

ONLINE MUTATION REPORT

SALL4 deletions are a common cause of Okihiro and acro-renal-ocular syndromes and confirm haploinsufficiency as the pathogenic mechanism

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The *SALL* genes, similar to the *Drosophila* gene *spalt*,¹ probably encode zinc-finger transcription factors. In humans, four such genes have been identified to date. Mutations at *SALL1* on chromosome 16q12.1 have been associated with Townes-Brocks syndrome and related phenotypes,^{2,3} and mutations at *SALL4* have been shown to be causative in patients with Okihiro/Duane-radial ray syndrome (OMIM No 607323).^{4,5} *SALL2*⁶ and *SALL3*⁷ have not yet been associated with human disease.

We previously reported frameshift and nonsense mutations in *SALL4* in five of eight families segregating the Okihiro syndrome phenotype.⁵ A further report⁴ identified two frameshift mutations and one nonsense mutation in three affected kindreds, including the family reported by Okihiro *et al.*⁸ In a recent study of patients with the clinical diagnosis of Holt-Oram syndrome (OMIM No 142900), one additional frameshift mutation was reported from a family which turned out to have Okihiro syndrome rather than Holt-Oram syndrome.⁹ Furthermore, we reported three novel and one already identified *SALL4* mutations in patients originally diagnosed as either Holt-Oram syndrome (later revised as Okihiro syndrome on the basis of the observation of a Duane anomaly in at least one of the affected family members in each family), acro-renal-ocular syndrome (OMIM No 102490), and Holt-Oram syndrome versus thalidomide embryopathy.¹⁰

In contrast to the report of Al-Baradie *et al.*,⁴ we found no *SALL4* mutations in three of eight families with clear Okihiro syndrome, the diagnosis being based on the presence of radial ray malformations in combination with a Duane anomaly. This finding led to the assumption that either mutations of another yet unidentified locus were responsible for the phenotype in those families, or that mutations within the *SALL4* gene were present but outside the region analysed (that is, they were promoter or intronic mutations).

SALL1 mutations causing Townes-Brocks syndrome and *SALL4* mutations in Okihiro and related syndromes show quite different distributions. Compared with the mutational spectrum of *SALL1* mutations,³ most of which reside in exon 2, 5' of the region encoding the first double zinc-finger domain, the *SALL4* mutations detected so far seem to be evenly distributed over the gene.^{4,5,9,10} It has not yet been possible to correlate the severity of the phenotype with the position of a *SALL4* mutation. It has been speculated that the different phenotypic severity in two families with the same mutation c.2593C→T is a result of epigenetic factors.¹⁰ The nature of all known *SALL4* mutations, all of which are truncating, suggested haploinsufficiency as the pathogenic mechanism.¹⁰ This is consistent with the observation that interstitial deletions of chromosome 20q, probably encompassing the *SALL4* locus, are associated with phenotypes similar to Okihiro syndrome.^{11,12}

Key points

- Point mutations of the *SALL4* locus, predicted to result in protein truncation, cause Okihiro syndrome and acro-renal-ocular syndrome in heterozygous individuals. However, several patients and families with these phenotypes have no identifiable *SALL4* mutation.
- Segregation analysis of intragenic single nucleotide polymorphisms was undertaken to detect loss of heterozygosity, consistent with an intragenic deletion, in families where no point mutation could be identified. Heterozygous *SALL4* deletion of at least exon 2 was suggested in four of six families by this method.
- Quantitative real time polymerase chain reaction was undertaken to confirm and map the intragenic deletions.
- Two families showed deletions of all four exons. Two families had deletions of exons 1–3; one family was heterozygous for a deletion of exon 4, and in a further family, a deletion of 8.9 kb including exon 1 was found. These deletions occurred de novo in two sporadic cases, and segregated with the phenotype in the remaining families.
- These data confirm that, in contrast to the likely dominant-negative action of *SALL1* mutations causing Townes-Brocks syndrome, Okihiro and acro-renal-ocular syndromes are clearly the result of *SALL4* haploinsufficiency.

Based on the fact that the *SALL1* knock-out mouse shows only kidney defects but no TBS-like phenotype,¹³ doubts were raised with respect to the hypothesis of *SALL1* haploinsufficiency causing TBS. As an alternative explanation 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.¹⁴ This would most probably result from dimerisation 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.^{1,6,7,15–22}

McLeskey Kiefer *et al* created a transgenic mouse harbouring a "typical" TBS mutation within the *Sall1* gene in order to

Abbreviations: FISH, fluorescence in situ hybridisation; OMIM, Online Mendelian Inheritance in Man; SNP, single nucleotide polymorphism

