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Clinical and Genetic Findings in Mexican Patients with Duane Anomaly and Radial Ray Malformations/Okihiro Syndrome

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ABSTRACT

Background: Okihiro syndrome is an autosomal-dominant condition characterized by radial ray malformations associated with Duane anomaly and other clinical characteristics. *SALL4* mutations have been identified in 80-90% of patients with Duane-Radial ray defects/Okihiro syndrome. We report the clinical findings and results of *SALL4* sequencing from a group of Mexican patients with this disorder. **Objective:** Clinical description and identification of *SALL4* mutations in Mexican subjects with radial defects and Duane anomaly. **Materials and methods:** Five unrelated index cases were studied. Complete ophthalmologic and general physical examination was performed in all patients. Polymerase chain reaction amplification and automated nucleotide sequencing of coding exons and intron-exon junctions of *SALL4* gene were carried out in genomic DNA. **Results:** A novel heterozygous deletion was identified in one patient. Intragenic heterozygous single nucleotide polymorphisms on *SALL4* gene ruled out deletions of some exons in other affected patients in whom non-pathogenic variants were identified by Sanger sequencing. Likewise, multiplex ligation-dependent probe amplification analysis ruled out large deletions in this gene. **Conclusion:** We observed a low frequency of *SALL4* mutations in Mexican patients with clinical criteria of Okihiro syndrome. (REV INVES CLIN. 2016;68:269-74)

Key words: Duane anomaly. DRS. Okihiro syndrome. Radial ray defects. *SALL4* disorder. *SALL4* gene. Strabismus.

INTRODUCTION

Duane retraction syndrome (DRS) is a rare congenital eye movement disorder, accounting for around 1-5% of all strabismus cases. This anomaly occurs almost always sporadically and is defined by the combination of limited abduction, variable limited adduction, and globe retraction with palpebral fissure narrowing in attempted adduction[1]. Numerous associated anomalies have been described in patients with DRS, including dysmorphic ears and hearing dysfunction, vertebral defects, and variable degrees of

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limb malformations[2,3]. Duane-radial ray syndrome/Okihiro syndrome is a syndromic form of DRS, predominantly defined by the presence of radial ray malformation and other clinical findings such as fused cervical vertebrae, spina bifida, renal and gastrointestinal anomalies, heart atrial or ventricular septal defects, and facial asymmetry[2-6]. Duane retraction syndrome/Okihiro syndrome has an autosomal-dominant inheritance with a highly variable clinical presentation and reduced penetrance. The disease has an unknown frequency and is caused by mutations in the *SALL4* gene on human chromosome 20q13.13-q13.2[7,8]. Gene *SALL4* encodes a transcription factor playing critical roles during the embryonic development of abducens motoneurons[8]. The *SALL4* mutations can be demonstrated in approximately 80-95% of patients with *SALL4*-related disorders: Okihiro syndrome and the allelic acro-renal-ocular syndrome. In this study, we reported a low frequency (1/5 patients) of *SALL4* mutations in Mexican patients with clinical criteria for Okihiro syndrome. The ophthalmologic and systemic findings of five index cases with this syndrome are described and a novel *SALL4* heterozygous mutation is reported.

MATERIALS AND METHODS

Subjects and diagnostic criteria

This study was approved by the Institutional Review Board of the Institute of Ophthalmology "Conde de Valenciana". Informed consent was obtained from each subject/parents. The defining characteristics of Okihiro syndrome in our study were Duane retraction anomaly and radial ray malformations. All subjects underwent a complete ophthalmologic/strabologic examination and a general physical assessment.

Polymerase chain reaction amplification and Sanger sequencing analysis of *SALL4* gene

Genomic DNA was extracted from venous blood leukocytes using the QuickGene system (Fujifilm, Tokyo, Japan). The complete *SALL4* coding region (four exons) and adjacent intronic boundaries were amplified by polymerase chain reaction (PCR) using pairs of primers derived from the normal gene sequence. Each 25 µl PCR amplification reaction contained 1 × buffer, 100 ng of genomic DNA, 0.2 mM of each dNTP, 2 U taq polymerase, 1 mM of forward and reverse primers, and 1.5 mM MgCl2. The PCR products were analyzed in 1.5% agarose gels from the bands with the amplified templates, were excised, and the DNA was subsequently purified with the QIAEX® II kit (Qiagen, Hilden, Germany). Direct automated sequencing of all exons was performed with the BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). All samples were analyzed on an ABI PRISM® 3130 Genetic Analyzer (Applied Biosystems). Wild-type (ENST 00000217086) and mutated *SALL4* sequences were compared manually. The novel *SALL4* mutation was investigated in the 1000 Genomes Project (www.1000genomes.org), Exome variant Server (www.evs.gs.washington.edu), and Exome Aggregation Consortium (ExAC) (www.exac.broadinstitute.org) databases. A total of 100 unrelated DNAs from Mexican Mestizo individuals without strabismus and/or radial ray defects were included as a mutational control group.

