

MUTATION IN BRIEF

Detection of Heterozygous *SALL1* Deletions by Quantitative Real Time PCR Proves the Contribution of a *SALL1* Dosage Effect in the Pathogenesis of Townes-Brocks Syndrome

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Townes-Brocks syndrome (TBS) is an autosomal dominantly inherited disorder characterized by ear, anal, limb, and renal malformations, and results from mutations in the gene *SALL1*. All *SALL1* mutations previously found in TBS patients create preterminal termination codons. In accordance with the findings of pericentric inversions or balanced translocations, TBS was initially assumed to be caused by *SALL1* haploinsufficiency. This assumption was strongly contradicted by a *Sall1* mouse knock-out, because neither hetero- nor homozygous knock-out mutants displayed a TBS-like phenotype. A different mouse mutant mimicking the human *SALL1* mutations, however, showed a TBS-like phenotype in the heterozygous situation, suggesting a dominant-negative action of the mutations causing TBS. We applied quantitative real time PCR to detect and map *SALL1* deletions in 240 patients with the clinical diagnosis of TBS, who were negative for *SALL1* mutations. Deletions were found in three families. In the first family, a 75 kb deletion including all *SALL1* exons had been inherited by two siblings from their father. A second, sporadic patient carried a de novo 1.9–2.6 Mb deletion including the whole *SALL1* gene, and yet another sporadic case was found to carry an intragenic deletion of 3384 bp. In all affected persons, the TBS phenotype is rather mild as compared to the phenotype resulting from point mutations. These results confirm that *SALL1* haploinsufficiency is sufficient to cause a mild TBS phenotype but suggest that it is not sufficient to cause the severe, classical form. It therefore seems that there is a different contribution of *SALL1* gene function to mouse and human embryonic development. ©2006 Wiley-Liss, Inc.

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KEY WORDS: SALL1; Townes-Brocks syndrome; TBS

INTRODUCTION

Townes-Brocks syndrome (TBS, MIM# 107480) is an autosomal dominantly inherited malformation syndrome characterized by anal, renal, limb, and ear anomalies (Powell and Michaelis, 1999). It is caused by mutations in the putative zinc finger transcription factor gene *SALL1* (Kohlhase et al., 1998). All disease-causing mutations reported from TBS patients to date are resulting in premature termination codons (Albrecht et al., 2004; Devriendt et al., 2002; Engels et al., 2000; Kohlhase, 2000; Kohlhase et al., 2003). Initially, based on this observation and the association of TBS with structural chromosomal anomalies i.e. a pericentric inversion inv(16)(p11.2;p12.1) (Powell et al., 1995) and a translocation t(5;16)(p15.5;q12.1) (Serville et al., 1993), *SALL1* haploinsufficiency was assumed to be the pathogenic mechanism leading to TBS (Kohlhase, 2000; Kohlhase et al., 1999c; Kohlhase et al., 1998; Marlin et al., 1999). In order to create an animal model for Townes-Brocks syndrome, Nishinakamura and coworkers established a *Sall1* knock-out mouse by deleting the essential second as well as the third exon, which harbor all zinc finger domains characteristic for SALL proteins (Nishinakamura et al., 2001). Unexpectedly, these *Sall1* knock-out mice did not show any phenotype in the heterozygous situation, and the homozygous mutants had only bilateral renal agenesis or severely hypoplastic kidneys with no preserved renal function (the same phenotype was observed in a knock-out mouse constructed by the authors group on two different genetic backgrounds; Kohlhase J, unpublished data). As an alternative explanation for the pathogenesis of TBS it was assumed that truncating *SALL1* mutations could lead to the TBS phenotype by a dominant-negative action, with truncated proteins interfering with nuclear transport of the wild type proteins (Sweetman et al., 2003) resulting from dimerization of wild type and mutant proteins mediated by the evolutionarily highly conserved glutamine-rich domain within the aminoterminal part of all known SAL-like proteins (Buck et al., 2000; Farrell and Munsterberg, 2000; Farrell et al., 2001; Hollemann et al., 1996; Kohlhase et al., 2000; Kohlhase et al., 1999a; Kohlhase et al., 1996; Köster et al., 1997; Kühnlein et al., 1994; Onuma et al., 1999; Ott et al., 1996). McLeskey Kiefer et al. created a transgenic mouse harboring a “typical” TBS-mutation within the *Sall1* gene in order to mimic the molecular defect in human patients. Indeed, they could show that mice heterozygous for this mutation displayed a phenotype similar to TBS and that this is likely to result from a dominant-negative action of truncated *Sall1* proteins (McLeskey Kiefer et al., 2003). However, this hypothesis could not integrate the observation of cytogenetic anomalies in TBS patients. We were therefore interested to find out if TBS can be caused by larger deletions within or including the *SALL1* gene. Here we report the first three families with heterozygous deletions of the *SALL1* gene detected by quantitative real time PCR.

