

Ultrastructural Analysis of Extraocular Muscle in Chronic Progressive External Ophthalmoplegia

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Extraocular muscles are primarily involved in many mitochondrial diseases, but no reports exist regarding the morphological appearance of the muscles in cases of long-standing ocular myopathies. For this reason, muscle samples obtained from surgery in a sporadic case of chronic progressive external ophthalmoplegia (CPEO) were used for ultrastructural investigation and molecular analysis of mitochondrial DNA. Genetic testing revealed a heteroplasmic macrodeletion of about 5.0 kilobases in length, localized between the 9570– and 14619–base pair regions. Electron microscopy revealed focal areas of both disruption and abnormality of mitochondria in only some of the muscle fibers, producing “selective vacuolization.” This ultrastructural pattern was highly selective and limited to some extraocular muscle fibers, sparing all the others. The “selective damage” observed in this case of CPEO resembles that case occurring in another mitochondrial disease, Leber hereditary optic neuropathy, where damage occurs only in the papillomacular bundle of the retina, sparing peripheral axons. It is possible that some anatomical and physiological factors play a leading role in both Leber hereditary optic neuropathy and ocular myopathies. The ultrastructural aspect herein observed needs to be further investigated to better understand whether a particular muscle fiber type is the target of mitochondrial impairment in CPEO.

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Chronic progressive external ophthalmoplegia (CPEO) is a mitochondrial disease that principally affects the extraocular muscles. Different types of macrodeletions in mitochondrial DNA (mtDNA) have been associated with CPEO and its variants, ranging from 3.0 to 8.5 kilobases (kb) in length.¹⁻³ Besides mtDNA testing, diagnosis of CPEO is supported by histopathological evidence of “ragged red fibers” on modified Gomori trichrome stain in muscle biopsy specimens. Biochemical abnormalities of oxidative phosphorylation may be found as well.^{4,5} However, few studies have been performed with attention to the ultrastructural features of the extraocular muscles. Indeed, the relationship between mtDNA abnormalities and the functional and morphological changes of mitochondria that lead to

the phenotypical manifestations of the disease is still poorly understood. To our knowledge, no studies correlating mtDNA deletion and muscle features in CPEO have been performed. To investigate the relationship existing between mtDNA abnormalities and the ultrastructural aspects of extraocular muscles in long-standing CPEO, we performed a molecular assessment of mtDNA and an electron microscopy study of the medial rectus in a patient with isolated CPEO who underwent ptosis and strabismus surgery.

MATERIALS AND METHODS

A 48-year-old white man visited the neuro-ophthalmology service of our hospital for surgical management of an almost complete bilateral ptosis with a high degree of a nonrestrictive form of exotropia. The patient was clinically diagnosed with CPEO at age 25 years. On Gomori modified stain-

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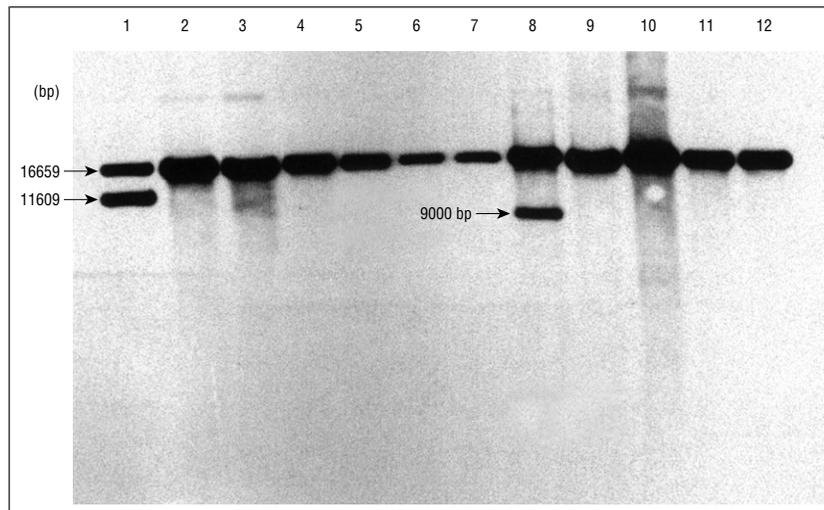


