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## **Characterisation of *TSCI* promoter deletions in Tuberous Sclerosis Complex patients**

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**Running title:** *TSC1* promoter deletions

**ABSTRACT**

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.

Here we describe the identification and characterisation of 8 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 4 families, deletions only affecting the noncoding 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 to the group of patients with small *TSC1* mutations.

**Key words:** *TSC1*, promoter, deletions, MLPA

## INTRODUCTION

Tuberous Sclerosis Complex (TSC, MIM#191100) is an autosomal dominant disorder characterised by seizures, mental retardation and hamartomas in multiple organ systems, including brain, skin, heart, lungs and kidneys<sup>1</sup>. 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<sup>2-7</sup>. The genes are categorised as tumour suppressor genes, since loss of heterozygosity has been shown in TSC-associated lesions<sup>8</sup>.

*TSC1* consists of 23 exons, of which exon 1 and 2 are noncoding. A core promoter has been defined by functional analysis<sup>9</sup>. 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 (SSCP), Protein Truncation Test (PTT), Denaturing High Pressure Liquid Chromatography (DHPLC) and direct sequencing<sup>3,5,10-14</sup>. 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<sup>15-18</sup>. Mutations in *TSC2* are more common than in *TSC1*, particularly in sporadic cases. Interestingly, whereas 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 8 different *TSC1* deletions have been described so far<sup>18-20</sup>.

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 4 cases a deletion of the noncoding exon 1 was identified and in a further 4 cases multi-exon deletions were detected. The deletions were characterised and it was demonstrated that deletion of exon 1 prevents *TSC1* expression.

## **MATERIAL 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 <sup>3</sup>.

### **Mutation analysis**

Extraction of DNA from peripheral blood cells was performed according to standard techniques. Mutation analysis of *TSC1* and *TSC2* was performed by DGGE <sup>3</sup> 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.

### **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<sup>21</sup>. Primers were designed with Primer Express 2.0.0 (Applied Biosystems) in the vicinity of single nucleotide polymorphisms (SNPs) mapping to the *TSCI* locus (Table 1). Primer specificity was checked by performing BLAST analysis. Taqman probes were synthesised with a melting temperature ( $T_m$ ) 8-10°C higher than the primers by incorporating Locked Nucleic Acid (LNA) monomers in the probe.  $T_m$  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).

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 x qPCR mastermix Plus - low ROX (Eurogentec: RT-QP2x-03-WOULR), 1 x 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 sec and 60°C for 1 min. All samples were analysed in triplicate and compared to a normal control sample

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 Dec 2004; build 36, NCBI).

### **RNA analysis**

Fibroblasts were cultured according to standard procedures. To increase the probability of recovering (truncating) mutant *TSCI* RNA, nonsense mediated decay of RNA was prevented by adding cycloheximide to the cells 4.5 hours 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:

Exon 20 Forward: 5'-TGTA AACGACGGCCAGTACAGGCAGCTGTTGGTTCTT-3'

Exon 23 Reverse: 5'-CAGGAAACAGCTATGACCGCCAGATGCCTCTTCATTGT-3'

Exon 20/21 Forward: 5'-TGTA AACGACGGCCAGTGC ACTCAGATAACCACAAAGGAA-3'

Exon 23 Reverse: 5'-CAGGAAACAGCTATGACCTCTGAGCACCCGTCATTACA-3'

A first round PCR was performed, followed by a nested PCR using 1µl of the first round PCR product. The PCR conditions were: 10 sec at 94°C, followed by 10 cycles of 30 sec 94°C, 30 sec 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 <sup>3</sup>. 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, since 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 30628, 21722, 21899 and 1264; Figure 1B - E) a deletion of the noncoding exon 1 was detected, 1 patient (31457; Figure 1F) had a deletion of exons 2 – 23, 2 patients (29445 and 28121; Figure 1G - H) had a deletion of exons 9 – 23 and one patient (14249; Figure 1I) was identified with a total gene deletion (Figure 1).

