

ORIGINAL ARTICLE

Mutations at the *SALL4* locus on chromosome 20 result in a range of clinically overlapping phenotypes, including Okihiro syndrome, Holt-Oram syndrome, acro-renal-ocular syndrome, and patients previously reported to represent thalidomide embryopathy

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We have recently shown that Okihiro syndrome results from mutation in the putative zinc finger transcription factor gene *SALL4* on chromosome 20q13.13-13.2. There is considerable overlap of clinical features of Okihiro syndrome with other conditions, most notably Holt-Oram syndrome, a condition in part resulting from mutation of the *TBX5* locus, as well as acro-renal-ocular syndrome. We analysed further families/patients with the clinical diagnosis of Holt-Oram syndrome and acro-renal-ocular syndrome for *SALL4* mutations. We identified a novel *SALL4* mutation in one family where the father was originally thought to have thalidomide embryopathy and had a daughter with a similar phenotype. We also found two novel mutations in two German families originally diagnosed as Holt-Oram syndrome and a further mutation in one out of two families carrying the diagnosis acro-renal-ocular syndrome. Our results show that some cases of "thalidomide embryopathy" might be the result of *SALL4* mutations, resulting in an increased risk for similarly affected offspring. Furthermore we confirm the overlap of acro-renal-ocular syndrome with Okihiro syndrome at the molecular level and expand the phenotype of *SALL4* mutations.

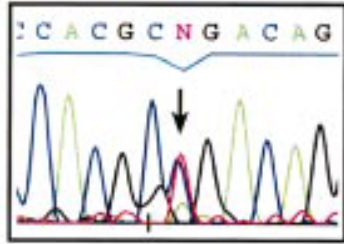
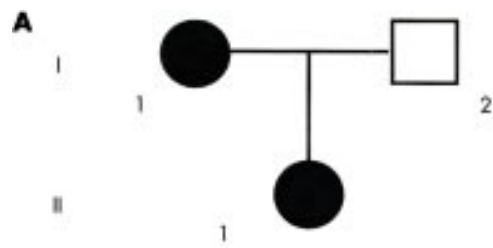
The *SALL* gene family, derived from the *Drosophila* gene *spalt*,¹ represents a related group of probable zinc finger transcription factors. Four such genes have been identified to date in man, of which *SALL2*² and *SALL3*³ remain to be associated with human disease. However, the importance of this gene family to human malformation syndromes is indisputable, mutations at *SALL1* on chromosome 16q12.1 being associated with Townes-Brocks syndrome and related phenotypes^{4,5} and, more recently, mutations at *SALL4* being shown to be causative in patients with Okihiro syndrome.^{6,7} Kohlhase *et al*⁷ reported frameshift and nonsense mutations in five of eight families segregating the Okihiro syndrome phenotype. A further report⁶ identified two frameshift mutations and one nonsense mutation in three affected kindreds, including the family reported by Okihiro *et al*.⁸

Okihiro syndrome (OMIM 607323) refers to an autosomal dominant condition characterised by radial malformations associated with Duane congenital eye movement disorder. The Duane eye anomaly is characterised by limitation of abduction and narrowing of the palpebral fissure with retraction of the globe on adduction. Okihiro syndrome has also been described by other authors and is known by other names in published reports. Temtamy *et al*⁹ used the term DR syndrome, D representing Duane anomaly and deafness, R identifying the radial and renal manifestations of the condition.⁹ The first report is likely to have been that of Crisp.¹⁰

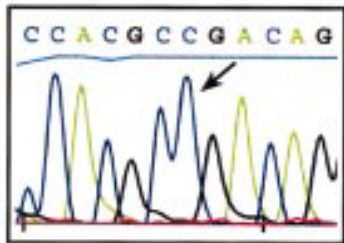
Familial occurrence of radial sided hand malformations, noteworthy for their variability, in association with Duane eye anomaly have been the central clinical features of several reports.^{10–13} The range of associated features observed in these patients is extensive, embracing anal stenosis, pigmentary disturbance, hearing deficit, renal malformations, external ear malformations, facial asymmetry, and cardiac lesions, particularly atrial septal defect. In reporting mutations of the *SALL4*

gene as the basis of Okihiro syndrome in their patients, Kohlhase *et al*⁷ similarly drew attention to associated clinical features of choanal atresia, triphalangeal thumb, renal agenesis, unilateral deafness, thumb reduplication, external ear malformations, and ventricular septal defect in their cohort.⁷ Not only do these clinical observations confirm that there is an extraordinary variability in phenotype in these patients with allelic conditions, but also prompt consideration as to what overlapping clinical entities, previously considered on a clinical basis to represent distinct conditions, might also be caused by mutation at the *SALL4* locus.

