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# Spectrum, frequency and penetrance of *OPA1* mutations in dominant optic atrophy

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**Dominant optic atrophy (DOA) is the commonest form of inherited optic neuropathy. Although heterogeneous, a major locus has been mapped to chromosome 3q28 and the gene responsible, *OPA1*, was recently identified. We therefore screened a panel of 35 DOA patients for mutations in *OPA1*. This revealed 14 novel mutations and a further three known mutations, which together accounted for 20 of the 35 families (57%) included in this study. This more than doubles the number of *OPA1* mutations reported in the literature, bringing the total to 25. These are predominantly null mutations generating truncated proteins, strongly suggesting that the mechanism underlying DOA is haploinsufficiency. The mutations are largely family-specific, although a common 4 bp deletion in exon 27 (eight different families) and missense mutations in exons 8 (two families) and 9 (two families) have been identified. Haplotype analysis of individuals with the exon 27 2708del(TTAG) mutation suggests that this is a mutation hotspot and not an ancient mutation, thus excluding a major founder effect at the *OPA1* locus. The mutation screening in this study also identified a number of asymptomatic individuals with *OPA1* mutations. A re-calculation of the penetrance of this disorder within two of our families indicates figures as low as 43 and 62% associated with the 2708del(TTAG) mutation. If haploinsufficiency is the mechanism underlying DOA it is unlikely that this figure will be mutation-specific, indicating that the penetrance in DOA is much lower than the 98% reported previously. To investigate whether Leber's hereditary optic neuropathy (LHON) could be caused by mutations in *OPA1* we also screened a panel of 28 LHON patients who tested negatively for the three major LHON mutations. No mutations were identified in any LHON patients, indicating that DOA and LHON are genetically distinct.**

## INTRODUCTION

Dominant optic atrophy (DOA) (MIM #165500) is the most common form of autosomally inherited optic neuropathy with a frequency of approximately 1:50 000 in most populations (1) but as high as 1:10 000 in Denmark (2). The disorder typically presents in childhood with variable bilateral slow visual loss, temporal optic nerve pallor, abnormalities of colour vision and centrocaecal visual field defects (3). The disease has been thought to be highly penetrant with previous estimates as high as 98% (4). However, a high degree of inter- and intra-familial phenotypic variability is observed in DOA (5).

The *OPA1* locus was first mapped to chromosome 3q28-qter using three extended Danish pedigrees (6), but with subsequent linkage studies (7–11) the locus was refined to a 1.4 cM interval flanked by the markers D3S3669 and D3S3562 (12). Although *OPA1* is clearly a major DOA locus with the majority of published pedigrees mapping to it, genetic heterogeneity has been suggested (13) and another locus has recently been identified on chromosome18q12.2–q12.3 (14).

Mutations responsible for DOA have been identified in a gene showing homology to a dynamin-related GTPase and subsequently designated *OPA1* (15,16). The *OPA1* mRNA is 5821 bp long and comprises 29 exons, 28 of which encode the 960 amino acid protein (17). The 11 mutations originally reported included insertions, deletions, nonsense, missense and splicing mutations (15,16). The function of the OPA1 protein is unknown but its similarities to the yeast Msp1 and Mgm1

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**Table 1.** Summary of mutations found in *OPA1* to date

| Location | cDNA change | Protein change | Family/ single | Frequency | Reference |
|---|---|---|---|---|---|
| Exon 6 | 635del(AA) | Frameshift codon 212, termination at codon 215 | Family | 1 | Present study |
| Exon 8 | 794del(TTGA) | Frameshift codon 265, termination at codon 306 | Single | 1 | Present study |
| Exon 8 | 869G→A | Arg290Gln | Both | 2 | Present study, (16) |
| Intron 8 | 870 + 5g→a | Altered splicing | Single | 1 | Present study |
| Exon 9 | 899G→A | Gly300Glu | Both | 2 | Present study, (15) |
| Intron 9 | 985 – 1g→a | Altered splicing | Family | 1 | (15) |
| Exon 10 | 1016del(C) | Frameshift codon 350, termination at codon 359 | Family | 1 | (16) |
| Exon 11 | 1096C→T | Arg366Stop | Family | 1 | (16) |
| Exon 12 | 1152A→C | Leu384Phe | Single | 1 | Present study |
| Exon 13 | 1296del(CAT) | Deletion of 432Ile | Family | 1 | (16) |
| Exon 14 | 1354del(G) | Frameshift codon 452, termination at codon 466 | Family | 1 | (16) |
| Exon 15 | 1508C→A | Thr503Lys | Family | 1 | Present study |
| Exon 15 | 1515A→C | Lys505Asn | Single | 1 | Present study |
| Intron 15 | 1516 + 1G→T | Altered splicing | Family | 1 | Present study |
| Exon 17 | 1644ins(T) | Frameshift codon 549, termination at codon 561 | Family | 1 | (16) |
| Exon 17 | 1669C→T | Arg557Stop | Single | 1 | Present study |
| Exon 19 | 1780del(TTTG) | Frameshift codon 594, termination at codon 607 | Single | 1 | Present study |
| Exon 19 | 1798G→T | Glu600Stop | Family | 1 | Present study |
| Exon 20 | 1848del(exon 20) | Frameshift codon 617, termination at codon 630 | Family | 1 | (16) |
| Intron 25 | 2613 + 1g→a | Altered splicing | Family | 1 | Present study |
| Exon 27 | 2708del(TTAG) | Frameshift codon 903, termination at codon 905 | Both | 8 | Present study, (15) |
| Exon 27 | 2713C→T | Arg905Stop | Family | 1 | Present study |
| Exon 27 | 2771del(A) | Frameshift codon 924, termination at codon 967 | Single | 1 | Present study |
| Exon 27 | 2800C→T | Gln934Stop | Family | 1 | Present study |
| Exon 28 | 2823del(AGTT) | Frameshift codon 942, termination at codon 966 | Family | 1 | (15) |