### **Characterisation of the breakpoints**

Direct sequence analysis of exon 1 of the 4 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 due to 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 4 exon 1 deletions had different breakpoints, all resulting in a complete loss of exon 1 (Figure 2). Three of the 4 deletions did not show a deletion only. In patients 30628 and 21722 also an inversion of 56 and 156 nucleotides, respectively, was present. In patient 21899 an even more complex rearrangement of two deleted regions and an insertion of 7 nucleotides was identified. The exon 1 deletion observed in patient 1264 and the multi-exon deletions in the other families (31457, 29445, 28121 and 14249) did not contain inserted or inverted nucleotides. Only the deletion in patient 28121 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 8 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 16271 and 15683 upstream of the ATG codon in exon 3<sup>9</sup>, indicating that 3 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 noncoding 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 (21899) 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, since the RNA showed a heterozygous pattern for the SNP (Figure 3B). In contrast, RNA from patient 21899 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 noncoding exon are therefore pathogenic mutations.

#### **Deletion specific PCR analysis**

Because the MLPA tests are sensitive to the quality of the DNA<sup>18</sup> (AvdO, unpublished observations), deletion specific PCRs were developed for diagnostic application of mutation analysis within the family, including prenatal testing. In all cases, 3 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 wildtype fragment (Figure 4). Using the deletion specific PCRs, the unaffected parents of patients 30628, 21722 and 14249 tested negative for the deletion, suggesting that these patients are *de novo* TSC cases. Patients 1264 and 28121 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 yrs). The mother of patient 28121 had facial angiofibroma, ungual fibroma, fibrous plaques, hypomelanotic macules and shagreen patches (diagnosed at

age 31 yrs.). None of the healthy relatives tested positive for the familial deletions and, thus, nonpenetrance was not encountered in these families. The parents of patient 31457 were tested elsewhere by MLPA analysis. Both parents showed a normal result (data not shown). The parents of individuals 21899 and 29445 were not available for testing.

### **Clinical details**

With one exception<sup>20</sup>, no clinical information was available on the other previously reported *TSC1* deletion cases. The clinical features of the 9 *TSC1* deletion index patients are summarised in Supplementary Table 3. All patients had a definite clinical diagnosis of TSC. The clinical findings of these 9 TSC patients with *TSC1* deletions were compared to other patients with a *TSC1* mutation<sup>3</sup>. 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 2 columns).

### **DISCUSSION**

In TSC patients, different types of mutations can be identified in *TSC1* or *TSC2*. Approximately one third are identified in *TSC1*<sup>3,4</sup>. 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*<sup>23-25</sup>. So far, only a small number of large rearrangements in *TSC1* have been described<sup>18-20</sup>. 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, 9 were large rearrangements (5.0%). The percentage large *TSC1* rearrangements in the patient group of Kozłowski et al. was lower (0.5% of all *TSC1* mutations) compared to our group<sup>18</sup>. 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 Kozłowski 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 to familial cases was observed (22 sporadic to 20 familial)<sup>3</sup>.

Since exon 1 is a noncoding 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 16271 - 16003 and 16002 - 15683 respectively, upstream of the start codon in exon 3<sup>9</sup>. 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 30628, 21722 and

21899. 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*.

Three of the 4 exon 1 abnormalities were complex events (Figure 1 and Table 2). A combination of a deletion and an inversion was detected in 2 patients, whereas the abnormality in the third patient consisted of 2 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 2 cases a very short repeat sequence (2 or 3 nucleotides) was observed. This is in contrast to the large rearrangements described in *TSC2*<sup>18</sup>, where in 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, while this was only observed in 1 of 5 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<sup>26</sup> (MN 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 to patients with point mutations in *TSC1*.

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#### **Titles and legends to figures and tables**

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 30628; C, patient 21722; D, patient 21899; E, patient 1264; F, patient 31457; G, patient 29445; H, patient 28121 and I, patient 14249.

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 dec 2004; build 36, NCBI)); ND: breakpoint is not determined; 2.3 kb: in the article of Kozlowski *et al.* only the length of the deletion is given<sup>18</sup>. 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.*<sup>18</sup>, \*\*Longa *et al.*<sup>19</sup>, \*\*\*Nellist *et al.*<sup>20</sup>.

Figure 3. RNA analysis of the coding SNP rs4962081 in exon 22 of *TSC1*.

(A) Genomic DNA of patient 21899. 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 21899. The boundary between exon 21 to exon 22 is given by a vertical line. Patient 21899, 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. F: father of the index patient; M: mother of the index patient; P: index patient; S; sibling of the index patient; C: negative control. \*: deletion fragment and <sup>0</sup>: wildtype fragment.

Table 1. Overview of oligonucleotides used for Q-PCR analysis and deletion specific breakpoint PCR.