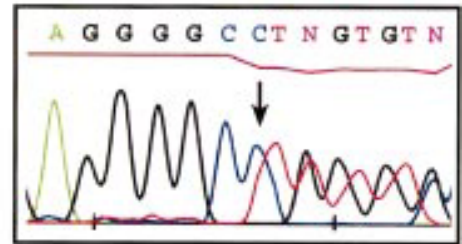
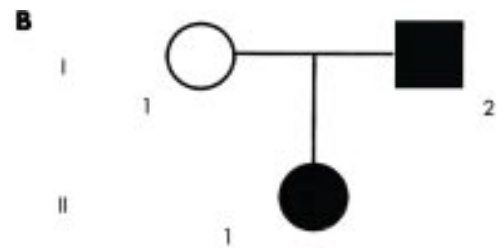
In reporting *SALL4* mutations in families with Okihiro syndrome, Kohlhase *et al*⁷ identified a number of possible candidate clinical conditions which might be tested for allelic mutations. Foremost among those conditions thus identified was Holt-Oram syndrome, an autosomal dominant disorder characterised by radial forelimb malformations, congenital heart abnormalities, and associated features.¹¹ Although a major locus for Holt-Oram syndrome has been identified at *TBX5*, only approximately 30% of clinically identified cases of Holt-Oram syndrome have been found to have mutations at that locus.¹² Attention was also drawn to a previous cytogenetic report of a de novo pericentric inversion of chromosome 20q13.2, where *SALL4* is located, associated with a clinical presentation of bilateral absence of the thumbs and an atrial septal defect.¹³ This phenotype was taken to represent Holt-Oram syndrome and the suggestion was made that there were likely to be other cases of apparent Holt-Oram syndrome but without *TBX5* mutation, in whom *SALL4* mutations might be expected to be observed.⁷ Similarly, other candidate clinical conditions which might represent mutation at the *SALL4* locus were suggested, comprising acro-renal-ocular syndrome¹⁴ and



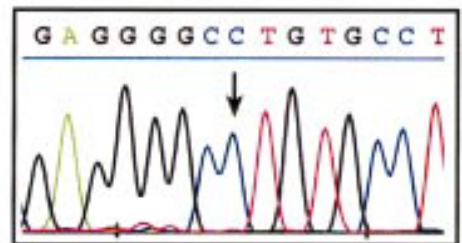
c.2593C>T



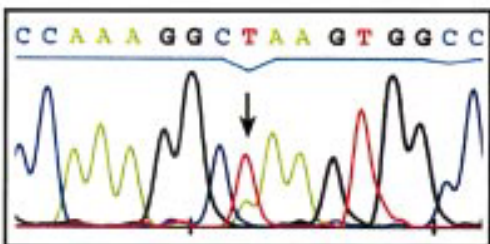
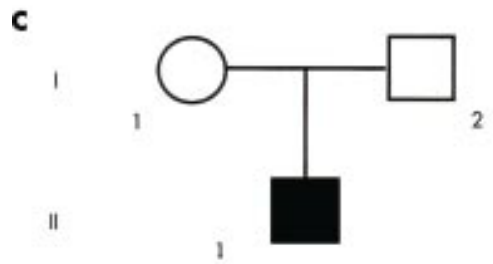
c.2593 wt



