ORIGINAL RESEARCH

Mitochondrial Genome Alterations, Cytochrome C Oxidase Activity, and Oxidative Stress: Implications in Primary Open-angle Glaucoma

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Abstract

Aim: To evaluate mitochondrial genome alterations, cytochrome C oxidase (COX) activity, and oxidative stress in primary open-angle glaucoma (POAG).

Methodology: Whole mitochondrial genome was screened in 75 POAG cases and 105 controls by polymerase chain reaction (PCR) sequencing. COX activity was measured from peripheral blood mononuclear cells (PBMCs). A protein modeling study was done to evaluate the impact of G222E variant on protein function. Levels of 8-hydroxy-2-deoxyguanosine (8-OHdG), 8-isoprostane (8-IP), and total antioxidant capacity (TAC) were also measured.

Results: A total of 156 and 79 mitochondrial nucleotide variations were found in the cohort of 75 POAG patients and 105 controls, respectively. Ninety-four (60.26%) variations spanned the coding region, and 62 (39.74%) variations spanned noncoding regions (D-loop, 12SrRNA, and 16SrRNA) of mitochondrial genome in POAG patients. Out of 94 nucleotide changes in coding region, 68 (72.34%) were synonymous changes, 23 (24.46%) non-synonymous, and three (3.19%) were found in the region coding for transfer ribonucleic acid (tRNA). Three changes (p.E192K in *ND1*, p.L128Q in *ND2*, and p.G222E in *COX2*) were found to be pathogenic. Twenty-four (32.0%) patients were positive for either of these pathogenic mitochondrial deoxyribonucleic acid (mtDNA) nucleotide changes. Majority of cases (18.7%) had pathogenic mutation in *COX2* gene. Patients who harbored pathogenic mtDNA change in COX2 gene had significantly lower levels of COX activity ($p < 0.0001$) and TAC ($p = 0.004$), and higher levels of 8-IP ($p = 0.01$) as compared to patients who did not harbor this mtDNA. G222E changed the electrostatic potential and adversely impacted protein function of COX2 by affecting nonpolar interactions with neighboring subunits.

Conclusion: Pathogenic mtDNA mutations were present in POAG patients, which were associated with reduced COX activity and increased levels of oxidative stress.

Clinical significance: POAG patients should be evaluated for mitochondrial mutations and oxidative stress and may be managed accordingly with antioxidant therapies.

Keywords: Case-control study, Cytochrome C oxidase, Mitochondrial genome alterations, Oxidative stress, Primary open-angle glaucoma.

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Introduction

Glaucoma is a neurodegenerative eye disease characterized by an array of optic neuropathies. POAG is associated with elevated intraocular pressure (IOP) and optic nerve degeneration due to retinal ganglion cell (RGC) death. Although several genes and single nucleotide polymorphisms are associated with POAG, etiology in the majority of POAG cases remains unexplained.[1,2]

There are several existing reports on the role of mitochondrial abnormalities in glaucoma. Mitochondrial genome exists as a 16,569 bp circular, double-stranded DNA.[3–5] Energy derived from mitochondrial respiration is utilized for nerve conduction in the unmyelinated part of ganglion cell axons.[6]

Impaired adenosine triphosphate (ATP) production due to defective respiratory complex I has been shown in POAG.[7] Pathogenic mitochondrial changes in Leber hereditary optic neuropathy (LHON) and primary congenital glaucoma (PCG) have been previously reported suggesting mitochondrial involvement in the pathogenesis of these ocular pathologies.[8,9] Oxidative stress has been documented in a number of ocular diseases, including glaucoma.[10–12] Mitochondria being the source of free radicals are the first target of oxidative damage which sets up a vicious cycle of oxidative mtDNA damage, impaired ATP production, and accumulation of sequence variations (pathogenic and nonpathogenic mtDNA nucleotide changes).[13] So, it becomes imperative to analyze the involvement of mtDNA variations and their correlation with oxidative stress and oxidative DNA damage in POAG.

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In this study, we screened POAG patients for mtDNA variations and investigated their correlation with oxidative stress and COX activity.

Methodology

Recruitment of Cases

It is a prospective case-control study, for which ethical clearance (Ref. No. IESC/T-359/28.09.2012) was obtained from the Institutional Review Board (IRB), All India Institute of Medical Sciences (AIIMS), New Delhi, India. POAG patients were examined and enrolled from the glaucoma services of a tertiary care center. All the participants signed written informed consent to participate in this study. This research was carried out according to the tenets of the Declaration of Helsinki. Inclusion criteria were patients with POAG of >40 years of age, baseline IOP of ≥21 mm Hg in at least one eye, open anterior chamber angle bilaterally on gonioscopy, and optic nerve suggestive of glaucomatous optic neuropathy. Patients with non-glaucomatous optic neuropathies or any other systemic illness were excluded from the study. Baseline IOP was defined as the first recorded IOP without medications. Seventy-five POAG patients and 105 age-matched unrelated controls of similar ethnicity without any ocular disorders were included. None of the controls had a history of any systemic or ocular disorders. All patients and controls were North Indians. Details of baseline IOP by Goldman applanation tonometry, optic nerve head status, and visual fields by using the 30–2 full threshold/SITA standard program of the Humphrey field analyzer, HFA™ II-i Series (Zeiss, San Leandro, California, USA), were recorded. The severity of glaucoma was categorized as early, moderate, and severe as per the Hodapp-Parrish-Anderson classification.[14]

