Role of Mitochondrial Mutations in Ocular Aggregopathy

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Abstract

Background
Mitochondria are essential cellular organelles that are responsible for oxidative stress-induced damage in age-dependent neurodegenerations such as glaucoma. Previous studies have linked mitochondrial DNA (mtDNA) mutations to cellular energy shortages that result in eye degeneration.

Methodology
To look for nucleotide variations in mtDNA in exfoliation syndrome/glaucoma (XFS/XFG), we performed a polymerase chain reaction (PCR) to amplify the entire coding region of the mitochondrial genome from peripheral blood of XFS/XFG (n = 25) patients and controls (n = 25).

Results
This study identified a total of 65 variations in XFS/XFG patients, of which 25 (38%) variations were non-synonymous single-nucleotide polymorphism (nsSNPs). Out of 25 nsSNPs, seven (five nsSNP in MT-ND4 and two in MT-ATP6 gene) were predicted as pathogenic using four different software, namely, SIFT, Polyphene2, mutation taster, and MutPred2. The pathogenic nsSNPs were then subjected to structural change analysis using online tools.

Conclusions
The pathogenic nsSNPs were found in both proteins’ transmembrane domains and were expected to be conserved, but with lower protein stability (ΔΔG < −0.5), indicating a possibly harmful effect in exfoliation. However, three-dimensional protein analysis indicated that the predicted mutations in MT-ND4 and MT-ATP6 were unlikely to alter the protein function.

Categories: Genetics, Ophthalmology, Other
Keywords: snps, aggregopathy, mitochondrial mutation, exfoliation glaucoma, exfoliation syndrome

Introduction
Exfoliation glaucoma (XFG) is an age-related fibrillopathy characterized by protein deposits on various ocular surfaces. Transforming growth factor beta-1 (TGF-β1) has been shown to be increased in exfoliation syndrome (XFS) and XFG [1, 2] eyes and is a key mediator for regulating extracellular matrix homeostasis [3], reactive oxygen species (ROS) production, and redox balance in the cell milieu [4]. ROS, in turn, induces/activates TGF-β1 and mediates many of the fibrogenic effects of TGF-β, forming a vicious cycle. An interplay loop is known to exist between ROS and proteinopathy [4, 5]. Oxidative stress can be either causative or consecutive to protein aggregation. Proteins appear to be a major target for oxidation due to their high reactivity with ROS [6]. In general, cysteine oxidation results in structural changes, for instance, through disulfide formation, which affects protein function. These structural changes can also provide a molecular switch to partially unfold and subsequently aggregate [6]. Evidence indicates that ROS plays a role in glaucoma pathogenesis in XFS [7-10] by stimulating apoptosis and inflammatory pathways. Both vascular and mechanical theories help to explain the formation of ROS in glaucoma [9]. The vascular theory is based on the ischemia-induced production of ROS due to compromised blood flow in retinal vessels [8, 9]. The mechanical pressure theory for the formation of ROS involves elevated intraocular pressure (IOP) inhibiting retrograde neurotrophin support for retinal ganglionic cells (RGC) axons [9, 10]. Intracellular ROS levels are maintained low within cells, ensuring redox homeostasis for proper cellular chemical reactions. Oxidative stress occurs when the ROS concentration exceeds the antioxidant capacities of the cell, leading to the oxidation of cellular molecules and their alteration [10].

Mitochondrial abnormalities such as defects in oxidative phosphorylation, increased accumulation of...
mitochondrial DNA (mtDNA) defects, defective calcium influx, accumulation of mutant mitochondrial proteins, and mitochondrial membrane potential dissipation are important cellular changes in both early and late onset of several neurodegenerative diseases such as amyotrophic lateral sclerosis, Alzheimer’s disease, and Parkinson’s disease [11,12]. Mishra et al. demonstrated a strong relationship between peripheral blood mtDNA damage and diabetic retinopathy and suggested the possible use of peripheral blood mtDNA as a non-invasive biomarker of diabetic retinopathy [13]. mtDNA has also been shown to be a potential biomarker in numerous other diseases [14-17]. Several studies have reported lower systemic levels of antioxidants with increased oxidative stress markers in XFS [18,19]. While increased ROS production and activation of stress markers are widely accepted to be a pathogenic mechanism for tissue damage or formation of protein complex aggregate formation in XFS, the role of mtDNA mutations in this disease remains unexplored.

This study is an effort to enquire into the possible involvement of the mitochondrial genomic variants in glaucoma (XFS/XFG) by direct sequencing of the entire mitochondrial genome.

