Original Article

Screening of *PEO1* and mitochondrial genes in sporadic cases of ophthalmoplegia

Abstract

Aim: To screen the gene *PEO1* and mitochondrial genes in sporadic cases of rare progressive external ophthalmoplegia (PEO) patients from North India. **Materials and Methods:** The nuclear and mitochondrial DNA was isolated from the sporadic PEO patients, and bi-directional sequencing was done in gene *PEO1* and mitochondrial genes to capture the mutations relevant to the PEO disease. **Results:** In the present study, none of the mutations were reported in the coding region of *PEO1* gene, while four mutations were observed in mtDNA genes, namely NADH dehydrogenase subunit 2 (*ND2*), *tRNA-Trp*, non-coding nucleotides (*MT-NC3*), and NADH dehydrogenase subunit 5 (*MT-ND5*). Our study revealed two novel mutations, one in *tRNA-Trp* and the other in *ND2* gene, which may have role in sporadic cases of PEO patients. The absence of novel conserved mutation in *tRNA-Trp* gene was also confirmed in 110 ethnically matched controls. **Conclusion:** The sporadic case of PEO disease is not associated with mutations in nuclear gene *PEO1*. The novel mutation in *tRNA-Trp* gene and *ND2* mutations may have role in the disease.

Key words:
Mitochondrial mutations, mtDNA, ophthalmoplegia, PEO1, rare disease, sporadic, tRNA genes

Introduction

Mitochondrial diseases have gained wide interest owing to their role in inherited diseases, aging,[1] and heterogeneous clinical symptoms including neurological manifestation,[2] resulting in considerable morbidity of undiagnosed/underdiagnosed cases. Following the discovery of first pathogenic mitochondrial mutation,[3] a number of nuclear and mitochondrial mutations have been reported in mitochondrial diseases till date. Recently, it has been hypothesized that there is continuous crosstalk between nuclear and mitochondrial genome to perform important cellular functions, and mis-communication may lead to diseases like progressive external ophthalmoplegia (PEO).

PEO is a rare neuromuscular disease (http://rarediseases.info.nih.gov) with heterogeneous clinical symptoms[2] and has no therapy as it affects a small number of human population. PEO has been associated with mutations in nuclear gene, chromosome 10 open reading frame 2 (*C10orf2*) or *PEO1*. The gene is located on chromosome 10 and encodes mtDNA maintenance protein Twinkle, which co-localize with mitochondrial nucleoid.[4] A number of mutations so far identified from the PEO patients are located in *PEO1* involved in subunit interactions of the hexameric helicase.[4] Furthermore, mutations of the 22 mitochondrial tRNAs of human (10% of mtDNA) are of particular interest because they harbor more than half of all known mitochondrial pathogenic mutations in humans.[5] Moreover, large mtDNA deletions, apart from mutations of tRNA genes, have also been found to be associated with PEO[6-11] and its chronic form.[12,13] Besides the *PEO1* gene, the PEO disease is also associated with *POLG* and *ANT1* genes.[14,15]

In the present study, nuclear gene *PEO1* along with mitochondrial tRNA genes (*tRNA-Leu*, *tRNA-Asn*, *TRNL2*, *tRNA-Ala*, *tRNA-Trp*, *tRNA-Cys*, *tRNA-Try*, *tRNA-His*, *tRNA-Ser*) and protein coding genes (NADH dehydrogenase

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subunit 2, NADH dehydrogenase subunit 5) were screened in PEO diagnosed patients of North Indian origin to find out nucleotide variations and the relation between *PEO1* and mitochondrial mutations.

Materials and Methods

Sample identification and collection

During the 1-year study period, totally five ophthalmoplegia patients were identified from Sitapur and Lucknow districts of Uttar Pradesh and blood samples were collected after written informed consent (in case of child, parents' consent) was obtained and were coded properly. The study protocol was approved by Institutional Ethics Committee of Central Drug Research Institute, Lucknow, UP. A total of 400 µl of blood samples was collected in 2 ml acid citrate dextrose (ACD) buffer collection tube and stored at 4°C till further use. Various specific and general body symptoms of the patients were checked and recorded in a questionnaire during clinical investigation by expert physician. All PEO samples were examined and diagnosed by physician and categorized as sporadic. The ethnically matched control samples (n=110) used for the confirmation of the absence of A/G mutation at position 5576 in *tRNA-Trp* gene were used from our lab DNA repository.

