PAPER

Two families with autosomal dominant progressive external ophthalmoplegia

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Objectives: We report here the clinical and genetic features of two new families with autosomal dominant progressive external ophthalmoplegia (adPEO).

Patients and methods: The examination of index patients included a detailed clinical characterisation, histological analysis of muscle biopsy specimens, and genetic testing of mitochondrial and nuclear DNA extracted from muscle and leucocytes.

Results: Index patients in both families presented with PEO and developed other clinical disease manifestations, such as myopathy and cardiomyopathy (patient 1) and axonal neuropathy, diabetes mellitus, hearing loss, and myopathy (patient 2), later in the course of illness. Both patients had ragged red fibres on muscle histology. Southern blot of mtDNA from muscle of patient 2 showed multiple deletions. In this case, a novel heterozygous missense mutation F485L was identified in the nuclear encoded putative mitochondrial helicase Twinkle. The mutation co-segregated with the clinical phenotype in the family and was not detected in 150 control chromosomes. In the other index patient, sequencing of *ANT1, C10orf2* (encoding for Twinkle), and *POLG1* did not reveal pathogenic mutations.

Conclusions: Our cases illustrate the clinical variability of adPEO, add a novel pathogenic mutation in Twinkle (F485L) to the growing list of genetic abnormalities in adPEO, and reinforce the relevance of other yet unidentified genes in mtDNA maintenance and pathogenesis of adPEO.

Patients with autosomal dominant progressive external ophthalmoplegia (adPEO) were first reported in the late 1980s.[1] Apart from chronic progressive external ophthalmoplegia, adPEO patients may suffer from generalised myopathy and exercise intolerance. In addition, some adPEO patients develop clinical signs suggestive of multi-organ involvement such as ataxia, peripheral neuropathy, encephalopathy, depression, cataracts, hearing loss, cardiomyopathy, and endocrine dysfunction.[2–5] Most patients show cytochrome c oxidase negative, ragged red fibres in muscle biopsy specimens, and multiple mtDNA deletions on Southern blot.[6 7] On a molecular level, adPEO arises from defects in mtDNA maintenance, and underlying mutations have been identified in three nuclear encoded genes: *ANT1*,[8–10] *C10orf2* (encoding for Twinkle),[11–13] and *POLG*.[14–16] However some adPEO families do not show mutations in any of these genes, indicating further genetic heterogeneity. We report here the clinical features and genetic findings in two new families with adPEO.

PATIENTS AND METHODS

Clinical and electrophysiological findings, and muscle histology of the two index patients are summarised in table 1. The pedigrees are shown in fig 1.

Patient 1

This 67 year old man had experienced slowly progressive external ophthalmoplegia from the age of 15 years. At 49 years of age, dyspnoea during exercise, fatigue, peripheral oedema, nocturia, and palpitations developed. Echocardiography showed an advanced dilated cardiomyopathy with marked reduction of ventricular wall movements and an ejection fraction of less than 25%. Mild improvement was achieved with diuretics, digitalis, and ACE inhibitor treatment. Because of atrial fibrillation with periods of paroxysmal ventricular tachyarrythmia, anticoagulation was started and because of severe symptoms the patient was placed on the waiting list for heart transplantation. Neurological evaluation revealed ptosis, mild facial weakness, and limitation of eye movements in all directions. Electromyography of right biceps and facial muscles showed short duration, low amplitude voluntary motor unit action potentials and an early recruitment of many motor units indicative of a myopathy. The family history was remarkable for progressive bilateral ptosis and ophthalmoplegia in the patient's grandfather, mother (+ diabetes mellitus) and one of his two sons, but none had cardiac disease manifestation (fig 1).