Amino acid and nucleotide numbering follows the cDNA sequence (GenBank accession no. AB011139), with nucleotide position 1 assigned to the first nucleotide of the ATG initiation codon in exon 1.
Bases in exons are denoted by upper case letters; bases in introns by lower case letters.
Family, the mutation segregated with the disease in a family; single, the mutation was found in a single patient for whom no further family DNAs were available.

proteins suggest that it may have a role in maintaining the integrity and function of mitochondria within cells (18–20).

The purpose of this study was 2-fold: to determine the types and frequency of mutations in *OPA1* which caused DOA in our clinical cohort and to determine whether a second condition, Leber's hereditary optic neuropathy (LHON) (MIM #535000) (21), with a similar pathology to DOA, could also be caused by mutations in *OPA1*. For this we screened a panel of LHON patients who were known to be negative for the three major Leber's mitochondrial mutations.

## RESULTS

### DOA patient screening

In order to determine the types and frequencies of *OPA1* mutations we tested 35 individuals with a clinical diagnosis of DOA. Oligonucleotide primers were designed to amplify each of the 28 coding exons and flanking splice junctions of the *OPA1* gene and these were analysed by single-stranded conformational polymorphism-heteroduplex analysis (SSCP/HA) and sequencing. We identified mutations in 20 out of 35 individuals with DOA (57%). Seventeen different mutations were found, of which 14 are novel and three have been reported previously (15,16). The characteristics of these mutations are presented in Figure 1 and Table 1.

### Deletions

Five different deletions were observed which were not detected in 150 normal control chromosomes. Mutation screening in a British family identified a 2 bp deletion leading to a frameshift in exon 6, 635del(AA). This deletion results in three incorrect amino acids after codon 211 (Lys212Arg, Glu213Glu and Lys214Asn) followed by a stop in codon 215. This mutation segregated with the disease in the family.

In exon 8, we identified a 4 bp deletion in a single British patient, 794del(TTGA). This mutation caused a frameshift resulting in the substitution of 41 amino acids after codon 264

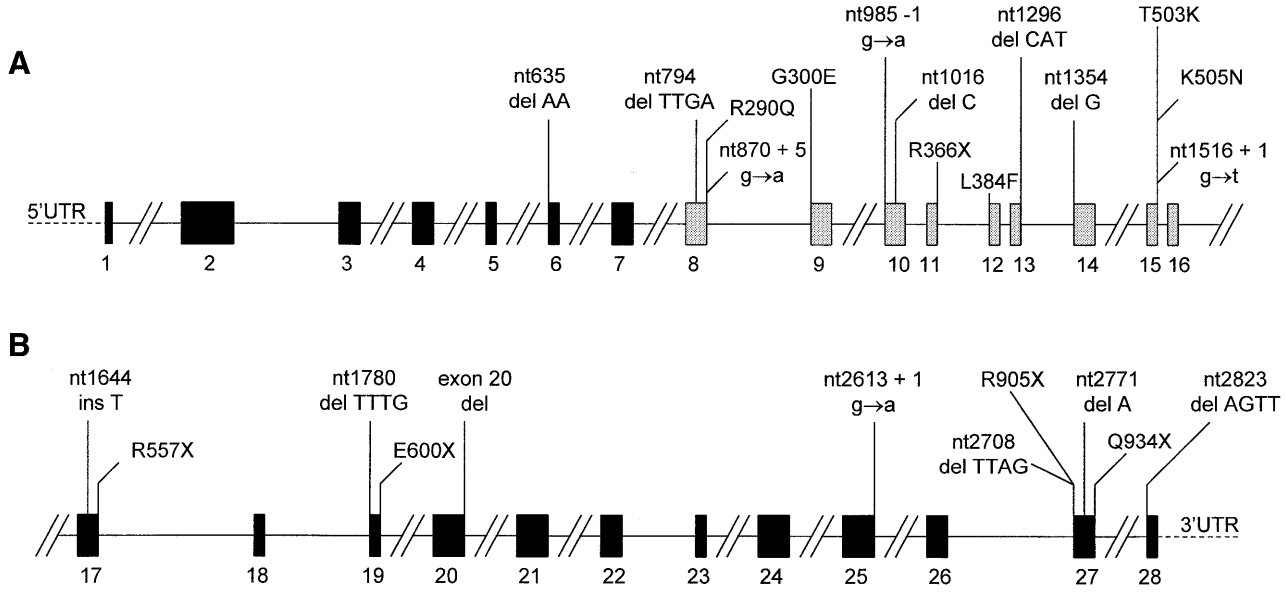


**Figure 1.** Schematic diagram of the *OPA1* gene showing the types and locations of the mutations detected to date. (**A**) The first portion of the gene, exons 1–16. (**B**) The last half of the gene, exons 17–28. All of the missense mutations identified are encompassed within the highly conserved GTPase domain (grey exons).