Figure 1. Southern blot with PvuII as restriction enzyme (gel of agarose 0.8% in Tris-acetate 1x). Lane 1 is our chronic progressive external ophthalmoplegia case where mitochondrial DNA (mtDNA) has been extracted from 50 mg of muscle. Two separate bands are clearly evident, corresponding to normal (upper band) and deleted (lower band) mtDNA, and accounting for 55% of heteroplasmy. Controls (lanes 2-5 and lanes 9-12) are from biopsy specimens of the quadriceps muscle obtained in cases of nonmitochondrial diseases (glycogenosis). Lanes 6 and 7 are mtDNA from peripheral lymphocytes of healthy age-matched controls. Lane 8 is a case of Kearns-Sayre syndrome, with macrodeletion of approximately 9000 base pairs (bp) (not quantified in length in this case). This latter has been used to compare the different migration of deleted mtDNA in 2 different cases of mitochondrial disease.

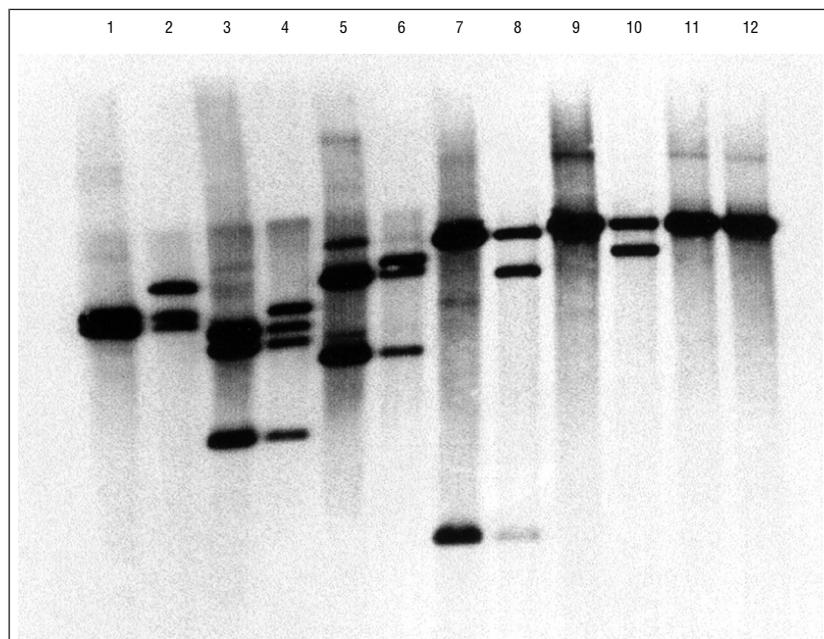


Figure 2. Southern blot with multiple restriction enzymes (0.8% agarose gel). Lanes 1, 3, 5, 7, and 9 are controls from quadriceps muscle biopsies of a patient affected by nonmitochondrial disease (glycogenosis). On lanes 11 and 12, 2 different patients are used as controls. The blotting of controls reveals the classic "wild-type" pattern in the presence of a normal mitochondrial DNA (mtDNA) amount. Lanes 2, 4, 6, 8, and 10 are from our chronic progressive external ophthalmoplegia case. The blotting reveals the typical fragmentation pattern of mtDNA in the presence of "common deletion." The different bands herein observed are secondary to the action of each restriction enzyme, which (for the presence of macrodeletion) cut in several irregular sites, giving a multiple mtDNA fragmentation. Restriction enzymes with their cutting sites are as follows: for lanes 1 and 2, the restriction enzyme is EcoRI, cutting sites are at the 4121, 5274, and 12640 positions; at lanes 3 and 4, the restriction enzyme is EcoRV, cutting sites at the 3181, 6736, and 12873 positions; at lanes 5 and 6, restriction enzyme is HindIII, cutting sites at 6203, 11680, and 12570; at lanes 7 and 8, the restriction enzyme is PstI, cutting sites at 6914 and 9024; at lanes 9, 10, 11, and 12, restriction enzyme is PvuII, cutting site at 2651.

ing technique, skeletal biopsy specimens of the left bicep muscle performed at age 28 years revealed ragged

red fibers. The patient was not previously tested for mtDNA. Visual acuity was 20/20 OU and fundoscopic ex-

amination results were within normal limits without any evidence of pigmentary changes. Family history was negative for systemic and ophthalmological diseases, so our case was classified as a sporadic form of CPEO.