MATERIALS AND METHODS

Patients

Patients from 240 different families with the clinical or differential diagnosis of Townes-Brocks syndrome but without detectable *SALL1* mutations were analyzed. Inclusion criteria for deletion analysis were (1) presence of at least one typical clinical major criterion for Townes-Brocks syndrome (Kohlhase et al., 1999c), (2) no other evident clinical diagnosis, and (3) previously negative *SALL1* mutation analysis by direct sequencing (Kohlhase et al., 1999c). Venous blood had been collected from patients and unaffected relatives after obtaining their informed consent. The study received approval by the ethics committee of the Faculty of Medicine at Freiburg University.

Quantitative PCR

Deletion detection and fine mapping of the deletions was performed using a quantitative PCR approach with SYBR-Green I detection (Boehm et al., 2004). We designed primers for amplification of small amplicons positioned 5' close to the translation start codon of exon 1, within intron 1, in exon 2, in intron 2 and within the 3' UTR in exon 3 (Fig. 2; primer sequences available on request). Amplicons could not be placed within the coding regions of exons 1 and 3 based on the presence of repetitive regions or high similarity to the *SALL1P* (Kohlhase et al., 1999b) sequence on chromosome Xp11.2. Three amplicons mapping to subtelomeric regions (2q24.2, 3p26.3, 4p15.2) were used as internal reference controls (Boehm et al., 2004). Genomic DNA from an unaffected individual was used as a deletion-negative control. *SALL1* genomic sequence including 100 kb upstream and

downstream of the coding region was retrieved from the NCBI nucleotide database (<http://www.ncbi.nlm.nih.gov/>) and masked for repeats (<http://www.woody.embl-heidelberg.de>). 11 primer pairs were designed to amplify fragments of 100-300 bp using the PRIME program (Genetic Computer Group, Wisconsin, USA) (sequences available on request). Amplicons were mapped relative to the *SALL1* coding region (Fig. 2A,B). The investigated genomic region comprises about 230 kb, approximately 100 kb on the 5' and on the 3' side of the *SALL1* gene (101-123 kb). The physical location of this region is 49620000–49849999 bp for the complete sequence (exactly 230.000 kb), and 49727830-49742653 bp for the *SALL1* coding region plus introns, calculated from the telomere of the p-arm of chromosome 16. Further amplicons were constructed for the fine mapping of the deletion in family 2 (Fig. 2B). Since *SALL1* is transcribed from telomere to centromere on the q arm, the startpoint of the analyzed sequence (bp 0 = bp 49849999) is more telomeric, and the end (bp 230,000 = bp 49620000) more centromeric. We used the ABI Prism 7900 Sequence Detection System (PE Applied Biosystems, Darmstadt, Germany) and white-colored 384-well plates (ABgene, Hamburg, Germany) for real time PCR. Reactions contained 0.25 mM each primer and 5 µl QuantiTect[®] SYBR[®] Green PCR Master Mix (Qiagen, Hilden, Germany) in a total of 10 µl. Assays included DNA standards in a final concentration of 5.0, 2.5, 1.25, or 0.625 ng/µl, a no-template control, or 2.5 ng/µl of the patient DNA in replicates (n=6). Cycling conditions were 50°C for 2 min, 95°C for 15 min, and 40 cycles of 94°C (15 sec), 60°C (15 sec), and 72°C (1 min). For all amplicons the same conditions were applied. In order to avoid the generation of unspecific products, a melting curve analysis of products was performed routinely following the amplification. A standard curve was constructed for each amplicon by plotting the cycle number (ct), at which the amount of target in standard dilutions reaches a fixed threshold, against the log of the amount of starting target. Absolute quantification of target amplicons in the patients was thereafter performed by interpolation of the threshold cycle number (Ct) against the corresponding standard curve. Quantitative data were further normalized against a normal diploid reference genome by calculating the ratio relatively to the average amount of reference amplicons for each amplicon. In this manner ratio-values of 1.0 indicate a diploid situation, values of 0.5 or 1.5 indicate partial haploidy or partial triploidy, respectively (Table 1). Experiments were performed in triplicates.