Isolation of genomic DNA

Briefly, each blood sample was divided into two parts, one for genomic DNA isolation and other remaining part for mtDNA isolation, as to get the pure quality DNA for subsequent amplification. Genomic DNA was isolated from all the 30 ethnically matched control samples and five patients' blood samples, using GenElute™ blood DNA isolation kit (Sigma, Saint Louise, MO, USA). The mtDNA from ophthalmoplegia patients was isolated using mtDNA isolation kit (Milpitas, BioVision Research Products, California, USA) and quantified using spectrophotometer (GE HealthCare, Port Washington, New York, USA).

Primers design and their details

Primers were designed for all the five exons of *C10orf2* gene with the help of Primer Select module of Lasergene v 6.0, (DNA STAR™). Two pairs of primer were designed for first exon of the gene *PEO1* (Exon 1A, FP/RP and Exon 1B, FP/RP) due to its large size and found suitable for amplification and sequencing [Table 1]. A total of six primers (5F/5R, 8F/8R, and 18F/18R) were selected for mtDNA amplification, as described earlier.[16] Primers 5F and 5R amplified *RNR2* and *ND1* genes. Similarly, primers 8F and 8R amplified *tRNA-Ala*, *tRNA-Trp*, *tRNA-Cys*, *tRNA-Try*, and *ND4* genes, while 18 F and 18 R primers amplified *tRNA-His*, *tRNA-Ser*, and *ND5* genes. All the primers were procured from Sigma. The primers for mitochondrial tRNA genes were chosen with an aim to cover most causative mutations linked with sporadic ophthalmoplegia (G12315A) and chronic PEO (A3243G, C3256T, A5692G, and G5703A).[5]

Table 1: The primer sequences and size of amplicons used for the amplification of progressive external ophthalmoplegia 1 gene

Exons	Primer sequences (5'....3')	Amplicon size
1A FP	GGAGGATCTGGAACCGAGCAATAC	477
1A RP	TAAGGGAGTAAGGCAGGGGGTAAG	
1B FP	GGCTGCCTACCCTTACTCTACCC	482
1B RP	AACCCACTTGCTTTTGTCACCTG	
2 FP	AGATCAGGTGACAAAACAAGTGG	435
2 RP	GGATATGTCTGGGAAAGCAAGGTG	
3 FP	GAACTCCCCCATCTCCTTAG	568
3 RP	TGTGGACAGCTGCTCGTGACC	
4 FP	GGTTGTGGTAGTTTGTGGGGAGAT	445
4 RP	CTGGGGGACAAGAACAGCATAAGA	
5 FP	ACCCAGCCCCTCTCCCCATTCTTA	451
5 RP	ACCAGCTCTGCACGGCCTTCACTT	

Polymerase chain reaction and electrophoresis

Gene-specific amplification of *PEO1* and mitochondrial genes was performed in thermal cycler (Waltham, MJ Research, MA, USA) using standard PCR reagents (Sigma, Bangalore). Briefly, in the PCR reaction of 25µl, 40-50ng template DNA; 2.5µl, 5X PCR buffer, 5pmol forward and reverse primers, 200µM dNTPs, 0.5U Taq DNA polymerase was used. The final PCR reaction volume was adjusted with Milli Q water. The PCR amplification conditions include 10 min denaturation at 95°C, 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 58.3°C for all primers, and 1 min extension at 72°C followed by final extension at 72°C for 4 min before holding the reaction at 4°C. Twenty-five microliters of amplified product was resolved on 1.2% agarose gel to verify the exact size of the PCR product.

