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OPA1 mutations in patients with autosomal dominant optic atrophy and evidence for semi-dominant inheritance

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We and others have shown recently that mutations in the *OPA1* gene encoding a dynamin-related mitochondrial protein cause autosomal dominant optic atrophy (ADOA) linked to chromosome 3q28–q29. Here we report screening of the *OPA1* gene in a sample of 78 independent ADOA families. *OPA1* mutations were identified in 25 patients (detection rate 32.1%) including 16 novel mutations. We successfully amplified *OPA1* cDNA prepared from leukocyte RNA of three patients, and found the amount of transcripts harboring the Arg366Stop mutation was significantly reduced compared with transcripts derived from the normal chromosome. Analysis of the distribution of *OPA1* mutations in ADOA revealed that most missense mutations cluster within the putative GTPase domain, and that there is a preponderance of mutations, which result in premature translation termination. These observations support the notion that haploinsufficiency may represent a major pathomechanism for ADOA. In addition, we identified an ADOA patient who is a compound heterozygote for two *OPA1* missense mutations. The fact that this patient is by far more severely affected than her simple heterozygotic parents and siblings implies that at least these *OPA1* alleles behave semi-dominantly rather than purely dominantly. Clinical examination revealed considerable variability in disease expression among patients carrying *OPA1* mutations and no strict correlation with either the position or the type of mutation.

INTRODUCTION

Autosomal dominant optic atrophy (ADOA) is the most frequent form of hereditary optic neuropathies with an estimated disease prevalence ranging between 1:12 000 (1,2) and 1:50 000 (3) in different populations. ADOA is a progressive disorder with onset in the first decade of life, and clinically characterized by a loss of visual acuity, development of central, paracentral or coecocentral scotomas, bilateral atrophy of the optic nerve and color vision disturbances (4–6). ADOA exhibits variable expression even within families, and in some families asymptomatic carriers have been observed (4,7,8). Histological post-mortem examination of affected donor eyes suggests that the fundamental pathology results from a degeneration of the retinal ganglion cells followed by ascending atrophy of optic nerve fibers (9,10). A first locus for ADOA (*OPA1*, OMIM 165500) was mapped to chromosome 3q28–q29 in Danish families (11). This locus was subsequently confirmed and refined by studies in British, American, Cuban and French families (12–16). However, ADOA is genetically heterogeneous and a second locus (*OPA4*) linked to markers on chromosome 18q12.2–q12.3 has been described in an American family of German descent (17).

Refined genetic mapping placed the *OPA1* gene within a 1–2 cM interval flanked by the marker loci D3S3669 and D3S3562 (18). Applying a positional cloning approach, yeast artificial chromosome and P1-derived artificial chromosome contigs were established and used to localize the expressed sequence tag SHGC37414, adopted from a brain cDNA of unknown function (KIAA0567), within the disease critical region (19). Subsequently, we and others were able to demonstrate that mutations in this gene, now designated the *OPA1* gene, cause ADOA linked to chromosome 3q28–q29 (20,21). The *OPA1* gene consists of 28 coding exons covering a genomic region >60 kb. The gene is widely expressed, but most abundant in the retina. The *OPA1* gene encodes a 960 amino acid polypeptide with similarities to specific GTP-binding proteins of the dynamin protein family (20). The presence of typical sequence features (i.e. basic leader, MPP and MIP cleavage sites) at the N-terminus suggests that the OPA1 polypeptide is imported into the mitochondrion, and may represent the human ortholog of the *Saccharomyces cerevisiae*

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*Mgm*I and the *Schizosaccharomyces pombe Msp*I proteins, which are important for mitochondrial inheritance and maintenance in these organisms (22,23).

In this report, we addressed the spectrum and distribution of *OPA1* mutations in a panel of 78 unrelated ADOA index patients, and the detectability of *OPA1* mutations in leukocyte cDNA. The identification of a considerable number of novel *OPA1* mutations enabled us to establish a first profound mutation spectrum of the *OPA1* gene which provides new insights into the disease mechanism of ADOA. Furthermore, we describe an example of a severely affected patient with two compound heterozygous *OPA1* mutations, which provides first evidence for a semi-dominant inheritance of ADOA.

RESULTS

Identification of *OPA1* mutations in ADOA patients

We screened 78 independent cases with a clinical diagnosis of ADOA for the presence of mutations in the *OPA1* gene. All 28 coding exons were analyzed by either complete direct sequencing of genomic PCR fragments (20 index patients) or combined single-strand conformation polymorphism (SSCP)/heteroduplex analysis followed by direct sequencing of samples showing apparent band shifts (58 unrelated patients).

In total, we identified *OPA1* mutations in 25 out of the 78 ADOA patients (detection rate 32.1%). Four of these cases have been described previously (20). Among the remaining patients, we identified 18 different mutations including seven missense mutations (808G→A/Glu270Lys, 818A→C/Asp273Ala, 868C→T/Arg290Trp, 869G→T/Arg290Gln, 1313A→T/Asp438Val, 1402A→G/Lys468Glu, 2354A→G/Gln785Arg), two stop codon mutations (6G→A/Trp2Stop and 629C→A/Ser210Stop), one in-frame deletion (1651–1653delTGC/Cys551del), five frameshift (fs) mutations (932delC/Ala311fs, 1072–1093del/Ala358fs, 1152–1153delAA/Leu384fs, 2125–2138del-insGGATCGACACAC/Ile709fs, 2708–2711delTTAG/Val903fs) and three putative splice site mutations (1065+3A→C, 2611+1G→C and 2707+2T→C) (Table 1). Of these mutations, only two, the Arg290Gln missense mutation found in family OAK51 and the 2708–2711delTTAG deletion detected in families OAK52 and OAK54, have already been found in other ADOA families (20,21). Additional novel recurrent mutations include one stop codon mutation, Trp2Stop, in families OAK55 and OAK87, and two missense mutations, Asp438Val in families OAK27 and OAK28 and Lys468Glu in families OAK8 and OAK16.

