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Whole Exome Sequencing identifies a splicing mutation in *NSUN2* as a cause of a Dubowitz-like syndrome

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Abstract

Dubowitz Syndrome is an autosomal recessive disorder characterized by the constellation of mild microcephaly, growth and mental retardation, eczema and peculiar facies, but causes are still unknown. We studied a multiplex consanguineous family with many features of Dubowitz syndrome using whole exome sequencing and identified a splice mutation in *NSUN2*, encoding a conserved RNA methyltransferase. NSUN2 has been implicated in Myc-induced cell proliferation and mitotic spindle stability, which might help explain the varied clinical presentations that can include chromosomal instability and immunological defects. Patient cells displayed loss of NSUN2-specific methylation at two residues of the aspartate tRNA. Our findings establish *NSUN2* as the first causal gene with relationship to the Dubowitz syndrome spectrum phenotype.

Keywords

Dubowitz; NSUN2; MISU; RNA methylation; microcephaly

Dubowitz Syndrome (DS, %223370) is a rare, developmental disorder characterized by small stature, intellectual disability, mild microcephaly, and a distinct facial appearance consisting of blepharophimosis, broad nasal bridge, sloping forehead and micrognathia [1, 2]. Although first reported in 1965 by Dubowitz as a condition characterized by familial low birthweight, dwarfism with unusual facies and skin eruption [3], the phenotypic spectrum

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Web Resources

The URLs for data presented herein are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.omim.org

SeattleSeq Annotation, http://gvs.gs.washington.edu/

Genome browser, http://www.genome.ucsc.edu/

has been expanded to include a wide variety of less frequent features such as congenital heart defects, frequent infections and chromosomal instability [4]. DS is distinguished from other blepharophimosis-mental retardation syndromes such as Say-Barber-Biesecker variant of Ohdo syndrome [2], recently linked to mutations in the *KAT6B* gene encoding a histone acetyltransferase [5]. To date there are approximately 140 DS reported cases with no causative mutations identified. Although there is a presumed recessive mode of inheritance in most cases, there are reports that suggest more complex modes of inheritance can occur [6].

We identified a consanguineous multiplex family 1455 in the United Arab Emirates of Lebanese origin (Fig. 1A). The eldest sibling was healthy with no pathological features. The three affected members displayed features consistent with DS, including low birth weight (5% or less), and oligohydramnios in the two older affected individuals (Table 1), which are compared with typical features of DS [2]. By 1 year of age, growth retardation, and developmental delay were noted. Facial features included blepharophimosis, telecanthus, hypertelorism, high or broad nasal bridge (Fig. 1B). Oral features included smooth philtrum, downturning upper lip and dental anomalies. Eczema was clearly apparent over the chest and back in the youngest affected at the time of evaluation, requiring treatment, although the two older affected individuals did not have a history of notable eczema. The neurological features included microcephaly (−2%ile occipital-frontal head circumference at birth, progressing to −4 SD 2 years of age), axial hypotonia, and autistic features. The eldest affected displayed epileptic seizures, controlled on valproate, a feature not observed in the younger two affected individuals by the time of evaluation. The brain MRI was unremarkable. The remainder of the history and physical was non-contributory, and serum chemistry, immune profiles and routine karyotype were normal. However, notable hoarse voice, triangular face, round nose tip and abnormal ears, occasionally observed in DS, were not reported. We therefore cautiously refer to this condition as a Dubowitz-like condition, although it may in fact represent Dubowitz syndrome, and the pedigree is consistent with a recessive mode of inheritance.

In order to identify the gene for this condition, we performed whole exome sequencing on two affected siblings. The study was approved by the Ethics Committee and the family provided informed consent for study. Blood DNA was extracted using Qiagen reagents, then subjected to exome capture with the Agilent SureSelect Human All Exome 50 Mb kit, sequenced on an Illumina HiSeq2000 instrument, resulting in ~94% target coverage at > 10X depth. GATK [7] was used for variant identification and intersected with identity-by-descent blocks identified by HomozygosityMapper [8], and then filtered for homozygous variants shared between the two affected individuals using custom Python scripts (available upon request).

We identified a homozygous variant in an intronic region of the gene *NSUN2*, located one base pair upstream of the start of exon 6 (chr. 5 base position 6,622,214 C>G; hg19) at the canonical splice acceptor site (Fig. 1C). The variant was found to be homozygous in all three affected cases, occurred with a 19.5 cM block of homozygosity in both affected members between chr. 5 base position 2,752,824–22,078,584 (Supplementary Fig. 1), was absent in

the single unaffected sibling, and heterozygous in each parent, and thus segregated according to a recessive mode of inheritance.

