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Infantile hepatocerebral syndromes associated with mutations in the mitochondrial DNA polymerase-γA

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Summary

We studied nine infant patients with a combination of progressive neurological and hepatic failure. Eight children, including two sibling pairs and four singletons, were affected by Alpers' hepatopathic poliodystrophy. A ninth baby patient suffered of a severe floppy infant syndrome associated with liver failure. Analysis of *POLG1*, the gene encoding the catalytic subunit of mitochondrial DNA polymerase, revealed that all the patients carried different allelic mutations in this gene. *POLG1* is a major disease gene in mitochondrial disorders. Mutations in this gene can be associated with multiple deletions, depletion or point mutations of mitochondrial DNA (mtDNA). In turn, these different molecular phenotypes dictate an extremely heterogeneous spectrum of clinical outcomes, ranging from adult-onset progressive ophthalmoplegia to juvenile ataxic syndromes with epilepsy, to rapidly fatal hepatocerebral presentations, including Alpers' syndrome.

Keywords: *POLG*; mitochondrial DNA polymerase; Alpers' hepatopathic poliodystrophy; valproate toxicity; mtDNA depletion

Abbreviations: ad = autosomal dominant; ar = autosomal recessive; MDS = mitochondrial DNA depletion syndrome; mtDNA = mitochondrial DNA; PCR = polymerase chain reaction; PEO = progressive external ophthalmoplegia; *POLG1* = gene encoding mitochondrial DNA polymerase-γA; pol-γA = DNA polymerase-γA; SANDO = sensory atactic neuropathy with dysarthria and ophthalmoplegia

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Introduction

Mutations in *POLG1*, the gene encoding the catalytic subunit of mitochondrial DNA (mtDNA) polymerase (pol-γA) are the prevalent cause of autosomal dominant (ad) or autosomal recessive (ar) forms of familial progressive external ophthalmoplegia (PEO) syndromes (Van Goethem *et al*., 2001; Lamantea *et al*., 2002; Agostino *et al*., 2003). However, additional clinical presentations associated with *POLG1* mutations have been reported recently, including autosomal recessive sensory atactic neuropathy with dysarthria and ophthalmoplegia (SANDO) (Van Goethem *et al*., 2003*a*), a juvenile-onset mixed sensory and cerebellar atactic syndrome complicated by epileptic seizures and myoclonus, (Van Goethem *et al*., 2004; Winterthun *et al*., 2005) and, very recently, Alpers' hepatopathic poliodystrophy (Naviaux and Nguyen, 2004). The latter is an early-onset, fatal disease, characterized by hepatic failure, intractable seizures and global neurological deterioration (OMIM #203700). It is commonly believed that the main target of *POLG1* mutations is mtDNA, and that lesions in this genome may ultimately determine the clinical phenotypes. For instance, both adPEO

and arPEO are characterized by the accumulation of multiple mtDNA rearrangements (mainly deletions) in post-mitotic tissues, notably skeletal muscle and brain (Zeviani *et al*., 1990; Servidei *et al*., 1991; Suomalainen *et al*., 1992; Moslemi *et al*., 1999). In contrast, much lesser amounts of mtDNA rearranged molecules are detected in the muscle tissue of patients affected by SANDO or sensory–cerebellar ataxia–epilepsy syndrome, but no information is available for these conditions on the integrity of mtDNA in the critical tissues (i.e. cerebellum, dorsal spinal ganglia and spinal cord). Finally, depletion of liver mtDNA, rather than accumulation of multiple deletions, has been reported in the case with Alpers' syndrome associated with a homozygous *POLG1* stop mutation (Naviaux *et al*., 1999; Tesarova *et al*., 2004).

We describe here nine patients (two sibling pairs and five singleton cases) with *POLG1* mutations associated with combined infantile fatal encephalopathy and hepatopathy. The two sibling pairs and four singletons were patients with typical Alpers' syndrome, while the ninth patient was affected by a severe floppy infant syndrome with signs of hepatic failure.

Patients and methods

All patients were of Caucasian origin. The families of patients 5, 6 and 7 (see below) were from Germany, and those of the other patients were from Italy.

