Progressive External Ophthalmoplegia and Vision and Hearing Loss in a Patient With Mutations in POLG2 and OPA1

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Objective: To describe the clinical features, muscle pathological characteristics, and molecular studies of a patient with a mutation in the gene encoding the accessory subunit (p55) of polymerase γ (POLG2) and a mutation in the OPA1 gene.

Design: Clinical examination and morphological, biochemical, and molecular analyses.

Setting: Tertiary care university hospitals and molecular genetics and scientific computing laboratory.

Patient: A 42-year-old man experienced hearing loss, progressive external ophthalmoplegia (PEO), loss of central vision, macrocytic anemia, and hypogonadism. His family history was negative for neurological disease, and his serum lactate level was normal.

Results: A muscle biopsy specimen showed scattered intensely succinate dehydrogenase–positive and cytochrome-c oxidase–negative fibers. Southern blot of muscle mitochondrial DNA showed multiple deletions. The results of screening for mutations in the nuclear genes associated with PEO and multiple mitochondrial DNA deletions, including those in POLG (polymerase γ gene), ANT1 (gene encoding adenine nucleotide translocator 1), and PEO1, were negative, but sequencing of POLG2 revealed a G1247C mutation in exon 7, resulting in the substitution of a highly conserved glycine with an alanine at codon 416 (G416A). Because biochemical analysis of the mutant protein showed no alteration in chromatographic properties and normal ability to protect the catalytic subunit from N-ethylmaleimide, we also sequenced the OPA1 gene and identified a novel heterozygous mutation (Y582C).

Conclusion: Although we initially focused on the mutation in POLG2, the mutation in OPA1 is more likely to explain the late-onset PEO and multisystem disorder in this patient.

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PROGRESSIVE EXTERNAL OPHthalmoplegia (PEO), with ptosis and weakness of extraocular muscles, is a common manifestation of mitochondrial diseases and is often associated with multisystem involvement.1 Progressive external ophthalmoplegia is best classified based on genetic transmission into sporadic, maternally inherited, or mendelian traits. Sporadic cases include Kearns-Sayre syndrome and adult-onset PEO with myopathy. In Kearns-Sayre syndrome, PEO is the clinical hallmark, together with pigmentary retinopathy and onset before the age of 20 years.2 Additional symptoms include ataxia and defects of cardiac conduction. Adult-onset PEO is characterized by the exclusive or prevalent involvement of skeletal muscle, although other clinical features may include deafness and cataracts. These patients harbor single heteroplasmic deletions of mitochondrial DNA (mtDNA).

Patients with maternal inheritance have point mutations in mtDNA, most commonly the A3243G transition, typically seen in mitochondrial encephalomyopathy, lactic acidosis, and strokelike episodes.1

The mendelian forms of PEO can be autosomal dominant or recessive, occur in about 15% of all cases, and are associated with multiple mtDNA deletions that are due to nuclear gene mutations.3,4 Autosomal dominant PEO has been associated with mutations in genes encoding adenine nucleotide translocator 1 (ANT1)5 and Twinkle, a putative mtDNA helicase (PEO1).6 Both protein products are involved in mtDNA maintenance. Mutations in the polymerase γ (poly) gene (POLG), encoding the α subunit of mtDNA poly, were identified in patients with either autosomal dominant PEO or autosomal recessive PEO,7,8 but approximately one-fourth of patients with PEO and multiple deletions do not have a positive family his-
tory of PEO. Of these sporadic cases, only 36% carry mutations in 1 of the 3 genes associated with familial autosomal recessive PEO or autosomal dominant PEO, and the remaining cases have no recognized molecular defect.9