Breakpoint cloning

Additional forward and reverse primers (sequences available on request) were designed for amplification of the breakpoint-spanning regions in families 1 and 3 with the TaKaRa LA Taq[™] (TaKaRa Bio Inc., Otsu, Shiga, Japan). The reactions contained 0.5 µg of genomic DNA, 5 µl LA PCR Buffer, 5 µl MgCl₂ (25 mM), 8µl dNTPs (2.5 mM each), 20 pmol of each primer and 2.5 units of TaKaRa LA Taq[™] enzyme to a total volume of 50 µl. An initial denaturation step at 94°C for 1 min was followed by 30 cycles of 94°C for 25 sec and 68°C for 15 min. The reaction terminated with a final elongation step at 72°C for 10 min. The PCR product was subcloned into pGEM-T Easy vector (Promega, Heidelberg, Germany) for further analysis. Plasmids were isolated from bacterial colonies by routine methods and sequenced using T7 and SP6 primers, respectively.

Fluorescence in situ hybridisation (FISH)

Metaphase spreads from peripheral blood lymphocytes were prepared by routine procedures. Prior to FISH, the slides were treated with RNase followed by pepsin digestion (Ried et al., 1992). FISH essentially followed the methods previously described (Schempp et al., 1995). Chromosome *in situ* suppression was applied to the following probes. As a gene specific probe, a 1.1 kb cDNA fragment of *SALL1* containing exon 2 sequence (Kohlhase et al., 1996) was chosen. After FISH, slides were counterstained with DAPI (0.14 µg/ml) and mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA).

Electronic database information

Accession numbers and URLs for data in this article are as follows: GenBank (<http://www.ncbi.nlm.nih.gov/>) accession numbers: NM_002968.1 (*SALL1* cDNA sequence), NT_010498.15 (genomic contig including *SALL1*). Online Mendelian Inheritance in Man (OMIM; <http://www.ncbi.nlm.nih.gov/OMIM/>): for Townes-Brocks syndrome, OMIM 107480. Repeat-masker at EMBL: <http://www.woody.embl-heidelberg.de>.