There were six other shared novel homozygous genetic variants, each leading to amino acid transversions annotated to be potentially damaging, occurring in the *CUL4A*, *RIMS2*, *TARP*, *PKHD1L1*, *CACHD1*, and *TRHR* genes. However, the reference amino acid for each was not fully conserved across evolution, and further upon direct testing was found to fail test of segregation in the family. There were no other likely deleterious variants that segregated according to the presumed recessive mode of inheritance, supporting pathogenicity of the *NSUN2* variant. The variant was not encountered in our in-house exome database of ~1000 Middle Eastern individuals, and was not reported in any public databases. We also found the variant absent in a panel of 96 ethnically matched Arab controls. The mutated position was highly conserved across species and annotation with SeattleSeq predicted it to be critical for splicing. Direct Sanger sequence analysis of four additional patients with DS-like phenotypes for all coding and splice sites yielded no obvious mutations, suggesting genetic heterogeneity.

The *NSUN2* gene is alternatively spliced with at least four splice variants and three proposed alternative start sites of transcription. To determine whether the exon 6 splice acceptor mutation leads to altered mRNA levels, we performed quantitative RT-PCR. Dermal fibroblasts were obtained from two affected individuals, the mother, and one unrelated control, cultured under similar conditions, and total RNA was reverse transcribed using SuperScript III (Invitrogen). Amplifications from exon 5 to 6 and exon 6 to 7 were compared with a control reaction against TATA binding protein (TBP) using the LightCycler 480 SYBR Green I (Roche) assay. All assays were normalized to GAPDH and then to the expression level of the unrelated control. We found a severe 95% reduction in normalized expression for both products from both affected patients, whereas the obligate carrier mother showed about a 50% reduction in amplification, suggesting a functional consequence of the mutation on the *NSUN2* mRNA (Fig. 2A). Because exon 6 is not present in all deposited *NSUN2* mRNAs, and its absence maintains the open reading frame, we additionally assessed amplifications from exon 5 to 7, 7 to 8 and 8 to 9. In all reactions, we found comparable 70–80% reduction in NSUN2 mRNA, which excluded the possibility of trivial exon skipping. The results suggest a global reduction in mRNA levels from altered splicing, resulting in either partial or complete loss of mRNA in the two affected individuals.

To determine the effect on splicing, we analyzed RT-PCR products from affected patient and control fibroblasts by Bioanalyzer (Agilent, Inc) electrophoresis. Amplification from exon 5 to 7 demonstrated an aberrant sized product from the two affected individuals of about 250 bp, also evident in the mother's sample, whereas the predicted size product of 210 bp was notably reduced in the two affected individuals. Products from exon 5 to 6 were reduced without an obvious difference in molecular weight (Fig. 2B). Analysis of the reference genome for possible cryptic splice acceptor and donor sites identified an AluY transposon located between exon 6 and 7. Replacement of exon 6 with a single arm of the transposon is predicted to yield a 250 bp cDNA product from exon 5 to 7. In order to determine if this AluY exon was incorporated in the mRNA, we sequenced the aberrant 250

bp band. As predicted, we identified a sequence corresponding precisely to the 3′ arm of the AluY transposon (Supplementary Fig. 2A). This sequence trace demonstrated splicing from exon 5 to the 3′ AluY arm to exon 7, which is predicted to maintain the reading frame (Supplementary Fig. 2B, Supplementary Fig. 3). Alu repeats have a two-part structure consisting of 5′ and 3′ arms separated by an 'A' rich linker region. The 3′ arm is terminated by a PolyA tail [9]. Novel insertions of Alu elements within the genome have been linked to disease, and their incorporation into mRNAs is frequently associated with microRNA-mediated degradation. Indeed dozens of human microRNAs are predicted to target conserved regions of Alu transposons[10].