**Materials And Methods**

**Patient recruitment**

Patients diagnosed with XFS/XFG and cataract (control) from 2018 to 2020 at glaucoma services of a tertiary eye care center were recruited for the study. We screened 298 cases with XFS/XFG and included only bilaterally severity-matched cases with no systemic diseases while excluding bilaterally asymmetric or unilateral cases. Age-matched control subjects without glaucoma who were scheduled for cataract surgery were also recruited as normals for the study. The study was performed in adherence to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board of L V Prasad Eye Institute (protocol code: 2016-60-1M-12; date of approval: May 28, 2019). Informed consent was obtained from all patients who underwent standardized ophthalmic examination including slit-lamp examination, gonioscopy, and fundus biomicroscopy, and IOP measurement by Goldman applanation. The definitions of XFS/XFG are detailed elsewhere [1-3].

**Sample collection and DNA isolation**

Ethylenediaminetetraacetic acid vials were used to collect a 4 mL blood sample from individuals. The samples were immediately stored at -80°C until experimentation. DNA was isolated using GSure® Blood DNA Mini Kit (G4626, India) from GCC Biotech following the manufacturer’s protocol. The purity and concentration of DNA were quantified using EPOCH microplate reader (BioTek, USA).

**Mitochondrial genome amplification by polymerase chain reaction**

The entire mitochondrial genome was amplified in 24 separate PCR reactions using 24 pairs of primers (Supplementary Table 3). PCR amplification for all primer sets was done in a 25 µL reaction volume containing 5 µL PCR master mix buffer, 0.5 µL of 10 µM stock of forward and reverse primer, and 200 ng of genomic DNA. The thermal cycling was performed for 35 cycles with the following reaction conditions: initial denaturation at 94°C for 30 seconds, annealing at 56°C for one second, extension at 72°C for one minute, and a final extension at 72°C for five minutes. The amplified PCR products were then sequenced. Sanger sequencing was used as the detection method. Both forward and reverse direction sequencing was done for all fragments. All variations in the sequence from both cases (XFS and XFG) and controls were compared to human mitochondrial reference sequence NC_012920 obtained from the National Centre for Biotechnology Information (NCBI) using ClustalW (multiple sequence alignment program for DNA); European Molecular Biology Laboratory (EMBL) - European Bioinformatics Institute (EBI). The corresponding amino acid positions of the nucleotide variation were identified using the Ensemble genome browser. The amino acid substitutions were then analyzed for functional and structural changes in the protein using various online tools. Figure 1 is a diagrammatic representation of the computational methods used in this study.
Identification of pathogenic non-synonymous SNPs

For prognostication of pathogenic attributes of all the obtained non-synonymous mtDNA variations, multiple homology-based programs including PolyPhen2 (Polymorphism Phenotyping), SIFT (Sorting Intolerant From Tolerant), and Mutation taster analysis tool were used. PolyPhen (http://genetics.bwh.harvard.edu/pph2/) structurally analyzes an amino acid polymorphism and predicts whether the amino acid substitution is likely to impair protein function [20-22]. To predict the potential functional impacts of mutation on the structure-function connection, it applies a unique empirical approach that integrates both comparative and physical aspects. Scores of 1.5-2.0 are possibly damaging, and scores of <1.5 are likely benign. SIFT (http://sift.jcvi.org/) is a sequence homology-based method that differentiates between intolerant and tolerant amino acid substitutions and predicts if a protein amino acid replacement will have phenotypic consequences. Positions with normalized probabilities less than 0.05 are predicted to be harmful and those greater than or equal to 0.05 are predicted to be tolerated. Mutation Taster (https://www.mutationtaster.org/) performs a battery of in silico tests to estimate the impact of the variant on the gene product/protein and estimates the disease-causing potential.

Verification of high-risk nsSNPs

The selected pathogenic nsSNPs were then put forward to the Mutpred2 server (http://mutpred.mutdb.org/) to calculate the probability score and prediction stature of the resultant protein due to mutations. A confident hypothesis has a g-value of >0.75 and a p-value of <0.05. Based on the prediction score, this method classifies a specific mutation as benign or pathogenic.

Determination of protein stability

The structural stability of the resulting amino acid substitution was predicted using I-Mutant 2.0 (https://folding.biofold.org/i-mutant/i-mutant2.0.html). The I-Mutant 2.0 output was indicated as a free-energy change value (ΔΔG) and a reliability index (RI). ΔΔG values of <0.5 were considered destabilizing.

Evolutionary conservation analysis

The conservation score of a specific amino acid can be used to infer its importance in the structure and
functions of a protein. The evolutionary conservation of each residue position in the native proteins was predicted using ConSurf (https://consurf.tau.ac.il/), an empirical Bayesian algorithm, and the phylogenetic relationships between closely related sequences were used to make the prediction. ConSurf evaluates the degree of conservation of each amino acid at a certain location as well as the evolutionary profile of the amino acid sequence and was used to identify the blueprint of amino acid conservation [27]. The tool calculates a colorimetric conservation score between 1 and 9 for each amino acid position and classifies the residue as variable (1–4), intermediately conserved (5–6), or highly conserved (7–9). Each residue position in the protein structure is also determined to be exposed (on the protein surface) or buried (inside the protein core). When a residue is highly conserved and exposed, it is predicted to be functional, whereas a structural residue is predicted to be buried.