Patient 1

Patient 1 was a boy, born at term from non-consanguineous Italian parents. He developed normally in the first months after birth, but head control was reached at 6 months, and autonomous deambulation at 14 months. An apparently myogenic torticollis was noticed after a few months of life; at 10 months, he had two episodes of sudden head drop, followed by the onset of psychomotor arrest/regression, hypotonia, ataxia and focal myoclonus of the right upper limb. In the following weeks, the myoclonus became subcontinuous, and was associated with rapidly progressive, severe generalized hypotonia and weakness requiring ventilatory assistance. Seizures were partially controlled with a combination of topiramate, clobazam and phenylbarbiturate. The EEG worsened in the following weeks, with diffuse disorganization of the basal activity and multiple foci of paroxysmal activity. A brain MRI showed symmetrical lesions of the basal ganglia, thalami, cerebellar dentate nuclei and left occipital cortical and subcortical regions (Fig. 1A and B). Proton magnetic resonance spectrometry (MRS) revealed an abnormal accumulation of lactic acid in the putamen and a reduction of the *N*-acetyl aspartate (NAA) peak, an index of neuronal loss. Light microscopy examination and the activities of the respiratory chain complexes were both normal in a muscle biopsy, while no liver and skin biopsies were taken. In the following months, increasingly high levels of γ-glutamyltransferase and transaminases, severe, predominantly cholestatic jaundice, hypoglycaemia and hypocoagulation were accompanied by dramatic worsening of the neurological symptoms, leading to the death of the child at 30 months of age. Autoptic examination was refused by the parents. The diagnostic conclusion was Alpers' syndrome.

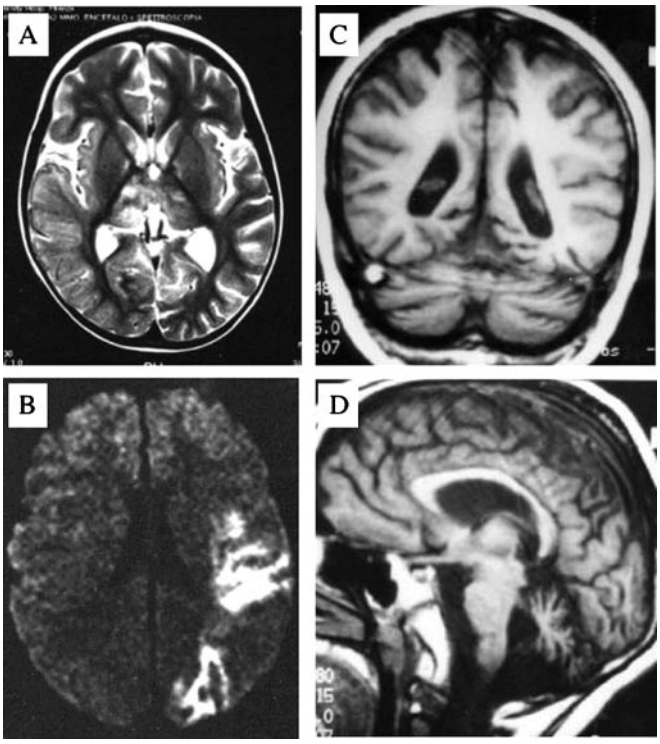

Fig. 1 Brain MRI of patients 1 (**A** and **B**) and 3 (**C** and **D**). (**A**) T2-weighted transversal section showing bilateral cortical atrophy more evident in the parietal lobe and peri-silvian regions. Bilateral lesions are present in thalamus, globus pallidus and caudate nucleus. The internal capsule and surrounding white matter are relatively normal. (**B**) Diffusion-weighted transversal section showing two areas of lesion in the left parieto-occipital region. (**C**) T1-weighted coronal section showing bilateral dilation of lateral ventricles and cortical atrophy. (**D**) T1-weighted inter-hemispheric sagittal section showing atrophy of both cerebral cortex and cerebellar vermis.

Patient 2

The elder sister of patient 1 had a very similar clinical course, characterized by a disease-free interval in the first 4 months after birth, followed by persistent vomiting, epileptic crises with loss of eye contact, and myoclonus in the upper limbs with secondary generalization and loss of consciousness. Similarly to her brother, she developed a fixed flexion of the neck with rotation toward the left side. Persistent myoclonus in the upper limbs was associated with profound hypotonia and loss of eye contact. A brain MRI at 5 months of age showed the presence of cortical atrophy and leukoencephalopathy in the subcortical areas of the frontal lobes. High levels of blood γ-glutamyltransferase and transaminases were firstly documented after the administration of valproate for the control of myoclonic seizures. The activities of the mitochondrial trifunctional protein (a component of the fatty acid β-oxidation pathway) and those of the respiratory chain enzymes were normal in cultured skin fibroblasts and muscle homogenate; unfortunately, liver tissue was not available for biochemical studies. A rapidly progressive hepatic failure with enlarged liver, hypoalbuminaemia and predominantly cholestatic jaundice led to the death of the child at 8 months of age. The autopsy showed diffuse encephalomalacia and severe liver steatosis with lobular fibrosis and bile ductule proliferation.