We performed 8 mm of right medial rectus resection for the high degree of exotropia, and ptosis correction. Tissue samples of the resected medial rectus were immediately stored at -80°C for mtDNA testing. To investigate mtDNA deletions, DNA was extracted from 50 mg of muscle. Southern blot with the restriction enzyme PvuII (RPN 3000 kit; Amersham Pharmacia Biotech, Uppsala, Sweden) was performed using about 7 to 8 μg of DNA. PvuII cuts the mtDNA at the 2651 position, thus allowing a linearization of the mtDNA ring. A Southern blot with polymerase chain reaction amplification was subsequently performed, using the following restriction enzymes: EcoRI, EcoRV, HindIII, and PstI. Mitochondrial DNA was purified from 0.8% agarose gel and sequenced (DNA sequencing kit; Perkin-Elmer, Norwalk, Conn) in an automated DNA sequencer (373A DNA sequencer; Perkin-Elmer) to identify the deleted fragment.

For conventional histological examination, specimens from the central part of the resected muscle were fixed in 10% buffered formalin, dehydrated in graded alcohol, and embedded in paraffin. Sections 5 μm thick were stained with hematoxylin-eosin and Gomori trichrome.

For ultrastructural examination, muscle specimens were immediately fixed in Karnovsky solution for 3 hours at room temperature, postfixed in 1% osmium tetroxide, dehydrated in graded acetone, and embedded in Araldite (Fluka Chemie AG, Buchs, Switzerland). Ultrathin (80-nm-thick) sections were stained with uranyl acetate-lead citrate, and examined with a Zeiss EM 109 electron microscope (Carl Zeiss Inc, Thornwood, NY).

RESULTS

Molecular assessment of muscle mtDNA revealed a heteroplasmic macrodeletion 5.0 kb in length (**Figure 1** and **Figure 2**). In particular, Figure 1 shows the presence of 2 clearly separate bands in lane 1 (CPEO case) accounting for heteroplasmy. The up-

per band corresponds to a normal mtDNA amount, whereas the lower band reveals the presence of deleted mtDNA. Heteroplasmy was estimated to be as high as 55%. In a second blotting with several restriction enzymes, deletion was localized between the 9570 and the 14619 positions (Figure 2). This type of deletion is called “common deletion” because it is the type most frequently associated with CPEO.² The deleted region (from position 9570 to 9990) encodes for several transfer RNAs (codons for glutamine, arginine, histidine, serine, and leucine), as well as for many subunits of complex III of cytochrome *c* oxidase. Furthermore, the deletion (from position 14149 to 14619) involves mitochondrial subunits for nicotinamide adenine dinucleotide activity (ND3, ND4, ND5, and pars of the ND6).

Low-magnification electron microscopy of the resected muscle revealed a heterogeneous ultrastructural pattern, with normal muscle fibers flanked by other fibers that have diffuse sarcoplasm vacuolization (Figure 3). At high magnification, ultrastructurally normal fibers displayed peripherally located nuclei, granular sarcoplasm containing regularly arranged myofibrils, tubules of smooth endoplasmic reticulum, glycogen deposits, and mitochondria characterized by numerous inner cristae (Figure 4). On gross estimation, the number of mitochondria was not increased. High-magnification analysis of the vacuolized fibers revealed alterations of the mitochondrial compartment, responsible for ultrastructural defects. In fact, the sarcoplasm of damaged muscle fibers was filled with ultrastructurally normal myofibrils, whereas mitochondria displayed different degrees of disruption of their cristae, leading from partial to complete mitochondrial matrix emptying, with preservation of the external membrane.

