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Molecular Analysis of *SALL1* Mutations in Townes-Brocks Syndrome

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Summary

Townes-Brocks syndrome (TBS) is an autosomal dominantly inherited malformation syndrome characterized by anal, renal, limb, and ear anomalies. Recently, we showed that mutations in the putative zinc finger transcription factor gene *SALL1* cause TBS. To determine the spectrum of *SALL1* mutations and to investigate the genotype-phenotype correlations in TBS, we examined 23 additional families with TBS or similar phenotypes for *SALL1* mutations. In 9 of these families mutations were identified. None of the mutations has previously been described. Two of these mutations are nonsense mutations, one of which occurred in three unrelated families. Five of the mutations are short deletions. All of the mutations are located 5′ of the first double zinc finger (DZF) encoding region and are therefore predicted to result in putative prematurely terminated proteins lacking all DZF domains. This suggests that only *SALL1* mutations that remove the DZF domains result in TBS. We also present evidence that in rare cases *SALL1* mutations can lead to phenotypes similar to Goldenhar syndrome. However, phenotypic differences in TBS do not seem to depend on the site of mutation.

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Introduction

Townes-Brocks syndrome (TBS; MIM 107480) was first described by Townes and Brocks (1972) as an association of imperforate anus, supernumerary thumbs, malformed ears, preauricular tags, and sensorineural hearing loss. Since the first description, several additional families have been described and several isolated cases have been reported (Reid and Turner 1976; Kurnit et al. 1978; Walpole and Hockey 1982; Barakat et al. 1988; de Vries–van der Weerd et al. 1988; O'Callaghan and Young 1990; Rossmiller and Pasic 1994; Wischermann and Holschneider 1997). However, the frequency of this condition has not yet been determined.

The clinical presentation of TBS is highly variable (de Pina-Neto 1984; O'Callaghan and Young 1995; Wischermann and Holschneider 1997) both within and between affected families. Characteristic features of TBS are anorectal abnormalities (imperforate anus), abnormalities of the hands (preaxial polydactyly, triphalangeal thumbs), abnormalities of the feet (syndactyly, club foot), deformities of the outer ear ("lop ears") and preauricular tags, and sensorineural hearing loss (Rossmiller and Pasic 1994). Renal malformations have also been reported in several cases (see O'Callaghan and Young 1995; Wischermann and Holschneider 1997) and can lead to renal failure in patients with TBS (Rossmiller and Pasic 1994; Newman et al. 1997). Cardiac malformations (O'Callaghan and Young 1995) and mental retardation (Cameron et al. 1991; Ishikiriyama et al. 1996) are rarely reported. In addition, in some families affected subjects show features typical both of TBS and of Goldenhar syndrome/oculoauriculovertebral spectrum

(Moeschler and Clarren 1982; Gabrielli et al. 1993; Johnson et al. 1996).

The variety of findings associated with TBS and their strong intrafamilial variability provide major problems for diagnosis of the syndrome and for genetic counseling. Furthermore, it is still unclear whether penetrance is complete in TBS (O'Callaghan and Young 1995). We have recently reported that TBS is caused by mutations in the putative zinc finger transcription factor gene *SALL1* (MIM 602218) (Kohlhase et al. 1998). The availability of a molecular diagnostic test now allows us to investigate whether phenotypic variation among different families with TBS is associated with a certain type of *SALL1* mutation and whether penetrance is complete in mutation-positive families. To solve these problems and to gain more insight into the mutational spectrum of *SALL1,* we searched for *SALL1* mutations in a total of 23 families in which at least one proband had clear TBS or features typical of TBS, including one patient in whom partial phenotypic overlap with Goldenhar syndrome was described (Gabrielli et al. 1993).

Subjects, Material, and Methods

Patients

A total of 23 families were investigated for the presence of *SALL1* mutations. In 12 families (group A), the diagnosis of TBS was based on the presence of typical (O'Callaghan and Young 1995; Wischermann and Holschneider 1997) malformations (major criteria) of the anus, hands (thumbs), and ears. Four of these families have been described elsewhere (de Vries–van der Weerd et al. 1988; O'Callaghan and Young 1990; Rossmiller and Pasic 1994; Newman et al. 1997). In six families (group B), the affected members showed only two of the major criteria in combination with at least one additional feature commonly found in TBS (minor criteria: hearing loss, kidney malformation or agenesis, impaired renal function, foot malformation, and congenital heart defect). One patient in this group has also been described previously because she displays features present in Goldenhar syndrome as well as in TBS (Gabrielli et al. 1993). The remaining five families (group C) were included because their affected members showed only one of the major criteria in combination with at least one minor criterion.

A thorough clinical examination was performed on all subjects. This examination included routine serum and blood analysis (including creatinine and electrolytes) as well as renal ultrasound. Further testing (x-rays of hands and feet, cardiac ultrasound, further urological investigations) was done if malfunctions or malformations were present or suspected. Audiography was performed on all subjects. In all subjects chromosomal analysis had revealed a normal karyotype. Appropriate informed consent was obtained from all subjects or from their parents prior to investigation.

