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Phenotypic spectrum associated with mutations of the mitochondrial polymerase γ gene

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Mutations in the gene coding for the catalytic subunit of the mitochondrial DNA (mtDNA) polymerase γ (*POLG1*) have recently been described in patients with diverse clinical presentations, revealing a complex relationship between genotype and phenotype in patients and their families. *POLG1* was sequenced in patients from different European diagnostic and research centres to define the phenotypic spectrum and advance understanding of the recurrence risks. Mutations were identified in 38 cases, with the majority being sporadic compound heterozygotes. Eighty-nine DNA sequence changes were identified, including 2 predicted to alter a splice site, 1 predicted to cause a premature stop codon and 13 predicted to cause novel amino acid substitutions. The majority of children had a mutation in the linker region, often 1399G→A (A467T), and a mutation affecting the polymerase domain. Others had mutations throughout the gene, and 11 had 3 or more substitutions. The clinical presentation ranged from the neonatal period to late adult life, with an overlapping phenotypic spectrum from severe encephalopathy and liver failure to late-onset external ophthalmoplegia, ataxia, myopathy and isolated muscle pain or epilepsy. There was a strong gender bias in children, with evidence of an environmental interaction with sodium valproate. *POLG1* mutations cause an overlapping clinical spectrum of disease with both dominant and recessive modes of inheritance. 1399G→A (A467T) is common in children, but complete *POLG1* sequencing is required to identify multiple mutations that can have complex implications for genetic counselling.

Keywords: mitochondrial encephalopathy; mitochondrial DNA; polymerase gamma; mtDNA; chronic progressive external ophthalmoplegia; Alpers syndrome

Abbreviations: mtDNA = mitochondrial DNA; PCR = polymerase chain reaction; PEO = progressive external ophthalmoplegia; pol γ = polymerase γ; SLE = stroke-like episodes

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Introduction

The 16 569-base pair human mitochondrial genome is replicated by the nuclear-encoded DNA polymerase γ, pol γ (Clayton, 1982). Like other A-type polymerases, pol γ has both polymerase and 3′→5′ proofreading exonuclease activity, and is translated from 22 exons of the *POLG1* gene on chromosome 15q25 (Kaguni, 2004). The first pathogenic mutations of *POLG1* were identified in families with autosomal dominant chronic progressive external ophthalmoplegia (adPEO, MIM 157640) associated with the accumulation of multiple mitochondrial DNA (mtDNA) deletions in clinically affected tissues (Van Goethem *et al.*, 2001). Subsequent work has shown compound heterozygous and homozygous *POLG1* mutations in recessive PEO (Van Goethem *et al.*, 2003*b*) and adult-onset cerebellar ataxia with multiple mtDNA deletions (Van Goethem *et al.*, 2004; Hakonen *et al.*, 2005; Winterthun *et al.*, 2005). Recessive *POLG1* mutations have recently been described in Alpers syndrome (also called the Alpers–Huttenlocher syndrome, MIM 203700) associated with mtDNA depletion (Naviaux and Nguyen, 2004; Davidzon *et al.*, 2005; Ferrari *et al.*, 2005). With the recent description of parkinsonism and premature ovarian failure in some dominant kindreds (Luoma *et al.*, 2004), the phenotypic spectrum associated with *POLG1* mutations continues to expand (Del Bo *et al.*, 2003; Gonzalez-Vioque *et al.*, 2006). At first sight, it appears that *POLG1* mutations cause a growing number of totally discrete clinical categories. Here, we present clinical and molecular findings in 38 previously unreported patients with mutations in *POLG1*, demonstrating a continuous spectrum of disease, with both dominant and recessive transmission.

Subjects, material and methods

Subjects

We studied a large collection of patients with clinical, biochemical and molecular genetic evidence of mitochondrial disease from several European research and diagnostic centres. Subjects were identified from three main clinical groups.

(i) *Sporadic and familial PEO with multiple mtDNA deletions.* Patients with (a) a clinical diagnosis of chronic PEO, (b) histochemical evidence of mitochondrial disease, with cytochrome *c* oxidase negative muscle fibres and/or ragged-red fibres in greater proportions than would be expected for age-matched healthy controls (Brierley *et al.*, 1998), (c) multiple deletions in skeletal muscle demonstrated by Southern blot or both long-range polymerase chain reaction (PCR) and real-time PCR (He *et al.*, 2002).

(ii) *Late-onset ataxia with multiple mtDNA deletions in skeletal muscle.* Patients with late-onset ataxia who fulfilled the following criteria: (a) no evidence of a neoplastic, metabolic, inflammatory and sporadic degenerative neurological disorder, (b) negative molecular genetic testing for spinocerebellar ataxia (SCA) 1, 2, 3, 6, 7, 17, dentatorubropallidoluysian atrophy and Friedreich's ataxia, (c) histochemical evidence of mitochondrial disease, with cytochrome *c* oxidase negative muscle fibres and/or ragged-red fibres in greater proportions than would be expected for age-matched healthy controls (Brierley *et al.*, 1998), (d) multiple deletions in skeletal muscle demonstrated with at least two of the following methods: Southern blot, long-range PCR and real-time PCR (He *et al.*, 2002).

(iii) *Alpers syndrome.* Children with (a) refractory seizures, (b) developmental delay, (c) liver failure, who had biochemical evidence of mitochondrial dysfunction and/or depletion of mtDNA in liver and/or skeletal muscle.