Patients 3 and 4

The clinical and neuropathological features of these two brothers, born from non-consanguineous Italian parents, have already been reported elsewhere (Simonati *et al*., 2003*a*). Briefly, patient 3 had a slowly progressive history with onset at 19 months, characterized by ataxia, spasticity and myoclonic seizures. At 9 years of age, atrophy of the occipital cortex was shown by MRI (Fig. 1C and D), and hepatic cirrhosis was documented on a liver biopsy. The clinical picture worsened thereafter, leading to a vegetative state and eventually to death at 15 years. The clinical history of the younger brother (patient 4) started with myoclonic fits in the right limbs at 5 months, followed by an atactic–dystonic syndrome with rapid global deterioration. Diffuse cortical atrophy was documented by CT scan at 6 months of age, and the EEG showed multifocal spikes in the left fronto-temporal region on a slow background activity. At 12 months, valproate therapy was started because of refractory seizures; however, the patient developed acute hepatic failure and died 1 month later. The neuropathological examination of the brain showed severe atrophy of the anterior portions of the frontal lobes, insula and parieto-occipital cortex, and necrotizing lesions with neuronal loss, neuropil microcysts and vascular proliferation in the brain cortex, thalamus, cerebellum and inferior olives. In both patients, the diagnosis was Alpers' syndrome.

Patient 5

This patient was a baby boy, born from non-consanguineous German parents. He developed normally in the first 6 months. Refusal of food and failure to thrive were noted from the 7th month of life. At 1 year of age, increased transaminase levels in the blood were accompanied by liver enlargement and hyperecogenicity documented by ultrasound examination. Motor development was delayed. His condition gradually deteriorated, with recurrent hypoglycaemic episodes and lactic acidosis. A liver biopsy at the age of 16 months revealed steatosis and fibrosis. At 20 months of age, he had numerous episodes of status epilepticus and epilepsia partialis continua. An increase of lactate and a decrease of choline were detected by MRS of the brain. The patient died at 2.5 years of age during an episode of status epilepticus. Autopsy showed liver fibrosis with transition to liver cirrhosis, spongiform changes in the cerebrum, focal infarcts in the cerebellar cortex, and mesial temporal sclerosis. Abnormal mitochondria were found in liver, heart and brain, but the mtDNA was not available for molecular studies. The diagnostic conclusion was Alpers' syndrome.

Patient 6

The clinical and neuroradiological features in this male patient, diagnosed as typical Alpers' syndrome, have already been reported (Flemming *et al*., 2002). He was born from non-consanguineous German parents. Mental and motor developmental delay was first noted at 4 years of age. At 7 years, generalized tonic–clonic seizures, myoclonus and recurrent focal motor seizures evolving into epilepsia partialis continua started after an operation for correction of a squint. An otherwise intractable focal motor status was treated with valproate, which was followed by life-threatening hepatopathy. The boy became blind and incontinent, and lost independent ambulation. He is now, at age 13 years, in a vegetative state.

Patient 7

The first child of non-consanguineous parents from Germany, this boy was born at term after a normal pregnancy and delivery. After a brief disease-free interval, mild motor retardation was first noted at 6 months. At the age of 16 months, he was admitted to a local hospital for status epilepticus. An MRI performed at 18 months of age showed mild generalized brain atrophy. He was treated with valproate, but during the following days he developed severe hepatopathy with an increase of transaminases in the blood, hypocoagulation and severe mixed jaundice. His neurological condition evolved into epilepsia partialis continua, which was partially controlled by carbamazepine, later switched to oxcarbazepine. A brain MRI showed hyperintense signals in the brain cortex, in both thalami and in the periventricular area of the mesencephalon. The MRS showed a lactate peak and a decrease of the choline peak. The blood lactate concentration was also increased. A diagnosis of Alpers' syndrome was given to this patient. He died at 2 years of age after severe complications from liver failure.

Patient 8

This male patient has already been reported as a juvenile case of Alpers' syndrome with central–peripheral axonopathy (Simonati *et al*., 2003*b*). He had recurrent vomiting episodes in infancy. At age 7 years, he developed epilepsia partialis continua, followed by progressive ataxia. At 18 years, neurological examination revealed sensory ataxia, absent deep tendon reflexes, cerebellar dysfunction, nystagmus, a peripheral vision defect and a pale optic disc. The blood lactate concentration was increased. An MRI showed mild brain cortical and cerebellar atrophy, with focal hyperintensity signals in the frontal and occipital cortex and in both thalami. Electrophysiological studies revealed the presence of abnormal central and peripheral conduction, and a sural nerve biopsy showed marked reduction of the myelinated fibres. The following months were characterized by numerous episodes of partial and generalized seizures and myoclonic fits, which were barely responsive to therapy. The administration of valproate to control myoclonus was followed by the development of severe acute hepatic failure, which required liver transplantation. However, relentless deterioration of the neurological conditions led to the patient's death at 19 years of age.

