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Large Contiguous Gene Deletions in Sjögren-Larsson Syndrome

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Abstract

Sjögren-Larsson syndrome (SLS) is an autosomal recessive disorder characterized by ichthyosis, mental retardation, spasticity and mutations in the *ALDH3A2* gene for fatty aldehyde dehydrogenase, an enzyme that catalyzes the oxidation of fatty aldehyde to fatty acid. More than 70 mutations have been identified in SLS patients, including small deletions or insertions, missense mutations, splicing defects and complex nucleotide changes. We now describe 2 SLS patients whose disease is caused by large contiguous gene deletions of the *ALDH3A2* locus on 17p11.2. The deletions were defined using long distance inverse PCR and microarray-based comparative genomic hybridization. A 24-year-old SLS female was homozygous for a 352-kb deletion involving *ALDH3A2* and 4 contiguous genes including *ALDH3A1*, which codes for the major soluble protein in cornea. Although lacking corneal disease, she showed severe symptoms of SLS with uncommon deterioration in oral motor function and loss of ambulation. The other 19-month-old female patient was a compound heterozygote for a 1.44-Mb contiguous gene deletion and a missense mutation (c.407C>T, P136L) in *ALDH3A2*. These studies suggest that large gene deletions may account for up to 5% of the mutant alleles in SLS. Geneticists should consider the possibility of compound heterozygosity for large deletions in patients with SLS and other inborn errors of metabolism, which has implications for carrier testing and prenatal diagnosis.

Keywords

ALDH3A1; fatty aldehyde; ichthyosis; mutation; *SLC47A1*; *ULK2*

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INTRODUCTION

Sjögren-Larsson syndrome (SLS; OMIM 270200) is an autosomal recessive inborn error of metabolism caused by mutations in the *ALDH3A2* gene for fatty aldehyde dehydrogenase (FALDH) [1,2]. Clinical features of SLS include ichthyosis, spastic diplegia or tetraplegia, mental retardation, seizures and a distinctive retinal crystalline maculopathy characterized by perifoveal glistening white dots. The ichthyosis is usually congenital in onset and is often pruritic in nature. Neurological symptoms of mental retardation and spasticity typically develop by the 2nd year of life and present with delay in achieving motor and cognitive milestones. The symptoms of SLS vary from mild to profound, and are generally non-progressive.

FALDH catalyzes the oxidation of fatty aldehyde to fatty acid, and is a necessary component of the fatty alcohol:NAD⁺ oxidoreductase enzyme complex that catalyzes the sequential oxidation of fatty alcohol to fatty acid [3,4,5]. SLS patients consequently have elevated fatty alcohols in plasma, urine and cultured cells [6-9]. The symptoms of SLS are thought to arise directly or indirectly from accumulation of fatty aldehyde, fatty alcohol or related lipid products in the skin and brain [10].

The *ALDH3A2* gene is located on chromosome 17p11.2. More than 70 mutations have been discovered in SLS patients, including small deletions or insertions, missense mutations, splicing defects and complex mutations composed of deletion/insertions and nucleotide substitutions [11]. Intragenic deletions of one or more exons have also been rarely described [12-14]. Most mutations in SLS are private, and many patients have been found to be homozygous due to consanguinity or founder effects.

We now present 2 unique SLS patients in whom the disease was caused by unusually large deletions involving *ALDH3A2* and surrounding genes on chromosome 17p11.2.

MATERIALS AND METHODS

The Institutional Review Board at the University of Nebraska Medical Center approved this research, and all subjects consented to the study.