Molecular genetic methods

Nuclear gene screening

Total genomic DNA was extracted from whole blood and the entire coding region and adjacent intronic regions of *POLG1* were amplified by PCR and sequenced using a fluorescent chain terminating sequencing kit (Applied Biosciences or Beckman Coulter Quickstart) and a fluorescent DNA analyser (primers and conditions available on request, Applied Biosciences 3100 or a Beckman Coulter CEQ 8000) and compared with the Genbank reference (accession number: BC050559). Substitutions were confirmed by reverse sequencing. *C10ORF2* and *ANT1* were also sequenced in seven individuals, where complete sequencing of all three genes was standard practice in one laboratory (indicated by an asterisk in Table 1, Genbank accession numbers: AF292005 and BC061589). The frequency of the novel sequence variants was determined in control chromosomes by denaturing high-performance liquid chromatography (DHPLC, Transgenomic Wave system, primers and conditions available on request), and direct sequencing. Genotyping of the novel central European variants was done by primer extension of multiplex PCR products with the detection of the allele-specific extension products by matrix-associated laser desorption/ionization time-of-flight (MALDITOF; Sequenom, San Diego, CA, USA) mass spectrometry.

Statistical analysis

Allele frequencies were compared using Fisher's exact test. Exact 95% confidence intervals were determined by the method of Clopper and Pearson (Armitage *et al.*, 2002).

Results

Clinical presentation

A wide range of phenotypes were observed in the patients with *POLG1* mutations, ranging from a fatal childhood hepatoencephalopathy to a mild clinical syndrome affecting

a single organ and presenting in adult life (Table 1). Of the 10 children who developed liver failure, the majority were male (90%, Fisher's exact $P = 0.001$) and presented in the first years of life (mean 1.8 years, range 0.5–7 years) with an encephalopathy and no family history. In five (50%), abnormal liver function developed within weeks of commencing sodium valproate for seizure control. Follow-up data were available for 10 cases, with a large proportion (40%) dying before 2 years of age. Four of the five individuals who developed a fluctuating encephalopathy without liver failure developed symptoms in childhood (mean: 13.3 years, range: 4–32 years). All were male and none were treated with sodium valproate. Stroke-like episodes and myoclonus were also common in the group presenting in childhood.

The 19 patients with ptosis and PEO generally presented in teenage years or adult life (mean: 36.1 years, range: 10–63). Nine of these individuals had relevant family history, which suggested dominant transmission in two. The majority of the 19 individuals developed additional features including a limb myopathy (79%), ataxia (42%), a peripheral neuropathy (32%) or dysphagia (37%). Fifty-three per cent were male (Fisher's exact $P = 1$). Additional features in this group included diabetes (11%), deafness (11%), young-onset dementia (11%) and one patient with cardiomyopathy (5%). No patients had parkinsonism. Of the individuals presenting in adult life, only one patient had ataxia and a peripheral neuropathy without PEO (5%). Additional presenting features included isolated muscle pain and isolated epilepsy as the only feature in two cases.

Overall, 13 subjects had a relevant family history (34%). There was no relevant family history in 66% of cases.

Molecular genetics

We identified 89 changes from the reference sequence, including 2 novel predicted splice-site mutations, 1 predicted to introduce a premature stop codon and 13 predicted to alter the amino acid sequence (Fig. 1, Tables 1 and 2). For clarity, each mutation is presented in terms of the predicted amino acid substitution, with the exception of predicted splice-site mutations.

Heterozygous alleles were found in four individuals with a family history consistent with dominant transmission, including the previously described mutations W312R, and T251I-p587L *in cis*. The only novel dominant mutation was G517V mutation found in three affected individuals in three generations of one family. The remaining 34 subjects had at least two mutated alleles. Nine were homozygous (26.5% of the recessive cases), including all three consanguineous children. Two individuals had mutations predicted to alter splice-sites (2.2% of substitutions, 5.3% of subjects), and one a novel stop-codon mutation (L965X). A467T was found in 12 of the 19 who presented in childhood (63.2%), and was homozygous in 4 (21.1% of children). Four substitutions were identified in six individuals (each with two pairs *in cis* confirmed by direct sequencing of the parents). The allele frequency corresponding to T251I, A467T, V1106I, E1143G and the novel substitutions is shown in Table 2.

Sequencing of *C10ORF2* and *ANT1* in seven individuals did not identify any changes from the reference sequence (Table 1, individuals indicated by an asterisk).

Discussion

Clinical features

Although our method of case ascertainment led to the identification of three prominent clinical groups, key individuals demonstrate the overlapping phenotypic spectrum associated with *POLG1* mutations (Table 1). Most patients with a fluctuating encephalopathy presented in childhood, but one adult presented in his third decade with a rapidly progressive fatal multi-system disorder with PEO, and one adult developed a slowly progressive dementing illness. Most adults with *POLG1* mutations developed PEO and a limb myopathy, often in association with ataxia and an axonal sensorimotor peripheral neuropathy [similar to the original description of sensory ataxic neuropathy with gastrointestinal dysmotility and ophthalmoplegia, SANDO (Fadic *et al.*, 1997)], but some presented with myopathy or ataxia without PEO. Although we did identify cardiomyopathy in two subjects and a cardiac conduction defect in one, none had a pigmentary retinopathy. We also observed isolated mild phenotypes broadening the clinical spectrum associated with *POLG1* mutations, including epilepsy as a presenting feature in two cases. Isolated ataxia with neuropathy was uncommon in this cohort, but myoclonus was noted especially in childhood onset cases. Parkinsonism was also uncommon, possibly because we did not find dominant *POLG1* polymerase domain mutations, which are the mutations that have been associated with parkinsonism to date (Luoma *et al.*, 2004).

