Mitochondrial DNA analysis in primary congenital glaucoma

Mukesh Tanwar,¹ Tanuj Dada,² Ramanjit Sihota,² Rima Dada¹

¹Laboratory for Molecular Reproduction and Genetics, Department of Anatomy, All India Institute of Medical Sciences, Ansari Nagar, New Delhi, India; ²Dr. R.P. Centre for Ophthalmic Sciences, All India Institute of Medical Sciences, Ansari Nagar, New Delhi, India

Purpose: To screen mitochondrial DNA (mtDNA) for nucleotide variations in primary congenital glaucoma (PCG).

Methods: The entire coding region of the mitochondrial genome was amplified by polymerase chain reaction from 35 PCG patients and 40 controls. The full mtDNA genome except the D-loop was sequenced. All sequences were analyzed against mitochondrial reference sequence NC_012920.

Results: MtDNA sequencing revealed a total of 132 and 58 nucleotide variations in PCG and controls, respectively. Of 132 nucleotide variations, 42 (31.81%) were non-synonymous and 82 (62.12%) were synonymous changes, and 8 were in RNA genes. The highest number of nucleotide variations were recorded in complex I followed by complex IV, then complex V. Eight patients (22.85%) had potentially pathogenic mtDNA nucleotide changes and twenty (57.14%) had mtDNA sequence changes associated with elevated reactive oxygen species (ROS) production. Mitochondria not only constitute the energy-generating system in the cell, but are also critically involved in calcium signaling and apoptosis. Mitochondrial function can be affected by mutations in mitochondrial and nuclear DNA, chemical insults to components of the electron transport chain, and a lack of substrates such as oxygen. Mitochondrial dysfunction results in an excessive generation of free radicals and reduced mitochondrial respiration. Developing trabecular meshwork (TM) is deficient in antioxidant enzymes, and thus is more susceptible to oxidative stress (OS) induced damage. Previous studies have documented certain mtDNA sequence variations associated with elevated ROS levels and OS. Three such changes (G10398A, A12308G, and G13708A) were present in our patients. Elevated ROS may cause OS. This OS may further damage mtDNA and may cause decreased mitochondrial respiration. This may lead to impaired growth, development and differentiation of TM and consequently trabecular-dysgenesis, which is a characteristic feature of PCG. OS affects both TM and retinal ganglion cells (RGCs) and may be involved in the neuronal death affecting the optic nerve in glaucoma. There are several studies which point that elevated hydrostatic pressure causes breakdown of the mitochondrial network by mitochondrial fission and induce cristae depletion and cellular ATP reduction in differentiated RGC-5 cells in vitro as well as in vivo.

Conclusions: A total of 44 novel mtDNA variations were identified in this study. Non-synonymous mtDNA variations may adversely affect respiratory chain, impair OXPHOS pathway result in low ATP production, high ROS production and impair growth, development and differentiation of TM lead to trabecular-dysgenesis and consequently RGC’s death. Such cases with mtDNA variations and consequent OS may benefit by early diagnosis and prompt management by antioxidant therapy. This may delay OS induced injury to TM and RGCs and hence improve visual prognosis.

Glaucomas are a heterogeneous group of eye conditions with manifestation as early as birth to very late age of onset and are among most common cause of blindness worldwide, accounting for 15% of cases. Primary congenital glaucoma (PCG; OMIM 231300; provided in the public domain by the National Centre for Biotechnology Information, Bethesda, MD) is a severe form of glaucoma with manifestation at birth or early childhood. It is characterized by elevated intraocular pressure (IOP), and enlarged cornea and globe (buphthalmos) [1]. The only observable anatomic defect in PCG is trabecular-dysgenesis. This leads to impaired aqueous drainage, increased intraocular pressure, optic nerve damage, and may consequently lead to partial/permanent visual impairment. Progressive degeneration of retinal ganglion cells (RGCs) and their axons is the primary cause of glaucomatous visual loss. However, many aspects of this blinding disorder are still unclear and current treatment options are not sufficient to block neurodegenerative injury in these patients.

PCG is bilateral in 80% cases. The majority of PCG cases present within the first year of life out of which 25% are diagnosed in the neonatal period and in about 60% within first six months of life. The majority of PCG cases are sporadic. PCG is the most common type of pediatric glaucoma and accounts for 55% of pediatric glaucomas. The prevalence of PCG varies across ethnic communities ranging from 1 in 10,000–20,000 in the western populations [2] to 1 in 2,500...
and 1 in 1,250 in the Saudi Arabian population [3] and Gypsy population of Slovakia [2], and 1 in 3,300 in Andhra Pradesh, India [4]. Early and reliable diagnosis of this disease is vital, so that appropriate and prompt treatment is initiated. This can improve the visual outcome and prevent visual loss.

Three genetic loci: GLC3A at 2p21, GLC3B at 1p36, and GLC3C at 14q24.3-q31.1 have been mapped for PCG [3,5,6]. Mutations in CYP1B1 (GLC3A locus) have been found in PCG patients from different populations [3,7-10] It is estimated that all known loci/genes of glaucoma account for the minority of total cases of glaucoma, and thus, many other genes remain to be identified.

The role of mitochondrial DNA (mtDNA) mutations and oxidative stress (OS) has been reported in primary open angle glaucoma (POAG) [11,12]. Recent studies reported an increased frequency of mtDNA sequence changes in primary open angle glaucoma (POAG), primary angle closure glaucoma (PACG), and pseudoexfoliation glaucoma (PEG) compared to controls [11,13,14]. Therefore this study was planned with the aim to screen PCG cases for mitochondrial DNA variations.

**METHODS**

Clinical examination and selection of cases: Primary congenital glaucoma cases (n=35) presenting at the Dr. R. P. Centre for Ophthalmic Sciences (AIIMS, New Delhi, India), were enrolled for this study, after ethical approval of the Institutional Review Board (IRB00006862; All India Institute of Medical Sciences, New Delhi, India). The diagnosis involved clinical ocular and systemic examination. Inclusion criteria of the patients were: increased corneal diameter (>12.0 mm), raised IOP (>21 mmHg) with presence/absence of Haab’s striae, and optic disc changes (where examination was possible). Symptoms of epiphora and photophobia were additional inclusion factors. The age of onset ranged from birth to 3 years. All patients with a history of blood transfusion, TORCH (Toxoplasmosis; Rubella; Cytomegalovirus; Herpes Simplex Virus) infection, and drug intake in the mother during pregnancy were excluded. Glaucoma cases other than PCG were also excluded. Detailed family history of ocular or other hereditary disorders up to three generations were taken, and pedigree charts were constructed. Forty ethnically matched normal individuals without any ocular disorders with IOP<20mmHg and corneal diameter <12×12mm were enrolled as controls.

Sample collection and DNA isolation: Peripheral blood sample was collected from patients and controls by venipuncture after informed consent. Blood samples were collected in EDTA (EDTA) vacutainers and stored in −80 °C (°C) until DNA isolation. DNA was isolated from whole blood using the phenol-chloroform method.

Polymerase chain reaction (PCR) amplification and sequence analysis of the mitochondrial DNA coding region: The whole mitochondrial genome was amplified in all patients and controls using 24 pairs of primers [15]. PCR amplifications for all primer sets were performed in a 40 μl volume containing 1.0 μl of 20 μM stock solution for each primer, 100 ng of genomic DNA, 1 unit of Taq polymerase (Bangalore Genei, Bengaluru, Karnataka, India), 0.1 mM of each dNTP, 4 μl of 10× PCR buffer (with 15 mM MgCl₂), by means of 30 cycles of amplification, each consisting of 30 s denaturation at 94 °C, 30 s annealing at 56 °C and 1 min extension at 72 °C. Finally, and extension for 5 min at 72 °C was performed. Amplified PCR products were purified using a gel/PCR DNA fragments extraction kit (catalog number DF100; Geneaid Biotech Ltd., Sijjh City, Taiwan). Purified PCR products were sent for sequencing to MCLAB (Molecular Cloning Laboratories, South San Francisco, CA). The full mtDNA genome was sequenced except D-loop as D-loop is a hyper-variable region. All fragments were sequenced in both forward and reverse direction for confirmation. All sequence variants from both PCG patients and controls were compared to Human Mitochondrial reference sequence NC_012920 provided by the National Center for Biotechnology Information (NCBI) using ClustalW2 (multiple sequence alignment program for DNA; European Molecular Biology Laboratory (EMBL) – European Bioinformatics Institute (EBI)).