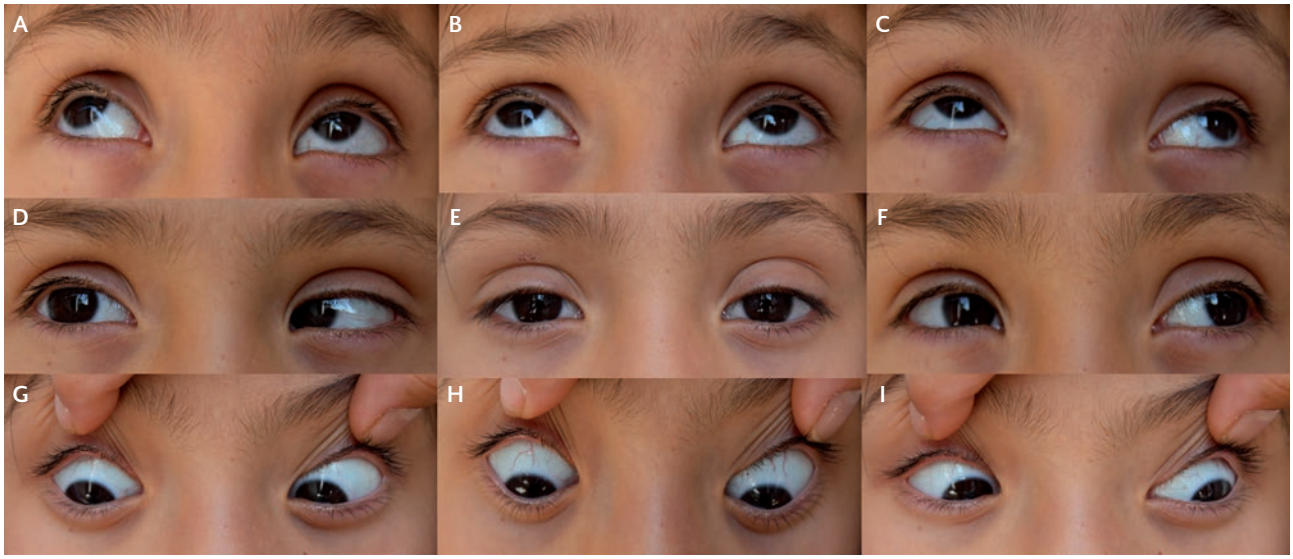
Multiplex ligation-dependent probe amplification analysis of *SALL4* gene

Multiplex ligation-dependent probe amplification (MLPA) was used to exclude gene deletions or duplications. The commercially available SALSA® MLPA® P180-B3 Limb-2/Heart probe mix (MRC-Holland, Amsterdam, Netherlands) contains four probes targeting *SALL4* gene exons 1, 2, 3, and 4.

The DNA samples were diluted to 50 ng/µl in deionized water, re-purified by 3M sodium acetate precipitation in 100% ethanol, washed in 70% ethanol, and rehydrated in Tris-EDTA pH 8.5 buffer to 20 ng/µl. The MLPA® assay was then completed according to the standard protocol supplied by MRC-Holland (MLPA® DNA Protocol version MDP-005; http://www.mrc-holland.com). The MLPA products (1 µl) were mixed with 13 µl of HiDi® formamide and 0.6 µl of GeneScan™ 600 LIZ® Size Standard (Applied Biosystems®, Foster City, USA). The fragment separation was performed on capillary electrophoresis system 3500 Genetic Analyzer (Applied Biosystems®) using POP7® polymer under the following conditions: run time 1,330 seconds at 19.5 kV, injection time 8 seconds at 1.6 kV, run current 6 µA and 60 °C run temperature.

The raw data analysis and the copy number ratio determination were performed using the Coffalyser. NET v.140721.1958 free software (MRC-Holland).