POLG1 mutations clearly do not exert their effect in isolation. The vast majority of severe childhood presentations were in boys. Gender bias is well recognized in other mitochondrial disorders, and recent evidence supports the role of additional nuclear genetic modifier loci interacting with the primary defect (Carelli *et al.*, 2003; Hudson *et al.*, 2005). It is intriguing that later-onset *POLG1* phenotypes are less susceptible to this bias, equally affecting men and women. In four infants the liver dysfunction began after treatment with sodium valproate, and the children with isolated encephalopathy were not exposed to this drug, nor were the adult cases. This demonstrates the potential effect of environmental factors modulating the phenotypic spectrum of the disorder, and suggests that this drug should be used with caution, or avoided in this group of patients. It is tempting to speculate that gender and environmental factors are more likely to influence the clinical expression of the more severe molecular defects presenting in childhood. Further clinical and functional studies will hopefully clarify the underlying mechanisms.

Table 1 Clinical phenotype and *POLG1* substitutions

Age at onset (years)	Current age (years)[a]	Sex	Family history	Primary clinical features							Other features	*POLG1* substitutions[e]				
				Liver[b]	Encephalopathy	PEO	Ataxia	Dysphagia	Neuropathy[c]	Myopathy[d]		cDNA substitution	Predicted amino acid change	Domain		
														Exo	Link	Pol
Group 1: Liver and encephalopathy																
0.5	1.3D	M	Nil	+	+						Cardiomyopathy	1399G→A *3573G→T*	A467T *K1191N*		+ +	+ +
0.5	1.3D	M	Nil	+	+							926G→A 1880G→A	R309H R627Q	+ 	 +	
0.6	1D	F	Nil	+	+							1399G→A 2542G→A	A467T G848S		+ +	+ +
0.8	1D	M	Epilepsy: father and cousin	+ (v)	+							*2209G→C* *2300C→A*	*G737R* *A767D*		 ++	
1	1.4D	M	Nil	+	+							1399G→A *IVS15-9_12Del*	A467T –		+ +	+ +
1.25	2.5	M	Nil	+	+						Cortical blindness	1399G→A *IVS21+1T→C*	A467T –		+ +	+ +
1.5	2	M	Nil	+ (v)	+						Cortical blindness	1399G→A *2740A→C*	A467T *T914P*		+ +	+ +
2	9	M	Consanguineous	+ (v)	+						Myoclonus achalasia	3286C→T 3708G→T* 3286C→T 3708G→T	R1096C Q1236H R1096C Q1236H			++ ++
2.5	3	M	Nil	+ (v)	+							1399G→A *2653A→T* * *2637T→G* 3428A→G	A467T *T885S* *Q879H* E1143G		+ +	+++ +
7	8D	M	Nil	+ (v)	+						SLE seizures	1880G→A *3287G→A*	R627Q *R1096H*			
Group 2: Encephalopathy																
4	8	M	Nil		+						Myoclonus and SLE	1399G→A *2740A→C*	A467T *T914P*		+ +	+ +
7.5	7.5	M	Nil		+		+					1399G→A 1399G→A	A467T A467T		++ ++	
8	8	M	Consanguineous		+						SLE	1399G→A 1399G→A	A467T A467T		 ++	
15	17	M	n/a		+						Myoclonus	1399G→A* 1399G→A	A467T A467T		 ++	
Group 3: PEO ± other features																
n/a	56	F	Nil			+				+		752C→T 1760C→T 752C→T 1760C→T	T251I P587L T251I P587L	 ++	 ++	
n/a	35	F	Nil			+				+		752C→T 1760C→T *3316G→A*	T251I P587L V1106I	 +	 +	 +
n/a	n/a	n/a	Sib			+	+	+	+	+	Diabetes	1399G→A*	A467T			