Patient Descriptions

Patient 1—This 24-year-old female was born at 32 weeks gestation to consanguineous first-cousin Pakistani parents. She was noted to have erythematous dry scaly skin at birth, but had no collodion membrane. She developed several seizures at 6 weeks of age, which were associated with hypocalcemia. Delays in achieving motor milestones and speech were noted in infancy. After 2 febrile illnesses at 6-7 months of age, she lost the ability to roll over and her limbs became very stiff. A brain CT at 9 months of age showed moderate cerebral atrophy. However, she began to sit unsupported and crawl at about 18 months. Physical examination at 3 years of age showed developmental delay, spastic diplegia and generalized ichthyosis. Speech consisted of occasionally saying “mama” and “baba”. Laboratory studies were normal, including karyotype, EEG, electroretinogram, thyroid function tests, plasma phytanic acid, urine amino acids and metabolic screen. Her ichthyosis responded well to etretinate therapy, which was subsequently switched to isotretinoin and then discontinued several years later because of concerns about retinoid toxicity. By 10 years of age, she was no longer speaking and began having difficulty swallowing. Physical exam showed a pruritic, generalized ichthyosis along with spastic diplegia, leg contractures and ankle clonus (Fig 1A). She had photophobia and avoided bright lights. She was able to ambulate only with a walker using a crouched gait. Brain MRI revealed bilateral, symmetrical abnormal T2-weighted signal involving the fronto-parietal and superior

periventricular regions. Several months later she developed increased drooling with tongue protrusion and dysphagia. Oral motor incoordination and loss of swallowing slowly progressed and necessitated placement of a gastrostomy tube at 12 years of age. Bilateral hamstring lengthening was also performed at this age. At 14 years, she was noted to have spastic tetraplegia and be non-ambulatory. At 17 years of age, ophthalmologic examination under sedation showed chronic blepharitis, but no cataracts, retinal or corneal abnormalities. The diagnosis of SLS was confirmed by demonstrating FALDH deficiency (8% of mean normal activity) in cultured fibroblasts.

Patient 2—This 19-month-old female infant was born at 36 weeks gestation to a 27-year-old mother and non-consanguineous father. Mother was of Irish and Cherokee Indian descent; father was English and American Indian. At birth, the infant was noted to have a collodion membrane with shiny, “tight” skin that transformed into dry, scaling skin after 2-3 weeks. Over time, she began scratching continually, often to the point of bleeding. At 10 months of age, a skin biopsy revealed hyperkeratosis, papillomatosis and acanthosis.

At 12 months of age, physical examination revealed height, weight, and head circumference within normal age limits. Significant findings included ichthyotic skin, ears adherent to the scalp bilaterally, and shortened fifth fingers bilaterally. She was noted to have spastic diplegia. Truncal hypotonia prevented her from sitting up for more than short periods of time. A Denver II Developmental Assessment found her to have social, language, fine motor, and gross motor abilities consistent with an age of 9, 7, 6, and 4 months old, respectively. Ophthalmologic examination revealed no ocular or visual abnormalities, including no evidence of macular glistening white dots. Her skin was dry with hyperkeratosis or scaling on the extremities, trunk, neck, palms, and soles (Fig 2D). The hyperkeratosis was most severe on the patient's legs, forearms, neck and trunk, where it was reminiscent of lamellar ichthyosis. Her hair and fingernails were normal. At 19 months of age, the patient suffered a tonic-clonic seizure, which subsequently recurred on several occasions.

Fibroblast Culture

Cultured skin fibroblasts were grown from skin biopsies in Dulbecco's minimal essential medium containing 10% fetal bovine serum, penicillin, and streptomycin at 37°C in an atmosphere of 5% CO₂. The cells were collected by trypsinization and washed twice with phosphate-buffered saline. Cell pellets were stored at -70°C for DNA isolation.

FALDH Assay

Fibroblasts were homogenized and assayed for FALDH activity using octadecanal as substrate as described [5], except that all reactions were run at 37° C in a 96-well fluorescent plate reader (Molecular Dynamics) in a total volume of 0.38 ml. Cell protein was determined according to Lowry et al [15]. Enzyme activity was determined as pmol/min/mg cell protein, and expressed as percentage of normal mean enzyme activity.

DNA Isolation

Genomic DNA was purified from cultured fibroblasts and blood using the Wizard Genomic DNA Purification kit (Promega). Buccal DNA was collected from controls and family members for PCR as described [16].