Patient 9

A baby girl, the second child of non-consanguineous Italian parents, was born at the 38th week by caesarian delivery after an otherwise uneventful pregnancy. However, both the patient and her healthy 4-year-old brother were conceived by *in vitro* fertilization because of hypofertility of the parents. The father was diagnosed as having severe astheno-terato-zoospermia, while the mother, at age 36 years, was found to have mild hypergonadotrophic hypogonadism with reduced levels of dehydroepiandrosterone (75.5 μg%, normal 120–360) and levels of follicle-stimulating hormone (FSH) in the upper part of the normal range (6.5 IU/l, normal 1.1–9.6), as typically found in pre-menopausal women. The body weight of the patient at birth was 2760 g, body length was 49 cm and head circumference was 34 cm. After a short disease-free interval, she started having frequent vomiting with increasing feeding difficulty leading to growth arrest at 5 months of age. From the third month of life, the patient suffered from progressive generalized hypotonia, with some signs of psychic regression but no loss of eye contact. The *SMN1* gene, responsible for the most common forms of hereditary spinal muscular atrophy (SMA) syndromes, including SMA type 1, was normal. Laboratory examination disclosed high levels of serum transaminases and γ-glutamyltransferase, high levels of lactate and pyruvate in both serum and CSF, and normal levels of blood glucose

and ammonia. High levels of lactic acid and dicarboxylic acids were found in the urine. An ultrasound examination of the abdomen showed a hyperecogenic liver with cholestasis and cholelithiasis, and moderate ascites, with no alteration of the kidneys and spleen. The ultrasound examination of the heart was normal. A brain MRI was normal as well, but the EMG examination showed a diffuse, marked reduction of the nerve conduction velocities (3.4 m/s in the left median nerve; 6.0 m/s in the right common peroneal nerve, normal values for a 5-month-child 30 m/s), suggesting the presence of a severe hypomyelinating peripheral neuropathy, accounting for the profound hypotonia, generalized weakness and deep tendon areflexia. No other abnormality was detected on the EMG. The visual evoked potentials were normal for the age. Activities of arylsulfatase A and cerebroside-β-galactosidase were normal in blood leukocytes. Light microscopy examination of a muscle biopsy did not disclose significant alterations. Biochemical investigation on the homogenate from a liver biopsy revealed the presence of multiple defects of the mtDNA-related respiratory chain activities (∼30% of age-matched controls), while no abnormality was found in muscle homogenate and in cultured fibroblasts. The child died at 6 months of age of ventilatory insufficiency due to generalized muscle weakness and profound hypotonia. She had persistent elevation of serum transaminases and γ-glutamuyltransferase, but no jaundice, hyperammonaemia, hypoglyacemia, abnormalities of blood coagulation or other signs of overt hepatic failure. No autopsy was performed.

Methods

Biochemical analysis of the respiratory chain complexes was performed on the 800 *g* supernatants obtained from muscle or liver homogenates and on digitonin-treated cultured skin fibroblasts, as previously described (Bugiani *et al*., 2004).

DNA extracted from fibroblasts, skeletal muscle, brain and liver was used for quantification of mtDNA versus nuclear DNA by real-time polymerase chain reaction (PCR) (He *et al*., 2002; von Wurmb-Schwark *et al*., 2002). Specific fluorochrome-labelled oligonucleotides (Applied Biosystems) were used as probes in real-time PCR experiments. In some cases, DNA was extracted from paraffin-embedded, formalin-fixed specimens of probands and controls, as described (Wright and Manos, 1990). Whenever possible, quantitative Southern blot analysis was also performed, as described (Zeviani *et al*., 1989). In both real-time PCR and Southern blot analyses, the amount of mtDNA was then compared with the amount of the nuclear gene cluster encoding the 18S rRNA on chromosome 21, contained in the same sample. The mtDNA/18S rDNA ratio obtained in the patients' samples was expressed as a percentage of the mean obtained in control samples, taken as 100%.

DNA from fibroblasts or lymphocytes was used as a template to amplify the 23 exons of the *POLG1* gene, as described (Lamantea *et al*., 2002). PCR fragments were analysed by automated nucleotide sequencing (Applied Biosystems).

Results

Analysis of mtDNA

Southern blot and real-time quantitative PCR analyses on muscle and fibroblast mtDNA in patients 1–3 and 9 failed to show large-scale rearrangements or abnormalities of the mtDNA copy number.

No liver or brain DNA was available for patients 1 and 4–7. Total DNA was extracted from paraffin-embedded post-mortem liver tissue from patients 2 and 3 and from two age-matched post-mortem paraffin-embedded control liver specimens. The results of real-time PCR showed a reduction of the mtDNA/18S rDNA ratio ranging from 25 to 40% of the controls' mean in different determinations for both patients. Likewise, a 30% reduction in mtDNA content was obtained by real-time PCR assays on post-mortem frozen frontal cortex of patient 8, compared with similar specimens from two age-matched control individuals. Finally, profound depletion (3–5% of the controls' mean) was demonstrated repeatedly in bioptic liver of patient 9 by both Southern blot and real-time PCR analyses.