										2243G→C	W748S		++		
10	14	F	Nil		+			+		*924G→C*	*Q308H*				
										2740A→C 3490T→A	*T914P F1164I*	+		++	
15	48	M	Sister with Alpers		+	+	+	+	+	Dementia	1399G→A	A467T			
											3428A→G	E1143G		+	+
25	53	M	Consanguineous		+		+		+	Thyroid disease	*1943C→G*	*P648R*			
											1943C→G	*P648R*		++	
25	50	M	Nil		+	+	+		+	Psychosis dementia	1298C→G*	S433C			
											3428A→G	E1143G		+	+
26	45	F	Nil		+	+		+		Myoclonus epilepsy	1880G→A 3708G→T	R627Q Q1236H			
											2894T→G 3428A→G	*L965X* E1143G	+	+	++
32	41D	M	Affected sibling	+	+	+	+	+	+	Cardiomyopathy	1399G→A	A467T			
										Hearing loss	1879C→T	R627W		++	
34	49	M	Affected sibling		+	+	+	+	+	Diabetes	1399G→A*	A467T			
											2243G→C	W748S		++	
37	62	M	Nil		+		+		+		934T→C	W312R			
											1720C→T	*R574W*		+	+
39	54	M	Sib		+	+			+	Hearing loss	1399G→A	A467T			
											1879C→T	R627W		++	
45	74	F	Sib		+	+			+		752C→T 1760C→T	T251I P587L			
											911T→G	L304R	++	+	
48	56	M	Nil		+						679C→T	R227W			
											752C→T 1760C→T	T251I P587L	++	+	
53	55	F	Nil		+				+		1943C→G	P648R			
											3286C→T	R1096C		+	+
60	62	F	Ptosis: father		+						934T→C*	W312R		+	
60	68	M	Affected sibling		+			+			752C→T 1760C→T	T251I P587L			
											911T→G	L304R	++	+	
62	70	F	Nil		+				+		752C→T 1760C→T	T251I P587L			
											752C→T 1760C→T	T251I P587L	++	++	
63	74	M	Nil		+				+	Chronic bronchitis	752C→T 1760C→T	T251I P587L			
											752C→T 1760C→T	T251I P587L	++	++	
Group 4: Other															
48	58	M	Possible dominant						+	Heart block, muscle pain	752C→T 1760C→T	T251I P587L	+	+	
4	26	F	Father and daughter			+		+		Exercise intolerance	1550G→T	G517V		+	+
											3428A→G	E1143G			
50	58	M	Daughter and granddaughter					+			*1550G→T*	*G517V*		+	
7	8	F	Mother and grandfather							Epilepsy	*1550G→T*	*G517V*		+	
15	20	F	Nil							Epilepsy	1399G→A	A467T			
											1399G→A	A467T		++	

Patients are arranged into four groups. Within each group the patients are ordered by increasing age of onset to illustrate the age spectrum and phenotypes associated with the different ages at presentation. Group 1 = encephalopathy with liver failure (Alpers–Huttenlocher syndrome). Group 2 = childhood encephalopathy without clinical evidence of liver involvement. Group 3 = patients with PEO ± additional features. Group 4 = other clinical presentations. Exo = exonuclease domain, link = linker domain, pol = polymerase domain (see Fig. 1), CC = corpus callosum, PEO = progressive external ophthalmoplegia, SLE = stroke-like episodes, n/a = not available. *C10ORF2* and *ANT1* also sequenced in individuals indicated by asterisk (*). [a]D = age of death; [b](v) = deterioration after starting sodium valproate; [c]axonal sensor-motor neuropathy; [d]proximal myopathy; [e]novel substitutions in italics.

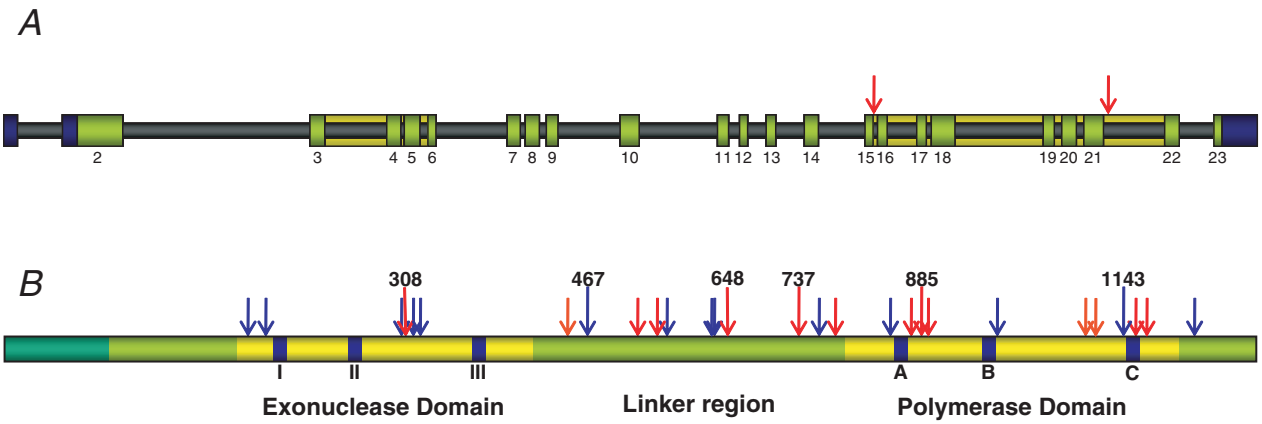

Fig. 1 *POLG1* substitutions in the 38 patients. Location of intronic/splice site variations (**A**) and amino acid substitutions (**B**). Red = novel substitutions; blue = previously described substitutions. Numbers above are examples of specific sites to illustrate the position of the amino acid residues. The exonuclease domain extends from amino acid residue 1–418. The polymerase domain extends from amino acid residue 756–1239. The linker region lies between amino acid residues 418 and 756 (Nguyen *et al.*, 2005).

