

ORIGINAL ARTICLE

POLG1 Mutations Associated With Progressive Encephalopathy in Childhood

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Abstract

We have identified compound heterozygous missense mutations in *POLG1*, encoding the mitochondrial DNA polymerase gamma (Pol γ), in 7 children with progressive encephalopathy from 5 unrelated families. The clinical features in 6 of the children included psychomotor regression, refractory seizures, stroke-like episodes, hepatopathy, and ataxia compatible with Alpers-Huttenlocher syndrome. Three families harbored a previously reported A467T substitution, which was found in compound with the earlier described G848S or the W748S substitution or a novel R574W substitution. Two families harbored the W748S change in compound with either of 2 novel mutations predicted to give an R232H or M1163R substitution. Muscle morphology showed mitochondrial myopathy with cytochrome *c* oxidase (COX)-deficient fibers in 4 patients. mtDNA analyses in muscle tissue revealed mtDNA depletion in 3 of the children and mtDNA deletions in the 2 sibling pairs. Neuropathologic investigation in 3 children revealed widespread cortical degeneration with gliosis and subcortical neuronal loss, especially in the thalamus, whereas there were only subcortical neurodegenerative findings in another child. The results support the concept that deletions as well as depletion of mtDNA are involved in the pathogenesis of Alpers-Huttenlocher syndrome and add 3 new *POLG1* mutations associated with an early-onset neurodegenerative disease.

Key Words: Alpers-Huttenlocher syndrome, COX deficiency, Mitochondrial myopathy, MtDNA deletions, MtDNA depletion, Polymerase gamma.

INTRODUCTION

POLG1 (MIM # 174763), encoding the catalytic subunit of mitochondrial DNA polymerase γ (Pol γ), is the only known polymerase for mitochondrial DNA (mtDNA) replication (1).

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Pol γ has 2 separate domains with catalytic activity, one polymerase domain and one 3'–5' exonuclease domain with proofreading activity. Between the 2 domains is a spacer region, which harbors 4 blocks with highly conserved sequences, and mutations herein severely compromise Pol γ processing (2).

Mutations in *POLG1* are associated with neurologic diseases with heterogeneous clinical presentations (3). It is the most commonly affected gene in dominant and recessive progressive external ophthalmoplegia (ad/ar PEO) with multiple mtDNA deletions and mitochondrial myopathy (4–7). The clinical features in PEO (MIM # 157640, # 258450) include exercise intolerance, weakness of the extraocular muscles, and various additional symptoms such as ataxia, hypogonadism, and parkinsonism (8). adPEO is caused by mutations in the polymerase domain, whereas arPEO is associated with mutations mainly in the exonuclease domain and/or the spacer region (3). *POLG1* mutations have also been found in recessively inherited sensory ataxic syndromes with various symptoms of central nervous system involvement with (9) or without (10–12) muscle involvement. Recently, *POLG1* mutations have been associated with Alpers syndrome (MIM # 203700) with liver involvement (13–16).

The onset of Alpers syndrome is usually at an early age, often within the first year of life. It is a rapidly progressive, neurodegenerative disease affecting mainly the grey matter (17). Clinical characteristics include failure to thrive, myoclonus, refractory seizures, psychomotor regression, spasticity, and cortical blindness. When the poliodystrophy is combined with liver failure, the disorder is usually called Alpers-Huttenlocher syndrome (18, 19). The disease occurs in a pattern consistent with a recessive mode of inheritance.

We describe 7 children (2 sibling pairs and 3 singletons) with progressive encephalopathy and compound heterozygous missense mutations in *POLG1* and present results that support the concept that deletions as well as depletion of mtDNA are involved in the pathogenesis of Alpers-Huttenlocher syndrome.

MATERIALS AND METHODS

Patients

Five of the children in the study were previously referred to us for investigation of mitochondrial disease. Two patients had neurologically affected siblings who were also included in

this study. Numbering of the families and patients are presented in Figure 1.

Patient 1

This girl was the fourth child of healthy, nonconsanguineous parents. The maternal grandmother had tremor and hepatopathy and her brother had tremor of unclear origin. The girl was healthy and developed normally until one year of age when, during a mild respiratory infection, she had repeated multifocal partial seizures with clonic jerking and unconsciousness. She was treated with phenobarbital. The episode was later followed by the development of muscular hypotonia and of myoclonus. At 18 months of age, slightly elevated lactate levels were found in blood and cerebrospinal fluid (CSF). Since that age, mild psychomotor regression, ataxia, and slightly elevated serum transaminases were noticed. At 2 years of age, investigations of the respiratory chain in muscle mitochondria showed normal results. The lactate levels were normal in blood, CSF, and urine and there were signs of blood-brain barrier (BBB) disruption (increased albumin CSF/serum ratio). The serum carnitine level was normal, but the muscle carnitine level was slightly reduced (7.1 μmol/g protein; reference range, 7.4–26 μmol/g). At 3 years of age, a mild infection provoked a prolonged status epilepticus, which lasted 3 weeks and included stroke-like features and rightsided hemiparesis. This was followed by a progressive deterioration of cognitive and motor functions and the development of ptosis and external ophthalmoplegia. Febrile infections provoked repetitive status epilepticus with seizures in the form of migrating epilepsia partialis continua (EPC). At 4 years of age, magnetic resonance imaging (MRI) of the brain showed supratentorial gray and white matter atrophy and increased T2 signal of the white matter (Fig. 2). At the same age, during status epilepticus, valproate was used for 10 days and then terminated because of signs of liver disease. The liver function continued to deteriorate to

severe liver failure and she died at 4 years 4 months of age of multiple organ failure.

Patient 2

This girl was an older sister of patient 1. She was healthy until 7 months of age when she, in association with pyelonephritis, had onset of myoclonic seizures and weakness of the left arm and leg, which lasted 2 1/2 weeks. The seizures disappeared on phenobarbital treatment. At 11 months of age, she had a mild respiratory tract infection accompanied by leftsided myoclonic seizures progressing to status epilepticus in the form of migrating EPC and unconsciousness. MRI of the brain showed supratentorial gray and white matter atrophy. She was treated with thiopental in ventilator and recovered. At 12 months of age, the seizures recurred and she was again treated with thiopental in a ventilator for status epilepticus with complicating pneumonia and colitis. Valproate was used for approximately one week after which she was found to have coagulopathy with a prothrombin level at 30% to 40% of normal mean. After another 3 weeks, she developed liver and renal failure. Analysis of the respiratory chain function in muscle mitochondria showed normal results. The serum carnitine level was normal. Blood lactate levels were occasionally slightly elevated and there were signs of BBB disruption. The girl died at 14 months of age of multiple organ failure.

