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Early-onset severe neuromuscular phenotype associated with compound heterozygosity for *OPA1* mutations

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ABSTRACT

Introduction: Pathogenic mutations in the *OPA1* gene are the most common identifiable cause of autosomal dominant optic atrophy (DOA), which is characterized by selective retinal ganglion cell loss, a distinctive pattern of temporal pallor of the optic nerve and a typical color vision deficit, with variable effects on visual acuity. Haploinsufficiency has been suggested as the major pathogenic mechanism for DOA. Here we present two siblings with severe ataxia, hypotonia, gastrointestinal dysmotility, dysphagia, and severe, early-onset optic atrophy who were found to be compound heterozygotes for two pathogenic *OPA1* mutations. This example expands the clinical phenotype of *OPA1*-associated disorders and provides additional evidence for semi-dominant inheritance.

Methods and results: Molecular analysis of the *OPA1* gene in this family by Sanger sequencing revealed compound heterozygosity for two mutations in *trans* configuration, a p.I382M missense mutation and a p.V903GfsX3 frameshift deletion in both affected siblings. Electron microscopy of a skeletal muscle biopsy of the older sibling revealed dense osmiophilic bodies within the mitochondria. Mitochondrial DNA (mtDNA) content was within normal limits, and electron transport chain analysis showed no deficiencies of the mitochondrial respiratory chain enzymes. Multiple mtDNA deletions were not found.

Conclusion: Compound heterozygosity of pathogenic *OPA1* mutations may cause severe neuromuscular phenotypes in addition to early-onset optic atrophy. While a role for OPA1 in mtDNA maintenance has been discussed, compound biallelic pathogenic *OPA1* mutations in our patients did not result in altered mtDNA copy number, mtDNA deletions, or deficiencies of the electron transport chain, despite the severe clinical phenotype.

1. Introduction

Autosomal dominant optic atrophy (ADOA, OMIM 165500) is the most common inherited optic atrophy, with an estimated prevalence of 1 in 35,000 or higher [1]. In Denmark, the prevalence is estimated as high as 1 in 10,000, likely attributable to a founder effect. The optic atrophy in ADOA is characterized by insidious onset of visual impairment, typically during the first two decades of life, leading to moderate to severe loss of visual acuity, temporal (more than nasal) optic disk pallor in the papillomacular bundle, characteristic color vision (tritan) deficits, and centrocecal, central, or paracentral visual field defects [2].

As many as 20% of individuals with ADOA have additional, extra-ocular neurological signs and complications, among them sensori-neural hearing loss, ataxia, and myopathy. The term "dominant optic atrophy plus" has been applied to these variants [3].

Most ADOA cases are caused by mutations in the *OPA1* gene, which encodes the human homolog of the *Schizosaccharomyces pombe* dynamin-related protein Msp1. More than 200 different mutations of the *OPA1* gene have been reported to date, most of them resulting in protein truncation. Consequently, haploinsufficiency has been proposed as the major pathogenic mechanism for ADOA. Interestingly, missense mutations have been associated with more severe phenotypes and most commonly with hearing loss (typically "ADOA plus") when compared to nonsense mutations [3], suggesting a dominant-negative mechanism for these variants.

A total of four ADOA patients have been reported who were found to each carry two mutations in the *OPA1* gene [3,4]. A 30 year-old

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woman, compound heterozygous for a p.E270K and a p.R290W mutation, had both severe impairment of visual acuity and total pallor of optic nerves in each eye. She was described to have a more severe clinical phenotype than either one of her heterozygous parents or her siblings, providing some evidence for semi-dominant rather than pure dominant inheritance of ADOA. Interestingly, the compound heterozygous subject was not described to have any non-ocular neurological features [4]. Two German siblings were found to both carry a p.K212fsX4 and a p.V548I mutation; however detailed clinical information was not available and in the absence of parental samples, it could not be determined if these mutations were on the same allele or compound heterozygous. The same applies to a 60 year-old Norwegian proband heterozygous for the p.S256R and p.Q285R mutations, for whom parental follow-up was not available [3].