In order to test for an effect on NSUN2 protein expression, we generated whole cell lysates from cultured fibroblasts and performed Western analysis by loading each sample in duplicate. We probed the blot for NSUN2 protein using a rabbit affinity-purified polyclonal antibody (Proteintech Catalog 20854-1), generated against full-length recombinant human protein. In control and mother cell line, we detected robust protein expression at the expected size of about 100 kD, similar to the published molecular weight [11]. Protein loading was controlled by probing the blot with anti-alpha-tubulin. In both patient cell lines, however, there was undetectable protein (Fig. 3A). To confirm these results, we fixed and stained these fibroblasts with NSUN2 antibody. Because NSUN2 is a nuclear and nucleolar protein, we counterstained nuclei with Hoechst. In control and mother lines, NSUN2 staining overlapped precisely with nuclei and nucleoli. In both patient lines, in images acquired with identical settings, there was striking reduction in staining for NSUN2, without detectable signal in either nucleoplasm or nucleoli (Fig. 3B). The data suggests severe reduction in NSUN2 protein levels in patient cells.

NSUN2 is an RNA methyltransferase known to operate downstream of c-MYC during cell proliferation. It contains a NOL1/NOP2/Sun domain, and was demonstrated to have in vitro activity to methylate cytosine in tRNA and hemimethylated DNA (polydI:dC) [11, 12]. In yeast, the closest orthologue is the gene SUN (Trm4/Ncl1), which is capable of methylating tRNAs at all four known sites (positions 34, 40, 48, 49) and where gene inactivation completely blocks tRNA methylation [13]. A recent study utilizing metazoan cells demonstrated a requirement for *NSUN2* in cytosine methylation (m^5C) of residue 34 in $tRNA^{Leu(CAA)}$ and residue 47 and 48 in $tRNA^{Asp(GTC)}$ [14], whereas residue 38 in $tRNA^{Asp(GTC)}$ is methylated by the alternative methyltransferase *DNMT2* [15]. NSUN2 deficient mice lack m^5C of $tRNA^{LEU(CAA)}$ at position C34. These mice also display a low body weight phenotype and partial alopecia, as well as altered cell cycle timing and self-renewal capacity in epidermal stem cells [16].

To test for similar defects in human *NSUN2*-mutant cells, RNA was isolated from dermal fibroblasts, and processed for bisulfite sequencing as previously described [16]. Primers were designed to amplify mature $tRNA^{ASP(GTC)}$ (Fig. 4A, Fw: 5′-GTTAGTATAGTGGTGAGTAT-3′, Rev: 5′-CTCCCCATCAAAAAATCAAAC-3′). Visualization of bisulfite sequencing data demonstrated the loss of cytosine-5 methylation at these NSUN2 target sites (C47 and C48) in affected individuals but not the unaffected mother (Figure 4B). Position C38, the target of *DNMT2*, was methylated in all individuals. These results suggest that loss of the *NSUN2* gene results in a loss of site-specific 5-cytosine

methylation in patient cells. *DNMT2* and *NSUN2* are the only known tRNA:m5C methyltransferases known, and although recently demonstrated to protect tRNA from cleavage and degradation [15], the significance of this post-translational modification is not well understood.