Prediction of pathogenicity: For prediction of pathogenic characteristics of all non-synonymous mtDNA changes two homology based programs PolyPhen (Polymorphism Phenotyping) and SIFT (Sorting Intolerant From Tolerant) analysis tool were used. PolyPhen structurally analyzes an amino acid polymorphism and predicts whether that amino acid change is likely to be deleterious to protein function [16-18]. The prediction is based on the position-specific independent counts (PSIC) score derived from multiple sequence alignments of observations in case of functional domain of protein and Predicted hydrophobic and transmembrane (PHAT) matrix element difference in case of transmembrane region of protein. PolyPhen scores of >2.0 indicate the polymorphism is probably damaging to protein function. Scores of 1.5–2.0 are possibly damaging, and scores of <1.5 are likely benign. SIFT is a sequence homology-based tool that sorts intolerant from tolerant amino acid substitutions and predicts whether an amino acid substitution in a protein will have a phenotypic effect [19-22]. SIFT is based on the premise that protein evolution is correlated with protein function. Positions important for function should be conserved in an alignment of the protein family, whereas unimportant positions should appear diverse in an alignment. Positions with normalized probabilities less than 0.05 are predicted to be deleterious and, those greater than or equal to 0.05 are predicted to be tolerated.

Statistical analysis: Pearson χ²/Fisher’s exact test was applied to make a comparison between two groups (cases versus controls). P-values less than 0.05 were considered as
significant. All tests were done using SPSS software for windows (version 11.5; SPSS Inc., Chicago, IL).

RESULTS

MtDNA sequencing following whole genome amplification of mitochondrial DNA revealed a total of 132 nucleotide variations (Table 1) in PCG patients and 58 in controls (Table 2). Of the 132 nucleotide variations, 42 (31.81%) were non-synonymous, 82 (62.18%) were synonymous changes, and 8 were in RNA genes. In total, 23.48% (31/132) variations were novel out of which 41.93% (13/31) were non-synonymous (Table 1). A total of 66/132 (50.00%) variations were observed in complex I, 12/132 (9.09%) in complex III, 26/132 (19.69%) in complex IV, and 20/132 (15.15%) were in complex V (Figure 1). Out of the total variations reported, complex I had 31.81% (21/66) non-synonymous base changes, complex III had 25.00% (3/12), complex IV had 23.07% (6/26), and complex V had 55.00% (11/20) non-synonymous base changes. Of 58 variations in the controls, 14 were non-synonymous changes.

Two non-synonymous changes (p.W239C in ND2 and p.A20T in ATPase6) were present both in cases as well as controls. The remaining 40 non-synonymous changes were limited to PCG cases only. All novel variations from patients and controls were submitted to the GenBank database and accession numbers were obtained (Table 1 and Table 2).

SIFT and PolyPhen analysis of all non-synonymous changes from cases and controls revealed five pathogenic changes (p.P2L, p.I10T, p.M31T in ND1 protein and p.M37I, p.W239C in ND2 protein). Eight patients (22.85%) were positive for either of these pathologic mtDNA nucleotide changes. Clinical features of patients and mtDNA variations identified in this study have been tabulated (Table 3).

DISCUSSION

The human mtDNA is a 16,569-base pair double-stranded, compact, circular molecule which lacks histones and is without introns. MtDNA has several overlapping genes and incomplete termination codons. It contains 37 genes which regulate oxidative phosphorylation (OXPHOS). Of these, 24 are needed for mtDNA translation (2 rRNAs [rRNAs] and 22 tRNAs [tRNAs]), and 13 encode subunits of the respiratory chain: seven subunits of complex I (ND1, 2, 3, 4, 4L, 5, and 6 [ND stands for NADH dehydrogenase]), one subunit of complex III (cytochrome b), three subunits of cytochrome c oxidase (COX I, II, and III), and two subunits of ATP synthase (ATPase6 and ATPase8; Figure 2).

MtDNA mutates 10 times more frequently as compared to nuclear DNA due to its proximity to the electron transport chain (ETC) and lack of histones and other protective proteins and has very basic repair mechanism [23]. Mitochondria are essential for ATP production by OXPHOS and are susceptible to oxidative damage because reactive oxygen species (ROS) damage mitochondrial enzymes directly and alter mitochondrial membrane permeability leading to cell death [24]. Most studies suggest that the majority of intracellular ROS produced by non-phagocytic cells are derived from mitochondria [25,26]. Thus mitochondria are both source and target of free radicals.

Several human diseases have been associated with mtDNA mutations, indicating that dysfunction of the components of oxidative phosphorylation encoded by the mitochondrial genome can be deleterious [27]. Abnormalities in mtDNA have proven to be associated with leber’s hereditary optic neuropathy (LHON) [28], POAG, pseudoexfoliation glaucoma (PEG), primary angle closure glaucoma (PACG), other spontaneous optic neuropathies [11,13,14,29], and male infertility [15].

In this study we screened 35 PCG cases for mtDNA variations. We found 42 non-synonymous mtDNA variations in PCG patients in different mitochondrial genes. The highest number of nucleotide variations were recorded in complex I, followed by complex IV and then complex V. Eight patients (22.85%) were found to be positive for pathogenic changes while in PEG patients this was 10.30% [14].

Complex I is responsible for pumping of protons (H+) from the matrix to the inter-membrane space in association with complex III and IV. Although the mitochondrial ETC is very effective in the reduction of oxygen to water, there is a constant “leak” of electrons from the ETC to oxygen and this results in the formation of superoxide anions. It is generally agreed that there are two main sites in the respiratory chain where superoxide anions are generated viz. complex I and complex III [30,31]. Dismutation of superoxide anions produces hydrogen peroxide as a secondary product, and in the presence of transition metals this can be converted to a highly reactive hydroxyl radical that can readily oxidize proteins, lipids, carbohydrates, DNA, and RNA [32]. Fifty percent nucleotide variations identified in our study were in complex I.

SIFT and PolyPhen analysis of missense changes showed that p.P2L, p.I10T, and p.M31T in ND1 protein and p.M37I and p.W239C in ND2 protein were deleterious to protein function. PHAT (predicted hydrophobic and transmembrane) score difference of p.P2L and p.I10T was >2 and PSIC score of p.M31T was >2. PSIC score of p.M37I and W239C was >2 and >1.5, respectively. All these changes (p.P2L, p.I10T, and p.M31T in ND1 and p.M37I and p.W239C in ND2) had SIFT scores <0.05 and were predicted to be deleterious. Pathogenic variants p.P2L, p.I10T, and p.M31T were present in 3 cases (1 case each) while p.M37I and p.W239C were present in one and four cases, respectively. However, frequency of the pathogenic variants (p.P2L, p.I10T, and p.M31T in ND1 and p.M37I and p.W239C in ND2) was not found to be statistically significant (p value >0.05) in our study population.

