Investigation on mtDNA deletions and twinkle gene mutation (G1423C) in Iranian patients with chronic progressive external ophthalmoplegia

M. Houshmand, M. Shafa Shariat Panahi, B. N. Hosseini, GH. Dorraj, A. R. Tabassi*

Department of Medical Genetics, National Institute for Genetic Engineering and Biotechnology (NIGEB), Tehran, *Farabi Eye’s Hospital, Tehran, Iran

Background: Chronic progressive external ophthalmoplegia (CPEO) is a phenotypic mitochondrial disorder that affects external ocular and skeletal muscles and is associated with a single or multiple mitochondrial DNA (mtDNA) deletions and also nuclear gene mutations. There are also some reports about the relationship between CPEO and the nuclear Twinkle gene which encodes a kind of mitochondrial protein called Twinkle. Aims: To study the mtDNA deletions and Twinkle gene G1423C point mutation in Iranian patients with CPEO. Materials and Methods: We collected 23 muscle samples from patients with CPEO, 9 women (mean age 34.3 years) and 14 men (36.7 years). Multiplex polymerase chain reaction (PCR) method was used to find the presence of single or multiple deletions in mtDNA. Single stranded conformational polymorphism (SSCP) and restriction fragment length polymorphism (PCR-RFLP) methods were carried out to investigate point mutation (G1423C) in the Twinkle gene in all DNA samples. Results: Different sizes of mtDNA deletions were detected in 16 patients (69.6%). Each of the 5.5, 7, 7.5 and 9 kb deletions existed only in 1 patient. Common deletion (4977bp) and 8 kb deletion were detected in 5 and 3 patients respectively. Multiple deletions were also present in 4 patients. Out of 23 patients included in our study, two cases (8.7%) had Twinkle gene mutation (G1423C) and 5 patients (21.7%) did not show any deletions in mtDNA or the Twinkle gene mutation. Conclusion: Our study provides evidence that the investigation of mtDNA and Twinkle gene mutations in CPEO may help with early diagnosis and prevention of the disease. Patients who did not show deletions in the mtDNA or G1423C mutation in the Twinkle gene may have other mtDNA, Twinkle or nuclear gene mutations.

Key words: CPEO, Mitochondria, mtDNA deletion, Twinkle gene.

Introduction

Chronic progressive external ophthalmoplegia (CPEO) is a disorder characterized by the slow progressive weakness of the extra ocular muscles. Patients usually experience bilateral, symmetrical and progressive ptosis, followed by ophthalmoparesis. Ciliary body and iris muscles are not involved in these patients.‡ Deletions of mitochondrial DNA (mtDNA), a few bases to 10 kb in size, were detected in more than 70% of patients with CPEO.‡ mtDNA is a small 16569bp molecule of double stranded DNA and encodes 13 protein subunits of multimeric oxidative phosphorylation, 2 ribosomal RNAs (rRNA) and all the translated RNAs (tRNA) required for the translation of its messenger RNA (mRNA).‡§ Sporadic germline and maternally inherited mutations of mtDNA, including single deletions/duplications and point mutations, are a frequent cause of human disease.‡§ Deletions of various lengths of mtDNA result in defective mitochondrial function, particularly in highly oxidative tissues (e.g., muscle, brain and heart). Extra ocular muscles are affected preferentially because their fraction of mitochondrial volume is several times greater than that of other skeletal muscles.‡[10]‡[10] The nuclear background of the autosomal dominant or recessive progressive external ophthalmoplegia (ad/arPPEO) with multiple mtDNA deletions has been already established.‡[11–14]

To date, mutations in three nuclear genes have been linked to this disorder. These include the muscle-brain and heart-specific isoform of the adenine nucleotide translocator 1 (ANT1) gene,‡[14] C10 or f2 encoding a putative mitochondrial helicase (Twinkle)‡[15] and the sole mtDNA polymerase (pol γ) gene (POLG).‡[16] The heterozygous, missense G→C transversion at bp 1423 in the C10 or f2 gene, results in an amino acid change of Ala-475 to Pro (A475P) has already been reported in a large Pakistani family.‡[14]

We studied 23 clinically diagnosed Iranian patients with CPEO to identify mtDNA deletions and Twinkle gene mutation (G1423C).
Materials and Methods

Consecutive patients with CPEO referred for evaluation to Medical Genetics Department of the National Institute for Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran over a period of 17 months (from June 2002 to December 2004) were the subjects of the study. The study protocol has the approval of the Institute Ethics Committee. Written informed consent was obtained from all the patients for the use of muscle biopsy for the study.

Muscle biopsies of 23 patients with CPEO were analyzed; 9 women, (mean age 34.3, range 30-40 yrs) and 14 men (mean age 36.7, range 32-43 yrs). Patients with CPEO with no family history of the disease and aged 18 years or above were included in the study. Patients were fully worked-up to exclude other causes of external ophthalmoplegia.