Figure 1. Eye movement examination in nine cardinal gaze positions in patient #1. **A.** Upshot at oblique right superior position. **B.** Upgaze divergence. **C.** Upshot evidenced at oblique left superior position. **D.** Moderate abduction limitation of right eye with narrowing of palpebral fissure on adduction with mild globe retraction of left eye. **E.** Both eyes are straight at primary position. **F.** Mild abduction limitation of left eye with narrowing of palpebral fissure on adduction with marked globe retraction in the right eye. **G, H, I.** Normal downgaze positions.



Nine reference probes included in the probe mix were used for intra-normalization. Three DNA samples of healthy individuals were included as reference for inter-normalization.

RESULTS

Five unrelated index cases born from healthy and non-consanguineous parents were studied. Their family histories were uneventful, and mothers of probands received regular prenatal care. Exposure to teratogenic agents was denied in all cases.

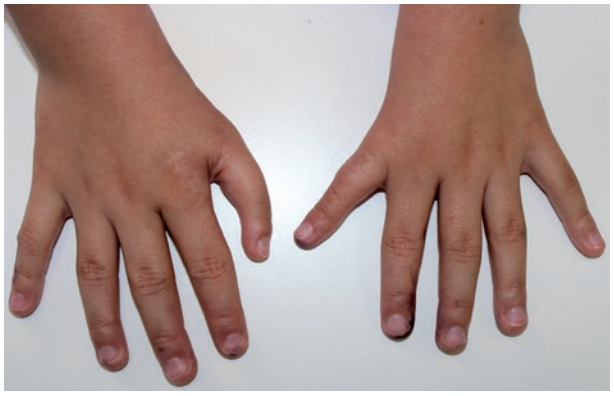
Patient #1 (mutation positive)

An 11-year-old female was referred to the Genetics Department due to esotropia and malformations in both hands. She was born by natural childbirth and her birth weight was 3,700 g; no other birth measurements were documented. Esotropia was noted since birth, although it appeared to decrease over time. Ophthalmic examinations by slit-lamp biomicroscopy and by fundoscopy were unremarkable in both eyes. Visual acuity measurement using Snellen chart revealed 20/20 for both eyes, while refraction was +0.25 +0.25 × 90º in both eyes. Stereopsis test measured by random-dot test revealed 20 seconds of arch. Eye movement examination disclosed a moderate abduction limitation of right eye and mild abduction limitation of left eye. Narrowing of palpebral fissure on adduction with marked globe retraction in right eye and mild in left eye were noted. An upshoot was found in superior oblique right and left positions during examination, and divergence was seen in upper gaze, supporting a bilateral Duane syndrome diagnosis (Fig. 1). Systemic examination disclosed hypertelorism, bilateral epicanthal folds, and midfacial hypoplasia (Table 1). Bilateral triphalangeal thumb or digitalized thumb was also found in the affected hands. Her right hand had a previous surgery (Fig. 2). Nucleotide analysis in this patient disclosed a novel heterozygous mutation c.1427delC in *SALL4* exon 2 (Table 2; Fig. 3). This one-base deletion originated a frame shifting and caused a premature stop signal (TAA) 4 codons downstream (p.P476L*4). Three synonymous changes including c.540T>C (p.N179=, homozygous), c.1056G>A (p.A351=, heterozygous), and c.2640G>C (p.S879=, heterozygous), were also identified in DNA from this subject. All *SALL4* nucleotide variants identified in this study are summarized in table 2.

Patients #2 to #5 (mutation negative)

Patient #2 is a one-year-old girl who had facial asymmetry, Duane syndrome, right thenar hypoplasia, and

Figure 2. Digitalized thumb in both hands (patient #1).



absence of left radius. Two nonpathogenic nucleotide variants, c.1520T>G (p.L507R) and c.1860A>G (p.T620=) were identified in heterozygous state in DNA from this subject. Patient #3 is an 18-year-old male who presented facial asymmetry, congenital strabismus (Duane syndrome), short neck, low posterior hair line, cervicothoracic kyphosis, ventricular septal defect, hypoplastic right humeral head, absence of right radius, absent thumbs, left radioulnar synostosis, syndactyly, and horseshoe kidney. Two heterozygous synonymous variants were found in DNA from this patient: c.1056G>A (p.A351=), and c.2037C>T (p.T679=). Patient #4 is a 17-year-old male referred due to