Patient 3

This boy was the first child of healthy, nonconsanguineous parents. The maternal grandfather died at 63 years of age of a rapidly progressive liver disease of unknown origin. The boy had feeding difficulties with failure to thrive since 3 months of age. The gross motor development was delayed. He could not hold his head up until 5 months of age and could not sit without support until 10 months of age. He never learned to roll over or crawl. At 1 year of age, he was found to

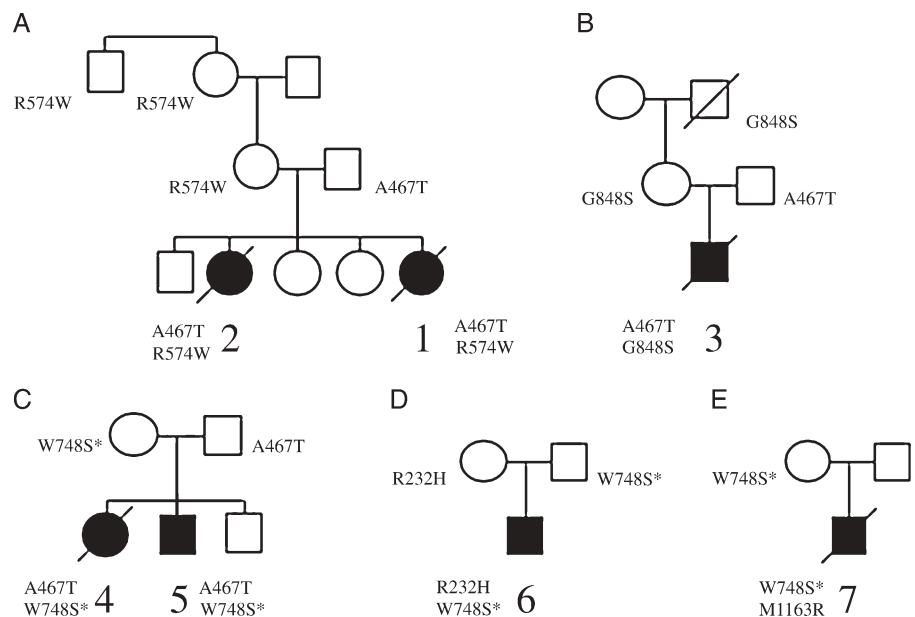


FIGURE 1. (A–E) Pedigrees of families showing numbers of affected children. Mutations in *POLG1* segregate with disease in all affected family members in a recessive mode of inheritance. The father of patient 7 was not available for genetic testing.

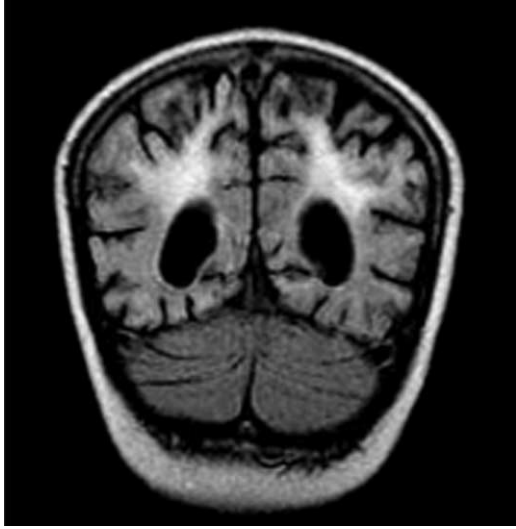


FIGURE 2. Magnetic resonance image of the brain of patient 1 at 4 years of age. Coronal FLAIR of the occipital region showing atrophy of both occipital lobes and high signal intensity in the white matter.

have increased levels of serum transaminases, whereas the blood lactate and urinary organic acids were normal. At 13 months of age, his condition deteriorated during a mild respiratory tract infection. He was drowsy, vomited, and there was general muscle weakness and ataxia. Laboratory investigations showed signs of compromised liver function. The prothrombin international normalized ratio was 1.5 (reference range, <1.2) and there was lactic acidosis. Serum and muscle carnitine levels were normal. CSF investigation showed signs of BBB disruption. At 14 months of age, investigations of the respiratory chain in muscle mitochondria showed generally decreased respiratory rate, and there was generally decreased activity (41–43% of the normal mean) of the respiratory chain enzyme complexes I–IV. At 15 months of age, he developed myoclonic seizures, which progressed to therapy-resistant EPC. After 10 days of extensive treatment with various anti-epileptic drugs, including pentobarbital in a ventilator, a computed tomography (CT) of the brain showed hypodense lesions. Cardiac investigations showed decreased contractility as a sign of cardiomyopathy. He was never treated with valproate. He died at 16 months of age of multiple organ failure.

Patient 4

This girl's medical history has been previously published as a patient with fatal deterioration from neurologic disease after orthotopic liver transplantation for valproate-induced liver damage (20). She was healthy and developed normally until her late preschool years when she developed slowly progressive ataxia. In her early teens, she developed occasional myoclonic seizures, and at 13 years of age, valproate treatment was started because of occipital seizures. Analyses of the respiratory chain complexes of muscle mitochondria were normal. Five months later, she developed acute liver failure and a liver transplantation was performed. Serum carnitine level was normal. Six weeks after the transplantation, her

condition rapidly deteriorated. There was aggravation of the ataxia, dementia, and cortical blindness. She developed refractory seizures, including multifocal EPC, status epilepticus, and myoclonus. Blood and CSF lactate levels were occasionally elevated and there were signs of BBB disruption. Repeated MRIs showed progressive multifocal lesions predominantly affecting the grey matter of the parietal and occipital lobes. She died at 14 years of age of multiple organ failure.