($NH_2$-TCILKFLMFSLIMMPVIIRKIICHGLLWLEIRVLER-LVCWK-COOH) and a premature stop in codon 306.

In exon 19 another 4 bp deletion, 1780del(TTTG), was identified in a single Australian patient with a family history of dominant disease. This mutation causes a frameshift after codon 593 replacing 13 amino acids ($NH_2$-KKLKMKSLM-KLSV-COOH) before introducing a stop at codon 607.

A 4 bp deletion, 2708del(TTAG), in exon 27 was detected in two British families, an Australian family, a single Australian patient and a single British patient from the LHON screen (see below). It has also been identified in three families originating from Northern France and Belgium (15). This frameshift mutation causes a 2 amino acid substitution after codon 902 (Val903Gly, Arg904Asp) and a premature stop at codon 905, deleting the last 58 amino acids of the protein. The mutation segregated with the disease in these families but asymptomatic carriers were detected in one of the British families and one of the Australian families.

A second deletion identified in exon 27 in a single British case was 2771del(A). This mutation causes a frameshift at codon 924 resulting in a delayed stop and the replacement of the last 37 amino acids of the protein with 42 different amino acids ($NH_2$-RLNCLLVNAFNWRKTSRKLEKFKKNLMLS-LKLFIRRNKLKSYS-COOH).

## Nonsense mutations

Four nonsense mutations were detected that were not found in 150 normal control chromosomes. One was identified in a single British DOA patient in exon 17, Arg557Stop. This change resulted from a C→T transition in the first base of the codon (1669C→T), the mutated C being part of a highly mutable CpG dinucleotide. The predicted effect of this mutation would be a severely shortened protein of 556 amino acids instead of the 960 amino acids found in the wild-type protein.

In exon 19 a Glu600Stop mutation was identified in an Australian family. This change resulted from a G→T transversion in the first base of the codon (1798G→T). This mutation, the effect of which would truncate the mutant protein to 599 amino acids, segregated with the disease in the family.

Two nonsense mutations were identified in exon 27 in two British families. The first is an Arg905Stop change caused by a C→T transition (2713C→T), within a highly mutable CpG dinucleotide, resulting in a truncated protein of 904 amino acids. The second is a Gln934Stop change also caused by a C→T transition (2800C→T) in the first base of the codon resulting in a truncated protein of 933 amino acids. Both of these changes segregated with the disease in these families although an asymptomatic carrier was detected with the Arg905Stop mutation.

## Splicing mutations

Three splice donor mutations were identified which were not found in 150 normal control chromosomes. The first one, found segregating with the disease in a large British family, was a substitution at the first nucleotide of intron 15. In this case the GT donor site was changed to TT (1516 + 1G→T). The second splice mutation was a substitution at the first nucleotide of intron 25, also found segregating with the disease in a British family. In this case the GT donor was changed to AT (2613 + 1G→A). The first base in the intron is 100% conserved and no functional splice site has been reported with this base of the intron substituted (22). The replacement of GT with either TT or AT at the beginning of an intron is believed to be permissive for 5′- cleavage but does not allow the second step of splicing. This leads to an accumulation of the lariat intermediate (23). An alternative theory for the effect of the GT→TT splice mutation suggests that a transition at this position of the intron may cause a shift in the splice junction by one

**Table 2.** Summary of benign sequence variants detected in *OPA1*

| Location | cDNA change | Protein change | Frequency | Reference |
|---|---|---|---|---|
| Exon 3 | 420G→T | Val140Val | G, 99%; T, 1% | Present study |
| Exon 4 | 473A→G | Asn158Ser | – | Present study, (15) |
| Exon 4 | 478G→C | Glu160Glu | – | Present study |
| Intron 4 | 557 – 19t→c | None | T, 48%; C, 52% | Present study |
| Intron 8 | 870 + 4c→t | None | C, 83%; T, 17% | Present study, (16) |
| Intron 8 | 870 + 32t→c | None | – | Present study |
| Intron 8 | 871 – 26a→g | None | A, 99.5%; G, 0.5% | Present study |
| Intron 16 | 1590 – 35a→t | None | A, 99.5%; T, 0.5% | Present study |
| Exon 17 | 1608A→C | Ala536Ala | A, 99%; C, 1% | Present study |
| Exon 18 | 1770 + 3a→g | None | A, 92%; G, 18% | Present study |
| Exon 21 | 2109C→T | Ala703Ala | C, 57%; T, 43% | Present study, (15) |
| Exon 26 | 2707 + 25t→a | None | T, 48%; A, 52% | Present study |
| Exon 27 | 2720A→G | Glu907Gly | – | (15) |

Amino acid and nucleotide numbering follows the cDNA sequence (GenBank accession no. AB011139), with nucleotide position 1 assigned to the first nucleotide of the ATG initiation codon in exon 1.
Bases in exons are denoted by upper case letters; bases in introns by lower case letters.

base, dependent on the surrounding nucleotides. Therefore, functional splicing would occur but with the introduction of a frameshift (23). Other splice defects that could result include the recognition of a cryptic splice-site instead of the mutated splice-site or failure to recognize the affected intron, such that it is retained as part of the final mRNA product (24). The precise effect of these splice donor mutations has not been confirmed in either family, as their RNA was unavailable for study.

