

MUTATION IN BRIEF**Multigene Deletions on Chromosome 20q13.13-q13.2 Including *SALL4* Result in an Expanded Phenotype of Okihiro Syndrome Plus Developmental Delay**Wiktor Borozdin,^{1,2} John M. Graham, Jr.,³ Detlef Böhm,¹ Michael J. Bamshad,⁴ Stefanie Spranger,⁵ Leah Burke,⁶ Michael Leipoldt,² and Jürgen Kohlhase^{1*}

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Okihiro syndrome results from truncating mutations in the *SALL4* locus on the chromosome 20q13.13-q13.2. Deletions of the whole *SALL4* coding region as well as single exon deletions are also a common cause of Okihiro syndrome and indicate haploinsufficiency as the disease causing mechanism. The phenotypes caused by *SALL4* deletions are not different from those caused by point mutations. No multigene deletion including *SALL4* has been documented to date. Here we report the detection and molecular characterization of four novel, overlapping microdeletions, all spanning *SALL4* and flanking genes, in four unrelated cases with features of Okihiro syndrome and variable degrees of psychomotor delay. All deletions were first identified and mapped by quantitative Real Time PCR. Subsequently, three of four deletions were mapped in further detail by high-resolution array CGH (244k oligo-arrays). All cases had larger deletions of varying size (1.76-1.78 Mb, 2.01-2.05 Mb, 2.16-2.17 Mb, and 1.3-2.8 Mb, respectively), which included *SALL4* plus 3 to 7 additional functional genes. While three cases with largely overlapping deletions are mildly developmentally delayed, the only patient with a more centromeric deletion is clearly mentally retarded. In this patient, four genes (*MOCS3*, *DPM1*, *ADNP*, *BCAS4*) are deleted, which were not affected in the other three cases, suggesting that the deletion of one or more of these genes contributes to the mental retardation. Since two of the four cases presented with choanal atresia, large deletions including *SALL4* should be considered in the differential diagnosis of children with suspected CHARGE syndrome but without detectable *CHD7* mutations. Published 2007 Wiley-Liss, Inc.

KEY WORDS: *SALL4*; Okihiro syndrome; microdeletion; mental retardation; array CGH; CHARGE syndrome

INTRODUCTION

Okihiro syndrome (OS, also called Duane-Radial ray syndrome, MIM# 607323) describes an autosomal dominant condition characterized by radial ray malformations associated with Duane anomaly (Okihiro et al., 1977) caused by mutations in the gene *SALL4* (MIM# 607343) on chromosome 20q13.13-q13.2. Acro-renal-ocular syndrome (AROS; MIM# 102490) is considered to be allelic to Okihiro syndrome in most if not in all cases based

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on the finding of truncating *SALL4* mutations or larger deletions in three families previously reported as AROS (Aalfs et al., 1996; Becker et al., 2002; Borozdin et al., 2004a; Borozdin et al., 2004b; Kohlhase et al., 2003). AROS has been characterized as a combination of radial ray, renal and structural eye anomalies such as iris or optic nerve colobomata (Aalfs et al., 1996; Halal et al., 1984), however, several authors had noted the extensive phenotypic overlap between OS and AROS (Becker et al., 2002; Chun et al., 2001). The clinical presentation of *SALL4*-related disorders is highly variable within and between affected families. Reduced penetrance has been described in one family prior to molecular testing (Hayes et al., 1985) and in a second family tested for *SALL4* mutations (Collins et al., 1993; Kohlhase et al., 2002). The frequency of the condition is not known.

The radial ray anomalies seen in OS/ AROS are very similar to those seen in Holt-Oram syndrome caused by mutations in *TBX5*. They include hypoplasia of the thenar eminences in the mildest cases, triphalangeal thumbs, occasionally preaxial polydactyly (Borozdin et al., 2004a; Kohlhase et al., 2002), hypoplasia or aplasia of the radius with concomitant ulna hypoplasia, shortening and radial deviation of forearms, phocomelia, and occasionally involvement of the shoulder girdle (Al-Baradie et al., 2002; Borozdin et al., 2004a; Borozdin et al., 2004b; Chun et al., 2001; Collins et al., 1993; Ferrell et al., 1966; Hayes et al., 1985; Kohlhase et al., 2005; Kohlhase et al., 2002; Kohlhase et al., 2003; MacDermot and Winter, 1987; Miertus et al., 2006; Temtamy et al., 1975; Terhal et al., 2006). 91.3 % of mutation carriers in *SALL4* show some kind of radial ray malformation (Kohlhase, 2004).