Predicted effects of high-risk nsSNPs on protein properties and three-dimensional (3D) protein modeling

HOPE was used to predict the effects of seven identified high-risk pathogenic nsSNP mutations on amino acid size, domains, hydrophobicity, conservation, and function. Structure comparisons between wild-type and mutant models, as well as predictive 3D modeling, were used to see if the five pathogenic nsSNPs in MT-ND4 and two in MT-ATP6 significantly alter the resultant protein structure. The 3D models for the wild-type proteins and their mutations were created using Pymol (https://pymol.org/2/). The best template used for the MT-ND4 protein structure was 1h88.1 and for MT-ATP6 was c5ldwM. Further validation of the structural integrity of the obtained wild-type and mutant protein structures was performed using a Ramachandran plot through the dihedral angles using PROCHECK. Structural comparison of wild-type MT-ND4 and MT-ATP6 proteins with their mutant forms was also done using Pymol.

Results

Prediction of pathogenicity

Whole-genome amplification of mtDNA sequencing revealed a total of 65 nucleotide variations in XFS/XFG patients (Figures 2a, 2b). The nsSNPs predicted to be harmful/disease-causing by any three sequence-based prediction methods were labeled as pathogenic nsSNPs. Out of 65 variants obtained in the cases, 14 (21%) were synonymous, 16 (24%) were non-synonymous SNPs, and 15 were in RNA genes. Few variations were also reported in the D-loop. Three non-synonymous changes (T2455G, T2760G in RNR2, and A12308G in TRNL) were common in both cases and controls. Polyphene2, SIFT, and mutation taster revealed seven mutations (out of 16 nsSNPS) in XFS/XFG patients to be pathogenic (Tables 1, 2). Five out of seven mutations were predicted in MT-ND4 protein and two in MT-ATP6 protein. The goal of employing multiple tools was to boost prediction confidence. The seven pathogenic nsSNPs identified were then verified by MutPred2. Supplementary Table 4 and Table 5 show the prediction scores and status (score >0.5 indicates disease). Out of the 16 non-synonymous mutations, seven (43.75%) were found to be pathogenic in nature (Table 2). One of the most unique observations of the study was the correlation between the age of the patients and the number of mutations. The number of mutations observed was higher in older XFG patients compared to XFS patients (Figure 2c).
FIGURE 2: Mutational landscape of the mitochondrial genome. (a) Venn diagram depicting the proportion of all mutations observed in exfoliation syndrome/glaucoma (XFS/XFG) and controls. (b) The landscape of mtDNA non-synonymous variations observed in XFS/XFG patients. (c) Correlation between the number of mtDNA mutations and patient (XFS/XFG) age at the time of diagnosis.

Authors’ own creation.

n: number of patients recruited; mtDNA: mitochondrial DNA; XFS: exfoliation syndrome; XFG: exfoliation glaucoma

| Group                     | XFS (n = 12) | XFG (n = 13) | Common between XFS and XFG | Control (n = 25) |
|---------------------------|--------------|--------------|----------------------------|------------------|
| Total variations identified | 26           | 22           | 17                         | 71 (3 common with XFS) |
| Synonymous SNPs           | 7            | 5            | 5                          | 28               |
| SNPs in D-loop            | 7            | 7            | 8                          | -                |
| Non-synonymous SNPs       | 12           | 10           | 4                          | 30               |
| Pathogenic SNPs           | 2            | 4            | 1                          | 6                |

TABLE 1: Mitochondrial DNA variations observed in XFS, XFG, and cataract (control) patients.

XFS: exfoliation syndrome; XFG: exfoliation glaucoma; SNP: single-nucleotide polymorphism
### Predicting the effect of amino acid substitutions on mutant protein stability

In total, all the seven nsSNPs identified in XFS/XFG patients were confirmed to decrease protein stability, with all nsSNPs predicted to have a $\Delta\Delta G$ value of $<0.5$, indicating a greater impact on the proteins (Tables 1, 2).

### Protein evolutionary conservation analysis

The evolutionary conservation of the mutated protein sequences was determined by running them through the ConSurf web server. Three out of five MT-ND4 nsSNPs were identified as highly conserved and buried residues, while the other two nsSNPs were variable. S273I mutation in MT-ND4 was predicted to be structural residues. On the contrary, both MT-ATP6 nsSNPs were predicted to be buried and variable. The importance of a given amino acid residue, as well as its localized evolution, is demonstrated by a relative study of amino acid residue conservation based on the protein sequence. As shown in Figure 3, the most conserved amino acids in MT-ND4 protein were 109-154, 199-210, 215-245, 268-277, 279-294, and 315-338 while in MT-ATP6 protein (Figure 4) were 85-99, 155-177, and 205-226; the remaining locations were more variable.