The third splicing mutation was observed in the splice donor site of intron 8 in a British patient, 870 + 5g→a. RNA from lymphocytes was available for this individual and RT–PCR analysis indicated that the mutation resulted in the skipping of exon 8.

### Missense mutations

Five putative missense mutations were identified. Two of the missense mutations identified in this study had been described previously. The exon 8 Arg290Gln substitution was found in a British patient in this study but had previously been described in a Cuban family (16), and the exon 9 Gly300Glu substitution, also found in a British patient in this study, had previously been described in a French family (15). In addition, in exon 12 we identified a novel putative missense mutation, Leu384Phe, in a single British patient. We identified two putative novel missense mutations in exon 15, Thr503Lys segregating with the disease in a large British family and Lys505Asn in a British singleton.

It is more complex to determine whether these missense mutations are disease-causing or benign variants than in the case of truncating mutations. However, all of these substitutions lie within the GTPase domain of *OPA1* and affect conserved amino acids, except Leu384Phe, which is not conserved in *Drosophila*, and Lys505Asn, which is not conserved in either *Caenorhabditis elegans* or *Drosophila* (Figure 2). None of these changes occurred in 150 normal control chromosomes and it is therefore likely that these changes cause the disease seen in the respective patients.

### Benign variants

In addition to the above mutations, a missense change that was not disease-specific has been detected; Asn158Ser (Table 2). A further four coding single nucleotide polymorphisms (SNPs) which do not change an amino acid and seven intronic SNPs were also identified (Table 2).

### LHON patient screening

We screened a total of 28 LHON patients who tested negative for three primary mitochondrial mutations at nucleotides 3460, 11778 and 14484. These three mutations are known to be responsible for 95% of LHON pedigrees of northern European descent (25). The common 4 bp *OPA1* deletion 2708del(TTAG) was identified in one of these LHON patients. However, upon re-examination, the patient had a clinical phenotype consistent with DOA rather than LHON. It is therefore likely that this mutation represented a misdiagnosis at the clinical level rather than a true case of LHON caused by mutations in *OPA1*. This individual was therefore treated as a DOA-affected patient in the haplotype analysis below.

### Haplotype analysis

Haplotypes around the *OPA1* gene were determined in members of the five families with the 2708del(TTAG) mutation identified in this study, to determine whether families with this mutation share a common ancestral haplotype or whether they represent independent mutation events. The microsatellite markers used, and their order on chromosome 3q28 in relation to *OPA1*, is: centromere-D3S3669-D3S1523-D3S3642-D3S3590-*OPA1*-D3S2305-D3S3562-D3S2748-telomere (26).