DNA Extraction Kit (Diatom, GenPanavaran, Tehran, Iran) was used to extract DNA from the skeletal muscle biopsy material. Primers used are given in Table 1. Using primers ONP 86F, ONP 89R, ONP 10R, ONP 74R, ONP 25F and ONP 99R investigated the deletion-prone regions between 5461bp of the light strand and 15000bp of the heavy strand. Amplification of mtDNA was only possible if a part of the DNA between respective primers was deleted. Primers ONP 86F and ONP 89R were used to amplify a normal internal mtDNA fragment in a region, which is seldom afflicted by deletions and served as a control for PCR analysis. Polymerase chain reaction (PCR) studies were performed in a total volume of 25 µl including 100 ng of total DNA, 0.2 mM of each dNTPs, 2 pmol of each primer, 5 mM KCl, 200 mM TRIS-HCL (PH=8.4), 1.5 mM MgCl₂ and 1.5 unit of Taq polymerase. (Roche, Inc) PCR reaction were carried out in a MWG Thermocycler (Germany) using the following program: 94°C for 5 min as initial denaturation and then amplification was accomplished in 36 cycles at following temperature: denaturing at 94°C for 1 min, annealing at 57°C for 50 sec and 1 min at 72°C for elongation, followed by a final extension at 72°C for 10 min.

PCR products were electrophoresed on 3% agarose gel and stained with ethidium bromide. In some patients, deletion break points were confirmed by sequencing of DNA fragments amplified by the PCR reactions, using the ABI 3100 and 3700 capillary sequencer. In order to investigate point mutation (G1423C) in the Twinkle gene, the method of single stranded conformational polymorphism (SSCP) was applied to all DNA samples. Then restriction fragment length polymorphism (PCR-RFLP) technique was applied to samples positive for single stranded conformational polymorphism (SSCP). All DNA samples were amplified by the PCR method. PCR was carried out with primers Tw-ex2-IR (5’-cctgactctattttg-3’) and Tw-ex2-2IR (5’-cccttacccgctgccc-3’). PCR program used was: 94°C for 5 min (initial denaturation), 94°C for 30”, 57°C for 45”, 72°C for 1 min (36 cycles) and 72°C for 10 min (final extension). SSCP was applied to the 413 bp amplified fragments following denaturation and separation on 8% poly acrylamide gels. Positive samples were further evaluated for the presence of the Twinkle gene (G1423C) mutation by using the HaeII restriction enzyme in the RFLP procedure. Samples positive for the G1423C mutation were further confirmed by sequencing with the Tw-ex2-If primer.

Results

Of the 23 patients with CPEO studied, 16 (69.6%) patients showed single deletion in the mtDNA, 2 (8.7%) patients showed G1423C mutation in the Twinkle gene without deletion in the mtDNA and the remaining 5 (21.7%) did not show any deletions in the mtDNA or mutation in the Twinkle gene. Five patients had common deletion (4977bp). While each of the 5.5, 7, 7.5 and 9 kb deletions was exclusively present only in a single patient, 8 kb deletion existed in 3 patients. Multiple deletions were found in 4 patients. [Table 2] In the SCCP and sequencing experiments, two patients showed different bands in comparison with the normal control. The G1423C mutation was detected in 2 women. Patients with the G1423C mutation showed the following fragments:

<table>
<thead>
<tr>
<th>Table 1: Primers used in diagnosis mt DNA deletion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Range</strong></td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

*NADH Dehydrogenase, L-strand Origin, Cytochrome c oxidase subunit II, Cytochrome b, Displacement Loop
The major aim of this study was to evaluate the prevalence of mtDNA deletions and Twinkle gene mutations (A475P) in Iranian patients with CPEO. It has been shown that POLG mutations cause adPEO and this gene has a crucial role in the mtDNA replication machinery. Single mtDNA deletions may occur in sporadic cases, but multiple deletions of mtDNA also occur in autosomal dominant disorders.

AdPEO was first diagnosed in several families. Generalized weakness of the skeletal muscles and sudden unexpected death are common clinical features of AdPEO. In a study of two families of adPEO, Kiechle et al. demonstrated a novel pathogenic mutation in Twinkle (F1485L). Deschauer et al. described a family with adPEO caused by a novel heterozygous A to C transversion at nucleotide 956 of the Twinkle gene. Mutations of ANT1, Twinkle and POLG1 in sporadic PEO have already been verified. To the best of our knowledge, this is the first study describing A475P mutation in Iranian patients. In our study 69.6% of patients showed single mtDNA deletions, 8.7% showed G1423C mutations in their Twinkle gene without mtDNA multiple deletions and the remaining 5 patients (21.7%) did not show any Twinkle gene mutations or deletions in the mtDNA. Arenas et al. analyzed Twinkle gene mutations in 11 Australian families with adPEO and investigated whether there were any distinct molecular and clinical features associated with this mutations. They found that multiple deletions in the mtDNA were not always prominent and there were significant variations in the clinical presentation within and between families with mutations in the Twinkle gene. We propose that patients with G1423C mutation in the Twinkle gene are heterozygotes with dominant phenotypes. They have a negative family history of adPEO, so it is possible that G1423C mutation has occurred sporadically as a germline mutation in the Twinkle gene. Further studies are required to reveal this in their siblings. Guley et al. had described this novel phenotype for twinkle gene mutations and multiple mtDNA deletion syndromes in a patient with no documented family history of PEO. These observations suggest either a sporadic or an autosomal-recessive syndrome. In our series two patients with Twinkle mutations had proximal muscle weakness, ataxia, neuropathy, depression or avoidant personality traits, pes cavus and tremors.

### Discussion

This project supported by National Institute for Genetic Engineering and Biotechnology, Tehran Iran (Project No 183) and Farabi eye's Hospital, Tehran, Iran.

### Acknowledgement

This project supported by National Institute for Genetic Engineering and Biotechnology, Tehran Iran (Project No 183) and Farabi eye's Hospital, Tehran, Iran.

### References

Houshmand et al.: mtDNA deletions and twinkle gene mutation in Iranian CPEO patients