Deletion Characterization by Long Distance Inverse-PCR (LDI-PCR)

LDI-PCR was performed essentially as described by Willis et al [17]. A PstI restriction enzyme cut site was identified in the DNA sequence flanking one end of the deletion. One

microgram of genomic DNA was therefore digested with 0.5 Unit of PstI (Patient 1) in a total volume of 25 μ l according to the manufacturer's instructions (New England Biolabs). The DNA digest was purified using a QIAquick PCR Purification Kit (Qiagen). Circular DNA was created by ligating 660-700 ng digested DNA with 30 Units of T4 ligase (Promega) in a total volume of 660-700 μ l overnight at 25° C. The ligated DNA was purified by a double ethanol precipitation and resuspended in TE buffer.

Using oligonucleotide primers within the known DNA sequence flanking the restriction enzyme cut site, we inversely amplified the circular DNA using the Expand Long Template PCR System (Roche). All PCR reactions were performed in a final volume of 50 μ l. The reactions contained 1 Unit *Taq* polymerase, 0.05 mM dNTPs, 12-20 ng purified ligated DNA, 17.5 mM MgCl₂, and 15-20 ng of each primer (primer sequence is available by request). The amplification occurred in three steps: 1) an initial denaturation at 94° C for 5 min; 10 cycles of denaturation at 94° C for 30 sec, annealing at 64° C for 1 min, and elongation at 72° C for 4 min; 2) 10 cycles of denaturation at 94° C for 30 sec, annealing at 64° C for 1 min, and elongation at 72° C for 5 min; 3) 20 cycles of denaturation at 94° C for 30 sec, annealing at 64° C for 1 min, and elongation at 72° C for 6 min; a final extension at 72° C for 1 min. The resulting PCR products were separated on a 1% agarose gel, purified with a MinElute Spin Column and sequenced.

Genomic DNA Amplification across the Deletion in Patient 1

After identifying deletion breakpoints by LDI-PCR, primers were designed to amplify across the breakpoint in undigested genomic DNA (Table 1). PCR amplification of the patient-specific DNA fragment was multiplexed with amplification of exon 8 of *ALDH3A2* as a control. The PCR contained 1 U *Taq* polymerase, 0.05 mM dNTPs, 50 ng of DNA, 17.5 mM MgCl₂, 10 ng of the patient-specific primers, and 20 ng of the exon 8 primers (Table 1). The cycling conditions consisted of 1) an initial denaturation at 94° C for 5 min; 2) 30 cycles of denaturation at 94° C for 30 sec, annealing at 68° C for 1 min, and elongation at 72° C for 1 min; 3) a final extension at 72° C for 5 min.

DNA Sequencing

LDI-PCR products and *ALDH3A2* exons amplified from genomic DNA [13] were sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Inc, Foster City, CA) and an ABI 377A sequencer.

Screening for the c.407C>T (P136L) Mutation in Patient 2

The c.407C>T (P136L) mutation in exon 3 creates a new *HindIII* restriction enzyme cut site. To screen for this mutation, a 102 bp fragment from exon 3 containing the mutation was amplified from genomic DNA and digested with *HindIII*. The PCR contained 0.75 U *Taq* polymerase, 2.5 mM dNTPs, 17.5 mM MgCl₂, 1 μ l buccal DNA and 10 ng of each primer (Table 1) in reaction volume of 50 μ l. The cycling conditions were 1) initial denaturation at 95° C for 7 min, 2) 40 cycles of 95° C for 30 sec, 62° C for 1 min and 72° C for 1 min, 3) final extension at 72° C for 5 min. The PCR product (0.25 μ g) was digested in 50 μ l reaction volume containing Buffer 2 and 0.5 Unit *HindIII* overnight at 37° C according to the manufacturer's instructions (New England Biolabs). The digestion products were separated on a 3% agarose gel. The c.407C>T mutation produces a *HindIII* cut site with generation of a 80bp fragment, whereas the wild-type DNA is undigested.