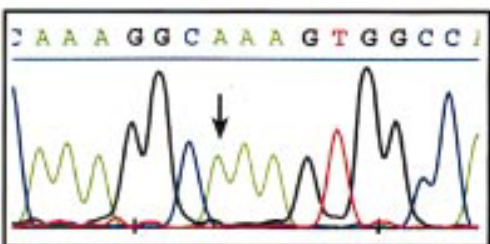
c.326delC



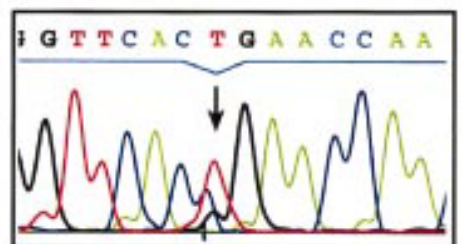
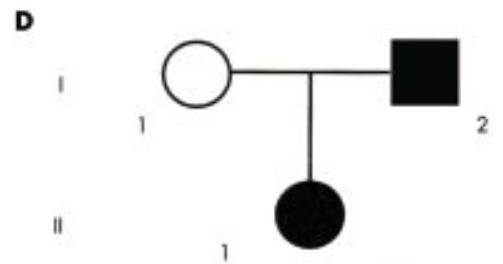
c.326 wt



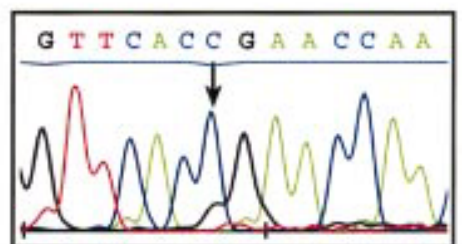
c.523A>T



c.523 wt



c.1849C>T



c.1849 wt

Figure 1 *SALL4* mutations identified in four families. (A) Family 1, showing c.2593C>T mutation, which segregates with the affected phenotype. The phenotype in this family¹⁴ included thumb aplasia, radial hypoplasia, hearing loss, unilateral dystopic kidney, as well as Duane anomaly. Limb anomalies were worse on the left than the right and worse in the daughter II.1 as compared to her mother I.1. (B) Mutation c.326delC segregating in family 2. The pedigree has already been reported.¹⁵ The index patient (II.1) was born with bilateral asymmetrical radial ray defects consisting of absence of the thumb and first fingers, radial aplasia, and ulnar hypoplasia. Her father (I.2) is similarly affected and also suffers from a left sided sensorineural and right sided mixed hearing loss. Both have structurally normal hearts with normal echo and normal ECG. Renal ultrasound and eye examination showed no abnormality. Thalidomide embryopathy was diagnosed in the father in childhood based on the nature of the abnormalities in the presence of a history of possible exposure during pregnancy. (C) Family 3, showing c.523A>T mutation in a sporadic case (II.1). The patient showed thumb aplasia with radial aplasia on the right and hypoplastic thumb and radius on the left. Bilateral Duane anomaly was present, also a broad, flat nasal bridge, epicanthic folds, a crossed renal dystopia, and a persisting foramen ovale. (D) Family 4 showing c.1849C>T nonsense mutation associating with the affected phenotype. The phenotype in this family comprised in I.2 unilateral Duane anomaly, bilateral thumb aplasia, hypoplastic radii, horseshoe kidney, slit-like openings of the auditory ducts, flat feet, and in II.1 no Duane anomaly, hypertelorism, slight epicanthic folds, severely shortened upper limbs with bilateral thumb aplasia, hypoplastic radius on the left and subtotal aplastic radius on the right, radial club hands, ventricular septal defects, slit-like openings of auditory ducts, and flat feet.

patients previously thought to represent thalidomide embryopathy but in whose offspring limb and cardiac malformations had also been observed.¹⁵

The purpose of this report is to communicate our initial experience of *SALL4* mutation analysis across a much wider range of patients representing several different clinical diagnoses which overlap with Okhiro syndrome. In doing so we identified three new mutations and one recurrent mutation also reported by Al-Baradie *et al*⁶ at this locus and confirm our hypothesis that mutation of *SALL4* results in a range of clinical conditions, hitherto considered to represent distinct entities.

MATERIALS AND METHODS

Patients

Venous blood was collected from patients and unaffected relatives after obtaining their informed consent.

Genetic analysis

Genomic DNA was prepared from peripheral lymphocytes by routine procedures. Mutation analysis of *SALL4* exons 1-4 (complete coding region) was performed as described previously.⁷ GenBank accession numbers were *SALL4* cDNA, NM_020436; genomic contig NT_011362.