5′ RACE

5′ RACE was performed on human adult kidney total RNA (Clontech) by using Superscript II reverse transcriptase (GIBCO BRL) according to the manufacturer's instructions. For first-strand cDNA synthesis, primer TR1.3 (table 1) was used. After first-strand synthesis, cDNA was poly-G–tailed by use of terminal transferase (Pharmacia) according to the manufacturer's protocol. The reaction products were ethanol precipitated and resuspended in 10 μl of water. A first round of PCR was carried out with primers AN-poly-C (5′-GCAAGCTTGAATTCCCGCGG$[C]_{14}$-3′), AN (5′-GCAAGCTTGATTCCCGCGGCC-3′), and 1R2 (table 1), after which 2.5 μl of tailed cDNA was mixed with 5 μl of dimethyl sulfoxide, 5 μl of 1 × PCR buffer (GIBCO BRL), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1.5 pmol of primer AN-poly-C, 15 pmol of primer AN, 15 pmol of primer 1R2, and 2.5 U of platinum *Taq* polymerase (GIBCO BRL) in a total volume of 50 μl. The following cycling conditions were used: 5 min at 94°C (initial denaturation), 1 min at 94°C, 2 min at 45°C, 3 min at 72°C (5 cycles), 1 min at 94°C, 2 min at 55°C, 3 min at 72°C (25 cycles), and 5 min at 72°C (final polymerization). A nested amplification was carried out according to the same procedure, with the following changes: 1 μl of the amplification product of the first round of PCR was used as the template and the primers were AN and TR1.2 (table 1), at 15 pmol each. The cycling conditions were 5 min at 94°C (initial denaturation), 1 min at 94°C, 2 min at 55°C, 3 min at 72°C (30 cycles), and 5 min at 72°C (final polymerization). The amplification products of first- and second-round PCR were analyzed by agarose gel electrophoresis, subcloned into pGEM-T (Promega), and sequenced on both strands.

Isolation of PAC clones

Screening of a human PAC library (Ioannou et al. 1994) was performed according to standard screening procedures (Sambrook et al. 1989) with a ^{32}P-labeled *SALL1* cDNA probe. Computer-spotted library filters (library no. 704) and cultures of isolated clones were supplied by the Resource Center of the German Human Genome Project. Preparation of PAC DNA was performed as described by Ioannou et al. (1994). For sequencing, 5 μg of PAC DNA was mixed with 10 pmol of primer E1/2 or E1/4R (table 1) and 8 μl of BigDyeTerminator Mix (Applied Biosystems), and water was added to a final volume of 20 μl. Cycle sequencing was performed in a Triblock Thermocycler (Biometra) for 5 min at 98°C (initial denaturation) followed by 40

Table 1

***SALL1* Primer Sequences**

Forward, 5′–3	Name	Reverse, 5′–3′	Name
Exon 1			
GCTCGATTTCCGTAATTTTGAG	E1/4[a]	aaagcagggacggagaagcc	E1R2[a]
TTCTGCCCCAGCTGATGTTTG	E1/2	GAGCGAGGCCACTTCGGGGTCGG	E1/4R
Exon 2, Part 1			
tacagttttgagttttgcaggtg	TF0.2[a]	AGGGTTGGTGAAGGTGGTGG	TR2.1[a]
gtcctggccccttttctaatc	TF1[b]	AGGCACAGGATAGGGGTTCAT	TR2[b]
CTTTCAGAACACAACGGACTTG	TF1.1	GGAGCCTGGATTTTCATTTACG	TR1.2
CTCGGGGACCTGACAACACT	1S2	TCCCCGAGTTGAGGTAGAGA	1R2
TTACGAACATCTGCCAACCCC	TF1.2	AAGTTGCCCAGTGTTGTCAGG	TR1.3
TATTGGCAGCGGCAGTTACC	1S3[a]	AATGGGAACTTAGCGTGGAC	1R3
		TTGGATGGAATGATGGTGTT	1R4
		CTGCAAGGCACTGTCACTCC	1R5[a]
Exon 2, Part 2			
GCAAGCCACCAAATGTCACTG	TF2[a]	ttcccttgcaagagctctctc	TR5.4[a]
ATCCACTTGCGTTCCCATACC	TF2.1[b]	ggacagggttgatggagcag	TR5[b]
CCCCCAGGCTCAGTCAAAAG	1S4	CTGATGGGGATGGGGGCTGG	1R6
GGACTCAGCTCAGGCATCAGA	TF3	AGGGTTGGTGAAGGTGGTGG	TR2.1
GTGCTATGCCCCCGCTCAG	TF3.1	CCTTCTTGTCAATGTTTTCTACC	TR3.1
CAGACGCCTCCCAAGACAGC	TF3.3	TGGACTCAGAGTAGCTGTCGG	TR3
GAGCGGTCCCAAGCGAGTTT	TF4	GGCAAAGGCGAAGAGGATAAG	TR3.2
AGTTTGCAATCGTGGCTTTTCC	TF4.1	CTGGGTGACTTGTGGAACTC	1R8
Exon 3			
GTAAGGACACCCCCACCAGT	1S6[a]	GGAGTAGGAGGCCACCATAG	TR5.6[a]
gttgccttcactgtctgtcac	TF5[b]	TCTGAACAGGAATGAATGCTATG	TR5.1[b]
GAGGCAATCCCGTCAAGTTC	1S7	TCCACAGAGAGCCGCCGACC	1R9
		TCTCCAGGTTTCCCGTCAGC	1R10

NOTE.—All primers were used for direct sequencing except for TF0.2 and TR5.4. Primers located in introns are lowercase, whereas exonic primers are capitalized.