Cosegregation analysis by PCR/restriction fragment length polymorphism (RFLP) analysis or SSCP analysis was performed in families with several affected members, for which samples were available (12 families). In these families, all affected members consistently carried the mutant allele. Furthermore, we excluded all mutations in a panel of 100 healthy controls (n = 200 chromosomes).

In addition, we identified a number of polymorphisms within the coding and flanking intron and untranslated region (UTR) sequences of the *OPA1* gene (Table 2).

Detection of mutations in leukocyte RNA

The *OPA1* gene is widely expressed in various tissues albeit at varying abundances (20). We therefore addressed the question of whether the *OPA1* gene is also expressed in blood leukocytes, which might allow simplification of mutation detection by analyzing *OPA1* cDNA. We isolated total leukocyte RNA from three ADOA patients, one carrying the 1313A→T/Asp438Val missense mutation, one with the 1096C→T/Arg366Stop nonsense mutation and, finally, one patient with the 1065+3A→C putative splice site mutation. Single-stranded cDNA was synthesized by reverse transcription and used to amplify overlapping cDNA fragments for subsequent direct DNA sequencing. Comparative inspection of genomic and cDNA sequence profiles revealed that the relative amount of transcripts from the missense allele is only slightly reduced while the transcripts derived from the nonsense mutation allele are considerably under-represented in leukocytes (Fig. 1A). Semi-quantitative RT–PCR/RFLP analysis further confirmed the reduced expression of the Arg366Stop allele (Fig. 1B). Sequencing of the *OPA1* cDNA prepared from the patient with the 1065+3A→C substitution revealed that this alteration induces the skipping of the preceding exon 10 in mature transcripts (data not shown).

Two mutant *OPA1* alleles are present in a severely affected ADOA patient

Two heterozygous missense mutations, 808G→A/Glu270Lys and 868C→T/Arg290Trp, both located in exon 8 of the *OPA1* gene, were identified in the index patient of family OAK86. Segregation analysis within the family revealed that the patient is compound heterozygous for these mutations since she inherited the Glu270Lys mutation from her father and the Arg290Trp mutation from her mother. Her siblings are simple heterozygotes for either one of the two mutations (Fig. 2). Clinical examination showed that the compound heterozygous index patient is the most severely affected family member. She presented with visual acuities of 0.2 and central scotomas on both eyes at 30 years of age. Pattern VEP showed prolonged latencies, and color vision testing revealed a slight dyschromatopsia (imperfect color discrimination) with no clear-cut axis of confusion. Fundus examination revealed total pallor of optic discs on both eyes. In contrast, both parents and the siblings presented with a much milder disease manifestation; visual acuities were normal or only slightly reduced. Visual fields were normal, as well as color vision. Pattern VEPs were mostly in the normal range or showed only slightly prolonged latencies. However, we consistently observed pallor of the optic discs which was taken as a main evidence for the presence of a pathological condition affecting the optic nerve. We thus suggest that both missense mutations observed in this pedigree represent, *per se*, mild disease alleles, with an additive effect if encountered in a single subject.

Types and distribution of *OPA1* mutations

The delineation of disease-causing mutations for a given gene provides a valuable data source for the identification of domains critical for the protein's proper function. This strategy is particularly useful for novel proteins, such as *OPA1*, on whose function one can only speculate.

Table 1. *OPA1* mutations detected in ADOA patients

No. of ADOA families[a,b]	Exon	Nucleotide change[c]	Alteration in polypeptide/mRNA	Reference
2	1	6G→A	Trp2Stop	This work
1	6	629C→A	Ser210Stop	This work
1*	8	808G→A	Glu270Lys	This work
1	8	818A→C	Asp273Ala	This work
1*	8	868C→T	Arg290Trp	This work
1	8	869G→T	Arg290Gln	(20); this work
	9	899G→A	Gly300Glu	(21)
1	9	932delC	Ala311fs	This work
	10	985–1G→A	Splice defect	(21)
	10	1016delC	Ala339fs	(20)
1	10	1065+3A→C	Splice defect	This work
1	11	1072–1093del	Ala358fs	This work
	11	1096C→T	Arg366Stop	(20)
1	12	1152–1153delAA	Leu384fs	This work
	13	1296–1298delCAT	Ile432del	(20)
2	14	1313A→T	Asp438Val	This work
	14	1354delG	Val452fs	(20)
2	14	1402A→G	Lys468Glu	This work
	17	1644–1645insT	Val548fs	(20)
1	17	1651–1653delTGC	Cys551del	This work
	20	Deletion of entire exon	Trp616fs	(20)
1	21	2125–2138del insGGATCGACACAC	Ile709fs	This work
1	23	2354A→G	Gln785Arg	This work
1	25	2611+1G→C	Splice defect	This work
1	26	2707+2T→C	Splice defect	This work
2	27	2708–2711delTTAG	Val903fs	(21); this work
	28	2823delAGTT	Lys941fs	(21)

[a]Number of ADOA families found in the present study.
[b]An asterisk (*) denotes the two mutations segregating in family OAK86 (Fig. 2).
[c]Nucleotide designation commencing 1 at position 56 (translation start) of GenBank accession no. AB011139.