NSUN2 has been shown to interact with the mitotic spindle and to play an important role in cell division, and interestingly, its effect on mitotic spindle stability is independent of this methyltransferase activity [17]. Thus it has been hypothesized to play a dual role in mammalian cells. Phosphorylation of NSUN2 by the cell-cycle related kinase Aurora reduces methyltransferase activity [11], further supporting this dual-role hypothesis. Cultured cells depleted of NSUN2 exhibit increased apoptosis, reduced proliferation, and a variety of spindle defects leading to abnormal mitoses [17], which might relate to the frequent reports of potential defects in immunological function and chromosomal instability in DS. Our discovery of a damaging mutation in *NSUN2* leading to a DS-like condition further highlights the role of cell proliferation genes in normal brain development and emphasizes the connection between abnormal cell cycle and microcephaly phenotypes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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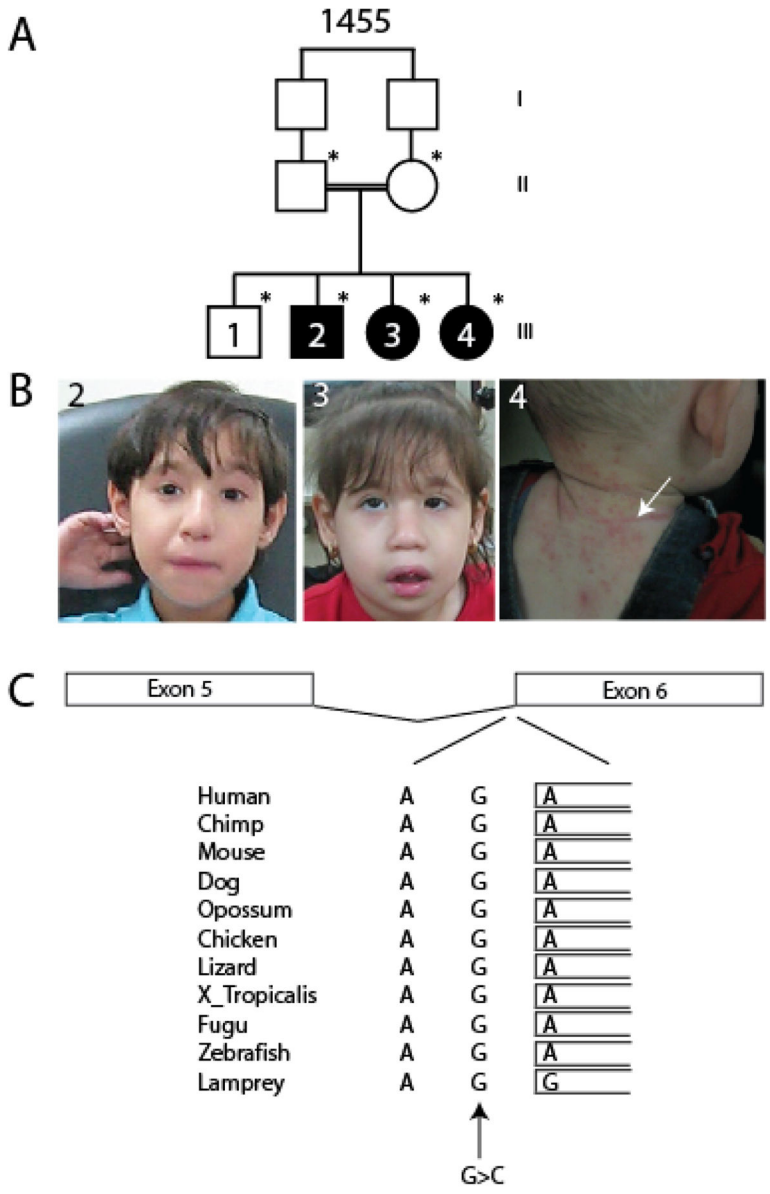

Figure 1.

Pedigree, phenotype, and predicted effect on splicing of the *NSUN2* mutation. (A) Family-1455 presents three affected children from a first-cousin marriage. (B) Photograph of affected individuals, demonstrating blepharophimosis, telecanthus, hypertelorism, high/broad nasal bridge, smooth philtrum, and downturning upper lip in 2 and 3. Eczema on the nape and back are visible in 4 (arrow). (C) Invariant conservation across species of the G residue at the predicted exon 6 splice acceptor site (arrow), with mutant G>C base indicated.