[a] Primers that were also used for primary PCR amplification.

[b] Primers that were also used for nested amplification.

cycles of 10 s at 96°C, 5 s at 50°C, and 4 min at 60°C. Sequencing reactions were analyzed on an ABI 377 automated sequencer (Applied Biosystems).

Genetic Analysis

Genomic DNA was prepared from peripheral lymphocytes or cultured fibroblasts by routine procedures. Primers for amplification of exons 2 and 3 and for direct sequencing are listed in table 1. PCR was performed essentially as described elsewhere (Kohlhase et al. 1998), with some modifications. A primary PCR was performed to amplify part 1 and part 2 of exon 2 and to amplify exon 3, followed by nested amplifications. For primary amplification of part 1 of exon 2, primers TF0.2 and TR2.1 were used. A nested PCR was performed with primers TF1 and TR2 under the following conditions: 4 min at 95°C (initial denaturation), 1 min at 94°C, 1 min at 66°C, 1 min 45 s at 72°C (35 cycles), and 5 min at 72°C (final polymerization). For part 2 of exon 2, primers TF2 and TR5.4 were used for the primary PCR, followed by nested amplification with primers TF2.1 and TR5 (4 min at 95°C [initial denaturation], 1 min at 94°C, 1 min at 66°C, 2 min 30 s at 72°C [35 cycles], and 5 min at 72°C). Exon 3 was primarily amplified with primers S6 and TR 5.6 (4 min at 95°C [initial denaturation], 45 s at 94°C, 1 min at 66°C, 1.5 min at 72°C [35 cycles], and 5 min at 72°C). Nested PCR was performed with primers TF5 and TR5.5 (4 min at 95°C [initial denaturation], 45 s at 94°C, 1 min at 66°C, 1 min at 72°C [35 cycles], and 3 min at 72°C).

The exon 1 coding region was amplified with forward primer E1/4 and reverse primer E1R2 (table 1). The cycling conditions were as follows: 4 min at 95°C (initial denaturation), 1 min at 94°C, 1 min at 64°C, 1 min at 72°C (35 cycles), and 2 min at 72°C).

All fragments were amplified in 1× PCR buffer (GIBCO BRL) (20 mM Tris-HCl at 8.4 pH and 50 mM KCl), 1.5 mM $MgCl_2$, 100 μM dNTPs, 10 pmol of each primer, and 1 U of platinum *Taq* polymerase (GIBCO BRL). The PCR products were analyzed on agarose gels, and the fragments were gel purified with QIAquick columns (Qiagen) and then cycle sequenced (for primers see table 1) under the following conditions: 1.5 min at 98°C (initial denaturation), 30 s at 98°C, 15 s at primer-specific annealing temperature, and 4 min at 60°C (25 cycles). PCRs were performed on Perkin-Elmer Cetus thermocyclers. Sequencing reactions were analyzed on an ABI 377 automated sequencer (Applied Biosystems).

For segregation analysis in family 8, a 368-bp fragment was amplified with primers 1S3 and 1R5 (4 min at 94°C, then 30 s at 94°C [35 cycles], 1 min at 64°C, 30 s at 72°C, and a final polymerization step of 3 min at 72°C). PCR products were ethanol precipitated and restriction digested with *Pst*I. Restriction fragments were separated on a 5% polyacrylamide gel and visualized by ethidium bromide staining.

Mutation Nomenclature

After cloning of the exon 1 sequence, both previously described mutations were renamed according to the guidelines for human gene mutation nomenclature (Antonarakis 1998). All mutation names presented herein are given with respect to the GenBank accession no. Y18265 (full *SALL1* coding sequence).

Results

Cloning of SALL1 *Exon 1*

To establish mutation analysis for the whole coding region, the exon 1 cDNA sequence, including the putative translation start codon, was isolated by 5′ RACE. Two cDNA fragments were obtained: a 603-bp fragment amplified with primers AN and 1R2 and a 301-bp nested fragment generated with primers AN and TR1.2. Subsequently, we used the 603-bp cDNA fragment as a molecular probe to screen a computer-spotted human PAC library (Ioannou et al. 1994). One positive clone was isolated. The DNA of this PAC clone was prepared as described by Ioannou et al. (1994) and sequenced with primers E1/2 and E1/4R to obtain the intronic sequence flanking the splice donor site and additional sequence information from the 5′ UTR. Chromosomal localization of the PAC clone to the *SALL1* locus at 16q12.1 was confirmed by FISH (data not shown).

Mutation Analysis

We first analyzed part 1 of exon 2, which includes the region encoding double zinc finger I (DZF I) from one affected person of each of the 23 families (fig. 1). If no mutation was found in this fragment, part 2 of exon 2, exon 3, and finally exon 1 were also amplified and sequenced (fig. 1). If a mutation was detected, all other family members available for investigation were likewise examined for the mutation by direct sequencing.