Combining our data with those of the two initial reports (20,21), 27 different *OPA1* mutations in 35 ADOA families have been identified so far (Table 1; Fig. 3). Frameshift-causing insertions and deletions account for the majority of the different mutations (10/27), followed by missense mutations (8/27), splice site mutations (4/27), stop mutations (3/27) and in-frame deletions (2/27). Thus, the spectrum of *OPA1* mutations points out a clear preponderance of mutations which result in truncated *OPA1* polypeptides. There is a considerable diversity of OPA1 mutations, which contrasts the proposed strong founder effects in British and Danish ADOA patients based on haplotype comparisons (24,25). Only the 2708–2711delTTAG mutation, which has been previously observed in three families originating from France and Belgium (21) and two further German families in this study, displays a notably increased frequency.

The mutations are not evenly distributed along the length of the OPA1 polypeptide. There is a significant clustering of more than half of all different mutations between residues 270 and 468 (Fig. 3). Interestingly, this segment, which corresponds to the putative GTPase domain of the OPA1 protein, includes all but one of the identified missense mutations. The GTPase domain represents the most conserved part of the OPA1 protein when compared with other members of the dynamin protein family. This domain includes the typical tripartite GTP-binding motif needed for phosphate binding (GXXXXGKS/T), coordination of Mg^{2+} (DXXG) and nucleotide binding (T/NKXD) (22,26), and the dynamin sequence signature (27). A minor cluster of mutations, including two frameshift and two splice site mutations, is located in close vicinity to the C-terminus of the polypeptide. This suggests that the C-terminal sequence is essential for effective

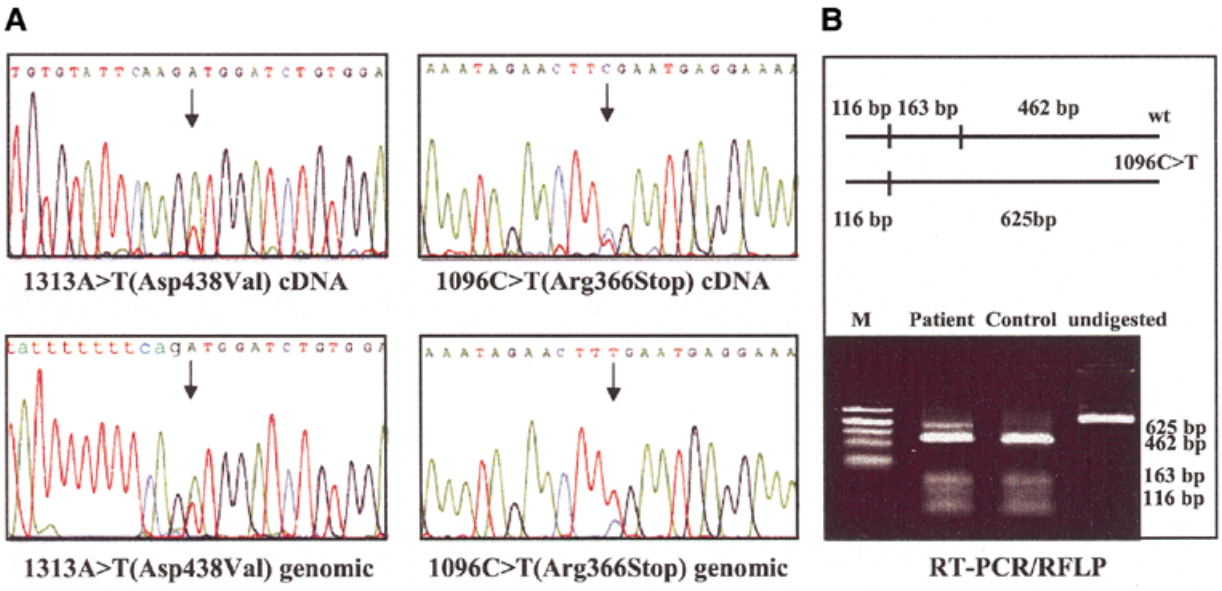

Figure 1. Mutant *OPA1* transcripts are reduced in amount in leukocytes. (**A**) Comparative sequencing of leukocyte cDNA (top panel) and genomic DNA (bottom panel) in patient WH with the Asp438Val missense mutation (left) and patient AS with the Arg366Stop mutation (right). Exon sequences are shown in upper case and intron sequences in lower case letters. Note that mutant transcripts are reduced in amount, in particular for the stop codon mutation. (**B**) Semi-quantitative RT–PCR/RFLP analysis of leukocyte *OPA1* transcripts in patient AS. Schematic delineation of *Taq*I sites on wild-type and 1096C→T mutant *OPA1* cDNA fragments (top) and corresponding electrophoretic separation of *Taq*I-digested RT–PCR products (bottom). Note the different abundancies of the mutant-specific 625 bp fragment relative to the wild-type-specific 462 bp fragment in the patient sample. M, marker DNA (pBR322/*Alu*I).

expression and/or stability of the *OPA1* transcript, or contains important elements for the structural or functional integrity of the polypeptide.

