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# Molecular analysis of *ANT1*, *TWINKLE* and *POLG* in patients with multiple deletions or depletion of mitochondrial DNA by a dHPLC-based assay

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*ANT1*, *TWINKLE* and *POLG* genes affect mtDNA stability and are involved in autosomal dominant PEO, while mutations in *POLG* are responsible for numerous clinical presentations, including autosomal recessive PEO, sensory ataxic neuropathy, dysarthria and ophthalmoparesis (SANDO), spino-cerebellar ataxia and epilepsy (SCAE) or Alpers syndrome. In this study, we report on the mutational analysis of *ANT1*, *TWINKLE* and *POLG* genes in 15 unrelated patients, using a dHPLC-based protocol. This series of patients illustrates the large array of clinical presentations associated with mtDNA stability defects, ranging from isolated benign PEO to fatal Alpers syndrome. A total of seven different mutations were identified in six of 15 patients (40%). Six different recessive mutations were found in *POLG*, one in *TWINKLE* while no mutation was identified in *ANT1*. Among the *POLG* mutations, three are novel and include two missense and one frameshift changes. Seventeen neutral changes and polymorphisms were also identified, including four novel neutral polymorphisms. Overall, this study illustrates the variability of phenotypes associated with mtDNA stability defects, increases the mutational spectrum of *POLG* variants and provides an efficient and reliable detection protocol for *ANT1*, *TWINKLE* and *POLG* mutational screening.

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## Introduction

Adenine nucleotide translocator 1 (*ANT1*; OMIM #103220), mitochondrial replicative helicase *Twinkle* (OMIM #606075) and *polymerase gamma* (*POLG*; OMIM #174763) genes affect mtDNA stability, and their mutations cause autosomal dominant progressive external ophthalmoplegia (adPEO).<sup>1–3</sup> *POLG* mutations have also been found in autosomal recessive PEO (arPEO) and it is thought that

**Table 1** Clinical features of patients

Patient	Sex	Age (years)	Onset (years)	Family history	Ptois/PEO	Other symptoms	MtDNA alterations
PA	F	63	50	Sporadic	+	None	Multiple deletions in muscle
PB <sup>†</sup>	M	15	8	<b>Sporadic</b>	+	<b>SANDO</b>	//
PC	M	63	40	<b>Sporadic</b>	+	<b>Weakness of lower limbs, ataxia, cognitive impairment</b>	//
PD	M	57	56	Sporadic	+	Exercise intolerance, dysphagia, diabetes, sensory axonal neuropathy	//
PE <sup>†</sup>	M	4	3	<b>Recessive</b>	–	<b>Alpers syndrome</b>	Depletion in liver
PF	M	82	78	Sporadic	+	Myopathy, dysphagia, dysphonia, ataxia, tremor, hypoacusia, diabetes	Multiple deletions in muscle
PG	F	45	18	<b>Dominant</b>	+	<b>Myopathy, dysphagia, dysphonia, dyspnea</b>	//
PH	F	52	Ch	Recessive	–	Myopathy	//
PI	M	62	58	Dominant	–	Myopathy, ataxia, frontal dementia	//
PJ	F	63	54	Dominant	–	Optic atrophy, myopathy, ataxia, sensory axonal neuropathy	//
PK	M	54	18	Sporadic	+	Cardiomyopathy, myopathy, strokes, epilepsy	//
PL	F	27	20	<b>Sporadic</b>	+	<b>SCAE</b>	//
PM	F	30	5	Sporadic	–	Epilepsy, bilateral deafness, sensory-motor axonal neuropathy	//
PN <sup>†</sup>	F	Nb	Nb	Sporadic	–	Hepatocerebral failure	Depletion in liver
PO <sup>†</sup>	F	15 m	7 m	<b>Sporadic</b>	–	<b>Alpers syndrome</b>	//

PEO: progressive external ophthalmoplegia; SANDO: sensory ataxic neuropathy dysarthria ophthalmoplegia syndrome; SCAE: spinocerebellar ataxia epilepsy syndrome; <sup>†</sup>: Died; m: months; Ch: childhood; Nb: newborn; (+): present; (–): absent; //: same as upper. Patients for whom the disease causing mutations were identified are in bold.