Here we report two siblings with severe, early-onset optic atrophy, ataxia, hypotonia, dysphagia, and gastrointestinal dysmotility, who were compound heterozygous for two pathogenic *OPA1* mutations, thereby expanding the clinical phenotype of *OPA1*-associated disorders and providing additional evidence for semi-dominant inheritance.

1.1. Clinical descriptions

An 8 year-old boy and his 3 year-old sister presented to the Pediatric Genetics Clinic (M.B.) for evaluation of ataxia and optic atrophy.

The boy was born at 30 weeks gestational age by spontaneous vaginal delivery to a 32 year-old G1 mother. The pregnancy was complicated only by preterm labor. His birth weight was 2097 g. His postnatal course was complicated by grade II intraventricular hemorrhage. Serial ophthalmoscopic examinations did not reveal retinopathy of prematurity. He had exotropia at age 1 year, for which he was treated with patching and corrective surgery. At the same time, he was noted to have optic nerve atrophy in each eye. By age 3 years, the boy was noted to have nystagmus, and his optic nerve atrophy had progressed to the point that he was declared legally blind. He was hypotonic during infancy and was noted to have substantial ataxia when attempting to stand at around 1 year of age. He was not able to walk without support until the age of 2.5 years. At the age 8 years, he is still able to walk independently but with profound ataxia and an unsteady, stomping gait with heavy heel strikes, as well as postural instability, suggestive of a sensory ataxia and peripheral neuropathy. His review of systems is significant for dysphagia, choking episodes, and vomiting, starting at age 4 years. Decreased intestinal motility with profound constipation ensued, requiring medication and intermittent disimpaction. He had brain MR imaging at age 2 years and was found to have mild periventricular leukomalacia. The cerebellum appeared normal in structure and size. Echocardiography revealed a structurally normal heart, and he had normal CK levels and a normal radiographic bone age study.

His 3 year-old sister was born at 36 weeks gestational age by spontaneous vaginal delivery to her then 36 year-old G2 mother. The pregnancy was uneventful and the postnatal course was uncomplicated. She was diagnosed with optic nerve atrophy O.U. at the age of 6 months and developed nystagmus at 1 year. She displayed profound hypotonia and ataxia and was not able to walk without support until the age of 3 years. Dysphagia and constipation were present clinically but not yet with the same severity observed in her older brother.

1.2. Ophthalmological and audiological evaluations

At age 8 years, the boy was able to count fingers at about 15 cm eccentrically with each eye. The anterior segment and biomicroscopic examination was structurally normal in each eye, except for the healed conjunctival scars over the medial and lateral rectus muscles in both eyes. Dilated fundus examination showed diffuse severe optic atrophy in each eye (Fig. 1A and B). No foveal light reflex or umbo was present in either eye, evidence of profound inner retinal atrophy. Attempts at formal color vision testing could not be completed because of his extremely poor acuity. Audiological testing revealed normal hearing sensitivity with normal middle-ear function. Ipsilateral acoustic reflexes were present but the contralateral acoustic reflexes were present only at 500 Hz with sound presented to the right ear. Distortion product otoacoustic emissions were present bilaterally but with reduced amplitudes, possibly reflecting subtle changes in cochlear mechanics not yet manifested in the behavioral audiogram.

For the 3 year-old girl, her poor visual acuity could not be measured because of her lack of cooperation; however, she avoided objects and moved around unaided. Her pupil function showed a slow direct response to light, but otherwise the anterior segments were structurally normal. Dilated fundus examination revealed diffuse advanced optic atrophy in an otherwise normal-sized optic disk of each eye. She had no foveal light reflex or umbo in either macula, consonant with her advanced optic atrophy. Due to her lack of behavioral cooperation, color vision testing and retinal photography were unobtainable. Audiological testing with condition-play audiometry revealed essentially normal hearing bilaterally. Distortion product otoacoustic emissions were present and robust. Acoustic immittance measures indicated normal middle-ear function; however there were subtle abnormalities in both the ipsilateral and contralateral acoustic reflexes.