Phenotypes of ADOA patients with *OPA1* mutations

Table 3 summarizes the clinical findings of index ADOA patients with *OPA1* mutations. In most patients, disease onset was within the first decade of life. However, two patients, OAK51 and OAK28, reported much later onset of visual complaints. All index patients examined consistently showed pallor of the optic disc, which was mostly confined to the temporal half. Visual acuities varied from 0.05 to 0.8 but were similar for both eyes. Central and/or coecocentral scotomas were the visual field defects most commonly detected. One patient (OAK26) presented with peripheral constriction of the visual field combined with bilateral central scotoma. Color vision (tested with the saturated and the desaturated Panel D 15 test) ranged from normal to severe dyschromatopsia (imperfect color discrimination) without clear-cut axis of confusion. Only a few patients presented with classical tritanopia (lack of short-wavelength sensitivity). Severity of symptoms correlated with neither the type nor the site of the mutation, nor the patient's age. Even patients carrying the same mutation showed highly variable disease expression (i.e. Val903fs mutation in OAK52 and OAK54). Whereas both index patients with the Trp2Stop mutation present with rather good visual acuities (0.7–0.8) and tritanopia, clinical examination of the affected father of subject OAK55/EK revealed a visual acuity of 0.5/0.4, similar to other ADOA cases.

Table 2. Polymorphisms identified in the *OPA1* gene

Exon	Nucleotide change	Alteration in polypeptide/ mRNA
2	321G→A	Silent
3	420G→T	Silent
4	473A→G	Asn158Ser
5	575C→T	Ala192Val
8	870+4C→T	–
14	1443+23G→A	–
17	1608A→C	Silent
17	1648G→A	Asp550Asn
21	2109C→T	Silent

DISCUSSION

We and others have shown recently that mutations in the *OPA1* gene encoding a dynamin-related mitochondrial protein are responsible for ADOA linked to chromosome 3q28–q29 (20,21). In this study, we address the prevalence of *OPA1* mutations in clinically defined ADOA cases, the spectrum of mutations and their distribution along the OPA1 protein, and the expression of mutant transcripts in leukocytes.

Among the 78 independent ADOA patients, we detected mutations in 25 cases, giving an overall detection rate of 32.1% of *OPA1* mutations in clinical ADOA patients. This rate did not differ significantly between the sub-sets of patients screened by complete DNA sequencing (20 index patients) or SSCP/heteroduplex analysis (58 index patients), indicating sensitive performance of our SSCP/heteroduplex protocol.

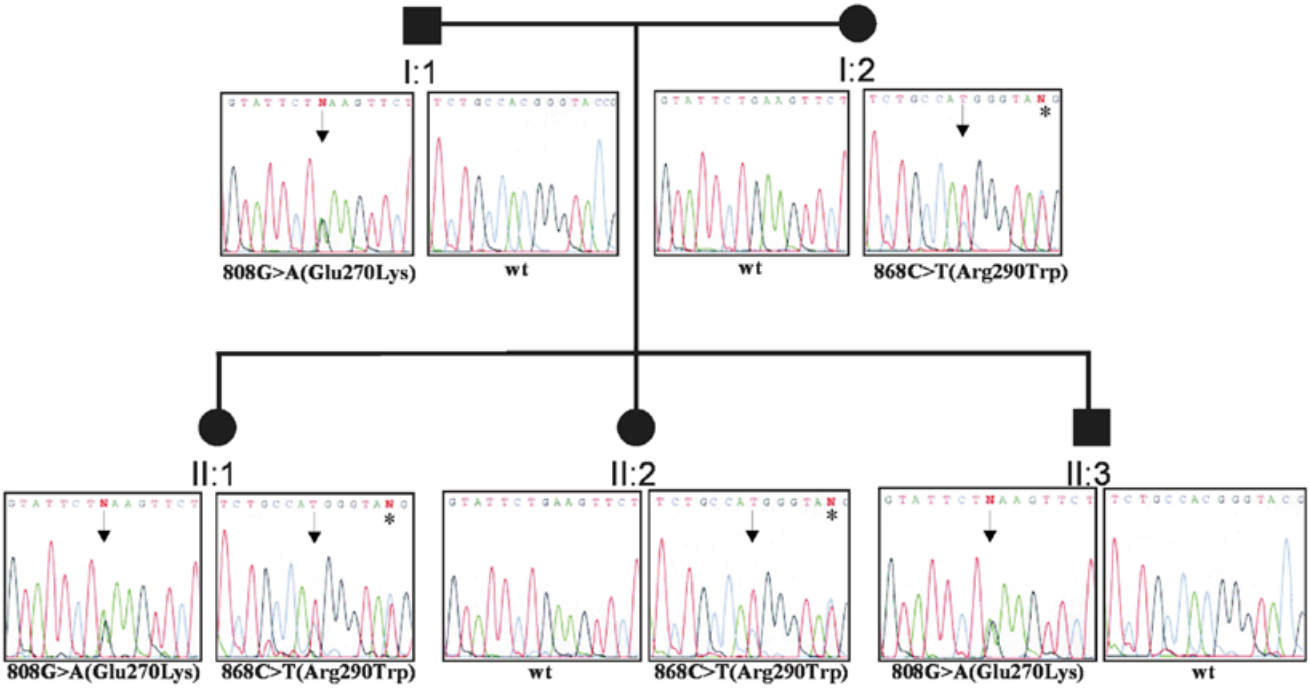

Figure 2. Two *OPA1* mutations segregate in family OAK86. Segregation of the 808G→A(Glu270Lys) and the 868C→T(Arg290Trp) mutations (arrows) is shown by sequence traces for all available family members. Closed squares and circles indicate that both parents and all children are affected by ADOA. Whereas subjects I:1, II:3, I:2 and II:2 are simple heterozygotes for the mutations 808G→A(Glu270Lys) and 868C→T(Arg290Trp), respectively, subject II:1 is a compound heterozygote for both *OPA1* mutations. An additional intronic polymorphism (870+4C→T) associated with the 868C→T(Arg290Trp) mutant allele is indicated by asterisks.