These “ghost” mitochondrial profiles were responsible for the selective vacuolization observed on low-magnification electron microscopy (Figure 5). Also, the cristae of many mitochondria were arranged in an onion ring-like shape (Figure 6). The histo-

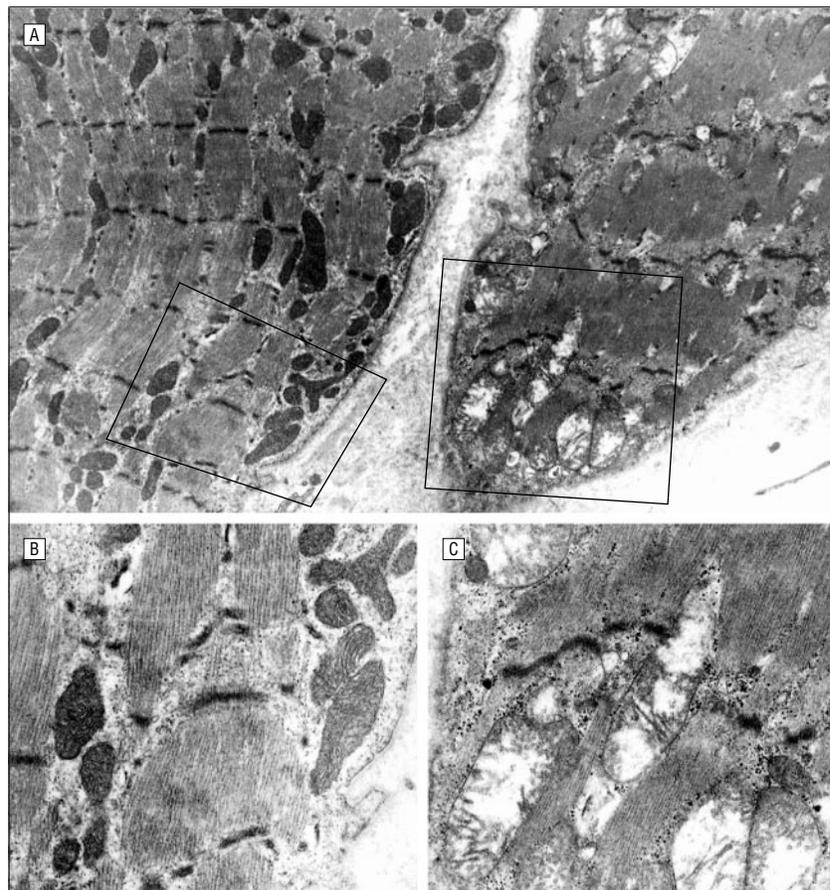


Figure 3. Electron micrograph showing an ultrastructurally normal muscle fiber. A, Left box, a fiber in close proximity to an altered, vacuolated fiber (right box) (original magnification $\times 12900$). At higher magnification, the fine structure of the mitochondrial compartment is displayed: B, a normal fiber; C, an altered fiber (original magnification $\times 27600$). In particular, the progressive emptying of mitochondria responsible for the selective vacuolization of muscle fibers can be appreciated in part C of this figure.

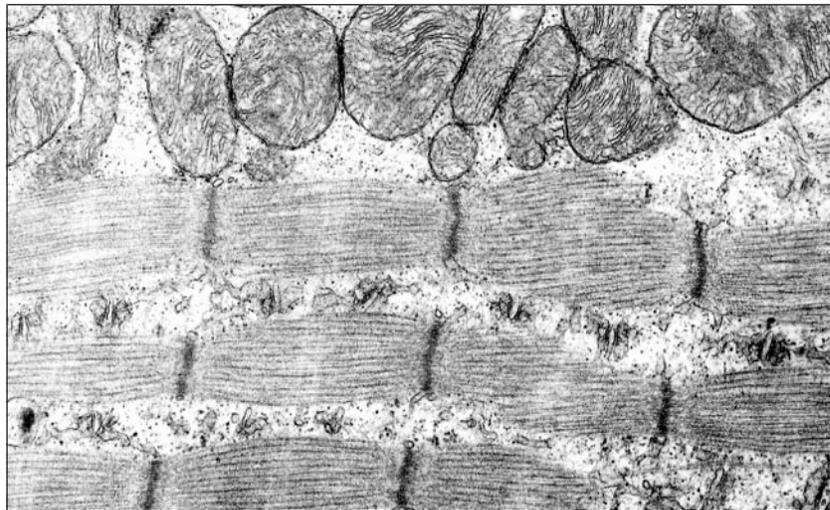


Figure 4. High-magnification detail of an ultrastructurally normal muscle fiber in which regularly ordered myofibrils, well preserved mitochondria, and tubules of the smooth endoplasmic reticulum can be appreciated (original magnification $\times 39600$).