Patient 5

This boy is the younger brother of patient 4. He was normal until 11 years of age when he developed mild tremor and ataxia. At 13 years of age, he reported occasional migraine-like headaches and was found to have mild ataxia and myoclonus. Later the same year, he had generalized seizures. At 14 years of age, analyses of the respiratory chain function in muscle mitochondria showed normal results. Serum carnitine level was normal. Blood and CSF lactate levels have been normal on most occasions, whereas there have been signs of disruption of the BBB. He has, with some difficulty, been able to follow a normal education and has had occasional generalized seizures, which have disappeared with lamotrigine and clonazepam treatment. He also had occasional episodes of unconsciousness of unclear origin. Presently, at 24 years of age, he has ataxia, myoclonus, mild left-sided hemiparesis, external ophthalmoplegia, and peripheral neuropathy. MRI of the brain has shown increased T2 signaling from the right motor cortex. He has never been treated with valproate and has never had any clinical or laboratory signs of liver disease. A liver biopsy has not been obtained.

Patient 6

This boy is the second child of healthy, nonconsanguineous parents. He was healthy and his development was normal until 5 months of age. He developed failure to thrive and muscular hypotonia after a *Bordetella pertussis* infection. His best psychomotor function was at 3 years of age, when he could walk with support, speak well, and had adequate fine motor skills. At 4 years of age, he developed myoclonus, which was first limited to the right eye and the right side of the mouth, but then progressed to EPC of the entire left side of the body without loss of consciousness. The seizures did not respond to anti-epileptic treatment. He was never treated with valproate. Since 9 months of age, the level of serum transaminases has been moderately elevated and the lactate levels have been repeatedly elevated in blood and CSF. MRI of the brain at 14 months of age showed a small lesion in the left parietal lobe. MRI of the brain at 26 months of age was normal. At 4 1/2 years of age, analyses of the respiratory chain in muscle mitochondria showed complex I deficiency. There were signs of BBB disruption. He had a low level of serum carnitine (21 $\mu\text{mol/L}$; reference range, 23–60 $\mu\text{mol/L}$) and muscle carnitine (2.8 $\mu\text{mol/g}$ protein; reference range, 7.4–26 $\mu\text{mol/g}$). He has since been treated with oral carnitine. He has developed a complex movement disorder and cognitive impairment, but contact, speech, and memory functions have been retained. At the last clinical examination at 5 years of age, he could talk in

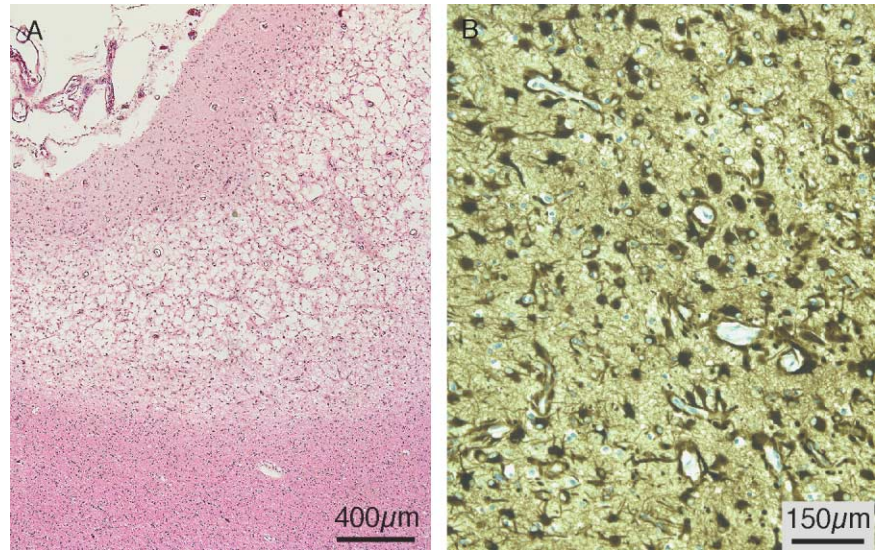


FIGURE 3. Occipital cortex of patient 1 demonstrating severe degeneration with spongiform changes and gliosis. **(A)** Hematoxylin and eosin. **(B)** Immunostaining of glial fibrillary acidic protein.

whole sentences and was able to sit and crawl but not stand. He had generalized muscular hypotonia. He had difficulty holding up his head and body as a result of axial muscle weakness. He also had ptosis and uncontrolled and uncoordinated movements, especially in his arms.

Patient 7

This boy was the first child of healthy, nonconsanguineous parents. During the first 6 months of life, he was considered healthy, but was irritable with colic-like symptoms. Since 6 months of age, he failed to thrive and his gross motor development was delayed. He sat without support at 9 months of age and had not learned to crawl at 12 months of age. At 9 months of age, after a mild upper airway infection, he developed myoclonus in the left arm and leg, which persisted for 16 hours, and a leftsided hemiparesis, which resolved after 2 weeks. On subsequent treatment with valproate and clonazepam, there were only occasional myoclonic seizures. A CT of the brain was normal. At 11 months of age, his

condition deteriorated. He lost his appetite, vomited, and stopped gaining weight. Laboratory investigations showed increased levels of serum transaminases and hyperlactatemia. The treatment with valproate was terminated. At 12 months of age, biochemical analyses of isolated muscle mitochondria showed complex I deficiency. Total serum carnitine was 24 μmol/L (reference range, 23–60 μmol/L) and the acylcarnitine fraction was increased (34%; reference range, <20%). The muscle carnitine level was slightly decreased (6.7 μmol/g protein; reference range, 7.4–26 μmol/g). He continued to deteriorate, developed lactic acidosis and liver failure, and died at 13 months of age.