mutations in this gene could explain 45% of sporadic or familial PEO cases with multiple mtDNA deletions.<sup>4</sup> Additional clinical presentations comprising mtDNA deletions have been described recently. They include autosomal recessive sensory ataxic neuropathy with dysarthria and ophthalmoplegia (SANDO), which is associated with mutations in either *POLG*<sup>5</sup> or *TWINKLE*<sup>6</sup> genes, parkinsonism with premature menopause, mitochondrial recessive ataxia syndrome (MIRAS), and juvenile spino-cerebellar ataxia-epilepsy syndrome (SCAE), all associated with *POLG* mutations.<sup>7,8</sup> Furthermore, mutations in *POLG* have also been associated with mtDNA depletion in patients affected by Alpers syndrome or by early hepatocerebral failure.<sup>9–11</sup> In order to improve the molecular diagnosis in such patients, we have developed a new dHPLC assay for the complete screening of *ANT1*, *TWINKLE* and *POLG* coding regions and exon–intron boundaries. We report here the results of this screening in a series of 15 patients, illustrating the heterogeneity of phenotypes associated with mtDNA instability. All selected patients carried either multiple mtDNA deletions in muscle or mtDNA depletion in liver (Table 1).

## Materials and methods

### Subjects

The clinical findings and family histories are summarized in Table 1. The pedigrees are given in Figure 1.

### DNA extraction and PCR conditions

Blood samples were obtained from patients and all available family members after informed consent was

given. Genomic DNA was extracted from leukocytes by standard procedure. *POLG*, *ANT1* and *TWINKLE* genes were PCR amplified with primers listed in Supplementary Tables 1, 2 and 3. Different Taq DNA polymerases were used in order to obtain dHPLC best results for each amplified exon (Supplementary Table 4).

### Cloning of wild-type DNA fragments

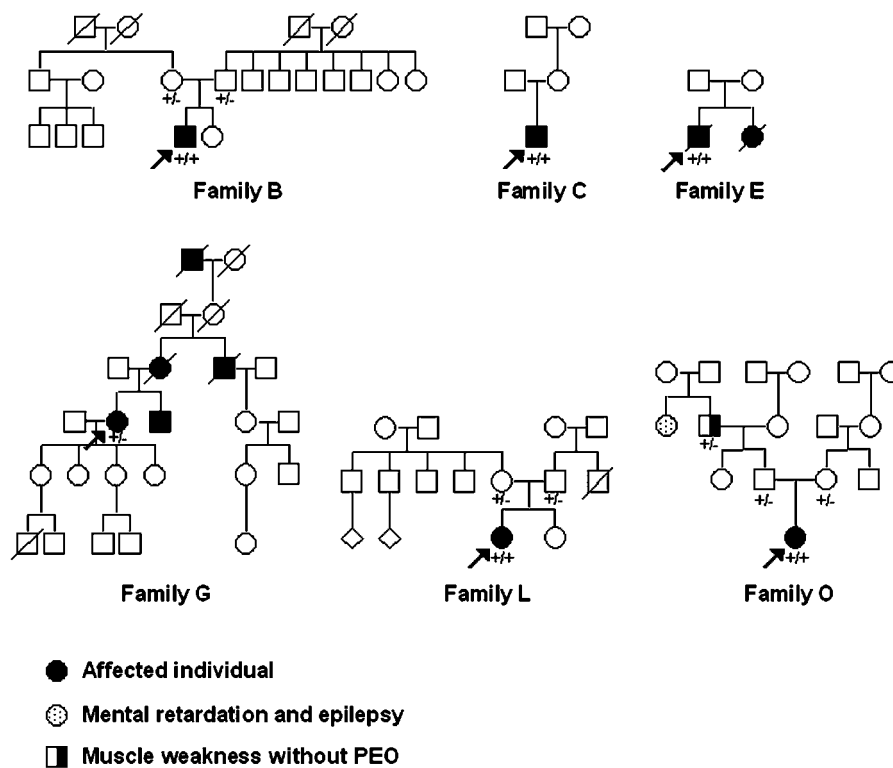
To detect homozygous variants, we generated wild-type DNA fragments. Each PCR product of *ANT1*, *TWINKLE* and *POLG* genes, resulting from control individuals, were subcloned into pGEM-T Easy Vector (Promega, Charbonnières-Les-Bains, France). All wild-type clones were sequenced and then, used as controls in dHPLC.