**TABLE 2: Free energy change ($\Delta\Delta G$-) and reliability index for the pathogenic non-synonymous SNPs.**

| Pathogenic nsSNPs       | Number of patients | Disease | $\Delta\Delta G$ | RI | Stability |
|-------------------------|--------------------|---------|-----------------|----|-----------|
| I191V in MT-ATP6        | 3                  | XFS     | 0.32            | 6  | Decreases |
| S273I in MT-ND4         | 1                  | XFS     | 0.23            | 1  | Decreases |
| A312V in MT-ND4         | 7                  | XFG     | -0.09           | 7  | Decreases |
| A300T in MT-ND4         | 5                  | XFG     | -0.75           | 5  | Decreases |
| Q304H in MT-ND4         | 4                  | XFG     | -3.15           | 8  | Decreases |
| F117C in MT-ATP6        | 1                  | XFG     | -0.82           | 2  | Decreases |
| A258P in MT-ND4         | 2                  | XFS/XFG | -0.71           | 3  | Decreases |

XFS: exfoliation syndrome; XFG: exfoliation glaucoma; nsSNP: non-synonymous single-nucleotide polymorphism
FIGURE 3: Evolutionary conservation analysis of MT-ND4.

FIGURE 4: Evolutionary conservation analysis of MT-ATP6. In addition to the conservation score, ConSurf considers the structural relevance of a given residue.

Effects of high-risk nsSNPs on protein properties

HOPE was used to predict the effects of the seven pathogenic MT-ND4 and MT-ATP6 nsSNP mutations on amino acid size, charge, hydrophobicity, conservation, and function. While five mutated amino acids in MT-ND4 were bigger than their wild-type counterparts, the two mutated amino acids in MT-ATP6 were smaller. Size differences can affect the contact with the lipid membrane. Bigger residues might lead to bumps and steric hindrance. Two mutations found in MT-ND4 and S273I increased hydrophobicity and A300T...
decreased hydrophobicity indicating that these changes could inhibit correct folding or could lead to loss of hydrophobic interactions in the core or surface of the proteins. The finding suggested that changes in physicochemical properties caused by amino acid mutations at these sites result in changes in protein structure and interactions between protein domains and other molecules, affecting protein function.

**Comparative modeling of wild-type MYB family proteins and their mutant structures**

Pymol was used to generate the structure of wild-type and mutant proteins (Figure 5 and Figure 6a). A Ramachandran plot through the dihedral angles was used to confirm the structural integrity of the generated wild-type and mutant protein structures using PROCHECK. The most favored section of the wild-type MT-ND4 includes 400 residues (95.9%) while the additional authorized region contains 17 residues (4.1%). Mutants A258P, S273I, A300T, Q304H, and A312V and the wild-type MT-ND4 have the same amino acid residue patterns. The structure of wild-type MT-ATP6 and mutants F117C and I191V is identical, with 196 residues (97%) in the most favored region and six residues (3%) in the additional allowed region, showing no substantial structural alterations. Figure 6b shows the particular position in the sequence where mutations are likely to affect function. For all the predicted seven pathogenic mutations, the amino acid substitution was unlikely to affect the function (indicated in blue).

**FIGURE 5:** Analysis of conformational changes in protein structure. We performed a structural homology-based comparative analysis of modeled tertiary structure of mutant proteins, (a) MT-ATP6.
FIGURE 6: To deduce putative structural and functional repercussions imposed by pathogenic nsSNPs in the proteins, we performed a structural homology-based comparison analysis of modeled tertiary structure of mutant proteins, (a, b) MT-ND4, with the wild-type (WT).

Discussion

The mitochondrial genome accumulates mutations faster than the nuclear genome. Consequently, mtDNA has a high degree of polymorphism, which is most likely due to two factors, namely, the lack of protective histones and repair mechanisms, which increases replication errors, and the proximity of mtDNA to the respiratory chain complexes [28]. The importance of mtDNA as a non-invasive biomarker is supported by its short length, comparatively simple structure, great abundance, and capacity to function as a liquid biopsy. According to clinical investigations and research findings, personalized medicine is becoming increasingly interested in mtDNA analysis, and there is hope that the number of overly aggressive and invasive diagnostic procedures will decline. Previous research has linked mtDNA mutations to cellular energy shortages that result in eye degeneration [29]. SNPs are a type of genetic mutation that has been linked to a number of disorders. Non-synonymous mitochondrial mutations impair oxidative phosphorylation, leading to decreased mitochondrial respiration and increased free radical generation [30].

This study effectively discovered high-risk pathogenic nsSNPs in mitochondrial genes using an in silico approach to better understand their association with XFS/XFG. Pathogenic mutations are discussed in terms of their functional significance, stability, and sequence conservation. We further expanded our research and examined the structural and functional effects of pathogenic mutations on proteins.