Pathology

Neuropathologic examination was performed in patients 1, 2, 4, and 7. Paraffin-embedded sections of paraformaldehyde-fixed brain specimens obtained postmortem were stained with hematoxylin and eosin and Luxol fast blue–cresyl violet

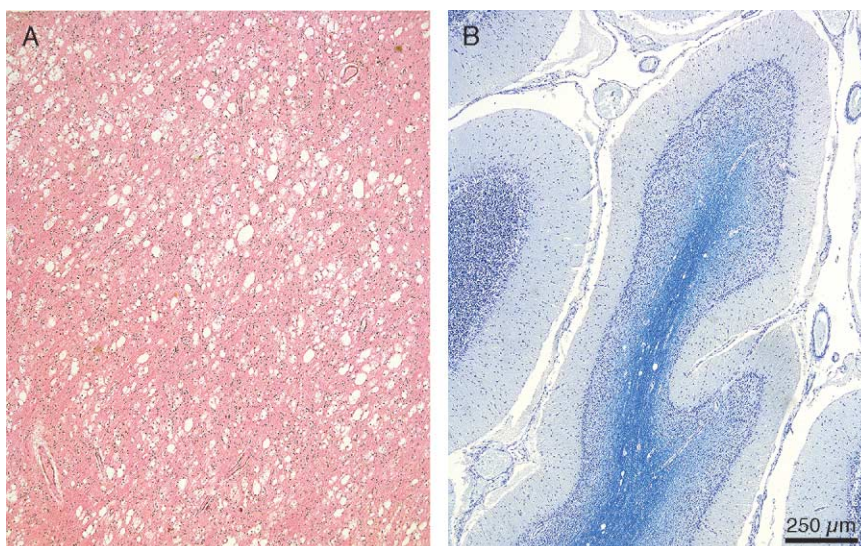


FIGURE 4. **(A)** Thalamus of patient 4 showing prominent vacuolization. Hematoxylin and eosin stain. **(B)** Cerebellum of patient 4 showing loss of Purkinje and granular cells and proliferation of Bergmann glia. Luxol fast blue–Cresyl violet.

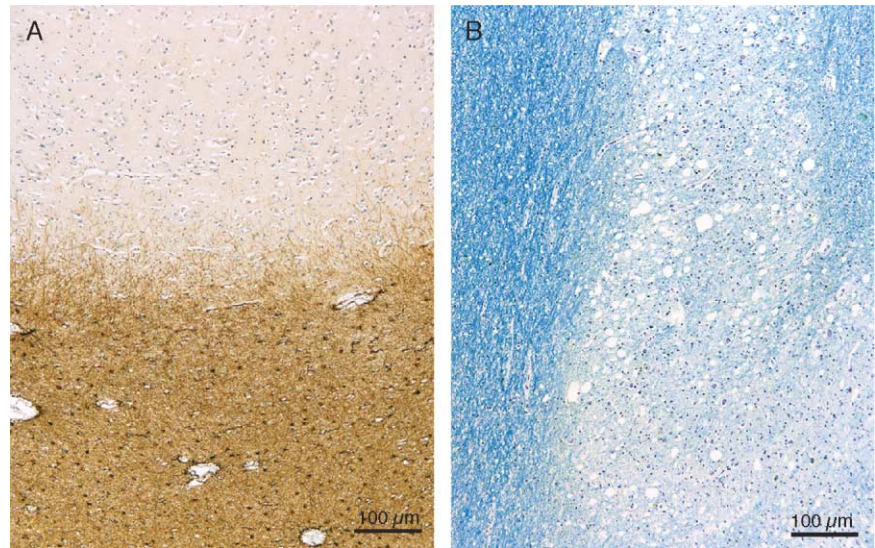


FIGURE 5. (A) Cortex and subcortical white matter of patient 7 demonstrating gliosis of the cerebral white matter. Immunostaining of glial fibrillary acidic protein. (B) Dentate nucleus of patient 7 demonstrating neuronal cell loss and spongiosis. Luxol fast blue-Cresyl violet.

and immunostained for glial fibrillary acidic protein. Cryostat sections of fresh-frozen skeletal muscle biopsies were investigated after histochemical stainings, including hematoxylin and eosin, Gomori trichrome, NADH-tetrazolium reductase, succinate dehydrogenase, cytochrome *c* oxidase, Sudan black, and Periodic acid and Schiff's reagent.

Liver biopsies were obtained by needle biopsy, open biopsy, or at postmortem investigation. Sections of paraffin-embedded, formalin-fixed liver specimens were investigated

after staining with hematoxylin and eosin. One liver biopsy was fresh-frozen in liquid nitrogen and subjected to enzyme-histochemical investigation of succinate dehydrogenase and COX.

Screening for *POLG1* Mutations

Total DNA was extracted from skeletal muscle tissue or blood using the DNA Extraction Kit (Qiagen, Hilden, Germany). The 22 coding exons of *POLG1* with flanking

TABLE 1. *POLG1* Mutations, Muscle Pathology, and mtDNA Alterations

Patient	Sex	Age at Onset/Death	<i>POLG1</i> Mutations	Amino Acid Change	Ragged-Red Fibers	COX-Deficient Fibers	Age at Biopsy	mtDNA Deletions	mtDNA Copy Number, Relative Amount*
1	F	12 months/4 years	1399 G→A 1720 C→T	A467T R574W	—	—	24 months	Yes	0.06†
2	F	7 months/14 months	1399 G→A 1720 C→T	A467T R574W	—	—	12 months	Yes	0.93
3	M	4 months/16 months	1399 G→A 2542 G→A	A467T G848S	+	++	15 months	No	0.07†
4	F	5 years/14 years	1399 G→A 2243 G→C 3428 A→G	A467T W748S E1143G‡	+	+	13 years	Yes	1.74
5	M	11 years/—	1399 G→A 2243 G→C 3428 A→G 695 G→A	A467T W748S E1143G‡ R232H	—	+	15 years	Yes	1.60
6	M	5 months/—	2243 G→C 3428 A→G 2243 G→C	W748S E1143G‡ W748S	+	++	4 years	No	0.12†
7	M	6 months/13 months	3428 A→G 3488 T→G	E1143G‡ M1163R	—	—	12 months	No	0.41

*. The value for mtDNA depletion is given in relation to the mean of a control group without neuromuscular disease 0 to 13 years old (n = 18). The mean is defined as 1 (range, 0.35–3.5).

†. Values below 0.3 are considered as mtDNA depletion.

‡. Reported as an SNP (3%), + = few fibers, ++ >10% of the fibers.

intronic sequences were amplified and sequenced using an ABI Prism 377 DNA sequencer and the Big Dye Terminator Kit 1.1 (Applied Biosystems, Foster City, CA). Primers and protocols are available on request.