Duane retraction syndrome (Duane anomaly) is a congenital eye movement disorder characterized by abnormal development of cranial nerve VI (the abducens nerve), resulting in restriction or absence of abduction, adduction, or both, and narrowing of the palpebral fissure and retraction of the globe on attempted adduction, and it occurs in 65% of *SALL4* mutation carriers. In Okhiro syndrome, Duane anomaly is mostly of type 1, resulting in minimal or no limitation of adduction due to absence of the abducens nerve, and it may occur unilaterally or bilaterally. Hearing loss (mostly sensorineural but also conductive or mixed) is common, occurring in 16% of mutation positive cases (Kohlhase, 2004) and 17% of clinically diagnosed cases (prior to gene identification) in a survey by Chun et al. (2001). Dysplastic ears with abnormal pinnae may be seen, as well as slit-like openings of the auditory canals (Kohlhase et al. 2003). Renal malformations are common and possibly underestimated in previous reports due to their mostly mild nature (Kohlhase, unpublished data). They include renal malrotation/ ectopia, horseshoe kidneys and less often renal hypoplasia or agenesis. Renal failure appears to be a rare complication. Urinary anomalies have also been reported (vesicoureteral reflux, bladder diverticula). Facial asymmetry may occur and present as severe hemifacial microsomia (Terhal et al., 2006). Other facial features include epicanthic folds, hypertelorism, flat nasal bridge and prominent nose tip (Kohlhase et al., 2003). Cardiac lesions, particularly ventricular but also atrial septal defects as well as patent ductus arteriosus and tetralogy of Fallot have been described (Borozdin et al., 2004b). Choanal atresia (Kohlhase et al., 2002), short neck and fused cervical vertebrae have been reported (Al-Baradie et al., 2002). Structural eye anomalies, also rare, include iris and optic nerve colobomata (Borozdin et al., 2004a; Borozdin et al., 2004b; Kohlhase et al., 2003). Gastrointestinal features include anal stenosis or imperforate anus, and rarely Hirschsprung disease (Al-Baradie et al., 2002). Although the homologous mouse gene *Sall4* is expressed in the brain, developmental delay or mental retardation has never been observed in a case with a *SALL4* mutation or deletion within or including the *SALL4* gene. Here we report for the first time the clinical and molecular characterization of multigene deletions that include *SALL4*.

MATERIALS AND METHODS

Cases

Venous blood was collected from cases after obtaining their informed consent or that of the parents.

Genetic Analysis

Genomic DNA was prepared from peripheral lymphocytes and other samples by routine procedures. Mutation analysis of *SALL4* was performed as described previously (Kohlhase et al., 2002). Deletion screening by quantitative Real Time PCR was performed as described (Borozdin et al., 2004a). For mapping of the identified deletions additional amplicons were designed analogous to the procedure outlined before (Borozdin et al., 2004a). The deletions were mapped with respect to the reference sequences GenBank accession numbers NM_020436.2 (*SALL4* mRNA) and NT_011362.9 (genomic contig on chromosome 20q13).

Array CGH

Genomic DNA. We obtained genomic reference DNA from a normal male 46,XY and a normal female 46,XX (Promega, Heidelberg, Germany). Array CGH was performed using premade Agilent oligo-CGH arrays (244 k arrays; Agilent Technologies, Palo Alto, CA, USA) consisting of ~244,000 *in situ* synthesized 60-mer oligonucleotides spanning the entire genome, resulting in an average genomic distance of ~12 kb. These probes included both coding and non-coding sequences on every human chromosome.

For each CGH-hybridization, we digested 3 µg of genomic DNA from the reference (46,XX or 46,XY) and the corresponding experimental sample with AluI (20 units) and RsaI (20 units) (Promega). All digests were done for a minimum of 2 h at 37°C, heat-inactivated at 65 °C for 20 min and then verified by agarose gel electrophoresis. Labeling reactions were performed with 3 µg restricted DNA and the Agilent Genomic DNA labeling Kit PLUS (Agilent Technologies) according to the manufacturer's instructions in a total volume of 50 µl with a modified dNTP pool containing dATP, dGTP, dCTP, and dTTP, and Cy5-dUTP (for the experimental sample) or Cy3-dUTP (for the 46,XX or 46,XY reference). Labeled targets were subsequently filtered twice by using 1 x TE-buffer pH 8.0 (Promega) through a Centricon YM-30 column (Millipore) and concentrated to 80.5 µl. The amount and the specific activity of the Cy3- and Cy5- labeled samples were determined by using the Nanodrop ND-1000 UV-VIS Spectrophotometer (PqLab, Erlangen, Germany) and the microarray measurement protocol. The manufacturer's recommendations (Agilent Technologies) were followed for selecting versus rejecting the probes for hybridization. Hybridization and features extraction was performed as described (Barrett et al., 2004; Spitz et al., 2006), and data were visualized by means of the CGHAnalytics 3.4 software (Agilent Technologies).