Recent studies have shown that G4580A (p.M37I) in ND2 and G10398A (p.A114T) in ND3 are associated with an
| Sample number | Genomic Position | Base Change | Gene /Location | Amino acid position | Codon change | Amino acid change | Change in protein | Syn/ Non-syn | Total number of patients with nt changes | GenBank accession number if novel |
|---------------|-----------------|-------------|---------------|---------------------|--------------|-------------------|------------------|-------------|------------------------------------------|----------------------------------|
| 1             | 2707            | G>A         | 16s RNA       | -                   | -            | -                 | -                | -           | 1                                        | -                                |
| 2             | 2790            | Ins T       | 16s RNA       | -                   | -            | -                 | -                | -           | 2 GU186097                                | -                                |
| 3             | 3311            | C>T         | ND1           | 2                   | CCC>CTC     | Pro>Leu          | p.P2L            | non-syn     | 1                                        | GU186068                         |
| 4             | 3316            | G>A         | ND1           | 4                   | GCC>ACC     | Ala>Thr          | p.A4T            | non-syn     | 1                                        | -                                |
| 5             | 3335            | T>C         | ND1           | 10                  | ATT>ACT     | IsoLeu-Thr       | p.I10T           | non-syn     | 1                                        | -                                |
| 6             | 3398            | T>C         | ND1           | 31                  | ATA>ACA     | Met>Thr          | p.M31T           | non-syn     | 2 GU186069                                | -                                |
| 7             | 3480            | A>G         | ND1           | 58                  | AAA>AAG     | Lys>Lys          | p.K58K           | syn         | 2                                        | -                                |
| 8             | 3645            | T>C         | ND1           | 113                 | GTT>GTC     | Val>Val          | p.V113V          | syn         | 1                                        | -                                |
| 9             | 3741            | C>T         | ND1           | 145                 | ACC>ACT     | Thr>Thr          | p.I145T          | syn         | 1                                        | -                                |
| 10            | 3826            | T>C         | ND1           | 174                 | TTA>CTA    | Leu>Leu          | p.L174L          | syn         | 1                                        | -                                |
| 11            | 3921            | C>T         | ND1           | 205                 | TCC>TCT    | Ser>Ser          | p.S205S          | syn         | 2                                        | -                                |
| 12            | 4216            | T>C         | ND1           | 304                 | TAT>CAT    | Tyr>His          | p.Y304H          | non-syn     | 1                                        | -                                |
| 13            | 4225            | A>G         | ND1           | 307                 | ATA>GTA    | Met>Val          | p.M307V          | non-syn     | 1                                        | -                                |
| 14            | 4502            | T>C         | ND2           | 11                  | TCT>TCC    | Ser>Ser          | p.S11S           | syn         | 1                                        | GU186070                         |
| 15            | 4561            | T>C         | ND2           | 31                  | GTA>GCA    | Val>Ala          | p.V31A           | non-syn     | 1                                        | -                                |
| 16            | 4580            | G>A         | ND2           | 37                  | ATG>ATA    | Met> iso         | p.M37I           | non-syn     | 1                                        | -                                |
| 17            | 4615            | A>G         | ND2           | 49                  | AAC>AGC    | Asn>Ser          | p.N49S           | non-syn     | 1                                        | GU186071                         |
| 18            | 4638            | A>G         | ND2           | 57                  | ACT>GTC    | Ile>Val          | p.I57V           | non-syn     | 1                                        | GU186072                         |
| 19            | 4703            | T>C         | ND2           | 78                  | AAT>AAC    | Asn>Asn          | p.N78N           | syn         | 1                                        | -                                |
| 20            | 4916            | A>G         | ND2           | 149                 | CTA>CTG    | Leu>Leu          | p.L149L          | syn         | 1                                        | -                                |
| 21            | 4917            | A>G         | ND2           | 150                 | AAC>GAC    | Asn>Asp          | p.N150D          | non-syn     | 1                                        | -                                |
| 22            | 4944            | A>G         | ND2           | 159                 | ATC>GTC    | Ile>Val          | p.I159V          | non-syn     | 1                                        | -                                |
| 23            | 5033            | A>G         | ND2           | 188                 | GGA>GAG    | Gly>Gly          | p.G188G          | syn         | 1                                        | -                                |
| 24            | 5097            | A>G         | ND2           | 210                 | ATC>GTC    | Ile>Val          | p.I210V          | non-syn     | 1 GU186073                                | -                                |
| 25            | 5186            | A>T         | ND2           | 239                 | TGA>TGT    | Trp>Cys          | p.W239C          | non-syn     | 4                                        | -                                |
| 26            | 5252            | G>A         | ND2           | 261                 | TGT>TTA    | Leu>Leu          | p.L261L          | syn         | 1                                        | -                                |
| 27            | 5360            | T>C         | ND2           | 297                 | ATC>ATT    | Ile>Ile          | p.I297I          | syn         | 1                                        | -                                |
| 28            | 6011            | T>C         | CO1           | 36                  | CTT>CTC    | Leu>Leu          | p.L36L           | syn         | 1                                        | -                                |
| 29            | 6023            | G>A         | CO1           | 40                  | GAG>GAA    | Glu>Glu          | p.E40E           | syn         | 1                                        | -                                |
| 30            | 6217            | T>C         | CO1           | 105                 | TTA>CTA    | Leu>Leu          | p.L105L          | syn         | 1                                        | GU186074                         |
| 31            | 6290            | C>T         | CO1           | 129                 | TAC>TAT    | Tyr>Tyr          | p.Y129Y          | syn         | 3                                        | -                                |
| 32            | 6366            | G>A         | CO1           | 155                 | GTC>ATC    | Val>Ile          | p.V155I          | non-syn     | 3                                        | -                                |
| 33            | 6584            | C>T         | CO1           | 227                 | GAC>GAT    | Asp>Asp          | p.D227D          | syn         | 1                                        | GU186075                         |
| 34            | 7280            | C>T         | CO1           | 459                 | TTC>TTT    | Phe>Phe          | p.F459F          | syn         | 2                                        | GU186076                         |
| 35            | 7598            | G>A         | CO2           | 5                   | GGC>GAC    | Ala>Thr          | p.A5T            | non-syn     | 1                                        | -                                |
| 36            | 7746            | A>G         | CO2           | 54                  | AAC>AAGC   | Asn>Ser          | p.N54S           | non-syn     | 1                                        | GU186077                         |
| 37            | 7843            | A>G         | CO2           | 86                  | ATA>ATG    | Met>Met          | p.M86M           | syn         | 3                                        | -                                |
| 38            | 7886            | C>T         | CO2           | 95                  | CTT>TGT    | Leu>Phe          | p.L95F           | non-syn     | 2                                        | -                                |
| 39            | 7961            | T>C         | CO2           | 126                 | TTA>CTA    | Leu>Leu          | p.L126L          | syn         | 1                                        | -                                |
| 40            | 7962            | T>C         | CO2           | 126                 | TTA>TCA    | Leu>Ser          | p.L126S          | non-syn     | 1                                        | GU186078                         |
| 41            | 8023            | T>C         | CO2           | 146                 | ATA>ATC    | Ile>Ile          | p.I146T          | syn         | 1                                        | GU186079                         |
| 42            | 8116            | A>G         | CO2           | 177                 | GGA>GAG    | Gly>Gly          | p.G177G          | syn         | 1                                        | -                                |
| 43            | 8136            | T>C         | CO2           | 184                 | TTC>TAT    | Phe>Phe          | p.F184F          | syn         | 32                                       | -                                |
| 44            | 8252            | G>A         | CO2           | 222                 | GGG>GGA    | Gly>Gly          | p.G222G          | syn         | 2                                        | -                                |
| 45            | 8346            | C>T         | tRNA Lys      | -                   | -            | -                 | -                | -           | 1 GU186096                                | -                                |
| Sample number | Genomic Position | Base Change | Gene / Location | Codon change | Amino acid change | Change in protein | Total number of patients with nt changes | GenBank accession number if novel |
|---------------|------------------|-------------|----------------|--------------|-------------------|------------------|-----------------------------------------|----------------------------------|
| 46            | 8396             | A>G          | ATPase8        | ATPase8      | Thr>Ala           | p.T11A non-syn   | 1                                        | GU186091                         |
| 47            | 8485             | G>A          | ATPase8        | ATPase8      | Lys>Thr           | p.K120K syn      | 1                                        | GU186092                         |
| 48            | 8502             | A>G          | ATPase8        | ATPase8      | Ala>Thr           | p.P111V syn      | 1                                        | GU186093                         |
| 49            | 8584             | G>A          | ATPase8        | ATPase8      | Thr>Thr           | p.V1121V syn     | 1                                        | GU186094                         |
| 50            | 8658             | C>T          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 51            | 8684             | C>T          | ATPase6        | ATPase6      | Ala>Thr           | p.A53T non-syn   | 1                                        | GU186089                         |
| 52            | 8697             | G>A          | ATPase6        | ATPase6      | Lys>Lys           | p.K120K syn      | 1                                        | GU186092                         |
| 53            | 8701             | A>G          | ATPase6        | ATPase6      | Thr>Thr           | p.V113V syn      | 1                                        | GU186093                         |