A total of 65 mtDNA variations were identified in XFS/XFG cases, out of which 16 were nsSNPs. The results of functional analysis by polyphen2, SIFT, mutation taster, and Mutpred of the nsSNPs revealed seven (out of 16) pathogenic nsSNPs. These pathogenic nsSNPs were A258P, S273I, A300T, Q304H, and A312V in MT-ND4 and F117C and I191V in MT-ATP6. MT-ATP6 mutations have been previously found in patients with primary open-angle glaucoma (POAG), primary angle-closed glaucoma, neuropathy, ataxia, retinitis pigmentosa, and mitochondrial DNA-associated Leigh syndrome [28-31]. The MT-ND4 gene is a protein-coding gene found in mtDNA that encodes complex I subunit 4 (NADH ubiquinone oxidoreductase). Complex I is the first enzyme in the respiratory chain, making it vulnerable to oxidative stress. It is also involved in cellular functions such as apoptosis [32]. SNPs in MT-ND4 can have an impact on the first step of the electron transport chain. Hence, these mutations may have an effect on mitochondrial respiratory chain function and may alter cellular energy metabolism. Indeed, due to increased mitochondrial ROS production and attenuation of the mitochondrial membrane, impairments in complex I have been reported to contribute to the gradual loss of trabecular meshwork (TM) cells in POAG patients. This decrease in ATP generation causes the cells to go into apoptosis [33]. However, additional research is needed to establish the regulatory function of mutation in MT-ND4, which could lead to an increase in oxidative stress and favor the
development of glaucoma. Protein stability is an important factor in determining whether a protein is biologically active and functional. A previous study on mutational analysis demonstrated that alterations in hydrophobic interactions are the primary cause of mutational impacts on protein stability [34]. The stability of the pathogenic nsSNPs was determined using I-Mutant 2.0, which identified all the seven nsSNPs with decreased stability. According to the ConSurf results, the majority of substitutions in MT-ND4 were highly conserved and buried while both MT-ATP6 substitutions were variable and buried. We also predicted the post-transcriptional modifications of the seven pathogenic nsSNPs using MsuiteDeep but we did not find any new modifications in the mutant residues.

XFS is a protein aggregopathy with protein complex aggregates being deposited in different ocular structures. These aggregates are believed to arise because of increased oxidative stress causing protein instability and exposure of hydrophobic portions on their surface triggering accumulation and binding of several low and high-molecular-weight proteins forming a complex aggregate. This study found several structural molecular changes in the predicted protein structure, which did not seem to have a structural impact on the proteins. The relevance and impact in aggregate formation are very complex and would need additional computing to discern how these structural changes in 3D protein structure may trigger aggregate formation in XFS/XFG. The protein models in this study were built using two templates (1h88.1 for MT-ND4 protein and cs1dwM for MT-ATP6). These templates were chosen for their high sequence similarity and high GMQE value, resulting in a high coverage. The mutants’ RMSD values suggest that the nsSNPs may not have a substantial structural influence on the proteins.

The only limitation of this study is that it looks at mtDNA sequence variations in a small group of patients with XFS/XFG of Indian ethnic origin, and these findings should be replicated in other populations.

**Conclusions**

The pathogenic A258P, S273I, A300T, Q304H, and A312V mutations in MT-ND4 and the F117C and I191V mutation in MT-ATP6 were expected to be pathogenic, highly conserved, and exposed to lower protein stability, indicating the most substantial harmful effect; however, these predictions need to be backed by proteomic analysis validation. Ideally, a larger sample size should be considered for a significant effect but we found age-related mutations in XFG while excluding other systemic diseases with age which makes it significant. Understanding the pathophysiology of glaucoma may be aided by knowledge of mtDNA mutations and/or mitochondrial dysfunction. Our findings suggest that the mitochondrial genome may be critical for deciphering the molecular patterns found in XFS/XFG and pinpointing putative driver events.