logical architecture of muscle fibers was otherwise normal, without any evidence of inflammatory infiltration. Moreover, we did not see any fibrosis among the muscle

fibers. No rectangular- or ring-shaped paracrystalline inclusions were found and “woolly bodies” were not observed in our case of CPEO.

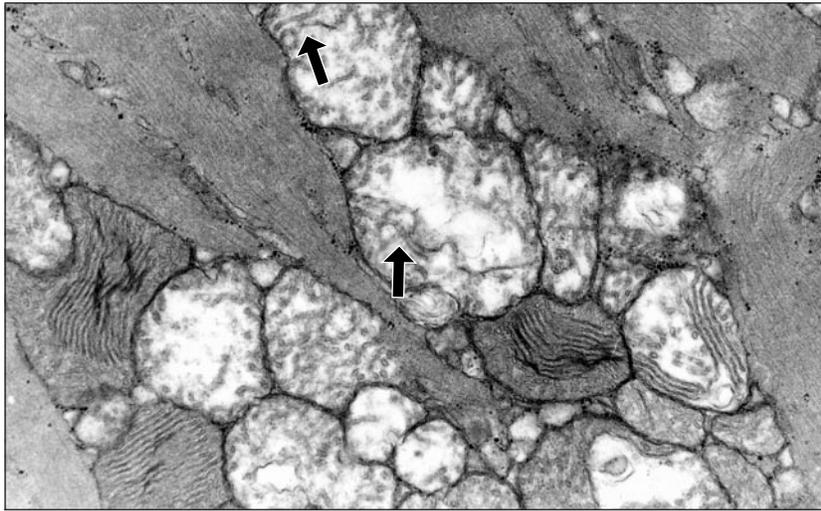


Figure 5. High-magnification detail of an ultrastructurally altered muscle fiber in which a variety of abnormal mitochondria can be seen, ranging from slightly damaged to highly damaged ones. In particular, many mitochondria have lost their inner cristae (arrows indicate some residual ones), creating the profile of "ghost mitochondria" (original magnification $\times 39600$).

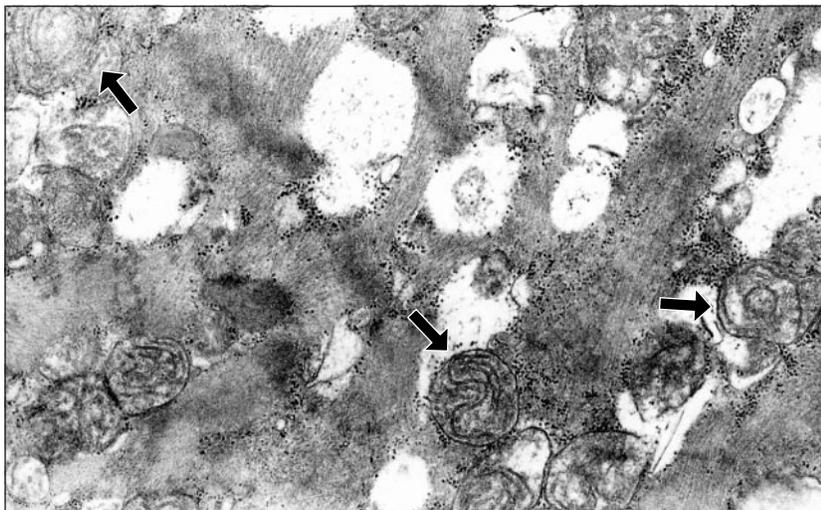


Figure 6. High magnification of an ultrastructurally altered muscle fiber showing onion ring-like arrangement (arrows) of mitochondrial cristae (original magnification $\times 38400$).