Patient 2

This patient is a 68 year old woman. Ptosis and limited eye movements were noted at the age 50. Later on, she developed progressive atrophy and weakness of the shoulder and quadriceps muscles as well as exercise intolerance. More recently, she also showed axonal polyneuropathy, hearing loss, dysphagia, diabetes mellitus, and idiopathic osteoporosis. On neurological examination, she had almost complete bilateral ptosis and ophthalmoplegia. Her fundi were normal. There was a moderate weakness and atrophy of her quadriceps and shoulder muscles. She was able to walk, but had problems in climbing stairs and lifting her arms over the shoulder level. Achilles tendon reflexes were absent. Electromyography recording from shoulder muscles showed a prominent myopathic pattern. An MRI scan of the brain was unremarkable. The patient's mother had had PEO as an isolated clinical feature. One brother had suffered from PEO,

Abbreviations: adPEO, autosomal dominant progressive external ophthalmoplegia; CS, citrate synthase; NCP, non-collagen protein; RC, respiratory chain; RFLP, restriction fragment length polymorphism

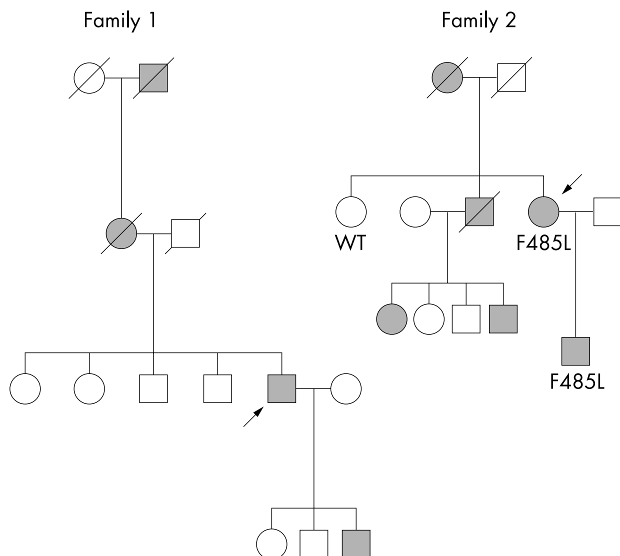


Figure 1 Family pedigrees. Solid symbols indicate clinically affected individuals. Barred symbols indicate deceased individuals. Arrows indicate the index patients. WT, wild type.

diabetes, cardiomyopathy, and myopathy of the respiratory muscles, and died from respiratory insufficiency. The patient's son and two children of her brother show incipient and isolated PEO.

Morphology and biochemistry of skeletal muscle

An open muscle biopsy was performed in both index patients (at age 55 and 65 years, respectively). Serial cross-sections (6 µm thick) were processed for histochemical stains according to standard procedures. A deep frozen part of the biopsy was used for biochemistry. Respiratory chain (RC) enzyme complex I–IV activities were determined in skeletal muscle as described.[17] In patient 1, muscle tissue was not sufficient for ultrastructural and biochemical analysis and a second biopsy was not considered because of long term anticoagulation.

DNA analysis

Total DNA was extracted from muscle and leucocyte samples using a commercial purification kit (Qiagen, Hildesheim, Germany). PCR, Southern blot and single strand conformational polymorphism analyses were performed as described previously.[18 19] Cycle sequencing was performed on gel purified fragments using the ABI Prism Big Dye Termination kit (Perkin Elmer, Langen, Germany). Sequencing of all 22 mitochondrial tRNA genes,[18] *ANT1*,[8] *C10orf2* (encoding for Twinkle),[11] and *POLG1*[15] were performed by standard methods. The required primers were designed according to the following Genbank sequences: J04982 for *ANT1*, AL133215 for *C10orf2*, and AC005317 for *POLG1*. In patient 2, the novel F485L mutation was confirmed by restriction fragment length polymorphism (RFLP) analysis of a PCR product containing exon 2 of Twinkle. The mutation F485L introduced a new *Mse*I restriction site. The patient's affected son, her unaffected sister, and 150 control chromosomes were similarly tested for this mutation.