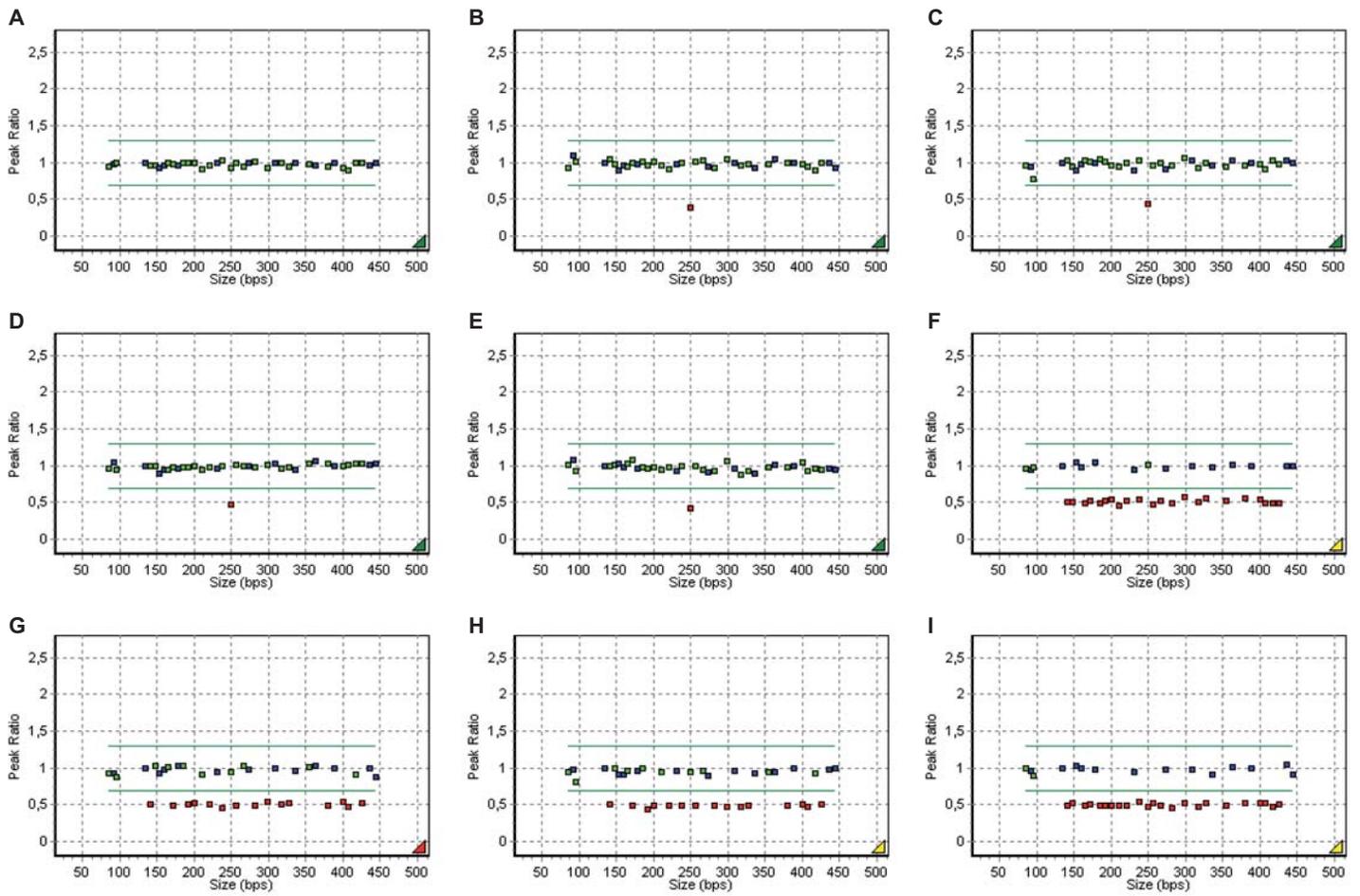
Table 2. Overview of the deletion junction sites and inserted sequences identified in individuals 30628, 21722 and 21899. Retained sequences are shown in capitals; deleted nucleotides are in lower case. On the left, the number of the first nucleotide in each row in the genomic reference sequence NM\_000368 (17 Dec 2004; build 36, NCBI) is given.