CASE REPORTS

Case 1 was a male born at term to healthy first-cousin parents with a negative family history. He had a membranous imperforate anus, choanal atresia, bilateral Duane anomaly and hypoplastic thumbs - small dysplastic left thumb composed of 3 ossicles located at the level of the 2nd metacarpal-phalangeal joint, with absence of the 1st metacarpal and a hypoplastic right thumb with a very short 1st metacarpal. He also had hypospadias, and posterior urethral valves. At 12 months, he had severe low frequency sloping hearing loss, with moderate high frequency hearing loss, worse on the left than the right. His bilateral conductive hearing loss was felt to be secondary to a congenital ossicular chain abnormality. At 5 years, laryngoscopy revealed a very small larynx (the size of a 12-month child). At 9 years, height and weight were <5 centile (116 cm, 18.7 kg), head circumference was <3rd centile (49 cm). He repeated syllables at 15 months, spoke single words at 18 months, was sitting at 18 months, and walking at 22 months. A Denver Developmental Screening Test at 36 months revealed gross motor and expressive language skills of 18 months, self-help, social, academic and receptive language skills of 24 months. At 6 years, the boy had borderline cognitive abilities. At 9.5 years, when cognitive assessment accommodated his hearing impairment, his IQ was 92 on the Leiter Scale, and WISC-R performance IQ was 96 (average cognitive abilities). A 625-banded karyotype and subtelomeric FISH analysis were read as normal in 2004. As an adult, he is now able to live independently and to work at least part-time.

Case 2 was a male born at term to unaffected, unrelated parents. Both upper arms and forearms were shortened. On the left, the radius and the thumb were absent. The right arm appeared less shortened than the left. He had synostosis of right proximal radius and ulna and a hypoplastic right thumb. He had short stature. Significant dysmorphic facial features included telecanthus, downslanting palpebral fissures, mild ectropion of the lateral lower eyelids, and a bifid nasal tip. Further anomalies: atrial septal defect, strabismus, bilateral hypoplastic kidneys with vesicoureteral reflux and severe gastroesophageal reflux. He was severely developmentally delayed. The karyotype was normal, as were chromosomal breakage studies.

Case 3 was a male born to unaffected, unrelated parents. He had bilateral radial aplasia with absent thumbs, an incomplete atrioventricular canal and unilateral (right) renal agenesis. Bilateral microtia with absent auditory canals was noted. The ocular exam was normal. He attended special education classes. The observed developmental delay was partly but not fully explained by the hearing deficit. The karyotype of the boy was normal.

Case 4 was a 14 months old female born to unrelated unaffected parents. At birth, she presented with bilateral triphalangeal thumbs with contracture of the metacarpophalangeal joint of the thumb on the left side and radial deviation of the hand on the right. The facial features appeared unremarkable. She was found to have bilateral choanal atresia and bilateral sensorineural hearing loss, more severe on the left side. Additional abnormalities included muscular hypotonia, broncho- and laryngeal malacia (infantile larynx), and thoracic scoliosis. Length was

81 cm (25 centile), head circumference 48.5 cm (75 centile) and weight 9.3 kg (3 centile). She showed a delayed psychomotor development as she could roll over and move around but could not crawl on her hands and knees. She was able to sit with only little support. Her behavior showed autistic tendencies. Her karyotype was normal.

RESULTS

Direct sequencing of the SALL4 coding region did not reveal a pathogenic mutation in any of the cases and further failed to demonstrate any heterozygous sequence variation. Subsequently, screening for *SALL4* deletions was performed by quantitative Real-Time PCR as described previously (Borozdin et al. 2004). For each case, we calculated normalized ratios around 0.5 for all 15 amplicons within and flanking the *SALL4* gene up to 100 kb up- and 100 kb downstream of the coding region, indicating heterozygous deletions of the whole analyzed region (Borozdin et al. 2004).

Several further amplicons were designed for walking along the chromosome to map the sizes of the deletions (primers available on request). By this method, the deletion size in case 1 was determined as 2.1-2.4 Mb and predicted to remove 5 known active genes in addition to *SALL4*. In case 2, qPCR revealed a *de novo* heterozygous deletion of approximately 1.9-2.3 Mb in size spanning *SALL4* plus 9 neighboring functional genes on chromosome 20q13.2. This deletion was overlapping with the one from family 1 by approximately 2.05 Mb, however, it was positioned more centromeric on the chromosome.



Figure 1. Phenotypic features of case 1 at age 9.5 years. Left upper and lower: Bilateral Duane anomaly associated with inhibited ab- and adduction of the eyes. Note the broad nasal root and downslanting palpebral fissures. Middle and right upper: small dysplastic ears with hearing aids. Right lower: hypoplastic thumb on the right hand on X-ray examination. Middle lower: flat feet.

In case 1, FISH was applied to narrow down the distal breakpoint of the deletion. Using two BAC clones, RP11-489N01 with the signal only on one chromosome and RP5-823G15 consistently binding to both chromosomes 20 (data not shown), we were also able to confirm this deletion by a second, independent method.

In case 3, a heterozygous deletion ranging from 1.3 to 2.8 Mb was detected, which overlapped with the telomeric ends of both previously described deletions. Unfortunately, the supplied DNA sample was sufficient only for performing crude deletion mapping and the parents denied collection of further samples. Therefore, the number of affected genes (including *SALL4*) could be determined as four definitely deleted and another 4 possibly

included within the telomeric breakpoint region of about 1.4 Mb (Table 2). In case 4, no further mapping was performed by qPCR prior to array CGH analysis.

In order to map the deletions in further detail, array CGH was performed, utilizing pre-made 244k oligomer CGH arrays (Agilent Technologies). By this method, the deletion size in case 1 was determined as 2.155794 – 2.170893 Mb, the deletion in case 2 as 2.010800 – 2.048667 Mb, and the deletion in case 4 as 1.764407 – 1.775181 Mb in size.

