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ORIGINAL COMMUNICATION

A new *POLG1* mutation with peo and severe axonal and demyelinating sensory-motor neuropathy

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Abstract *Background* Progressive external ophthalmoplegia (PEO) is a mitochondrial disorder associated with defective enzymatic activities of oxidative phosphorylation (OXPHOS), depletion of mitochondrial DNA (mtDNA) and/or accumulation of mtDNA mutations and deletions. Recent positional cloning studies have linked the disease to four different chromosomal loci. Mutations in *POLG1* are a frequent cause of this disorder. *Methods* We describe two first-cousins: the propositus presented with PEO, mitochondrial myopathy and neuropathy, whereas his cousin showed a Charcot-Marie-Tooth phenotype. Neurophysiological studies, peroneal muscle and sural nerve biopsies, and molecular studies of mtDNA maintenance genes (*ANT1, Twinkle, POLG1, TP*) and non dominant CMT-related genes (*GDAP1, LMNA, GJB1*) were performed. *Results* A severe axonal degeneration was found in both patients whereas hypomyelination was observed only in the patient with PEO whose muscle biopsy specimen also showed defective OXPHOS and multiple mtDNA deletions. While no pathogenetic mutations in *GDAP1*, *LMNA*, and *GJB1* were found, we identified a novel homozygous *POLG1* mutation (G763R) in the PEO patient. The mutation was heterozygous in his healthy relatives and in his affected cousin. *Conclusions* A homozygous *POLG1* mutation might explain PEO with mitochondrial abnormalities in skeletal muscle in our propositus, and it might have aggravated his axonal and hypomyelinating sensory-motor neuropathy. Most likely, his cousin had an axonal polyneuropathy with CMT phenotype of still unknown etiology.

Key words POLG1 · PEO · neuropathy · mitochondrial disorders

Introduction

Progressive external ophthalmoplegia (PEO) is a mitochondrial disorder associated with depletion of mitochondrial DNA (mtDNA) and/or accumulation of mtDNA mutations and deletions. In most cases PEO is transmitted as an autosomal dominant trait (adPEO) but mutations are also described in autosomal recessive (arPEO) sib-ship and in singleton, apparently sporadic

cases [3]. Recent positional cloning studies have linked the disease to four different chromosomal loci: a gene at locus 4q34–35 encodes adenine nucleotide translocator 1 (*ANT1*), chromosome 10q24 harbors a mitochondrial helicase (*C10ORF2*, Twinkle gene), a PEO3 locus on chromosome 15q22–26 encodes the catalytic subunit of mitochondrial DNA polymerase (*POLG1*) and a fourth locus at chromosome 22q13.32–qter, corresponding to the thymidine phosphorylase (*TP*) gene, is associated with mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) [1, 8].

Mutations in *POLG1* are a common cause of PEO in both familial and sporadic cases [1, 9, 16]. However, *POLG1* is also a significant cause of additional inherited degenerative phenotypes, including sensory ataxic neuropathy, dysarthria, myoclonus, seizures, parkinsonism, premature aging, and male infertility [10]. Recently reported clinical outcomes of *POLG1* mutations are also Alpers' syndrome [5, 13] characterized by progressive neurological disorder and liver failure, and mitochondrial recessive ataxia syndrome (MIRAS), a common form of recessive ataxia in the European population [7].

We here describe two related patients: the propositus, homozygous for a *POLG1* mutation, presented with PEO, mitochondrial myopathy and neuropathy, whereas his first-cousin, heterozygous for the same mutation, showed a typical Charcot-Marie-Tooth phenotype.

Patients and methods

Clinical features

The propositus (III.01, Fig. 1) was a 37-year-old man born to consanguineous, clinically and neurophysiologically healthy parents (II.01 and II.02). His 8-year-old son is also normal. Patient III.01 did well until he was age 20, when he presented with progressive weakness and wasting in both proximal and distal limb muscles, bilateral ptosis, opthalmoparesis with residual minimal lateral movements, dysphonia, and dysphagia. Gastrointestinal dysmotility was not referred and brain magnetic resonance image (MRI) was normal. Neurological examination at the age of 37 years showed gait possible only with support and bilateral "steppage", mild proximal weakness in all limbs, and weak facial and neck flexor muscles. The patient also had absent deep tendon reflexes in the lower limbs, bilateral pes cavus, reduced ankle vibration sense, and stocking hypoesthesia. III.02 is a maternal cousin to III.01 (Fig. 1). The patient did well until age 20 when he had difficulty in walking and progressive distal weakness and wasting in the lower limbs. At age 27 years, the patient showed bilateral "steppage", muscles weakness and wasting which was more severe in distal muscle, pes cavus and absent deep tendon reflexes in the lower limbs. Moreover, dysphonia, reduced vibration sense at the ankles, and stocking hypoesthesia were present. There was no involvement of extraocular muscles. Subjects II.03 and II.04 are neurologically healthy. They denied formal consanguinity, though they bear the same surname and come from the same small village in Molise, a region in central Italy.