The 1399 G→A mutation abolished a restriction site for the endonuclease *MscI* and was analyzed for the allele frequency in 200 control chromosomes from healthy blood donors among the Swedish population. The 3 novel mutations were screened for in the same 200 control chromosomes. The 695 G→A abolished a restriction site for *MaeIII*, 1720 C→T for *BsrWI*, and finally the 3488 T→G mutation for the endonuclease *NspI*. Only wild-type DNA was cleaved in the amplified polymerase chain reaction (PCR) products in all reactions.

mtDNA Analyses in Muscle

Quantitative PCR was performed as described (21) using an ABI prism 7700 sequence detection system and the comparative C_T method (www.appliedbiosystems.com, products and services, User Bulletin #2). With this method, the relative amount of mtDNA molecules and nDNA molecules in one sample are compared with the relative amount in a calibrator sample.

To investigate the presence of large-scale mtDNA deletions, a long expand polymerase chain reaction (LX-PCR) was performed as previously described (22). With this method, a 10.8-kb product between mtDNA positions nt 5420–16259 is amplified. In addition, a standard PCR analysis with mtDNA primers L8197–8216 and H13640–13621 was

performed. This widely separated primer pair will only amplify mtDNA with deletions (23). Presence of the so-called “common deletion” (breakpoints at nt 8469–13447) will give rise to a 467-bp product. After running the gel electrophoresis, bands at the size of approximately 500 bp were cut out and sequenced.

Muscle biopsy specimens from 25 children without evidence of Alpers-Huttenlocher syndrome served as controls for LX-PCR analysis.

RESULTS

Pathology

Neuropathologic examination was performed in patients 1, 2, 4, and 7.

In patient 1, only parts (280 g) of the brain were available for examination. Macroscopically, there was reduction in cortical thickness in the occipital lobes and focal cortical softening. Microscopic examination showed widespread changes in the cortex, most prominent in the occipital lobes, with regions showing neuronal loss, astrogliosis, and spongiform changes (Fig. 3). Astrogliosis and some vacuoles were present in the subcortical white matter, mainly in the occipital lobes. In the hippocampus, there was loss of neurons, especially in the Sommer sector. Loss of neurons, increased number of astrocytes, and vacuoles were present in the thalamus. Some vacuoles were also found in the globus pallidus. Severe reduction of Purkinje cells with proliferation of Bergmann glia was seen in the cerebellum and there was focal loss of granular cells as

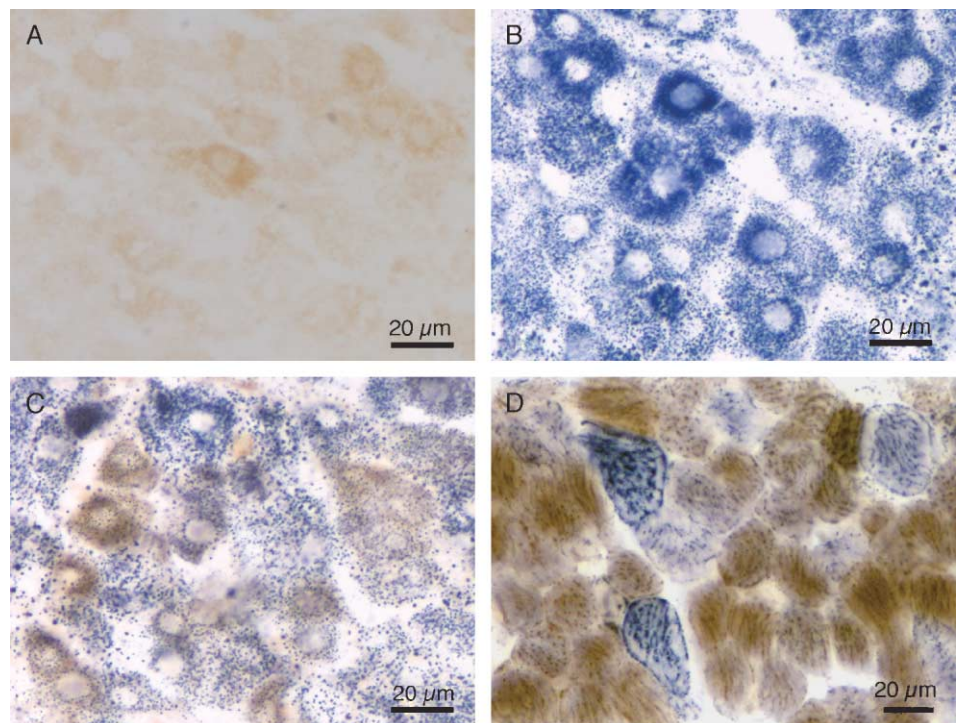


FIGURE 6. (A–C) Liver and (D) muscle tissue of patient 3 showing a mosaic pattern of cytochrome c oxidase (COX) deficiency. The reduced COX (complex IV) activity despite preserved succinate dehydrogenase (complex II) activity is highly suggestive for a mitochondrial defect secondary to mtDNA alterations such as mutations or depletion. (A) Enzyme histochemical staining of COX. (B) Enzyme-histochemical staining of succinate dehydrogenase. (C, D) Combined staining of COX and succinate dehydrogenase illustrating COX-deficient (blue) cells among cells without evidence of COX deficiency.

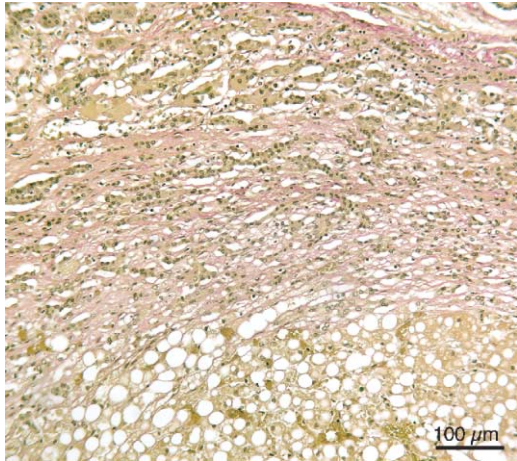


FIGURE 7. Liver biopsy of patient 1 showing marked fibrosis, degeneration of hepatocytes, and bile ductular proliferation in addition to a regeneration nodule (bottom) with steatosis. Van Gieson stain.

well. There was also loss of neurons in the dentate nucleus where vacuolization was present. Astrocytes of Alzheimer type II were found mainly in the cerebral cortex and basal ganglia.