| 54            | 8704             | C>T          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 55            | 8708             | C>T          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 56            | 8710             | G>A          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 57            | 8712             | C>T          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 58            | 8715             | G>A          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 59            | 8717             | C>T          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 60            | 8720             | G>A          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 61            | 8722             | C>T          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 62            | 8724             | G>A          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 63            | 8726             | C>T          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 64            | 8728             | G>A          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 65            | 8730             | C>T          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 66            | 8732             | G>A          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 67            | 8734             | C>T          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 68            | 8736             | G>A          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 69            | 8738             | C>T          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 70            | 8740             | G>A          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 71            | 8742             | C>T          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 72            | 8744             | G>A          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 73            | 8746             | C>T          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 74            | 8748             | G>A          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 75            | 8750             | C>T          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 76            | 8752             | G>A          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 77            | 8754             | C>T          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 78            | 8756             | G>A          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 79            | 8758             | C>T          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 80            | 8760             | G>A          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 81            | 8762             | C>T          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 82            | 8764             | G>A          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 83            | 8766             | C>T          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 84            | 8768             | G>A          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 85            | 8770             | C>T          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 86            | 8772             | G>A          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 87            | 8774             | C>T          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 88            | 8776             | G>A          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 89            | 8778             | C>T          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| 90            | 8780             | G>A          | ATPase6        | ATPase6      | Thr>Thr           | p.T44T syn       | 1                                        | GU186081                         |
| Sample number | Genomic Position | Base Change | Gene / Location | Amino acid position | Codon change | Amino acid change | Change in protein | Syn/ Non-syn | Total number of patients with nt changes | GenBank accession number if novel |
|---------------|-----------------|-------------|-----------------|---------------------|-------------|------------------|-----------------|------------|--------------------------------------|----------------------------------|
| 91            | 12308           | A>G         | tRNA Leu        | -                   | -           | -                | -               | -          | 3                                     | -                                |
| 92            | 12372           | G>A         | ND5             | 12                  | CTG>CTA     | Leu>Leu         | p.L12L          | syn        | 4                                     | -                                |
| 93            | 12477           | T>C         | ND5             | 47                  | AGT>AGC     | Ser>Ser         | p.S47S          | syn        | 1                                     | -                                |
| 94            | 12498           | C>T         | ND5             | 54                  | TTC>TGT     | Phe>Phe        | p.F54F         | syn        | 2                                     | -                                |
| 95            | 12561           | G>A         | ND5             | 75                  | CAG>CAA     | Gln>Gln        | p.Q75Q          | syn        | 1                                     | -                                |
| 96            | 12681           | T>C         | ND5             | 115                 | AAT>AAC     | Asn>Asn        | p.N115N         | syn        | 1                                     | -                                |
| 97            | 12705           | T>C         | ND5             | 123                 | ATT>ATC     | Ile>Ile        | p.L123I         | syn        | 10                                    | -                                |
| 98            | 12793           | T>C         | ND5             | 153                 | TTG>TGT     | Leu>Leu        | p.L153L         | syn        | 1                                     | GU186084                         |
| 99            | 12810           | A>G         | ND5             | 158                 | TGA>TGG     | Thr>Trp        | p.W158W         | syn        | 1                                     | -                                |
| 100           | 12849           | A>T         | ND5             | 171                 | GCA>GCT     | Ala>Ala        | p.A171A         | syn        | 8                                     | GU186085                         |
| 101           | 12879           | T>C         | ND5             | 181                 | GGT>GGC     | Gly>Gly        | p.G181G         | syn        | 1                                     | -                                |
| 102           | 12906           | C>T         | ND5             | 190                 | ATT>ATT     | Ile>Ile        | p.I190I         | syn        | 1                                     | -                                |
| 103           | 13020           | T>C         | ND5             | 228                 | GGT>GGC     | Gly>Gly        | p.G228G         | syn        | 1                                     | -                                |
| 104           | 13135           | G>A         | ND5             | 267                 | GCA>GCA     | Ala>Thr        | p.A267T         | non-syn    | 1                                     | -                                |
| 105           | 13138           | G>A         | ND5             | 268                 | GAA>GAA     | Ser>Ser        | p.E268K         | non-syn    | 20                                    | GU186083                         |
| 106           | 13188           | C>T         | ND5             | 284                 | ACC>ACC     | Thr>Thr        | p.T284T         | syn        | 1                                     | -                                |
| 107           | 13194           | A>T         | ND5             | 286                 | CTG>CTA     | Leu>Leu        | p.L286L         | syn        | 1                                     | -                                |
| 108           | 13215           | T>C         | ND5             | 293                 | CTT>CTC     | Leu>Leu        | p.L293L         | syn        | 1                                     | -                                |
| 109           | 13368           | G>A         | ND5             | 344                 | GGG>GGG     | Gly>Gly        | p.G344G         | syn        | 1                                     | -                                |
| 110           | 13500           | T>C         | ND5             | 388                 | GGT>GGG     | Gly>Gly        | p.G388G         | syn        | 1                                     | -                                |
| 111           | 13539           | A>G         | ND5             | 401                 | ATA>ATG     | Met>Met        | p.M401M         | syn        | 2                                     | -                                |
| 112           | 13542           | A>G         | ND5             | 402                 | TCA>TCA     | Ser>Ser        | p.S402S         | syn        | 1                                     | -                                |
| 113           | 13651           | A>G         | ND5             | 439                 | ACC>ACC     | Thr>Thr        | p.T439A         | non-syn    | 1                                     | -                                |
| 114           | 13656           | T>C         | ND5             | 440                 | CTT>CTC     | Leu>Leu        | p.L440L         | syn        | 4                                     | -                                |
| 115           | 13708           | G>A         | ND5             | 458                 | GCA>GCA     | Ala>Thr        | p.A458T         | non-syn    | 1                                     | -                                |
| 116           | 13768           | T>C         | ND5             | 478                 | TTC>TTC     | Phe>Leu        | p.F478L         | non-syn    | 1                                     | GU186086                         |
| 117           | 14000           | T>A         | ND5             | 555                 | CCT>CCT     | Leu>Pro        | p.L555P         | non-syn    | 2                                     | -                                |
| 118           | 14022           | A>N         | ND5             | 562                 | TTA>TTG     | Leu>Leu        | p.L562L         | syn        | 1                                     | -                                |
| 119           | 14935           | T>C         | CYB             | 63                  | TTT>TTC     | Phe>Phe        | p.F63F          | syn        | 1                                     | -                                |
| 120           | 15038           | A>G         | CYB             | 98                  | ATC>ATC     | Ile>Ile        | p.I90I          | syn        | 1                                     | -                                |
| 121           | 15043           | G>A         | CYB             | 99                  | GGG>GGA     | Gly>Gly        | p.G99G          | syn        | 12                                    | -                                |
| 122           | 15061           | A>G         | CYB             | 105                 | GGA>GGA     | Gly>Gly        | p.G105G         | syn        | 1                                     | -                                |
| 123           | 15097           | T>C         | CYB             | 117                 | ATT>ATT     | Ile>Ile        | p.I117I         | syn        | 1                                     | -                                |
| 124           | 15148           | G>A         | CYB             | 134                 | CCG>CCA     | Pro>Pro        | p.P134P         | syn        | 3                                     | -                                |
| 125           | 15301           | G>A         | CYB             | 185                 | TTG>TGT     | Leu>Leu        | p.L185L         | syn        | 16                                    | -                                |
| 126           | 15317           | G>A         | CYB             | 191                 | GCC>GCC     | Ala>Thr        | p.A191T         | non-syn    | 1                                     | -                                |
| 127           | 15452           | C>A         | CYB             | 236                 | CTT>ATT     | Leu>Ile        | p.L236I         | non-syn    | 2                                     | -                                |
| 128           | 15607           | A>G         | CYB             | 287                 | AAA>AAA     | Lys>Lys        | p.K287K         | syn        | 1                                     | -                                |
| 129           | 15670           | T>C         | CYB             | 308                 | CAT>CAT     | His>His        | p.H308H         | syn        | 1                                     | -                                |
| 130           | 15683           | A>G         | CYB             | 312                 | CAA>CAG     | Gly>Gln        | p.Q312Q         | syn        | 2                                     | GU186093                         |