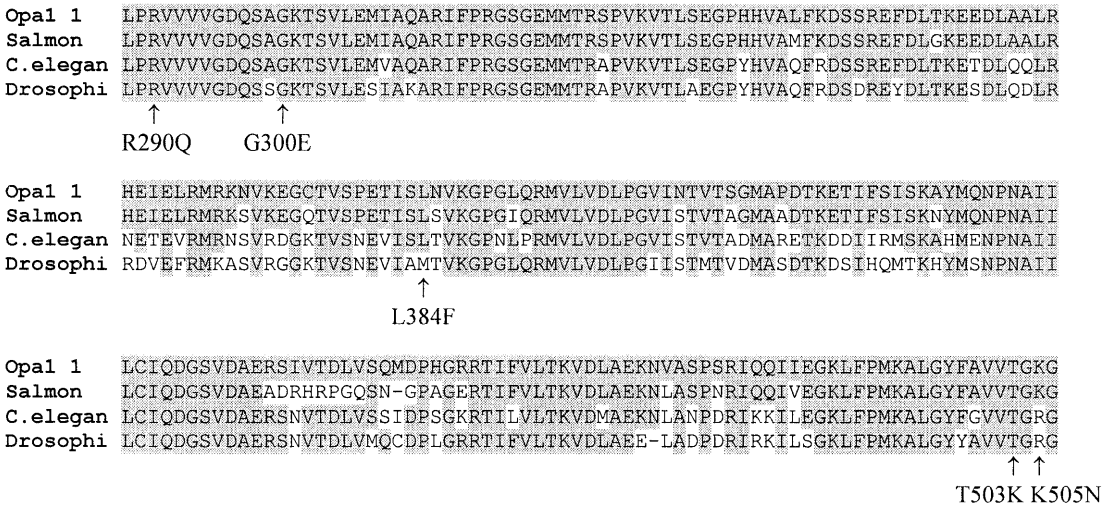


**Figure 2.** Protein alignment depicting the similarity of human OPA1 to salmon GTP-binding protein, *C.elegans* GTP-binding protein and *Drosophila* CG8479 gene product. Only amino acid residues 288–506, the part of the OPA1 GTPase domain containing the missense mutations, are shown. Conserved amino acid residues are highlighted. The positions of the missense mutations Arg290Gln, Gly300Glu, Leu384Phe, Thr503Lys and Lys505Asn are indicated. All the missense mutations alter conserved amino acids in all species, except Leu384Phe, which is not conserved in *Drosophila*, and Lys505Asn, which is not conserved in *C.elegans* or *Drosophila*.

The disease haplotypes determined are shown in Table 3. Where the phase of the marker alleles and the mutation could not be determined, both possibilities are given. The marker D3S2305 lies nearest to *OPA1* and the linked haplotypes in these five families/cases include at least three different alleles for this marker.

To confirm this result we typed one of the newly identified coding SNPs occurring within exon 21 of the gene. The SNP 2109C→T was typed using the restriction enzyme *Hae*III. This cuts the PCR products of exon 21 with a C at position 2109 (producing bands of 149 and 184 bp) but not those with a T. The results are also shown in Table 3. This SNP is <10 kb away from the mutation and still no common genotype is shared between these individuals. These data suggest that the 2708del(TTAG) mutation arose independently in multiple families.

### Calculation of penetrance

We calculated the penetrance of *OPA1* mutations in our patients. We were unable to calculate penetrance based on our full patient cohort as this primarily consists of clinically affected patients. We therefore only calculated penetrance on pedigrees in which every individual (affected and unaffected) had been sampled. Only two such pedigrees were available to us, both segregating the exon 27 deletion 2708del(TTAG). Figure 3 shows one of these pedigrees in which a total of seven individuals carry the mutation but only three are symptomatic, resulting in a penetrance figure of 43% (3/7). Similar analysis on a second family in which 13 individuals carried the mutation but only eight showed symptoms of the disease gave a penetrance figure of 62% (8/13).

## DISCUSSION

To determine the types and frequencies of disease-causing mutations in *OPA1*, we tested 35 affected DOA patients for mutations. We identified 17 different disease-causing mutations in 20 of these patients (57%). Eleven of these mutations are predicted to result in truncation of the protein, the proportion of the protein deleted ranging from 77% for the

**Table 3.** Haplotype analysis in the five patients with the 2708del(TTAG) mutation

| | D3S3669 | D3S1523 | D3S3642 | D3S3590 | 2109C/T | D3S2305 | D3S3562 | D3S2748 |
|---|---|---|---|---|---|---|---|---|
| 1 | 194 | 232 | 149 | 211 | T | 196 | 220 | 110 |
| 2 | 196 | 234 | 149 | 211 | T | 180 | 220 | 94 |
| 3 | 198 | 232 | 149 | 211 | T | 180/184 | 220/224 | 80/94 |
| 4 | 192/194 | 232 | 149 | 209/215 | C/T | 180/196 | 220/224 | 108/110 |
| 5 | 194/200 | 230/232 | 149 | 211 | C | 192/184 | 220/224 | 80 |

The patients were genotyped using genetic markers in the *OPA1* region and an intragenic SNP contained within exon 21 of *OPA1*. The disease haplotypes are shown. Where the phase of the marker alleles and the mutation could not be determined, both possibilities are given. No one common disease haplotype was identified within these individuals.