PCR Amplification and Sequence Analysis of the Whole mtDNA

Peripheral blood samples (5 mL, blood drawn by venepuncture) of all participants were collected in ethylenediaminetetraacetic acid vacutainers (Greiner Bio-One Catalogue no. 455036; Frickenhausen, Germany) and frozen at –80°C until further used. DNA was extracted by the phenol–chloroform method.[15] The whole mitochondrial genome was amplified and sequenced (Sanger sequencing) in POAG patients and controls using 24 sets of primers and analyzed for sequence variants following the protocol explained elsewhere.[13]

Prediction of Pathogenicity of Missense Mutations

Four pathogenicity prediction tools (MutationTaster, PolyPhen-2 HumDiv, PolyPhen-2 HumVar, and SIFT) were used to predict the impact of identified missense mtDNA nucleotide changes on protein function. Non-synonymous mtDNA nucleotide changes were called pathogenic only when atleast three of these softwares predicted the change to be disease-causing, and the mtDNA nucleotide change was absent in controls.

Mitochondria Isolation and Protein Estimation

Mitochondria were isolated from PBMCs isolated by Ficoll's gradient. Mitochondria isolation was carried out using mitochondria isolation kit (BioVision, Catalog No. K288-50, California, USA) following manufacturer's protocol. Briefly, to the PBMC pellet, 1 mL of mitochondria isolation buffer was added and then vortexed for 5 seconds, followed by incubation on ice for 2 minutes. Ten µL of Reagent A was added and vortexed for 5 seconds. The tubes were kept in ice for 5 minutes and were centrifuged (600 rpm, 10 minutes, 4°C). The supernatant was centrifuged (7000 rpm, 10 minutes, 4°C) in another tube. The pellet was washed with mitochondria isolation buffer, and mitochondria were collected in storage buffer provided in the kit. Protein estimation was done by Bradford's method.

COX Activity Assay

Cytochrome c oxidase (EC 1.9.3.1) or complex IV is the fourth complex of the mitochondrial respiratory chain. It couples the electron transport with oxidative phosphorylation. COX activity (EC 1.9.3.1) was measured by COX activity colorimetric assay kit (BioVision, Catalog No. K287-100, California, USA). The assay was carried out following the protocol described by the manufacturer. The efficiency of reduction of cytochrome c was analyzed by mixing 20 µL of reduced cytochrome c in a 96-well plate with 100 µL of cytochrome oxidase assay buffer. A parallel well was taken as blank with only assay buffer. Optical density (OD) was taken at 550 nm. The OD at 550 nm of reduced cytochrome c should be between 0.2 and 0.6. Otherwise, 5 µL of dithiothreitol/mL of reconstituted cytochrome c was added again, and OD was taken after 15 minutes. The mitochondrial protein extract was diluted by enzyme dilution buffer supplied with the kit. For the activity assay, 5 µg of protein was taken. Cytochrome c was diluted 1:6 times with pre-warmed cytochrome oxidase assay buffer in a separate tube. A 120 µL of diluted cytochrome c was prepared per reaction. The spectrophotometer was set at 550 nm on the kinetic program for 45 minutes at 30 seconds intervals. The test samples were added to each well of a 96-well plate. For negative control (blank), equal volume of enzyme dilution buffer was added. A 120 µL of diluted cytochrome c was added to each sample and controlled using a multichannel pipette. The plate was shaken and read immediately, and a decrease in OD over a period of 30–45 minutes was recorded. Rate of reaction was calculated by change in OD: change in OD/minute by using a maximum linear rate. The COX activity was normalized for both protein concentration (absolute COX activity) and citrate synthase activity (relative COX activity).

Citrate Synthase Activity Assay

Citrate synthase (EC 2.3.3.1) is an enzyme of Krebs cycle. It converts acetyl-coenzyme A and oxaloacetate into citrate, and its activity remains constant in mitochondria. So, citrate synthase activity is used as a predictive marker of intracellular density of mitochondria.[16] Citrate synthase activity was measured in mitochondrial protein extract by Citrate Synthase Activity Colorimetric Assay Kit (BioVision, Catalog No. K318-100, California, USA). The assay was performed following the manufacturer's protocol. Briefly, an intermediate product is formed by the interaction of citrate synthase with the substrate mix. When this intermediate product comes in contact with the developer, a colored product is formed. The enzyme activity is directly proportional to the rate of color development. Citrate synthase activity was expressed as µmoL/min/mg protein.