Given the suspicion for an inherited optic atrophy, both parents underwent ophthalmologic evaluation and dilated ophthalmoscopic examination. The father had been wearing glasses since his early 20s and was aware of his difficulty with color discrimination. His best corrected visual acuity was 20/25 OD and 20/30 + 2 OS. Upon examination, results of his anterior segment, motility, and biomicroscopic examination were normal. Dilated fundus examination was normal except for the optic nerves and macular areas. The optic nerves on each side were normal in size, cup, and contour, but there was distinct temporal pallor of the papillomacular bundle of each optic nerve, with a clear demarcation between the striated ganglion cell layer, which was missing from approximately 8 o'clock to approximately 10:30 o'clock in the right eye, and in the comparable temporal sector of the left eye. The umbo was minimally detectable and there was no foveal light reflex in either eye. Formal color vision testing demonstrated a distinct tritan error with minimal effect on the protan and deutan axes. Audiological evaluation revealed mild high frequency sensorineural hearing loss in the right ear and mild low frequency sensorineural hearing loss in the left. Acoustic immittance measures indicated normal middle-ear function. Distortion product otoacoustic emissions were present and of normal amplitude in the right ear but of reduced amplitude above 4 kHz in the left ear.

The mother had a myopic refractive error of approximately −4.75 sphere in each eye yielding normal visual acuity (20/20 O.U.). External, adnexal, and biomicroscopic examination were normal. Dilated fundus examination including each optic nerve was normal. Formal color vision testing was normal. Audiological evaluation was significant for bilateral, mild low frequency sensorineural hearing loss with normal middle-ear function. Otoacoustic emissions amplitude was below 2 kHz, suggestive of decreased outer hair cell function.

2. Materials and methods

2.1. Patients and samples

Both probands were reassessed through the Pediatric Genetics Clinic at Texas Children's Hospital, Houston, TX. The probands and their parents were enrolled in research protocols approved by the Institutional Research Boards for Human Subject Research at Baylor College of Medicine, Houston, TX. Total DNA was extracted from muscle and blood specimens with DNA isolation kits (Gentra Systems Inc., Minneapolis, MN, USA) according to the manufacturer's protocols.

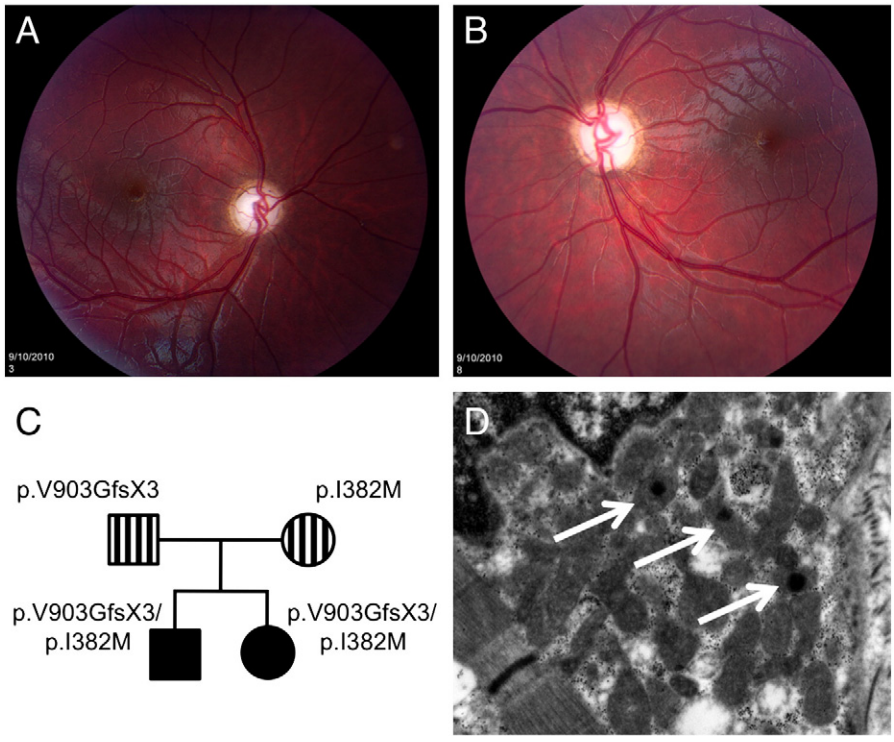

Fig. 1. Compound heterozygosity of *OPA1* mutations. A and B: Images of the right (A) and left (B) fundus show bilateral diffuse, advanced optic atrophy in the reported 8 year-old boy. C: The pedigree shows segregation of respective *OPA1* mutations within this family. D: Dense osmiophilic bodies (marked by arrows) seen on electron microscopy of the boy's skeletal muscle.