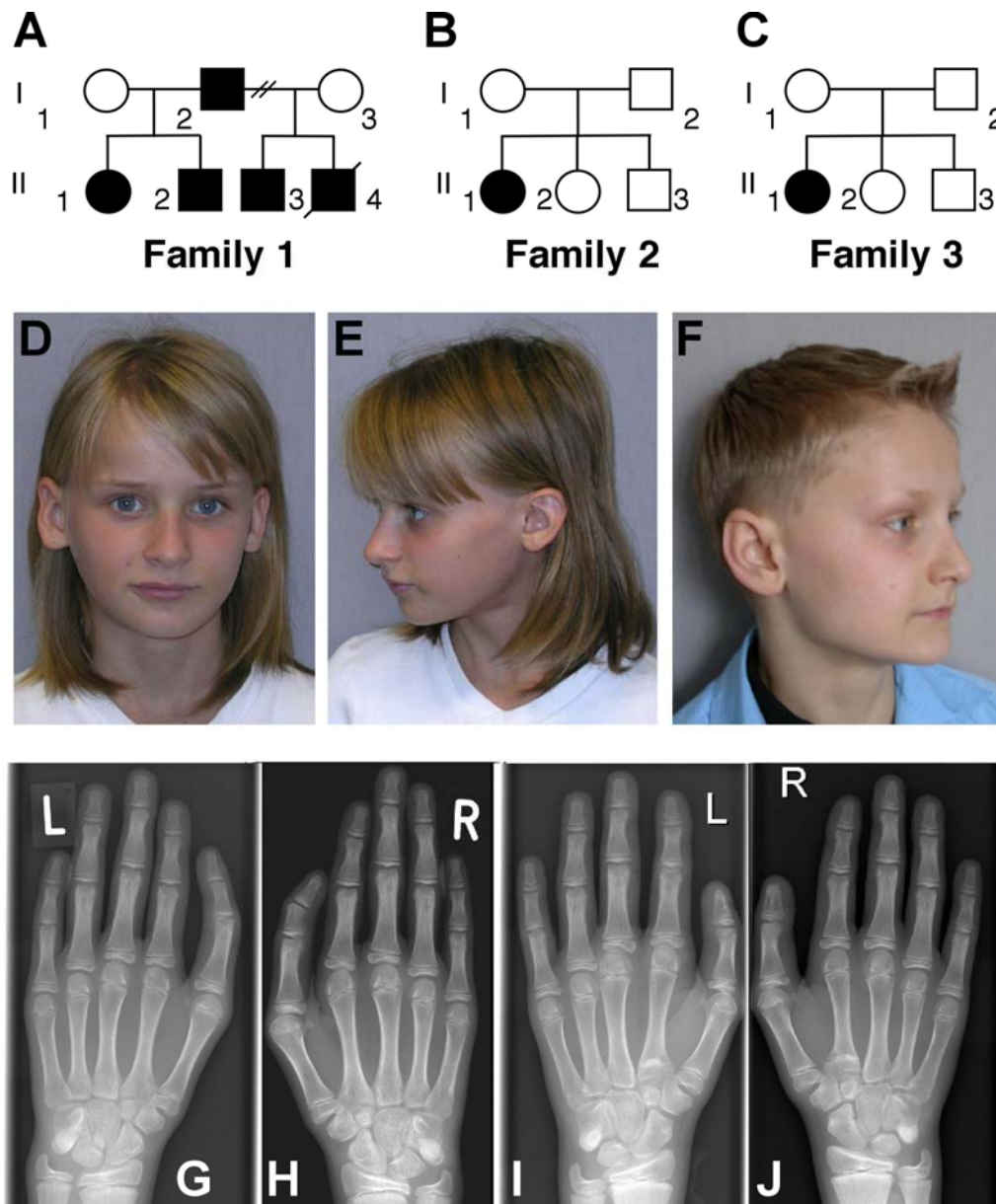


Figure 1. Pedigrees of the families in which *SALL1* deletions were identified (A-C). Filled symbols indicate affected or possibly affected ((A) II.3, II.4) individuals. See text for details. Frontal (D) and left side (F) view of the index patient in family 1 (A; II.1) and right side view of her brother (A; II.2). Hand X-rays of the girl of family 1 showing bilateral triphalangeal thumbs (G,H) and her brother (I, J) showing broader thumbs with a perpendicular calcification in the middle of the terminal phalanx of the right thumb corresponding to a fine groove in the middle of the right thumb nail (J).