Protein Modeling Study for Role of Mutation

In order to understand the role of mutations on structural changes and their functional implication, we need atomic details of three-dimensional (3-D) structures of the wild-type and mutant proteins. Since there was no experimental 3-D structure of human mitochondrial COX available, we have developed its model structure with the help of homology modeling program. This is the most reliable way to model a protein structure. 3-D model structure of COX was built using crystal structure of bovine COX (PDB: 1V54) with the

help of MODELLER program (v-9.2) in the modeling environment of Discovery Studio (DS) 2.0 (Accelrys Inc.).[17–21] Structural constraints were removed, and the model was relaxed by energy minimization program and short molecular dynamics simulation. The model structure was validated by PROCHECK for stereochemical quality and then was used for structural analysis.[22]

"Build Mutant" protocol available in DS 2.0 was used to create model structure of *COX2* mutant (G222E). This protocol uses MODELLER 9.2 and changes the selected residue (wild-type) into a desired residue (mutant) and checks for optimization of the conformation of mutated residue with residues nearby. The mutant model was also refined and validated similar to wild-type.

Similarly, model structures of *ND1* and *ND2* have been built, refined, and validated for wild-type and mutant form. Models were developed using crystal structure of corresponding protein of bacterial electron transport chain complex I (PDB: 4HE8, Chain H for *ND1* and 4HE8, Chain N for *ND2*).[23]

8-IP and 8-OHdG Estimation

To evaluate oxidative stress, 8-IP; a lipid peroxidation product, and 8-OHdG; which is an oxidative DNA damage marker, were quantified. Venous blood samples were collected in heparin-coated vials from each subject. The blood samples were placed on ice and centrifuged at 3500 rpm, 4°C for 15 minutes, and separated plasma was stored at –80°C until assayed. 8-IP and 8-OHdG were quantified by Cayman's EIA ELISA kit (Catalog No. 516351 for 8-IP and Catalog No. 589320 for 8-OHdG). Manufacturer's protocol was strictly followed for the quantification of different markers.

TAC Estimation

Total antioxidant capacity of a system is the sum of enzymatic and nonenzymatic antioxidant capacities. TAC levels were evaluated to assess the total cellular antioxidant capacity. Venous blood samples were collected in heparin from each subject. The blood samples were placed on ice and centrifuged at 3500 rpm, 4°C for 15 minutes, and separated plasma was stored at –80°C until assayed. TAC was quantified by Cayman's colorimetric kit (Catalog No. 709001) following the protocol given by the manufacturer.

Statistical Analysis

Continuous data were expressed in mean ± standard deviation and categorical variables in frequency (%). Statistical analysis was performed using STATA 12.1 (College Station, Texas, USA). The two-sample *t*-test was used to analyze quantitative data. Chi-square test was used to analyze categorical data. A *p*-value of <0.05 was considered statistically significant.

Results

The mean age of onset of POAG was 48.5 ± 5.7 years, and the mean duration of the disease was 6.2 ± 3.0 years. Of the 75 POAG cases, 59 (78.67%) had bilateral, and 16 (21.33%) had unilateral glaucoma. None of the cases had a family history of glaucoma. Mean vertical cup-disk ratio was 0.80 ± 0.08/0.81 ± 0.11 oculus dextrus/oculus sinister (OD/OS) and mean baseline IOP was 30.88 ± 3.52/30.28 ± 2.26 mm Hg (OD/OS). Ten patients (13.33%) had a history of glaucoma surgery. Mean deviation (MD) on HFA was –14.25 ± 8.85/–14.41 ± 9.70 (OD/OS). Forty-two males and 33 females were present among 75 cases, and 55 males and 50 females were present among 105 controls. There was no statistically significant difference between the age of cases (54.7 ± 5.70 years) and controls (55.50 ± 6.45 years) ($p = 0.45$).

Mitochondrial Genome Sequence Analysis

A total of 156 and 79 mitochondrial nucleotide variations were found in the cohort of 75 POAG patients and 105 controls, respectively. Ninety-four (60.26%) variations spanned the coding region and 62 (39.74%) variations spanned the noncoding regions (D-loop, 12SrRNA, and 16SrRNA) of mitochondrial genome in POAG patients. Out of 94 nucleotide changes in the coding region, 68 (72.34%) were synonymous changes, 23 (24.46%) were non-synonymous, and three (3.19%) were found in the region coding for tRNA. Out of 23 non-synonymous nucleotide changes, three changes (p.E192K in *ND1*, p.L128Q in *ND2,* and p.G222E in *COX2*) were predicted to be pathogenic. The nucleotide changes, p.E192K and p.L128Q were predicted to be disease-causing by three of the mutation testing tools (PolyPhen-2 HumDiv, PolyPhen-2 HumVar, and SIFT) and p.G222E was predicted to be disease-causing by all the four mutation testing tools (PolyPhen-2 HumDiv, PolyPhen-2 HumVar, SIFT, and MutationTaster) (Table 1).