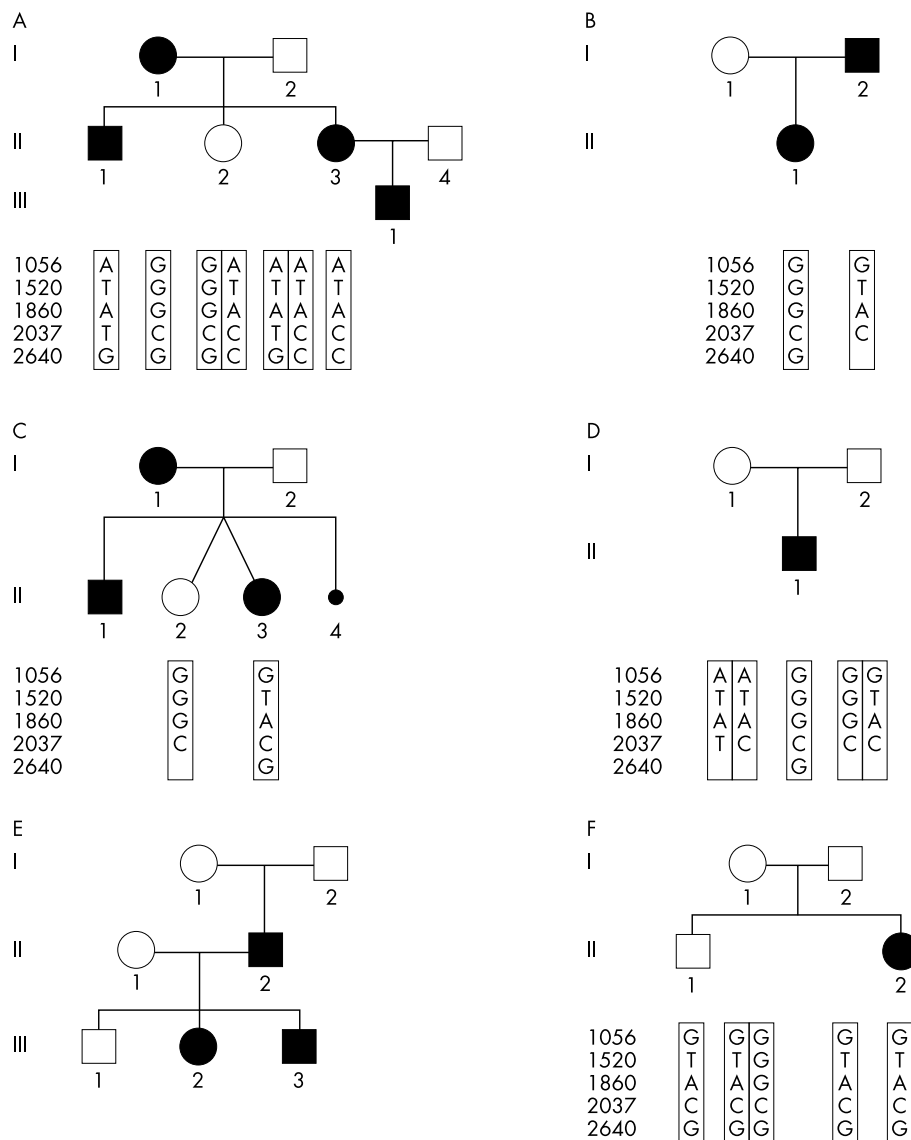


Figure 1 Pedigrees of the families analysed and results of the segregation analysis of the intragenic single nucleotide polymorphisms (SNPs) c.1056G→A, c.1520T→G, c.1860A→G (all exon 2), and c.2640G→C (exon 3) of *SALL4*. See table 2 for clinical details. (A) Family 1 with Okihiro syndrome²⁴ showing that the affected family members II.1 and II.3 only inherited one paternal *SALL4* allele (with respect to exons 2 and 3) but no allele from their affected mother. II.4 and III.1 were unavailable for the study. Note that in the other families, SNP c.2640G→C was only genotyped in all available family members if exon 2 SNPs were not informative. (B) In family 2²⁵ the diagnosis was acro-renal-ocular syndrome. The affected girl II.1 only inherited one maternal *SALL4* allele (with respect to exon 2) but not the single *SALL4* allele from her affected father. (C) In family 3 the diagnosis was Okihiro syndrome. The affected girl II.3 only inherited one paternal *SALL4* allele (with respect to exon 2) but not the single *SALL4* allele from her affected mother. (D) Family 4, showing de novo deletion of one *SALL4* allele in a sporadic patient with Okihiro syndrome. The boy only inherited one paternal *SALL4* allele (with respect to exon 2) but no maternal *SALL4* allele. (E) In family 5 (diagnosis Okihiro syndrome), the index patient II.2 was found to be heterozygous for SNPs c.1520T→C and c.1860A→G. Thus no segregation analysis was carried out. (F) In family 6 (diagnosis, Okihiro syndrome), segregation analysis was done but was not informative for all analysed SNPs.

mimic the molecular defect in human patients. Indeed, they could show that mice heterozygous for this mutation display a phenotype similar to TBS and that this is likely to result from a dominant-negative action of truncated Sall1 proteins.²³ In view of these results, the authors assumed that a similar mechanism for *SALL4* mutations should explain the pathogenesis of Okihiro syndrome. However, in our view the differing distributions of *SALL1* and *SALL4* mutations suggested that a different pathogenic action should still be considered. To approach this question, we sought further clarification for the role of *SALL4* in six families in which affected members were diagnosed as having Okihiro syndrome or acro-renal-ocular syndrome but were found negative for *SALL4* mutations.