DNA sequencing

Following electrophoresis, the total PCR products were eluted using MinElute™ Gel Extraction Kit (Qiagen, Valencia, CA, USA) and sequenced bi-directionally (ABI 3100) using exon-specific primers at The Centre for Genomic Application (TCGA), Okhla, New Delhi. Briefly, sequencing primer (2 pmol/µl) and 50-150 ng/µl amplicons were added to 4 µl reaction mix, and the volume made up to 10 µl with autoclaved Milli-Q water as per the Big Dye Terminator kit instructions (v 3.1, Applied Biosystem, Foster city, ABI, California, USA). The sequencing reaction was carried out for 30 cycles of denaturation at 96°C for 10 s followed by annealing for 5 s and an extension at 60°C for 4 min. The annealing temperature was 58°C or 60°C depending on the primer utilized for sequencing. After the sequencing reaction, the products were precipitated, followed by washing (twice) with 70% ethanol and finally resuspended in 10 µl Hi-Di formamide (Applied Biosystem, CA, USA). The tubes were incubated at 94°C for 5 min and snap frozen in ice for denaturation before loading in the automated sequencer (Applied Biosystem, CA, USA).

Restriction digestion

To confirm the absence of a novel heteroplasmic mutation in T stem of *tRNA-Trp* where allele A is replaced by G at position 5576 in mtDNA, restriction digestion was performed in 110 ethically matched samples. The PCR was performed in 110 control samples for amplification of *tRNA-Asn* gene using 5F/5R primers. PCR conditions were similar as for the other mitochondrial primers.

Briefly, 2.5 µl of buffer, 0.3 µl bovine serum albumin (BSA), 0.2 µl TspRI restriction enzyme (Ipswich, New England Biolabs, MA, USA), 10 µl of PCR product, and 12 µl molecular grade water were added to make up a total of 25 µl restriction digestion reaction. The restriction digestion was subjected to restriction enzyme TspRI at 65°C for 6 h and resolved on 1.2% agarose gel. TspRI enzyme was able to differentiate the allele A and G in PCR product following restriction digestion. The total length of PCR product was 777 bp. If the allele is A, TspRI produces the fragment of 108 and 669 bp, while three fragments of 322, 346, and 108 bp are produced in case of G allele. In case of heterozygous condition of the allele, TspRI produces four bands of 669, 322, 346, and 108 bp size.

Sequence analysis

DNA sequences were analyzed with the help of SeqMan module of Lasergene v 6.0 (DNA STAR™) and compared with the reference sequence (NT_030059.12) for *PEO1* (http://www.ncbi.nlm.nih.gov/). In case of mitochondrial sequences, Revised Cambridge Reference Sequence or rCRS [Genbank accession number: NC_001807 (http://www.ncbi.nlm.nih.gov/)] was used as a reference sequence.[17]

Results

Clinical presentation of the patients

Presently, all ophthalmoplegia cases had commonly observed symptoms like ptosis, eye muscle pain, and weakness. None of the subjects had any family history of ophthalmoplegic or any other eye disease up to their first cousin level, hence all samples were categorized as sporadic. Except third patient, all others developed the disease symptoms during early age of their childhood [Table 2]. Few of the rarely observed ophthalmoplegic symptoms like mental changes, cardiomyopathy, rhabdomyolysis, sensorineural deafness, cataracts, and endocrinopathies were absent in our patients.[18] We did not find any large mitochondrial DNA deletions in blood mitochondrial DNA.

Mitochondrial DNA deletions

We did not find any large mitochondrial DNA deletion in our patients' blood mitochondrial DNA samples when it was run along with a lambda mix marker, 19 (Fermentas, Hanover, Maryland, USA).

Mutational analysis of *PEO1* and mitochondrial genes

Mutation analysis in five coding regions of *PEO1* revealed no nucleotide variation in any ophthalmoplegia patient, in comparison to 30 ethnically matched controls.

However, mtDNA analysis revealed mutations in *ND2* (5450, A/G, synonymous, novel) [Figure 1], *tRNA-Trp* (5576, A/G, T stem of *tRNA-Trp*, novel heteroplasmic mutation) [Figure 2], non-coding nucleotide region or *MT-NC3* (5585, G/A, reported at MITOMAP) [Figure 3], and in NADH dehydrogenase subunit 5 (*MT-ND5*) (12705, C/T, synonymous, reported at MITOMAP) genes. Mutations A5450G and C12705T were observed in fifth patient; A5576G, G5585A, and C12705T were observed in second; and C12705T in fourth patient [Table 3, Figures 1-4].