Among these two changes (p.E192K and p.G222E) were novel, and one was a previously reported change (p.L128Q). Twenty-four (32%) patients were positive for either of these pathogenic mtDNA nucleotide changes, and out of which 14 patients (18.67%) had p.G222E mutation in *COX2*, three patients (4.0%) had p.E192K mutation in *ND1*, and seven patients (9.33%) had p.L128Q mutation in *ND2* genes. None of these cases had all three pathogenic mutations together. None of the controls had these pathogenic mtDNA nucleotide changes. A total of 39/156 (25%) variations were observed in complex I, 12/156 (7.69%) in complex III, 20/156 (12.82%) in complex IV, 10/156 (6.41%) in complex V, and 75/156 (48.07%) in other regions (D-Loop, rRNA, tRNA, and noncoding regions) of the mitochondrial genome. Out of total non-synonymous variations reported, complex I had (12/23) 52.17%, complex III had (3/23) 13.04%, complex IV had (2/23) 8.7%, and complex V had (6/23) 26.09% non-synonymous base changes.

Seventy-nine mitochondrial nucleotide variations found in controls consisted of 58 changes (44 synonymous, 13 non-synonymous, and one in tRNA) in the coding region and 21 in the noncoding region. While there were no changes found in the *ND6* and *COIII* genes in controls, patients harbored 10 changes in both genes. The noncoding region and the RNA coding regions showed significantly higher number of nucleotide changes in patients as compared to controls ($p = 0.008$).

COX Activity

Both absolute and relative COX activity was significantly lower in POAG patients compared to controls (Table 2). Patients with G222E mutation in *COX2* gene had significantly lower COX activity as compared to patients without this mutation (Table 3) and controls as well.

Protein Modeling Study of *COX2, ND1,* and *ND2*

COX2 is an important subunit in multiprotein complex COX like other enzyme systems involved in the respiratory chain. Sequence analysis of this protein from several species indicates that residue G222 in human is conservatively substituted by nonpolar residues and lie in a region that is surrounded by nonpolar residues. Structural analysis of available crystal structures of COX and model shows that they are present on surface and make a groove-like interface (Fig. 1A). This gives a presumption that G222 and surrounding nonpolar residues are involved in interaction with protein or inner

Table 1: Non-synonymous mitochondrial nucleotide variations in POAG cases

Sl. no.	*Gene; nucleotide change*	*Amino acid change*	*PolyPhen-2 HumDiv*	*PolyPhen-2 HumVar*	*SIFT*	*MutationTaster*	*Pathogenicity*
1	*ND1*; G3880A	*p.E192K	0.999 (PD)	0.999 (PD)	0 (D)	Polymorphism	Pathogenic
2	*ND1*; T4216C	p.Y304H	0 (B)	0.006 (B)	0.82 (T)	Polymorphism	Nonpathogenic
3	*ND2*; A4824G	p.T119A	0.771 (PD)	0.381 (B)	0.16 (T)	Polymorphism	Nonpathogenic
4	*ND2*; T4852A	p.L128Q	0.998 (PD)	0.981 (PD)	0 (D)	Polymorphism	Pathogenic
5	*ND2*; A4917G	p.N150D	0.129 (B)	0.060 (B)	0.14 (T)	Polymorphism	Nonpathogenic
6	*ND2*; G5046A	p.V193I	0.026 (B)	0.024 (B)	1 (T)	Polymorphism	Nonpathogenic
7	*ND2*; A5186T	p.W239C	0.999 (PD)	0.958 (PD)	0.13 (T)	Polymorphism	Nonpathogenic
8	*ND2*; G5460A	p.A331T	0 (B)	0 (B)	0.37 (T)	Polymorphism	Nonpathogenic
9	*COX2*; G8250A	*p.G222E	1 (PD)	0.999 (PD)	0 (D)	Disease Causing	Pathogenic
10	*ATPase8*; A8537G	p.I58V	0.326 (B)	0.467 (B)	0.09 (T)	Polymorphism	Nonpathogenic
11	*ATPase6*; A8537G	p.N4S	0.004 (B)	0.003 (B)	0.74 (B)	Polymorphism	Nonpathogenic
12	*ATPase6*; C8794T	p.H90Y	0.002 (B)	0.003 (B)	1 (T)	Polymorphism	Nonpathogenic
13	*ATPase6*; A8860G	p.T112A	0 (B)	0.001 (B)	1 (T)	Polymorphism	Nonpathogenic
14	*ATPase6*; G9064A	p.A180T	0.011 (B)	0.020 (B)	0.37 (T)	Polymorphism	Nonpathogenic
15	*ATPase6*; C9094T	p.L190F	0.967 (PD)	0.749 (PD)	0.73 (T)	Polymorphism	Nonpathogenic
16	*COX3*; G9300A	p.A32T	0 (B)	0.002 (B)	0.22 (T)	Polymorphism	Nonpathogenic
17	*ND3*; A 10398G	p.T114A	0 (B)	0 (B)	1 (B)	Polymorphism	Nonpathogenic
18	*ND4*; C 10845T	p.T29I	0.999 (PD)	0.997 (PD)	0.6 (T)	Polymorphism	Nonpathogenic
19	*ND5*; T 13154C	*p.I273T	0.51 (PD)	0.265 (B)	0.09 (T)	Polymorphism	Nonpathogenic
20	*ND5*; G 13889A	p.C518Y	0 (B)	0 (B)	1 (T)	Polymorphism	Nonpathogenic
21	*CYB*; A 15329G	*p.L195V	0.996 (PD)	0.996 (PD)	1 (T)	Polymorphism	Nonpathogenic
22	*CYB*; G 15431A	p.A229T	0.049 (B)	0.147 (B)	0.03 (D)	Polymorphism	Nonpathogenic
23	*CYB*; C 15452A	p.L236I	0.029 (B)	0.244 (B)	1 (T)	Polymorphism	Nonpathogenic