Key: A – Adenine; T – Thymine; G- Guanine; C- Cytosine; Syn- synonymous; Non-syn- non-synonymous; *- novel change; ND1 - NADH dehydrogenase subunit 1; ND2 - NADH dehydrogenase subunit 2; ND3 - NADH dehydrogenase subunit 3; ND4 - NADH dehydrogenase subunit 4; ND4L - NADH dehydrogenase subunit 4L; ND5 - NADH dehydrogenase subunit 5; CO1 - cytochrome c oxidase 1; CO2 - cytochrome c oxidase 2; CO3 - cytochrome c oxidase 3; ATPase6 - ATP synthase subunit a (F-ATPase protein 6); ATPase8 - ATP synthase protein 8; CYB - cytochrome B; tRNA - transfer ribonucleic acid; rRNA - ribosomal ribonucleic acid.
| Sample number | Genomic position | Base change | Gene/ Location | Codon change | Amino acid change | Change in protein | Syn/ Non-syn | GenBank accession number if novel |
|---------------|------------------|-------------|----------------|--------------|-------------------|-------------------|-------------|----------------------------------|
| 1             | 3591             | G>A         | ND1            | 95           | CTG>CTA           | Thr>Thr           | syn         | -                                |
| 2             | 3915             | G>A         | ND1            | 203          | GGG>GGA           | Gly>Gly           | syn         | -                                |
| 3             | 3918             | G>A         | ND1            | 204          | GAG>GAA           | Glu>Glu           | syn         | -                                |
| 4             | 3933             | A>G         | ND1            | 209          | TCA>TGC           | Ser>Ser           | syn         | -                                |
| 5             | 3970             | C>T         | ND1            | 222          | CTA>TTA           | Leu>Leu           | syn         | -                                |
| 6             | 3996             | C>T         | ND1            | 230          | AAC>AAT           | Asn>Asn           | syn         | -                                |
| 7             | 4029             | C>A         | ND1            | 241          | ATC>ATA           | Ile>Ile           | syn         | -                                |
| 8             | 4093             | A>G         | ND1            | 263          | ACC>GCC           | Thr> Ala          | syn         | -                                |
| 9             | 4793             | A>G         | ND2            | 108          | ATA>ATG           | Met>Met           | syn         | -                                |
| 10            | 4852             | T>A         | ND2            | 128          | CTG>CAG           | Leu>Gln           | syn         | -                                |
| 11            | 5186             | A>T         | ND2            | 239          | TGA>TGT           | Trp>Cys           | syn         | -                                |
| 12            | 5348             | C>T         | ND2            | 293          | TAC>TAT           | Tyr>Tyr           | syn         | -                                |
| 13            | 5351             | G>A         | ND2            | 294          | CTA>CTG           | Leu>Leu           | syn         | -                                |
| 14            | 6305             | G>A         | ND1            | 134          | GGG>GGA           | Gly>Gly           | syn         | -                                |
| 15            | 6719             | T>C         | ND1            | 272          | GGT>GGC           | Gly>Gly           | syn         | -                                |
| 16            | 6962             | G>A         | ND1            | 353          | CTG>CTA           | Thr>Thr           | syn         | -                                |
| 17            | 7316             | G>A         | ND1            | 471          | AGT>ATA           | Met>Met           | syn         | -                                |
| 18            | 7738             | T>C         | ND2            | 51           | ACT>ACC           | Thr>Thr           | syn         | -                                |
| 19            | 7762             | G>A         | ND2            | 59           | CAG>CAC           | Gln>Gln           | syn         | -                                |
| 20            | 8143             | T>C         | ND2            | 186          | GGT>GCC           | Ala>Ala           | syn         | -                                |
| 21            | 8251             | G>A         | ND2            | 222          | GGG>GGA           | Gly>Gly           | syn         | -                                |
| 22            | 8503             | T>G         | ATP8           | 46           | AAT>AAG           | Asp>Lys           | syn         | -                                |
| 23            | 8584             | G>A         | ATP6           | 20           | GCA>GCA           | Ala>Thr           | syn         | -                                |
| 24            | 8594             | T>C         | ATP6           | 23           | ATC>ACC           | Ile>Thr           | syn         | -                                |
| 25            | 8650             | C>T         | ATP6           | 42           | CTA>TTC           | Leu>Leu           | syn         | -                                |
| 26            | 8718             | A>G         | ATP6           | 64           | AAA>AAG           | Lys>Lys           | syn         | -                                |
| 27            | 8812             | G>A         | ATP6           | 96           | ACC>GCC           | Thr>Ala           | syn         | -                                |
| 28            | 8886             | G>A         | ATP6           | 120          | AAG>AAG           | Lys>Lys           | syn         | -                                |
| 29            | 8925             | A>G         | ATP6           | 133          | ACA>ACC           | Thr>Thr           | syn         | -                                |
| 30            | 10310            | G>A         | ND3            | 84           | CTG>CTA           | Thr>Thr           | syn         | -                                |
| 31            | 10609            | T>C         | ND4            | 47           | ATA>ACA           | Met>Thr           | syn         | -                                |
| 32            | 11467            | A>G         | ND4            | 236          | TTA>TGC           | Leu>Leu           | syn         | -                                |
| 33            | 11914            | G>A         | ND4            | 385          | ACG>ACA           | Thr>Thr           | syn         | -                                |
| 34            | 12007            | G>A         | ND4            | 416          | TGG>TAG           | Trp>Trp           | syn         | -                                |
| 35            | 12073            | C>T         | ND4            | 438          | TCT>TTC           | Phe>Phe           | syn         | -                                |
| 36            | 12107            | G>A         | ND4            | 449          | CTC>CTT           | Thr>Thr           | syn         | -                                |
| 37            | 12133            | C>T         | ND4            | 458          | TCC>TCT           | Ser>Ser           | syn         | -                                |
| 38            | 12237            | G>A         | ND5            | 12           | CTG>CTA           | Thr>Thr           | syn         | -                                |
| 39            | 12373            | A>G         | ND5            | 13           | ACT>GCT           | Thr>Ala           | syn         | -                                |
| 40            | 12406            | G>A         | ND5            | 24           | GTT>ATT           | Val>Ile           | syn         | -                                |
| 41            | 12486            | C>T         | ND5            | 50           | CCC>CCT           | Pro>Pro           | syn         | -                                |
| 42            | 12498            | C>T         | ND5            | 54           | TTC>TTC           | Phe>Phe           | syn         | -                                |
| 43            | 12561            | G>A         | ND5            | 75           | CAG>CAC           | Gln>Gln           | syn         | -                                |
| 44            | 13204            | G>A         | ND5            | 290          | GTC>ATC           | Val>Ile           | syn         | -                                |
| 45            | 13299            | A>G         | ND5            | 321          | CAA>CAG           | Gln>Gln           | syn         | -                                |
| 46            | 13676            | A>G         | ND5            | 447          | AAC>AGC           | Asn>Ser           | syn         | -                                |
| 47            | 13731            | A>G         | ND5            | 465          | GAA>GAA           | Gly>Gly           | syn         | -                                |
| 48            | 13860            | C>T         | ND5            | 490          | GCC>GCT           | Ala>Ala           | syn         | -                                |
| 49            | 14058            | C>T         | ND5            | 574          | GCC>GCT           | Ala>Ala           | syn         | -                                |
| 50            | 14782            | C>T         | CYB            | 13           | TTA>CTA           | Leu>Leu           | syn         | -                                |
| 51            | 15119            | G>A         | CYB            | 125          | GCA>ACA           | Ala>Thr           | syn         | -                                |
| 52            | 15172            | G>A         | CYB            | 142          | GGG>GGA           | Gly>Gly           | syn         | -                                |
| 53            | 15217            | G>A         | CYB            | 157          | GGG>GGA           | Gly>Gly           | syn         | -                                |
| 54            | 15385            | C>T         | CYB            | 213          | GCC>GCC           | Ala>Thr           | syn         | -                                |
| 55            | 15431            | A>G         | CYB            | 229          | GCC>GCC           | Ala>Thr           | syn         | -                                |
| 56            | 15484            | A>G         | CYB            | 246          | TCA>TGG           | Ser>Ser           | syn         | -                                |
| 57            | 15670            | T>C         | CYB            | 308          | CAT>CAC           | His>His           | syn         | -                                |