Figure 2 Clinical features of family 4. (A) Patient II.1 showing hypertelorism and slight epicanthic folds. (B) Flat feet of the same patient showing broad, short first toes and large sandal gap. (C) Patient I.2 and (D) his daughter II.1 showing slit-like opening of the auditory ducts. (E, F) Forearms of I.2 with bilateral thumb aplasia and hypoplastic radii. (G, H) Forearms of II.1. Note subtotal radial aplasia on the right and radial club hand on the left as well as bilateral aplasia of the thumbs.

Case reports

In family 1, the index patient (II.1, fig 1A) showed bilateral absent thumbs, absent radii, and shortened humeri, the left arm being more severely shortened.¹⁴ She has a bilateral Duane anomaly, fine nystagmus, and slightly dysplastic optic discs. A mild bilateral conductive hearing loss was noted. Her affected mother (I.1, fig 1A) showed absent thumbs and shortened forearms. She has a hypoplastic dystopic left kidney and a moderate conductive and significant sensorineural hearing loss.

In family 2, the index patient (II.1, fig 1B) was born with bilateral asymmetrical radial ray defects consisting of absence of the thumb and first fingers, radial aplasia, and ulnar hypoplasia. Her father is similarly affected and also suffers from a left sided sensorineural and right sided mixed hearing loss. Both have structurally normal hearts with normal echo and normal ECG. Renal ultrasound and eye examination showed no abnormality. Thalidomide embryopathy was diagnosed in the father in childhood based on the nature of the abnormalities in the presence of a history of possible exposure during pregnancy. Occurrence of abnormalities in a subsequent generation was attributed to mutagenicity of thalidomide.¹⁵

In family 3, the affected boy (II.1, fig 1C) was born to unaffected non-consanguineous parents. A left thumb and radial hypoplasia together with a right sided club hand and aplasia of the thumb and radius was noted. Further abnormalities were a persistent foramen ovale and a crossed dystopia of the kidneys. He showed a simian crease on the left hand, deep creases of the feet, broadened and slightly elongated first toes, elongated fourth toes, mild epicanthic folds, and a broad flat nasal bridge. Psychomotor development was normal at the age of 2 years. Growth was on the 90th centile, weight on the 3rd, and head circumference on the 50th centile. An initial diagnosis of Holt-Oram syndrome was suggested, but after *SALL4* mutation detection and re-examination, a bilateral Duane anomaly was noted and the diagnosis revised to Okihiro syndrome.

In family 4, the affected girl (II.1, fig 1D) showed bilateral thumb aplasia and radial club hand (fig 2G, H). Both arms were shortened. On the left side, all carpal bones were present and the radius was hypoplastic. On the right, a subtotal aplasia of the radius was noted together with a malformed olecranon. Further abnormalities were a VSD, hypertelorism with slight epicanthic folds, slit-like openings of the external auditory meatus (fig 2C), flat feet with large sandal gaps, and broad, short first toes. She had no kidney malformation or Duane anomaly. The initial diagnosis was Holt-Oram syndrome. Her father (I.2, fig 1D) showed less severe limb malformations, with bilateral thumb aplasia and hypoplastic radii (fig 2E, F). On re-examination, a right sided Duane anomaly was noted. He also had slit-like openings of the external auditory meatus (fig 2D), flat feet with large sandal gaps, and broad, short first toes. Facial abnormalities were not seen. Further anomalies include a bifid uvula, horseshoe kidney, and limited movements of the joints of fingers 2-5 bilaterally. Following mutation analysis and detection of the Duane anomaly in the father, a revised diagnosis of Okihiro syndrome was made in this family. The phenotypic features of all participating patients are summarised in table 1.

RESULTS

Mutation analysis

SALL4 mutation analysis was carried out in two families presenting with overlapping features of Okihiro syndrome and acro-renal-ocular syndrome.¹⁴ In one of these families (family 1 in this report and in the report of Becker *et al*¹⁴) we found the heterozygous nonsense mutation c.2593C>T (R865X) in exon 3 of the *SALL4* gene. The mutation was detected in both affected family members (fig 1A).