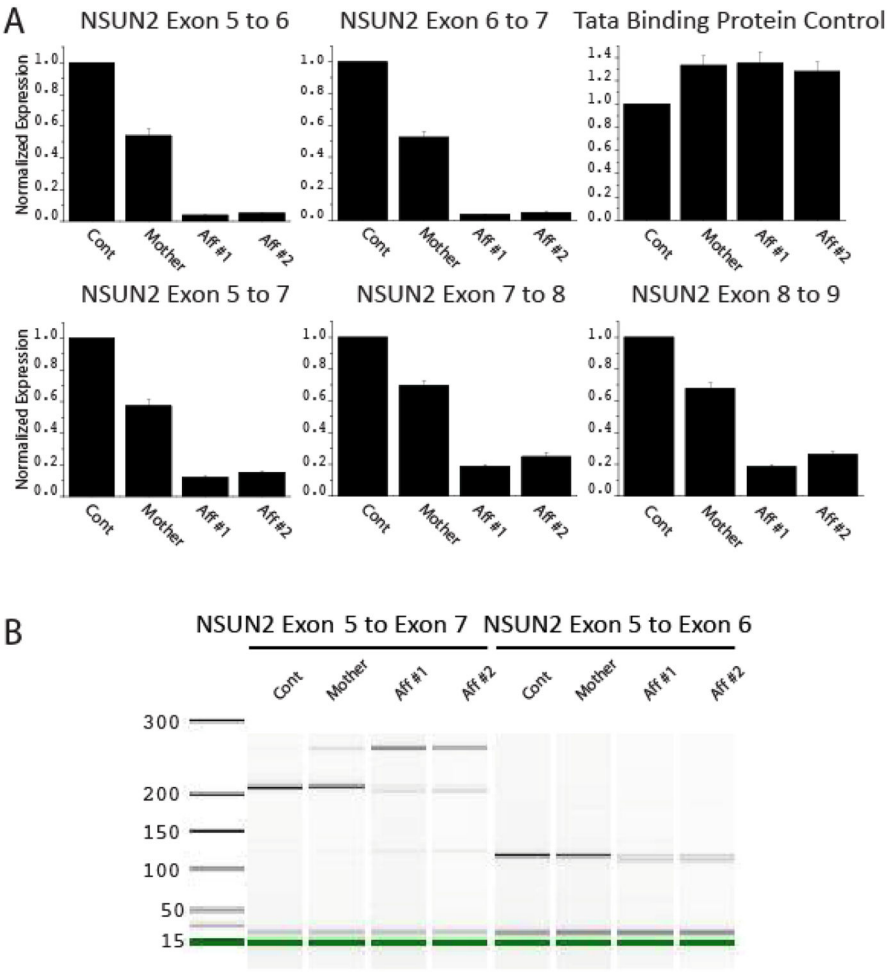

Figure 2.

Altered *NSUN2* mRNA levels and aberrant splicing in affected individuals from Family-1455. (A) Each experiment compares qRT-PCR levels from healthy unrelated control to mother and two affected members. Severely reduced incorporation of exon 6 was detected from exon 5 to 6 and 6 to 7 reactions, whereas mother displayed about 50% of control. TATA Binding Protein qRT-PCR, used as control. Reactions from exons 5 to 7, 7 to 8 and 8 to 9 showed overall reduction in *NSUN2* mRNA levels. (B) Agilent Bioanalyzer visualization of aberrant splicing of *NSUN2* mRNA in Family-1455. Amplification from exon 5 to 7 produced a 210 bp product in control and mother, severely reduced in the affected individuals, who instead show a 250 bp product, also evident in mother at reduced level, containing an AluY exon.

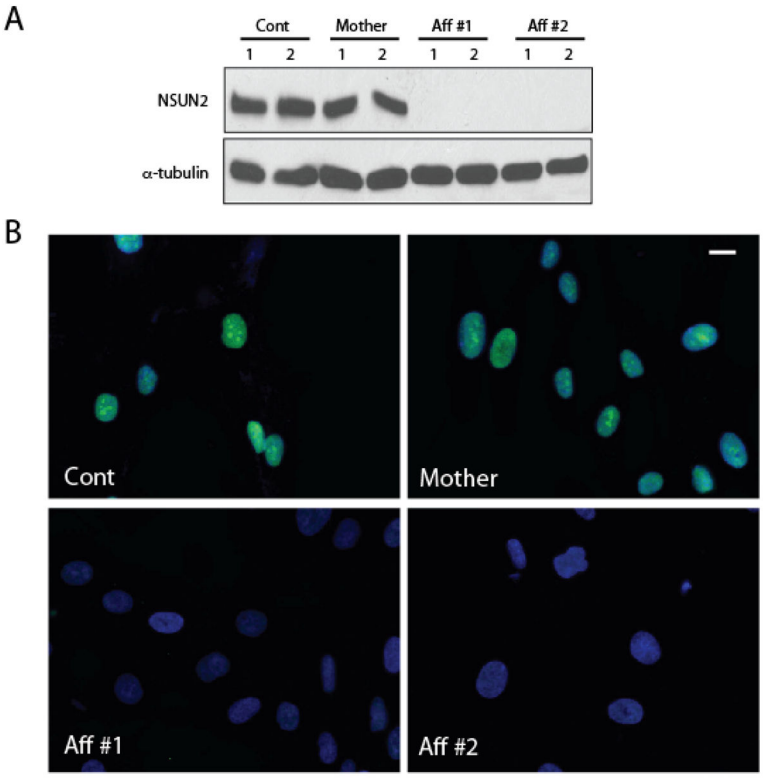

Figure 3.

Reduced NSUN2 protein levels in affected cell lines from Family-1455. (A) Western blot from whole cell extracts of control, mother and two affected individuals (Aff #1 and Aff #2), loaded in duplicate, show undetectable protein, compared with alpha-tubulin loading control. (B) Immunofluorescence of same cells showing undetectable NSUN2 reactivity (green) but intact Hoechst (blue) staining in affected cells compared with cells from control and mother. Staining is specific to nuclei and is enriched at nucleoli as previously described. Scale bar 10 uM.