Key: A – Adenine; T – Thymine; G- Guanine; C- Cytosine; Syn- synonymous; Non-syn- non-synonymous; ND1- NADH dehydrogenase subunit 1; ND2- NADH dehydrogenase subunit 2; ND3- NADH dehydrogenase subunit 3; ND4- NADH dehydrogenase subunit 4; ND4L- NADH dehydrogenase subunit 4L; ND5- NADH dehydrogenase subunit 5; CO1- cytochrome c oxidase I; CO2- cytochrome c oxidase II; CO3- cytochrome c oxidase III; ATPase6- ATP synthase subunit a (F1-ATPase protein 6); ATPase8- ATP synthase protein 8; CYB- cytochrome B.
increase in production of ROS due to altered complex I function [33-35]. G4580A (p.M37I) was present in 1 patient and G10398A (p.A114T) in 16 patients in our study. The frequency of G10398A (p.A114T) alteration was found to be statistically significant (p value <0.001) in our study population. Twenty patients (57.14%) had changes associated with elevated ROS production. It has been reported that alterations in mitochondrial complex I causes cytochrome c oxidase deficiency and OS [36]. Pathogenic mutations in ND genes have been reported in POAG, PACG, and PEG [11,13,14].

In this study we found mtDNA sequence changes which were different from other types of glaucoma. When compared between cases and controls, frequency of non-synonymous sequence variations in ND2 and ND3 were found to be statistically significant (p value <0.05; Table 4). Point mutations in ND1, ND4, and ND6 have been reported in association with LHON [37,38]. Moreover, mutations in complex I genes are also associated with Leigh syndrome, mitochondrial encephalomyopathy, lactic acidosis stroke-like episodes (MELAS), and infertility [15,39-41].

Cytochrome c oxidase (COX or complex IV), the terminal enzyme of the respiratory chain (RC) catalyzes the reduction of molecular oxygen by reduced cytochrome c. This complex is composed of 13 subunits [42]. Twenty six variations (19.69%) identified in this study were present in complex IV of which six were non-synonymous. Frequency of non-synonymous sequence variations in COI and COII in PCG cases were found to be statistically significant (p value <0.05; Table 4). Human diseases associated with COX mutations include POAG, PACG, PEG, and Leigh syndrome [11,13,14,43]. The frequency of non-synonymous sequence variations in CYB was not found to be statistically significant (p value >0.05; Table 4).

In the current study, 15.15% mtDNA variations (20/132) were observed in complex V (ATPase6 and ATPase8). Mutations in ATPase6 have been reported in POAG, PACG, PEG, neuropathy, ataxia, retinitis pigmentosa (NARP), and mitochondrial DNA-associated Leigh Syndrome (MILS) patients [11,13,14,44,45]. Mitochondrial variations in ATPase6 and ATPase8 have been reported in spinocerebellar ataxias [46].