Table 1 Clinical synopsis of participating family members

Patient	Eyes	Arms	Ears	Hearing	Kidneys	Feet	Heart	Other
Family 1	I.1	Bil AT, short forearms	N	Bil CHL/SNHL	L: HPL, pelvic, R: N	NR	NR	Bil latent fine nystagmus, slightly dysplastic optic discs
	II.1	Bil AT + AR, short humeri, L>R	N	Bil CHL	N	NR	NR	
Family 2	I.2	Bil AT + AR, ulnar HPL	N	L: SNHL, R: CHL/SNHL	N	NR	N	Thalidomide exposure of mother during pregnancy
	II.1	Bil AT + AR, ulnar HPL	N	N	N	NR	N	
Family 3	II.1	L: HPL of thumb and radius, R: club hand, AT + AR	N	NR	Crossed dystopia	Deep creases, broad, elongated 1st toes, elongated 4th toes	Persistent foramen ovale	Mild epicanthic folds, broad, flat nasal bridge
	I.2	Bil radius HPL + AT	Slit-like openings of EAM	N	HS kidney	Flat feet, large sandal gaps, broad, short 1st toes	N	Bifid uvula, limited movements of joints fingers II-V
Family 4	II.1	Short forearms, L: radius HPL + AT, R: subtotal AR + AT	Slit-like openings of EAM	N	N	Flat feet, large sandal gaps, broad short 1st toes	VSD	Mild epicanthic folds, broad flat nasal bridge

AR: absent radius/ radii; AT: absent thumb(s); Bil: bilateral; CHL: conductive hearing loss; DA: Duane anomaly; EAM: external auditory meatus; HPL: hypoplasia; HS: horseshoe; L: left; N: normal; NR: no abnormality reported; R: right; SNHL: sensorineural hearing loss.

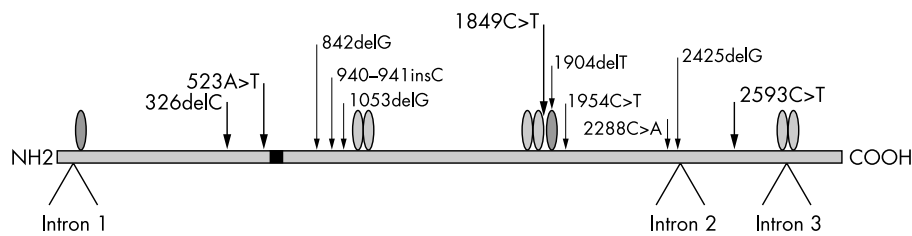


Figure 3 Schematic representation of the SALL4 protein and localisation of all mutations identified. Zinc finger motifs are shown as oval symbols. Mutations reported in this paper are shown in larger font and previously identified mutations in smaller font. The mutation c.2593C>T has also been described by Al-Baradie *et al.*⁶ The black box represents the conserved Glu rich domain thought to mediate interaction with other SALL proteins. Note that the mutations are distributed over the gene and that mutation c.2593C>T can cause both severe and mild phenotypes in different families.

We also analysed three families with the clinical diagnosis of Holt-Oram syndrome. Two of these families were negative for a *TBX5* mutation and one had not been tested. Family 2 had been described previously as a possible example of thalidomide mutagenicity.¹⁵ Because of the clinical overlap with Holt-Oram syndrome, *TBX5* mutation analysis was performed, but no mutation was detected. We found the heterozygous frameshift mutation c.326delC in exon 2 of *SALL4* in both affected persons (father and daughter), resulting in a premature stop codon 31 bp 3' of the mutation (fig 1B). In family 3, both parents are unaffected. Their only son was diagnosed with Holt-Oram syndrome, but was negative for a *TBX5* mutation. We detected the heterozygous nonsense mutation c.523A>T (K175X) in exon 2 of *SALL4* (fig 1C) in the patient, but not the parents. Family 4 had also been diagnosed with Holt-Oram syndrome. Here, the father and his daughter are affected. We identified the heterozygous nonsense mutation c.1849C>T (R617X) in exon 2 of *SALL4*. The mutation segregates with the abnormal phenotype in both affected subjects (fig 1D).

DISCUSSION

In this report we show that *SALL4* mutations cause a range of phenotypes. Three out of four families reported here show that kidney anomalies are part of the phenotypic spectrum of *SALL4* mutations, thereby confirming the hypothesis that Okihiro syndrome and acro-renal-ocular syndrome are allelic to some extent. However, we did not find a mutation in family 2 reported by Becker *et al.*¹⁴ leaving open the possibility that another locus might be involved in this family.