Herein, we describe a sporadic adult patient with an unusual clinical history characterized by PEO, hearing loss, macrocytic anemia, hypogonadism, central vision loss, and mild ataxia. Skeletal muscle histochemistry revealed variation in fiber size because of the presence of atrophic fibers. There were no ragged-red fibers, but several fibers had no cytochrome-c oxidase activity. Because we detected multiple mtDNA deletions in muscle, we looked for a mutation in ANTI, PEO1, and POLG1 (the gene encoding the catalytic subunit of poly(H9253) but found none. Genetic testing for Leber hereditary optic neuropathy excluded the typical mtDNA mutations. The recent report10 of a similar patient harboring a single mutation in the gene encoding the accessory subunit (p55) of poly(POLG2) prompted us to sequence this gene: we identified a different heterozygous mutation, which, however, did not significantly impair enzyme function. We, therefore, sequenced OPA1, which encodes a dynamin-related GTPase, and identified a novel heterozygous missense mutation that better explains this patient’s features.

**METHODS**

**PATIENT REPORT**

This 42-year-old man underwent successful cochlear implantation at the age of 31 years after a 3-year history of progressive hearing loss. At that time, he also had macrocytic anemia and hypogonadism, requiring hormonal therapy. A bone marrow biopsy specimen showed mild hypocellularity (20%-50%), with megaloblastic erythropoiesis and increased iron store. Blood levels of vitamin B12 and folate were normal. At the age of 37 years, he complained of sudden nonprogressive and painless loss of central vision. There was no family history of vision loss.

An ophthalmologic examination at the age of 37 years showed that his visual acuity with correction was 20/100 OD and 20/160 OS. On confrontation, a visual fields test confirmed the presence of central scotomata in both eyes, with a small area of preserved vision in the center of the scotoma. His pupils were normal, with no afferent pupillary defects. His intraocular pressure was normal bilaterally. Ocular motility was impaired, with a mild restriction in all directions of gaze. There was no nystagmus. Funduscopy showed no pallor or edema of the optic nerve and no obvious pigmentary abnormality. Optical coherence tomography indicated loss of nerve fiber layer in both optic nerves, especially in the maculopapillary bundle, which subserves central vision. An electroretinogram showed that electrical...
responses were just below normal for the dark-adapted bright flash stimulus and normal for other stimuli. The results of multifocal electrotoretinography were normal. On neurologic examination, he showed mild ataxia and tremor. An electrocardiogram and rhythm strip were normal, with no conduction block.

A complete blood cell count confirmed significant macrocytic anemia. A subsequent bone marrow smear analysis revealed that erythroid precursors were decreased (13%) and showed megaloblastic changes. Granulopoiesis showed a full range of maturation. Lactic and pyruvic acid levels in serum were normal. Magnetic resonance imaging was not performed because of cochlear implants, but the result of imaging performed at the age of 30 years was normal. A muscle biopsy specimen from the left gluteus maximus was devoid of cytochrome-c oxidase activity (Figure 1). The amount of neutral lipid seemed normal.

Genetic testing for Leber hereditary optic neuropathy excluded mutations at nucleotide positions 3460, 11778, and 14484 in mtDNA. These mutations account for 90% of Leber hereditary optic neuropathy cases.

BIOCHEMICAL AND MOLECULAR ANALYSES

Respiratory chain enzymes in muscle biopsy specimens were assayed by direct sequencing. Total DNA from the patient’s muscle was extracted using standard protocols. Southern blot analysis was performed by standard techniques. The ANT1, Twinkle, and POLG1 genes were screened by direct sequencing, as described.

To amplify the coding region of human POLG2, we designed primers from intronic sequences flanking the exons, as described in Table 1. Polymerase chain reaction amplification was performed in a volume of 20 µL, containing 50 ng of genomic DNA, 12.5 pmol of each of the primers, 200 µmol of each deoxyribonucleotide triphosphate, and 0.5 U of Taq polymerase. Cycling conditions are given in Table 1. DNA sequencing of the new plasmid was determined by monitoring extension of a 5’-end–labeled 35-mer hybridized to M13mp18 DNA, as previously described. Reaction products at low and high salt concentrations were resolved by denaturing polyacrylamide gel electrophoresis and were visualized with an imager (Typhoon 9400 Phosphorimager; Molecular Dynamics, Sunnyvale, California) and National Institutes of Health software (Image 1.63).