Array Comparative Genomic Hybridization (CGH) and Fluorescence In Situ Hybridization (FISH)

CGH was performed using a whole genome 44K custom oligonucleotide array (Agilent Technologies, Inc) as described [18]. The deletion was confirmed via FISH, using a bacterial artificial chromosome corresponding to the deleted region (RP11-147i5) and alpha satellite DNA from chromosome 17. Probes were labeled and hybridized to metaphase chromosomes as described previously [18].

RESULTS

Patient 1

Patient 1 was first suspected to have a large gene deletion when *ALDH3A2* exons could not be amplified from genomic DNA. Southern blot analysis subsequently confirmed a complete deletion of the gene. The deletion boundaries were initially narrowed down by generating PCR products from flanking DNA sequences and sequentially walking closer to the deletion until no PCR products were produced; however, attempts to amplify across the deletion using multiple combinations of primers were unsuccessful. We therefore used LDI-PCR to determine the precise deletion breakpoints. This method depends on knowing DNA sequence flanking one end of the deletion breakpoint. Restriction enzymes that cut within the known sequence (and presumably somewhere on the other sides of the deletion breakpoints) were used to generate DNA fragments that were subsequently ligated into circular DNA. Primers situated within the known DNA sequence were then used to inversely amplify across the deleted DNA region in the circular DNA and locate the DNA sequence from the other side of the deletion to determine the precise breakpoints.

Using this approach, a 4.1 kb PCR product was produced by LDI-PCR of the patient's circularized DNA, and sequencing of the PCR product identified the deletion breakpoints on chromosome 17 at nucleotide 19,446,110 and nucleotide 19,798,450 (in GRCh37/hg19 of the human genomic assembly). This 352 kb deletion includes *ALDH3A2*, *ALDH3A1*, *ULK2*, *SLC47A1* and *SLC47A2* (Fig 3). The homozygous deletion was confirmed by array CGH (data not shown).

Patient 1 was a child of consanguineous parents. Using primers that flanked the deletion breakpoints, a 496 bp PCR product was amplified from the patient's genomic DNA and her parents, thus confirming the parental carrier status for this deletion, but not from controls (Fig 1B). A control PCR product of exon 8 was produced using DNA from the parents and her unaffected brother, but not from the patient, consistent with her homozygous genotype.

Patient 2

Because of the patient's constellation of clinical findings, CGH analysis was initially undertaken using a 44K oligonucleotide genomic microarray. Patient 2 was found to carry a heterozygous 1.44 Mb interstitial deletion of 17p11.2 [chr17:18,716,455 - 20,160,197] in NCBI36/hg18 of the human genomic assembly that spans 15 genes, including *ALDH3A1* and *ALDH3A2* (Fig 2A, Fig 3). The deletion lies within the recurrent Smith-Magenis syndrome (SMS) deletion region, but does not include the complete SMS critical region. FISH analysis confirmed the heterozygous deletion (Fig 2B).

Sequence analysis of the patient's only remaining *ALDH3A2* gene copy identified a novel hemizygous missense mutation (c.407C>T, P136L) in exon 3 (Fig 2C). This mutation generates a new *HindIII* restriction enzyme cut site. By screening a PCR amplicon of exon 3 and digesting with *HindIII*, we did not detect the mutation in 50 unrelated Caucasian control subjects, suggesting that it is not a common polymorphism. The P136 amino acid residue is

invariantly conserved among FALDH proteins in vertebrate species ranging from zebra fish to humans and undoubtedly has a critical function in the protein. In this regard, the P136 residue in human FALDH corresponds to P138 in a related rat class 3 ALDH protein, for which the crystalline structure has been reported [19]. P138 of the rat protein initiates a kink between the $\beta 2$ β -strand and the αB α -helical domain within the Rossmann fold, which is important for NAD binding.

Investigation of the parents by microarray and DNA sequencing revealed that the patient's father carried the deletion mutation and her mother was heterozygous for the P136L mutation.