Analysis of the POLG1 gene

Mutations in the *dGK* or *TK2* genes, which are known to be responsible for some cases of liver or muscle mtDNA depletion syndromes (MDS) (Mandel *et al*., 2001; Saada *et al*., 2001), were first ruled out.

The recent identification of *POLG1* mutations in three children with Alpers' hepatopathic poliodystrophy belonging to two families (Naviaux and Nguyen, 2004) prompted us to investigate the *POLG1* gene in our patients. The results are reported in Table 1 and Fig. 2.

Patients 1 and 2 had two identical *POLG1* mutations, including (i) a 2243G→C transversion in exon 13, predicting a W748S change in the spacer region of the pol-γA protein; and (ii) the insertion of a cytosine at position 3630 of the *POLG1* cDNA (3630Cins) in exon 22. The first mutation was inherited from the father, while the second was inherited from

Table 1 *POLG1 mutations*

Patient no.	Mutations*	Gene position	Amino acid
1 and 2	2243G→C	Exon 13	W748S[+]
	3630Cins	Exon 22	Y1210fs1216X
3 and 4	731T→C	Exon 3	L244P
	2243G→C	Exon 13	W748S[+]
5	1399G→A	Exon 7	A467T
	2542G→A	Exon 16	G848S
6	1399G→A	Exon 7	A467T
	3482 + 2T→C	Exon 21/ intron 21	Splice site
7	1399G→A	Exon 7	A467T
	2869G→C	Exon 18	A957P
8	1399G→A	Exon 7	A467T
	1399G→A	Exon 7	A467T
9	694C→G	Exon 3	R232G
	752C→T–1760C→T[†]	Exon 3–Exon 10	T251I-P587L[†]

*The nucleotide and amino acid positions are indicated for both alleles of each patient according to the nomenclature of the human *POLG1* cDNA and pol-γA sequences in Lamantea *et al*. (2002); [+]Associated with the SNP 3428A→G (E1143G); [†]Syntenic mutations.

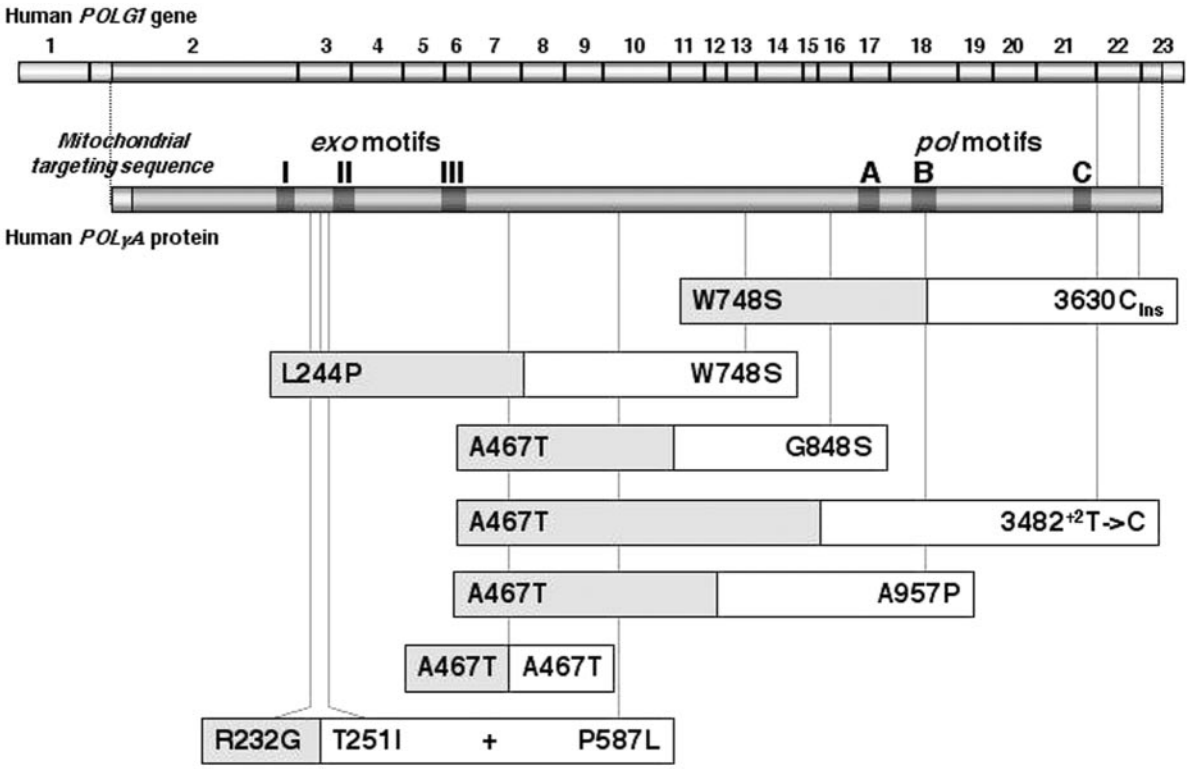

Fig. 2 *POLG1* mutations. Each pair of mutations is plotted against a scheme of the *POLG1* gene and corresponding protein. Exons of the gene are indicated by numbers; the ''exo'' and ''pol'' motifs indicate conserved regions in the exonucleolytic (proofreading) and polymerase (catalytic) domains of the protein.