The protection by the inhibitor N-ethylmaleimide (NEM) was measured on the reconstituted 2-subunit form of pol

<table>
<thead>
<tr>
<th>Enzyme (Complex)</th>
<th>Controls, Mean (SD)</th>
<th>Muscle Extracts of the Patient (% of Controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH-cytochrome-c reductase (I plus III)</td>
<td>103 (38)</td>
<td>73 (71)</td>
</tr>
<tr>
<td>Succinate-cytochrome-c reductase (II plus III)</td>
<td>71 (20)</td>
<td>70 (99)</td>
</tr>
<tr>
<td>Cytochrome-c oxidase (IV)</td>
<td>283 (50)</td>
<td>238 (84)</td>
</tr>
<tr>
<td>Succinate dehydrogenase (II)</td>
<td>101 (31)</td>
<td>107 (108)</td>
</tr>
</tbody>
</table>

Abbreviations: CS, citrate synthase; NADH, nicotinamide adenine dinucleotide.

DNA POLYMERASE ASSAYS

The processivity of pol was determined by monitoring extension of a 5’-end–labeled 35-mer hybridized to M13mp18 DNA, as previously described. Reaction products at low and high sodium chloride conditions were resolved by denaturing polyacrylamide gel electrophoresis and were visualized with an imager (Typhoon 9400 Phosphorimager; Molecular Dynamics, Sunnyvale, California) and National Institutes of Health software (Image 1.63).

The protection by the inhibitor N-ethylmaleimide (NEM) was measured on the reconstituted 2-subunit form of pol at 75-mmol/L sodium chloride, as described.

BIOCHEMICAL AND MOLECULAR ANALYSIS

Using pET-p55CHIS as the template and the mutagenic primer 5’-ATTCTGGTGGCCGCTATTATGGAAACT-3’ and its complement (Oligos Etc, Wilsonville, Oregon) using a kit (QuikChange Site-Directed Mutagenesis kit; Stratagene, Loyola, California). DNA sequencing of the new plasmid pET-G416A p55CHIS confirmed the G416A substitution. The G416A was grown, expressed, purified, and stored, as described.

PROTEIN PREPARATION

The catalytic and accessory subunits of human poly were overexpressed and purified, as described. The G416A derivative of p55 was constructed by site-directed mutagenesis, using pET-p55CHIS as the template and the mutagenic primer 5’-ATTCTGGTGGCCGCTATTATGGAAACT-3’ and its complement (Oligos Etc, Wilsonville, Oregon) using a kit (QuikChange Site-Directed Mutagenesis kit; Stratagene, Loyola, California). DNA sequencing of the new plasmid pET-G416A p55CHIS confirmed the G416A substitution. The G416A was grown, expressed, purified, and stored, as described.

BIOCHEMICAL AND MOLECULAR ANALYSIS

Southern blot of muscle mtDNA revealed multiple bands in addition to the 16.5-kilobase band of normal mtDNA (data...
not shown). The activities of respiratory chain enzymes were essentially normal (Table 2). The patient did not harbor mutations in the ANT1, Twinkle, or POLG1 genes.

Analysis of the POLG2 gene showed a heterozygous G→C transversion at nucleotide 1247 in exon 7, which changes a highly conserved glycine at codon 416 to alanine (Figure 2). The G1247C mutation eliminates an ScrFI restriction site detectable by polymerase chain reaction restriction length fragment polymorphism. This polymorphism was not detected in more than 100 control subjects. Sequencing of OPA1 revealed a novel 1741 A→G transition that causes a Tyr582Cys amino acid substitution.