### dHPLC analysis

The dHPLC system used in this study is a Transgenomic Wave Nucleic Acid Fragment Analysis System (Transgenomic<sup>™</sup>, Crewe, UK). Optimal buffer gradients and mobile phase temperatures, which were determined by the Transgenomic software (Navigator<sup>™</sup> software) are indicated in Supplementary Tables 1, 2 and 3. For homozygous variant detection, PCR products amplified from patients were combined with equal amounts of PCR products amplified from control DNA.

### Sequencing analysis

PCR fragments were purified on Montage PCR columns (Millipore SA, Saint-Quentin, France) and sequenced on a ABI 310 automated sequencer with D-Rhodamine cycle



**Figure 1** Pedigrees of six patients with *POLG* or *TWINKLE* mutations. +: presence of *POLG* mutation; -: absence of *POLG* mutation (families B, C, E, L, O); +: presence of *TWINKLE* mutation; -: absence of *TWINKLE* mutation (family G).

**Table 2A** Mutations detected in the study

Patient	Gene	Nucleotide change	Amino-acid change	Type of mutation
B	<i>POLG</i>	[c.911T>G]*	[L304R]*	Missense
C	<i>POLG</i>	<b>c.1139G&gt;A</b> ; c1399G>A	<b>G380D</b> ; A467T	<b>Missense</b> ; Missense
E	<i>POLG</i>	<b>c.975_976insC</b> ; c1399G>A	<b>T326fsX387</b> ; A467T	<b>Frameshift/Truncation</b> ; Missense
G	<i>TWINKLE</i>	c.1121G>A	R374Q	Missense
L	<i>POLG</i>	c.911T>G; c.2243G>C	L304R; W748S	Missense; Missense
O	<i>POLG</i>	<b>c.2740A&gt;C</b> ; c1399G>A	<b>T914P</b> ; A467T	<b>Missense</b> ; Missense

Homozygous variations are indicated with an asterisk.  
New sequence variations are in bold face.

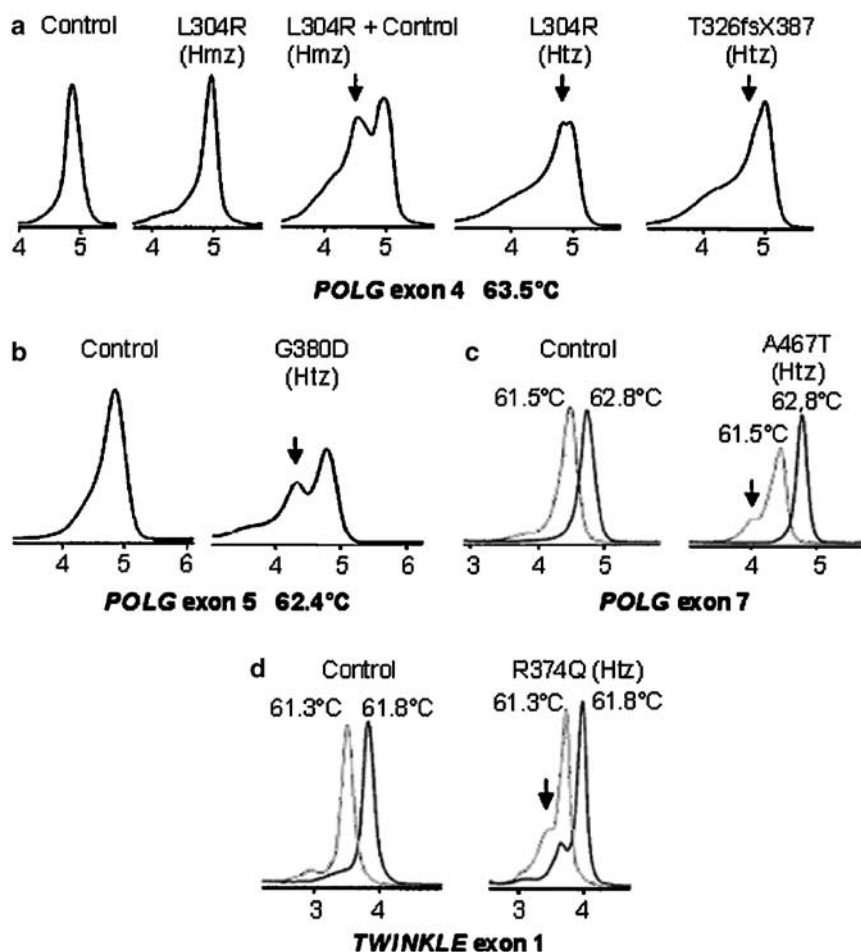
sequencing ready reaction kit (PE Applied Biosystems, Foster City, CA).