2.2. Molecular analysis

Sequence-specific oligonucleotide primers linked to M13 universal primers were designed to amplify all coding exons and at least 50 nucleotides of the flanking intronic regions of the *OPA1* gene. Sequencing reactions were performed with the BigDye Terminator cycle sequencing kit (version 3.1), purified with Performa DTR 96-well V3 short plates (Edge Bio-Systems), and analyzed on an ABI3730XL automated DNA sequencer with Sequencing Analysis Software version 5.1 (Applied Biosystems) and Mutation Surveyor version 2.6.1 (SoftGenetics®, State College, PA). The GenBank sequence NM_015560 was the reference sequence. The mtDNA copy number in muscle was determined by real time quantitative polymerase chain reaction (RT-qPCR) with primers specific for the mitochondrial and the nuclear genes, according to published protocols [5]. MtDNA multiple deletions were analyzed by Southern blot and PCR as previously described [6,7].

2.3. Electron transport chain (ETC) enzyme assay

Spectrophotometric analysis of the respiratory chain complexes was performed on skeletal muscle. Skeletal muscle was immediately frozen in liquid nitrogen after collection, stored at −80 °C, and shipped on dry ice. The electron transport chain (ETC) enzymes were assayed at 30 °C with a temperature-controlled spectrophotometer (Tecan M200 Microplate Reader. Tecan US, Inc. Durham, NC). Each assay was performed in duplicate. The activities of nicotinamide adenine dinucleotide (NADH):ferricyanide reductase (complex I), succinate dehydrogenase (SDH; complex II), rotenone sensitive NADH:cytochrome c reductase (complex I + III), succinate:cytochrome c reductase (complex II + III), ubiquinol: ferricytochrome C oxidoreductase (complex III) and cytochrome c oxidase (complex IV) were measured with appropriate electron acceptors and donors [8,9].

The increase or decrease in the absorbance of cytochrome c at 550 nm was measured for complex I + III, complex II + III, complex III, or complex IV. The activity of NADH: $Fe(CN)_3$ reductase was measured by the oxidation of NADH at 340 nm. For SDH, the reduction of 2,6-dichloroindophenol at 600 nm was measured. Citrate synthase (CS) was used as a marker for the mitochondrial content. Enzyme activities are expressed in nanomoles/min/mg of protein with respect to both the total protein and CS activity [10].

2.4. Audiological

Audiological evaluations for all subjects were completed in a single-wall sound booth with condition play and standard behavioral measurement techniques by a Madsen Itera II (ANSI S3.6-1989) audiometer with insert earphones (Entymotic Research, Elk Grove Village IL). Acoustic immittance measures and acoustic reflex measures were obtained with a Madsen Otoflex 100 (GN Otometrics, Schaumburg, IL). Distortion product otoacoustic emissions were measured by the ERO*SCAN (Maico Diagnostics—Entymotic Research Elk Grove Village IL) with primaries at 65/55 dB SPL from 1.5 kHz through 6 kHz.

3. Results

Sanger sequencing of DNA obtained from peripheral blood revealed compound heterozygosity of a frameshift mutation, c.2708_2711delTTAG (p.V903GfsX3) in exon 27, and a missense mutation, c.1146A>G (p.I382M) in exon 12, in both the 8 year-old boy and his 3 year-old sister. Both mutations have been reported previously in heterozygous state to be associated with ADOA [11,12]. Concurrent parental analyses revealed that the father is heterozygous for the p.V903GfsX3 mutation and the mother is heterozygous for the p.I382M missense mutation (Fig. 1C).

