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ORIGINAL COMMUNICATION

Novel Twinkle (*PEO1*) gene mutations in mendelian progressive external ophthalmoplegia

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Abstract Multiple deletions of mitochondrial DNA (mtDNA) are associated with different mitochondrial disorders inherited as autosomal dominant and recessive traits. Causative mutations have been found in five genes, mainly involved in mtDNA replication and stability. They include *POLG1*, the gene encoding the catalytic subunit of mtDNA polymerase (polγ), *POLG2* encoding its accessory subunit, *ANT1* coding the adenine nucleotide translocator and *PEO1* which codes for Twinkle, the mitochondrial helicase. Finally *OPA1* missense mutations are involved in phenotypes presenting optic atrophy as a major feature.

To define the relative contribution of *POLG1*, *POLG2*, *ANT1* and *PEO1* genes to the mtDNA multiple deletion syndromes, we analysed them in a cohort of 67 probands showing accumulation of multiple mtDNA deletions in muscle. The patients were predominantly affected with a mitochondrial myopathy with or without progressive external ophthalmoplegia (PEO). Genetic analysis revealed that 1) *PEO1* has a major role in determining familial PEO, since it accounts for 26.8 % of familial cases, followed by *ANT1* (14.6 %) and *POLG1* (9.8 %); 2) no mutations in any of the known genes were found in 53.7 % of probands of this series. Six novel missense mutations contributing to the mutational load of *PEO1* gene (p.R334P, p.W315S, p.S426N, p.W474S, p.F478I, p.E479K) were associated with an adult onset PEO phenotype.

Key words mtDNA deletions · progressive external ophthalmoplegia

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Introduction

The Mendelian forms of progressive external ophthalmoplegia (PEO) are clinically and genetically heterogeneous disorders characterized by the accumulation of multiple deletions and point mutations of mitochondrial DNA (mtDNA) in postmitotic tissues. Most of the autosomal dominant PEO (adPEO) families carry heterozygous mutations in either one of five genes. They include *POLG1* that encodes the catalytic subunit of DNA polymerase γ (pol γ) [1], *POLG2* encoding the p55 accessory polγ subunit [2], *ANT1* coding for the mitochondrial adenine nucleotide translocator [3], and *PEO1* (formerly *C10ORF2*) which codes for Twinkle, a mitochondrial protein with structural similarity to the phage T7 primase/helicase [4]. Heterozygous missense mutations in the *OPA1* gene leading to accumulation of mtDNA deletions in muscle tissue have been observed in families with optic atrophy, PEO and other variable signs such as ataxia, deafness and a sensory-motor neuropathy [5–7].

Mutations in *POLG1* can also cause autosomal recessive PEO (arPEO) [8]. In addition, recessive *POLG1* mutations are responsible for different neurological disorders, including sensory-ataxic neuropathy, dysarthria and ophthalmoplegia (SANDO) [9], juvenile spino-cerebellar ataxia-epilepsy syndrome (SCAE) [10], Alpers-Huttenlocher hepatopathic poliodystrophy [11, 12] and levo-dopa responsive parkinsonism with premature ovarian failure [13]. So far, a lower degree of clinical pleiomorphism has been observed in patients with *PEO1* gene mutations, although recessive mutations have been observed in infantile onset spinocerebellar ataxia [14], encephalopathic and hepatocerebral forms of mtDNA depletion syndrome [15, 16], while a heterozygous dominant mutation has been associated with familial parkinsonism and ophthalmoplegia [17].

As far as autosomal dominant or recessive PEO are concerned, *POLG1* is believed to represent the most commonly mutated gene, while mutations in *ANT1* and *PEO1* are relatively rare [18].

Mendelian PEO patients usually present ptosis and ophthalmoparesis due to the accumulation of multiple mitochondrial DNA (mtDNA) deletions in extraocular skeletal muscle. Many patients also develop limb weakness and some have multisystemic involvement including hearing loss and psychiatric abnormalities [19].

To define the relative contribution of the known genes to the etiology of Mendelian PEO, we reviewed the clinical and molecular features of a large sample of mitochondrial myopathy patients with muscle multiple mtDNA deletions, with special reference to inheritance, age of onset and progression, type of tissue and organ involvement. The complete screening of the *POLG1*, *POLG2*, *PEO1* and *ANT1* showed that 1) *PEO1* gene has a major role in determining familial PEO and 2) undetermined familial cases may represent up to the 48.8 %.