## Results and discussion

Our principal aims were to determine the *ANT1*, *TWINKLE* and *POLG* mutational spectrum in a series of patients, and to develop an efficient molecular test for mutational analysis of genes involved in mtDNA stability. The entire *ANT1*, *TWINKLE* and *POLG* coding regions and exon-intron boundaries were screened by dHPLC in patients with neuromuscular symptoms. *POLG* only was analyzed in the three children deceased from hepatocerebral failure. All fragments giving an abnormal dHPLC profile were

further analyzed by direct sequencing and a total of seven different probable pathogenic alleles were identified in six patients (Table 2A). Some of the dHPLC elution profiles are presented in Figure 2. Interestingly, the detection of A467T mutation in exon 7 of *POLG* was seen with one temperature only (Figure 2c). Several additional polymorphisms were also identified (Table 2B). In order to evaluate the reliability of our method, all fragments giving a normal dHPLC profile were also systematically sequenced and no discrepancy was observed between sequencing and dHPLC analysis.

Five patients out of 15 (33%) had *POLG* recessive mutations. *POLG* contains two domains, a DNA polymerase and a 3'-5' exonuclease domain separated by a conserved inter-domain region known as spacer region.<sup>12,13</sup>



**Figure 2** dHPLC elution profiles of four *POLG* and one *TWINKLE* mutations. Patient status is indicated above elution profiles. DNA-heteroduplexes are indicated by an arrow. Amplicons and oven temperature are indicated. Hmz: Homozygous; Htz: Heterozygous. (a) Elution profiles of *POLG* exon 4 amplicons. From left to right: amplicon obtained from a control; amplicon from patient B, alone and mixed with a wild-type amplicon; amplicons from patient L and E, respectively. (b) Elution profiles of *POLG* exon 5 amplicons. Amplicon from a control (left) and patient C (right). (c) Elution profiles of *POLG* exon 7 amplicon. Amplicon from a control (left) and patient E (right) at 61.5°C (gray) and 62.8°C (black). (d) Elution profiles of *TWINKLE* exon 1. Amplicon from a control (left) and patient G (right) at 61.3°C (gray) and 61.8°C (black).

Patient B, who presented with a SANDO phenotype, resulted to be homozygous for the c.911T>G mutation leading to a L304R amino-acid change (Table 2A). This highly conserved amino acid is located between exonuclease domains II and III of *POLG*. This mutation has already been reported in arPEO Belgian families presenting with features of the SANDO syndrome, but only in combination with the c.1399G>A substitution (A467T).<sup>5</sup> We describe, for the first time, the L304R mutation in a homozygous state in a patient presenting with a SANDO phenotype.

The A467T mutation was found in a heterozygous state in patient C, in combination with a new c.1139G>A substitution. This new mutation leads to a G380A amino-acid change affecting a highly conserved residue lying between the exonuclease motifs *ExoII* and *ExoIII*. This patient, who presented with a PEO phenotype, was a

sporadic case and the pathogenicity of this new mutation was impossible to be assessed by segregation analysis. Nevertheless, we did not find the c.1139G>A substitution (G380D) in 300 healthy french control chromosomes. The A467T substitution was also found in patient E, in combination with a new c.975\_976insC mutation, which leads to a premature termination codon (T326fsX387). The A467T mutation, which maps within the conserved  $\gamma 1$  element of *POLG* spacer, has already been described in Alpers disease in a homozygous state or in combination with other mutations.<sup>10,11</sup> Another patient, who presented with an Alpers syndrome (patient O) also carried the A467T mutation in combination with a new c.1399G>A substitution, leading to a highly conserved T914P amino-acid change, between DNA polymerase domains A and B. The first mutation was inherited from the father, while the second was inherited from the mother. Recently, it has