Nonsense Mutations

In a total of four families, we identified nonsense mutations in the affected family members. In three of the families (families 3–5; table 2), we found a heterozygous 826C→T mutation (fig. 2*c*) resulting in the transition of an arginine to a stop codon (R276X). All affected children had "sporadic" TBS, and, as expected, all clinically unaffected parents did not show the mutation. In another family (family 6), we identified the mutation 1115C→G (fig. 2*e*) at the same site as the previously reported mutation 1115C→A (formerly known as 1222C→A) (Kohlhase et al. 1998). Like 1115C→A, the 1115C→G mutation causes the identical transition from a serine codon to a stop (S372X). Unfortunately, in this family the parents were not available for investigation.

Frameshift Mutations

In five additional families (families 7–11; table 2), we identified heterozygous short deletions in the *SALL1* coding sequence, each of which resulted in a frameshift. In family 7, the affected patient had been previously described because of the association of TBS with renal failure (Newman et al. 1997). In this patient we found a 7-bp deletion (1200-1206del; fig. 2*i*). Subsequently, we analyzed his parents and his two unaffected siblings for the mutation. All but the mother showed wild-type *SALL1* sequence. In the lymphocyte DNA of the mother, the mutation was found in repeated examinations (fig. 2*k*). To exclude DNA contamination, DNA preparation and mutation analysis were repeated from a second blood sample, and identical results were obtained. Subsequently, a fibroblast cell line was established from a skin biopsy specimen, and mutation analysis was performed on fibroblast DNA. Here, the mutation was also detectable (fig. 2*l*). However, comparison of electro-

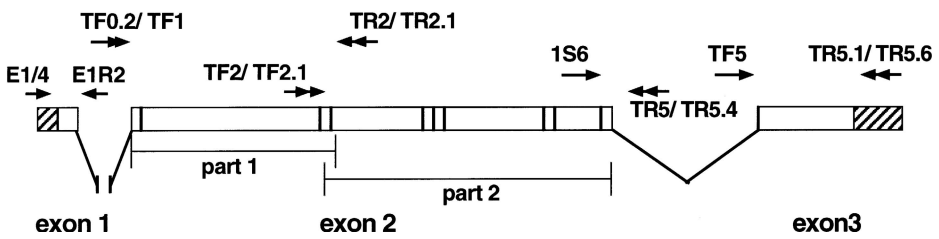

Figure 1 PCR strategy for mutation analysis of *SALL1* exons 1–3. Exons are shown as boxes, introns as lines. Zinc fingers are indicated as vertical bars within the exons. Hatched regions = 5′/3′ UTR. For primers (arrows), see table 1.

Table 2

***SALL1* Mutations and Clinical Findings**

Patient/Sex	Mutation	Anus	Hands	Ears	Hearing	Mental Development	Kidneys	Renal Function	Feet	Urogenital Region	Heart
1-1/M	1115C→A	IA/ fistula	PP, TTs	DPL	NR	NR	Borderline small	…	…	…	ASD
2-1/F	1268delC	IA	PP, TTs	DPL	SNHL	NR	…	…	HPL nails	…	NR
2-2/M	1268delC	IA/ fistula	PP, TTs	DPL	SNHL	NR	…	…	SD IV-V	…	NR
2-3/F	1268delC	IA/ fistula	PP, TTs	DPL	SNHL	NR	…	…	Dorsoflexion	…	NR
3-1/M	826C→T	IA/ fistula	PP	DPL	SNHL	MMR	…	…	CFT	HSP	NR
4-1/M	826C→T	IA	TTs	DPL	SNHL	NR	PC	RC	CFT	HSP	NR
5-1/M	826C→T	"Bucket handle"	TTs	DPL	SNHL	NR	Unilateral agenesis	…	Overlapping toes, dorsoflexion	…	VSD
6-1/F	1115C→G	IA	Partial PP, misplaced Ts	DPL	SNHL	NR	Pyelum fissum	…	Vertical talus HPL D III	…	NR
7-1/M	1200-1206del	IA	PP	DPL	SNHL	NR	Bilateral HPL	RF	SD III-IV	…	MTR
7-2/F	1200-1206del	…	…	…	…	NR	…	…	…	…	NR
8-1/M	1291-1300del	…	PP, TTs, SD III-IV	…	SNHL	NR	Bilateral HPL	RC	SD III-IV Hallux valgus	…	NR
8-2/M	1291-1300del	IA/ fistula	PP, TTs	DPL	SNHL	NR	Bilateral HPL	RC	SD III-IV	HSP	NR
9-1/M	419delC	…	Misplaced fingerlike Ts	DPL	…	NR	Unilateral agenesis	RC	…	…	NR
10-1/F	1146delT	…	Medially rotated Ts	…	SNHL	NR	…	PU	Pes planus	…	Murmur
10-2/F	1146delT	IA/fistula	Elongated proximal phalanges	DPL	SNHL	NR	Borderline small	RC	Overlapping toes	…	NR
10-3/M	1146delT	IA/fistula	…	DPL	SNHL	NR	Borderline small	RC	Underfolded D III	…	NR
11-1/F	1277-1278del	Anteriorly placed	…	DPL	SNHL	NR	…	ULRC	CFT	…	NR