Patients and families analyzed in this study were collected mainly on the basis of clinical diagnosis and included cases in which autosomal dominant inheritance could not be unequivocally established. Of the 25 index patients with *OPA1* mutations, 21 belonged to the group of cases with evidence for dominant inheritance [21/50 (42%) detection rate], three patients were simplex cases [3/16 (18.75%) detection rate] and one patient belonged to a family with affected individuals in only one generation [1/3 (33.33%) detection rate]. This observation indicates that *OPA1* mutations are predominantly present in multi-generation families, and it illustrates the suspicious genetic nature of the disease in sporadic cases and families with affected siblings only. These may represent examples of recessively X-linked or mitochondrially inherited disorders, clinically similar to ADOA [i.e. Leber's hereditary optic neuropathy (LHON).] The actual prevalence of *OPA1* mutations might be even higher if we take into consideration possible mutations in the, as yet undefined, promoter region of the *OPA1* gene, and larger deletions, which remain undetectable by PCR-based screening approaches. On the other hand, it is reasonable to suggest that non-allelic genetic heterogeneity contributes to the large fraction of ADOA patients lacking *OPA1* mutations in our screen. Recently, a second locus for ADOA has been mapped to chromosome 18q12 (17). The fact that this linkage study was performed on a family of German descent implies that some of our mostly German ADOA families might also be linked to this locus.

The sensitivity and practicability of *OPA1* screening could perhaps be improved by cDNA analysis. We were able to show that *OPA1* cDNA can be reliably amplified from leukocyte RNA and that putative splicing defects can be confirmed by this approach. We found that in leukocytes the 1313A→T/Asp438Val allele and particularly the 1096C→T/Arg366Stop allele are expressed at reduced levels in comparison to the wild-type allele (Fig. 1).

Studies in other genetic disorders and yeast have shown that transcripts containing premature stop codons are frequently subject to degradation (28). It has been observed that if the position of the premature termination is located >50 bp proximal to the final exon/intron junction, the mutant mRNA will fall to degradation. More distally terminated transcripts are usually stable (29,30). Whereas such a nonsense decay mechanism holds true at least for the nonsense mutation for *OPA1* leukocyte transcripts, we did not observe selective skipping of the exon containing the stop mutation as described by Dietz and Kendzior Jr (31). Although cDNA-based screening is an attractive alternative to conventional exon-by-exon procedures, further studies will be necessary to determine its actual sensitivity for the screening of the *OPA1* gene in ADOA.

Nonsense mutations including stop codon, frameshift and splice site mutations represent the majority of all *OPA1* mutations involved in ADOA. At least some of them obviously represent functional null mutations since they produce severely truncated polypeptides, lacking the putative GTPase domain and the dynamin-related core region. The Trp2Stop mutation, for instance, results in an instant termination of translation and, thus, practically does not produce any product. Moreover, our

Table 3. Clinical phenotype of ADOA index patients

Patient	Mutation[a]	Age (years)	Age of onset[b] (years)	Visual acuity OD/OS	Color vision[c]	Visual fields	Optic disc	Fundus presentation
OAK87/HT	Trp2Stop	18	ch	0.7/0.8	Tritanopia	Relative central scotoma	Temporal pallor	Normal
OAK55/EK	Trp2Stop	14	ch	0.8/0.8	Tritanopia	Normal	Temporal pallor	Normal
OAK19/SD	Ser210Stop	13	1	0.2/0.2	NA	NA	Temporal pallor	NA
OAK86/WH	Glu270Lys Arg290Trp	30	ch	0.2/0.2	Dyschromatopsia	OD/OS central scotoma	Complete pallor	Normal
OAK41/PH	Asp273Ala	32	6	0.2/0.2	Dyschromatopsia	OD/OS central-coecocentral scotoma	Temporal pallor	Normal
OAK51/GE	Arg290Gln	50	46	0.4/0.4	Dyschromatopsia	Central scotomas	Temporal pallor	Normal
OAK59/E	Ala311fs	35	ch	0.2/0.3	NA	Relative scotoma	Temporal pallor	NA
OAK30/PD	1065+3A→C	41	NA	0.1/0.3	OD dyschromatopsia, OS tritanopia	OS unspecific scotoma	Temporal pallor	Normal
OAK58/E	Ala358fs	41	ch	0.16/0.2	NA	Relative scotoma	Temporal pallor	NA
OAK18/AS	Arg366Stop*	35	8	0.6/0.6	Dyschromatopsia	Normal	Temporal pallor	Normal
OAK32/LB	Leu384fs	42	ch	0.5/0.5	Tritanopia	Normal	Temporal pallor	Normal
OAK28/WH	Asp438Val	57	29	0.3/0.3	Dyschromatopsia	OD/OS central scotoma	Temporal pallor	NA
OAK9/SG	Val452fs*	56	ch	0.5/0.5p	Dyschromatopsia	NA	Temporal pallor	Normal
OAK8/BW	Lys468Glu	27	12	0.4/0.3p	Dyschromatopsia	NA	Temporal pallor	Normal
OAK16/RA	Lys468Glu	12	ch	0.3/0.4	Dyschromatopsia	NA	Temporal pallor	Normal
OAK34/BA	Val548fs*	43	8	0.2/0.2	Dyschromatopsia	Paracentral scotoma	Temporal pallor	Normal
OAK26/PC	Cys551del	38	2	0.04/0.04	NA	OD/OS central scotoma, constricted peripheral visual fields	Temporal pallor	Macula alterations
OAK36/E	Ile709fs	26	4	0.05/0.05	Deuteranopia	Centrocoecal scotoma	Temporal pallor	NA
OAK80/HJ	Gln785Arg	38	NA	0.4/0.5	Tritanopia	Central scotoma	Temporal pallor	Normal
OAK37/E	2611+1G→C	14	6	0.3/0.3	Tritanopia	Paracentral scotoma	Temporal pallor	NA
OAK54/KA	Val903fs	27	6	0.125/0.125	Dyschromatopsia	Coecocentral scotoma	Temporal pallor	Normal
OAK52/TE	Val903fs	44	5	0.5/0.5	NA	Relative scotoma	Temporal pallor	NA