POLG1 mutations

Following the identification of the first pathogenic *POLG1* mutations (Van Goethem *et al.*, 2001), a range of nucleotide substitutions have been described in patients and control subjects (http://dir-apps.niehs.nih.gov/polg/ and http://www.ncbi.nlm.nih.gov/SNP/). This presents a diagnostic challenge: in individual patients it can be difficult to decide which substitutions are directly responsible for the disease, and which are polymorphic variants. The possibility that frequent polymorphic genetic variants modify the phenotype further complicates the situation (Hisama *et al.*, 2005). We considered a nucleotide substitution to be pathogenic when (i) it was predicted to alter a conserved amino acid in a functionally relevant site of pol γ; (ii) it was close to other established pathogenic *POLG1* mutations; (iii) compound heterozygous mutations were never homozygous in control subjects; (iv) dominant mutations were never found in control subjects. This study has shown that the population frequency of heterozygous carriers varies throughout Europe (Table 2). Given the polymorphic nature of *POLG1*, studying a large, ethnically matched control group is of fundamental importance. Final confirmation of pathogenicity is only possible when the same substitution segregates with disease in different families, and the proposed mechanism is supported by functional data.

We identified 16 novel mutations. These were either not detected in large control groups from corresponding geographical regions, or they were extremely rare (<0.5%, Table 2). Conservation tables showing the position of the predicted amino acid substitutions are shown in the online supplement.

Novel mutations predicted to truncate pol γ

The two predicted to alter splice sites (IVS15-9-c.2485 Del12bp, IVS21+1T→C) were compound heterozygotes with previously reported pathogenic mutations. The only stop-codon mutation (L965X) was found in a compound heterozygote with a previously described recessive mutation [R627Q (Luoma *et al.*, 2005)] and is predicted to remove part of the catalytic domain of pol γ. A similar stop-codon mutation (E873X) was shown to cause nonsense associated alternative splicing and nonsense mediated decay with Alpers syndrome in a compound heterozygote with A467T (Chan *et al.*, 2005*a*).

Novel recessive missense mutations

Of the remaining 13 novel missense mutations, T914P was found in three unrelated compound heterozygotes. This mutation alters a conserved amino acid residue and is close to a published pathogenic mutation (G923D) in the polymerase domain (Lamantea *et al.*, 2002). P648R was homozygous in one consanguineous patient with PEO, ataxia and a neuropathy, and in a compound heterozygote with a previously identified pathogenic mutation. P648R alters a conserved residue in the linker region of pol γ, close to the previously published R627W and R627Q (Van Goethem *et al.*, 2003*b*; Luoma *et al.*, 2005). The substitutions G737R, A767D, Q879H, T885S and K1191N were also found in compound heterozygotes, altered conserved residues, and were close to published pathogenic mutations in the polymerase domain [W748S, R853W, N864S, A889T, and D1184N (Agostino *et al.*, 2003; Filosto *et al.*, 2003; Van Goethem *et al.*, 2003*c*; Van Goethem *et al.*, 2004; Gonzalez-Vioque *et al.*, 2006)]. The R1096H substitution affects the same codon as the previously reported R1096C (Agostino *et al.*, 2003). S433C and R574W were found in different compound heterozygotes with a previously published substitution. Both are close to previously reported recessive mutations in the linker region [G431V (Agostino *et al.*, 2003); R562Q and R579W (Di Fonzo *et al.*, 2003; Filosto *et al.*, 2003)]. One mutation in the exonuclease

Table 2 Allele frequencies in control subjects

Nucleotide change	Predicted amino acid substitution	Geographic origin of the patients*	Allele frequency in control subjects							
			British		German		Italian		Finnish	
			n/*N*	% (95% CI)	*n*/*N*	% (95% CI)	*n*/*N*	% (95% CI)	*n*/*N*	% (95% CI)
752T→C	T251I	C, I, F					5/450**	1.11 (0.36–2.57)		
924G→C	Q308H	I					0/200	0 (0–1.49)		
1399A→G	A467T	C, I, UK	3/432	0.69 (0.14–2.02)	2/1066	0.19 (0.02– 0.73)	0/674	0 (0–0.44)	1/602	0.17 (0–0.92)
1550G→T	G517V	C			1/672	0.15 (0–0.83)				
1720C→T	R574W	I					0/200	0 (0–1.49)		
1943C→G	P648R	I					0/200	0 0–1.49)		
2209G→C	G737R	C			2/666	0.3 (0.04–1.08)				
2300C→A	A767D	C			0/678	0 (0–0.44)				
2637G→A	Q879H	UK	0/192	0 (0–1.09)						
2653A→T	T885S	UK	0/192	0 (0–1.09)						
2740A→C	T914P	C, I			0/664	0 (0– 0.45)	0/200	0 (0–1.49)		
3316G→A	V1106I	I					0/500	0 (0–0.60)		
3428A→G	E1143G	C, UK	4/192	2.08 (0.57–5.25)					8/300	2.67 (1.16–5.19)
3490T→A	F1164I	I					0/200	0 (0–1.49)		
3573G→T	K1191N	C			0/664	0 (0– 0.45)				
IVS21+1T→C	–	C			0/666	0 (0– 0.45)				
IVS15-9_12Del	–	C			1/666	0.15 (0–0.83)				

n = number of chromosomes with the allele; *N* = total number of chromosomes screened. 95% CI = exact 95% confidence interval.
*C = central European, F = Finnish, I = Italian, UK = United Kingdom; ** = P587L was found in all Italian cases with T251I.

domain (Q308H) was also in a compound heterozygote, and is adjacent to a previously reported recessive mutation [R309L (Lamantea *et al.*, 2002)].