NOTE.—The first column identifies the patients and their families. For example, "2-1/F" reads: family 2, patient 1, female. ASD = atrial septum defect; CFT = club feet; DPL = dysplastic; HPL = hypoplastic; HSP = hypospadia; IA = imperforate anus (type not specified); MD = mental development; MMR = mild mental retardation; MTR = mitral/tricuspidal regurgitation; NR = no abnormality reported (clinically normal; no further testing performed); PC = polycystic; PP = preaxial polydactyly; PU = proteinuria; RC = reduced clearance; RF = renal failure; SD = syndactyly; SNHL = sensorineural hearing loss; T = thumb; TT = triphalangeal thumb; UG = urogenital; ULRC = unilateral reduced clearance; and VSD = ventricular septum defect. … = normal (clinically and by further testing if required). Families 1 and 2 were described elsewhere by Wischermann and Holschneider (1997) and Kohlhase et al. (1998) (mutations have been renamed: position 1115 was formerly position 1222; position 1268 was formerly position 1377); family 7 was described by Newman et al. (1997); family 8, by de Vries–van der Weerd et al. (1988); family 10, by Rossmiller and Pasic (1994); and family 11, by Gabrielli et al. (1993).

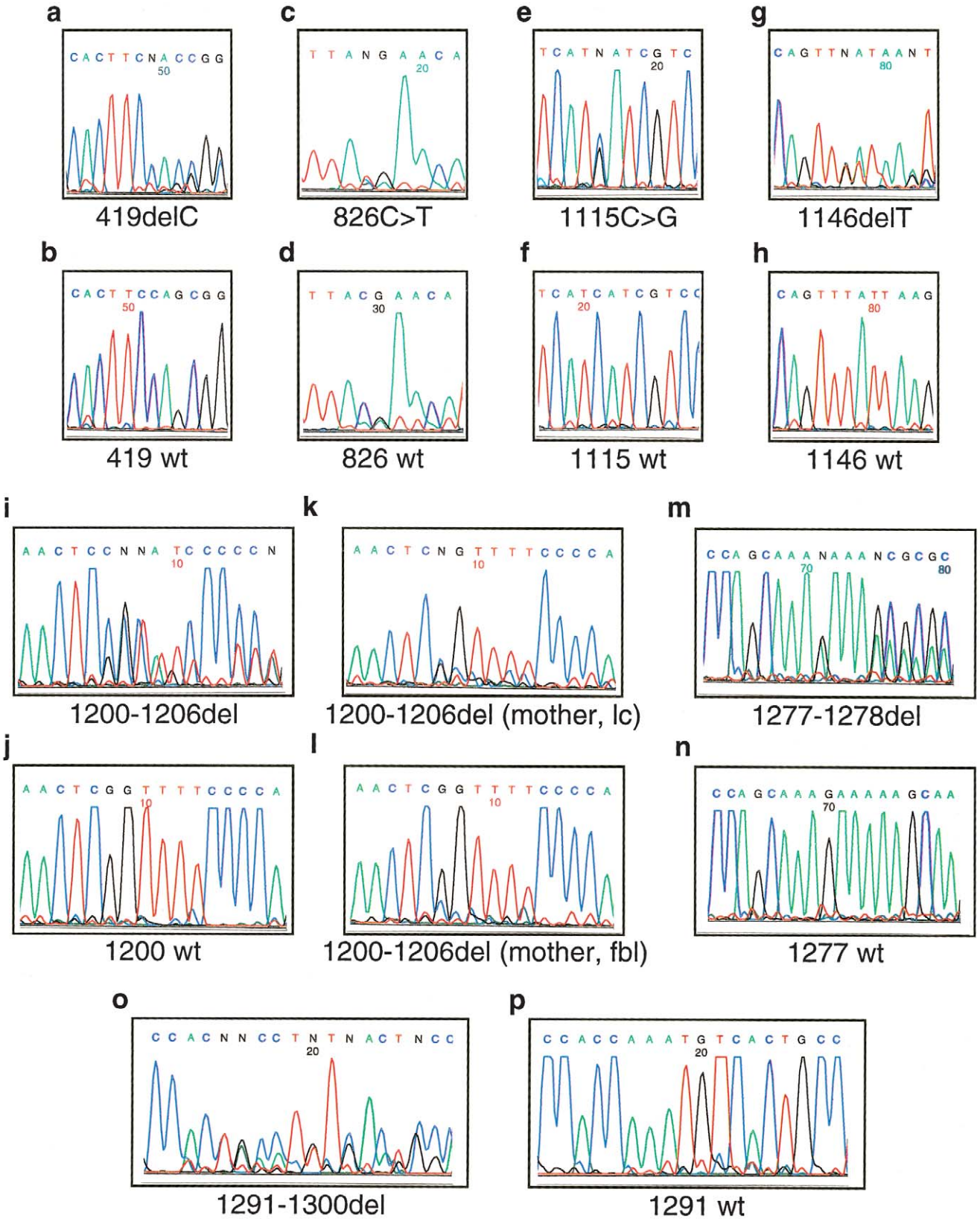

Figure 2 Electropherograms of the mutations (*a*, *c*, *e*, *g*, *i*, *m*, and *o*) detected in families 3–11 and the corresponding wild-type (wt) sequences (*b*, *d*, *f*, *h*, *j*, *n*, and *p*). The 1200-1206del mutation in family 7 (*i*, *k*, and *l*) is easily detectable in the patient (*i*) but much weaker in his mother's lymphocyte (lc) DNA (*k*) and barely visible in her fibroblast (fbl) DNA (*l*). Therefore, a mosaicism for the mutation was suspected in the mother.

pherograms of lymphocyte and fibroblast DNA sequencing showed that the mutated allele seemed to be less frequent in the mother's DNA than in her son's (fig. 2*k, l*). To exclude insufficient amplification of the mutated allele because of a mutation in the primer binding sites for TF1 or TR2, we amplified the mutated region with primers 1S3 and 1R5 and then performed direct sequencing, but the results showed no difference (data not shown). In addition to the extensive mutation analysis, the mother was reexamined for minor manifestations of TBS. A clinical examination was performed, as well as hand x-rays, renal ultrasound, and audiography. However, no feature of TBS was found.