METHODS

Patients

Patients from six different families were analysed in this study on the basis of the presence of typical clinical criteria for Okihiro or acro-renal-ocular syndrome. Two of these families have been reported previously.^{24, 25} Three families without mutations were mentioned in our first report.⁵ 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 carried out as described.⁵

Table 1 Primers used for quantitative real time polymerase chain reaction of the *SALL4* gene as well as of the genomic region 100 kb 5' to the ATG and 100 kb 3' to the stop codon.

Primer name	Position	Sequence 5'-3'	Amplicon length
10F	10656–10682	CCTTTTCCAACACCCATCATCCCAGCA	230
10R	10885–10860	CCAAGTCACCCCTCACTCTCCTCACCA	
16F	16234–16257	CATGGGGTGGAGGAGGCTGGAGA	163
16R	16396–16373	AGGGGGTGGAGGGAAGGAGAAAGA	
41F	41734–41760	GCCCAGGGACCTACTTCCGTCTTGCCA	150
41R	41883–41857	TTCTGAGCCCTCGCTTCTCACTGCC	
49F	48877–48901	AAGGTGTGGGGGGCAGTGGAGCAG	161
49R	49037–49011	TGGAGAGAATGAGGCAGCCAGTGGGGG	
65F	65489–65513	GGCACAGTGGAAAGTCTCCCCAAC	113
65R	65601–65579	GCTAGGCGGTGGCTCACACACAA	
81F	81354–81376	TCCCCAGTCCCCAAATCCACA	233
81R	81586–81563	GGGAGGAGGAGCAGAAGCAGTGA	
Ex1-F	101935–101958 exon 1	GAGTTGAGATGGGTGGGAGGGCTG	206
Ex1-R	102118–102141 exon 1	GATGTCCACCAGCCGAGAAGGGAG	
104F	104044–104069 intron 1	GGGGTTTCCCGAGAAGACGGTCGCCA	175
104R	104218–104194 intron 1	AAACGCCAGCCATCCCTCCGTCA	
111F	111150–111175 intron 1	TCCCTGACCCACCCCGCCTACAC	150
111R	111300–111274 intron 1	ACTTTTGACAGCACAGTCCACCCACGG	
Ex2-F	113309–113330 exon 2	GGCAGGGGCGGACACTCTGAAG	254
Ex2-R	113543–113563 exon 2	GGAAGCAAAGCGCTCGGGAGG	
Ex3-F	115957–115980 exon 3	GTTCTGGCACATTTGTGGGACCC	244
Ex3-R	116178–116201 exon 3	GCTGAAAGCCACACAAACCCACC	
Ex4-F	120368–120392 exon 4	CACACGGGGCGAACAATAACTCAGC	250
Ex4-R	120594–120618 exon 4	CAAGGAAACCGGGAGGGTAGGAACC	
135F	134925–134952	GGTGGCTCCATCTGTGTACAGCAAA	142
135R	135067–135042	TCACCAGAAGCCTTCCCTGCTCCC	
146F	146514–146540	GGTTTTTGAGGCAAGGCATGGAACCG	164
146R	146678–146652	CCCAACCCCTCCACCCCTCAAAGGCA	
161F	161653–161679	TCTTGACGCCATCTCCCAGCCTGTC	178
161R	161830–161805	TGCCCCAGGGTTCAGAGGAGCAGTG	
172F	172750–172775	TGAGGTGAGCGGTCCAGCGAGGGACG	199
172R	172948–172923	ACCCCACTCCAGGTCACCTCCCA	
175F	175123–175149	GGCACCCCGAGAAGAGAGACCAGAGT	100
175R	175221–175195	CCCTGCTGCACGTACCCAGGAAGAA	
194F	194058–194082	GCAACCAACAAAAGCCCCATCGGAG	284
194R	194341–194316	GCCGCAAAGAGAGACCCACAAAGGAA	
217F	217607–217633	GCAAGGAGAGGGAATGAAGCAGCACAA	296
217R	217902–217879	GGAAAGTGGCAAGGAGGGGCAGGA	

Position numbers refer to the 220 kb region as described in Methods.

Sequencing reactions were analysed on a Megabace 500 automated sequencer (Amersham Biosciences, Amersham, Buckinghamshire, UK). Segregation analysis was done by sequence analysis for the *SALL4* single nucleotide polymorphisms (SNPs) c.1056G→A, c.1520T→C, c.1860A→G, 2037C→T (all exon 2, analysed in all families) and c.2640G→C (exon 3, analysed in families 1 and 6 only).