The A12308G variation in tRNA leu gene is also associated with increased ROS production [34] and this variation was detected in three patients in this study. However, the frequency of the A12308G variation and non-synonymous variations in ATPase8 was not found to be statistically significant (p value >0.05) while that of non-synonymous
| Patient number | ND1 | ND2 | CO1 | CO2 | ATPα6 | ATPα8 | CO3 | ND4L | ND4 | ND5 | CYB | Other changes | Last cup disc ratio (OS/OD) at presentation | Corneal diameter (mm) OS/OD | IOP (mmHg) OS/OD |
|---------------|------|-----|-----|-----|-------|-------|-----|------|-----|-----|-----|---------------|--------------------------------|------------------|---------------|
| 1             | 1020Y | 1080Y | T135 | T1215 | W57W | W112L | P114T | -    | -    | 1135 | 1185 | G646 | Ins. of T at 2790, 91 (16sRNA) | Hazy MEDIA | 15x15/15x15 | 15  |
| 2             | 1020Y | 1080Y | T135 | T1215 | W57W | W112L | P114T | -    | -    | 1135 | 1185 | G646 | Ins. of T at 2790, 91 (16sRNA) | Hazy MEDIA | 15x15/15x15 | 15  |
| 3             | M31T | V155 | I   | -    | V113 | V | W57W | P114A | W416 | -    | 1135 | 1185 | G646 | Ins. of T at 2790, 91 (16sRNA) | Hazy MEDIA | 15x15/15x15 | 15  |
| 4             | D227D | G222 | G   | -    | 1195 | T | W57W | P114A | W416 | -    | 1135 | 1185 | G646 | Ins. of T at 2790, 91 (16sRNA) | Hazy MEDIA | 15x15/15x15 | 15  |
| 5             | Y304H | M307 | F184 | T187 | W57W | L112L | A114T | -    | -    | 1135 | 1185 | G646 | Ins. of T at 2790, 91 (16sRNA) | Hazy MEDIA | 15x15/15x15 | 15  |
| 6             | N150T | I123 | I   | -    | W416 | W | E268K | P134F | L185 | -    | 1135 | 1185 | G646 | Ins. of T at 2790, 91 (16sRNA) | Hazy MEDIA | 15x15/15x15 | 15  |
| 7             | G177 | P134 | F184 | L126 | W57W | L112L | A114T | -    | -    | 1135 | 1185 | G646 | Ins. of T at 2790, 91 (16sRNA) | Hazy MEDIA | 15x15/15x15 | 15  |
| 8             | L174 | L105 | S205 | T145 | S11 | S | W57W | P114A | W416 | -    | 1135 | 1185 | G646 | Ins. of T at 2790, 91 (16sRNA) | Hazy MEDIA | 15x15/15x15 | 15  |
| 9             | T115 | T145 | S11 | T135 | W57W | L112L | P114T | -    | -    | 1135 | 1185 | G646 | Ins. of T at 2790, 91 (16sRNA) | Hazy MEDIA | 15x15/15x15 | 15  |
| 10            | V113 | T145 | T135 | T1215 | W57W | L112L | P114T | -    | -    | 1135 | 1185 | G646 | Ins. of T at 2790, 91 (16sRNA) | Hazy MEDIA | 15x15/15x15 | 15  |
| 11            | W394 | L105 | T126 | L105 | W57W | L112L | A114T | -    | -    | 1135 | 1185 | G646 | Ins. of T at 2790, 91 (16sRNA) | Hazy MEDIA | 15x15/15x15 | 15  |
| 12            | P2L | N49 | -    | -    | A97; 1065 | -    | P114T | -    | G320 | -    | 1135 | 1185 | G646 | Ins. of T at 2790, 91 (16sRNA) | Hazy MEDIA | 15x15/15x15 | 15  |
| 13            | M31T | V155 | I   | -    | V113 | V | W57W | P114A | W416 | -    | 1135 | 1185 | G646 | Ins. of T at 2790, 91 (16sRNA) | Hazy MEDIA | 15x15/15x15 | 15  |
| 14            | -    | -    | -    | -    | P114A | -    | W416 | -    | W416 | -    | 1135 | 1185 | G646 | Ins. of T at 2790, 91 (16sRNA) | Hazy MEDIA | 15x15/15x15 | 15  |
| 15            | L261 | L126 | T11A | N460 | W57W | P114A | -    | -    | -    | 1135 | 1185 | G646 | Ins. of T at 2790, 91 (16sRNA) | Hazy MEDIA | 15x15/15x15 | 15  |
| Patient number | ND1 | ND2 | CO1 | CO2 | ND3 | ND4 | ND4L | ND5 | CYB | Other changes | Last cup disc ratio (OS/OD) at presentation | Corneal diameter (mm) OS/OD | IOP (mmHg) OS/OD |
|----------------|-----|-----|-----|-----|-----|-----|------|-----|-----|---------------|---------------------------------|--------------------------|-----------------|
| 16             |     |     |     |     |     |     |      |     |     |               | p.K40K – p.W57W p.A114A – p.W416W | 0.4:10.5:1               | 11x11/12x12.5  |
| 17             |     |     |     |     |     |     |      |     |     |               | p.S205S | p.T44T – p.W57W p.A114A – p.F457F | 0.5:1 – p.G99G | NA | 14x14/14x14.5 |
| 18             |     |     |     |     |     |     |      |     |     |               | p.A59T | p.W57W p.A114T – p.G320G | 0.4:1 – p.L185L | NA | 14.5x14/14x14 |
| 19             |     |     |     |     |     |     |      |     |     |               | p.V155I | p.F17T p.W57W p.A103T – p.F457F | 0.5:1 – p.G99G | 0.5:1 | 12x12.5/12x12 |
| 20             |     |     |     |     |     |     |      |     |     |               | p.J10T | p.Y129Y p.M86M – p.G99G | NA | NA | 12x13/13x13 |
| 21             |     |     |     |     |     |     |      |     |     | p.I59V – p.Y129Y | p.B59V | p.A59T | 0.7:1 – p.L185L | NA | 15x16/15x16 |
| 22             |     |     |     |     |     |     |      |     |     |               | p.K58K | p.V31A | 0.7:1 – p.L185L | NA | 14x15/14x15.5 |
| 23             |     |     |     |     |     |     |      |     |     |               | p.K120K | p.L164L; p.G170G | 0.7:1 – p.L185L | NA | 13x13.5/13x13.5 |
| 24             |     |     |     |     |     |     |      |     |     |               | p.K120K | p.A114A; p.L123M | 0.7:1 – p.L185L | NA | 14x14/14x14 |
| 25             |     |     |     |     |     |     |      |     |     |               | p.K120K | p.A114A – p.M27M | 0.7:1 – p.L185L | NA | 14x15/14x15 |
| 26             |     |     |     |     |     |     |      |     |     |               | p.K120K | p.L123M | 0.7:1 – p.L185L | NA | 14x14/14x14 |
| 27             |     |     |     |     |     |     |      |     |     |               | p.K120K | p.A114A – p.L446L | 0.7:1 – p.L185L | NA | 14x14/14x14 |
| 28             |     |     |     |     |     |     |      |     |     |               | p.K120K | p.A114A | 0.7:1 – p.L185L | NA | 14x14/14x14 |
| 29             |     |     |     |     |     |     |      |     |     |               | p.K120K | p.A114A | 0.7:1 – p.L185L | NA | 14x14/14x14 |
| 30             |     |     |     |     |     |     |      |     |     |               | p.K120K | p.N78N | 0.7:1 – p.L185L | NA | 14x14/14x14 |
| 31             |     |     |     |     |     |     |      |     |     |               | p.K120K | p.A4T | 0.7:1 – p.L185L | NA | 14x14/14x14 |
| 32             |     |     |     |     |     |     |      |     |     |               | p.K120K | p.A4T | 0.7:1 – p.L185L | NA | 14x14/14x14 |
| 33             |     |     |     |     |     |     |      |     |     |               | p.K120K | p.A4T | 0.7:1 – p.L185L | NA | 14x14/14x14 |
| 34             |     |     |     |     |     |     |      |     |     |               | p.K120K | p.A4T | 0.7:1 – p.L185L | NA | 14x14/14x14 |
| 35             |     |     |     |     |     |     |      |     |     |               | p.K120K | p.A4T | 0.7:1 – p.L185L | NA | 14x14/14x14 |

Key: NA- not available; OD- right eye; OS- left eye; OU- both eyes; mmHg- millimeter of mercury; IOP- intra ocular pressure; Syn- synonymous; Non-syn- non-synonymous; - novel change; ND1 - NADH dehydrogenase subunit 1; ND2 - NADH dehydrogenase subunit 2; ND3 - NADH dehydrogenase subunit 3; ND4 - NADH dehydrogenase subunit 4; ND4L - NADH dehydrogenase subunit 4L; ND5 - NADH dehydrogenase subunit 5; CO1 - cytochrome c oxidase I; CO2 - cytochrome c oxidase II; CO3 - cytochrome c oxidase III; ATPase6 - ATP synthase subunit a (F-ATPase protein 6); ATPase8 - ATP synthase protein 8; CYB - cytochrome B; tRNA- transfer ribo nucleic acid.
variations in ATPase6 and others (16s RNA, tRNA), as shown in Table 4, were statistically significant (p value <0.05).

Non-synonymous mitochondrial variations adversely affect oxidative phosphorylation resulting in decreased mitochondrial respiration and increased free radical (FR) production [47]. Thus, we hypothesize that mtDNA variations with resultant lower ATP levels may impair growth, development, and differentiation of TM and result in trabecular-dysgenesis (Figure 3); a characteristic feature of PCG. Trabecular-dysgenesis leads to impairment in aqueous drainage hence causes elevation in IOP. ROS levels may increase to supraphysiological levels in TM endothelial cells and due to low ATP levels these cells are unable to eliminate the reactive oxygen intermediates (ROI). MtDNA mutations are also associated with optic neuropathies like LHON [38], NARP [48,49] or Leigh syndrome [50]. The mechanisms by which mitochondrial abnormalities may place the optic nerve at risk remain uncertain [51].

Distribution of high number of mitochondria in the optic nerve head reflects the high energy requirement of the human optic nerve head. Neurons, because of their high energy requirement, are heavily dependent on mitochondria for survival [52]. Mitochondria not only constitute an energy-generating system, but are also critically involved in calcium signaling and apoptosis. Mitochondrial function is impaired by mutations in mitochondrial and nuclear DNA, chemical insults to components of the electron transport chain, and a lack of substrates such as oxygen. The latter is relevant to tissue hypoxia that is believed to be present in the glaucomatous retina and optic nerve head either primarily or secondary to elevated IOP. Any malfunction of the mitochondrial electron transport chain results in excessive generation of free radicals and low ATP production. In our

Table 4. With p-value and relative risk at 95% confidence interval by using Pearson $\chi^2$/Fisher’s exact test for non-synonymous sequence variations in different mitochondrial genes in PCG and controls.