RESULTS

Morphology and biochemistry of skeletal muscle

In muscle biopsy specimens stained with Gomori trichrome, both patient 1 and 2 showed ragged red fibres (8 and 10%, respectively). These fibres were histochemically negative for cytochrome c oxidase. Histochemistry of a representative section of muscle from patient 1 is shown in fig 2A.

Biochemical and ultrastructural examinations were performed for patient 2 only. The activities of all RC complexes were within normal ranges per gram of non-collagen protein (NCP) and citrate synthase (CS), but the activity of CS (a mitochondrial marker enzyme) itself was slightly increased (111 U/g NCP; normal range: 45–105) suggesting mitochondrial proliferation. On electron microscopy, ultrastructural abnormalities of mitochondria including paracrystalline inclusions were found (fig 2B).

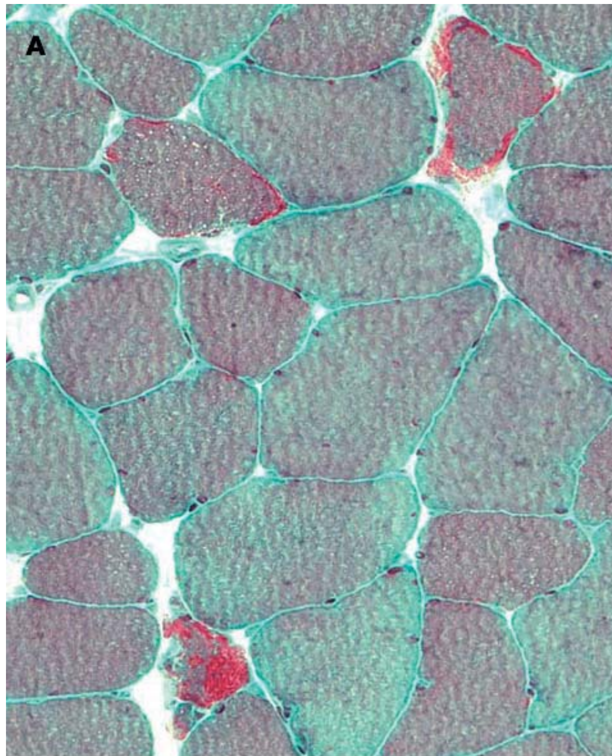
Table 1 Clinical characteristics, structural finding, biochemical and genetic analyses in two patients with adPEO

	Patient 1	Patient 2	Normal value
Clinical characteristics and routine analyses			
Age at onset of PEO/gender	15/M	50/F	–
Age of onset of systemic manifestations	49	55	
Exercise intolerance	–	+	
Additional clinical findings	Severe cardiomyopathy, mild myopathy	Generalised myopathy, axonal neuropathy, diabetes, hearing loss	
Creatine kinase (U/L)	32	54	<80 (men); <60 (women)
Electromyography	M	M/N	–
Muscle biopsy			
Structural findings			
Ragged red fibres (%)	8%	10%	–
Mitochondrial abnormalities on electron microscopy	ND	Yes	
Biochemical analysis			
NADH/coQ-oxidoreductase (U/g NCP)	ND	22.3	12.0–40.0
Succinate/cytochrome c oxidoreductase (U/g NCP)	ND	9.9	6.0–24.0
Cytochrome c oxidase (U/g NCP)	ND	126.0	90.0–281.0
Citrate synthase (U/g NCP)	ND	111.0	45.0–105.0
Genetic analysis			
MtDNA			
tRNA genes	Normal	Normal	
Southern blot	ND	Multiple deletions	
Nuclear genes			
Sequencing of *ANT1*	Normal	Normal	
Sequencing of *C10orf2* (Twinkle)	Normal	F485L	
Sequencing of *POLG1*	Normal	Normal	

M, myopathic; N, neuropathic; NCP, non-collagen protein; ND, not done.