Family 8 has been described elsewhere by de Vries–van der Weerd et al. (1988). In this family, TBS was transmitted from father to son. The father's parents as well as his wife are clinically unaffected. In both affected family members we identified the 10-bp deletion 1291-1300del (fig. 2*o,* fig. 3), which did not occur in the unaffected relatives (fig. 3).

In family 9, we found a 419delC deletion (fig. 2*a*) in the affected boy. His parents were not available for examination.

In family 10 (Rossmiller and Pasic 1994), nine subjects within four generations are affected. Three affected and four unaffected family members were available for investigation. All affected patients (the female proband, her mother, and her maternal cousin) showed the 1-bp deletion 1146delT (fig. 2*g*), but the unaffected relatives (the proband's father and three half-siblings) did not.

The affected girl of family 11 had been previously described (Gabrielli et al. 1993) because she showed features present in both Goldenhar syndrome and TBS. In this girl we identified a heterozygous 2-bp deletion, 1277-1278del (fig. 2*m*). Her parents were not available for examination.

Polymorphisms

In addition to the truncating mutations, three different changes of the *SALL1* coding sequence were detected: 475A→G (S159G), 447-448insAGC (S149-150ins), and 448-450delAGC (S150del) (data not shown), which are all predicted to result in changes of the *SALL1* amino acid sequence. However, we expect these mutations to be silent. 475A→G occurs within a stretch of 10 serine codons followed by 4 glycines (10/4). In contrast, the transformed allele is predicted to encode 9 serines and 5 glycines (9/5). Both the 10/4 and the 9/5 alleles occur homozygously as well as heterozygously both in patients and in their unaffected parents (not shown). The 447-448insAGC/448-450delAGC mutations insert or remove a serine from the same repeat, leading to 9/4 and 11/4 alleles, respectively. Since the 11/4 allele was also found in unaffected parents, we presume that this mutation is also silent. To date, the 9/4 allele has been identified in only one patient, whose parents were not available for analysis, but on the basis of the findings for the other mutations within the repeated region, we also expect this mutation to be silent.

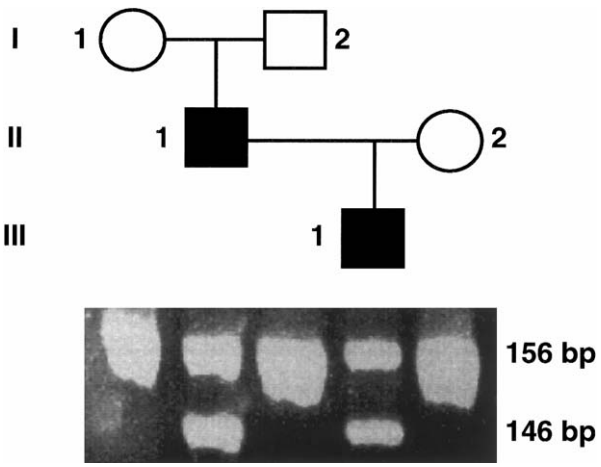

Figure 3 Segregation of the mutation 1291-1300del in family 8. A 368-bp amplicon was digested with *Pst*I and analyzed on a 5% PAGE gel. The digestion results in wild-type fragments of 212 bp and 156 bp in the unaffected family members 1-1, 1-2, and 2-2, whereas the mutated fragment of 146 bp is visible only in the affected members 2-1 and 3-1. The 212-bp fragments are not shown.

Discussion

We have now analyzed a total of 14 families with TBS (according to our major criteria; see Subjects, Material, and Methods) for *SALL1* mutations, including the 2 families already described (Kohlhase et al. 1998). An additional 11 families were investigated in which TBS is a consideration among other differential diagnoses. In a total of 9 (64.3 %) of 14 group A families (7 of the 12 families described in this study [see Subjects, Material, and Methods] plus the 2 families described elsewhere), we detected heterozygous mutations. Within group B of this study, we detected mutations in 2 (33.3%) of 6 families, and within group C, no mutation was found (0 of 5 families).