According to the nomenclature guidelines for human mutations, the deletions can be described as case 1, g.(49,058,969_49,063,303)_ (51,219,097_51,229,862)del(chromosome 20, NCBI Build 36.1), case 2, g.(48,825,400_48,831,934)_ (50,842,734_50,874,067)del(chromosome 20, NCBI Build 36.1), case 3, g.(49,737,083_49,760,644)_ (51,096,325_52,526,086)del(chromosome 20, NCBI Build 36.1), case 4, g.(49,759,845_49,765,695)_ (51,520,102_51,531,026)del(chromosome 20, NCBI Build 36.1).

The deletion in case 1 contains the genes *TSHZ2*, *ZFP64*, *SALL4*, *ATP9A*, *NFATC2*, and *KCNIG1* (Fig. 2, Table 2). Case 2 has lost one copy each of *ZFP64*, *SALL4*, *ATP9A*, *NFATC2*, *KCNIG1*, *MOCS3*, *DPM1*, *ADNP*, and *BCAS4* (not *TSHZ2* as predicted by qPCR) (Fig. 2, Table 2). In case 4, the deletion was found to include only the genes *TSHZ2*, *ZFP64*, *SALL4*, and *ATP9A* (Fig. 2, Table 2).

TABLE 1. Summary of Clinical Features

Case	Mutation	Okhiro syndrome feature	Atypical features
1	Del 2.16 - 2.17 Mb	Duane anomaly, hypoplastic thumbs, hearing loss, choanal atresia, dysplastic ears, posterior urethral valves, hypospadias, flat feet.	infantile larynx, downslanting palpebral fissures, developmental delay in early childhood but average non-verbal intelligence at age 9.5 years
2	Del 2.01 - 2.05 Mb	Radial aplasia, bilateral thumb hypoplasia, right radioulnar synostosis, short stature. atrial septal defect, strabismus, hypoplastic kidneys, vesicoureteral reflux, severe gastroesophageal reflux.	telecanthus, downslanting palpebral fissures, mild ectropion of the lateral lower eyelids, bifid nasal tip, severe developmental delay
3	Del 1.3 - 2.8 Mb	Bilateral radial aplasia with absent thumbs, incomplete atrioventricular canal, right renal agenesis, bilateral microtia with absent auditory canals. Normal ocular exam.	attended special education classes, developmental delay not fully explained by the hearing deficit
4	Del 1.76 - 1.78 Mb	Bilateral triphalangeal thumbs, hearing loss, choanal atresia.	bronchomalacia, infantile larynx, developmental delay, muscular hypotonia, autistic tendencies

Summary of phenotypic features in the cases reported here. See case reports for further details. Bil.: bilateral, L: left, R: right.

DISCUSSION

Here we present the first clinical and molecular characterization of four microdeletions, which include *SALL4* and several neighboring genes. Of the 10 genes which are heterozygously deleted in at least one of the cases, *SALL4* is the only for which an associated phenotype is known. None of the deletions shares a breakpoint with another, but case 1 and 4 have in common that the telomeric breakpoint lies within the *TSHZ2* gene.

Okhiro syndrome features. In all cases, radial ray defects are present, ranging from triphalangeal thumbs to bilateral radial and thumb aplasia. Three out of four cases have hearing loss, One has bilateral Duane anomaly and another strabismus. Two have renal malformations, one urogenital malformations, and two were born with a heart defect. Short stature is present in one case and choanal atresia in two. Although these features are all known to be associated with *SALL4* mutations, the rates for choanal atresia and hearing loss appear especially high. However, with the small number of cases, this might just be due to chance. Nevertheless, the presence of choanal atresia together with ear defects and developmental delay points to an overlap between CHARGE and Okhiro syndromes. Since limb malformations do occur in CHARGE syndrome (Brock et al., 2003), contiguous gene deletions including *SALL4* should be considered in cases diagnosed with CHARGE syndrome but without *CHD7* mutation.

TABLE 2. Genes Included in the Microdeletions on Chromosome 20q13.13-q13.2 (From Telomer to Centromer)