Confirmation of the absence of novel mtDNA mutation *tRNA-Trp* (A/G) in control

The novel heteroplasmic mitochondrial mutation in T stem of *tRNA-Trp* at position 5576 (A/G) was absent in 110 ethnically matched human mitochondrial DNAs [Figure 5].

Discussion

Mutation analysis of *PEO1* revealed no change in any ophthalmoplegia patient, in comparison to 30 ethnically matched controls. On the contrary, previous reports describe pathogenic mutations in coding regions of *PEO1* in different ethnic populations of ophthalmoplegia[4] and autosomal dominant PEO in Australian families.[2] The results of

Table 2: Associated symptoms along with age, sex, and age of onset of disease in progressive external ophthalmoplegia patients

Patients of ophthalmoplegia	Associated symptoms	Age/sex	Onset of disease
1	Single eye ptosis, limited eye movement, and symptoms of eye ptosis	16 years/female	At the age of 6 months
2	Ophthalmoparesis, limited eye movement in all gazes except abduction, single eye ptosis	9 years/female	At the age of 2 years
3	Single eye ptosis, diplopia, limited eye movement except down gaze	25 years/female	At the age of 25 years
4	Single right eye ptosis	8 years/female	At birth
5	Left eye ptosis since birth, eye muscle pain, and limited eye movement except the down gaze, eye pain	20 years/male	At birth

Table 3: Mitochondrial mutations observed in progressive external ophthalmoplegia patients

Nucleotide position	rCRS	Variation in blood mtDNA	Affected sample number	Gene affected	Amino acid change	Significance	References
5450	A	G	5	NADH dehydrogenase subunit 2 (*ND2*)	Synonymous	Novel	–
5576	A	G	2	T stem of *tRNA-Trp*	–	Novel	–
5585	G	A	2	Non-coding nucleotides	Non-coding region	Reported	MITOMAP
12,705	C	T	2, 4, 5	NADH dehydrogenase subunit 5 (*MT-ND5*)	Synonymous	Reported	MITOMAP

rCRS – Revised cambridge reference sequence

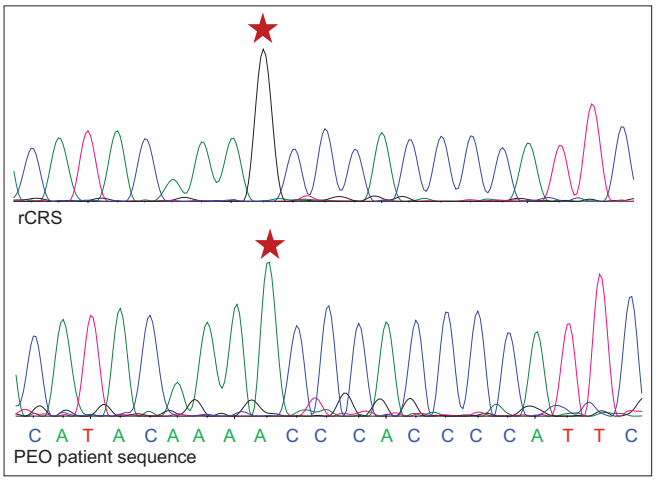

Figure 1: Chromatograms showing mutation at nucleotide position 5450 where allele "A" is replaced by "G" in ND2 gene of mtDNA. Revised Cambridge Reference Sequence is used as a reference sequence for progressive external ophthalmoplegia (PEO) patient