[a]Asterisks represent patients described by Alexander *et al.* (20).
[b]ch, childhood.
[c]Dyschromatopsia, with no clear-cut axis of confusion.
NA, data not available.

analysis on leukocyte cDNA shows that the level of transcripts harboring nonsense mutations is considerably reduced at least in these cells. These observations suggest that haploinsufficiency for *OPA1* might be a mechanism in ADOA. A single functional allele might be insufficient to maintain the maximum level of *OPA1* transcripts observed in the retina, and thus explains why the clinical phenotype is restricted to the visual system although the gene is widely expressed (20). Preliminary data from *in situ* hybridization experiments indicate that within the retina *OPA1* is predominantly expressed in the ganglion cell layer—an observation that is consistent with the suggested cellular pathology in ADOA (unpublished data). Although the function of the OPA1 polypeptide is not known, the presence of a typical leader sequence and the cellular colocalization of tagged OPA1 proteins with mitochondrial markers strongly support that the OPA1 polypeptide is imported into mitochondria (20,21). Interestingly, OPA1 shares significant homology with *Mgm1* from *S.cerevisiae* and *Msp1* from *S.pombe*, genes known to be important for mitochondrial inheritance and maintenance in these organisms (22,23). Using antibodies against the mitochondrial marker protein HSP60, Delettre *et al.* (21) observed disorganization of the mitochondrial network in monocytes of two ADOA patients with the *OPA1* 2708–2711delTTAG mutation. These observations suggest that ADOA caused by *OPA1* mutations is a mitochondriopathy. This relationship is remarkably similar to LHON, which is caused by mutations in mtDNA-encoded genes for subunits of the complex I of the respiratory chain (32). It has been suggested that in this case the synergistic effect of mtDNA mutations and environmental factors eventually result in a decrease of oxidative phosphorylation (OXPHOS) capacity below a threshold that is critical for the functional integrity of retinal ganglion cells (33). However, this model does not explain why only certain mitochondrial