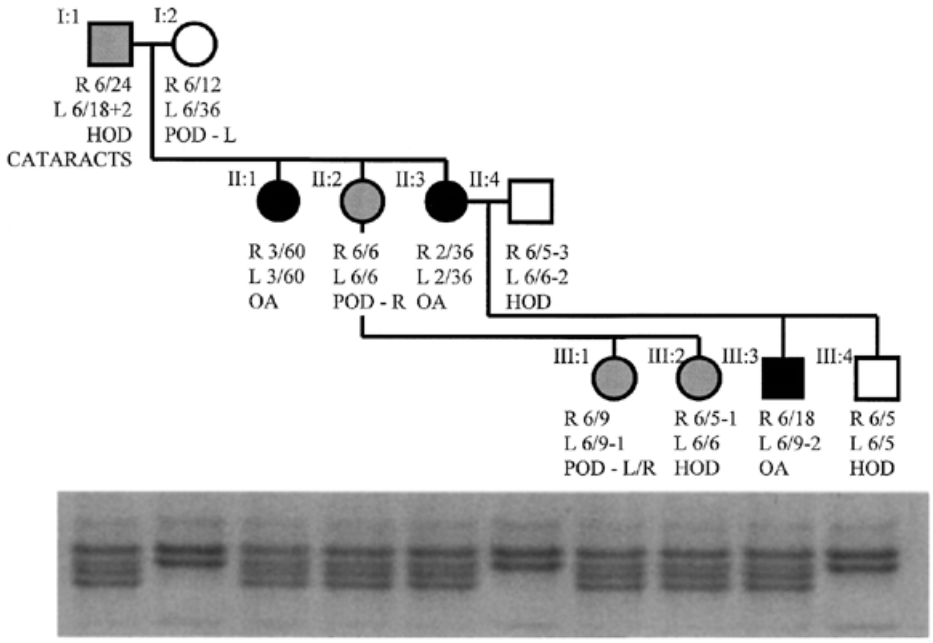


**Figure 3.** *OPA1* mutation 2708delTTAG tracking in a family with reduced penetrance. The figure shows the SSCP result for exon 27. The grey symbols represent asymptomatic individuals. Best corrected Snellen visual acuity figures for left (L) and right (R) eyes are listed below symbols for each individual, and the appearance of the optic discs is described. OA, classic optic atrophy discs; HOD, healthy optic discs; POD–R/L, pale optic disc affecting left or right eye. Individual I:1 male has cataracts and his low visual acuity figure is consistent with this diagnosis rather than DOA.

exon 6 deletion, 635del(AA), to 3% for the exon 27 nonsense mutation, Gln934Stop. The exon 27 mutation, 2771del(A), is expected to result in the elongation of the protein to 966 amino acids instead of the normal 960. However, this mutation changes the last 37 codons of the gene, therefore altering 4% of the protein. The remaining five mutations identified were substitutions altering amino acids within the highly conserved GTPase domain of the protein (Figure 2). Although the pathogenic effects of all these missense mutations have not been proven, the conserved or semi-conserved nature of the changes and their location in the GTPase domain suggest that they are likely to be pathogenic. There are now a total of 25 different mutations reported to cause DOA (Table 1), including 10 deletions, five nonsense, five missense, four splicing and an insertion mutation. Translation-terminating mutations are distributed throughout the gene with the exception of exons 1–5 and 21–24. However, missense mutations are restricted to the GTPase domain (Figure 1).

Due to the large size of the *OPA1* gene we opted to screen genomic DNA from affected individuals by SSCP/HA. This approach has allowed us to identify causative mutations in 57% of the DOA patients screened. We anticipate that a few mutations may have been missed by this choice of mutation detection approach (27,28). Large rearrangements or deletions within the gene have not been examined in this study and one such mutation (deletion of exon 20) has previously been described (16). Also, mutations may have occurred in the promoter, introns, or the 5′- or 3′-UTRs, which have not yet been examined. Despite this, we did identify mutations in all four of our known chromosome 3q linked families. It is therefore likely that most of the patients in whom *OPA1* mutations were not detected have mutations in other genes. Unfortunately, the individuals in whom mutations were not identified are either single cases or from families too small at present to test for linkage to chromosome 18q. It may be significant that we identified mutations in 11 of our 16 families (68%) and nine of our 19 single cases (47%). The higher identification rate in families as opposed to single cases may imply that single cases are more likely to include X-linked (although only two are male), mitochondrial or non-inherited forms of optic atrophy.

The mutational spectrum observed in DOA strongly suggests that the disorder may result from haploinsufficiency, with all of the mutations likely to cause loss of function of the mutant allele. Although haploinsufficiency is relatively rare in dominant disorders, there are several well documented examples (29), such as type I Waardenburg syndrome, which has been shown to be due to mutations in the *PAX3* gene (30,31) and aniridia, which results from mutations in the *PAX6* gene (32).

The majority of the mutations identified are unique to a family, although three mutations were detected in more than one family (Table 1). One of these mutations, which was detected in five families in this study and previously described in three others (15), was a 4 bp deletion 2708(TTAG) occurring in exon 27. The other mutations that have occurred in more than one family were Arg290Gln in exon 8 and Gly300Glu in exon 9. Both of these mutations were detected once in this study and have been reported once previously (15,16). The Arg290Gln mutation resulted from a G→A transition within a CpG dinucleotide, and is therefore likely to be a mutational hotspot. We were unable to check haplotypes for these two common missense mutations as we only had

access to a single patient with each mutation. However, haplotype analysis using several chromosome 3 microsatellite markers and an SNP within exon 21 of *OPA1*, in five families with the common exon 27 deletion, suggests that they are not all related, so this mutation is likely to be a mutational hotspot. It is therefore evident from the present study that there is no common founder mutation for DOA at this locus, as had been proposed previously by others (33,34).

Mutation analysis in three of our families revealed clinically unaffected mutation carriers. Two of these families have the exon 27 deletion 2708del(TTAG) and the third family segregated another exon 27 mutation, Arg905Stop. Delettre *et al.* (15) also reported an asymptomatic carrier with the mutation 2708del(TTAG) and Alexander *et al.* (16) reported unaffected carriers with 1354del(G) and 1848del(exon 20) mutations. This high number of asymptomatic carriers indicates that the original figure of 98% penetrance for DOA (4) was an overestimation. Any new calculation of penetrance based on full patient cohorts will now be biased towards clinically affected individuals, because in the majority of families, not all the clinically unaffected patients are sampled. However, in the two full pedigrees available to us, penetrance figures as low as 43% (3/7) (Figure 3) and 62% (8/13) have been observed for the 2708del(TTAG) deletion. Further analysis will be necessary to determine whether these low penetrance figures are specific for this mutation. A recent study of variable penetrance at the RP11 locus for dominant retinitis pigmentosa indicated that polymorphisms within the 'wild-type' allele at the disease locus influenced the penetrance of the disease caused by a mutation (35). Although only a small number of families were studied, our initial findings indicate that this is probably not the mechanism for the observed phenotypic variation in DOA. Other reasons for the wide variability in the phenotype between individuals with identical mutations may include environmental influences, polymorphisms in interacting genes and the effects of different chromosomal and mitochondrial backgrounds.