Gene	Case 1	Case 2	Case 3	Case 4
<i>DOK5</i> ; docking protein 5	not deleted	not deleted	not deleted	not deleted
<i>PFDN4</i> ; prefoldin subunit 4	not deleted	not deleted	possible	not deleted
<i>CYP24A1</i> ; cytochrome P450, family 24, subfamily A, polypeptide 1	not deleted	not deleted	possible	not deleted
<i>BCAS1</i> ; breast carcinoma amplified sequence 1	not deleted	not deleted	possible	not deleted
<i>ZNF217</i> ; zinc finger protein 217	not deleted	not deleted	possible	not deleted
<i>TSHZ2</i> ; teashirt family zinc finger 2	deleted	not deleted	deleted	deleted
<i>ZFP64</i> ; zinc finger protein 64 homolog (mouse)	deleted	deleted	deleted	deleted
<i>SALL4</i>	deleted	deleted	deleted	deleted
<i>ATP9A</i> ; ATPase, Class II, type 9A	deleted	deleted	deleted	deleted
<i>NFATC2</i> ; nuclear factor of activated T-cells 2	deleted	deleted	not deleted	not deleted
<i>KCNKG1</i> ; potassium voltage-gated channel, subfamily G	deleted	deleted	not deleted	not deleted
<i>MOCS3</i> ; molybdenum cofactor synthase 3	not deleted	deleted	not deleted	not deleted
<i>DPM1</i> ; dolichyl-phosphate mannosyltransferase polypeptide 1, catalytic subunit	not deleted	deleted	not deleted	not deleted
<i>ADNP</i> ; activity-dependent neuroprotector	not deleted	deleted	not deleted	not deleted
<i>BCAS4</i> ; breast carcinoma amplified sequence 4	not deleted	deleted	not deleted	not deleted
<i>PAR6B</i> ; par-6 partitioning defective 6 homolog beta (<i>C. elegans</i>)	not deleted	not deleted	not deleted	not deleted
Severity of retardation/ developmental delay	mild	severe	mild	unclear

Physical features not reported with *SALL4* defects. Of the features reported herein, some have not yet been observed in cases with *SALL4* mutations and are therefore more likely to be associated with haploinsufficiency of one or more of the genes included in the deletion. Among these features, the most obvious is the developmental delay. None of the known cases with *SALL4* mutations has been reported to have any degree of developmental delay. Among the cases reported here, all have or had developmental delay. The observation that larger deletions are associated with a much higher risk for developmental delay resembles the situation in Saethre-Chotzen syndrome, where larger deletions including the *TWIST* gene confer a much higher risk for developmental delay than intragenic point mutations (Cai et al., 2003).

The degree of the delay was clearly mild in cases 1 and 3, while case 2 is the most severely affected. Case 4 is too young for a final statement, but her development appears similar to that of case 1. Since all deletions extending further towards the telomeres are associated with a milder degree of developmental delay but not with mental retardation, it seems likely that the heterozygous deletion of one or more of the genes *MOCS3*, *DPM1*, *ADNP*, and *BCAS4* is responsible for the mental retardation in case 2 (although we cannot exclude that larger mechanisms and not just deletion one or more of these additional genes is responsible for the developmental delay).