**Appendices**
| Serial number | Mts DNA variations (genomic position) | Codon change | Name of gene | Polyphene-2 score | Polyphene-2 HUmVAr score | SIF score | Mutation tester score | Pathogenic/Non-pathogenic |
|---------------|--------------------------------------|--------------|--------------|-------------------|--------------------------|---------|-----------------------|--------------------------|
| 1             | A2438G                               | ALA>ALA      | RNR2         | -                 | -                        | -       | -                     | -                        |
| 2             | A2451G                               | LYS>GLU      | RNR2         | -                 | -                        | -       | -                     | -                        |
| 3             | A2480G                               | ALA>ALA      | RNR2         | -                 | -                        | -       | -                     | -                        |
| 4             | A2539G                               | HIS>ARG      | RNR2         | -                 | -                        | -       | -                     | -                        |
| 5             | T2455G                               | VAL>GLY      | RNR2         | -                 | -                        | -       | -                     | -                        |
| 6             | A2467T                               | LYS>STOP     | RNR2         | -                 | -                        | -       | -                     | -                        |
| 7             | G2534T                               | STOP>TYR     | RNR2         | -                 | -                        | -       | -                     | -                        |
| 8             | T12149G                              | VAL>VAL      | TRNH         | -                 | -                        | -       | -                     | -                        |
| 9  | A12158G | LYS>LYS | TRNH | - | - | - | - | - |
| 10 | C12187T | GLA>ILE | TRNH | - | - | - | - | - |
| 11 | C12231G | LEU>VAL | TRNS2 | - | - | - | - | - |
| 12 | A1811G | STOP>STOP | RNR2 | - | - | - | - | - |
| 13 | T10873C | PRO>PRO | ND4 | - | - | - | - | - |
| 14 | A2706G | ASN>ASP | RNR2 | - | - | - | - | - |
| 15 | A9097G | ATC>GTC | ILE>VAL | TRNL2 | Possibly damaging with a score of 0.689 (sensitivity: 0.86; specificity: 0.92) | Possibly damaging with a score of 0.736 (sensitivity: 0.77; specificity: 0.86) | Tolerated | Polymorphism | Pathogenic |
| 16 | G1719A | ALA>THR | RNR2 | - | - | - | - | - |
| 17 | G12372A | LEU>LEU | ND5 | - | - | - | - | - |
| 18 | A12308G | LYS>GLU | TRNL2 | - | - | - | - | - |
| 19 | G6899A | MET>ILE | COX1 | Benign with a score of 0.000 (sensitivity: 1.00; specificity: 0.00) | Benign with a score of 0.000 (sensitivity: 1.00; specificity: 0.00) | Tolerated | Disease-causing | Non-pathogenic |
| 20 | A2833G | ASN>SER | RNR2 | - | - | - | - | - |
| 21 | A9251G | PRO>PRO | COX3 | - | - | - | - | - |
| 22 | A3029G | STOP>STOP | 1 | RNR2 | - | - | - | - | - |
| 23 | A3052G | LYS>ARG | RNR2 | - | - | - | - | - |
| 24 | C12106T | LEU>LEU | ND4 | - | - | - | - | - |
| 25 | A11467G | LEU>LEU | ND4 | - | - | - | - | - |
| 26 | G11531C | ALA>PRO | ND4 | Probably damaging with a score of 1.000 (sensitivity: 0.00; specificity: 1.00) | Probably damaging with a score of 0.996 (sensitivity: 0.36; specificity: 0.97) | Not tolerated | Polymorphism | Pathogenic |
| 27 | C11563T | GLY>GLY | ND4 | - | - | - | - | - |
| 28 | G11577T | SER>ILE | ND4 | Probably damaging with a score of 1.000 (sensitivity: 0.00; specificity: 1.00) | Probably damaging with a score of 0.995 (sensitivity: 0.45; specificity: 0.96) | Not tolerated | Polymorphism | Pathogenic |
| 29 | G1598A | THR>THR | RNR1 | - | - | - | - | - |
| 30 | A9218G | GLN>GLN | COX3 | - | - | - | - | - |
| 31 | A12163G | GLN>ARG | RNR2 | - | - | - | - | - |
| 32 | G8950A | VAL>ILE | ATP6 | Benign with a score of 0.000 (sensitivity: 0.1; specificity: 0.00) | Benign with a score of 0.000 (sensitivity: 1.00; specificity: 0.00) | Tolerated | Polymorphism | Non-pathogenic |
| 33 | T11460C | VAL>ALA | ND4 | - | - | - | - | - |
| 34 | G3010A | ARG>GLN | RNR2 | - | - | - | - | - |
| 35 | G8790A | LEU>LEU | ATP6 | - | - | - | - | - |
| 36 | C9094T | LEU>PHE | ATP6 | Possibly damaging with a score of 0.855 (sensitivity: 0.83; specificity: 0.93) | Benign with a score of 0.433 (sensitivity: 0.84; specificity: 0.80) | Tolerated | Polymorphism | Non-pathogenic |
| 37 | G11651C | VAL>LEU | ND4 | Benign with a score of 0.002 (sensitivity: 0.99; specificity: 0.30) | Benign with a score of 0.007 (sensitivity: 0.97; specificity: 0.46) | Tolerated | Polymorphism | Non-pathogenic |
| 38 | G11657A | ALA>THR | ND4 | Probably damaging with a score of 0.999 (sensitivity: 0.999; specificity: 0.999) | Probably damaging with a score of 0.988 (sensitivity: Not | Polymorphism | Pathogenic |
| Serial number | MtDNA variations | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
|---------------|-----------------|---|---|---|---|---|---|---|---|---|----|---|---|----|---|---|---|
| 1             | A750G RNR1      | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 2             | A1438G RNR1     | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 3             | A8630G ATP6     | Benign with a score of 0.351 (sensitivity: 0.90; specificity: 0.89) | Benign with a score of 0.052 (sensitivity: 0.93; specificity: 0.63) | Tolerated | Polymorphism | Non-pathogenic |
| 4             | A8860G ATP6     | Probably damaging with (score of 0.978 sensitivity: 0.76; specificity: 0.96) | Probably damaging with a score of 0.967 (sensitivity: 0.81; specificity: 0.93) | Tolerated | Polymorphism | Pathogenic |
| 5             | A11719G ND4     | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 6             | A9180G ATP6     | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 7             | G11719A ND4     | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 8             | T2302G RNR2     | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 9             | A2473G RNR2     | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 10            | T12477C ND5     | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 11            | T1187C RNR1     | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 12            | C4883T ND2      | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 13            | C5187A ND2      | Possibly damaging with (score of 0.513 sensitivity: 0.88; specificity: 0.90) | Benign with a score of 0.393 (sensitivity: 0.84; specificity: 0.79) | Tolerated | Polymorphism | Non-pathogenic |