**Table 2B** Polymorphisms and variants detected in the study

Location	Nucleotide change	Amino-acid change	Patient	NCBI SNP ID
<i>POLG</i>				
Exon 2	c.156_157insCAG	—	K	
	<b>c.159A&gt;G</b>	<b>Q53Q</b>	B*	
Intron 2	c.660 -46G>A	—	F	rs3176170
Exon 5	<b>c.1126C&gt;T</b>	<b>L376L</b>	J	
Intron 6	c.1250 -43C>T	—	J	rs2307444
Exon 10	<b>c.1780C&gt;T</b>	<b>L594L</b>	K	
Intron 11	c.2071 -22T>C	—	B*, E, F, J*, K*	rs2072267
Intron 12	c.2157 +92T>C	—	B*, D, E, I, J*, K	rs2072266
Exon 13	<b>c.2254C&gt;T</b>	<b>L752L</b>	I	
Intron 17	c.2734 +39_40insGTAG	—	B*, D*, E, F, I, J*, K*	rs2307433
Intron 19	c.3105 -11T>C	—	D, I, J*, K	rs2302084
	c.3105 -36A>G	—	B*, D, I, J, K	rs2246900
Exon 21	c.3428A>G	E1143G	L	
Intron 21	c.3483 -19T>G	—	D, I, J, K	rs2307438
Exon 23	c.3708G>T	Q1236H	J	rs3087374
3'UTR	c.3769_3770insG	—	B*, D, E, I*, J, K	rs3087377
<i>TWINKLE</i>				
Exon 1	c.1102G>A	V368I	K	rs17113613

*POLG* and *TWINKLE* polymorphisms and sequence variants detected in patients.

Novel variants appear in bold type.

Patients who carry the variant in a homozygous state are marked with an asterisk.

The references of variants already described are indicated. Single nucleotide polymorphism (SNP) database: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=snp>.

been shown that a single allele with the A467T substitution may manifest in a dominant manner, leading to late-onset ptosis.<sup>14</sup> In our study, pedigree O analysis is consistent with this observation since the paternal grandfather, who is heterozygous for A467T, presented with muscular weakness but without PEO symptomatology (Figure 1). Finally, patient L was found to be a compound heterozygous carrying the c.911T>G (L304R) mutation in combination with the recently described c.2243G>C substitution (W748S). Our patient carried the W748S mutation *in cis* with a c.3428A>G (E1143G) variant. The L304R mutation was inherited from her mother while the W748S mutation, associated with E1143G, was inherited from her father. The W748S mutation is located in the conserved  $\gamma 4$  region of the *POLG* spacer, has been associated with SCAE, Alpers and infantile hepatocerebral syndromes,<sup>7,9,10,15</sup> and has always been described in combination with the E1143G variant *in cis*. From a practical point of view, the case of this young woman illustrates the interest of molecular analysis in mitochondrial diseases. She was pregnant and the identification of two *POLG* mutations *in trans* allowed us to give a reassuring genetic counselling.

One patient harbored a *TWINKLE* mutation which has already been described.<sup>1</sup> Patient G was found to carry a c.1121G>A transition, predicting a highly conserved R374Q amino-acid change of the *TWINKLE* protein sequence. R374Q was not found in the patient's daughter, the only healthy individual available in the pedigree. In our study, the R374Q mutation is associated with severe respiratory failure due to respiratory muscle weakness

(pedigree G). No other *TWINKLE* or *ANT1* mutations were found in the remaining pedigrees.

Overall, this study, which illustrates the variability of phenotypes associated with mtDNA stability defects, increases the mutational spectrum of *POLG* variants and provides an efficient and reliable detection protocol for *ANT1*, *TWINKLE* and *POLG* mutational screening.

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**Conflicts of interest declared:** none

#### Databases:

*ANT1* – OMIM: 103220; 609283 (PEOA2); GenBank: NT\_022792.  
*TWINKLE* – OMIM: 606075; 609286 (PEOA3), 607459 (SANDO); GenBank: NT\_030059, AF29004.  
*POLG* – OMIM: 174763; 157640 (PEOA1), 258450 (PEOB), 607459 (SANDO), 203700 (PNDC), 251880 (MDS); GenBank: NT\_033276, NM\_002693.

**Website:** <http://dir-apps.niehs.nih.gov/polg/index.cfm>

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Supplementary Information accompanies the paper on European Journal of Human Genetics website (<http://www.nature.com/ejhg>)