RESULTS

SALL1 mutation analysis in all 240 patients had revealed no heterozygous *SALL1* mutation. In order to test the patients for *SALL1* deletions, quantitative real time PCR was applied. Deletions were detected in 3 out of 240 index patients. Their phenotypic features are as follows: in family 1, a 10-year old girl (II.1; Fig. 1A,D,E) was referred for clinical genetic evaluation. She presented with bilateral triphalangeal thumbs (Fig. 1G,H), ventrally positioned anus, a dysplastic left ear (Fig. 1E), bilateral sensorineural hearing loss, and bilateral metatarsus

adductus. Her mental development was normal, and she attended a normal school. Her brother (II.2; Fig. 1A,F) presented with broad thumbs (Fig. 1I,J), and slightly dysplastic right ear. At birth, he had membranous anal atresia. The father of the children (I.2; Fig. 1A) lived far away from the family and could not be examined by the authors. He was reported to have normal hands but bilateral hearing loss. Interestingly, two further children of his from a previous marriage had died in early childhood and were reported to be dysmorphic. The reason for their death is not known. In family 2, the unaffected non-consanguineous parents of Portuguese origin sought a diagnosis for their oldest mentally delayed daughter (II.1; Fig. 1B). She was born at term with a weight of 3590 g, a length of 49 cm and an OFC of 35 cm. A ventrally-displaced anus with a rectoperineal fistula was noted at birth and surgically corrected at the age of 6 months. Early milestones were normal (sitting at 7 months, walking at 12 months, first words at 12 months), but speech was delayed, the first sentences being said between 36 and 42 months. Because of learning disabilities and difficult adaptation in mainstream schools, the patient underwent special education. At first genetic evaluation at the age of 10 years normal growth and OFC parameters were noted, as well as asymmetric ear length, the right one being smaller and with an overfolded helix. Her thumbs were long with ulnar deviation of the terminal phalanges but not triphalangeal on radiographs. The diagnosis of TBS was considered at the time and bilateral sensorineural deafness (40-50 dB at 1000 Hz) detected concomitantly. Idiopathic hyperprolactinemia with normal brain MRI was found at the age of 15 years. When last examined at the age of 18 years, the patient had a height of 153 cm (p3-10), a weight of 40 kg (<p3) and an OFC of 54.5 cm (p50). Despite being fluent in 2 languages, she did not know how to read, write, or count and had no knowledge of time. She is currently employed in a sheltered environment, learning daily household tasks. Her mental age was estimated by the parents to be at 8-9 years. Routine chromosomal analysis revealed a normal karyotype 46,XX. In family 3 (Fig. 1C), the sporadic index case (II.1) is again a girl, the oldest of three children. Parents are of Turkish origin, and non-consanguineous. There is no family history of congenital malformations. She was born by C-section because of breech position at 38 weeks of gestation with a birth weight of 3750 g and a length of 56 cm. Head circumference was not recorded. Milestones in development were normal: she walked at the age of 12 months, and first words were said at the normal age. At birth, the anus was found to be ectopic and too narrow, for which she had surgery. The ears were protruding and small, for which cosmetic surgery was performed at 3 years of age. She suffered from recurrent urinary infection, caused by vesicoureteric reflux, which was treated by means of Teflon injection at the age of 13 years. The patient developed reflux nephropathy leading to renal failure. She also had bilateral hearing loss: sensorineural on the right side (35 dB), mixed on the left side (65dB). A CT scan of the middle and inner ear did not reveal structural anomalies. Clinical examination at 13 years revealed a weight of 36 kg (p3-10), a length of 148 cm (p3-10), a preauricular pit and a supernumerary nipple on the right side, hammer toes and hallux valgus on the right side and relatively broad thumbs. X-rays of the hands and feet did not reveal any skeletal anomalies except for the broad first rays. Ophthalmological examination at the age of 14 yrs revealed an anomaly of both papillae with borders of the papillae that were not well delineated, and an unusual vascular pattern. She followed regular school. Pubertal development was normal. At age 20, she was 162 cm tall and weighed 50 kg. High resolution karyotype was normal 46,XX.