Materials and methods

Sample

We first analysed Twinkle and *POLG2* genes in 39 patients, previously resulted negative for mutations in *POLG1* and *ANT1* genes. Then we reviewed clinical and genetic data of an entire series of 82 patients (45 with an autosomal dominant transmission and 37 sporadic), collected at our department over the last 20 years and belonging to 67 independent families and presenting muscle multiple mtDNA deletions.

Gender distribution was as follows: 40 males and 42 females. Mean age at the diagnosis was 54.1 years (range 18–75 yrs) and mean age at disease onset of 43.9 yrs (range 17–75 yrs). All patients underwent a detailed clinical characterization and a muscle biopsy that showed the presence of ragged red fibres and cytochrome c oxidase negative fibres.

Written informed consent was obtained from all subjects or their caregivers at the moment of primary diagnostic procedures, with explicit consent to future uses for research purpose, according to the Declaration of Helsinki. This protocol was approved by our Institutional Review Board.

Molecular studies

Southern Blot analysis and long-range polymerase chain reaction (PCR) of the muscle DNA showed multiple mtDNA deletions. Therefore patients with a low level of mtDNA deletions were not included in this study. The coding sequences and splicing sites of *ANT1*, *PEO1* (*C10ORF2*), *POLG1* and *POLG2* genes were analysed as described [1–4].

All newly identified mutations were confirmed by restriction fragment length polymorphisms (RFLP) analysis on independent PCR products using the following restriction enzymes (New England Biolabs) with appropriate buffer: *Hpy188I* (W313S), *NlaIV* (R334P), *MseI* (E479K), *BsrI* (W474S), *TspRI* (S426N), *Transgenomics Surveyor™ Nuclease* (F478I).

To prove that W474S and E479K are de novo mutations, patient F II-1 and G II-1 and their parents were examined by microsatellite analysis on chromosome X using ABI PRISM Linkage Mapping Sets 2.5 (Applied Biosystems). The following markers were checked: DXS1227, DXS990, DXS987, DXS993, DXS1073, DXS8091, DXS1106, DXS1001, DXS1068, DXS1214, DXS8055, DXS8043, DXS1060, DXS991.

In order to evaluate the possibility of a common allele in patients who presents R303W, we analysed the following intronic SNPs: c.1485–5, c.1485–3, c.1734+16 and the microsatellite markers D10S185, D10S192 and D10S1668.

Results

PEO1 and *POLG2* gene analysis

We identified seven heterozygous missense mutations in the *PEO1* gene in ten independent families. Six of them are novel (c.G1001C leading to p.R334P amino acid substitution, c.G944C resulting in p.W315S, c.G1277A: p.S426N, c.G1421C: p.W474S c.T1432A: p.F478I, c.G1435A: p.E479K) (Fig. 1). A further mutation (R303W) has been previously described, but its pathogenetic role needed confirmation. The R303W mutation was found in four unrelated Italian PEO patients. Evidence of disease segregation with the mutation was present in three pedigrees, one of whom showed several affected members