Quantitative PCR

Deletion detection and fine mapping of the deletions was carried out using a quantitative polymerase chain reaction (PCR) approach with SYBR-Green I detection (Boehm *et al*, 2004). We designed primers for amplification of small amplicons within each of the *SALL4* coding exons (table 1). Four amplicons mapping to subtelomeric regions of 3p26.3, 2q24.2, 4p16, and 4q35.2 were used as reference.²⁶ The *SALL4* genomic sequence including 100 kb upstream and downstream of the coding region from NCBI databases (<http://www.ncbi.nlm.nih.gov/>) was masked for repeats, and 15 primer pairs were designed to amplify fragments of 100–300 base pairs (bp) using the PRIME program (Genetic Computer Group, Madison, Wisconsin, USA) (table 1). Amplicons were mapped relative to the *SALL4* coding region (see fig 2). The investigated genomic region comprises about 220 kb, approximately 100 kb on the 5 side and 100 kb on the 3 side of the *SALL4* gene (102–120 kb). The physical location of this region is 50987000–51206999 bp for the complete sequence (exactly 222 000 kb), and 51086226–51104369 bp

for the *SALL4* coding region plus introns, calculated from the telomere of the p-arm of chromosome 20. As *SALL4* is transcribed from telomere to centromere on the q-arm, the startpoint of the analysed sequence (bp 0 = bp 51206999) is more telomeric, and the end (bp 220 000 = bp 50987000) more centromeric.

We used the ABI Prism 7900 system (PE Applied Biosystems, Norwalk, Connecticut, USA) and white coloured 384-well plates (ABgene, Hamburg, Germany) for real time detection. Reactions contained 0.25 mM of each primer and 5 µl of QuantiTect SYBR® Green PCR Master Mix (Qiagen) 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 two minutes, 95°C for 15 minutes, and 40 cycles of 94°C (15 seconds), 58°C (15 seconds), and 72°C (one minute). For all amplicons the same conditions were applied.

In order to avoid the generation of non-specific products, a melting curve analysis of products was routinely undertaken 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 done by interpolation of the threshold cycle number (Ct) against the corresponding standard curve. Quantitative data were further normalised against a normal

Table 2 Phenotypic features of the participating family members

Patient	Eyes	Arms	Ears	Hearing	Kidneys	Feet	Heart	Other
<i>Family 1</i>								
I.1	N	Bilat AT, HPLH L, AR L, HPLR + HPLU R	N	N	N	NR	NR	
II.1	Bilat DA	Bilat AT, AR, HPLU	N	N	N	NR	N	White hairlock, hyper/depigmented skin patches
II.3	Bilat DA	Bilat AT+AR, HPLU, HPLH, HPL deltooid muscle	N	N	N	NR	N	
III.1	NR	Bilat radial ray defects	NR	NR	NR	NR	NR	
<i>Family 2</i>								
I.2	DA R, struct anom	Bilat AT + AR, HPLU	N	NR	N	NR	NR	Thalidomide exposure of mother
II.1	DA L, struct anom	AT L + HPLT R	N	Bilat CHL	PC dilat L, VUR	NR	NR	
<i>Family 3</i>								
I.1	Bilat DA	Bilat AR, AU + AT	N	N	Pelvic kidney L		NR	
II.1	N	Bilat AT	N	N	NR	NR	NR	
II.3	Bilat DA	R: HPLT, HPL metacarp I + thenar L: AT	N	N	HS kidney	NR	NR	Temporo-mandibular joint instability
<i>Family 4</i>								
II.1	Bilat DA	L: AT, R: HPLT, bilat HPLR	N	N	HPLK R	Bilat talipes	NR	Bilat epicanthic folds, hypertelorism, low hCG (PSS)
<i>Family 5</i>								
II.2	Bilat DA	Bilat AT + HPLR	N	NR	NR	NR	NR	Hip dislocation
III.2	Bilat DA	Bilat AT + HPLR	N	Mild HL L	Pelvic kidney R, HN L	NR	N	Subluxated hip L, disloc shoulder, lumbo-sacral meningocele
III.3	Bilat DA	Bilat AT + HPLR	NR	NR	NR	Tibial hemimelia	NR	
<i>Family 6</i>								
II.2	Bilat DA	Bilat HPLR, PRPD L	Bilat dyspl	Bilat SNHL	NR	NR	NR	