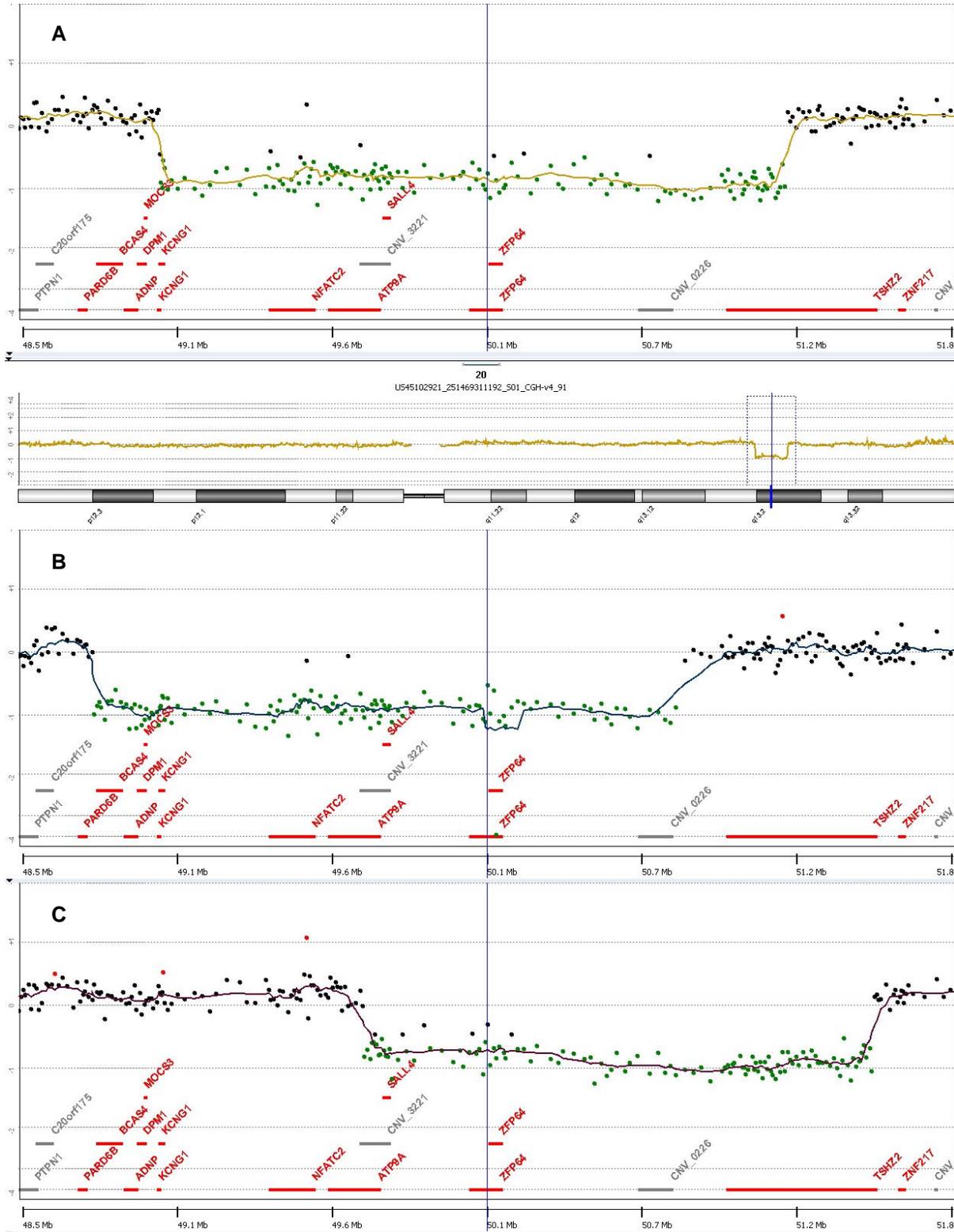


Figure 2. Array CGH results (CGHAnalytics 3.4 software, Agilent Technologies) of case 1 (A) case 2 (B), and case 4 (C). Genes included in and bordering the deletion are depicted in red. Note that all deletions include *SALL4*, *ATP9A*, and *ZFP64*. Case 2 is severely mentally retarded, possibly due to the deletion of the genes *ADNP*, *BCAS4*, *MOCS3*, and *DPM1*, which are not deleted in the other cases with developmental delay but not mental retardation. See discussion for details.

Mutations in *MOCS3* have not been described before, but *MOCS1* and *MOCS2* mutations result in Molybdenum cofactor deficiency (MIM# 252150), a severe autosomal-recessive disorder characterized by severe and progressive neurological damages, including brain abnormalities, mental retardation and intractable seizures (Reiss and Johnson, 2003). The phenotype presented in Molybdenum cofactor deficiency is usually more severe than that exhibited by case 2, involves seizures and is progressive, but rarely cases with mild presentation have been described (Johnson et al., 2001). However, if molybdenum cofactor deficiency would be associated with *MOCS3* mutations at all, it would be predicted to result only from homozygous inactivation of the *MOCS3* gene. This would require another mutation affecting the second allele in case 2.

Mutations in *DPM1* are causative for the congenital disorder of glycosylation, type Ie (CDG-Ie) (Kim et al., 2000). CDG-Ie is an autosomal recessive disorder with a very broad range of clinical presentations. Most frequently cases are characterized by developmental delay and severe neurologic features, among which seizures and acquired microcephaly are prominent. The cases often exhibit dysmorphic features. To exclude the possibility of a CDG, serum transferrin (Tf) isoelectric focusing (IEF) analysis was normal in case 2, confirming that the second *DPM1* allele was intact. This excluded homozygous inactivation of *DPM1* as a cause of his phenotype.

ADNP is a protein discovered in murine neuroglial cells (Bassan et al., 1999). It contains a unique neuroprotective peptide sequence (NAPVSIPQ), which binds to tubulin facilitating the microtubule assembly and leading to enhanced cellular survival, associated with fundamental cytoskeletal elements. NAPVSIPQ (NAP) shares structural and immunological homology with the previously reported, activity-dependent neurotrophic factor (ADNF) (Bassan et al., 1999), but acts more efficacious than peptides derived from ADNF. The protein has nine zinc fingers, a proline-rich region, a nuclear bipartite localization signal, and a homeobox domain profile, all suggesting transcription factor function. The human *ADNP* gene expression is enriched in cerebellum, cortex, hippocampus, medulla, midbrain, and in cerebral cortical astrocytes *ADNP* expression is upregulated by the vasoactive intestinal peptide (VIP) (Bassan et al., 1999; Gozes et al., 1999). VIP is another neuropeptide, which protects electrically blocked neurons from death, prevents beta amyloid toxicity, and blocks the envelope protein of the human immunodeficiency virus (Gozes et al., 2003). These same functions were also proven for ADNP (Gozes et al., 2003), which was also shown to play a crucial role in proper neurodevelopment in mice (Pinhasov et al., 2003). Homozygous *ADNP* knockout embryos revealed cranial neural tube defects and died at E8.5-9.0. Interestingly, in other studies, daily NAP injections into newborn ApoE-deficient mice (a breed prone to brain damage during hypoxic insult) accelerated the acquisition of developmental reflexes and prevented short-term memory deficits (Rotstein et al., 2006). Moreover, NAP-treated mice with closed head injuries as well as rat models of stroke presented reduced mortality and more efficient neurobehavioral and brain-tissue recovery (Gozes et al., 2003). To date, no disease phenotype has been linked to mutations in the *ADNP* gene. However, concerning the functions of the gene and the phenotypes presented in animal models, it is tempting to hypothesize that an ADNP dosage effect could have a major contribution to the mental retardation of case 2. This scenario would be also supported by the observation of delayed neural tube closure in the heterozygous *ADNP* knockout embryos as compared to wild type animals, and by the observation that ADNP enhances the expression of genes associated with organogenesis/neurogenesis (Mandel et al., 2006).