Our results show that the likelihood of mutation detection increases depending on the number of major criteria (anorectal, thumb, and ear malformations) present. However, in approximately one-third of the TBS patients of group A we did not detect a mutation within the coding region. This could be explained by the hypothesis that these patients do have a *SALL1* mutation that cannot be detected with our current strategy (i.e., promoter mutations, intronic mutations, or larger deletions) or that in these families TBS is caused by mutations in a different gene. Analysis of the promoter region and the

introns in patients in whom no mutation has yet been found will show the extent to which such mutations contribute to TBS. Quantitative Southern blotting or FISH analysis as well as the analysis of intragenic STS markers should reveal whether heterozygous deletions of larger parts of the *SALL1* coding region or of the whole gene are also part of the mutational spectrum in TBS. Furthermore, we cannot yet exclude the possibility that TBS is genetically heterogeneous. The *spalt* (*sal*) gene of *Drosophila* and its counterpart in the fish medaka have been shown to be integrated within the *hedgehog* signaling pathway (de Celis et al. 1996; Lecuit et al. 1996; Nellen et al. 1996; Köster et al. 1997). If we assume that this regulation is also conserved for human *spalt*-like genes, we might conclude that mutations in other genes implicated in the *hedgehog* signaling pathway upstream or downstream of *SALL1* could cause similar phenotypes.

Like the mutations reported previously (Kohlhase et al. 1998), all mutations reported herein are predicted to result in truncated SALL1 proteins translated from the mutated alleles. Nonsense mutations were found in 5 of 11 families and short deletions in 6. All the deletions occur at different sites, but all nonsense mutations reside either at nucleotide 826 or at nucleotide 1115. None of the seven mutations reported in this article have been described before. All nine mutations presented here and in our previous report (Kohlhase et al. 1998) reside in exon 2 within the 932 bp preceding the region encoding DZF I (fig. 4). No mutation was found within part 2 of exon 2 (fig. 1) or within exons 1 and 3. Of the 11 mutations, 10 have been found within the 525 bp preceding DZF I. Therefore, this region seems to represent a "hot spot" for mutations within *SALL1*.

All identified *SALL1* mutations in TBS are predicted to remove all DZF domains from the mutated proteins. One explanation for the lack of *SALL1* mutations in exon 1 or 3′ of DZF I in patients with TBS could be that, although the region preceding DZF I is especially prone to mutations, mutations in other regions of *SALL1* also occur in TBS patients but at much lower frequencies. One could also argue that truncating *SALL1* mutations that leave any of the DZFs intact would either have no effect at all or lead to a phenotype different from TBS. The latter hypothesis is inspired by the mutation spectrum observed in *GLI3*. Here, a truncating mutation removing the DNA binding domain leads to Greig cephalopolysyndactyly syndrome (Wild et al. 1997), whereas mutations resulting in truncated proteins with an intact zinc finger domain cause different phenotypes such as Pallister-Hall syndrome (Kang et al. 1997) or postaxial polydactyly type A (Radhakrishna et al. 1997), depending on the site of mutation (Biesecker 1997). The SALL1 protein, however, contains four putative DNA binding domains (Kohlhase et al. 1996), and it remains to be shown which and how many of these are essential for gene function.

Genotype-Phenotype Correlations

The clinical features of the subjects in whom *SALL1* mutations were found are listed in table 2. We calculated the occurrence rates of clinical findings in TBS patients positive for *SALL1* mutations and compared the numbers with rates derived from another study (O'Callaghan and Young 1995). Patient 7-2 (table 2) was omitted from this calculation because she seems to carry the mutation in a mosaic state (see below). In our subgroup of mutation-positive TBS patients, we observed higher rates for malformations of hands (thumbs), feet, kidneys, and ears, for sensorineural hearing loss, and for impaired renal function than observed by O'Callaghan and Young 1995) (table 3). Only anal malformations have been found slightly less frequently in our mutation-positive group of patients. However, if we compare the families instead of the patients (in which case a feature is part of the phenotypic spectrum within a family if it is present in at least one affected member), the frequencies of clinical features do seem quite similar between the two studies, with the exception of renal malformations or impaired renal function. Therefore, it appears that the families reported here and the families listed by

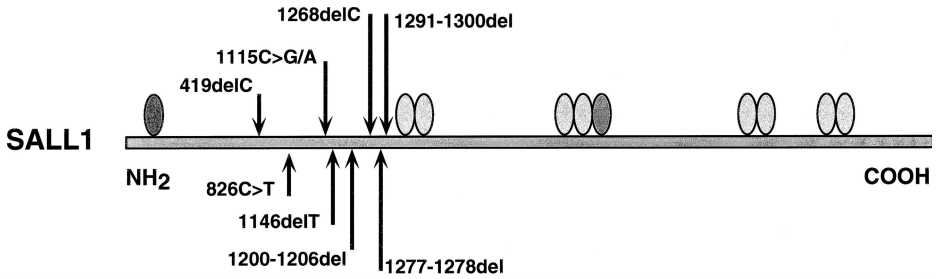

Figure 4 Positions of the *SALL1* mutations in TBS with respect to the encoded protein. All mutations are truncating and result in the putative proteins, lacking all DZF motifs (oval symbols), that are characteristic of *SALL1*.