Structural eye anomalies in family 2 are bilateral segmented disc hypoplasia (I.2) and retinal coloboma on left, and right dysplastic disc with bilateral nystagmus (II.1). References: family 1, MacDermot K, Winter R. *Am J Med Genet* 1987;27:313–19; family 2, Becker K, et al, *J Med Genet* 2002;39:68–71. AR, absent radius/radii; AT, absent thumb(s); Bilat, bilateral; CHL, conductive hearing loss; DA, Duane anomaly; dilat, dilatation; disloc, dislocated; HL, hearing loss; HN, hydronephrosis; HPL, hypoplasia; HPLH, humerus hypoplasia; HPLK, hypoplastic kidney; HPLR, radius hypoplasia; HPLU, ulna hypoplasia; HPLT, thumb hypoplasia; HS, horseshoe; L, left; N, normal; NR, no abnormality reported; PC, pelvicceal; PRPD, preaxial polydactyly; PSS, prenatal serum screening; R, right; SNHL, sensorineural hearing loss; struct anom, structural anomaly.

diploid reference genome by calculating the ratio relative to the average amount of reference amplicons for each amplicon. In this manner ratio values of 1.0 indicate a diploid situation, and values of 0.5 or 1.5 indicate partial haploidy or partial triploidy, respectively (see table 3).

Breakpoint cloning

An additional forward primer (92F, 5' CTTCGTGTTCCAT CCCATCCTAGCC 3') was designed at position 92309–92335 bp for amplification of the breakpoint spanning region in family 6 with the Long PCR enzyme mix (Fermentas, Vilnius,

Lithuania). The reaction contained 0.2 µg of genomic DNA, 5 µl Long PCR buffer with MgCl₂, 5 µl dNTPs (0.2 mM each), 10 pmol of each primer (92F and 104R), and 2.5 units of Long PCR enzyme mix to a total volume of 50 µl. An initial denaturation step at 94°C for three minutes was followed by 10 cycles of 94°C for 15 seconds and 68°C for 10 minutes. The next 25 cycles were done under the same conditions with a 10 second time increment per cycle during the elongation step. The reaction terminated with a final elongation step at 72°C for 10 minutes. The PCR product was subcloned into pGEM-T Easy vector (Promega, Madison, Wisconsin, USA) for further

Table 3 Results of the *SALL4* real time polymerase chain reaction applied to patient DNA samples of six families with Okhiro/DRR or acro-renal-ocular syndrome

Family	Amplicon position																		
	10 kb	16 kb	41 kb	49 kb	65 kb	81 kb	Ex 1	104 kb	111 kb	Ex 2	Ex 3	Ex 4	135 kb	146 kb	161 kb	172 kb	175 kb	194 kb	217 kb
1	0.99	1.03	1.05	1.05	0.50	0.52	0.55	0.54	0.54	0.52	0.49	0.96	1.02	1.01	1.03	1.03	1.05	0.97	0.96
2	0.56	0.49	0.55	0.56	0.46	0.47	0.51	0.47	0.49	0.50	0.50	0.53	0.55	0.99	0.99	0.99	0.99	1.00	1.02
3	1.03	0.97	1.02	0.97	0.97	0.98	0.52	0.49	0.47	0.52	0.52	1.04	0.49	0.97	0.99	1.05	0.98	1.00	1.05
4	1.03	1.05	1.02	1.01	1.00	0.99	0.47	0.55	0.51	0.54	0.47	0.50	0.54	1.02	1.03	0.97	1.04	1.02	0.98
5	1.05	1.02	0.98	1.01	0.96	0.96	0.98	1.00	0.99	1.04	1.02	0.51	0.57	1.05	1.00	1.01	1.00	1.06	0.97
6	1.02	0.98	1.05	0.98	1.00	0.95	0.52	1.04	0.96	1.02	1.05	0.98	1.05	1.03	1.05	1.05	1.03	0.97	0.98

The normalised ratios (*SALL4* amplicon/ reference amplicon) are given. Values interpreted as haploid situation (deletion) range from 0.46 to 0.57, whereas diploid situation was assumed for values from 0.95 to 1.06. Exonic amplicons are printed in bold; the grey shading indicates deleted regions. The position numbers refer to the 220 kb region as described in Methods