Supplementary Table 3. Clinical features of the index patients

The identification numbers for the patients with *TSC1* deletions identified in our cohort and of family 1219 of which clinical data were published<sup>20</sup> are given in the top row. The clinical data from these families were compared to the overall *TSC1* mutation positive families previously described<sup>3</sup>. Gender: M, male and F: female. + Clinical feature present; 0 absent; NE: not examined; ND: no data. If the age of

diagnosis is not known, the age of diagnosis is calculated by subtracting the year of birth of the patient of the year the laboratory received the request for mutation analysis.

Figure 1



**Figure 2**



—————	c.[-24893_-15354del9540;-24902_-24847inv56]	30628/S
—————	c.[-21656_-14846del6811;-18011_-17856inv156]	21722/S
—————	c.[-18348_-18313del36;-18267_-11107del7161ins7]	21899/ND
—————	c.-16116_-15364del753	1264/F
-----	ND	ONK104-1/ND*
—————	c.-12499_*67438del112575	31457/S
-----	ND	ONK150-1/ND*
—————	c.738-1380_*101485del119088	29445/ND
—————	c.738-1292_*4009del21524	28121/F
—————	c.2625+353_3197-190del3419	78.3/S**
—————	c.2626-442_3196+136del1005	82.3/F**
—————	c.2625+305_*3459del7635	86.3/F**
—————	c.2625+803_*39306del42986	1219/F***
—————	2.3 kb	BMD8901/ND*
—————	c.-38403_*17484del88525	14249/S
-----	ND	TS00-276/ND*