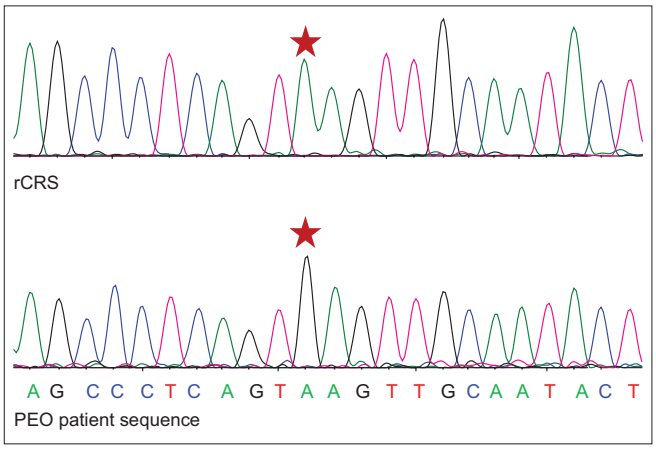

Figure 2: Chromatograms showing mutation at nucleotide position 5576 where allele "A" is replaced by "G" in T arm of tryptophan gene of mtDNA. This alteration at nucleotide position 5576 is highly conserved in animal kingdom, and was reported for the first time by us as novel mtDNA mutation in PEO patient of North Indian origin

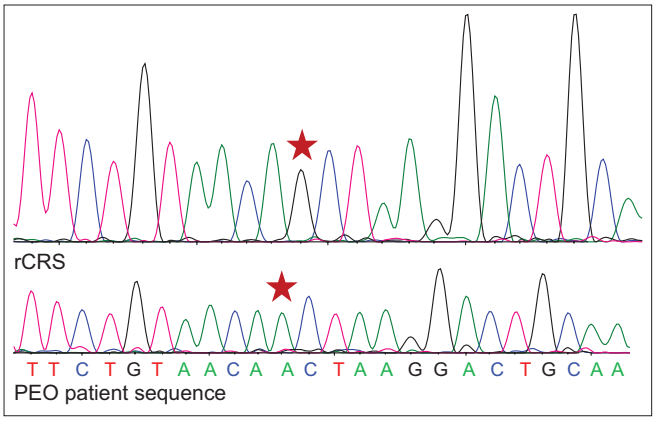

Figure 3: Chromatograms showing mutation at nucleotide position 5585 where allele "G" is replaced by "A" in non-coding region of mtDNA

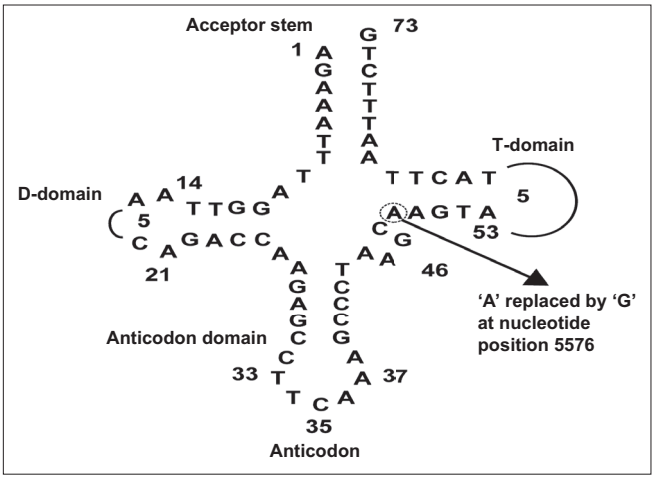

Figure 4: Illustration of *tRNA-Trp* sequences along with a novel mutation at position 5576 at highly conserved region of mtDNA sequence, where allele "A" is replaced with "G." The location of mutation in T stem of tRNA is also illustrated (circled nucleotide). The typical number of nucleotide is enclosed in the loop of the *tRNA-Trp*

PEO1 are in corroboration with a study on 38 sporadic PEO patients with multiple mtDNA deletion.[19] The other plausible explanation may be the population-specific rare occurrence of pathogenic mutation.