| Gene name | Cases (n=35) | Controls (n=40) | p-value | Relative risk at 95% confidence interval |
|-----------|--------------|----------------|---------|----------------------------------------|
| ND1       | 6 (17.14%)   | 2 (5.0%)       | 0.136   | 1.73 (1.07–2.81)                       |
| ND2       | 10 (28.57%)  | 2 (5.0%)       | 0.005   | 2.10 (1.41–3.12)                       |
| ND3       | 17 (48.60%)  | 0 (0%)         | <0.001  | 3.22 (2.19–4.72)                       |
| ND4       | 0 (0%)       | 0 (0%)         | —       | —                                       |
| ND4L      | 0 (0%)       | 2 (5.0%)       | 0.495   | 1.92 (1.54–2.39)                       |
| ND5       | 21 (60.00%)  | 4 (10.0%)      | <0.001  | 3.00 (1.86–4.83)                       |
| CO1       | 4 (11.42%)   | 0 (0%)         | 0.043   | 2.29 (1.75–2.98)                       |
| CO2       | 5 (14.30%)   | 0 (0%)         | 0.019   | 2.33 (1.78–3.05)                       |
| CO3       | 1 (2.90%)    | 0 (0%)         | 0.467   | 2.17 (1.70–2.78)                       |
| CYB       | 4 (11.42%)   | 8 (20.00%)     | 0.360   | 0.57 (0.18–1.73)                       |
| ATPase6   | 18 (51.42%)  | 7 (17.50%)     | 0.002   | 2.12 (1.34–3.34)                       |
| ATPase8   | 1 (2.85%)    | 1 (2.5)        | 1.00    | 1.07 (0.26–4.40)                       |
| Others    | 8 (22.85%)   | 0 (0%)         | 0.001   | 2.48 (1.85–3.32)                       |

Abbreviations: ND1 - NADH dehydrogenase subunit 1; ND2 - NADH dehydrogenase subunit 2; ND3 - NADH dehydrogenase subunit 3; ND4 - NADH dehydrogenase subunit 4; ND4L - NADH dehydrogenase subunit 4L; ND5 - NADH dehydrogenase subunit 5; CO1 - cytochrome c oxidase I; CO2 - cytochrome c oxidase II; CO3 - cytochrome c oxidase III; ATPase6 - ATP synthase subunit a (F-ATPase protein 6); ATPase8 - ATP synthase protein 8; CYB - cytochrome B; tRNA- transfer ribonucleic acid; rRNA- ribosomal ribonucleic acid.
study we identified 50.00% variations in complex I, 9.02% in complex III, 19.54% in complex IV, and 15.03% in complex V. The presence of primary LHON mutations has been investigated previously in normal tension glaucoma and POAG [12,53] but not in PCG. None of PCG cases had primary LHON mutations (3460G>A, 11778G>A, 14484T>C) in the current study.

It has already been reported that OS leads to oxidative damage to cellular macromolecules such as mitochondrial and nuclear DNA, proteins, and lipids, along with energy depletion and a local dysregulation of calcium homeostasis, resulting in neuronal degeneration [54]. OS is the underlying etiology in several ocular diseases [11,54-59] and plays an essential role in early retinal ischemic injury [60] and glaucoma pathogenesis [61,62]. Mitochondrial dysfunction leads to RGC death through caspase-dependent and caspase-independent pathways initiated by the loss of mitochondrial membrane potential, release of cell death mediators and OS [54]. Glaucomatous eyes have a significant increase in OS and decreased antioxidant activity [62]. Seppet et al. [63] reported that OS is a critical factor in injury to anterior segment of eye. OS has also been reported to induce human trabecular meshwork degenerative changes that favor increased intraocular pressure [64]. Oxidative DNA damage is significantly increased in the trabecular-meshwork (TM) of glaucomatous patients compared to controls [11]. The pathogenic role of ROS in glaucoma is supported by various experimental findings, including (a) resistance to aqueous humor outflow is increased by hydrogen peroxide by inducing TM degeneration and (b) intraocular-pressure increase and severity of visual loss in glaucoma patients parallel to the amount of oxidative DNA damage affecting TM [11]. Oxidative damage constitutes an important pathogenic step triggering TM degeneration which results in intraocular hypertension. OS thus affects both TM and retinal ganglion cells, and may be involved in the neuronal cell death affecting the optic nerve in glaucoma (Figure 3).

Further evidence of oxidative damage in trabecular meshwork in glaucoma [57] and neural degeneration is that many retinal proteins exhibit oxidative modifications in experimental glaucoma [65], and may lead to important structural and functional alterations. Thus, the structure and function of mitochondria are critical determinants of endothelial cells and neuronal health. Essentially, once the mitochondrial lipid bilayer is compromised after the mitochondrial translocation of Bel-2-associated X protein (Bax), cell death is inevitable, because of already triggered events.

It has been established that pathogenic mitochondrial mutations can cause mitochondrial dysfunction and enhance OS, which in turn lead to apoptosis in affected tissue and primary culture of human cells that harbor mtDNA mutations [66]. There are several studies which point to mitochondrial dysfunction in glaucoma and RGC death [66-68]. One hypothesis suggests that progressive optic nerve damage in POAG is the result of optic nerve fiber apoptosis [67]. Mitochondria-induced apoptosis, which may be a mechanism of injury in experimental glaucoma [67] and other optic neuropathies [66], may also be a pathological factor in PCG. Recent study by Abu-Amero et al. [11] reported mitochondrial dysfunction-associated OS as a risk factor for glaucoma. MtDNA alterations result in reduced mitochondrial respiration [11] and OS [36]. Thus reduced ATP levels secondary to mitochondrial damage may impair development and differentiation of TM. Endothelial cells are also damaged due to supraphysiological ROS levels.

These findings suggest that elevation of IOP is related to oxidative degenerative processes affecting the TM specifically endothelial cells. Much evidence indicates that in this region ROS play a fundamental pathogenic role by reducing local antioxidant activities inducing outflow resistance. TM is neural crest in origin [69,70] and developing TM is deficient in antioxidant enzymes and more susceptible to OS induced DNA damage [71]. OS disturbs Ca²⁺ homeostasis and so raised Ca²⁺ levels activate endonucleases.
which cause nuclear DNA damage [63]. OS, early in development and/or throughout life could precipitate both metabolic and anatomic sequelae that cause trabecular dysgenesis and ultimately optic nerve damage in PCG.

Elevated IOP is a characteristic feature of glaucoma and an important risk factor for optic nerve damage [72]. However, the precise relationship between among elevated IOP, glaucomatous optic nerve (ON) damage, and retinal ganglion cell death are poorly understood. Growing evidence indicates that mitochondrial structural and functional dynamics play an important role in cell and animal physiology. Imbalance in the control of mitochondrial fusion and fission dramatically alters overall mitochondrial morphology [73-76]. Elevated IOP in glaucoma induces reduction of cytochrome c oxidase (COX) activity, mitochondrial fission, mitochondrial matrix swelling, cristae depletion, triggers release of optic nerve atrophy type-I (OPA1), and induces subsequent apoptotic cell death in differentiated RGC-5 cells [77,78] (Figure 4). Similar findings were also confirmed in a mouse model [79].

In summary, frequency of mtDNA sequence variations in PCG was significantly higher as compared to controls. Five pathogenic changes (3 in ND1 and 2 in ND2) and 3 other changes (G10398A, A12308G, and G13708A) associated with elevated ROS were present in our patients. Nonsynonymous mtDNA alterations may lead to mitochondrial dysfunction which leads to reduced mitochondrial respiration, OS, damage to mtDNA, altered mitochondrial morphology, alterations in mitochondrial fission and fusion, and ultimately cell’s demise. OS impairs development and differentiation of trabecular meshwork that favor increased intraocular pressure in PCG and consequently RGC death.

This study describes mtDNA sequence variations in a relatively small number of patients with PCG of north Indian ethnic origin. However, these results should be confirmed in other populations. Knowledge of mtDNA mutations and/or mitochondrial dysfunction in PCG may lead to a better understanding of glaucoma pathophysiology [80]. Novel approaches are now available for studying mitochondrial disease in the eye, and a novel in vitro treatment has already been devised for the metabolic defect of at least one mtDNA mutation in LHON [81]. PCG cases with mtDNA variations and consequent OS may benefit by early diagnosis and prompt management with antioxidant therapy.

**Conclusion:** A total of 44 novel mtDNA variations were identified in current study. MtDNA variations adversely affect respiratory chain, impair the OXPHOS pathway resulting in low ATP production, and impair growth, development, and differentiation of TM. Mitochondrial DNA variations also lead to increased ROS production, oxidative injury to TM and RGCs. Thus, early diagnosis of mitochondrial DNA variations and prompt antioxidant administration may delay OS induced injury to TM and RGCs and hence improve visual prognosis.

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