Dominant mutations

One novel heterozygous linker-region mutation was found (G517V) in one family with clear evidence of dominant transmission. Intriguingly, most dominant mutations reported to date affect the polymerase domain (Luoma *et al.*, 2004; Van Goethem *et al.*, 2004). In two families with dominant transmission, previously published recessive alleles [W312R and T251I-P587L (Lamantea *et al.*, 2002; Agostino *et al.*, 2003)] were the only *POLG1* mutations identified.

Relationship between genotype and phenotype

Our observations confirm that pathogenic mutations in *POLG1* are scattered throughout the coding region (Fig. 1). Although there is not a strict genotype–phenotype relationship, a pattern is emerging (Table 1). Most cases with a severe disease onset in childhood are associated with at least one mutation in the linker region, and one in the polymerase domain (Table 1). In contrast, in this series, exonuclease domain mutations are more commonly found in patients presenting in teenage or adult life. However, onset in childhood has been previously noted for W748S homozygotes (Hakonen *et al.*, 2005). Finally, all but one of the patients with two linker-region mutations presented in adult life. It should be noted, however, that the conventional subdivision of the *POLG1* gene into three distinct regions implies a strict genotype–phenotype relationship at the biochemical level. This appears not to be the case, because mutations in the linker region can affect catalytic efficiency of pol γ (Chan *et al.*, 2005*b*).

58.9% of the childhood-onset cases harboured the A467T linker-region mutation, but only 21.1% of the remaining cases haboured A467T. In this study, A467T was found in 0.69% of British control alleles, providing a reservoir for recessive disease. In contrast, A467T was found in 0.17% of Finnish control alleles, 0.19% of 1066 German control alleles, but not in 674 Italian control chromosomes, demonstrating geographic differences in the carrier frequency around Europe (Table 2). It is not clear why more childhood-onset cases are associated with A467T than adult-onset cases. Intriguingly, the four individuals homozygous for A467T had a milder phenotype when compared with compound heterozygotes with the same mutation. This is surprising given recent *in vitro* data demonstrating only 5% polymerase activity with the A467T mutant protein (Chan *et al.*, 2005*b*; Luoma *et al.*, 2005), suggesting that other factors contribute to the phenotypic variability, as for the W748S mutation (Hakonen *et al.*, 2005). Further observations on a larger cohort are needed to substantiate this finding. Recent clinical studies highlight the potential role of A467T in late-onset mild ptosis and PEO (Luoma *et al.*, 2005). Given the frequency of this mutation in

the general population, it is likely that many cases remain undiagnosed.

In contrast, we only identified E1143G in five patients. E1143G is found at a higher frequency than A467T in the general population [2.1% of British controls in this study, 3.3% of Belgians (Van Goethem *et al.*, 2001), 2.1% of Finns (Van Goethem *et al.*, 2004) and 2.9% of the North American population (http://www.ncbi.nlm.nih.gov/SNP/)]. However, E1143G homozygotes have never been described in patients with disease, although we have detected them in control subjects (Suomalainen A, Ahola S, Luoma P, unpublished). In contrast, the frequency of E1143G in our patient cohort was significantly greater than controls (Fisher's exact $P = 0.007$). In addition, in the three-generation family with G517V the clinical presentation of the heterozygous carriers was very variable (epilepsy, neuropathy and myopathy, mild neuropathy). The most severe phenotype was observed in the mother, who also carried the E1143G substitution on the other allele. When taken together, this suggests that, on its own, E1143G is not sufficient to cause disease (either in a homozygous or heterozygous state), but it can cause disease when in combination with a different compound heterozygous mutation. It may also act as a phenotypic modifier through an interaction with other mutations (Hisama *et al.*, 2005).

Multiple mutations in *POLG1*

In 11 individuals we identified more than 2 substitutions (see Table 1, where mutations *in cis* are shown on the same line). Seven of these individuals harboured T251I and P587L *in cis*, as compound heterozygotes with other putative pathogenic mutations as described previously (Di Fonzo *et al.*, 2003; Ferrari *et al.*, 2005). In two previous studies, T251I was reported in compound heterozygotes *without* P587L (Lamantea *et al.*, 2002; Di Fonzo *et al.*, 2003). However, in one individual, this was a technical artefact, and further analysis identified P587L (Lamantea and Zeviani, 2004). Moreover, all five of the Italian carriers of T251I detected in this study also had P587L (Table 2). Thus, in the vast majority of cases reported to date, T251I and P587L have been described *in cis*, and it is currently not possible to determine whether T251I or P587L is the primary pathogenic allele, or whether both substitutions are required to cause disease in these patients. On the other hand, P587L was recently described in a compound heterozygote with R853W presenting with PEO and ptosis *without* T251I (Gonzalez-Vioque *et al.*, 2006). On balance, it is therefore likely that P587L is the pathogenic allele, but in most affected individuals the allele is also transmitted with T251I. The functional significance of the T251I substitution therefore remains uncertain at present. T251I-P587L was detected in 1.1% of Italian control chromosomes, thus providing a reservoir for recessive disease in this population.