the mother. Both parents were heterozygous carriers for the corresponding mutations.

Patients 3 and 4 were compound heterozygotes for the same (paternal) mutation 2243G→C (W748S) which was found in patients 1 and 2, and for a (maternal) 731T→C mutation, predicting a L244P change in the amino acid sequence. In both sibling pairs, the 2243G→C (W748S) mutation was syntenic to a known single-nucleotide polymorphism (SNP), 3428A→G, predicting an E1143G amino acid change. The latter is relatively frequent (3–4%) in Caucasians and can be found in homozygosity in normal adult individuals. The allele carrying both 2243G→C and 3428A→G has been reported in other *POLG1* mutant patients of European origin (Van Goethem *et al*., 2004; Winthertun *et al*., 2005).

Patient 5 was a compound heterozygote for a (paternal) 1399G→A mutation predicting an A467T amino acid change, and a (maternal) 2542G→A mutation predicting a G848S amino acid change. The *POLG1* gene in a healthy brother had neither mutation.

Patient 6 was a compound heterozygote for the same 1399G→A (A467T) mutation, which was inherited from the mother, and for a paternal 3482 + 2T→C splicing mutation affecting an invariant position in the 3′ exon/intron donor site of exon 21. A healthy brother had neither mutation.

Patient 7 was a compound heterozygote, again carrying the 1399G→A (A467T) mutation, which was inherited from the father, and a new maternal 2869G→C transversion predicting an A957P amino acid change.

Patient 8 was homozygous for the 1399G→A (A467T) mutation.

Finally, allelic mutations were also found in patient 9. The paternal mutant allele contained the *cis* mutations 752C→T in exon 3 and 1760C→T in exon 10, predicting a T251I and a P587L amino acid change in the same pol-γA protein molecule. The maternal mutant allele contained a 694C→G transversion in exon 3, predicting an R232G amino acid change. The parents were heterozygous carriers for the corresponding mutations; the healthy brother of the patient, now 4 years old, was also a heterozygous carrier for the paternal allele, while the maternal allele was normal.

Discussion

Clinical considerations

We report here the association of *POLG1* mutations with two severe infantile syndromes, both characterized by a combination of hepatic and neurological failure. The first syndrome meets the clinical and pathological hallmarks of Alpers' hepatopathic poliodystrophy (Blackwood *et al*., 1963; Huttenlocher *et al*., 1976), including (i) refractory seizures, in particular epilepsia partialis continua; (ii) progressive neurological deterioration with poliodystrophic changes; and (iii) progressive hepatic failure. The association between *POLG1* mutations and Alpers' syndrome was shown recently in two apparently unrelated families (Naviaux and Nguyen, 2004). We found *POLG1* mutations in eight out of 10 patients

that were diagnosed in, or referred to our Institute as affected by typical Alpers' syndrome over the past 10 years, suggesting that *POLG1* is a prevalent disease gene in this condition. The classification of Alpers' syndrome as a mitochondrial disorder has long remained controversial. This uncertainty may explain the scarcity of biochemical or molecular studies on mitochondrial metabolism carried out in patients with Alpers' syndrome, including most of those reported here. In the very few cases in which quantitative analysis of mtDNA was performed, a decrease of mtDNA content ranging from 10 to 30% of the normal mean was documented in liver and brain, while results in skeletal muscle were less consistent (Naviaux *et al*., 1999; Tesarova *et al*., 2004). Our own results suggest the presence of a partial decrease of mtDNA content in the only two liver specimens (from patients 2 and 3) that could be examined, although the value of these data is limited by the fact that they were obtained from, and compared with, formalin-fixed autoptic specimens, i.e. in conditions in which DNA can be damaged or degraded. We had more reliable results by studying a frozen brain autoptic specimen from patient 8, which showed a partial but clearly detectable reduction in mtDNA content compared with two control brains. Taken together, these findings suggest that tissue-specific, partial mtDNA depletion is indeed a molecular feature of Alpers' syndrome. However, more work is necessary to establish whether depletion alone can account for the damage to liver and brain typical of this condition, or if additional pathogenetic mechanisms, for example the accumulation of mtDNA point mutations, may cooperate with mtDNA depletion in determining the clinical phenotype.