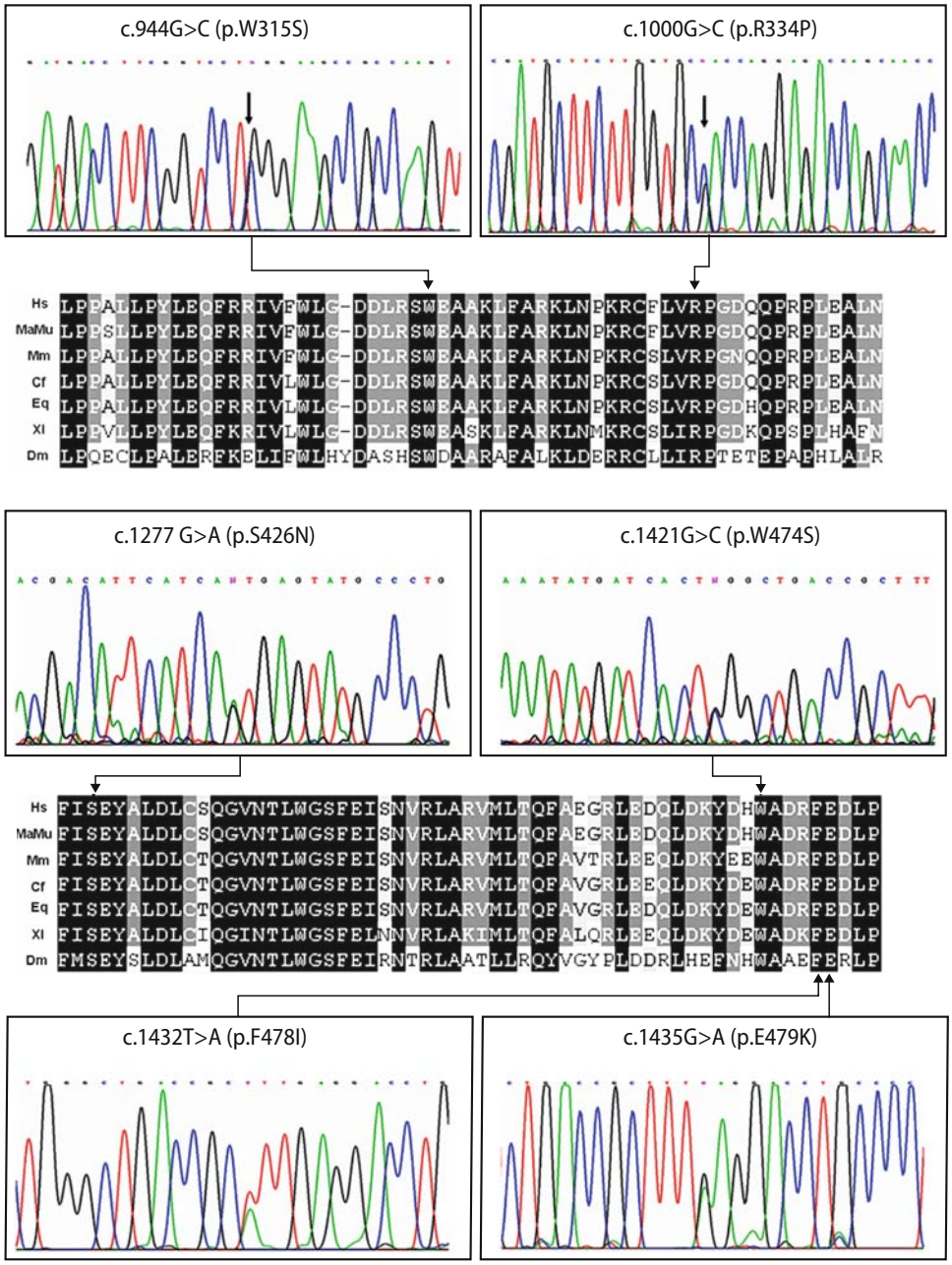

Fig. 1 New *PEO1* gene mutations in PEO probands. Electropherograms of the heterozygous nucleotide sequence changes observed in each proband are shown, in a 5′-3′ order. The corresponding patients are as follows. Proband of family H carried the W315S; patient II-3 of family D carried the R334P mutation; patient III-4 of family E showed the S426N mutation, the sporadic patient III-3 of family F carried the W474S mutation; proband I carried F478I, and the sporadic patient G II-1 presented the de novo E479K mutation. A table of phylogenetic conservation is also shown: *Hs* Homo sapiens: seq. ref. *MaMu* Macaca Mulatta; *Mm* Mus musculus; *Cf* canis familiaris; *Eq* Equus Caballus; *Xl* Xeanopus laevis; *Dm* Drosophila Melanogaster

along four generations (Fig. 2, families A–C). Notably, all patients carrying this mutation share a common haplotype on chromosome 10 from marker D10S185 to D10S1668. Their clinical features are presented in Table 1.

The novel R334P mutation co-segregated with a pure phenotype in a Greek family (Fig. 2, family D). The S426N amino acid change was found in a 66 year-old female proband, affected with severe PEO, upper and lower limb proximal weakness and hypothyroidism; her disease was transmitted as an autosomal dominant trait (Fig. 2, family E, III-4).