**BIOCHEMICAL ANALYSIS OF THE G416A MUTANT p55 PROTEIN**

We tested whether the phenotypic changes caused by the G416A substitution would also result in measurable biochemical defects. The wild-type p55 (WT-p55) and the G416A p55, both lacking the mitochondrial targeting sequence, were overproduced in Escherichia coli and purified to apparent homogeneity, as described in the “Protein Preparation” subsection of the “Methods” section, and their in vitro biochemical properties were compared. Figure 3 shows a Coomassie-stained gel of the purification profile for the mutant G416A protein. The G416A mutant protein eluted from the MonoS column as a single peak at the identical position as the WT-p55 protein. The mutant G416A-p55 had identical chromatographic properties as the WT-p55, indicative of correct folding. The peak fraction was chosen for further analysis.

**MUTANT G416A PROTECTS THE CATALYTIC SUBUNIT FROM NEM SIMILAR TO WT-p55**

With the purified protein, we assayed the ability of the G416A-p55 to protect the p140 catalytic subunit of poly from inactivation by NEM (Figure 4), because it was previously documented that the isolated catalytic p140 subunit is sensitive to inactivation by NEM.14 N-ethylmaleimide covalently binds to solvent-assessable sulfhydryl groups (cysteine side chains) and sterically interferes with activity. However, the WT-p55 accessory subunit protects the catalytic subunit from NEM, indicating a physical shielding of any critical cysteine residues by the bound p55. Thus, this assay measures the extent of p55 binding to the p140 subunit.

Figure 4 shows that the profile of the NEM curve with G416A-p55 was similar to that with WT-p55, indicating that mutant and wild-type proteins bind to the catalytic subunit in a similar manner. In fact, the WT-p55 stimulated the activity of the catalytic subunit by 2-fold, whereas the G416A-p55 stimulated the p140 activity by 2.5-fold. The comparable ability of mutant and WT-p55 proteins to protect the catalytic subunit indicates that there is a normal physical interaction between the mutant accessory subunit and the catalytic subunit of poly.

**PROCESSIVITY**

The role of the p55 accessory subunit is to increase processivity (ie, the number of nucleotides synthesized on
a DNA template per binding event). Wild-type p55 increases the processivity of the isolated catalytic subunit from approximately 100 nucleotides to several thousand nucleotides, suggesting that the holoenzyme can synthesize almost the whole mitochondrial genome in a single binding event. The high processivity conferred by the p55 subunit to the catalytic subunit is caused by increased binding affinity of the complex to DNA and by enhanced salt tolerance. To assess the effect of the mutant G416A protein, we extended a phosphorus-32 end-labeled oligonucleotide annealed to M13 single-stranded DNA in the presence and absence of a DNA trap. The sequencing gel in Figure 5 depicts the processivity of the p140 catalytic subunit in the presence or absence of the p55 subunit (mutant or wild type). Lanes 1 through 6 show the products of reactions performed in the presence of a DNA trap and reflect true processivity, whereas lanes 7 through 12 show reaction products obtained without a DNA trap and represent multiple binding events. Odd-numbered lane reactions were performed at a low salt concentration, while even-numbered lane reactions were performed at a high salt concentration (150-mmol/L sodium chloride). Comparing lanes 3 and 4 with lanes 1 and 2 demonstrates the degree of stimulation and the length of products in the presence of the WT-p55 subunit. Comparing lanes 4 and 5 shows that the stimulation by G416A-p55 was similar to, if not better than, that by WT-p55, indicating that the mutation did not impair the stimulation of processivity.

**COMMENT**

Multiple deleted molecules of mtDNA accumulate in postmitotic tissues in a group of mitochondrial disorders because of mutations in nuclear genes controlling mtDNA synthesis and maintenance. The clinical hallmark of these disorders is PEO, usually associated with a variety of other symptoms.