One individual had the novel mutation Q308H as compound heterozygote with T914P and F1164I *in cis*. Since we also identified two compound heterozygotes with T914P in the absence of F1164I, this suggests that T914P is the primary pathogenic mutation in this case, and not F1164I. We also identified homozygous substitution Q1236H in a consanguineous child *in cis* with the previously published pathogenic mutation (R1096C) (Agostino *et al.*, 2003). Q1236H has been found in patients with PEO (Di Fonzo *et al.*, 2003), and up to 4% of control subjects (http://www.ncbi.nlm.nih.gov/SNP), and 15% of Finns (Luoma *et al.*, 2005), suggesting that it is a polymorphism with no functional consequences. It is likely that R1096C is primarily pathogenic in this individual.

Finally, in one boy we found four substitutions. Segregation analysis confirmed that A467T was *in cis* with the novel mutation T885S (from the mother), and Q879H was *in cis* with E1143G (from the father). Although tempting to speculate that the four mutations act synergistically to cause the severe phenotype in this individual, he was not the most severely affected case in our series, with other individuals having only two compound heterozygous mutations. On the other hand, given emerging data from functional (Chan *et al.*, 2005) and clinical studies (Hisama *et al.*, 2005), it is clear that A467T contributes directly to the pathogenesis, and E1143G may modify the phenotype.

Mutations in *C10ORF2* and *ANT1*

Recently, a patient/family with PEO transmitted both a mutation in *POLG1* (G848S) and *C10ORF* (R334Q), and it was suggested that the two mutated alleles interacted to cause the disease (Van Goethem *et al.*, 2003*a*). We therefore sequenced *C10ORF2* and *ANT1* in seven of the individuals described here. No substitutions were identified, demonstrating that co-mutation is not common. Moreover, we have also identified the R334Q *C10ORF2* mutation in a family with dominant PEO with no associated mutation in *POLG1*, demonstrating that R334Q is primarily pathogenic on its own, and questioning the role of the *POLG1* mutation in the earlier report. Thus, clear-cut digenic interaction of *POLG1* and *C10ORF2* has yet to be demonstrated.

Conclusions—diagnosis and clinical genetic implications

Identifying which individuals to screen for *POLG1* mutations can be difficult. The majority of subjects in this study had no relevant family history, including those with limited phenotypes, indicating the importance of considering *POLG1* mutations in a broad range of sporadic neuromuscular diseases. All of the cases in this study either had clear biochemical evidence of mitochondrial dysfunction in muscle, liver or fibroblasts, and/or a secondary defect of mtDNA in affected tissues, but this is not always the case. *POLG1* mutations have been described in adults with normal muscle histochemistry and a normal Southern blot of muscle mtDNA (Van Goethem *et al.*, 2004). However, retrospective analysis of the literature and the cases presented here confirm

that a sensitive PCR-based assay detected a small amount of deleted mtDNA in all *POLG1* patients presenting in adult life (Van Goethem *et al.*, 2003*c*; Luoma *et al.*, 2005). On the basis of the available data, if muscle is available, mtDNA PCR analysis is an effective primary screening method to identify patients for *POLG1* sequencing. It should, however, be noted that low levels of deleted mtDNA also occur in healthy aged individuals. The muscle mtDNA screening approach is therefore sensitive, but not specific. Selecting which patients to screen ultimately relies on clinical suspicion, and hence the importance of our careful documentation of the relationship between genotype and phenotype. Our current diagnostic approach is outlined in Fig. 2.