Only parts (140 g) of the brain were available for examination in patient 2. Macroscopic examination disclosed regions with slight reduction of cortical thickness. By microscopy, prominent changes more or less throughout the cortex were observed with loss of neurons, astrogliosis, spongiform changes, and increased number of capillaries. The subcortical white matter was focally affected by gliosis. In the basal ganglia and thalamus, a decreased number of neurons and some vacuoles were observed. In the mammillary bodies, there was loss of neurons, astrogliosis, and an increased number of capillaries. Some vacuoles were also present. In the cerebellum, there was severe loss of Purkinje cells and proliferation of Bergmann glia. The number of granular cells was focally reduced. Astrocytes of Alzheimer type II were present and most frequent in the cerebral cortex, basal ganglia, and thalamus.

In patient 4, the fixed brain weighed 1230 g. Macroscopic examination revealed softening of the cortex with reduced thickness but with sharp demarcation to the white

matter in both occipital lobes. Similar changes were also present focally in the right parietal lobe. The basal ganglia and thalami were without obvious changes. Microscopic investigation showed prominent reduction of neurons in the occipital lobes. There was also severe astrogliosis, spongiform changes, and proliferation of capillaries. Similar but milder lesions were also detected in other regions of the cortex such as in the superior part of the frontal lobes. Between these lesions, the cortex appeared without morphologic changes. The subcortical white matter in the affected areas showed increased number of astrocytes and mild spongiosis. The hippocampus was well preserved. In the thalami, prominent vacuolization of the tissue was found without any major loss of neurons (Fig. 4A). Similar but much milder lesions were also seen in the basal ganglia, especially in the globus pallidus. Some vacuoles were also present in the mammillary bodies and in the mesencephalon. In the cerebellum, there was severe loss of Purkinje cells and proliferation of Bergmann glia (Fig. 4B). Prominent loss of the granular cells was found focally (Fig. 4B). Some vacuoles were seen in the granular cell layer and adjacent white matter. In the dentate nucleus, loss of neurons with gliosis was found as well as a few vacuoles, and astrocytes of Alzheimer type II were present in the cerebral cortex and basal ganglia.

Neuropathologic examination of patient 7 showed no macroscopic changes and no obvious neuronal loss, spongiosis, or gliosis in investigated regions of the cerebral cortex. The cerebral white matter, on the other hand, showed marked gliosis and areas with mild spongiosis (Fig. 5A). Mild spongiosis was also present in the thalamus but there was no apparent nerve cell loss. Marked neuronal cell loss and gliosis was found in the Sommer sector of the hippocampus. In the cerebellum, there was focal loss of Purkinje cells in addition to marked loss of nerve cells and spongiosis in the nucleus dentatus (Fig. 5B). Spongy change was also present focally in the cerebellar white matter.

Muscle pathology is summarized in the Table. COX-deficient muscle fibers were present in 4 of the 7 cases and were frequent in patients 3 and 6. Figure 6D illustrates the mosaic pattern of COX-deficient fibers among muscle fibers with apparently normal COX activity in sections from patient 3.

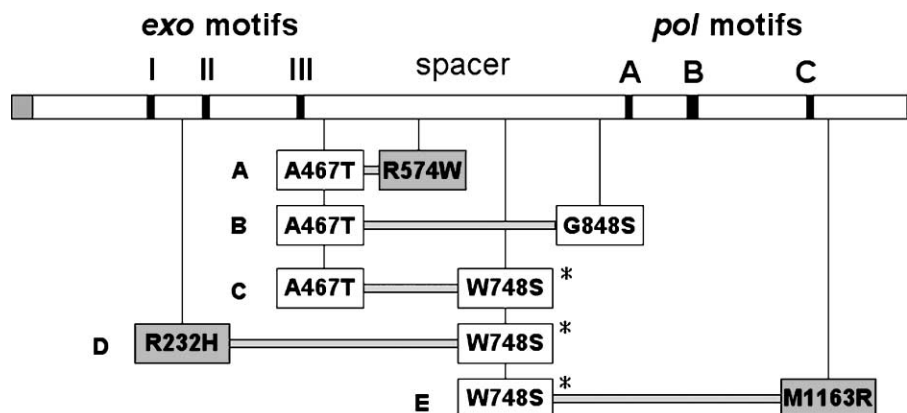


FIGURE 8. Illustration of Pol γ showing its 2 catalytic domains, the spacer region and the identified mutations in family A–E. Only 2 of the identified mutations do not reside in the spacer region. Gray boxes represent new mutations. *, Associated with the SNP E1143G.

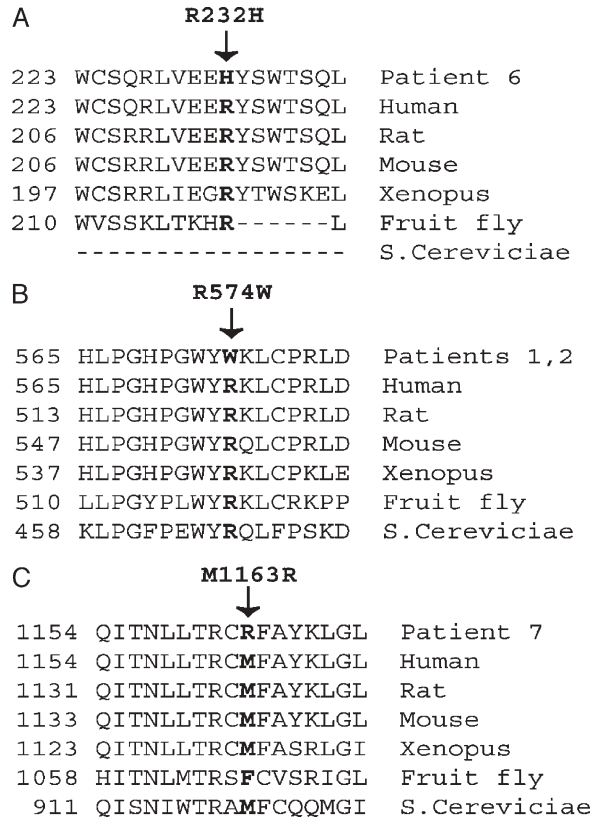


FIGURE 9. Alignment showing the Pol γ regions in which the 3 novel amino acid substitutions in our study were detected. **(A)** R232 and **(B)** R574 are invariant and **(C)** M1163 is well conserved.