The W474S mutation was observed in a 34 year-old woman affected with migraine since her menarche, with PEO by the age of 20 years, premature ovarian failure at the age of 29 years, followed by depression and proximal myopathy (Fig. 2, F, III-3). Her parents were healthy as well as her first and second degree relatives. Molecular analysis showed a wild-type *PEO1* sequence in both parents; paternity was confirmed.

Another "de-novo" E479K mutation was identified in a 19 year-old female PEO patient; indeed, her parents had a normal clinical examination and molecular analysis of *PEO1* gene (Fig. 2, G). Also in this case, paternity

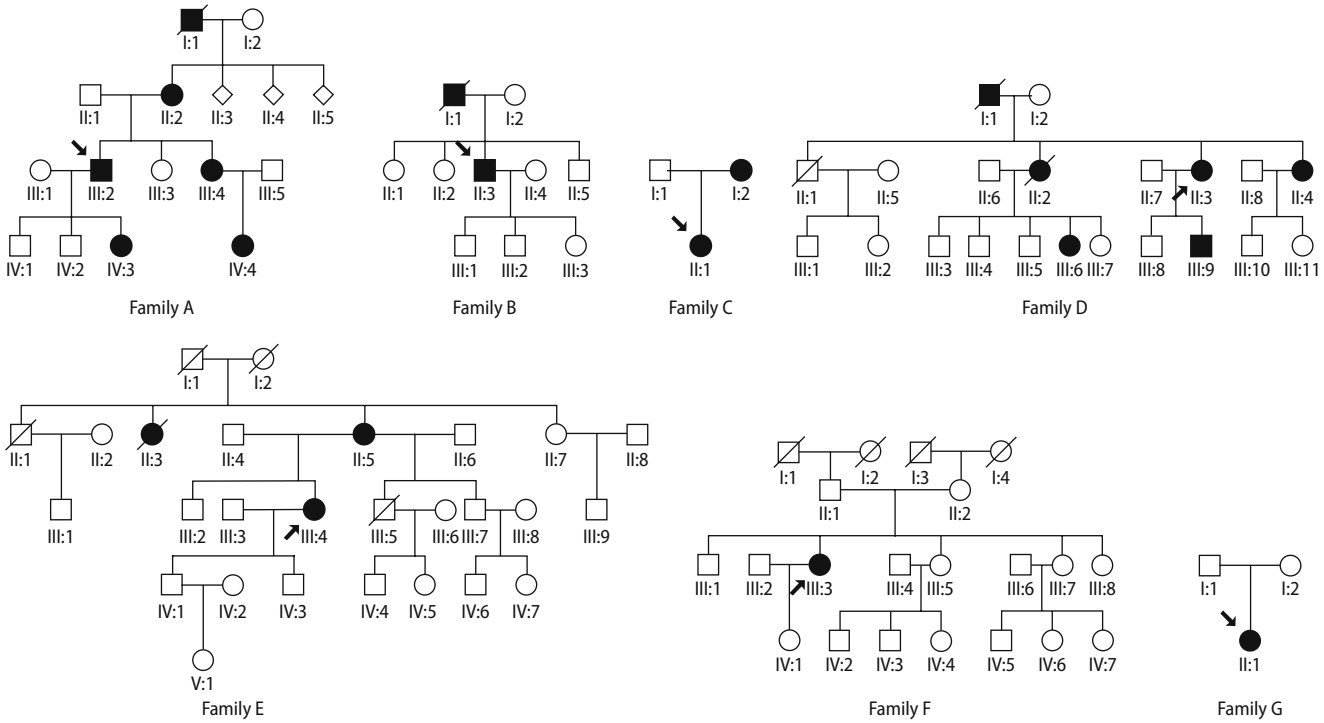

Fig. 2 Genealogic trees of *PEO1* gene mutated families. Dark signs denote affected individuals

Table 1 Clinical features of probands and affected relatives carrying *PEO1* gene mutations