Table 1. Clinical findings in patients with Okihiro syndrome

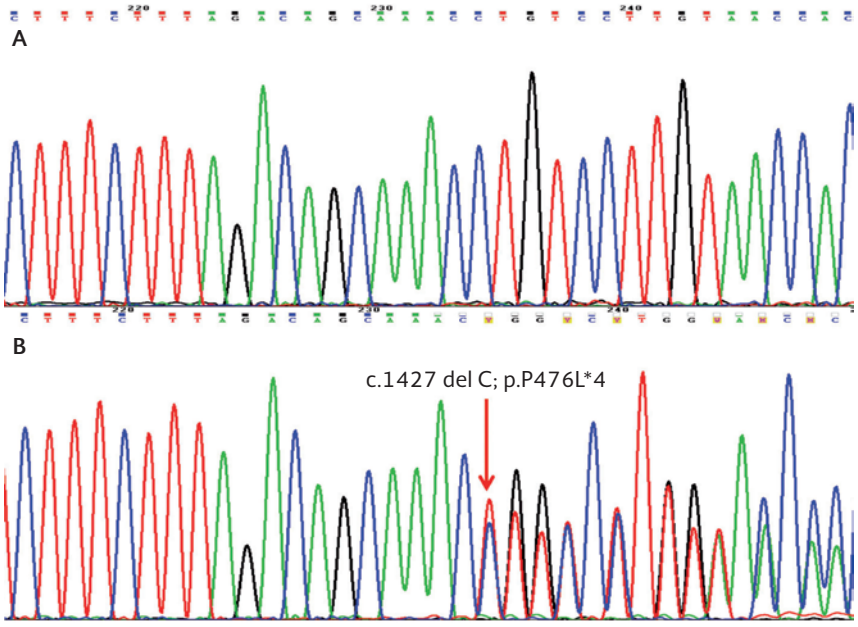
Finding	Patient #1	Patient #2	Patient #3	Patient #4	Patient #5
Duane anomaly	+	+	+	+	+
Radial ray anomaly	+	+	+	+	+
Facial asymmetry	+	+	+	+	–
Hypertelorism	+	–	–	–	–
Epicanthal folds	+	–	–	–	–
Ptosis	–	–	–	+	–
Midfacial hypoplasia	+	–	–	–	–
Microtia	–	–		+	–
Dental anomalies	–	–	–	+	–
Short neck	–	–	+	–	–
Congenital heart defects	–	–	+	–	–
Renal anomalies	–	–	+	+	–
Spine anomalies	–	–	+	+	–
Other limb anomalies	–	–	+	–	–
Syndactyly	–	–	+	+	–
Intellectual disability	–	–	–	+	–

+: present; –: not present.

Table 2. Nucleotide variants identified in this study

Patient #	Pathogenic variant	Non-pathogenic variants (Genotype) (Exon)
1	c.1427delC; p.P476L*4	c.540T>C; p.(N179=) (homozygous) (E2) c.1056G>A; p.(A351=) (heterozygous) (E2) c.2640G>C; p.(S879=) (heterozygous) (E3)
2		c.1520T>G; p.L507R (heterozygous) (E2) c.1860A>G; p.(T620=) (heterozygous) (E2)
3		c.1056G>A; p.(A351=) (heterozygous) (E2) c.2037C>T; p.T679=) (heterozygous) (E2)
4		c.540T>C; p.(N179=) (homozygous) (E2) c.1520T>G; p.L507R (heterozygous) (E2) c.1860A>G; p.(T620=) (heterozygous) (E2)
5		c.540T>C; p.(N179=) (homozygous) (E2) c.1056G>A; p.(A351=) (heterozygous) (E2) c. 2640G>C; p.(S880=) (heterozygous) (E3)

Figure 3. Partial DNA sequence in *SALL4* exon 2 from control father (A) and patient #1 carrying a heterozygous c.1427delC (p.P476L*4) mutation (B). The arrow indicates the frameshift mutation.



intellectual disability and dysmorphic features including facial asymmetry, right Duane anomaly, right palpebral ptosis, right microtia, absence of mandibular incisors, thoracic scoliosis, telethelia, segmentation defect of the cervical vertebra (C5-C7), left kidney agenesis, hypoplastic right radius, triphalangeal thumb in right hand, syndactyly in left hand, and sandal gap between first and second toes bilaterally. Three nonpathogenic variants were identified in DNA from this patient: c.540T>C (p.N179=, homozygous), c.1520T>G (p.L507R, heterozygous), and c.1860A>G (p.T620=, heterozygous). Patient #5 is a six-year-old female who was referred due to Duane syndrome and bilateral radial ray defects (radioulnar synostosis, first metacarpal hypoplasia, thumb hypoplasia, and thenar hypoplasia). One homozygous c.540T>C (p.N179=) and two heterozygous c.1056G>A (p.A351=) and c.2640G>C (p.S880=) nonpathogenic variants were identified in DNA from this patient.