DNA analysis

PCR, RFLP, and sequencing analysis of mtDNA tRNA genes in muscle of the two index patients revealed no abnormalities. Southern blot analysis could not be performed in patient 1 because of small sample size. In patient 2, multiple mtDNA deletions were observed on Southern blot. Sequencing of the *ANT1, C10orf2*, and *POLG1* genes in leucocyte DNA did not show any mutations in patient 1. In patient 2 in the *C10orf2* gene, encoding for Twinkle, a heterozygous missense mutation at nucleotide position C1640A was detected (according to the Genbank accession number AF292004). The mutation exchanged a leucine for a



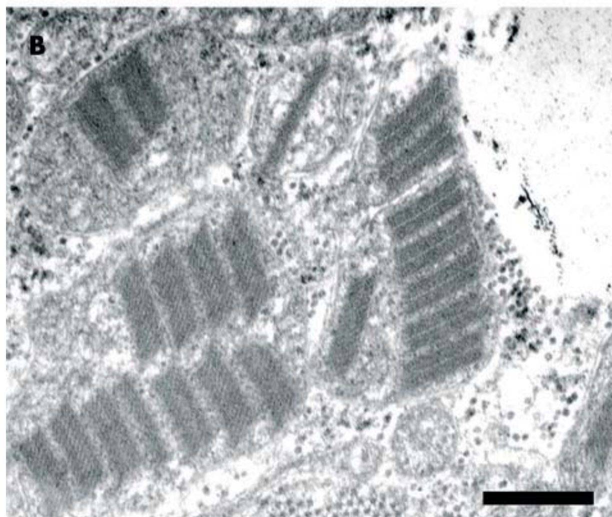


Figure 2 (A) Modified Gomori trichrome staining of a representative section of muscle from patient 1 showing numerous ragged red fibres and mild myopathic changes with increased variability of fibre size and internalised myonuclei, original magnification ×20. (B) Ultrastructure of a muscle fibre from patient 2 containing enlarged mitochondria with crystalloid inclusions (bar = 0.4 μm). The electron micrograph was kindly provided by Professor H Budka, Institute of Neurology, University of Vienna.

conserved phenylalanine at the aminoacid 485 (F485L) (fig 3A). RFLP analysis of Twinkle exon 2 confirmed the mutation in the patient and in her affected son (fig 3B), which was not present in the unaffected sister of the patient or in 150 normal control chromosomes. The mutation is located in a conserved region between Walker motifs A and B of the helicase domain of Twinkle, close to two other previously described pathogenic mutations (W474C and A475P)[11] and co-segregated with the clinical phenotype in the pedigree (fig 1).

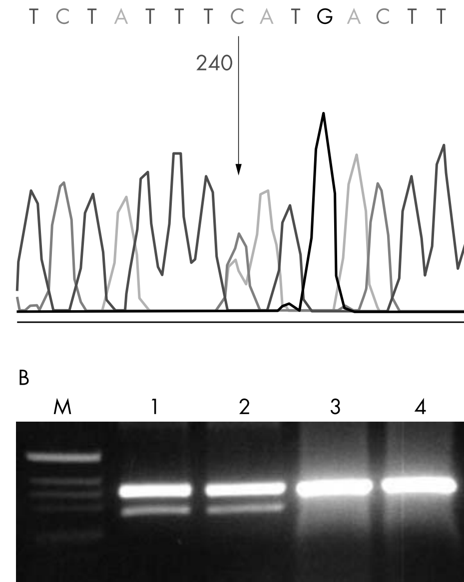


Figure 3 (A) Sequencing of Twinkle revealed a heterozygous missense mutation (arrow). (B) RFLP analysis of Twinkle exon 2 confirmed the mutation in the patient (lane 1) and in her affected son (lane 2), the unaffected sister and a normal control were negative (lane 3, 4). Marker, 1 kb; wild type, 342 bp; mutant, 269 bp.