DISCUSSION

The SLS patients reported here have unusually large deletions, including the entire *ALDH3A2* gene on chromosome 17p11.2. Previously reported intragenic deletions in SLS have ranged from 1 or 2 nucleotides to as large as 6 kb [11], but no complete gene deletions have been reported. Our patients carried contiguous gene deletion alleles that included *ALDH3A2* and multiple flanking genes. Indeed, this region of chromosome 17 is associated with large *de novo* contiguous gene mutations that are a well-known marker of SMS [20], which is caused by haploinsufficiency for the *RAI1* gene [21].

Patient 1 represents a unique “experiment in nature”. She is homozygous for a contiguous gene mutation that deletes *ALDH3A2* and 4 neighboring genes, including *ALDH3A1*, *ULK2*, *SLC47A1* and *SLC47A2*. *ALDH3A1* encodes an aldehyde dehydrogenase that is expressed at high levels in stomach and comprises up to 40% of the total soluble protein in cornea [22]. The enzyme oxidizes medium-chain aliphatic aldehydes [23], and is thought to eliminate toxic aldehydes generated during lipid peroxidation [24]. However, its unusual abundance in cornea suggest that the protein may also have a non-catalytic role in absorbing UV light and protecting the underlying lens from damage [25]. A naturally occurring strain of mice (SWR/J) that is deficient in ALDH3A1 enzyme activity [26] and *Aldh3a1*^{-/-} gene knockout mice [25] are abnormally susceptible to cataract formation when exposed to UV light. The *ULK2* gene codes for a UNC-51-like serine/threonine protein kinase that is widely expressed in tissues [27] and is involved in cell signaling pathways for apoptosis [28], autophagy [29,30], axonal outgrowth and endocytosis [31], and fibroblast growth factor receptor substrates [32]. The *SLC47A1* and *SLC47A2* genes encode multidrug and toxin extrusion proteins (MATE1 and MATEK-2, respectively) that are localized in the kidney and are important for actively secreting certain organic cationic molecules and drugs, including metformin and cimetidine, from the blood into the urine [33,34,35]. Non-synonymous polymorphisms in the genes may be responsible for decreasing, or even eliminating, renal clearance of these ionic drugs and thereby affect drug pharmacokinetics and toxicity [35,36]. Mice naturally lack the *SLC47A2* gene, but genetic knockout of the murine *SLC47A1* gene results in a profound reduction in metformin excretion and increased blood concentrations of this drug [37].

Although missing *ALDH3A2* and 4 additional genes, the clinical phenotype of Patient 1 was within the spectrum seen in other SLS patients, albeit at the more severe end. Some of her symptoms, however, may originate from the deleted neighboring genes. Her oral motor dysfunction and excessive drooling is not usually seen in SLS, and most patients do not require a gastrostomy tube for feeding. Despite lacking the ALDH3A1 protein, she did not exhibit corneal or lens opacities. Her long-standing photophobia and squinting, which are common features of SLS patients, may have limited her ocular exposure to UV light and prevented eye damage. The absence of perimacular glistening white dots, which are often seen in SLS, indicates that her photophobia does not arise from light hitting and refracting

off these abnormal structures. Without the MATE1 and MATEK-2 transporters, Patient 1 should be at higher risk for adverse effects of MATE-dependent drugs. The lack of ULK2 would seem to impair several cell signaling pathways and may contribute to her more severe SLS phenotype with progressive loss of speech, oral motor function and worsening spasticity. Most SLS patients have a static disease with little or no symptom progression over time. Additional subtle phenotypic effects of her deleted genes could be missed on her clinical background of severe SLS. Moreover, the extent to which the clinical phenotype of Patient 2 is influenced by haploinsufficiency for the many deleted genes is also not known.

The genetic analysis of Patient 2 underscores the importance for considering large gene deletions in patients with ostensibly homozygous *ALDH3A2* mutations and the need for parental testing to confirm carrier status. If this patient had only undergone standard mutation analysis by sequencing genomic exons amplified by PCR, her genotype could have been initially misidentified as homozygous P136L. In the absence of parental testing, a heterozygous deletion or duplication would have gone undetected, and subsequent genetic testing of family relatives or prenatal diagnosis could have led to erroneous results. Mutation analysis of her parents, however, would have raised the possibility of a co-existing deletion mutation in the patient and prompted further DNA analyses by array CGH or high-density single nucleotide polymorphism (SNP) chips.