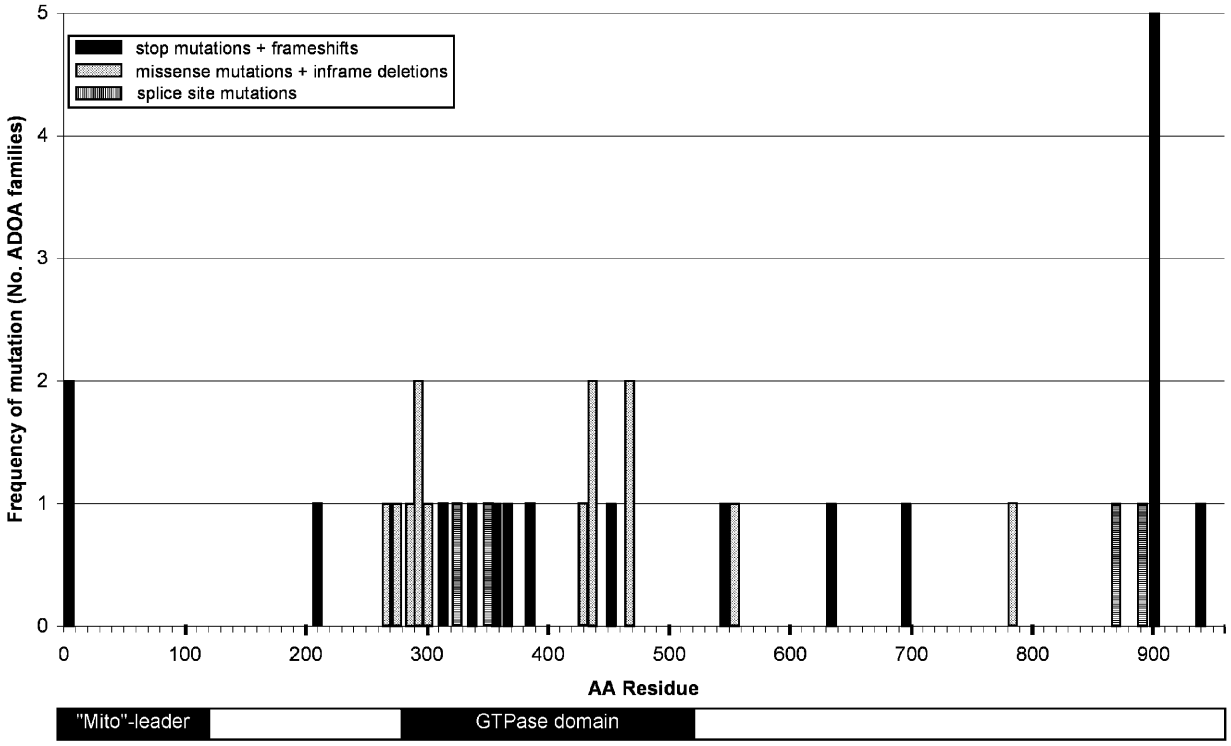

Figure 3. Delineation of all known disease-causing mutations in the *OPA1* gene.

defects result in optic atrophies and others do not. Further studies will be necessary to elucidate the higher susceptibility of retinal ganglion cells for these specific defects.

Most missense mutations cluster within or close to the putative GTPase domain and may reduce or prevent GTP-binding and/or GTP-hydrolysis. This suggests that GTPase activity is of particular importance for the functioning of the OPA1 protein. Whether such missense mutations represent functional null alleles compatible with the hypothesis of haploinsufficiency or rather induce a dominant-negative effect cannot be resolved at this stage. If the latter holds true, specific targets for such aberrant OPA1 proteins must exist in retinal ganglion cells. Remarkably, we did not observe any missense mutations at the N-terminus of the OPA1 protein, which is thought to represent the signal sequence for the import of the OPA1 protein into the mitochondrion (20,21). However, since these signal sequences are less conserved in primary sequence but rather an accumulation of basic residues followed by a weakly conserved cleavage site for mitochondrial import peptidases, amino acid substitutions may be more tolerable in the signal sequence than elsewhere in the OPA1 polypeptide sequence.

Interestingly, we found two distinct *OPA1* mutations segregating in one ADOA pedigree. While both affected parents and two of the affected children are simple heterozygotes for one or the other mutation, the third, most severely affected child is a compound heterozygote for both mutations. This, to our knowledge, is the first case of a patient carrying two mutant *OPA1* alleles. Given an incidence of 1:12 000 to 1:50 000 for ADOA, this coincidence of mutant alleles represents a very rare situation. However, there are examples of preferential mating of subjects with the same condition due to social adherence. This case leads to two important conclusions: taking into account the severity of the clinical picture in the compound heterozygous patient, ADOA must be considered as a semi-dominant condition rather than a purely dominant disorder. The case also documents that the presence of two mutant *OPA1* alleles is not lethal, *per se*, and even the clinical presentation can still be restricted to a loss of visual function. However, since both mutations cause only amino acid substitutions and, obviously, represent mild disease alleles, it may be hypothesized that these mutations merely disturb rather than completely inhibit OPA1 function.

ADOA patients with *OPA1* mutations present with highly variable expression of visual symptoms. Visual acuities ranged between 0.04 and 0.8, and were mostly correlated with the presence of central or near central scotomas. Whereas pallor of optic discs was common in all patients, tritanopia was not a consistent clinical feature. In most patients, we rather noticed a general reduction in color matching capabilities without any clear axis of confusion.

Genotype–phenotype comparisons were mostly inconclusive. Neither the type of mutation (missense versus nonsense) nor the location of the mutation within the polypeptide follows a conclusive pattern. This may be explained by the variability in clinical expression between affected family members as reported in previous clinical studies and as also seen in this study between non-related patients carrying the same *OPA1* mutation. Further studies in ADOA families and affected twins will be necessary to address the question of whether other genetic and/or epigenetic factors are responsible for the variability in clinical expression.