LHON is another major inherited optic neuropathy which is clinically distinct from DOA as it involves a sudden onset of visual loss in two eyes asynchronously and a later age of presentation (typically 18–35 years). However, in the atrophic stage it has been reported to be difficult to make the distinction between DOA and LHON without a family history (36). LHON can be caused by mitochondrial mutations in genes encoding subunits of complex I of the respiratory chain (37). Studies have shown that three primary mitochondrial mutations at nucleotides 3460, 11778 and 14484 are responsible for 95% of LHON cases of northern European descent (25). The characteristics of the pathology of DOA resemble those of LHON, and many studies have shown that a range of different clinical phenotypes can be caused by mutations in the same gene (38–40). We therefore screened a panel of 28 LHON patients who tested negative for the three common mutations, to determine whether a subset of LHON patients were allelic with DOA patients harbouring *OPA1* mutations. Although a mutation was identified in one of these patients, re-evaluation of the patient clinically indicated that he had a phenotype more consistent with DOA than LHON and had originally been misdiagnosed. Therefore, no mutations were identified in any LHON patients, indicating that the two disorders are indeed genetically distinct.

This study offers insights into the type, distribution and frequency of *OPA1* mutations that cause DOA as well as evidence of a much lower penetrance for *OPA1* mutations than had been observed previously. Future studies will determine whether the variable phenotype observed within families can be attributed to the inheritance of the second modulating allele at the same locus or whether other mechanisms are involved, and whether all *OPA1* mutations behave in this way. Continued identification of mutations will allow accurate diagnosis of asymptomatic individuals and subsequently permit better genetic counselling of these patients.

## MATERIALS AND METHODS

### Subjects

Genomic DNA samples from 16 DOA families and 19 single cases diagnosed with DOA were analysed in this study. Clinical diagnosis was made on the basis of reduced visual acuity, abnormal colour discrimination (City Universal plates, Farnsworth Munsell D15 Test, or Farnsworth Munsell 100 Hue Test) and temporal pallor of the optic disc, on examination and photography. Electrophysiology was performed in selected cases to confirm the diagnosis. A further 28 DNA samples from patients diagnosed with LHON were also screened. These patients were obtained from the Yorkshire Regional Clinical Genetics service. They had been referred by ophthalmologists throughout the Yorkshire region for mitochondrial testing. These patients had tested negative for the three common mitochondrial mutations known to cause LHON. Informed consent was obtained from all subjects tested. Ethical approval was provided by the Leeds Teaching Hospitals Trust Research Ethics Committee. Control subjects were normal partners from optic atrophy and other retinal degeneration pedigrees.

### SSCP/HA

The coding exons and the flanking splice junctions of one affected individual from each family were PCR-amplified with the primers detailed in Table 4. The method used was the same as that described elsewhere (41) with the exception that $^{33}P$ isotope was substituted for $^{35}S$. The amplified products were mixed with an equal volume of formamide loading buffer, heated to 95°C for 2 min and quenched on ice prior to loading on a non-denaturing, glycerol-containing gel (8% acrylamide:5% glycerol:0.5× TBE). The gels were run at 6 W for 18–22 h, transferred to Whatmann 3 mm filter paper and dried. Autoradiography was performed for 24 h using Hyperfilm MP (Amersham).

### Sequence analysis

Where the SSCP/HA showed a mobility shift, PCR amplification of the coding exon and flanking intronic sequence was performed to provide a template for sequencing. Reactions were carried out in a 50 µl volume with 50 ng of genomic DNA, 20 pmol of each primer, 200 µM each of dATP, dCTP, dGTP and dTTP, 10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin and 1 U *Taq* DNA polymerase (Gibco BRL). After the initial denaturation step at 96°C for 3 min, the samples were processed through 35 cycles of 92°C