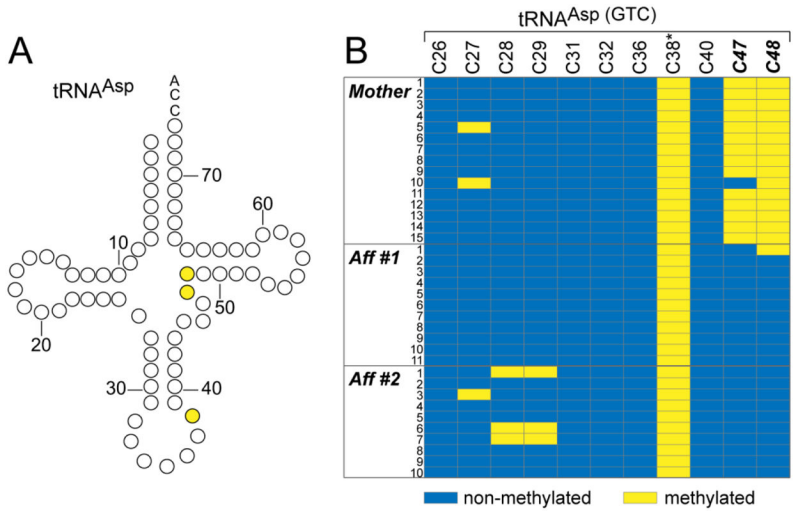

Figure 4.

Site-specific loss of cytosine-5 methylation (m^5C) in two affected members. (A) Secondary structure of $tRNA^{Asp}$. Prediction is that C47 and C48 require *NSUN2* for m^5C, whereas C38, methylated by *DNMT2*, will be unaffected. (B) RNA bisulfite sequencing of $tRNA^{Asp(GTC)}$ in the mother as a control and two affected members. In affected members m^5C is specifically lost at *NSUN2*-dependent sites C47 and C48 (highlighted in bold) whereas m^5C of C38 (highlighted with a star) remains unchanged. Blue indicates cytosine-5 not methylated; yellow indicated cytosine-5 methylated.

Table 1

Features	Reported in Dubowitz S.	1455-3-2	1455-3-3	1455-3-4
Pregnancy/neonatal				
IUGR	69%	2.200 kg (<5%)	2.900 kg (5%)	2.900 kg (5%)
Oligohydramnios	2%	+	+	–
Respiratory problems	15%	–	–	–
Feeding difficulties/GER	22%	–	–	–
Frequent infections	32%	–	–	–
Growth retardation (PN)	86%	+	+	+
Developmental/mental delay	72%	+	+	+
Speech delay	67%	+	+	+
Craniofacial				
Microcephaly	79%	+	+	+
Blepharophimosis	43%	+	+	+
Ptosis	38%	–	–	–
Upward palpebral fissures	11%	–	–	–
Telecanthus	25%	+	+	+
Hypertelorism	19%	+	+	+
Broad/depressed nasal bridge	22%	high bridge	+	+
Anteverted nares	–	–	–	–
Low-set/dysplastic ears	25%	–	–	–
Preauricular pits	–	–	–	–
Cleft palate	8%	–	–	–
Micrognathia	57%	–	+	–
Tooth problems	29%	+	+	+
Small mouth	Rare	–	–	–
Mask like/immobile face	–	–	+	–
smooth philtrum		+	+	+
downturning upper lip/absent cupid bow	+	+	+	+
Neurological				
Abnormal brain image	3%	–	–	–
Axial hpotonia	40%	+	+	+
Ocular impairment	22%	–	–	–
Hearing impairment	–	–	–	–
Cutaneous				
Sparse hair/thin eyebrows	41%	–	+	+
Eczema	42%	–	–	+
Thin skin	–	–	–	–
Cardiac	9%	–	–	–
Gastrointestinal				

Features	**Reported in Dubowitz S.**	**1455-3-2**	**1455-3-3**	**1455-3-4**
Gastrectasia	–	–	–	–
Constipation	9%	–	–	+
Distension of gall-bladder	–	–	–	–
Skeletal/limb				
Long thumbs	Rare	–	–	–
Clinodactyl of 5th fingers	34%	–	–	–
Contractures	–	–	–	–
Urogenital abnormalities	41%	–	–	–
Thyroid abnormalities	Rare	+	–	–
Other	HA, HD	Self Stimulation, Convulsions	Self Stimulation	Self Stimulation
Inheritance	AR	AR	AR	AR

AR: Autosomal Recessive; HD: Hematological disturbance; HA: Hyperactivity