Patient	Sex	Age (years)	Onset (years)	Family history	Clinical features	Country of origin	Aminoacid changes	cDNA[a]
A III 2	M	55	45	AD	Ptosis, severe restriction of horizontal and vertical eye movements, progressive diplopia in right lateral and vertical position	Italy	R303W	C907T
B	M	73	68	AD	Ptosis, severe restriction of horizontal and vertical eye movements, bilateral cataract	Italy	R303W	C907T
C I	F	81[b]	60	AD	Ptosis, diplopia, dysphagia and dysphonia	Italy	R303W	C907T
C II	F	65	48	AD	Ptosis, diplopia, dysphagia and dysphonia			
D II 3	F	64	62	AD	Ptosis and mild restriction of horizontal eye movements	Greece	R334P	G1001C
D II 4	F	61	55	AD	Ptosis and mild restriction of horizontal eye movements	Greece	R334P	G1001C
D III 9	M	49	36	AD	Ptosis	Greece	R334P	G1001C
D III 6	F	40		AD	Mild ptosis	Greece	R334P	G1001C
E III 4	F	66	50	AD	Ptosis, ophthalmoplegia, upper and lower limb proximal weakness and hypothyroidism	Italy	S426N	G1277A
E II 5	F	87	70		Ptosis , ophthalmoplegia	Italy	S426N	G1277A
F	F	34	20	Sporadic	Ptosis , ophthalmoplegia, migraine, premature ovarian failure, depression and proximal myopathy	Italy	W474S	G1421C
G	F	20	18	Sporadic	Ptosis and severe mild restriction of horizontal eye movements	Italy	E479K	G1435A
H	F	78[b]	64	AD	Ptosis and ophthalmoplegia	Greece	W315S	G944C
I	M	75	50	AD	Ptosis and ophthalmoplegia	Greece	F478I	T1432A
L	F	64	56	AD	Ptosis and severe restriction of horizontal and vertical eye movements	Italy	R303W	C907T

[a] All nucleotide changes are heterozygous; [b] Age at death

was confirmed by molecular analysis. As observed with the W474S mutation, this mutation determines an early disease onset, as well, although the clinical picture was relatively stable after a five-year follow-up period.

Other two mutations (W315S, F478I) were observed in Greek late-onset PEO patients: their family history was positive, but family members were unavailable for further investigations.

All these *PEO1* mutations were missense changes, located within exon 1 and 2 coding sequence, while no mutation was found in other exons of the gene (Fig.3). The amino acid changes predicted by the novel coding sequence mutations affect conserved amino acids within evolutionarily conserved Twinkle domains (Fig.1). Furthermore, all mutations were not observed in 200 Italian and 100 Greek control subjects.

Finally, all probands had a normal *POLG2* gene analysis.

Genetic heterogeneity of patients with multiple mtDNA deletions

To estimate the relative frequency of mutations of genes responsible for ad/ar-PEO within a larger series of patients, we reviewed all the consecutive cases from our department over the last 20 years (67 probands) and screened for all known genes.

This extended series includes two familial *PEO1* mutations (R345P, A359T) that have been previously described [4]. Therefore, the incidence of *PEO1* gene mutations in independent probands of our cohort is 17.9 % of cases.

The screening of *POLG1* gene in cases of new occurrence revealed a new mutation: it was a heterozygous nucleotide change (c.G3556C, p.D1186H) (not shown) that affects a highly conserved amino acid and was absent in 200 Italian control subjects. This mutation occurs in trans- with the relatively common heterozygous haplotype T251I-P587L. The affected patient is an apparently sporadic 74 year-old Italian male, whose illness started at the age of 64 years and presented ptosis, dysphagia and neurosensorial hypoacusia. Interestingly, in another newly diagnosed patient, the common haplotype T251I-P587L was present in homozygosis: the affected patient is a 50 year-old female with a recent onset of myopathy, without PEO.

In the past, we and others have identified 13 mutations in the *POLG1* gene in 15 of these independent families [5, 20]. Nine of them showed autosomal recessive transmission, while four have an autosomal dominant pattern of inheritance. Overall the *POLG1* gene is mutated in 19.4 % of our probands. Clinically, *POLG1*-mutated patients are more heterogeneous; in fact they usually present PEO phenotype, but signs of other tissue involvement are also observed: axonal neuropathy (4 patients), myopathy (7 patients), neurosensorial hypoacusia (1 patient), cerebellar signs (3 patients), retinitis pigmentosa (1 patient), lactic acidosis (two patients).