DISCUSSION

We report here two families with dominant transmission of progressive external ophthalmoplegia. In one, a new mutation in Twinkle helicase was identified, whereas the other family had no mutations in the known PEO genes. Presence of multiple mtDNA deletions was verified in patient 2. Multisystem manifestations and morphological findings in our two families were typical for mitochondrial disease. The first symptom in both patients was PEO, but various other manifestations developed at a later stage of disease (10 to 34 years after onset of PEO) such as severe progressive dilated cardiomyopathy, generalised myopathy, axonal polyneuropathy, diabetes mellitus, and hearing loss. Involvement of extraocular tissues and organs showed high intra-familial variability in both cases. In previous adPEO families, similar systemic manifestations have been described including axonal polyneuropathy, ataxia, depression, dysarthria, tremor, hearing loss, cardiomyopathy, diabetes mellitus, hypogonadism and growth failure.[1–16] The variety of clinical symptoms closely resembles that seen in mitochondrial encephalomyopathies caused by primary mtDNA mutations, which suggests that acquired mtDNA deletions caused by nuclear gene defects are actually responsible for the tissue dysfunction. Multiple deletions probably arise as somatic mutations and accumulate in non-dividing cells, but do not occur in cultured—that is, dividing, cells from adPEO patients.[2] This may also explain the late onset and the variable outcome of the disease.

Recent breakthroughs in molecular genetics of PEO allow a more specific genetic classification. Sporadic or maternally inherited forms of PEO are often caused by single mtDNA deletions and several mutations in mitochondrial tRNA genes.[20] Autosomal disorders of intergenomic communication cause multiple mtDNA deletions or depletion (for review see Moslemi *et al*[4]). The estimated frequencies of mutations in adPEO have been reported to be 4–10% for *ANT1*[8] and 15–35% for *C10orf2* (Twinkle),[2 11 15] and 45% for *POLG1*.[14 15] Mutations in *ANT1, C10orf2* (Twinkle), and *POLG1* were recently also described in sporadic patients with PEO.[21] Cases of adPEO

still exist in which sequencing or linkage analysis excluded all the three genes as causative.[13]

In both patient 2 and her affected son, a new missense mutation in the gene *C10orf2* encoding Twinkle, a putative mitochondrial ring helicase, was found to change a conserved amino acid at position 485. This mutation is located between Walker motifs A and B, next to two other previously described pathogenic mutations (W474C and A475P).[11] Interestingly, in the Twinkle homologue of the T7 bacteriophage, a gene4 primase helicase, the amino acids corresponding to W474 and A475 in Twinkle are located in a helical loop that contacts the ''linker region'' of an adjacent subunit in the hexameric complex. Up to date, 8 of 13 published pathogenic mutations cluster in the linker region.[11 13] According to previous functional studies and crystal structure data of the hexameric T7 gene4 protein, this linker region seems to be involved in the multimerisation process and/or to affect catalytic properties of the primase/helicase function. Furthermore, the adjacent subunit interfaces, joined together by the linker helix loop, form the nucleotide binding pocket. Therefore, mutations W474C, A475P, or the newly discovered F485L in the adjacent helical loop could likewise affect subunit interactions or nucleotide binding, or disturb catalytic activities of the complex. Previous functional studies suggest that the multimerisation of the protein is not drastically altered in linker mutants (a 352–364 duplication and W474C), which agrees with the late disease onset, most likely associated with moderate functional defect.[10]

In conclusion, our families illustrate the clinical and genetic variability of adPEO. In one family, sequencing of the gene encoding Twinkle unravelled a novel pathogenic mutation F485L, whereas in the other index patient with PEO and severe dilated cardiomyopathy, no genetic abnormalities in the *ANT1, C10orf2* (Twinkle), or *POLG1* genes could be detected, indicating the existence of a yet unknown nuclear gene defect. Genetic testing has become an important tool in the difficult diagnosis of inherited PEO.

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