COMMENT

To date, investigations of extraocular muscles in mitochondrial diseases reported in the literature describe abnormalities of the mitochondria in the muscle analyzed.⁶⁻¹⁰ Ultrastructural studies of these abnormalities revealed longer than normal and irregularly shaped inner cristae, increased surface-volume ratio, paracrystalline inclusions of uncertain nature, increased number of mitochondria filling up to two thirds of the muscle cell cytoplasm, and presence of vacuoles in the mitochondria. All those abnormalities are presumed to be related to cell energy needs in re-

sponse to a genetically determined impairment of adenosine triphosphate production.

In our patient, we found several other features never reported in previous studies: mtDNA macrodeletion involving genes encoding for cytochrome *c* oxidase and for several transfer RNAs, is associated with a selective vacuolization of some muscle fibers as observed under electron microscopy (Figure 3, A). Also, high magnification of the same areas showed that the vacuolization is caused either by total disruption of the mitochondrial structure or of their inner cristae (Figure 3, C). Moreover, we did not detect any evidence of muscle fibrosis. Indeed, we

were unable to find any paracrystalline inclusions or vacuoles within the mitochondria. Paracrystalline inclusions have been reported as early ultrastructural changes in CPEO,^{6,9} whereas they have never been described in long-standing cases of mitochondrial diseases,^{6,7,9} nor in our case. The significance of paracrystalline inclusions is still a matter of debate since they are assumed to be an early sign of mitochondrial matrix changes, or abnormal condensation of the matrix produced during laboratory procedures. In contrast, cristae disruption and autolytic digestion are almost always present during the entire clinical evolution of those diseases.⁶⁻¹⁰ The selective damage found in the medial rectus of our patient may be only partially explained by heteroplasmy, since this is estimated to be as high as 55%, whereas a lower percentage of fibers is involved. The selective damage found in our case resembles the selective damage occurring in LHON, where point mutations of mtDNA are associated with progressive atrophy of the papillomacular bundle, sparing all other axons in the retina.

Why the damage is so selective remains unclear. A possible explanation is that in LHON, as well as in CPEO, the thinnest structures (axons and muscle fibers) are the targets of the mitochondrial impairment. Also, some anatomical features may play a key role. In fact, the papillomacular bundle fibers are the thinnest axons in the human organism. In LHON, this may give rise to an impossibility of mitochondrial duplication in such a small neuronal bag in response to an energy request, thus creating a block of the axonal flow with subsequent optic atrophy (catastrophe theory).¹¹ Perhaps some unknown physiological features such as energy request, heteroplasmy, and molecular dimension of the muscle fibers involved play a similar role in our case of CPEO, and may be responsible for the selective damage we observed.

To our knowledge, no studies correlating mtDNA testing and ultrastructural aspects of extraocular muscles in cases of long-standing CPEO have been previously published. In this regard however, 2 re-

cent studies using Gomori stain compared mtDNA defects with light microscopy appearance of muscle biopsy specimen,^{12,13} but ultrastructural features have not been investigated. Kiyomoto et al¹² found a decrease in cytochrome *c* oxidase subunit IV activity in some muscle fibers in skeletal biopsy specimens of patients with CPEO with heteroplasmic common deletion. Lindner et al¹³ found cytochrome *c* oxidase-negative fibers in 74% of their patients affected by mitochondrial encephalomyopathies; among those patients, 42% tested positive for mtDNA common deletion. Anyway, the architectural structure of the muscle itself has not been tested so we cannot conclude that only some fibers are selectively damaged in these studies. Although our case shares the same common deletion with these 2 cited studies, we did not perform any biochemical study on cytochrome *c* oxidase activity to characterize muscle fibers. In planning future studies, further morphological investigations in mitochondrial diseases are important as they may provide insight about the pathogenesis of those clinical entities. With regard to CPEO, to confirm the ultrastructural pattern observed in our case and to identify the

muscle fiber type that may be selectively damaged, additional studies correlating mtDNA testing with ultrastructural and biochemical features of extraocular muscles are needed.

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