Finally *ANT1* mutations were relatively rare and occurred in 8.9 % of the sample. We did not identify new *ANT1* mutations in recently diagnosed patients. The previously identified *ANT1* mutations consisted in two heterozygous missense mutations in six independent families (L98P, A114P) [3, 21]. All *ANT1* mutated patients

Table 2 Relative proportion of genetically diagnosed cases

	Probands	Familial cases	Sporadic cases
PEO1	12 (17.91 %)	11 (26.8 %)	1 (3.8 %)
ANT1	6 (8.95 %)	6 (14.6 %)	0 (0 %)
POLG1	13 (19.40 %)	4 (9.8 %)	9 (34.6 %)
POLG2	0	0	0
ND	36 (53.73 %)	20 (48.8 %)	16 (61.5 %)
TOTAL	67	41	26

ND not diagnosed

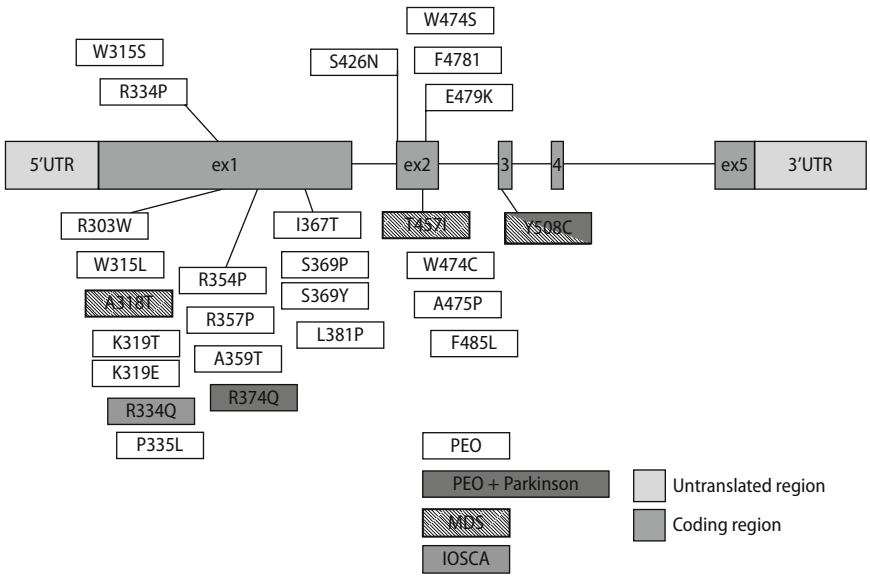

Fig. 3 Schematic representation of intron/exon organization of *PEO1* gene and distribution of mutations along the gene. The six new mutations described in the present manuscript are shown above the scheme, while the previously described are below the scheme. Phenotypes are also associated with mutations: *MDS* mitochondrial DNA depletion syndrome; *IOSCA* infantile onset spino-cerebellar ataxia

belonged to families with autosomal dominant transmission: these patients were predominantly affected by PEO, with facial and proximal upper limb myopathy, with the possible additional features of axonal polyneuropathy and hypothyroidism.

Finally, this series does not include any mutation in *POLG2*.

Thirty-six of the screened patients resulted negative for *ANT1*, *POLG1*, *POLG2* and *PEO1* gene mutations, but presented a phenotype and diagnostic exams that are characteristic of a mtDNA multiple deletions syndrome. This subgroup is composed by 20 familial and 16 sporadic cases. Clinical features of patients without mutations and unlinked to known genes are relatively heterogeneous. In particular, muscle weakness is the most frequent symptom (86.3 %), followed by other features such as ptosis (75.0 %), ophthalmoplegia (54.5 %), hyperCKemia (64.4 %), axonal sensori-motor neuropathy (50 %), lactic acidosis (30 %), neurosensorial hypoacusia (30 %), cerebellar signs (33 %), diabetes (27.8 %), cognitive impairment (25 %), bulbar symptoms (22 %), short stature (15.8 %), cataract (20 %), extrapyramidal signs (10.5 %) and cardiopathy (8 %). A major cluster of clinical features was represented by patients with PEO, myopathy, axonal neuropathy, hypoacusia and diabetes; nine patients had isolated proximal myopathy without PEO.