The frequency of large gene deletions in SLS is not precisely known. We are aware, however, of two other SLS patients who also carry unique large gene deletions that involve the complete *ALDH3A2* gene or most of it (unpublished observations). Together with the two mutations reported here, these large deletions account for approximately 5% of the mutant alleles reported so far.

Large deletion mutations have been detected in several other inborn errors of metabolism, including Canavan disease [38-40], cystinosis [41], steroid sulfatase deficiency [42-44], X-linked adrenoleukodystrophy [45], phenylketonuria [46], pyruvate dehydrogenase deficiency [47] and ornithine transcarbamylase deficiency [44,49]. Although CGH and SNP arrays are rarely done for the evaluation of suspected metabolic disorders, they are becoming a standard diagnostic test for patients with non-specific developmental delay. Our findings in SLS suggest that these tests also have utility for detecting contiguous gene deletions in an increasing number of inborn errors of metabolism.

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Abbreviations

CGH	comparative genomic hybridization
FALDH	fatty aldehyde dehydrogenase
FISH	fluorescence <i>in situ</i> hybridization
LDI-PCR	long distance inverse-PCR
OMIM	Online Mendelian Inheritance in Man
SLS	Sjögren-Larsson syndrome
SMS	Smith-Magenis syndrome

SNP single nucleotide polymorphism

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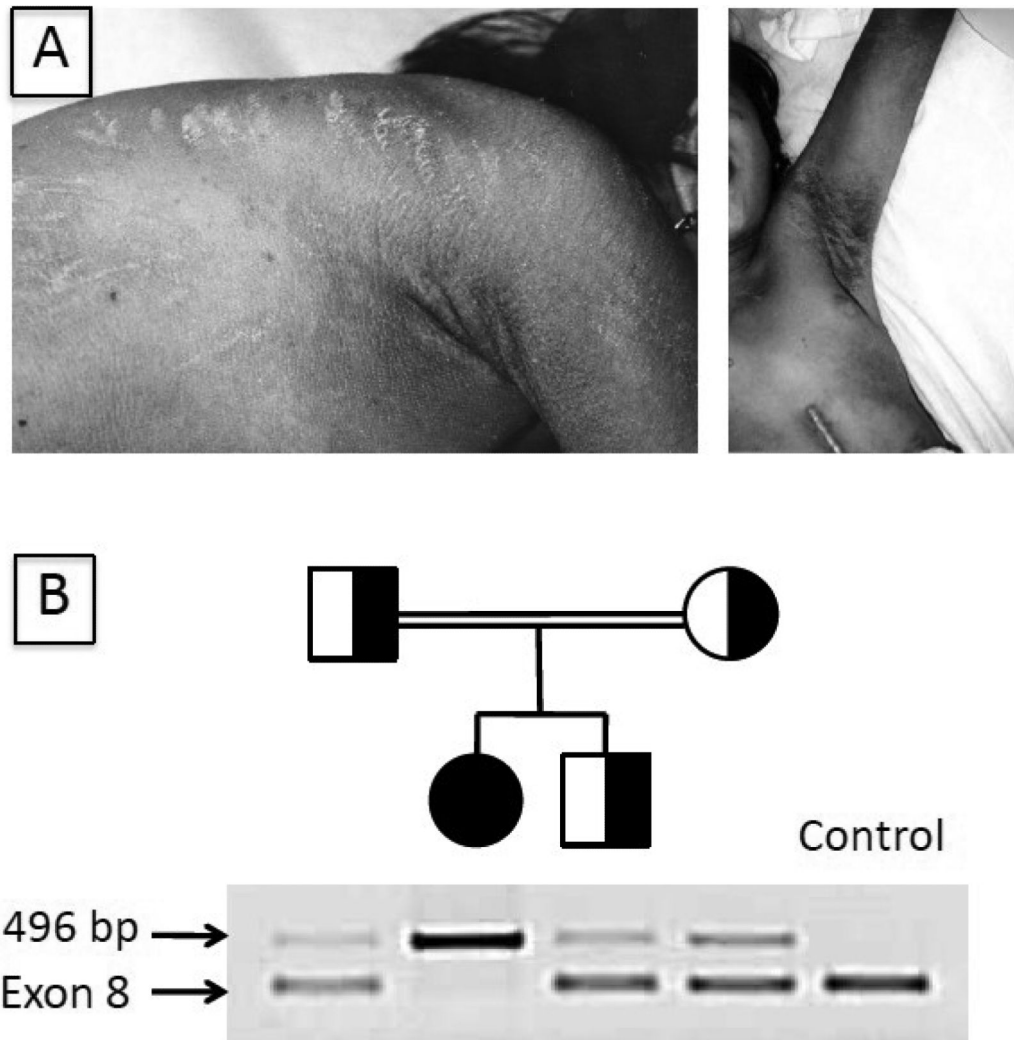