In family 1, quantitative real time PCR revealed a heterozygous deletion (Fig. 2A; Table 1) spanning exons 2 and 3 as well as a large part of intron 1. The deletion was present in the DNA of the index patient, her brother and their father but not in the mother. The deletion size was estimated to be 68 to 89 kb. In family 2, the index patient was found to carry a *de novo* deletion spanning all coding exons and all amplicons ranging from 100 kb upstream until 100 kb downstream of *SALL1* showed that the whole region was deleted on one chromosome. Additional amplicons helped to map the deletion size as to be 1.9 Mb to 2.6 Mb maximum (Fig. 2A,B). In family 3, the index patient had only two amplicons heterozygously deleted, positioned in exon 2 and intron 2. The deletion could therefore be determined to be 2 kb minimum and 15 kb maximum in size (Fig. 2A,B).

In family 1, real time PCR results predicted the expected 5' breakpoint between the 5'UTR and intron 1 within a region of approximately 2 kb 3' of exon 1. By long range PCR we amplified a deletion-spanning fragment of approximately 2.5 kb from the index patient. Sequencing of the subcloned PCR fragment (Fig. 2C) revealed a deletion size of 74593 bp (g.4727273_4801865del with respect to GenBank NT_010498.15), containing the whole *SALL1* gene except for part of the 5'UTR. The 5' breakpoint resides upstream of the ATG but not within a predicted repetitive element but the 3' breakpoint lies within a predicted THE1D repeat. The deletion was confirmed by FISH with an exonic *SALL1* probe. Indeed, this probe consistently detected only one chromosome 16 in all examined metaphases (Fig. 2C). In family 3, long range PCR revealed a breakpoint-spanning fragment of approximately 750 bp. Sequencing of the subcloned fragment (Fig. 2D) revealed a deletion size of 3384 bp

(g.4785579_4788962del), consisting of 2164 bp exon 2, 1137 bp intron 2 and 83 bp exon 3 sequence. The deletion removes or interrupts the coding sequence for all double zinc finger domains of *SALL1*.

DISCUSSION

Our data show for the first time that Townes-Brocks syndrome may also be caused by heterozygous deletions either of the whole *SALL1* coding region or of a larger part of it. In one of the families, we could prove a deletion by three independent methods, FISH, quantitative real time PCR and long range PCR/ sequencing. In one family, *de novo* occurrence of the deletion in the index patient was proven by real time PCR and long range PCR/ sequencing, and in the remaining family by real time PCR only. Nevertheless, this method has been successfully applied to other genes like *SALL4* (Borozdin et al., 2004) and *MECP2* (Laccone et al., 2004) as well as for detection of subtelomeric copy changes (Boehm et al., 2004). Therefore no doubt remains that the deletion in family 2 is also a reliable finding, which explains the phenotype.

Real time PCR proved to be a valuable tool to approximately determine the sizes of the deletions. Two of the three deletions would likely have escaped a strategy using a conventional FISH probe, i.e. a larger genomic clone with an insert of >100 kb from the *SALL1* region, for detection. Therefore, the applied real time PCR seems to be the method of choice for diagnostic purposes. However, with only three deletions being detected among 240 patients with a differential diagnosis of Townes-Brocks syndrome, larger *SALL1* deletions do not seem to contribute to a larger extent to the mutational spectrum. This might be due to the relative sparcicity of repetitive elements in the genomic region of *SALL1* as compared to *SALL4*, which is situated within a region rich in such elements (Borozdin et al., 2004).

Most importantly, our results show that heterozygous deletions of *SALL1* do occur and lead to Townes-Brocks syndrome or at least a milder form of it. Based on animal model data, *SALL1* mutations causing Townes-Brocks syndrome were predicted to have dominant (-negative) effects (McLeskey Kiefer et al., 2003; Nishinakamura, 2003; Sweetman et al., 2003), and resulting truncated *SALL1* proteins could interfere with other *SALL* proteins. Our results shown here prove that *SALL1* is required for proper development of thumbs, ears, hearing (i. e. inner and/ or middle ear) and anus in humans, and (2) that *SALL1* haploinsufficiency leads to TBS.