Discussion

Our data increase the genetic and clinical heterogeneity of progressive external ophthalmoplegia associated with muscle multiple mtDNA deletions. It has been described that mutations in three nuclear genes (*POLG1*, *PEO1*, which codes for the protein Twinkle, and *ANT1*) are found in 70 % of families with dominant PEO and 1/3 of sporadic cases of PEO with multiple mtDNA deletions [18]. A heterozygous dominant mutation in *POLG2* was also shown to cause PEO [2]. Mutations in *OPA1* gene are also emerging as a new cause of autosomal dominant "optic atrophy plus" phenotype with muscle accumulation of multiple mtDNA deletions [5–7]. Although the involvement of this gene in patients without optic nerve atrophy as a prominent clinical feature cannot be excluded at this moment, the absence of optic neuropathy in the undiagnosed patients of the present study makes this hypothesis unlikely.

We identified one *POLG1* and six *PEO1* novel heterozygous mutations: all of them are likely to be pathogenetic. Several lines of evidence support their role. As far as the *PEO1* gene is involved, the R334P, W315S, S426N, W474S, F478I and E479K amino acid changes affect position highly conserved among several eukaryotic homologues, within conserved regions of the protein (Fig. 2). All mutated amino acid positions are encoded by Twinkle exon 1 and 2, suggesting that mutations 3' in the gene are either lethal or more tolerated than mutations in the NH2 terminus. Second, the mutations were not present in 600 control chromosomes. Third, the R334P and S426N mutations co-segregated with ad-PEO in the affected families. Similar evidence could not be obtained for two other familial mutations (W315S and F478I), while we demonstrated that the two apparently sporadic mutations (W474S and the very close E479K) were indeed de novo changes. The complete coding sequences of the *POLG1*, *POLG2* and *ANT1* genes were normal in all these cases, strongly suggesting a causative role for the new Twinkle amino acid changes. However, their mutational status should remain provisional, until new cases are described. Finally, the novel recessive D1186H *POLG1* mutation is likely to be pathogenetic, since it is absent in controls and affects a region of the protein conserved among several eukaryotic homologues.

Twinkle and *ANT1* genes mutations are almost exclusively associated with adult onset PEO phenotypes, in this selected series. Usually these PEO patients belong to ad families. However, the identification of de novo sporadic mutations in Twinkle suggests that also sporadic patients should be investigated for *PEO1* as well as for the other genes.

Reviewing our data, we confirmed that *POLG1* gene mutations may cause a relatively heterogeneous phenotype [21]. Most of our patients presented PEO, but a large proportion of them had signs of multisystemic involvement (neuropathy, myopathy, hypoacusia, cerebellar signs, retinitis pigmentosa, lactic acidosis). This contrasts with the predominantly milder phenotype seen in Twinkle and *ANT1* mutated patients.

Considering the relative causative role of PEO-associated genes, *POLG1* is considered the most commonly mutated nuclear gene. In this relatively large series of patients with muscle mtDNA multiple deletions, 19.4 % of the probands carry mutations in the *POLG1* gene. Instead *PEO1* mutations occur in 17.9 % of cases. Finally *ANT1* mutations are relatively rare and occur in 8.9 % of this sample. As pointed out previously, we did not find any mutations in the *POLG2* gene. Similar data regarding the *POLG1* gene were reported in a U.S.A. resident group of PEO patients (13 % of 30 probands irrespective of inheritance pattern and 15 % of those with a positive family history [21]), while the relative proportions were different in another study involving 27 Italian and British patients was as follows: *POLG1* 26 %, *PEO1* 7.4 %, *ANT1* 3.7 % [17].

However, taking into consideration a positive family history, Twinkle results as the most commonly mutated gene in ad-PEO, with its mutation occuring in 26.8 % of patients, while *ANT1* and *POLG1* account for 14.6 % and for 9.8 % of familial PEO, respectively. On the contrary, *POLG1* is the most commonly mutated gene in our spo-

radic cases of PEO (34.6 %), while Twinkle mutations occur in 3.8 % of these patients.

In this series no recessive mutations in *ANT1* and *PEO1* genes have been identified. The number of patients unlinked to any known genes is relatively high and the percentage is higher in sporadic cases (61.53 %) compared to familial cases (48.8 %). This high proportion of genetically undiagnosed patients suggests that other genes cause autosomal dominant and recessive adult-onset mitochondrial myopathy with mtDNA multiple deletions, often without PEO. The clinical characterization in clusters of undiagnosed patients and the identification of multigenerational families may support the search of new causative genes.

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