POLG1 is the nuclear master gene of mtDNA replication and repair. It encodes poly, the only DNA polymerase found in animal cell mitochondria and, thus, bears sole responsibility for DNA synthesis in all replication and repair transactions involving mtDNA. Poly is synthesized as a longer precursor containing an amino-terminal leader sequence that targets the protein to mitochondria and is then cleaved off. Mature poly is a 140-kDa polypeptide composed of 2 functionally different domains, a polymerase domain encompassing the C-terminus and an exonuclease domain with proofreading activity, located on the N-terminus. In addition, poly has a 5'-deoxyribosephosphatase lyase activity that can remove a 5'-deoxyribosephosphate group during base excision repair. In human mitochondria, poly is part of an enzyme complex containing an accessory subunit of 55 kDa, p55. This subunit forms a dimer in solution, which binds tightly to the poly monomer to form a heterotrimer with the structure AB2. The accessory subunit p55 stabilizes binding to primer template DNA and stimulates the catalytic activity of poly under physiological conditions. It seems that p55 does not increase the polymerization rate but enhances the base excision repair function of poly by stimulating 2 subreactions in the repair process.

The tertiary structure of poly B can be divided into 3 domains (Figure 6). Domain 1 contains a 7-stranded...
β sheet, which is antiparallel, except for strand 1, which is located at one edge of the sheet. One face of the sheet is covered with helices C and G. The other face of the twisted sheet has a pocket formed by helices F and H. Domain 2 is inserted between strand 1 and helix F of domain 1 and contains secondary structure elements that interact with their symmetry counterparts in the dimer. Domain 3 contains a 5-stranded mixed β sheet that is sandwiched between helices J and M on one side and a β hairpin and helix K on the other side.20,21

Recently, a mutation was identified in a 60-year-old woman with apparent autosomal dominant PEO starting in her 40s, multiple mtDNA deletions in muscle, and cytochrome-c oxidase–deficient muscle fibers.10 This mutation changed a conserved Gly451 to glutamic acid near the C-terminus of domain 3 on the surface of the protein between helix L and β-sheet 19. Biochemical analysis of the recombinant protein demonstrated a dramatic loss of processivity, decreased NEM protection, and loss of interaction with the catalytic subunit. Thus, this domain provides the subunit interaction with the poly-catalytic subunit, enhancing DNA binding and processivity of the poly complex.

Our patient was younger but also had PEO, multiple mtDNA deletions in muscle, and cytochrome-c oxidase-negative fibers. He seemed to be a sporadic case. The mutation in this patient changes a glycine to an alanine at position 416. The highly conserved glycine residue is at the end of a short helix before a loop structure in domain 3 of the p55 monomer. This domain is not involved in dimerization of the second accessory subunit, but may play an important role in the structure of this domain, thereby affecting the interaction with the catalytic subunit. Biochemical analysis of the recombinant G416A-p55 protein showed that it protects the poly-catalytic subunit from NEM and can stimulate processive DNA synthesis, implying that protein-protein interaction with the catalytic subunit is not impaired. Recently, the National Center for Biotechnology Information reported this same G1247C transversion in exon 8 of the POLG2 gene in 1 of 8 sequences, but no frequency was reported for this mutation, which cannot be a frequent polymorphism because it was not found in more than 100 controls. Because the pathogenicity of the POLG2 mutation appears dubious, while this article was in press we also sequenced the gene encoding OPA1, a dynamin-related GTPase involved in mitochondrial fusion. This choice was prompted by the recently described association of mutations in OPA1 with optic atrophy, PEO, hearing loss, multiple mtDNA deletions and COX-negative fibers in muscle, all features present in our patient.22,23 We identified a heterozygous missense mutation (Y582C) that

![Figure 6. Position of the G416A side chain on the ribbon structure of the human accessory subunit in one monomer within the p55 dimer. The ribbon drawing was generated with Swiss PDB viewer from Protein Data Bank file 2G4C. The inset is an expanded view of the structure around G416 in domain 3.](http://archneur.jamanetwork.com/pdfaccess.ashx?url=/data/journals/neur/7497/ on 06/18/2017)
changes a highly conserved tyrosine to a cysteine. Although not yet fully characterized, this mutation is more likely than the POLG2 mutation to explain the clinical and laboratory features of this patient.

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REFERENCES