| 14            | C8562T ATP8     | Benign with a score of 0.000 (sensitivity: 1.00; specificity: 0.00) | Benign with a score of 0.000 (sensitivity: 1.00; specificity: 0.00) | Tolerated | Disease-causing | Non-pathogenic |
| 15            | C8573A ATP6     | Benign with a score of 0.015 (sensitivity: 0.96; specificity: 0.79) | Benign with a score of 0.011 (sensitivity: 0.96; specificity: 0.51) | Not tolerated | Disease-causing | Non-pathogenic |
| 16            | C6020T COX1     | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
|   | 17 T9098C | ATP6 | Probably damaging with (score of 0.999 sensitivity: 0.14; specificity: 0.99) | Probably damaging with a score of 0.999 (sensitivity: 0.09; specificity: 0.99) | Not tolerated | Polymorphism | Pathogenic |
|---|-----------|------|-------------------------------------------------------------------------|--------------------------------------------------------------------------------|---------------|--------------|------------|
| 18 | C8137T    | COX2 | -                                                                       | -                                                                               | -             | -            | -          |
| 19 | C11674T   | ND4  | -                                                                       | -                                                                               | -             | -            | -          |
| 20 | A11947G   | ND4  | -                                                                       | -                                                                               | -             | -            | -          |
| 21 | A5204G    | ND2  | -                                                                       | -                                                                               | -             | -            | -          |
| 22 | C5229G    | ND2  | Probably damaging with (score of 0.998 sensitivity: 0.27; specificity: 0.99) | Probably damaging with a score of 0.962 (sensitivity: 0.62; specificity: 0.92) | Not tolerated | Polymorphism | Pathogenic |
| 23 | A6461G    | COX1 | -                                                                       | -                                                                               | -             | -            | -          |
| 24 | T620C     | TRNF | -                                                                       | -                                                                               | -             | -            | -          |
| 25 | G8896A    | ATP6 | Benign with a score of 0.000 (sensitivity: 1.00; specificity: 0.00)       | Benign with a score of 0.002 (sensitivity: 0.99; specificity: 0.18)            | Tolerated     | Polymorphism | Non-pathogenic |
| 26 | G1888A    | RNR2 | -                                                                       | -                                                                               | -             | -            | -          |
| 27 | G4991A    | ND2  | -                                                                       | -                                                                               | -             | -            | -          |
| 28 | A2468G    | RNR2 | -                                                                       | -                                                                               | -             | -            | -          |
| 29 | G6305A    | COX1 | -                                                                       | -                                                                               | -             | -            | -          |
| 30 | C8431T    | ATP8 | -                                                                       | -                                                                               | -             | -            | -          |
| 31 | T980C     | RNR1 | -                                                                       | -                                                                               | -             | -            | -          |
| 32 | G11963A   | ND4  | -                                                                       | -                                                                               | -             | -            | -          |
| 33 | G12561A   | ND5  | -                                                                       | -                                                                               | -             | -            | -          |
| 34 | T2498G    | RNR2 | -                                                                       | -                                                                               | -             | -            | -          |
| 35 | A8396G    | ATP8 | Possibly damaging with a score of 0.955 (sensitivity: 0.79; specificity: 0.95) | Possibly damaging with a score of 0.974 (sensitivity: 0.59; specificity: 0.93) | Not tolerated | Polymorphism | Pathogenic |
| 36 | A8502G    | ATP8 | Possibly damaging with a score of 0.955 (sensitivity: 0.79; specificity: 0.95) | Possibly damaging with a score of 0.879 (sensitivity: 0.71; specificity: 0.89) | Tolerated     | Polymorphism | Pathogenic |
| 37 | A8842G    | ATP6 | Benign with a score of 0.003 (sensitivity: 0.98; specificity: 0.44)      | Benign with a score of 0.002 (sensitivity: 0.99; specificity: 0.18)            | Tolerated     | Polymorphism | Non-pathogenic |
| 38 | G11963A   | ND4  | Benign with a score of 0.000 (sensitivity: 1.00; specificity: 0.00)      | Benign with a score of 0.000 (sensitivity: 1.00; specificity: 0.00)            | Tolerated     | Polymorphism | Non-pathogenic |
| 39 | G12501A   | ND5  | Benign with a score of 0.000 (sensitivity: 1.00; specificity: 0.00)      | Benign with a score of 0.000 (sensitivity: 1.00; specificity: 0.00)            | Tolerated     | Disease-causing | Non-pathogenic |
| 40 | C4058G    | ND1  | Benign with a score of 0.367 (sensitivity: 0.90; specificity: 0.89)      | Benign with a score of 0.366 (sensitivity: 0.85; specificity: 0.78)            | Tolerated     | Polymorphism | Non-pathogenic |
| 41 | C4197T    | ND1  | -                                                                       | -                                                                               | -             | -            | -          |
| 42 | T4231C    | ND1  | Benign with a score of 0.003 (sensitivity: 0.99; specificity: 0.15)      | Benign with a score of 0.000 (sensitivity: 0.95; specificity: 0.57)            | Tolerated     | Polymorphism | Non-pathogenic |