NS nucleotide change was called pathogenic only if it was predicted to be pathogenic by at least three of four protein prediction algorithms (PolyPhen-2 HumDiv, PolyPhen-2 HumVar, SIFT, and MutationTaster); *Novel and others reported; SYN, synonymous; NS, non-synonymous; ND1, NADH dehydrogenase subunit 1; NA, not applicable; ND2, NADH dehydrogenase subunit 2; ND3, NADH dehydrogenase subunit 3; ND4, NADH dehydrogenase subunit 4; ND5, NADH dehydrogenase subunit 5; COX2, cytochrome c oxidase subunit 2; ATPase6, ATP synthase subunit a (F-ATPase protein 6); ATPase8, ATP synthase protein 8; CYB, cytochrome B; mtDB, human mitochondrial database; PD, probably damaging; B, benign; D, deleterious; T, tolerated

Table 2: COX and oxidative stress markers (POAG vs controls)

Parameters	*POAG (n = 75)*	*Controls (n = 105)*	*p-value*
Absolute COX activity (μM/min/mg)	0.04 ± 0.008	0.06 ± 0.004	<0.0001
Relative COX activity (μM/min/mg)	0.56 ± 0.11	0.70 ± 0.05	<0.0001
8-IP (pg/mL)	330.70 ± 78.17	213.97 ± 93.22	<0.0001
TAC (mM)	6.13 ± 2.06	10.41 ± 2.83	<0.0001
8-OHdG (ng/mL)	19.52 ±1.49	17.09 ±3.26	<0.0001

$p < 0.05$ considered significant

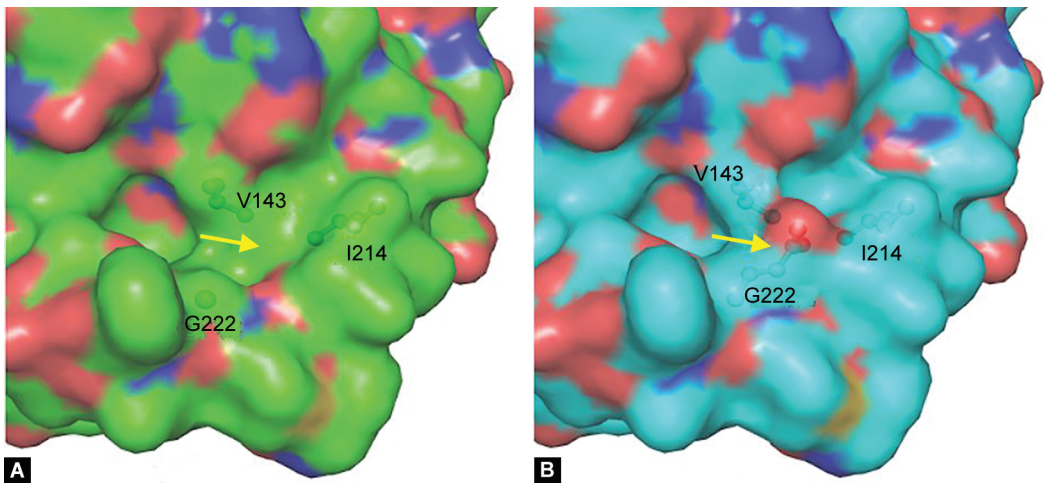
membrane of mitochondria through nonpolar interactions. This region is known to interact with one of the subunits of this complex through non-covalent interactions.[24] Substitution of G222 by E due to mutation changes the topography of the region as well as electrostatic potential (Fig. 1B), since G is a small residue lacking any side chain while E is an acidic residue having negatively charged side chain. Hence this mutation may affect the nonpolar interactions with the neighboring subunit or membrane and consequently might adversely affect the function of COX as indicated by its activity assay.