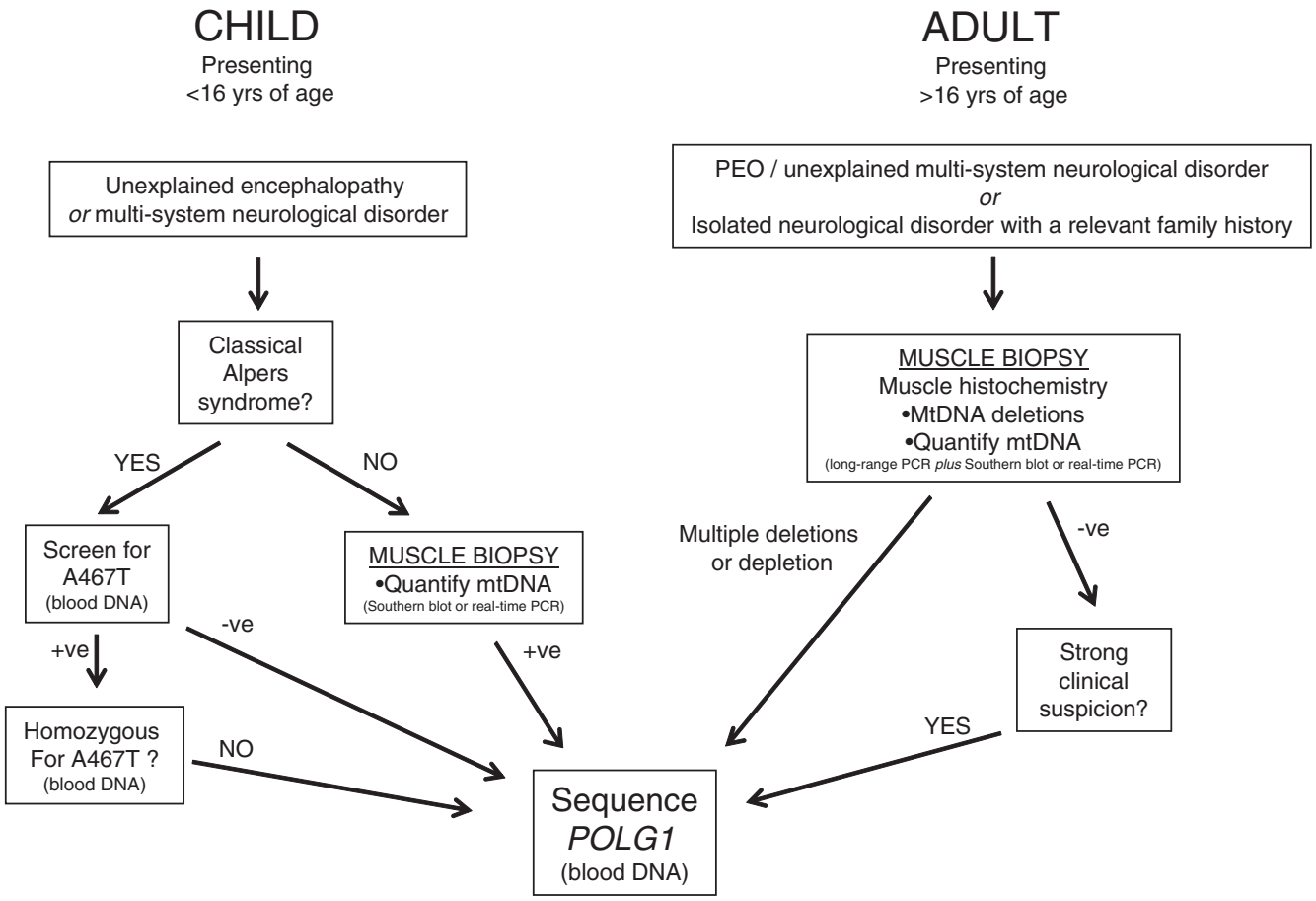

Fig. 2 Algorithm for the investigation of suspected *POLG1* mutations. Children (presenting at <16 years of age) with an unexplained encephalopathy or multi-system neurological disorder should be investigated for a possible *POLG1* mutation. If the child has classical Alpers–Huttenlocher syndrome (encephalopathy with refractory seizures, developmental delay and liver failure), then a blood sample should be screened for the 1399G→A (A467T) substitution. If the patient is heterozygous for A467T, then *POLG1* should be sequenced in blood DNA. If the patient is homozygous for A467T, this confirms the diagnosis. It would be prudent to sequence *POLG1* in a subject homozygous for A467T to determine whether there are additional mutations/substitutions that could influence genetic counselling. If the child does not have classical Alpers syndrome, then a muscle biopsy is indicated, and muscle mtDNA should be quantified by Southern blot or real-time PCR. *POLG1* should be sequenced if there is evidence of mtDNA depletion. It is possible to quantify mtDNA in other affected tissues (e.g. a liver biopsy or post-mortem brain tissue), but the results are often difficult to interpret because of a lack of control data. Adults (presenting >16 years of age) should have a muscle biopsy if they have PEO, an unexplained multi-system neurological disorder, or an isolated neurological disorder (such as ataxia, epilepsy or myopathy) with a family history suggesting mitochondrial disease. Muscle mtDNA should be analysed for mtDNA deletions and mtDNA depletion. Long-range PCR can be used to screen for mtDNA deletions but the preferential amplification of smaller deleted mtDNA molecules means that this technique is not quantitative. mtDNA deletions develop in skeletal muscle as part of normal ageing. The presence of significant levels (>5%) of deleted mtDNA should therefore be confirmed by Southern blot or real-time PCR. *POLG1* should be sequenced in patients with significant depletion or multiple deletions of mtDNA. Some patients with *POLG1* mutations have normal skeletal muscle histochemistry and no evidence of a secondary mtDNA abnormality in skeletal muscle (Van Goethem *et al.*, 2004). If there is therefore a strong clinical suspicion of *POLG1* related disease (such as PEO, or a family history consistent with the disorder), then *POLG1* should be sequenced in these individuals when the muscle histochemistry and muscle mtDNA analysis is normal. *POLG1* sequencing is commercially available (March 2003). Details can be found on www.genetests.org and www.ednal.com. If *POLG1* sequencing is negative, then other nuclear genes should be screened, including *C10ORF2* and *ANT1*. +ve = positive. −ve = negative.

Collecting clinical and molecular data from 38 new cases with *POLG1* mutations highlights the complexity of genetic counselling for this group. This is illustrated by linker-region mutation A467T, which can cause a severe fatal childhood encephalopathy as a compound heterozygote with a mutation in the polymerase domain, but possibly a milder phenotype in homozygotes. In one juvenile homozygous individual A467T/A467T, isolated epilepsy was the only feature at 20 years of age. This has been observed before in subjects with a homozygous linker-region mutation, but additional features developed later in life (Hakonen *et al.*, 2005). A467T was found in control subjects in some populations, and the parents of the affected children in this study were clinically normal. However, clinical genetic and functional studies suggest that A467T can also behave as a dominant mutation causing a mild phenotype of late-onset ptosis (Luoma *et al.*, 2005). This observation, coupled with emerging evidence that *POLG1* contains putative genetic modifiers, which are common in the background population (Hakonen *et al.*, 2005), makes genetic counselling difficult for families with more than two substitutions. Counselling the family with four *POLG1* mutations will be particularly challenging. Further clinical and functional studies will help resolve these issues.

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