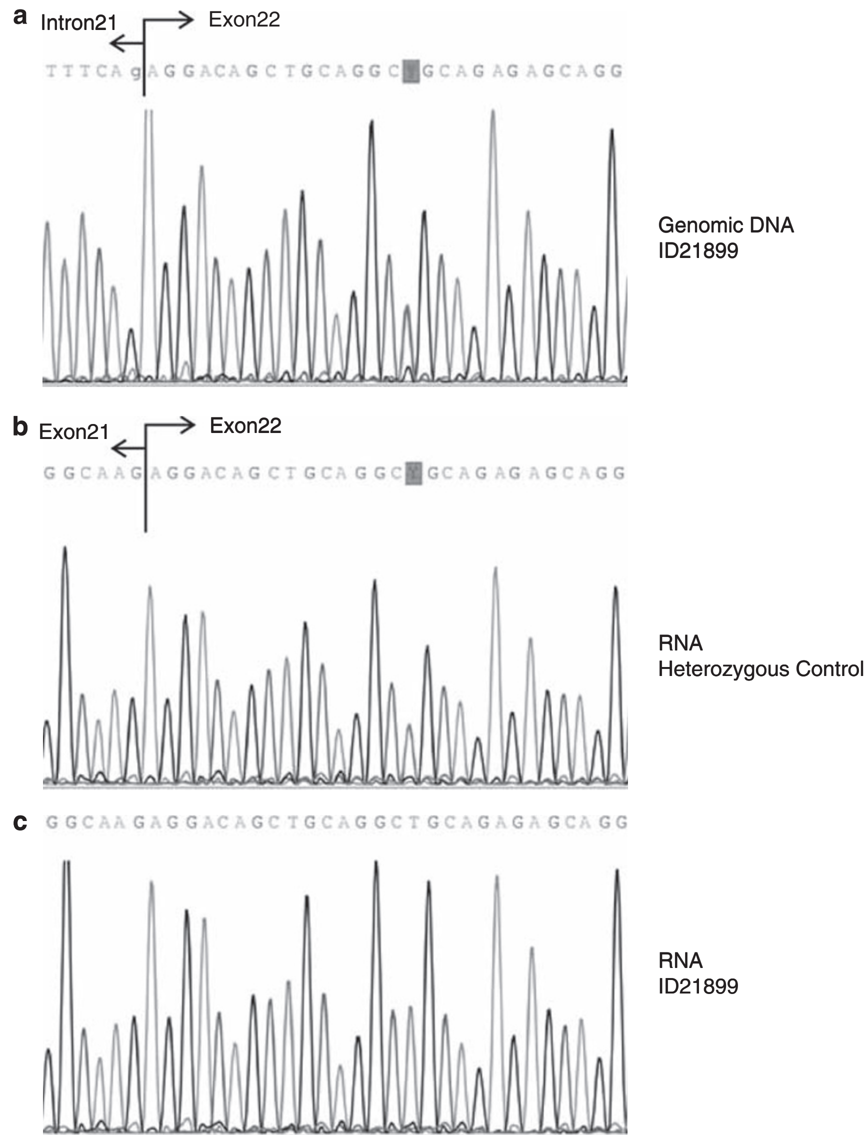

Figure 3 RNA analysis of the coding SNP rs4962081 in exon 22 of *TSC1*. (**a**) Genomic DNA of patient 21 899. The intron–exon boundary is given by a vertical line. The heterozygous pattern of SNP rs4962081 is recognised by 'Y' (C/T combination). (**b**) RNA of a control individual heterozygous for SNP rs4962081. The boundary between exons 21 and 22 is given by a vertical line. The heterozygous pattern is called by 'Y' (C/T combination). (**c**) RNA of patient 21 899. The boundary between exon 21 to exon 22 is given by a vertical line. Patient 21 899, who is heterozygous for SNP rs4962081 in genomic DNA (see **a** above), shows expression of only the 'T' allele, indicating monoallelic expression of *TSC1*.

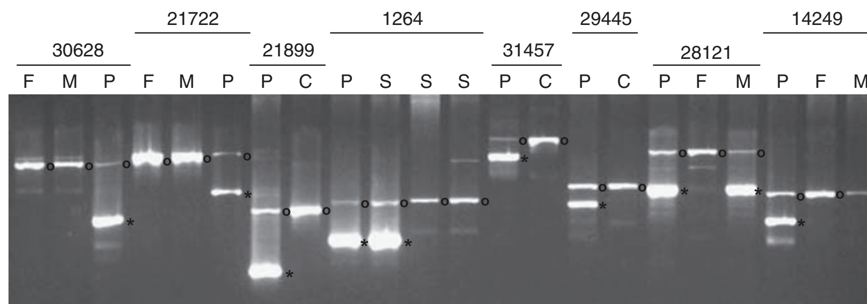

Figure 4 Agarose gel electrophoresis of the deletion PCR products. The identification number of the patients are indicated at the top of the figure. Abbreviations: *, deletion fragment; ^{0}, wild-type fragment; C, negative control; F, father of the index patient; M, mother of the index patient; P, index patient; S, sibling of the index patient.

Three of the four exon 1 abnormalities were complex events (Figure 1 and Table 2). A combination of a deletion and an inversion was detected in two patients, whereas the abnormality in the third patient consisted of two deletions separated by 45 nucleotides and an insertion of unknown origin. It is not clear why exon 1 deletions account for almost half of the large *TSC1* rearrangements in our cohort. Most deletions were not associated with specific repeat sequences and in only two cases a very short repeat sequence (two or three nucleotides) was observed. This is in contrast to the large rearrangements described in *TSC2*,[18] wherein 70% of the abnormalities very short sequence repeats were present at the junction of the deleted segments.

Although genotype–phenotype comparisons with such a small number of TSC patients should be made with caution, the clinical phenotype of the patients with a *TSC1* deletion was slightly less severe overall than that of patients with other *TSC1* mutations. In addition, we noted that all patients with a deletion of exon 1 had epilepsy, whereas this was only observed in one of the five individuals with a deletion affecting other exons of *TSC1*. We demonstrated that deletions encompassing exon 1 are true null alleles. Therefore, one possible explanation for our observation is that the expression of truncated or mutant TSC1 isoforms may modify the TSC phenotype. Mutant TSC1 isoforms could either have a dominant negative effect by competing with wild-type TSC1 to form inactive TSC1–TSC2 complexes, or have a protective effect by retaining some functionality and maintaining some TSC1–TSC2 activity in the cell[26] (M Nellist, unpublished observations).

In our cohort, 5.0% of all *TSC1* mutations were identified using MLPA, indicating that it is necessary to screen for large *TSC1* rearrangements in TSC patients. Although the number of patients identified with a large (complex) deletion is relatively small, it might be that these patients show less severe symptoms compared with patients with point mutations in *TSC1*.

CONFLICT OF INTEREST

The authors declare no conflict of interests.

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