The index patient in family 1 has typical thumb malformations but very mildly dysplastic ears and mild anal malformations. Her brother and father are even milder effected. Although TBS shows considerable inter- and intrafamilial variability, only one of the five affected persons in this study shows the complete and fully expressed trias of typical thumb defects, imperforate anus and dysplastic ears. It seems therefore that *SALL1* deletions are associated with a milder expression of TBS and possibly a higher phenotypic variability as compared to TBS caused by point mutations, especially to that associated with p.R276X (c.826C>T) (Kohlhase et al., 2003).

If it holds true that the most severe TBS phenotype is only seen with point mutations, it could be that a dominant (-negative) effect of point mutations may result in a more severe phenotype. However, experimental evidence is still lacking for the existence of truncated *SALL1* proteins and the capability of mutated *SALL1* transcripts carrying premature termination codons to escape nonsense mediated mRNA decay.

Even though there seems to be a milder malformation phenotype associated with heterozygous *SALL1* deletions, a risk for mental retardation/ learning disabilities cannot be excluded if the deletion removes not only *SALL1* but also neighboring genes, as illustrated by the index patient in family 2. A "mild" TBS phenotype associated with mental retardation could therefore be an indicator of a larger deletion including *SALL1*. Further cases with deletions similar to the one in family 2 might help to identify the gene(s) responsible for the mental retardation. At present, we cannot predict if a heterozygous deletion of *SALL1* alone also harbors a risk for mental retardation.