The MLPA analysis did not detect the presence of deletions or duplications in *SALL4* gene in any of the patients (patients #2 to 5).

DISCUSSION

SALL4-related disorders comprise a spectrum of phenotypes previously recognized as distinct entities including Duane-radial ray syndrome or Okihiro syndrome, acro-renal-ocular syndrome, and rarely, Holt-Oram syndrome[6]. In this study, we have selected as key inclusion criteria Duane anomaly and ray radial malformation due to the fact that these two features are present in up to 45% of all the subjects carrying *SALL4* mutations[9]. Similarly, previous studies have shown a *SALL4* mutational frequency of 90% in patients associating Duane syndrome and radial ray defects, which sharply contrasts with a 20% mutation frequency (1/5 cases) in our group of Mexican patients with these anomalies[9].

Okihiro syndrome presents high phenotypic variability and anomalies of the eyes (microphthalmia, cataracts, optic disc hypoplasia and dysplasia, retinal coloboma, epicanthal folds, and mild hypertelorism)[10,11], upper extremities (hypoplastic humerus and ulnae, syndactyly, hypoplastic deltoid muscle)[11,12], kidney (renal agenesis, and dystopian or ectopic kidney)[10,12], ears (sensorineural and/or conductive deafness, abnormal pinnae, microtia)[4,12], and heart (atrial or ventricular septal defects, tetralogy of Fallot)[6] have been reported. Patients #2 to #4 presented facial asymmetry, and one of them (patient #4) had also microtia, fused vertebrae, and renal agenesis, features that may resemble oculo-auriculo-vertebral spectrum. Terhal, et al. described a

family with similar characteristics and carrying a *SALL4* stop mutation, raising the possibility that some patients with hemifacial microsomia might have pathogenic *SALL4* mutations[13]. Patient #3 had a history of congenital strabismus, congenital heart defect, and renal malformation (horseshoe kidney), a phenotype that is similar to the Holt-Oram syndrome. However, the Holt-Oram syndrome does not include eye anomalies[14]. Thus, all our patients had phenotypic features compatible with the Okihiro syndrome, and although a *SALL4* mutation was identified only in one patient, other molecular mechanisms, such as whole or partial deletion of the *SALL4* gene, could explain the low mutation frequency. Several patients and families with SALL4-related phenotypes have no identifiable *SALL4* mutations by direct sequencing[7], but subsequent deletion/duplication analysis demonstrated causal heterozygous deletions of the whole gene, of exons 1-3, of exon 1, or of exon 4[15]. In our patients, some identified variants (single nucleotide polymorphisms) were used to exclude heterozygous deletions of some exons (Table 2). Thus, two heterozygous variants, c.1520T>G and c.1860A>G, in exon 2 in patients #2 and #4 excluded deletion of this exon. Similarly, three heterozygous variants, c.1056T>G, c.2037C>T, and c.2640G>C, ruled out deletion of *SALL4* exon 2 in DNA from patients #3 and #5. Finally, a heterozygous c.2640G>C nucleotide change in patient #5 excluded deletion of *SALL4* exon 3 (Table 2). Mental retardation, which was observed in patient #4, is a feature described in patients carrying large deletions in the *SALL4* gene[16]. Nevertheless, MLPA analysis of *SALL4* gene completely ruled out any deletion or duplications in this gene in patients #2 to #5.

SALL4 encodes sal-like protein 4, a zinc finger transcription factor essential for developmental regulation that cooperates with *SALL1* and *TBX5* in anorectal, heart, brain, and kidney development[17]. In our study, we reported a novel c.1427delC heterozygous one-base deletion, which predicts premature protein truncation that probably led to *SALL4* haploinsufficiency. To date, 48 *SALL4* mutations have been reported, most of which are non-sense mutations and gross deletions[18]. Locus heterogeneity or a misdiagnosis of the syndrome may be other possibilities when mutations in *SALL4* are not identified in individuals with a clinical diagnosis of Okihiro syndrome.

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