BCAS4 together with *BCAS3* represent two genes with no homology to any other known gene or protein. Both genes undergo amplification, overexpression, and fusion in the MCF7 breast cancer cell line (Barlund et al., 2002). The *BCAS4* gene is overexpressed in nine additional breast cancer cell lines. No other *BCAS4* function or activity is known to date. Based on the reports, it is very unlikely that loss of one copy of the *BCAS4* gene would contribute to the phenotype in case 2.

Strategy for deletion detection. All deletions reported here were initially identified by quantitative Real-Time PCR. Although qPCR is a reliable method for this purpose as reported previously by us, it proved to be laborious to achieve fine mapping of these extended deletions by walking along the chromosome. The oligonucleotide array CGH platform with the 244k array confirmed the deletions detected by qPCR and helped to identify the exact extent of the deletions and the exact number of genes affected within a relatively short time.

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REFERENCES

- Aalfs CM, van Schooneveld MJ, van Keulen EM, Hennekam RCM. 1996. Further delineation of the acro-renal-ocular syndrome. *Am J Med Genet* 62:276-281.
- Al-Baradie R, Yamada K, St Hilaire C, Chan WM, Andrews C, McIntosh N, Nakano M, Martonyi EJ, Raymond WR, Okumura S, Okihiro MM, Engle EC. 2002. Duane Radial Ray Syndrome (Okihiro syndrome) maps to 20q13 and results from mutations in SALL4, a new member of the SAL family. *Am J Hum Genet* 71:1195-1199.
- Barlund M, Monni O, Weaver JD, Kauraniemi P, Sauter G, Heiskanen M, Kallioniemi OP, Kallioniemi A. 2002. Cloning of BCAS3 (17q23) and BCAS4 (20q13) genes that undergo amplification, overexpression, and fusion in breast cancer. *Genes Chromosomes Cancer* 35:311-317.
- Barrett MT, Scheffer A, Ben-Dor A, Sampas N, Lipson D, Kincaid R, Tsang P, Curry B, Baird K, Meltzer PS, Yakhini Z, Bruhn L, Laderman S. 2004. Comparative genomic hybridization using oligonucleotide microarrays and total genomic DNA. *Proc Natl Acad Sci U S A* 101:17765-17770.
- Bassan M, Zamostiano R, Davidson A, Pinhasov A, Giladi E, Perl O, Bassan H, Blat C, Gibney G, Glazner G, Brenneman DE, Gozes I. 1999. Complete sequence of a novel protein containing a femtomolar-activity-dependent neuroprotective peptide. *J Neurochem* 72:1283-1293.
- Becker K, Beales PL, Calver DM, Matthijs G, Mohammed SN. 2002. Okihiro syndrome and acro-renal-ocular syndrome: clinical overlap, expansion of the phenotype, and absence of PAX2 mutations in two new families. *J Med Genet* 39:68-71.
- Borozdin W, Boehm D, Leipoldt M, Wilhelm C, Reardon W, Clayton-Smith J, Becker K, Mühlendyck H, Winter R, Giray Ö, Silan F, Kohlhase J. 2004a. SALL4 deletions are a common cause of Okihiro and acro-renal-ocular syndromes and confirm haploinsufficiency as the pathogenetic mechanism. *J Med Genet* 41:e113.
- Borozdin W, Wright M, Hennekam R, Hannibal M, Crow Y, Neumann T, Kohlhase J. 2004b. Novel mutations in the gene SALL4 provide further evidence for Acro-Renal-Ocular and Okihiro syndromes being allelic entities, and extend the phenotypic spectrum. *J Med Genet* 41:e102.
- Brock KE, Mathiason MA, Rooney BL, Williams MS. 2003. Quantitative analysis of limb anomalies in CHARGE syndrome: correlation with diagnosis and characteristic CHARGE anomalies. *Am J Med Genet A* 123:111-121.
- Cai J, Goodman BK, Patel AS, Mulliken JB, Van Maldergem L, Hoganson GE, Paznekas WA, Ben-Neriah Z, Sheffer R, Cunningham ML, Daentl DL, Jabs EW. 2003. Increased risk for developmental delay in Saethre-Chatzen syndrome is associated with TWIST deletions: an improved strategy for TWIST mutation screening. *Hum Genet* 114:68-76.
- Chun BB, Mazzoli RA, Raymond WR. 2001. Characteristics of Okihiro syndrome. *J Pediatr Ophthalmol Strabismus* 38:235-239.
- Collins A, Baraitser M, Pembrey M. 1993. Okihiro syndrome: thenar hypoplasia and Duane anomaly in three generations. *Clin Dysmorphol* 2:237-240.
- Ferrell RL, Jones B, Lucas RV, Jr. 1966. Simultaneous occurrence of the Holt-Oram and the Duane syndromes. *J Pediatr* 69:630-634.
- Gozes I, Bassan M, Zamostiano R, Pinhasov A, Davidson A, Giladi E, Perl O, Glazner GW, Brenneman DE. 1999. A novel signaling molecule for neuropeptide action: activity-dependent neuroprotective protein. *Ann N Y Acad Sci* 897:125-135.
- Gozes I, Divinsky I, Pilzer I, Fridkin M, Brenneman DE, Spier AD. 2003. From vasoactive intestinal peptide (VIP) through activity-dependent neuroprotective protein (ADNP) to NAP: a view of neuroprotection and cell division. *J Mol Neurosci* 20:315-322.
- Halal F, Homsy M, Perreault G. 1984. Acro-renal-ocular syndrome: autosomal dominant thumb hypoplasia, renal ectopia, and eye defect. *Am J Med Genet* 17:753-762.
- Hayes A, Costa T, Polomeno RC. 1985. The Okihiro syndrome of Duane anomaly, radial ray abnormalities, and deafness. *Am J Med Genet* 22:273-280.