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| N | SNP | Gene | Chromosome | Position | Effect | Disease-Causing | Tolerated | Polymorphism | Pathogenic |
|---|-----|------|------------|----------|--------|----------------|----------|--------------|------------|
| 43 | A8887G | ATP6 | 1 | 179997129 | Benign with a score of 0.000 (sensitivity: 1.00; specificity: 0.98) | Tolerated | Polymorphism | pathogenic |
| 44 | A12507G | ND5 | 1 | 179997129 | Benign with a score of 0.006 (sensitivity: 0.97; specificity: 0.44) | Tolerated | Polymorphism | Non-pathogenic |
| 45 | A12507G | ND5 | 1 | 179997129 | Benign with a score of 0.006 (sensitivity: 0.97; specificity: 0.44) | Tolerated | Polymorphism | Non-pathogenic |
| 46 | A4093G | ND1 | 1 | 179997129 | Benign with a score of 0.000 (sensitivity: 1.00; specificity: 0.44) | Tolerated | Polymorphism | pathogenic |
| 47 | C6164T | COX1 | 1 | 179997129 | Benign with a score of 0.000 (sensitivity: 1.00; specificity: 0.44) | Tolerated | Polymorphism | pathogenic |
| 48 | T6293C | COX1 | 1 | 179997129 | Benign with a score of 0.000 (sensitivity: 1.00; specificity: 0.44) | Tolerated | Polymorphism | pathogenic |
| 49 | T1180G | RNR1 | 1 | 179997129 | Benign with a score of 0.000 (sensitivity: 1.00; specificity: 0.44) | Tolerated | Polymorphism | pathogenic |
| 50 | G6480A | COX1 | 1 | 179997129 | Benign with a score of 0.000 (sensitivity: 1.00; specificity: 0.44) | Tolerated | Polymorphism | pathogenic |
| 51 | T1243C | RNR1 | 1 | 179997129 | Benign with a score of 0.000 (sensitivity: 1.00; specificity: 0.44) | Tolerated | Polymorphism | pathogenic |
| 52 | C6173T | COX1 | 1 | 179997129 | Benign with a score of 0.000 (sensitivity: 1.00; specificity: 0.44) | Tolerated | Polymorphism | pathogenic |
| 53 | T5082C | ND2 | 1 | 179997129 | Benign with a score of 0.000 (sensitivity: 1.00; specificity: 0.44) | Tolerated | Polymorphism | pathogenic |
| 54 | C6290T | COX1 | 1 | 179997129 | Benign with a score of 0.000 (sensitivity: 1.00; specificity: 0.44) | Tolerated | Polymorphism | pathogenic |
| 55 | A11947G | ND4 | 1 | 179997129 | Benign with a score of 0.000 (sensitivity: 1.00; specificity: 0.44) | Tolerated | Polymorphism | pathogenic |
| 56 | C1530T | RNR1 | 1 | 179997129 | Benign with a score of 0.000 (sensitivity: 1.00; specificity: 0.44) | Tolerated | Polymorphism | pathogenic |
| 57 | T6676G | COX1 | 1 | 179997129 | Benign with a score of 0.000 (sensitivity: 1.00; specificity: 0.44) | Tolerated | Polymorphism | pathogenic |

**TABLE 5: Mitochondrial DNA mutation analysis in control (cataract) patients by various online tools.**

**Additional Information**

**Disclosures**

**Human subjects:** Consent was obtained or waived by all participants in this study. Institutional Review Board of L V Prasad Eye Institute issued approval 2016-60-IM-12. **Animal subjects:** All authors have confirmed that this study did not involve animal subjects or tissue. **Conflicts of interest:** In compliance with the ICMJE uniform disclosure form, all authors declare the following: **Payment/services info:** This work was funded in full by “DBT Wellcome Trust India alliance, grant ref number IA/CPHI/15/1/502031” awarded to Dr. Aparna Rao. **Financial relationships:** All authors have declared that they have no financial relationships at present or within the previous three years with any organizations that might have an interest in the submitted work. **Other relationships:** All authors have declared that there are no other relationships or activities that could appear to have influenced the submitted work.

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