Table 4. *OPA1* primers used in mutation analysis

Exon/cDNA segment	Forward primer (5′→3′)	Reverse primer (5′→3′)
1	ACT TCC TGG GTC ATT CCT GG	TCT GGG AAT TCT CCA ACT GC
2	TGC TCT TTT AAT GCC ATT TCC	CAT CCA ATT GTA TTC CAC TAC ACA A
3	AAT TTT TCT TTA CAT GTT TAT TTG GC	TTT CTC TTT CCT CGA GAT GAC C
4	TTT TGT AGT GGT TGT CAT GAG G	AAA AAT GTC CTG TTT TTC ATT GG
5	TGG AGA ATG TAA AGG GCT GC	TCT TTC AAG ACT ACC TAC ATG AAC AA
6	AAA AAT TTA ACT TGC TGT ACA TTC TG	CAC CTT CCA AAT TTT GCT CTG
7	ACT ATT TGA TAA CCA TCT TTT GC	CAG CTC CTT AGA AAC TGG TAC TGA
8	CCG TTT TAG TTT TTA CGA TGA AGA	TTT TTG CTA GTT GGC AAG TTC A
9	AGA GCA GCA TTA CAA ATA GGT TTT	CAG GTT TCC CTG AAG CAG TT
10 + 11	CTG TCT AGA CCA CAT ACG GGC	CCA TAA AAC GTC ACT GAA ATG AA
12 + 13	AAA TTC TTG ACA AAT TCC CCC	CGA AGA GAA GGC AAA AAT GC
14	TTG CTA TAA TGT AGA CAC AGG GG	TAT CAC AGC TGA GCT TTT ACA
15 + 16	AGC ATT ATT TTG CTT TCT AAA TTG T	TGA AAA CAG TTC AAT TTA AGC TAC TC
17	CTG TTA GCA AGC ACA TTC GC	TAT GGA TGC CAA AGA TTG CC
18	ACA TCT GGA AAG AAG GAG GG	CCC ACT AAA TTA CAG GAA TAC ACG
19	CAG CCT AGT CAA AAA CCT CCC	CAA GGC AAC AAT AAA TCA CTG C
20	TCT AAA ATT CAC AGC TCC TAC TCC	TGA CTG GTG CGA TTT ACA GG
21	TTT GGC TTG AGC TCG TGT TA	CCT ATG AAA AAG TAT CAA TTT GAG AAG
22	TTT TTC CAT ATT TAC TAA GCT GTC AA	GAC TCC TTC ACC ACT GTG AAC TC
23	TTT TTC CTT TAT TTC AAC TGC C	TGG TCT AGA GCC ACA AAA AGG
24	TTG AGA CTG TTT TTC AAG CAC C	CAC GTG ACA AAA GTC AAA TTA AGC
25	TTT TTG TAC AAC TTC TCA GTG TGG	TTT CCC CAG ATG ATC AAA GG
26	ATG CTG AAT TTC ATG GCT CC	TGG GAA GTA TTT TGG CAT CC
27	TTC ATT TAT AAA AAC GAT GCT CC	GAT TAC AAG CGT GAG CCA CC
28	CCT CCT GAT TTG TGA TAC CTT TG	CAA GCA GGA TGT AAA TGA AGC A
Segment 1	CTC CCG CGT GGC CGT CT	CAG ATG ATC TTG CGT ATT A
Segment 2	ATC GTG GAT CTG AAA GTG ACA AGC	TGT TGT TCA ACA GAC TCT CGT ACC AT
Segment 3	TCA GCA GAT AAT TGA AGG AAA GC	ATG TCA TCA TGC TCT TTC CC
Segment 4	CTT CAA CAA TCT TTG TGG GAA AGA G	AAA GAG GCT GGA CAA AAG ACG TTG
Segment 2*	GAT GTT CTC TCT GAT TAT GAT GCC	TTC ATA TTC TCT TAT AGC TTC AAT GC

MATERIALS AND METHODS

Patients and DNA samples

Patients were seen at various ophthalmological institutions, and the diagnosis of ADOA was based on ophthalmological examinations including visual acuity, visual field and color testing, fundoscopy and electrophysiology. In total, our sample comprises 78 independent cases with a clinical diagnosis of ADOA. Fifty patients were from pedigrees with affecteds in at least two subsequent generations, three were from families with multiple affected siblings and 16 patients were sporadic. For nine cases no family history was available. None of the families underwent prior marker analysis for linkage to the *OPA1* locus on chromosome 3q28. Blood samples were taken from patients and family members after informed consent had been obtained. Genomic DNA was extracted from the blood samples according to standard procedures.

Genomic DNA amplification

Oligonucleotide primers (Table 4) for the amplification of all 28 coding exons were designed from flanking intron or UTR sequences using the Primer Picking Program available at the MIT/Whitehead Institute web site (http://www-genome.wi.mit.edu/). Standard 50 µl PCR reactions were carried out in 10 mM Tris–HCl pH 8.9, 50 mM KCl, 1.5–3 mM $MgCl_2$ and 200 µM of each dNTP including 50–100 ng genomic DNA, 10 pmol of corresponding forward and reverse primers and 1 U AmpliTaq polymerase (Applied Biosystems, Weiterstadt, Germany). Typical cycling conditions were 94°C for 4 min, 35 cycles of 30 s at 94°C, 30 s at 53°C and 30 s at 72°C, and a final extension step of 7 min at 72°C.

SSCP/heteroduplex analysis

For SSCP/heteroduplex analysis, PCR fragments were separated on 20 × 20 cm, 10% non-denaturing polyacrylamide gels either for 12 h at room temperature or for 17 h at 4°C for gels including 10% glycerol. Gels were silver-stained and samples showing band shifts for single- or double-strand products underwent direct DNA sequencing.

Mutation detection in leukocyte RNA

Leukocytes were isolated from total venous blood by Ficoll-Paque density centrifugation (Pharmacia Biotech, Freiburg, Germany) and total RNA was extracted with Trizol reagent (Life Technologies, Eggenstein, Germany). Oligo(dT)-primed total leukocyte RNA was reverse transcribed into single-stranded cDNA and used to amplify four overlapping cDNA segments (segments 1–4, PCR primers; Table 4) according to the manufacturer's recommendations (RNA PCR Kit; Takara, Shiga, Japan). For semi-quantitative RFLP analysis of the 1096C→T mutation, a 741 bp cDNA fragment was amplified with segment 2* primers (Table 4). RT–PCR products were purified, digested with *Taq*I and separated on a 1.8% agarose gel.

Sequencing

PCR and RT–PCR products were either purified by ultra-filtration (Centricon-100 cartridges; Amicon, Bedford, USA) or Qiaquick columns (Qiagen, Hilden, Germany). The samples were sequenced using Big Dye Terminator chemistry (Applied Biosystems, Weiterstadt, Germany) and separated on an ABI 377 DNA sequencer. The Lasergene Software package (DNASTAR, Lasergene Cooperation, London, UK) was used for editing and alignment of sequences.

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