The reported association of *POLG1* mutations and hepatic mtDNA depletion (Naviaux *et al*., 1999; Tesarova *et al*., 2004) prompted us to extend the analysis of the gene to patients with hepatocerebral MDS. A well-established cause of this syndrome is mutations in the *dGK* gene (Mandel *et al*., 2001); the clinical features are characterized by a more severe and rapid involvement of the liver compared with Alpers' syndrome (Filosto *et al*., 2004). Of the two *dGK*-negative patients with early onset, rapidly progressive hepatic MDS that were included in our study, only one patient (patient 9) resulted as a compound heterozygote for *POLG1* mutations. Her clinical phenotype was dominated by signs of progressive hepatic damage associated with profound depletion of mtDNA in liver and by early-onset muscle hypotonia. SMA1-like presentations have been reported in cases of muscle-specific MDS (Mancuso *et al*., 2002), and depletion of mtDNA associated with denervation has been documented in the skeletal muscle of SMA patients. However, no mtDNA depletion in either skeletal muscle or cultured fibroblasts was found in our patient 9, possibly because of the rapidly fatal course of her disease. On the other hand, we found markedly decreased nerve conduction velocities in this patient, which suggested the presence of a severe hypomyelinating peripheral neuropathy. Peripheral neuropathy is frequent in syndromes associated with mutations in *POLG1*, including adPEO, arPEO, SANDO and the ataxia–epilepsy syndrome, (DiFonzo *et al*., 2003; Van Goethem *et al*., 2003*a*, 2004; Lamantea and Zeviani, 2004; Luoma *et al*., 2004; Winthertun *et al*., 2005). To our knowledge, this is the first case of early-onset hepatocerebral MDS with electrophysiological signs of predominant myelin abnormalities in the peripheral nerves. Unfortunately, early death and lack of autoptic examination prevented us from verifying whether the EMG signs found in this patient were accompanied by morphological alterations in the motor nerves and/or in the motor areas of the spinal cord, and in other regions of the CNS.

Interestingly, both parents of patient 9 suffered from severe hypofertility, which required *in vitro* insemination. Asthenozoospermia has been associated with length variations of the triplet repeat stretch encoding a polyglutamine tract located in the N-terminal portion of the pol-γA protein (Rovio *et al*., 2001). Hypofertility in males and precocious menopause in females were reported in *POLG1*-positive adPEO patients (Luoma *et al*., 2004), and severe hypofertility has been documented in male and female *POLG1* knock-in mutant mice (Trifunovic *et al*., 2004). Our observation supports the idea that a search for heterozygous *POLG1* mutations should be included in the genetic screening for non-syndromic hypofertility in both male and female subjects.

It is unclear why the syndromes associated with mutations in *POLG1* are tissue specific. Pol-γA is a housekeeping protein essential for life, and appears to be the only mtDNA-specific polymerase in mammals. Nevertheless, mtDNA depletion was present in the liver, but not in the skeletal muscle, or in cultured skin fibroblasts of our patients. Clinical signs of liver damage or failure, including exquisite sensitivity to valproate hepatotoxicity, was a consistent finding in our patient series and has been reported previously by others (Van Goethem *et al*., 2004). Unfortunately, in only one case could nervous tissue be investigated. Because of this limitation, we also cannot establish whether the cerebral involvement in our patients was secondary to the liver failure or rather, and more probably, could be the consequence of a primary involvement of neural cells. This latter hypothesis is supported by the progression of the neurological symptoms which occurred in patient 8 after liver transplantation.

Molecular considerations

Our findings confirm that recessive *POLG1* mutations can determine hepatocerebral syndromes, possibly associated with MDS, and further expand the spectrum of clinical presentations associated with defects in this gene.

Nine mutant alleles were found in our patients, two carrying nonsense and seven carrying missense changes (Table 1). The first nonsense mutation, 3630Cins, predicts the synthesis of an aberrant sequence of five amino acid residues, downstream from position Y1210 in the extreme C-terminus of the pol-γA protein, followed by a premature opa stop codon. A second nonsense mutation, 3482 + 2T→C, alters an invariant position in the donor splicing site between exon 21 and the adjacent intron. Each nonsense mutation was allelic to an

already reported missense mutation, namely the W748S and the A467T mutations (Table 1).