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- Johnson JL, Coyne KE, Rajagopalan KV, Van Hove JL, Mackay M, Pitt J, Boneh A. 2001. Molybdopterin synthase mutations in a mild case of molybdenum cofactor deficiency. *Am J Med Genet* 104:169-173.
- Kim S, Westphal V, Srikrishna G, Mehta DP, Peterson S, Filiano J, Karnes PS, Patterson MC, Freeze HH. 2000. Dolichol phosphate mannose synthase (DPM1) mutations define congenital disorder of glycosylation Ie (CDG-Ie). *J Clin Invest* 105:191-198.
- Kohlhase J. 2004. SALL4-related disorders. In: University of Washington S, editor. GeneReviews at GeneTests.
- Kohlhase J, Chitayat D, Kotzot D, Ceylaner S, Froster UG, Fuchs S, Montgomery T, Rosler B. 2005. SALL4 mutations in Okhiro syndrome (Duane-radial ray syndrome), acro-renal-ocular syndrome, and related disorders. *Hum Mutat* 26:176-183.
- Kohlhase J, Heinrich M, Schubert L, Liebers M, Kispert A, Laccione F, Turnpenny P, Winter RM, Reardon W. 2002. Okhiro syndrome is caused by SALL4 mutations. *Hum Mol Genet* 11:2979-2987.
- Kohlhase J, Schubert L, Liebers M, Rauch A, Becker K, Mohammed SN, Newbury-Ecob R, Reardon W. 2003. Mutations at the SALL4 locus on chromosome 20 result in a range of clinically overlapping phenotypes, including Okhiro syndrome, Holt-Oram syndrome, acro-renal-ocular syndrome, and patients previously reported to represent thalidomide embryopathy. *J Med Genet* 40:473-478.
- MacDermot K, Winter R. 1987. Radial ray defect and duane anomaly: report of a family with autosomal dominant transmission. *Am J Med Genet* 27:313-319.
- Mandel S, Rechavi G, Gozes I. 2006. Activity-dependent neuroprotective protein (ADNP) differentially interacts with chromatin to regulate genes essential for embryogenesis. *Dev Biol* epub ahead of print.
- Miertus J, Borozdin W, Frecer V, Tonini G, Bertok S, Amoroso A, Miertus S, Kohlhase J. 2006. A SALL4 zinc finger missense mutation predicted to result in increased DNA binding affinity is associated with cranial midline defects and mild features of Okhiro syndrome. *Hum Genet* 119:154-161.
- Okhiro MM, Tasaki T, Nakano KK, Bennett BK. 1977. Duane syndrome and congenital upper-limb anomalies. A familial occurrence. *Arch Neurol* 34:174-179.
- Pinhasov A, Mandel S, Torchinsky A, Giladi E, Pittel Z, Goldsweig AM, Servoss SJ, Brenneman DE, Gozes I. 2003. Activity-dependent neuroprotective protein: a novel gene essential for brain formation. *Brain Res Dev Brain Res* 144:83-90.
- Reiss J, Johnson JL. 2003. Mutations in the molybdenum cofactor biosynthetic genes MOCS1, MOCS2, and GEPH. *Hum Mutat* 21:569-576.
- Rotstein M, Bassan H, Kariv N, Speiser Z, Harel S, Gozes I. 2006. NAP enhances neurodevelopment of newborn apolipoprotein E-deficient mice subjected to hypoxia. *J Pharmacol Exp Ther* 319:332-339.
- Spitz R, Oberthuer A, Zapatka M, Brors B, Hero B, Ernestus K, Oestreich J, Fischer M, Simon T, Berthold F. 2006. Oligonucleotide array-based comparative genomic hybridization (aCGH) of 90 neuroblastomas reveals aberration patterns closely associated with relapse pattern and outcome. *Genes Chromosomes Cancer* 45:1130-1142.
- Temtamy SA, Shoukry AS, Ghaly I, El-Meligy R, Boulos SY. 1975. The Duane radial dysplasia syndrome: an autosomal dominant disorder. *Birth Defects Orig Artic Ser* XI:344-345.
- Terhal P, Rösler B, Kohlhase J. 2006. A family with features overlapping Okhiro syndrome, hemifacial microsomia and isolated Duane anomaly caused by a novel SALL4 mutation. *Am J Med Genet* 140A:222-226.