ND1 is highly conserved protein of electron transport chain complex I (NADH-ubiquinone oxidoreductase) known to form a part of proton channel. It consists of eight transmembrane helices forming hydrophobic membranous core while its cytosolic surface helps in ubiquinone binding.[24] The residue E192 lies on one of the transmembrane helix, and sequence analysis indicates that residue E192 is highly conserved from prokaryotes to higher eukaryotes. The E192 lines the channel formed by helices which also includes crucial residue E143 protruding into the channel (Fig. 2A). Mutation of E143 to K143 is known to be associated with LHON.[25] This indicates that conserved negatively charged E might be essential in the transport of proton across the inner mitochondrial membrane. Mutational substitution of negatively charged E192 by positively charged K might have a severe effect on the function of proton transport due to drastic changes in the electrostatic environment of the channel. Model structure of mutant reveals that K192 forms a hydrogen bond with another crucial residue E143 (Fig. 2A), due to its longer side chain while this interaction was absent in wild-type protein (Fig. 2B). Hence, this hydrogen bond might lead to occlusion

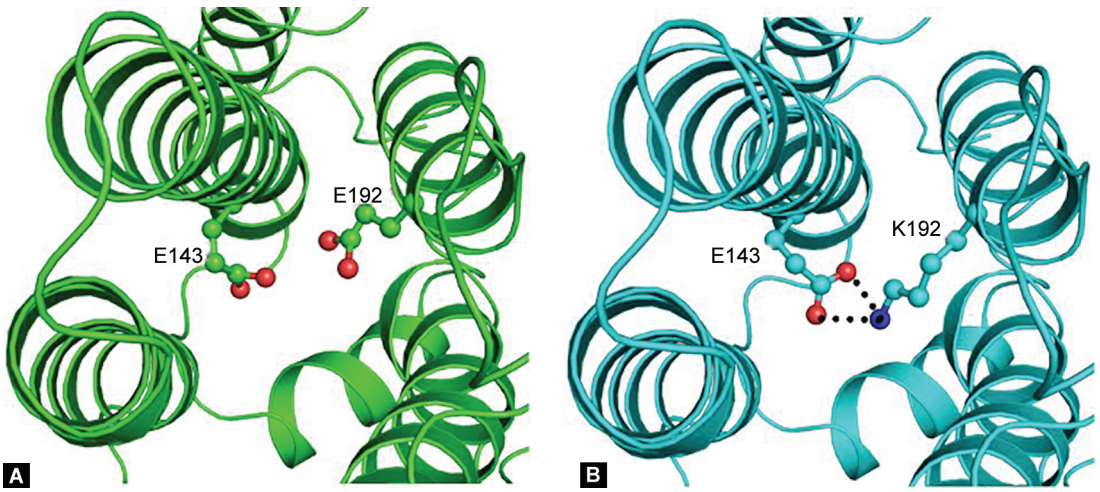
Table 3: COX and oxidative stress markers (patients with p.G222E mutation in COX2 vs patients without p.G222E mutation)

Parameters	*Patients with p.G222E mutation (n = 14)*	*Patients without p.G222E mutation (n = 61)*	*p-value*
Absolute COX activity (µM/min/mg)	0.03 ± 0.001	0.05 ± 0.004	<0.0001
Relative COX activity (µM/min/mg)	0.36 ± 0.01	0.61 ± 0.06	<0.0001
8-IP (pg/mL)	390.42 ± 15.98	351.64 ± 58.58	0.01
TAC (mM)	4.74 ± 0.62	5.83 ± 1.16	0.004
8-OHdG (ng/mL)	19.84 ± 1.21	19.45 ± 1.09	0.21

$p < 0.05$ considered significant



Figs 1A and B: Model structure of human COX represented in surface rendering (A) wildtype (green) and (B) mutant G222E (cyan). The side chain of E222 and its interacting residues I214 and V143 are shown as ball and stick



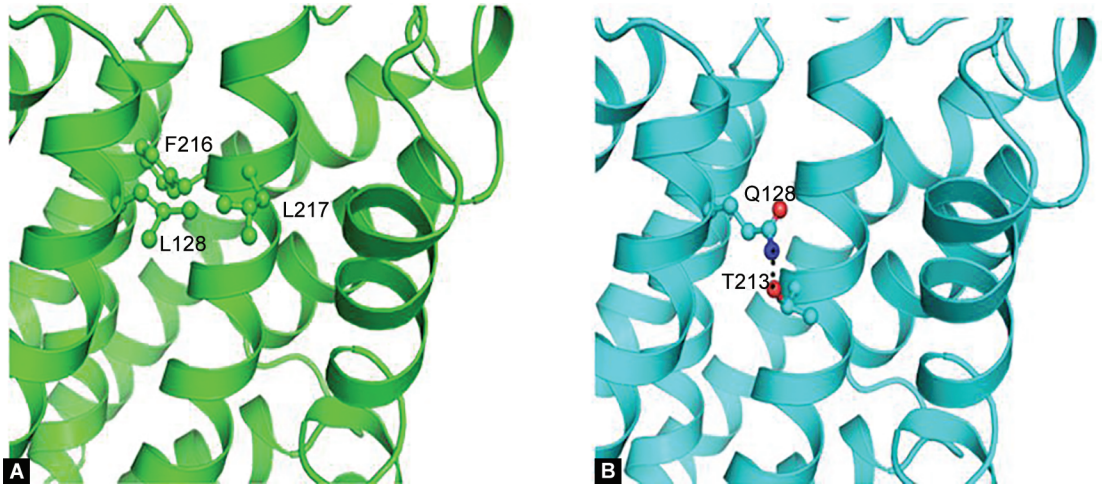
Figs 2A and B: Cartoon representation of model structure of human ND1 (A) wild-type (green) and (B) mutant E192K (cyan). The side chain of E192/K192 and E143 are shown as ball and stick. Hydrogen bonded interactions are shown in black dotted lines

of the proton channel in addition to adversely affecting its electrostatic environment.