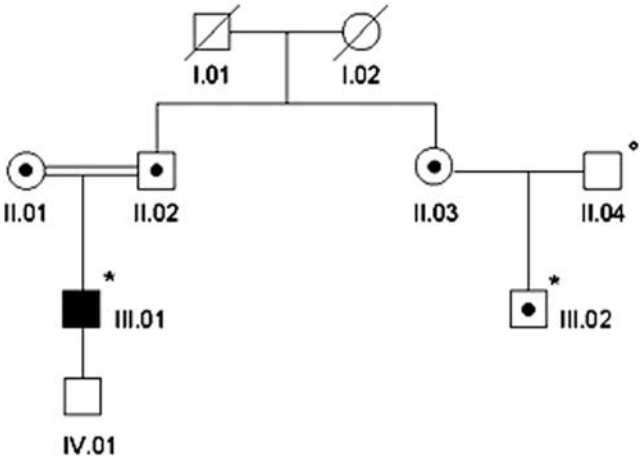

Fig. 1 Pedigree of family. Squares represent male subjects; circles, females; slashed symbols, deceased subjects; solid black square, the subject bearing the homozygous *POLG1* mutation; centred symbols, individuals harboring a heterozygous *POLG1* mutation; ° = wild-type alleles only; * = CMT phenotype subjects

Electrophysiological Studies

A battery of neurophysiological tests was performed, including needle electromyography (EMG) in selected muscles (i.e., biceps brachialis, triceps brachialis, rectus femori, tibialis anterior and abductor digiti minimi muscles), antidromic sensory (sural and median nerves) and orthodromic motor (median, ulnar, tibial nerves) nerve conduction velocities (NCVs).

Muscle and Nerve Biopsy Studies

Sural nerve and peroneal muscle biopsies were performed under local anaesthesia. Tissues were prepared for light and electron microscopic examination. Ten mm-thick frozen sections of peroneal muscle specimens were stained with hematoxylin-eosin (HE), modified Gomori's trichrome stain (GT), adenosine triphosphatase (pH 9.4, 4.3 and 4.6) (ATPase), NADH-tetrazolium reductase, succinate dehydrogenase (SDH), cytochrome c oxidase (COX), combined COX-SDH stain, periodic acid-Schiff, Sudan black, alkaline phosphatase and non specific esterase using standard techniques.

Biochemical analyses

Spectrophotometric analysis of the activities of respiratory chain enzymes in muscle was performed according to standard procedures. Values were referred to the activity of citrate synthase (CS), an index of total mitochondrial mass.

Molecular Genetic analyses

Genomic DNA extraction from tissues, Southern blotting, investigation of mtDNA rearrangements by means of a "long-template" amplification, and mapping of deletions employing a PCR "primer shifting" strategy adopted methods already reported by us [15]. PCR-based direct sequencing of the *ANT1*, *C10ORF2*, *POLG1*, *TP*, *LMNA*, *GDAP1*, and *GJB1* genes used intronic primers (sequences available on request). PCR products were sequenced in both strands using a 3100 Avant Genetic Analyser (PE Applied Biosystems, Foster City, CA) according to ABI Prism Dye Terminator chemistry. Available family members were also genotyped with microsatellite markers surround-

ing the CMT4A locus on chromosome 8q21 and the PEO3 locus on chromosome 15q22–26.

Results

Electrophysiological studies

Patient III.01. In all the explored muscles EMG examination showed a moderate reduction of motor units at maximal effort with marked reduction of amplitude. Mean duration of motor unit action potentials (MUAPs) was reduced. Compound muscle action potentials (CMAPs) were not recordable in the abductor hallucis by stimulation of tibial nerve. CMAP amplitude and motor NCVs were markedly reduced in the median and ulnar nerves. Nerve sensory action potentials (SAPs) were not recordable in all the explored nerves. Nerve conduction study was consistent with a mixed axonal and demyelinating sensory-motor polyneuropathy.