The 8 year-old boy had a normal plasma thymidine level (<300 nanomoles/L) requested due to his gastrointestinal dysmotility, ruling out mitochondrial neurogastrointestinal encephalomyopathy (MNGIE). He underwent a skeletal muscle biopsy as part of his

evaluation for hypotonia, ataxia, gastrointestinal dysmotility, dysphagia, and optic atrophy. The tissue was sent for histology and electron microscopy. Cryostat sections stained with hematoxylin and eosin revealed mild variation in fiber size and shape, with occasional small fibers. Gomori trichrome stain showed increased numbers of mitochondria and ragged red fibers. Rimmed vacuoles and abnormal inclusions were noted. Oxidative enzyme reactions (NADH and COX) demonstrated diffusely increased oxidative activity with scattered fibers with a mild increase in subsarcolemmal oxidative activity. Rare COX depleted fibers were observed. Oil red O staining showed many fibers with increased lipid droplets. Electron microscopic analysis was significant for a mild increase in sub-sarcolemmal and inter-myofibrillar mitochondria, mild increased in lipid droplets, and round osmiophilic dense bodies (Fig. 1D).

Skeletal muscle was used for mitochondrial studies, which revealed absence of multiple deletions of mtDNA by both Southern blot and PCR analyses. Mitochondrial respiratory chain enzyme assays were performed; no enzyme deficiencies were identified (Supplemental Table 1). The mtDNA content in muscle specimen was evaluated by real time qPCR and was within normal limits compared to age- and tissue-matched controls (114% of normal controls, Supplemental Table 2).

4. Discussion

We present the clinical and molecular characterization of two siblings who are compound heterozygotes for disease-causing mutations of the *OPA1* gene. To our knowledge, these represent the second and third reported cases of *OPA1* compound heterozygosity.

While a total of four individuals with two *OPA1* mutations have been reported, compound heterozygosity has only been proven for one of these four. Pesch et al. described a female who was compound heterozygous for two missense mutations (p.E270K and p.R290W) in the *OPA1* gene and documented how compound heterozygosity for these two mutations caused a more severe clinical phenotype at 30 years of age with decreased visual acuity with central scotomas in both eyes. Pattern visual evoked potentials (VEP) demonstrated prolonged latencies, and color vision testing revealed a slight dyschromatopsia with no clear-cut axis [4]. This proband was not described to have any neurological phenotype aside from her optic nerve atrophy. In contrast, both of our probands exhibit severe neurological phenotypes of infantile onset, including hypotonia and ataxia, and profound bilateral optic atrophy of very early onset, which may be explained by either the combinatorial effects of the specific mutations involved or putative genetic modifiers at other loci that are yet undiscovered.

The clinical phenotype of both probands involves dysphagia and lower gastrointestinal dysmotility to the point that the 8 year-old boy requires intermittent manual disimpaction. This could reflect mitochondrial dysfunction in smooth muscle, as it has been observed in individuals with mitochondrial neurogastrointestinal encephalopathy disease (MNGIE), POLG deficiency, *RRM2B* mutations, and other mitochondrial cytopathies [13]. Neither dysphagia nor gastrointestinal dysmotility has been associated with *OPA1* mutations in any prior reports.

To date, no correlation has been observed between the degree of visual impairment and the type of mutation in the *OPA1* gene or the alteration of a specific OPA1 domain. While an increased risk for multisystem neurological disease has been suggested for missense mutations with an odds ratio of 3.06 [3], this inference cannot be generalized and does not imply that individuals with missense mutations are necessarily affected with more severe clinical phenotypes. While the father of our index patients (heterozygous p.V903GfsX3) was diagnosed with mild optic atrophy and bilateral sensorineural hearing loss as part of this study, the mother (heterozygous p.I382M) did not show evidence of optic atrophy, dyschromatopsia, or any detectable change in hearing at age 40 years. This missense mutation was reported in association with autosomal dominant optic atrophy before, but no detailed clinical information regarding the actual phenotype and age of onset is available for that case [11]. The isoleucine at amino acid position 382 of the OPA1 protein is evolutionarily conserved from *Caenorhabditis elegans* to human. While not validated for clinical use, the computer-based algorithms SIFT and PolyPhen-2 predict p.I382M to be deleterious. However, given the clinical non-penetrance of optic atrophy in the mother, one might speculate that p.I382M represents a mild mutation, but that it may still display strong additive effects in these cases of compound heterozygosity, as seen in her children. In addition, if this is a mild mutation, it may go undetected in other clinical situations and thus be missed unless sought at the DNA level. Overall penetrance of *OPA1* mutations varies from family to family, even within a family, and from mutation to mutation. It has been reported as high as 100% (c.1065 + 1 G>T)[14] and as low as 43% (c.2708_2711delTTAG)[15].