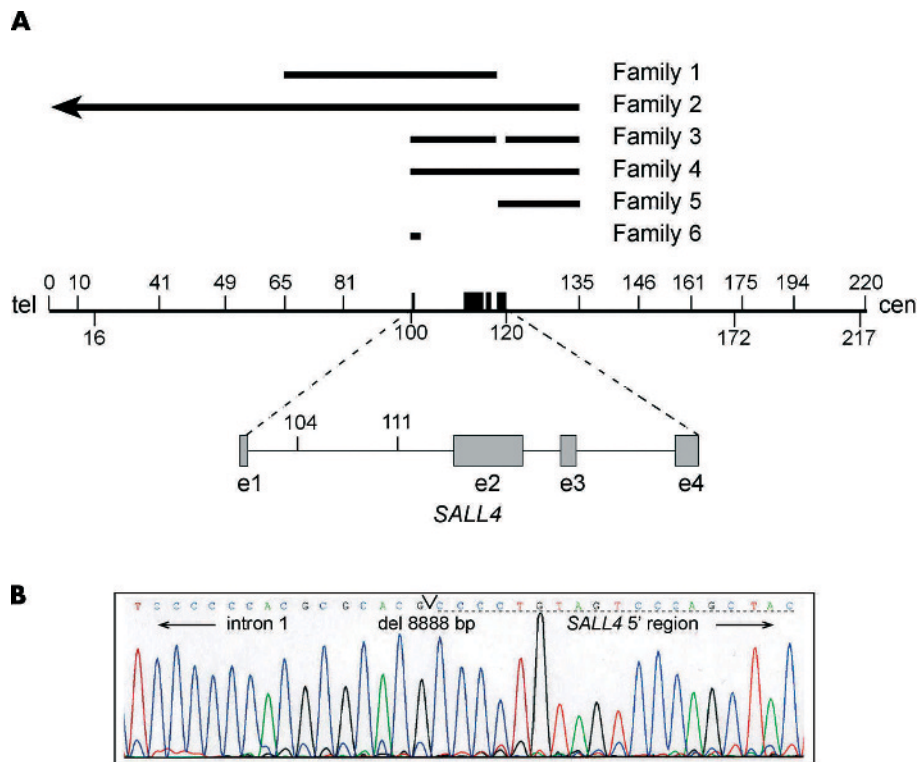


Figure 2 (A) Schematic diagram of the *SALL4* genomic region on chromosome 20q13.13–13.2 with positions of the deletions detected in the six families analysed; 220 kb of genomic DNA sequence (line) consisting of the *SALL4* gene plus 100 kb flanking sequence on each side were analysed by quantitative real time polymerase chain reaction (PCR) for deletions. The numbers indicate the positions of amplicons. The black boxes on top of the line indicate the *SALL4* exons enlarged below. Note the considerable size differences of the deletions as well as the presumed common breakpoint regions. (B) Sequence (reverse complementary) of the breakpoint spanning long range PCR fragment obtained from the index patient in family 6. The dotted line indicates part of an AluY element 7.6 kb upstream of the *SALL4* ATG within which the 5' break occurred. No Alu element was found near the 3' breakpoint positioned within intron 1 of the *SALL4* gene.

analysis. Plasmids were isolated from bacterial colonies by routine methods and sequenced using T7 and SP6 primers, respectively.

Fluorescence in situ hybridisation

Metaphase spreads from peripheral blood lymphocytes were prepared by routine procedures. Before fluorescence in situ hybridisation (FISH), the slides were treated with RNase followed by pepsin digestion.²⁷ FISH essentially followed the methods previously described.²⁸ Chromosome

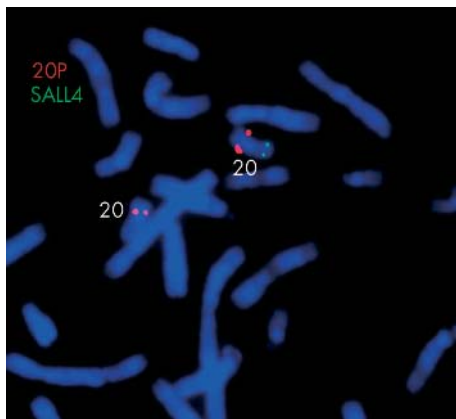


Figure 3 FISH analysis in the index patient of family 4. A *SALL4* specific probe (green) and a PAC clone hybridising to chromosome 20p11.2 (red) detect both chromosomes 20 but only one *SALL4* allele, thereby confirming the deletion.

in situ suppression was applied to the following probes. As a gene specific probe, a 5.1 kb PCR product of *SALL4* spanning part of intron 1 as well as exons 2 and 3³ was chosen and subcloned into pCR[®] II-TOPO[®] (Invitrogen, San Diego, California, USA). As a marker to tag chromosome 20 we used a human PAC (PI artificial chromosome) clone derived from the same well of the Human Male Genome PAC library (RPCII, 3, 4, 5)²⁹ as the Y chromosome specific clone RP4-529G14.³⁰ This clone hybridised constantly to chromosome 20p11.2 with a single signal (Wimmer R, personal communication). After FISH, the slides were counterstained with DAPI (0.14 µg/ml) and mounted in Vectashield (Vector Laboratories, Burlingame, California, 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_020436 (*SALL4* cDNA sequence), NT_01132 (genomic contig including *SALL4*). Online Mendelian Inheritance in Man (OMIM; <http://www.ncbi.nlm.nih.gov/OMIM>): for Okihiro/ Duane Radial Ray syndrome, OMIM 607323; for *SALL4*, OMIM 607343; for acro-renal-ocular syndrome, OMIM 102490. Repeat-masker at EMBL: <http://www.woody.embl-heidelberg.de>