Patient III.02. EMG examination showed signs of chronic denervation in lower limb muscles. CMAP amplitude was severely reduced in the tibial nerve, markedly reduced in the median nerve, and normal in the ulnar nerve. NCVs were markedly reduced in lower limbs and normal or slightly reduced in the median and ulnar nerves respectively. SAPs were not recordable in all the explored nerves (Table 1). According to the electrophysiological data the neuropathy was defined as axonal.

Muscle biopsy

Patient III.01. Both atrophic and hypertrophic fibers were observed by light microscopy. Atrophic fibers were of different shape, either angulated or circular. GT stain showed several ragged-red fibers (RRF) (Fig. 2A). RRF also presented increased subsarcolemmal SDH reactivity. There was type I fiber prevalence, with some type II grouping. Many fibers (about 20 %) were COX negative (Fig. 2B). Some fibers showed small lipid droplets and increased amount of glycogen.

Patient III.02. Light microscopy examination showed type-grouping, signs of denervation and central nuclei (Figs. 2C, D). No signs of mitochondrial abnormalities were observed.

Nerve biopsy

Patient III.01. Light microscopy examination of transverse sections showed a severe loss of myelinated fibers (Fig. 3A). The residual fibers were of small and intermediate diameter with disproportionately thin myelin sheaths in relation to the axon caliber (Fig. 3B). No "onion bulbs" were present. Some clusters of regenerating axons were also observed. The perineurium, endoneurium and blood vessels were normal. Electron microscopy showed axonal degeneration and several fibers with signs of hypomyelination. There were no abnormal mitochondria in Schwann cells.

Patient III.02. Light and electron microscopy examination revealed a severe loss of myelinated fibers. The residual fibers, of small and intermediate diameter, showed normal myelin sheaths in relation to the axon calibers. No "onion bulbs" were present. Some clusters of regenerating axons were also observed (Figs. 3C, D).

Biochemical analyses

After the values were corrected for the activity of CS, complexes I, III and IV were partially reduced in muscle biopsy from patient III.01 as compared to normal controls. Conversely, respiratory chain enzyme activities were normal in patient III.02 (Table 2).

Molecular genetic analyses

Southern blotting in skeletal muscle from patient III.01 disclosed multiple mtDNA deletions, accounting for about 38 % of total mtDNA. Using a sensitive PCR strategy, we identified several rearranged mtDNA species,

Table 1 Motor and Sensory Conduction Studies

Patient	Median					Ulnar			Tibial			Sural	
	MCV (m/s) (range)	DML (ms) (range)	CMAP (mV) (range)	SCV (m/s) (range)	SAP (μV) (range)	MCV (m/s) (range)	DML (ms) (range)	CMAP (mV) (range)	MCV (m/s) (range)	DML (ms) (range)	CMAP (mV) (range)	SCV (m/s) (range)	SAP (μV) (range)
III.01	**31** (54–73)	**4.5** (2.5–3.9)	**1.1** (8–43)	***NV***	***NR***	**33** (61–74)	**4** (2–3.1)	**2.5** (8–43)	***NV***	***NV***	***NR***	***NV***	***NR***
III.02	57 (55–74)	**4.5** (2.4–3.8)	**3.7** (9–46)	***NV***	***NR***	**50** (61–74)	**3.9** (2–3)	25 (8–44)	**34** (45–60)	**8.2** (3–4.8)	**0.5** (6–32)	***NV***	***NR***

MCV, SCV motor, sensory conduction velocity; *DML* distal motor latency; *CMAP* amplitude of compound muscle potential; *SAP* amplitude of sensory action potential; *NV* not valuable; *NR* not recordable