**Table 4.** Sequences of oligonucleotide primers used in the present study

| Exon | Forward primer (5′→3′) | Reverse primer (5′→3′) | Product size (bp) |
|---|---|---|---|
| 1 | CTCTGTGCCCTTGCTGCTGAG | GGCCTGGCCTGAGAGTCACC | 260 |
| 2a | CTTTGTACTGTTACCCTCTCTG | GTCAGAGAAGAGAACTGCTGAA | 221 |
| 2b | AGCTTCAACGACCCCAATTAAG | CATCCAATTGTATTCCACTACAC | 301 |
| 3 | TGTTTATTTGGCATGCAGAGC | CTCTTTCCTCGAGATGACCA | 199 |
| 4 | TTTGTAGGGTTGTCATGAGG | CCTCCATGGTTCTTAAATTTTT | 268 |
| 5 | TAGGCTGTTGACATCACTGG | TATTTGCTTCTCAGATGTTATCA | 223 |
| 6 | CTTGCTGTACATTCTGTATAAACC | GTCCATATCACCAAAGTTACATTC | 186 |
| 7 | GACTTAATACTATTTGATAACCC | GACAGAATACTTTACAGCTCC | 264 |
| 8 | TTTCTCTTGACACATCTGTTAT | TTAGGAAGCTAAGCTGCTTC | 226 |
| 9 | AAAACTCAGAGCAGCATTACAA | TGAAGCAGTTACTCTCCTAAG | 251 |
| 10 | CTAGACCACATACGGGCTG | CTTTAACTGTCTTTTATAAGCTC | 230 |
| 11 | GACAGTTAAAGAATATTTGTGAA | TCTACATATCTAGATAGCAGC | 256 |
| 12 | GTGAGCGTCTTATCTGAATGG | CCATGCCTGATGTCACAGTC | 294 |
| 13 | CTTGTTGACTTACCAGGTGTG | CTTGTATGTTTACATAACACAAAC | 306 |
| 14 | TGCTATAATGTAGACACAGGG | AACAAATCCCTATCACAGCTG | 296 |
| 15 | CTTATGTAAACTATATCTCACAT | CAATGCTTTCAGAGCTCTTC | 244 |
| 16 | GCTGTTGTAACAGGAAAAGGT | CAGTTCAATTTAAGCTACTCTC | 255 |
| 17 | GAGTGGCTGTTAGCAAGCAC | AGAATCGTATGGATGCCAAAGA | 292 |
| 18 | TTCAGTTAATACAGAGGATATG | GATAACTGCTCCTAGAGATG | 315 |
| 19 | CTCTGAAAATCATGACAGGGT | CAAGGCAACAATAAATCACTGC | 251 |
| 20 | TAATGATACTTCAGTCAAGCTG | AAAATTCACAGCTCCTACTCC | 310 |
| 21 | ATCTGTTTGGCTTGAGCTCG | TTAGTCATATAAAGTCTCCTATG | 333 |
| 22 | CATATTTACTAAGCTGTCAATTTG | CTCAGAACTCAATAAGCCGTG | 229 |
| 23 | GTTAAGAAAGCAAGACCATTATTAG | CTGAGACTGGTCTAGAGCCAC | 263 |
| 24 | TTCAAGCACCAAATTATGAACC | ATCTAACTAACACATGTAGTAAC | 281 |
| 25 | GAATATACAGTAACTCTGGGC | GGACTTAAAATTAATGCTTAAGC | 253 |
| 26 | GTAGTTGTATGTGTTTACGATG | GGCATCCTTCTATTAAATATGG | 249 |
| 27 | GTATTAGCAATAGTTCTAACATG | GTAGTAAGGTAGAGGGCAGT | 264 |
| 28 | CTCCTGATTTGTGATACCTTTG | AGCAGGATGTAAATGAAGCAGA | 222 |

for 30 s, 50–60°C for 30 s and 72°C for 30 s. A final extension step was performed at 72°C for 10 min. Following amplification, PCR products were processed using the Qiaquick PCR purification kit (Qiagen). Both the forward and reverse strands of the PCR products were directly sequenced on an ABI 377 sequencer using the ABI Prism BigDye Terminator cycle sequencing ready-reaction kit (Applied Biosystems). Sequencing reactions were set up according to the manufacturer's instructions using either the forward or reverse primers detailed in Table 4. DNA from 75 normal controls was screened by SSCP/HA to check that they did not contain any of the mutations identified in this study.

## Haplotype analysis

Five individuals with the 2708del(TTAG) mutation, and other family members where available, were genotyped for the markers D3S3669, D3S1523, D3S3642, D3S3590, D3S2305, D3S3562 and D3S2748 as described elsewhere (26). These individuals were also typed for the SNP 2109C/T. PCR products for exon 21 were generated with the primers in Table 4 using the method for sequencing detailed above. Following *Hae*III digestion, products containing a C at position 2109 produced bands of 149 and 189 bp, whereas products of 338 bp with a T at position 2109 remained uncut.

## RT–PCR analysis

RNA was extracted from fresh blood using the QIAamp RNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. One microgram of this RNA was incubated with100 ng of random primer at 70°C for 10 min. The samples were chilled on ice and MMLV reverse transcriptase buffer, 10 mM DTT, 1 mM dNTPs (all Gibco BRL) and 0.5 U RNAsin (Promega) were added. The reactions were equilibrated at 37°C for 2 min, 200 U MMLV reverse transcriptase was added

and the samples were incubated at 37°C for 1 h. The samples were heated to 95°C and 2 µl of cDNA was used in PCR reactions to generate templates for sequencing.

### Entrez accession numbers

KIAA0567 protein, BAA25493; Salmon GTP-binding protein, BAA32279; *Drosophila melanogaster* CG8479 gene product, AAF58275; *C.elegans* GTP-binding protein, CAA87771.

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