ND2 along with *ND3* and *ND4* is an important subunit of complex I involved in proton translocation in addition to its provisional role in quinone binding.[26,27] The residue L128 in wild-type protein remains buried due to its hydrophobic nature. Its side chain is surrounded by other hydrophobic residues and interacts with F216 and L217 (Fig. 3A). This hydrophobic residue is substituted by polar residue Q128 due to mutation. Though its side chain has the propensity to be in the surrounding of polar residues, it interacts with another polar residue T213 through hydrogen bonding and gets stabilized (Fig. 3B). This substitution is not as favorable and stable as wild-type protein, but this does not alter the overall conformation of protein since residue 213 lies on highly stable helical region of protein. Hence this mutation appears to have mild effect on structure and function of protein.

Oxidative Stress

To assess oxidative stress, we measured 8-IP and TAC levels in patients and controls. The 8-IP levels were significantly higher, and



Figs 3A and B: Cartoon representation of model structure of human ND2 (A) wild-type (green) and (B) mutant L128Q (cyan). The side chain of L128/Q128 and important interacting residues are shown as ball and stick. Hydrogen bonded interactions are shown in black dotted lines

TAC levels were significantly lower in POAG patients than in controls, respectively (Table 2). Patients with p.G222E mutation in *COX2* gene had significantly higher 8-IP levels and lower TAC levels as compared to patients without this mutation, respectively (Table 3).

Oxidative DNA Damage

The 8-OHdG levels were significantly higher in POAG patients than in controls (Table 2). The 8-OHdG levels in patients with p.G222E mutation in *COX2* gene were higher as compared to patients negative for this mutation, but the difference was not statistically significant (Table 3).

Discussion

The mitochondrial genome is more prone to mutations than the genomic DNA. The mtDNA does not have histone proteins and the rate of mtDNA replication is faster than the nuclear genome replication making it more prone to replication errors. Since the mtDNA repair mechanism is not efficient, the replication error-induced mutations are less repaired. The mtDNA owing to their proximity to free radicals is susceptible to oxidative stress-induced damage.[28]

The role of mitochondria has been investigated in many age-related neurodegenerative diseases like Parkinson's disease, Alzheimer's disease, and glaucoma.[29–31] The prevalence and incidence of open-angle glaucoma increase exponentially with age. So, there is a high chance of involvement of mtDNA in glaucoma.

Glaucoma is associated with accelerated loss of RGCs. RGCs have high metabolic rates and thus may be the first to show atrophic changes when energy demands are not met due to dysfunctional mitochondria. This mitochondrial dysfunction due to accumulation of pathogenic mtDNA sequence variations can affect RGC survival, apoptosis, and calcium signaling and impair RGC function. It has been shown in rat models of glaucoma that mtDNA mutations contribute to the progressive loss of RGCs.[32]

There is a higher energy requirement in optic nerve head which is evident from the abundance of mitochondria in optic nerve head.[33] Damage to mtDNA can cause energy crisis in RGCs, making them susceptible to glaucomatous injury. It is already established that mtDNA mutations are the major underlying pathology in LHON and PCG.[13,34] A study in East Indian POAG patients reported that complex I, especially ND5 gene had the highest mutation rate as compared to other mitochondrial complexes/genes and controls.[35] Consistent with this finding in present study, it was observed that complex I had the highest number of mtDNA variants in POAG patients. Recently mitochondrial gene-sets were examined for association with POAG, normal-tension glaucoma, and high-tension glaucoma, and mitochondrial lipid metabolism was observed to be associated with POAG.[36] Another study in African American POAG patients showed that African mtDNA haplogroups L1c2, L1c2b, and L2 are risk factors for POAG.[37] A similar study recently showed that non-L haplogroup was associated with a higher risk of POAG than L3 haplogroup.[38]

In the present study, we found mtDNA sequence changes different from already reported changes seen in other types of glaucoma. Mutations in complex I genes are reported to be associated with Leigh syndrome, mitochondrial encephalomyopathy, lactic acidosis stroke-like episodes, and infertility. Defects in mitochondrial complex I have also been reported in POAG.[39] A study performed on Irish and Indian POAG patients reported complex I, as a hotspot for mtDNA mutations in POAG patients, and 50% of patients had pathogenic mtDNA mutations.[40] In the present study, 52.17% of total non-synonymous changes found in cohort of 75 POAG patients were in complex I and 26.09% in complex V. Since these two complexes are the crucial complexes in respiratory chain, these alterations may cause impaired or reduced respiration and lead to RGC death and eventually POAG. The mtDNA nucleotide change (T4852A, p.L128Q) in *ND2* is already reported in LHON patients, but for the first time reported in POAG in this study.[34] However, these patients showed no features consistent with LHON. All the mtDNA mutations reported in this study are homoplasmic, as is evident from single peaks observed in the sequence chromatograms, or the heteroplasmy levels were too low to be detected by Sanger sequencing method.