RESULTS

SALL4 mutation analysis revealed no heterozygous *SALL4* mutation and no heterozygous SNP in the index patients of five families with the clinical diagnosis of Okihiro syndrome, among these being a family with Okihiro syndrome reported previously,²⁴ and one family reported as acro-renal-ocular syndrome²⁵ (fig 1). The clinical features of the affected family

members are summarised in table 2. In the first family (fig 1A), three affected and two unaffected subjects (I.1, 2; II.1, 2, 3) were available for the study. In the latter (family 2; fig 1B), both the affected father and the affected daughter (I.2, II.1) were analysed. In a further family (family 3; fig 1C), mother, son, and one daughter were affected. Samples were available from mother and daughter. In family 4 (fig 1D), an affected boy was born to unaffected parents. Family 6 (fig 1F) is another sporadic case with healthy parents and an unaffected brother. In family 5 (fig 1E), no mutation was found in the index patient (II.2), but he was heterozygous for the SNPs c.1520T→C and c.1860A→G within exon 2. Samples were available from affected father and daughter.

In family 1, segregation analysis was done by analysis of exons 2 and 3 in all available family members and was informative for SNPs c.1056G→A, c.1520T→G, c.1860A→G (all exon 2), and c.2640G→C (exon 3), suggesting a heterozygous deletion of at least exons 2 and 3 (fig 1A). Subsequently, segregation analysis was carried out for exon 2 SNPs in the other families reported here; c.2640G→C (exon 3) was only analysed in the remaining families if exon 2 SNPs were not informative. In family 2 (fig 1B), the results were informative for SNPs c.1520T→G and c.1860A→G, suggesting that at least exon 2 was deleted in both patients. In family 3, samples were available of affected mother and daughter. Segregation analysis was again informative for SNPs c.1520T→G and c.1860A→G, suggesting a heterozygous deletion of at least exon 2 of *SALL4*. In family 4 (fig 1D), segregation analysis was informative for SNPs c.1056G→A, c.1520T→G, and c.1860A→G, suggesting a *SALL4* de novo deletion which must have occurred on the maternally inherited chromosome 20. In family 5 (fig 1E), segregation analysis was not done because SNPs c.1520T→G and c.1860A→G were found to be heterozygous in the index patient II.2. In family 6, the index patient did not show any heterozygous SNP of exon 2 or 3 but segregation analysis was not informative for all SNPs (fig 1F).

To confirm the suspected deletions and to determine their size, primer pairs (table 1) were designed for all four exons of *SALL4* to be used for quantitative real time PCR. In this analysis, all six families were included. Quantitative real time PCR confirmed all exonic deletions predicted by SNP genotyping (table 3). Furthermore, it revealed a heterozygous deletion (see Methods for details) of all exons in the index patients of families 2 and 4, whereas in the index patients of families 1 and 3, a deletion was observed only for exons 1–3 but not for exon 4. Interestingly, in the index patient of family 5 exons 1–3 were amplified normally, whereas exon 4 was found to be deleted on one allele. In contrast, only exon 1 amplification products were reduced in the index patient of family 6. The procedure was repeated three times, now including all available affected as well as unaffected family members, and the results fully segregated with the phenotype in all families (data not shown) or proved de novo occurrence of deletions in the sporadic cases.

To map the size of the deletions, additional primer pairs were designed for amplification of non-repetitive regions upstream and downstream of the *SALL4* coding region (tables 1 and 3; fig 2). The results of real time PCR (table 3) suggested minimum deletion sizes ranging from approximately 0.2–24 kb in family 6 to more than 135 kb in family 2 (fig 2). Unexpectedly, analysis of family 3 showed that, while exon 4 was preserved, the following amplicon at 135 kb was again deleted. This result was confirmed by three additional repetitions.

To corroborate our results with a third method, we applied FISH to detect the deletion on the chromosome in situ. Based on the deletion sizes, families 1–4 seemed suitable for application of this method, but cells were available only

from family 4. As a cosmid clone must lie entirely within the deleted region in order to allow unambiguous detection of the deletion, we chose to use a subcloned *SALL4* PCR fragment spanning exon 2, intron 2, and exon 3 as the specific probe. Indeed, this probe consistently detected only one chromosome 20 in all examined metaphases (fig 3).

In order to determine the exact size of the deletions in families 1 and 3–6, long range PCR was carried out with forward and reverse primers of the amplicons located in the non-deleted regions neighbouring the deletions, but without success. In family 6, real time PCR results predicted the expected 3' breakpoint in intron 1 within a region of approximately 2 kb 3' of exon 1. By using the forward primer 92F and the reverse primer 104R in family 6 with the smallest predicted deletion, we could amplify the deletion spanning fragment of approximately 3 kb (expected wild type size 11.8 kb) from the index patient of family 6. Sequencing of the subcloned PCR fragment (fig 2B) with flanking primers revealed a deletion size of 8888 bp, consisting of 1139 bp of intron 1, 130 bp exon 1 coding sequence, and 7619 bp sequence upstream of the ATG. The 5' breakpoint resides within an AluY element but the 3' breakpoint does not lie within a predicted repeat.