Fig 1. Phenotypic appearance and mutation detection in Patient 1. A. Left photo: At 14 years of age, note the generalized pruritic ichthyosis with excoriations on the trunk. Right photo: Dark scales in the axillary region and an abdominal scar from gastrostomy tube placement are evident. B. Multiplex PCR amplification of the deletion mutation in genomic DNA generated a 496 PCR product from Patient 1, her mother and father, and heterozygous brother. A control PCR product (exon 8) was produced using DNA from the parents and heterozygous brother, but not from Patient 1.

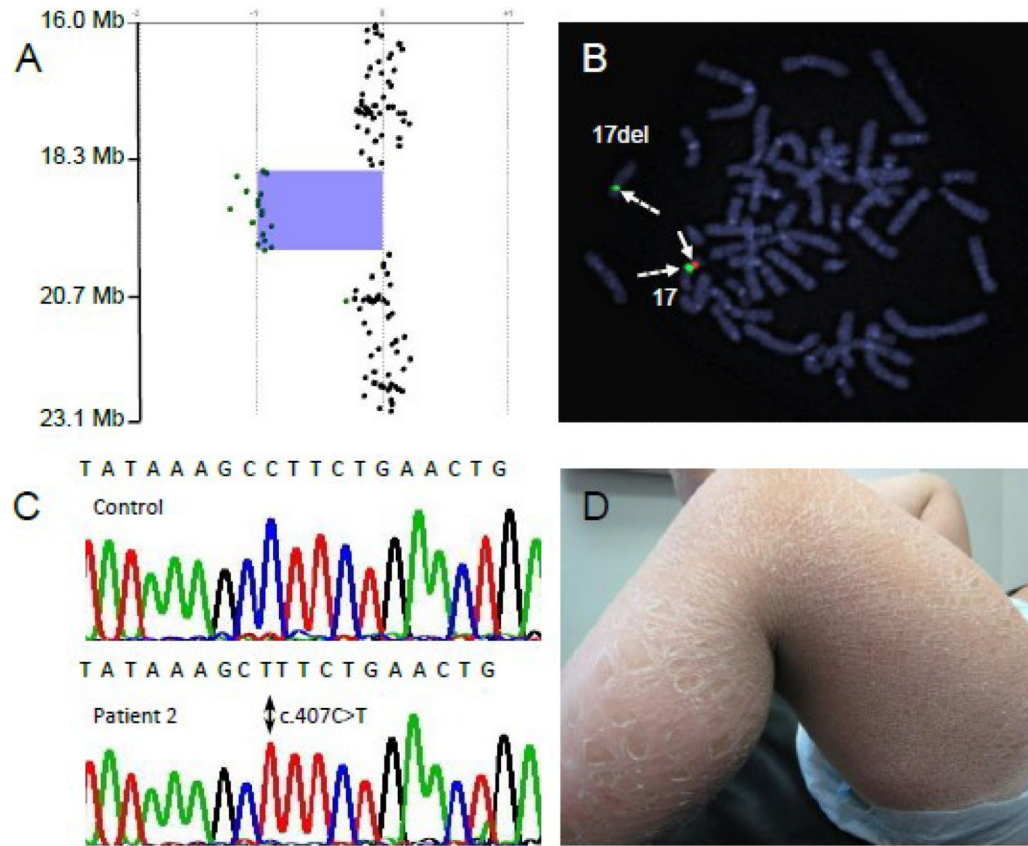


Fig 2. Genetic analysis and phenotypic appearance of Patient 2. A: Microarray analysis of chromosome 17p11.2 showing heterozygous deletion. B: FISH using a chromosome 17-specific centromeric probe (green signal, dashed arrows) identifies both 17 chromosomes. The RP11-147i5 probe (red) corresponds to the deleted region and hybridizes to only the non-deleted chromosome 17 (solid arrow). C: DNA sequencing chromatogram demonstrates a hemizygous c.407C>T (p.P136L) mutation in