The majority of the cases 20/24 (83.3%) carrying pathogenic mtDNA changes were severely affected (MD > –12.00), and the remaining 4/24 (16.7%) were moderate glaucoma (MD = –6.00 and –12.00). All patients who carried p.G222E mutation in *COX2* showed severe POAG phenotype. Among patients who did not harbor any mtDNA pathogenic mutations, 25/51 (49%) were affected severely, 12/51 (23.5%) were affected moderately, and 14/51 (27.5%) patients were affected mildly. Comparative structure modeling revealed

the effect of p.G222E mutation on structure and consequently on functions of COX. The mutation p.G222E in *COX2* disturbs the topography as well as electrostatic environment of the region and hence affects the interaction of this protein with other subunits. COX (complex IV) is the fourth complex of respiratory chain. Cytochrome c chain couples the electron transport with oxidative phosphorylation and helps in generation of ATP, the energy source of neuronal function. Improper interaction of this enzyme with other subunits may affect the coupling process and result in inefficient respiration and generation of free radicals. So, we did COX activity assays to evaluate and compare the activity of COX between POAG patients and controls. We also showed COX activity in patients with p.G222E mutation in *COX2* gene. Blood PBMCs were chosen to study mitochondrial enzyme activity. There are several reports about mitochondrial abnormalities in PBMCs in many diseases that involve neurodegeneration like Alzheimer's disease, bipolar disorder, and schizophrenia and depression.[16,41,42] Thus, studies in PBMC can be informative about the central nervous system.[42,43] We observed lower COX activity and higher oxidative stress in patients carrying p.G222E mutation in *COX2* gene. The findings of protein modeling studies and activity assays indicate that the mutation p.G222E in *COX2* may affect structure and activity of this crucial enzyme and consequently may be causal in POAG pathogenesis through oxidative stress. To the best of our knowledge, we are the first to highlight lower levels of COX activity in Indian POAG patients. As COX is involved in coupling of oxidative phosphorylation with ATP generation, its decreased activity in POAG patients may hinder ATP production in POAG patients.

Impaired complex I linked respiration has been reported in lymphoblasts of POAG patients.[7] He et al., also have documented higher oxidative stress in trabecular meshwork (TM) accompanied by lower complex I activity and ATP production and subsequent loss of TM cells due to enhanced apoptosis in POAG.[39] They have also shown that antioxidant supplementation rescued TM cells from cytotoxic effects of oxidative stress by reducing the production of free radicals. Our study substantiates the role of oxidative stress through mitochondria in glaucoma. There are studies that report decreased antioxidants and increased lipid peroxidation and oxidative stress enzymes in aqueous humor of POAG patients.[44] A recent study also documented the role of lower systemic antioxidant capacity in pathogenesis of POAG by affecting IOP.[45] In our study, higher levels of systemic oxidative stress markers (8-IP), DNA damage marker (8-OHdG), and lower TAC in patients strongly support the role of oxidative stress in POAG. Higher levels of oxidative stress markers and lower TAC in patients carrying pathogenic mutation p.G222E implies an association of this mutation with oxidative stress. Oxidative stress has been documented to be an important inducer of DNA damage in TM, which ultimately leads to IOP elevations and visual field defects POAG.[46] Although we found a significantly higher level of 8-OHdG in patients as compared to controls, there was no significant effect of mutation p.G222E on 8-OHdG levels. DNA-based modifications in the nuclear and mitochondrial genome are major contributors to genomic instability and mitochondrial dysfunctions in neurodegenerative diseases. Extensive studies to explore the role of mtDNA damage and dysfunctional mitochondria in neurodegenerative disorders may provide molecular targets for developing pharmacological agents for these diseases. Along with studying mtDNA sequence alterations, efficiency of mtDNA repair pathways should also be analyzed in POAG patients to develop a complete perception of the role of mitochondrial dysfunction in POAG.

Recently stress reduction by mindfulness meditation has been shown to ameliorate oxidative stress (by improving the antioxidant capacity), reduce IOP, and improve quality of life in POAG patients.[47] Meditation leads to a physiological relaxation response driven by the parasympathetic nervous system which has been reported to upregulate the expression of genes associated with mitochondrial function and downregulate the expression of genes related to stress pathways. This can lead to an improvement in mitochondrial function by upregulation of ATP synthase.[48] Considering the wide range of positive effects on the mitochondrial function and oxidative stress, mindfulness meditation may be advised as an adjunctive therapy in POAG patients with altered mitochondrial functions and elevated oxidative stress, although further investigations with long-term outcomes are required on this intervention.

Conclusion

Mutations in the mitochondrial genome are associated with POAG. These can lead to impairment in respiratory chain complexes (especially in *COX2* gene) and also mitochondrial dysfunction, which consequently leads to oxidative stress-induced glaucomatous changes in POAG patients.

Clinical Significance

Primary open-angle glaucoma patients should be evaluated for mitochondrial mutations and oxidative stress and may be managed accordingly with antioxidant therapies.

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