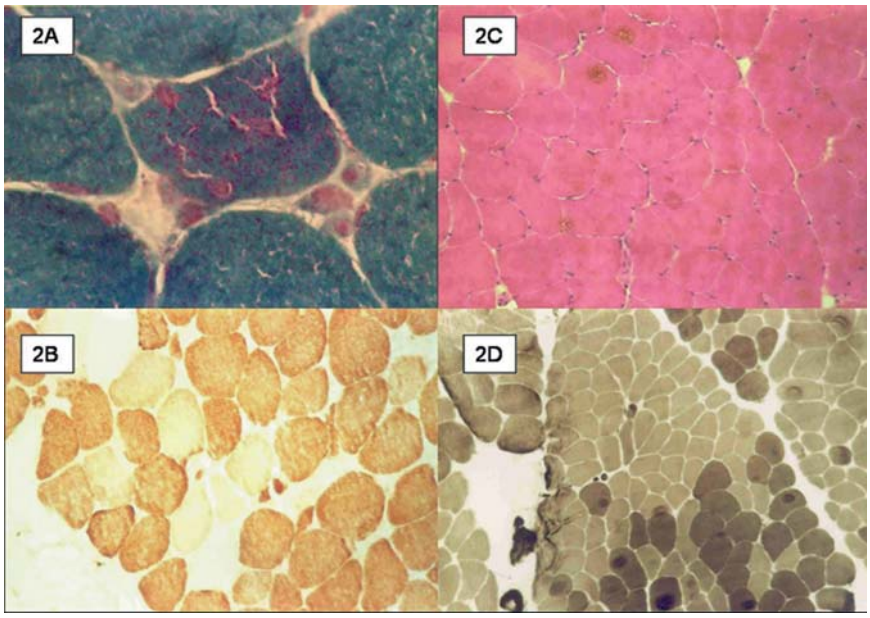

Fig. 2 Light microscopy examination of 10-µm skeletal muscle transverse sections. A (Pt. III.01) Gomori's modified Trichrome stain showing RRFs (40x). **B** (Pt. III.01) COX reaction showing the presence of COX-negative fibers (20x). **C** (Pt. III.02) HE stain showing moderate variability of fiber size with both round and angular hypotrophic fibers and increase in central nuclei. **D** (Pt. III.02) ATPase 9.4 reaction showing fiber type II-grouping

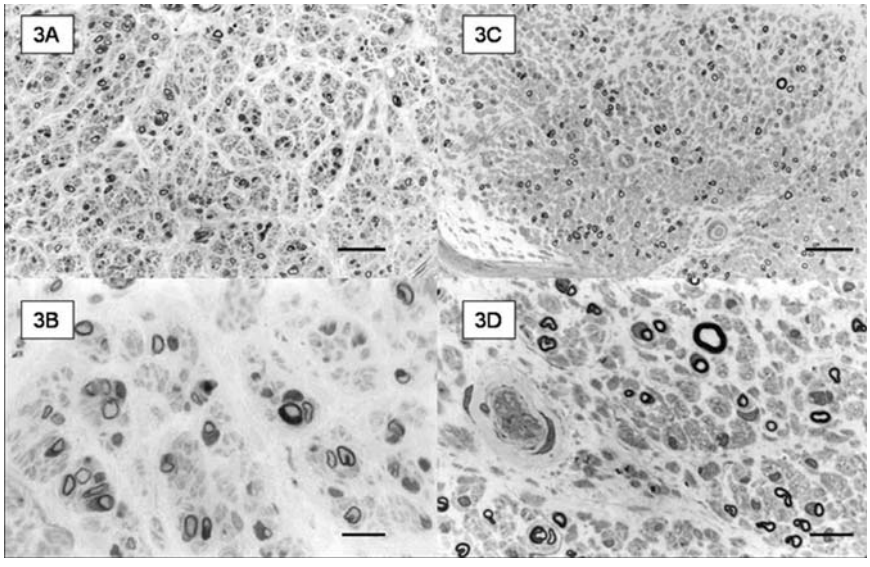

Fig. 3 Light microscopy examination of transverse epoxy one-µm sections of sural nerve stained with toluidine blue. A (Pt. III.01) severe loss of myelinated fibers with some clusters of regenerating axons (X 110; bar: 100 µm). **B** (Pt. III 0.01) residual fibers of small and intermediate diameter with disproportionately thin myelin sheaths in relation to the axon caliber (X 440; bar: 20 µm). **C** (Pt. III.02) severe loss of myelinated fibers (X 110; bar: 100 µm). **D** (Pt. III.02) residual fibers of small and intermediate diameter with normal myelin sheaths in relation to the axon calibres (X 440; bar: 20 µm)

Table 2 Respiratory chain enzyme complexes in skeletal muscle biopsy

	Complex I/CS	Complex II/CS	SDH/CS	Complex III/CS	Complex IV/CS
III.01	0.13	0.05	0.16	0.13	1.35
III.02	0.60	0.09	0.14	0.25	2.06
Controls (n = 50)	0.37 ± 0.11 (0.15–0.46)	0.09 ± 0.02 (0.05–0.13)	0.19 ± 0.03 (0.14–0.23)	0.31 ± 0.09 (0.19–0.54)	2.80 ± 0.70 (1.86–4.09)