Two mouse models of ADOA have been published, designated B6;C3-*Opa1*^{Q285STOP} and B6;C3-*Opa1*$^{329\text{-}355del}$. Both models show ~50% reduction in *Opa1* transcript in retinal tissue and a ~50% reduction in Opa1 protein across a range of tissues, suggesting that haploinsufficiency underlies the pathophysiologic mechanism. Both mutant mouse models are embryonic lethal when homozygous [16,17]. Clinical data presented here suggest that humans are viable with alterations of both *OPA1* alleles. It remains to be seen, however, if other individuals with homozygous or compound heterozygous nonsense or frameshift mutations will be identified.

Given an estimated prevalence of 1 in 35,000 for ADOA in the general population, the chances for compound heterozygosity of two disease-causing mutations would represent a rare event with a projected frequency of 2 in 10^{10}. Since, including this article, three individuals with confirmed compound heterozygosity of *OPA1* mutations have been described, one might suspect that the actual frequency of these events is higher than expected based on sheer statistical grounds. Possible explanations include, but would not be limited to, the failure to detect extremely mild phenotypes of some mutations, or, equivalently, incomplete penetrance of disease-associated mutations and assortative mating among individuals with vision loss, even though vision loss was not present (mother) or clinically significant (father) in the parents reported here.

A role for *OPA1* in mtDNA maintenance has been suggested. This is based on the finding of cytochrome *c* oxidase (COX)-negative fibers in skeletal muscle biopsies from *OPA1*-positive patients, together with the presence of multiple mtDNA deletions on long-range PCR analysis of homogenate skeletal muscle [18]. Interestingly, the mtDNA analysis of the skeletal muscle of our 8 year-old proband did not reveal multiple mtDNA deletions, and electron transport chain analysis and mtDNA content were both within normal limits. This could be related to the fact that whole muscle was used for analysis rather than single-muscle fiber analysis of COX-negative fibers, as it has been shown that mtDNA deletion levels are significantly higher in COX-negative fibers when compared to type I and type II COX-positive fibers [19]. Interestingly, not all patients with dominant optic atrophy and multisystem neurological disease harbor multiple mtDNA deletions and COX deficiency. Moreover, while multiple mtDNA deletions contribute to the pathophysiology of dominant optic atrophy, they seem not to be the sole mechanism responsible for triggering cellular dysfunction, and especially optic nerve degeneration. Another caveat is that molecular observations in skeletal muscle may not be a true reflection of the status in retinal ganglion cells [3]. Lastly, it could be hypothesized that mtDNA depletion and multiple mtDNA deletions may manifest age-dependent penetrance and may become apparent as the disorder progresses. The role of OPA1 in oxidative phosphorylation has been subject to discussion and while some *OPA1* mutations may affect oxidative phosphorylation, others may not [20,21]. Mouse models suggest that the optic atrophy is not caused by deficiencies in oxidative phosphorylation [22]. Mayorov et al. proposed that the

pathophysiology of autosomal dominant optic atrophy stemmed from OPA1's role in mitochondrial structure and fusion and not from OPA1 support of oxidative phosphorylation [21].

In summary, our data strengthen the concept that ADOA is inherited in semi-dominant rather than pure dominant fashion. Compound heterozygosity for disease-causing mutations of the *OPA1* gene can be related to severe optic atrophy of infantile or early childhood onset, accompanied by severe neurological impairment with hypotonia and ataxia.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ymgme.2011.04.018.

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