Three missense mutations, 694C→G, predicting an R232G amino acid change, 731T→C (L244P) and 2869G→C (A957P) were never reported before. Both the R232 and the L244 residues are located between Exo-I and Exo-II conserved motifs in the N-terminal proofreading domain of the protein. Interspecies comparison demonstrates that R232 is invariant in vertebrates and in *Drosophila melanogaster*, while L244 is invariant in many organisms, from vertebrates to yeast (Ropp and Copeland, 1996). Both mutations, which were found in Italian patients, were absent in 350 DNA samples from consecutive, ethnic-matched control individuals, for a total of 700 alleles, indicating a frequency of <0.14% for each mutation. The novel 2869G→C mutation (A957P) affects a highly conserved alanine residue in the polymerase domain of the protein. Interestingly, an A957S amino acid change, affecting the same amino acid residue, was associated with a very mild, late-onset phenotype in heterozygous Italian individuals with adPEO (Lamantea *et al*., 2002). The young mother of patient 7, who carries the A957P allele in heterozygosity, has no neurological abnormality for the time being, but, given the dominant, albeit very mild, behaviour of the A957S mutation, we cannot exclude that she may eventually develop symptoms later in life. The A957P mutation, which was found in a patient from Northern Germany, was absent in 200 consecutive control individuals from Northern continental Europe as well as in the Italian controls.

Of the four already reported missense mutations, W748S is located in the 'linker' region between the N-terminal proofreading domain and the C-terminal polymerase domain of the protein (Fig. 2). This mutation was found recently in homozygosity in Finnish and Norwegian patients, and in a heterozygous British patient, all affected by ataxia–epilepsy syndrome (Van Goethem *et al*., 2004; Winthertun *et al*., 2005). Likewise, both the A467T and the G848S mutations affect highly conserved positions in the spacer region of the protein. The G848S mutation was reported previously in heterozygosity in two unrelated patients: an Italian PEO patient housing a T251I-P587L mutation in the second allele (Lamantea *et al*., 2002), and a Belgian patient with PEO that housed a heterozygous mutation in the *C10orf2/Twinkle* gene, suggesting digenic inheritance (Van Goethem *et al*., 2003*b*). The A467T mutation was found in several patients affected by arPEO, SANDO or ataxia–epilepsy syndrome (Van Goethem *et al*., 2003*a*, 2004; Winterthun *et al*., 2005), as well as in the two Alpers' syndrome patients originally reported by Naviaux and Nguyen (2004) (see also the Human Polymerase Gamma Mutation Database website http://dir-apps.niehs.nih.gov/polg/).

Finally, a ninth mutant allele, found in patient 9, carried two mutations, T251I-P587L, which have been reported in several cases of arPEO. Since T251I and P587L were consistently found as syntenic changes, it is not yet established whether the pathogenicity of this allele is brought about by either one mutation or by a combination of the two. The T251I-P587L variant is a relatively frequent allele. It is indeed the most common recessive mutation in *POLG1*-related phenotypes, and we found it in heterozygosity, in combination with a wild-type allele, in ~1% of Italian controls.

We have reported previously the presence of the T251I-P587L mutation in an arPEO compound heterozygous patient, whose second allele carried a nonsense mutation predicting the virtual destruction of the corresponding protein (Lamantea *et al*., 2002; Lamantea and Zeviani, 2004). A second arPEO patient was homozygous for T251I-P587L (Lamantea and Zeviani, 2004). Therefore, both homo- and hemizygosity of T251I-P587L are associated with a relatively mild phenotype. The results of molecular analysis in our patient 9 suggest that the phenotype determined by T251I-P587L can be substantially worsened by the R232G change, from a relatively benign, juvenile- or adult-onset skeletal myopathy with accumulation of multiple mtDNA deletions, to a rapidly progressive infantile hepatocerebral syndrome associated with profound depletion of mtDNA. One possibility is that the R232G mutation may have a partially dominant-negative effect over a second allele expressing an abnormal protein, such as that carrying the T251I-P587L mutation. Interestingly, depletion of mtDNA can be produced by the inducible expression of a dominant-negative pol-γA variant in human cells (Jazayeri *et al*., 2003), as well as by exposing cultured cells (Janes *et al*., 2004) or individuals to pol-γ inhibitors (Divi *et al*., 2004; Walker *et al*., 2004).

Different functional effects on the catalytic activity, processivity and fidelity of pol-γA are expected to occur in the presence of mutations affecting different domains of the enzyme. For instance, mutations in the C-terminal polymerase domain are clearly associated with decreased catalytic activity and reduced processivity, with or without abnormalities in the accuracy of nucleotide selectivity and fidelity to the template (Ponomarev *et al*., 2002; Graziewicz *et al*., 2004). On the other hand, mutations of the N-terminal proofreading domain are expected to cause an increase in copying errors, while mutations in the linker region could possibly affect the stability and processivity of the enzyme. The multiple functions carried out by the protein could account, at least in part, for the bewildering heterogeneity of the mtDNA abnormalities and clinical phenotypes identified in pol-γA mutations. The functional characterization of the different mutant variants of the enzyme will contribute to understanding the pathogenesis of the different syndromes and also make progress in the elucidation of the basic mechanisms of mtDNA replication.

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