exon 3 of the *ALDH3A2* gene. D: Note the variable appearance of the ichthyosis on the leg of patient 2 with large lamellar-like scales on the lower leg and smaller scales on the thigh.

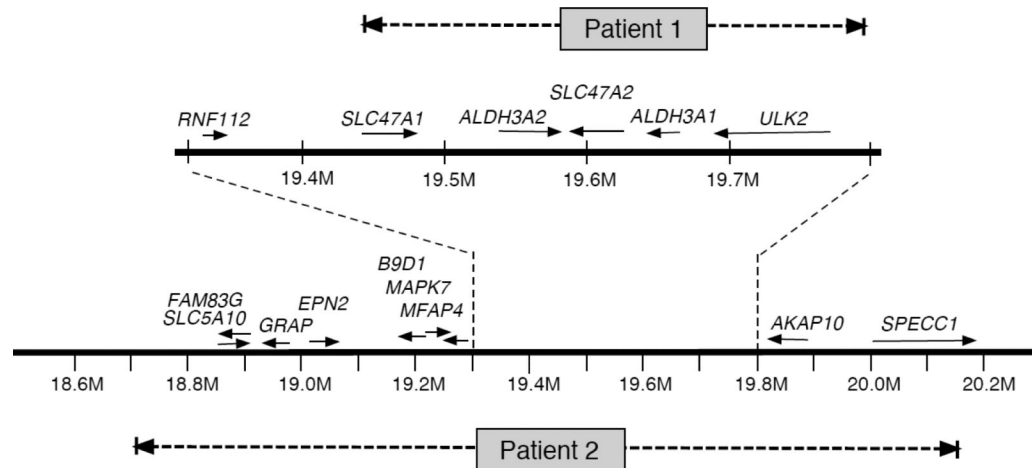


Fig 3. *ALDH3A2* genetic locus at chromosome 17p11.2 showing the large deletions in the patients. The horizontal solid lines indicate chromosome DNA with an expanded region delimited by the vertical dashed lines. Arrows represent orientation of genes from 5' to 3' direction. Horizontal dashed lines correspond to deleted regions in Patient 1 and Patient 2. Nucleotide positions are numbered in megabases (M).

Table 1

Oligonucleotide primers used in this study.

DNA Product	Primer	Oligonucleotide Primer Sequence
Patient 1 Genomic Deletion	Forward	5'-TGTGTAACCTTGCAGATTCCTAGGTTC-3'
	Reverse	5'-ATCTCAGTGGAAATCTGGACAGTGACAC-3'
<i>ALDH3A2</i> Exon 8	Forward	5'-TTGACACATAACTGAGCACACAGCCCTC-3'
	Reverse	5'-AGCAGCCATACAATCCACTCATGA-3'
<i>ALDH3A2</i> Exon 3 for Detection of c.407C>T	Forward	5'-GAGCTGCAGAAATAATTGGGAGTACCTAGC-3'
	Reverse	5'-CTTGGCTGTATTTTCACTCAGTTC-3'