Table 3

Frequency (%) of Clinical Findings in Patients with TBS

	Present Study		O'Callaghan and Young (1995)	
Clinical Feature	Patients (n = 16)	Families (n = 11)	Patients (n = 44)	Families (n = 10)
Anorectal anomaly	81.3	90.9	95.4	90
Thumb anomaly	87.5	90.9	50	100
Auricular anomaly	87.5	100	61.4	90
Hearing loss	87.5	81.8	29.5	80
Kidney anomaly	62.5	72.7	20.5	40
Impaired renal function	56.3	54.5	NE	NE
Foot anomaly	87.5	81.8	25	50
Hypospadias (males)	30	37.5	NE	NE
Heart anomaly	25	36.4	9.1	40
Mental retardation	1 Case	1 Case	NE	NE

Note.—The columns give the percentages at which the corresponding malformations occur with respect to the total number of mutation-positive patients and with respect to the number of families in which mutations were detected (our study). For comparison, we calculated percentages of malformations based on the number of families and numbers of mutation-positive patients described by O'Callaghan and Young (1995). In their article, the frequency of malformations was presented for a total of 44 TBS patients from 10 families. NE = no estimate.

O'Callaghan and Young (1995) do not differ considerably with respect to the phenotypic range within a family, except for renal involvement. However, the clinical variability seems to be higher within the families listed by O'Callaghan and Young (1995), with the exception of one family that was included in their study but in which we found a mutation (de Vries–van der Weerd et al. 1988). It remains to be shown in how many of the families listed by O'Callaghan and Young (1995) *SALL1* mutations are found to be the cause of TBS.

Renal failure has been reported in different TBS patients (Rossmiller and Pasic 1994; Newman et al. 1997). Interestingly, in our study, among the 10 mutation-positive patients with changes of renal morphology, 7 show impaired renal function or renal failure (tables 2 and 3). Renal function might also deteriorate even if ultrasound examination reveals normal morphology, as shown for two of our patients (10-1 and 11-1). Impaired renal function might therefore be more common in TBS patients than previously thought. This indicates that, even in the absence of kidney malformations, kidney function should be routinely monitored in patients with TBS.

Rare Findings

Mild mental retardation was diagnosed in one of our mutation-positive patients. This boy underwent a HAWIK-R test and reached a total IQ of 59. Furthermore, some mutation-positive patients showed features unusual for TBS. In patient 11-1, some symptoms pointed toward Goldenhar syndrome rather than toward TBS. Apart from the symptoms listed in table 2, this girl also has facial asymmetry and costal malformations. She has, however, neither epibulbar dermoids nor vertebral anomalies (Gabrielli et al. 1993). Other features found in this girl are unilateral palsies of cranial nerves VI and III, hypoplasia of the parotid glands, constipation, and an unstable bladder. Patient 10-1 has bilateral iris and choroidal-retinal inferior colobomata plus a small coloboma of the left lens. Her daughter's eyes show Duane's contracture phenomena. Patient 5-1 has constipation. Patient 4-1 has hypothyroidism.

The identification of a mutation in the girl with Goldenhar-like symptoms is especially interesting, since an overlap between TBS and Goldenhar syndrome has been reported in two additional families (Moeschler and Clarren 1982; Johnson et al. 1996). Mutation analysis of *SALL1* in these families should reveal whether symptoms thought to be pathognomonic for Goldenhar syndrome, such as epibulbar dermoids, are indeed part of the phenotypic spectrum caused by *SALL1* mutations.

Mutation Position and Phenotype

All *SALL1* mutations found so far in TBS can roughly be grouped as mutations distant (position 419), intermediate (position 826), or close (position 1115-1398) in relation to the DZF I coding region. The shortest putative truncated protein results from 419delC in patient 9-1. He has very slight hand malformations, no anal defect, and no hearing loss, but the function of his only kidney is impaired. The 1291-1300del allele results in the longest putative truncated protein. Here, the hand malformations are more severe in both patient 8-1 and patient 8-2. Anal defects, however, are not present or are slight (imperforate anus type 1) in these patients. Patient 3-1 has imperforate anus type 3, preaxial polydactyly, hearing loss, and mental retardation, but no renal symptoms. In patient 5-1 with the same mutation, 826C→T, the kidneys are severely affected. We therefore find no difference associated with the position of the mutation with respect to severity of renal manifestations. Furthermore, there is no significant difference in severity in other TBS symptoms within our study group with respect to the site of mutation when we exclude patient 9-1. We therefore conclude that the severity of the phenotype for mutation-positive offspring of our patients cannot be predicted on the basis of the site of the mutation.

Penetrance in TBS

In all mutation-positive families except family 7, only affected members carried a *SALL1* mutation. In family 7, the 1200-1206del mutation was also found in the unaffected mother (fig. 2*k*). This finding raises the question of whether penetrance of TBS is incomplete in this

family. The reproducible results of our mutation analysis indicate that the proportion of lymphocytes carrying a *SALL1* mutation is <100% in the mother. Furthermore, the mutation analysis of her fibroblast DNA (fig. 2*l*) suggests that the proportion of fibroblasts carrying the mutated chromosome is even less than that of the lymphocytes. Therefore, we think that the *SALL1* mutation in the mother is present in a mosaic state. However, in the absence of experimental evidence, we cannot yet exclude the possibility of reduced penetrance of the *SALL1* mutation in this family.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, http://www.ncbi.nlm.nih.gov (for accession nos. Y18264 [*SALL1* exon 1 and intron 1 genomic sequence {partial}], Y18265 [full *SALL1* coding sequence], and X98833 [*SALL1* genomic sequence of intron 1 {partial}, exons 2 and 3, and intron 2])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/OMIM (for TBS [MIM 107480] and *SALL1* [MIM 602218])

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