Values are expressed as nmol/min/mg protein and normalized to the activity of citrate synthase (CS), a matrix enzyme used as a good index of total mitochondrial mass

deletions ranging in size from 4.0 to 7.4 kb and mostly located in the major arc between the two replication origins. Using the "long-template" PCR method, we did not find evidence for duplicated molecules. *POLG1* sequencing identified a novel, homozygous c.2287G > C mutation which replaces glycine 763 for arginine (G763R). Mutation nomenclature follows the format indicated in << www.hgvs.org/mutnomen >> and refers to the cDNA sequence (NT_ 010274). The mutation lies in exon14 and it was homozygous in patient III.01 and heterozygous in subjects II.01, II.02, and II.03, and in patient III.02 whereas it was absent in subject II.04. Although not tested functionally, indirect arguments in favor of a disease-causing character of the novel variant were the following: i) the mutation affects a highly conserved residue; ii) it was functionally related to a mitochondrial disorder in the patient but not in his clinically healthy parents; iii) the mutation was not found in 500 ethnically-matched control chromosomes, though it is present in two different EST tumoral clones (http://image.llnl.gov). Haplotype reconstruction did not support involvement of the CMT4A region on chromosome 8q21. In addition, complete sequencing of *GDAP1* excluded pathogenic mutations in patients III.01 and III.02. Genetic analysis did not show any mutation in *ANT1*, *C10ORF2*, *TP*, and *GJB1* genes and any variation either at the heterozygous or homozygous state in *LMNA* gene.

Discussion

We have identified a new homozygous *POLG1* mutation (G763R) in a PEO patient in whom clinical, morphological, biochemical, and molecular genetic analyses pointed to a mitochondrial disorder. We did not find this specific amino acid change in a large number of Italian control chromosomes. According to canonical criteria, this might point to a causative mutation, though low frequency in ethnically-matched controls cannot be completely ruled out. In fact, this has already been observed for the A467T mutation which is the commonest *POLG1* alteration in Belgian AR-PEO patients but it is also detected at lower frequency in control alleles (allele T frequency 0.6%) [16]. Other genes involved in mtDNA maintenance (*ANT1*, *Twinkle*, *TP*) have been also ruled out by genetic analysis. Moreover, the MNGIE syndrome has been excluded by absence of gastrointestinal dysmotility and brain MRI abnormalities in the propositus.

Under the assumption of *POLG1* mutation pathogenicity, the severe axonal and hypomyelinating sensory-motor neuropathy still remains a debatable point. In fact, although peripheral neuropathy is a described clinical aspect in dominant or recessive PEO kindred bearing *POLG1* mutations, it is reported as purely axonal without features of hypomyelination [6, 9, 11, 17]. Nevertheless, Ferrari and colleagues [5] have recently described an infant bearing *POLG1* mutations in association with hypotonia, liver failure and hypomyelinating peripheral neuropathy.

The clinical and electrophysiological investigations of our patient' relatives disclosed a cousin who had an axonal neuropathy with a CMT phenotype. This patient (III.02), as his healthy relatives, harbored a heterozygous G763R mutation in *POLG1* and did not show any evidences for an OXPHOS-related disorder. An explanation of these data appears to be the unusual and unfortunate occurrence in patient III.01 of two genetic diseases, PEO and CMT. Consistent with this hypothesis is the geographical origin of the family: parental couples (subjects II.01 and II.02, and subjects II.03 and II.04) shared a common *POLG1* haplotype which appears to favor the hypothesis of common ancestry. Moreover, the patients originate from a region in central Italy with a high rate of consanguinity and a high incidence of neurological defects of the peripheral nervous system [12]. Pursuing this hypothesis, we have screened for known genes associated with axonal, non dominant CMT but failed to detect mutations in *LMNA* and *GJB1* genes [2, 4]. Moreover, we also ruled out alterations in *GDAP1*, which is known to be associated with both demyelinating and axonal AR-CMT [14].

In conclusion, the simplest explanation of our data is the unusual, coincidental occurrence in our propositus of two overlapping genetic distinct diseases: *POLG1* might explain the PEO phenotype and its related mitochondrial abnormalities in skeletal muscle, and might act as an aggravating factor in modulating the expression of the CMT phenotype. Another still unidentified AR-CMT gene might well be invoked in this family.

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