DISCUSSION

Our data show for the first time that Okhiro syndrome may also be caused by heterozygous deletions either of the whole *SALL4* coding region or of single exons. In four of six families analysed, among them being the three mutation negative families of our initial report on *SALL4* mutations,⁵ we were able to prove a deletion by two independent methods—segregation analysis of intragenic SNPs, and quantitative real time PCR. In one family, a third method, FISH, was applied to confirm and illustrate the results. In families 1–3, both SNP segregation analysis and real time PCR confirmed segregation of the phenotype with the deletion. In family 5, segregation of the deletion and its de novo occurrence in the index patient was proven by real time PCR only, and in families 4 and 6 the deletions occurred de novo as expected for sporadic cases. Therefore, no doubt remains that the deletions are pathogenic.

Real time PCR proved to be a valuable tool to determine the approximate sizes of the deletions. Most of these deletions would have escaped a strategy using a conventional FISH probe for detection—that is, a larger genomic clone from the *SALL4* region. Therefore, the applied real time PCR seems to be the method of choice for diagnostic purposes. The deletions range from 8.9 kb in family 6 up to more than 135 kb in family 2. Looking at the preliminary breakpoints (fig 2), it seems that there are hotspot regions for breakpoints close to the *SALL4* gene. Four of six deletions (families 2–5) end between 135 and 146 kb, respectively. Three of six deletions (families 3, 4, 6) start between 81 kb and exon 1. Possibly, a breakpoint between exons 3 and 4 is shared by families 1, 3, and 5. Cloning of the breakpoint in family 6 showed that an AluY element contributes to the break on the 5' side. On the 3' side, no Alu element is involved. Cloning of the other breakpoints is required to investigate if the respective breakpoints are indeed shared by the different deletions and if there are special recombination associated motifs³¹ present at those sites.

How do the deletions arise? Analysis of the investigated 220 kb region—that is, the *SALL4* coding region with an additional 100 kb genomic sequence on either end—by repeat masker revealed that 314 Alu elements are present within the region, contributing to 38.58% of the whole sequence. The total amount of interspersed repeats makes up for 59% of the sequence. Looking at the region around the common breakpoint region 3' to the *SALL4* gene—that is, 11 000 bp from

135 to 146 kb—this region contains 13 Alu elements (35.02% of the sequence), and repetitive sequences of all kind make up for 54.96% of this region. Thus Alu mediated recombination might be responsible at least for some of the deletions,^{32,33} and the richness in Alu elements and other repetitive regions might make the *SALL4* gene especially prone to deletions. The breakpoint in family 6, however, shows that an Alu element is involved on one but not on the other side of the deletion, and the process leading to the deletion remains unclear.

Our results show for the first time that Okihiro and acro-renal-ocular syndromes are not only caused by truncating *SALL4* mutations as shown previously^{4,5,9,10,34} but also by deletions of either the whole gene or single exons, all of which are coding. While it seems clear that a deletion removing the whole coding region (as observed in families 2 and 4), or most of the coding region (exons 1 to 3 deleted) including the regions encoding the double zinc finger domains (in families 1 and 3), results in non-functional alleles, the deletions in family 5 and 6 remove the exon harbouring the start and the stop codon, respectively. It seems that this cannot be compensated for by alternative splicing or by the use of alternative ATGs or stop codons. Neither of those possibilities is known for the *SALL4* gene or its murine homologue *Sall4*.³⁵ Nevertheless, it is likely that the 8.9 kb deletion harbouring exon 1 not only removed the ATG but also part if not all of the *SALL4* promoter sequence. The similar clinical picture among the different families and the presumed deletion sizes strongly indicate that all deletions result in *SALL4* haploinsufficiency.

As the phenotypes caused by deletions are not different from those caused by *SALL4* point mutations, we speculate that the truncating *SALL4* point mutations have the same pathogenic effect. In all families reported here, at least one affected member had limb malformations typical for Okihiro/DRR syndrome in combination with a Duane anomaly. Renal anomalies are present in four out of six families (families 2–5), showing that acro-renal-ocular syndrome could also have been diagnosed in families 3–5. Sensorineural hearing loss and dysplastic ears as in family 6, structural eye malformations as in family 2, and foot malformations and facial features as in family 4 have also been seen in families with *SALL4* point mutations.^{4,5,9,10,34} Interestingly, patient I.2 in family 2 represents another example of a patient in whom thalidomide embryopathy was initially discussed. The only difference in the phenotypic features between patients with point mutations and larger deletions seems to be the rate of congenital heart defects. In our most recent study,³⁴ we found congenital heart defects in three of 13 mutation carriers (three of five families), and in a former study, two of seven patients (two of four families) had a congenital heart defect. Analysis of further deletion cases will show if this represents a true difference between the two groups.

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