Liver disease was present in all cases except in patient 5. Morphologic changes were variable from steatosis and slight fibrosis to widespread degeneration with necrosis and fibrosis/cirrhosis. Bile ductular proliferation was conspicuous in most cases (Fig. 7). In patient 3, examination of fresh-frozen liver tissue displayed COX-deficient hepatocytes mixed with hepatocytes with normal COX activity (Fig. 6A–C).

Screening for POLG1 Mutations

Direct sequencing of the 22 coding exons in *POLG1* revealed 6 different missense mutations in the 7 children and in their parents (Table; Figs. 1 and 8). Three of these mutations have not been previously reported (Fig. 9). Patients 1 and 2 harbored a 1720 C→T transition indicating a novel R574W change in compound heterozygosity with a 1399 G→A transition predicting an A467T substitution. Patient 3 was compound heterozygous for a 2542 G→A transition (G848S) and the 1399 G→A transition (A467T). In patient 4, we identified a 2243 G→C transversion (W748S) in compound heterozygosity with the 1399 G→A transition (A467T). The same mutations were also identified in the patient’s neurologically affected brother (patient 5). Patient 6 was compound heterozygous for the 2243 G→C transversion (W748S) and a novel transition, 695 G→A (R232H). Patient 7

was compound heterozygous for the 2243 G→C transversion (W748S) and another novel transversion, 3488 T→G (M1163R). In addition, patients 4, 5, 6, and 7 all harbored a heterozygous 3428 A→G transition (E1143G) reported as an SNP (<http://egp.gs.washington.edu>). The 1399 G→A mutation (A467T) was found at a frequency of 0.5% in a healthy control group, but the novel mutations 695 G→A, 1720 C→T, or 3488 T→G were not present in the 200 control alleles.

mtDNA Analyses

Quantitative PCR analysis showed that 3 of the children (patients 1, 3, and 6) had reduced copy numbers of mtDNA in skeletal muscle (Table). By LX-PCR analysis, multiple mtDNA deletions in muscle were identified in patients 1 and 5 (Fig. 10A). Standard PCR analysis also demonstrated mtDNA deletions in patients 2 and 4 (Fig. 10B). Both the LX-PCR and the conventional PCR were repeatedly performed with identical results. Sequencing of the 500-bp PCR product (Fig. 10B) verified breakpoints in mtDNA in accordance with the “common” deletion (nt 8469–13447). Deletions of mtDNA were not identified in any of the controls.

DISCUSSION

Characteristic neurologic features of the 7 children in whom we have identified compound missense mutations in *POLG1* were early-onset psychomotor regression with a relapsing and remitting course, which was frequently worsened

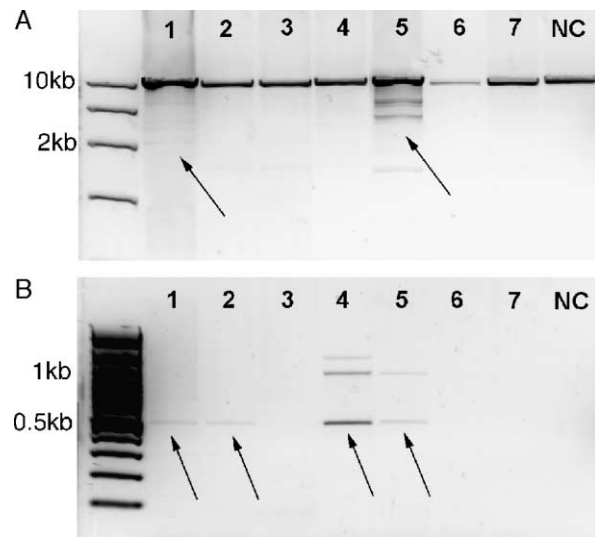


FIGURE 10. **(A)** GelStar staining of long expand polymerase chain reaction (LX-PCR) amplifications separated on a 0.7% agarose gel that illustrates a normal 10.8-kb amplified fragment from the patients. Smaller bands, corresponding to multiple deleted mtDNA fragments, are present in patients 1 and 5 (arrows). **(B)** Standard PCR with mtDNA primers L8197–H13640. PCR products are formed only in the presence of mtDNA deletions. With this method, it is obvious that patients 1, 2, 4 and 5 harbored deletions in mtDNA. The arrows point at the PCR products harboring the common deletion, which was verified with sequencing. NC, normal control.

during infections. They also had refractory seizures in the form of myoclonus and EPC, stroke-like episodes, and ataxia. Liver involvement, which was found in 6 of the children, progressed to fatal failure in 5 of them. Hyperlactatemia was not a consistent feature, and the results from biochemical investigations of the respiratory chain were frequently normal.

The temporal relationship of onset of deteriorating liver function with valproate treatment in the majority of these cases clearly suggests a triggering mechanism induced by this drug. In fact, one child had an initial diagnosis of valproate-induced liver disease. Valproate treatment is known to be associated with fatal hepatotoxicity (24). The exact mechanism is not known but may involve secondary carnitine deficiency, depression of intramitochondrial fatty acid oxidation, and/or inhibition of the oxidative phosphorylation (25–27). The reason for an increased susceptibility to valproate in patients with *POLG1* mutations is unclear. However, the results from enzyme histochemical analysis of liver tissue in one child clearly demonstrated that defective mitochondrial metabolism is a major effect of the *POLG1* mutations.