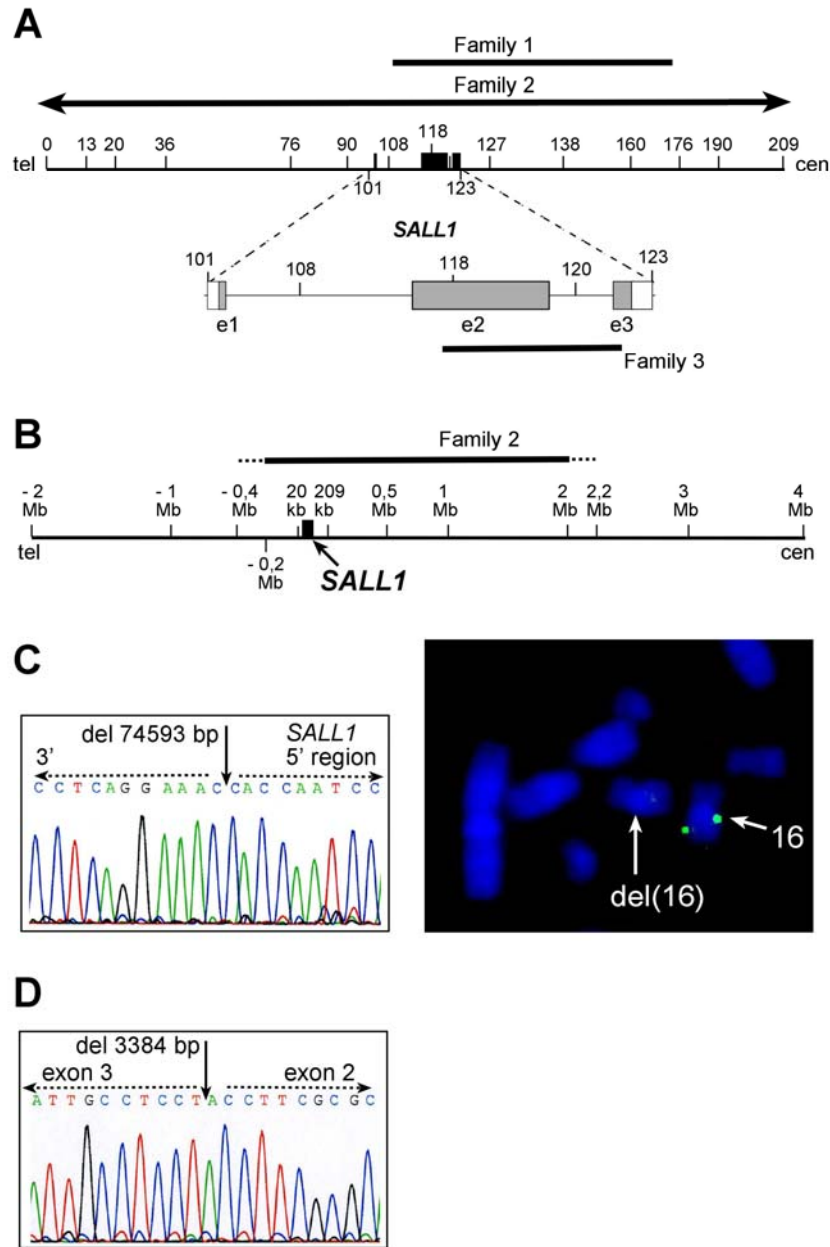


Figure 2. (A) Schematic diagram of the SALL1 genomic region on chromosome 16q12.1 with positions of the deletions detected in the three families analyzed. 230 kb of genomic DNA sequence (line) consisting of the SALL1 gene plus 100 kb flanking sequence on each side were analyzed by quantitative real time PCR for deletions. The numbers indicate the positions of amplicons. The boxes on top of the line indicate the SALL1 exons enlarged below with black indicating coding and white UTR regions. Note the considerable size differences of the deletions. The deletion in family 3 removes or interrupts the coding sequence for all double zinc finger domains of SALL1. (B) Schematic map of the deletion in family 2. The solid line indicates definitely deleted regions, the dotted line indicates that this sequence might or might not be deleted. (C) Left: reverse complementary (in relation to SALL1 sense strand) sequence of the breakpoint in family 1 with the 5' breakpoint residing in the 5'UTR and the 3' breakpoint within a THE1D repetitive element. Right: FISH analysis in the index patient of family 1. A SALL1-specific probe hybridizing to chromosome 16q12.1 detects only one chromosome 16, thereby confirming the deletion. (D) Sequence of the breakpoint-spanning fragment in family 3 with the 5' breakpoint residing in the exon 2 and the 3' breakpoint in exon 3. Mutation description with respect to reference sequence (GenBank NT_010498.15): g.4727273_4801865del (family 1; C), g.4785579_4788962del (family 3; D).

Table 1. Real-time PCR results

Family	Amplicon position															
	13 kb	20 kb	36 kb	76 kb	90 kb	101 kb	108 kb	118 kb	120 kb	123 kb	127 kb	138 kb	160 kb	176 kb	190 kb	209 kb
1						1.03	<i>0.46</i>	<i>0.47</i>	<i>0.47</i>	<i>0.49</i>	<i>0.51</i>	<i>0.49</i>	<i>0.51</i>	<i>0.58</i>	1.10	1.01
2	<i>0.46</i>	<i>0.56</i>	<i>0.45</i>	<i>0.53</i>	<i>0.45</i>	0.51	0.51	0.49	0.50	0.49	<i>0.41</i>	<i>0.51</i>	<i>0.46</i>	<i>0.57</i>	<i>0.47</i>	<i>0.56</i>
3						1.01	0.99	0.50	0.44	0.90						
C	1.03	0.99	1.05	1.01	0.97	0.97	1.04	0.95	0.98	1.06	1.04	0.99	1.00	1.03	0.98	1.01

Results of the *SALL1* real-time PCR applied to patient DNA samples of three families with Townes-Brocks syndrome. The normalized ratios (*SALL1* amplicon/ reference amplicon) are presented. Values interpreted as haploid situation (deletion) range from 0.41 to 0.58 whereas a diploid situation was assumed for values from 0.90 to 1.10. Amplicons within the *SALL1* coding region and UTR's are printed in bold lettering; larger and italic lettering indicates deletions.

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