The variation at nucleotide position A5576G of T stem of tryptophan gene is highly conserved in animal kingdom (http://mamit-trna.u-strasbg.fr/Table/Tryptophane.html). This sequence variation present in one sample, i.e., second, has never been reported in any literature, MITOMAP and mtSNP (GIFU International) database [Figures 2 and 4]. There are many other mutations

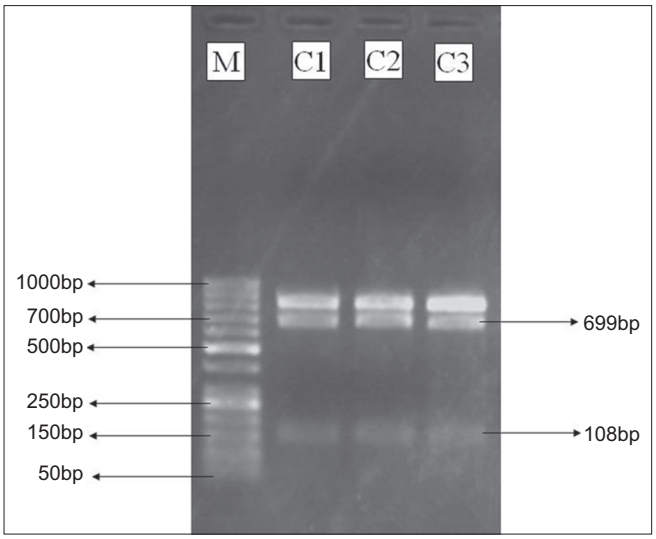

Figure 5: Illustration of the results of restriction digestion showing absence of G allele in control samples. The first lane M is a 50 bp ladder (Fermentas); C1, C2, and C3 are control samples showing the presence of two digested fragments of 669 and 108 bp. The restriction site for TspRI is at 5558, A/G. Absence of 322 and 346 bp bands indicates the absence of G alleles in all the control samples

reported in *tRNA-Trp* gene by different groups in various mitochondrial diseases like G5521A in mitochondrial myopathy,[20] G5532A in gastrointestinal syndrome,[21] A5537T (T-insertion) in severe multisystem mitochondrial disorder,[22] G5540A in sporadic encephalomyopathy,[23] and G5549A in progressive dementia.[24] All these mutations were reported in highly conserved regions of *tRNA-Trp* gene. However, MITOMAP describes these mutations as provisional because they have been reported pathogenic in single studies. Occurrence of A5576G mutation at T-stem of *tRNA-Trp* in the present study may be a contributing factor for PEO, as tRNAs have very specific structural properties that allow an optimal positioning of signals for interaction with various partners, translational initiation or elongation factors, and the ribosomal machinery to accomplish their biological role. It has been suggested that deviations exist within the tRNA structures due to absence of conserved nucleotides in D-loop and T-loop of tRNAs. Moreover, these point mutations in human tRNA genes may lead to reduced functionality with subsequent disease state.[25] Additionally, a mutation has higher probability of being clinically relevant when conserved nucleotides get modified as in the case of A5576G [Figure 4].

Another novel and synonymous transition (A5450G) in *ND2* gene has been observed for the first time by us. Mutation A5450G lies in third nucleotide position of a codon and does not change resultant amino acid. Mutation G5585A was an already reported mutation at MITOMAP in non-coding nucleotides (region 5580-5586 bp) in second sample.

We also observed segregation of a synonymous mutation, C12705T, in second, fourth, and fifth cases, which is a reported mutation that has not been associated with common mitochondrial diseases.[26,27] Mutations A5576G, G5585A, and C12705T were present in second case only. Unfortunately, due to limited samples in this study, we are unable to correlate any of these mutations to the disease pathogenicity of PEO. Mutations A5450G and A5576G have not been reported in any other population across the world. We have also confirmed the absence of the mutation A5576G in 110 ethnically matched controls [Figure 5]. These data confirm the novelty of the A5576G in opthalmoplegia sample. However, occurrence of novel mutation in important mitochondrial genes like *ND2* and *tRNA-Trp* in PEO patients may have a role in disease progression and observed phenotype. Identification of novel mutation in mitochondrial genes of blood tissue also suggests that the blood samples should not be neglected in mutational screening. Similar studies will open new avenue for etiological, clinical, and genetic analysis in undiagnosed/underdiagnosed patients of mitochondrial disorder in India.

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