Five of the 7 children presented with signs and symptoms of Alpers-Huttenlocher syndrome (18). The clinical features of patient 4 started as an ataxic syndrome and she developed an Alpers-Huttenlocher-like phenotype after the initiation of valproate treatment. Patient 5 did not have any liver symptoms and had a phenotype more similar to the previously described *POLG1*-associated ataxic syndrome (10). This indicates that the clinical phenotype is variable. It is obvious that infections and valproate treatment have an impact on the course of the disease and there might be additional genetic and environmental factors of importance for the phenotypic expression of the disease.

The neuropathologic examination showed widespread degeneration and gliosis of the cortex, neuronal loss, and spongiosis in the thalamus and gliosis of the white matter in 3 cases (patients 1, 2, and 4). These changes, together with characteristic liver pathology, are frequent findings in Alpers-Huttenlocher syndrome (19). In patient 7, the investigation did not reveal macroscopic cortical changes and microscopic examination of various neocortical regions did not show any spongiosis, apparent neuronal loss, or gliosis, whereas the white matter of the brain hemispheres showed marked gliosis comparable to that of the other cases. On the other hand, this case showed mild spongiosis of the thalamus and marked spongiosis of the dentate nucleus, findings that are consistent with Alpers-Huttenlocher syndrome. These findings suggest that, in cerebral degeneration of childhood-associated *POLG1* mutations, the morphologic changes may be quite variable and that white matter changes are not only secondary to cortical degeneration.

We identified 6 different missense mutations in *POLG1*. Three amino acid substitutions, A467T, W748S, and G848S, have already been reported in various neurologic conditions. The other 3 mutations, R232H, R574W, and M1163R, have not been reported.

The 1399 G→A mutation (A467T) appears to be the most frequent *POLG1* mutation associated with early-onset progressive neurodegenerative disease. This mutation, which

was present in 5 of our 7 patients, has additionally been reported in 12 patients with Alpers syndrome (13, 14, 16). Moreover, it has been reported also in juvenile ataxic syndromes (10, 11) and in adult-onset arPEO (5, 28). The A467 residue is well conserved among species, and functional studies have shown that a substitution of alanine by threonine severely compromises the catalytic activity of Pol γ (29). A threonine at this position has a frequency of approximately 0.6% in the Belgian population (5) and 0.5% in Norwegian (11) and Swedish populations (present study).

The *POLG1* mutation leading to the W748S substitution was first described in 5 patients with ataxia and central nervous system features in 3 unrelated families (10). Additionally, it has been reported in 2 cases with ataxic syndromes (11) and recently also in association with Alpers syndrome (13, 16). Four of our patients harbored this amino acid change. A recent haplotype analysis showed that patients with this mutation originate from a common ancient founder (30), and it is possible that our patients belong to the same Northern haplotype group. The allele frequency in Finland is 0.4% (30). In our study as well as in all other reports, the W748S change has been syntenic with another amino acid substitution, E1143G, which has been classified as a rare single nucleotide polymorphism.

A *POLG1* mutation leading to G848S has been reported in association with arPEO (4, 31, 32) and recently also in Alpers syndrome (13, 15, 16). This residue is highly conserved and located very close to motif A in the polymerase domain of Pol γ (33).

The 3 novel mutations affect highly conserved amino acid residues in Pol γ (Fig. 7) and were not present in 200 control alleles. A mutation affecting position 232 was previously reported (16), but in that case, there was a 694 C→G transversion giving a predicted R232G change, and in our case, it was a 695 G→A transition leading to the R232H substitution.

The R574W substitution was present in both siblings (patients 1 and 2) from family A in combination with A467T (Fig. 6). Some of the family members on the maternal line have unspecific liver disease, and it is possible that the R574W substitution alone may cause liver disease with variable penetrance. The combination of R574W and A467T appears to cause a severe phenotype with rapid progression and deterioration after disease onset.

In this study, M1163R and R232H were the only identified amino acid substitutions that do not reside within the spacer region of Pol γ . M1163R affects the polymerase domain and is located close to motif C (33). Because the father of patient 7 was not available for genetic testing, it is still unclear if the 3488 T→G mutation (M1163R) arose de novo or was inherited.

The sibling pair in family C, patients 4 and 5, with the combination of A467T and W748S/E1143G had a somewhat milder phenotype with later onset than the other children in the study. The same combination of mutations and amino acid substitutions was described in a recent report on ataxia (10).

Three children, patients 1, 3, and 6, had clearly reduced copy number of mtDNA in skeletal muscle in

relation to an age-matched control group. Our results thus support the concept that *POLG1* mutations may cause childhood mtDNA depletion (15, 34). Whereas mtDNA depletion syndromes are associated with severe conditions in childhood (35, 36), multiple mtDNA deletions are frequently associated with adult-onset mitochondrial diseases such as ad/arPEO, which in most cases are associated with *POLG1* mutations (6, 7). Multiple mtDNA deletions are uncommon in children and have to our knowledge not previously been reported in children younger than 11 years old. In this study, there was a small but distinct amount of multiple mtDNA deletions in muscle tissue from the 2 sibling pairs of families A and C. The amount of deletions that was readily amplified with the LX-PCR technique would probably be too small to be visualized with the less sensitive Southern blot analysis that is often used to detect and quantify mtDNA deletions. Thus, although the LX-PCR method is not quantitative, it can be a useful tool to detect small amounts of deletions that otherwise might be overlooked. We were not able to investigate mtDNA in brain tissue, which is more severely affected than muscle in children with Alpers-Huttenlocher syndrome. It is possible that organs with a higher degree of clinical symptoms harbor a higher proportion of mtDNA abnormalities, but this hypothesis remains to be further studied. Liver was severely affected in all children except for patient 5, and indeed, the enzyme histochemical staining of liver sections from patient 3 displayed even more prominent mitochondrial defects than did muscle tissue from the same patient (Fig. 5). Nevertheless, the presence of COX-deficient muscle fibers and COX-deficient hepatocytes has not been previously reported in this disorder.

Because Pol γ is essential for mtDNA replication, our findings of mtDNA depletion and mtDNA deletions support the concept that *POLG1* mutations in Alpers-Huttenlocher syndrome compromise mtDNA maintenance. This is further supported by the COX deficiency in muscle and liver tissue. Our results indicate that Alpers-Huttenlocher syndrome is a mitochondrial disease.

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