Our results further show that in some cases reported as possibly resulting from thalidomide mutagenicity, *SALL4* mutations could be the true cause of the phenotype. The mutation in family 2 reported here (case 2 in the report of McBride¹⁵) was found in both affected family members. The heritability of the abnormal phenotype in this pedigree has been previously represented as evidence for thalidomide as a mutagen. However, in view of the extended phenotype of limb malformations we now observe with *SALL4* mutations, it appears much more likely that the phenotype in generation I of this family was misdiagnosed as representing thalidomide embryopathy when the phenotype was in fact the result of a mutation of *SALL4*.

The families described here also expand the phenotype of *SALL4* mutations. We observed facial characteristics (hypertelorism, epicanthic folds) in two of our patients and not previously described slit-like openings of the external auditory meatus, which might help to discriminate Okihiro syndrome from Holt-Oram syndrome if no Duane anomaly is present. We also describe characteristic flat feet and large sandal gaps in both affected members of one family.

Our results suggest that all patients with Duane anomaly and radial ray malformations should be investigated for *SALL4* mutations. Furthermore, as shown by our cohort, patients with typical radial ray malformations without Duane anomaly

might carry a *SALL4* mutation as well. These patients obviously might be diagnosed with Holt-Oram syndrome instead and require *TBX5* mutation analysis. However, if *TBX5* mutation analysis fails to show a mutation, *SALL4* analysis should be considered. Our results further suggest that in such cases all affected family members should be clinically examined for Duane anomaly and other features associated with Okihiro syndrome because only one of the affected family members might have a Duane anomaly.

Together with this report, in total 11 different *SALL4* mutations have now been described.^{6,7} These are one small insertion (c.940-941insC), five deletions (c.326delC, c.842delG, c.1053delG, c.1904delT, c.2425delG), and five nonsense mutations (c.523A>T, c.1849C>T, c.1954C>T, c.2288T>A, c.2593C>T).

Compared to the mutational spectrum of *SALL1* mutations,⁵ most of which reside in exon 2, 5' of the region encoding the first double zinc finger domain, the *SALL4* mutations detected seem to be distributed over the gene.

It is not possible to correlate the severity of the phenotype with mutation position. Severe limb malformations with shortening of the upper limbs have been described for the mutations c.523A>T, c.842delG, c.1849C>T, and c.1904delT. Milder malformations have been seen with the mutations c.940-941insC, c.1053delG, and c.2405delG, and the mildest (thenar hypoplasia only) with c.1954C>T. The observation of a strong limb phenotype in family 1 of this report and a mild phenotype in pedigree DA in the report of Al-Baradie *et al.*⁶ with the same causative *SALL4* mutation c.2593C>T suggests that the phenotypic variability in these two families is the result of differences in the genetic background. With respect to the pathogenesis of the anomalies caused by *SALL4* mutations, the nature of these mutations, all causing premature termination of translation, would suggest haploinsufficiency. Also, the similar phenotype of patients with cytogenetic deletions of chromosome 20q probably encompassing the *SALL4* locus would be consistent with this.^{16,17} However, the fact that the *SALL1* knock-out mouse shows only kidney defects but no TBS-like phenotype¹⁸ raises serious doubts about the assumption that *SALL1* mutations which are exclusively truncating⁵ cause Townes-Brocks syndrome via haploinsufficiency. An alternative explanation which is being discussed now is that truncating *SALL1* mutations could lead to the TBS phenotype by a dominant negative action,¹⁹ with truncated proteins interfering with nuclear transport of the wild type proteins. This is thought to result from dimerisation of wild type and mutant proteins mediated by the evolutionarily highly conserved glutamine rich domain within the amino-terminal part of all known SAL-like proteins (fig 3).^{1-3,20-27} With respect to *SALL4*, the mutations c.326delC and c.523A>T show that the Glu rich domain (encoded by nucleotides 661-714 of the *SALL4* cDNA) cannot be crucial for a possible dominant negative action since the domain would be absent from truncated proteins resulting from these mutations.

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