Figure 3

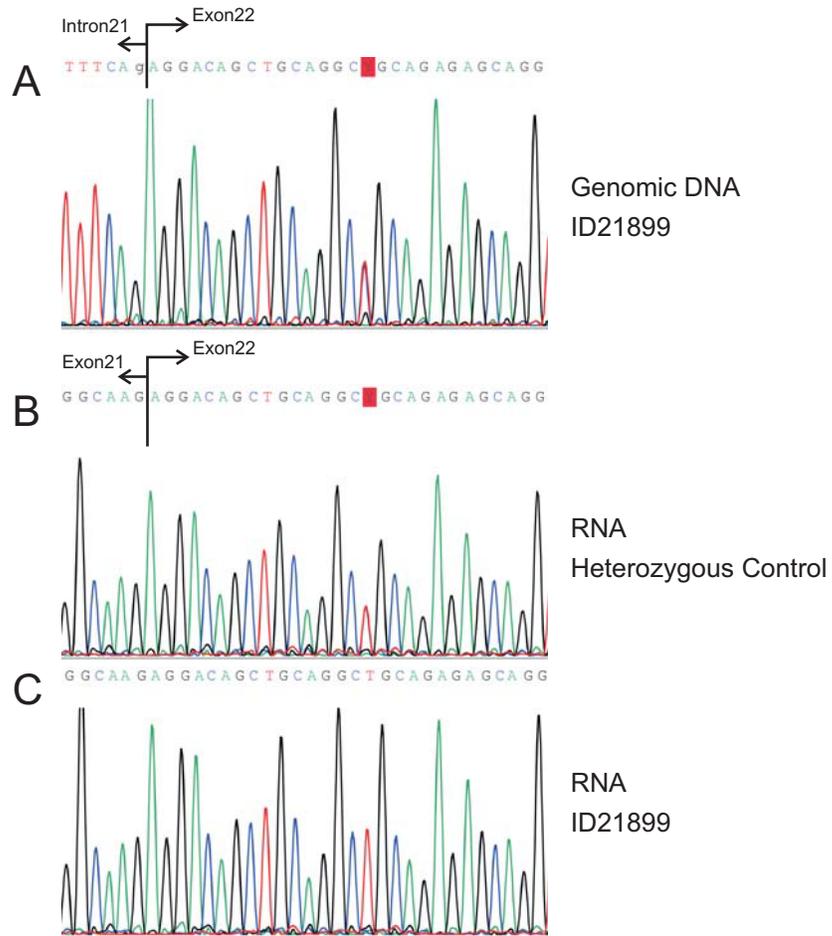


Figure 4

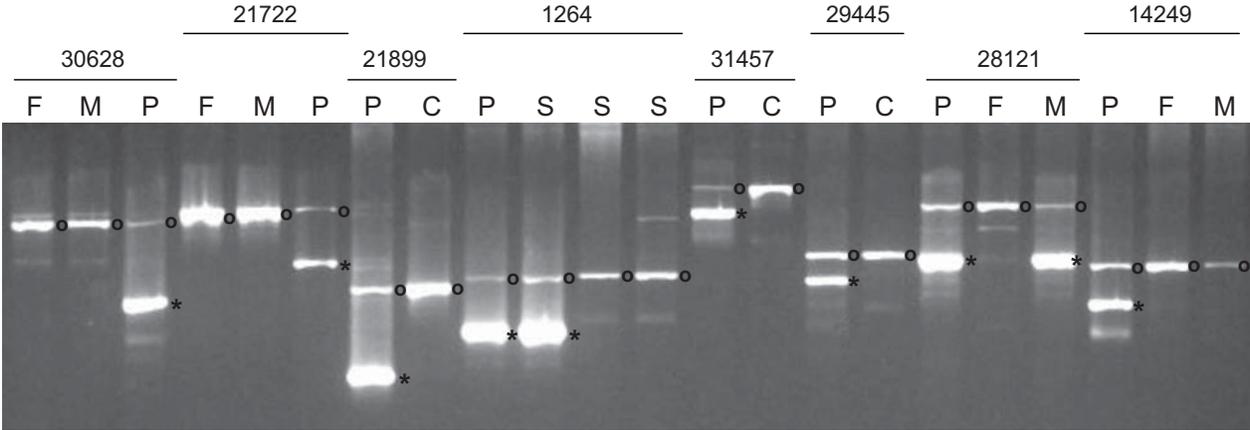


Table 1 Oligonucleotides used in this study.

SNP for Q-PCR analysis	Sequence 5'-3'	Ref. seq. NM_000368.3 5'-3'
rs36021960	ttcctgtcttgctcggttactca tttcggtgagactggg catgacgttctgcccttaag	134.838.750 134.838.712 134.838.670
rs2905078	ggctggaagtcggaatcaa gtcggcaggattg cctcggagactccagaaggaa	134.831.005 134.830.983 134.830.948
rs3011289	cctgcaaagtcacgcagtttg ctccaagtgcaccctt tccagcaacaggtggtacatt	134.821.751 134.821.773 134.821.812
rs7040593	accctgacagtgaggacatt cgagaactctcatcgac ttgcagttccaagagaggttga	134.816.600 134.816.626 134.816.670
rs869116	ctccctcgcaccagatg agcctgtgctggta cccagacagagaagggcaaa	134.814.011 134.814.037 134.814.078
rs12380834	agttccaccaatctgcaactacttt aggaacagactcttccct gaatccctccctggtgtgaa	134.811.775 134.811.803 134.811.846
rs11796704	ccagcctctgtttccataaaaat agtgttcaggtcctg ccagtgaggaaggacaactga	134.807.844 134.807.817 134.807.775
rs700797	tggactcagttgccctctgaa ctgtgggaagctatgg tgggacataaagggtagaagagaaa	134.805.668 134.805.644 134.805.598
rs13300390	gctcttcacagctcataatgcat ctagggcagagatatgtgaa caggaccctgacgccattt	134.803.943 134.803.914 134.803.874
rs5203101	ttaggaggagccaaaggtagactct taggctcaggaatggg ccttggtctagccacatgct	134.801.009 134.800.982 134.800.946
rs12337302	ctggttctcgtctgcttagtac cagctgtcatcctagtct cacaggagagaggcgaagga	134.784.975 134.784.950 134.784.903
rs2809244	tcaactggctcctctaccaaa ccctgcctcagctg gcctaagaactgtggtctggtgtt	134.760.050 134.760.075 134.760.113
rs2073869	tctcgcagataccctcatgatg tcagctgtgacgaggc acgccgccgtagtggtt	134.753.491 134.753.524 134.753.560
rs2231405	tgcttggcatcccacagtt ctcaaccccagtgga ggatcctgtccttcccata	134.749.724 134.749.704 134.749.666
rs2519759	agagggaaaatggcacagtca tgcagacagcccc aagcggagccagaactgaa	134.744.564 134.744.586 134.744.621
rs2072058	tgaaacccaacaggcgacat agggcaggcgaag cagaatgacctatggaatcaatta	134.739.974 134.739.999 134.740.042
rs12555164	cacccaacacaaaagagtgaacg tgggacagagcacg caggcagaacttccgtcatg	134.693.461 134.693.486 134.693.523
rs35602700	tggcaactaaggctgcatga ttaagctcgaccctgtt caggagtttgctctgcatacga	134.673.378 134.673.399 134.673.439
rs4962216	tgaattcatttctctcacattttcc ccccatcttgagc ttctaagcatgacacagcattggt	134.654.970 134.655.000 134.655.039
<b>Deletion specific PCR</b>		
c.[-24893_-15354del9540; -24902_-24847inv56]	agctcctgggaaacaggat gggctctgcaaatagctgtc aaacagggggcaggaaatag	134.810.337 134.809.122 134.819.205

c.[-21656_-14846del6811; -18011_-17856inv156]	ccccatacaaacagagaaagc actgcatcccaaaaaag ttcgagaaggaggggaggt	134.816.026 134.808.562 134.809.913
c.[-18348_-18313del36;-18267_-11107del7161ins7]	tggactcagttgccctctgaa gggcagtggttctcaaatg ctggggctgggtactgtgatt	134.805.668 134.805.005 134.812.518
c.-16116_-15364del753	gggatccctaccaagcaagt gggctctgcaaatagctgtc agctcctgggaaacaggat	134.809.625 134.809.122 134.810.337
c.-12499_*67438del112575	tggacataaagggtagaagagaaa caccacaacacaagagtgtaacg ggtgaatttgggctctgaa	134.805.598 134.693.461 134.807.378
c.738-1380_*101485del119088	tttgtctctccccactct tggaaggctctatggcagat ccattttccctgcctagaa	134.779.656 134.660.735 134.659.847
c.738-1292_*4009del21524	accctcctgtccaaactg taaaaatattctggccgggtaca gcagggaaaaatgtcctttg	134.757.047 134.779.420 134.777.973
c.-38403_*17484del88525	cacttcaccatactggagc ctggttctgtcagtgctccc aagcggagccagaactgaa	134.832.923 134.743.808 134.744.621

## Table 2

<b>ID 30628</b>	
134818993	TTAGTAGAGACAGGGTTTCaccatgttagccaggatggtc
134809453	ggggcgaggtgggccccttcGTCAGTCCCTCACTTGGTGG
134818982	inversion: AGGGTTTCACCATGTAGCCAGGATGGTC TCAATCTCCTGACCTCGTGATCTGCC
<b>ID 21722</b>	
134815756	TTGGGATTACAGGCGTGAGCaccsagcctggcctgttcc
134808945	taacgttaatggagtgtctctCTGTGTGCCAGGCACTGTGA
134812091	inversion: TGTAGAAACAGTGACAATGTGGTTATGGC CCCTGGAAAGTGAGCAGTTGATGAGCTCTGCTTATGGCT GCTCTCCTCCATGGCAGAATGCATTCCTGCAGGATGAGGT CATGGAGCCAAGGAAGGTCAGGGTGTGGGGGGCTTCCTG GGTCAGA
<b>ID 21899</b>	
134812448	AGAAGGCGAGGGGAGATGTcacacagccaaatgcagcag first breakpoint
134812412	gcagatgtcctccaccacgtGCATCCTGTCAGCACGCCCG second breakpoint
134812367	GGAGGGCTCAGCCCTTTCATgctcagacagggcggtcagg third breakpoint
134805206	gaatgaatgtttttaccttCATCCTTCTTTTCCTAGAC insertion:AATACAT
<b>ID 1264</b>	
134810216	CAGGCGCCGCCAGCTTGTTTacgcctctccgccggtccc
134809463	ccctcgggcagggcgaggtGGGCCCTTCGTCAGTCCCT
<b>ID 31457</b>	
134806599	TTTCTCTGGTTTACTTTTTAagactgtagttgctagaaat
134694024	tttctataaagctattatatTCCTTTAAAATAATTCCAA
<b>ID 29445</b>	
134779065	CATTGGCTCACTTTTTTTTTtttttttttttttgagacgga
134659977	tggttccccaaagtctggtCAAGTTGATTTTAAACAGAGT
<b>ID 28121</b>	
134778977	ACTGCAAATTCGCCTCCTGggttcacgcattctcctgc
134757453	ttaggtcgaggactggccccTAGGCTGCTGCTGTGACCCT
<b>ID 14249</b>	
134832503	TAACAGAATGCCACAGACTGggtcacttataaagaaaaga
134743978	csactctaggagtgggctgAGGATTTCCACCCGATCCTC