Recessive Twinkle mutations in early onset encephalopathy with mtDNA depletion

Anna H. Hakonen,1,* Pirjo Isohanni,1,2 Pirjo Isohanni,1,2 Anders Paetau,3 Riitta Herva,4 Anu Suomalainen1,5 and Tuula Lönnqvist2

1Research Program of Molecular Neurology, Biomedicum-Helsinki, University of Helsinki, 2Department of Child Neurology, Hospital for Children and Adolescents, Helsinki University Central Hospital, Helsinki, 3Department of Pathology, University of Helsinki, and HUSLAB, Helsinki, 4Oulu University Hospital, Oulu and 5Department of Neurology, Helsinki University Central Hospital, Finland

*These authors contributed equally to this work.

Correspondence to: Anu Suomalainen, Biomedicum-Helsinki, Research Program of Molecular Neurology, r. c523B, P.O. Box 63, University of Helsinki, 00290 Helsinki, Finland
E-mail: anu.wartiovaara@helsinki.fi

Twinkle is a mitochondrial replicative helicase, the mutations of which have been associated with autosomal dominant progressive external ophthalmoplegia (adPEO), and recessively inherited infantile onset spinocerebellar ataxia (IOSCA). We report here a new phenotype in two siblings with compound heterozygous Twinkle mutations (A318T and Y508C), characterized by severe early onset encephalopathy and signs of liver involvement. The clinical manifestations included hypotonia, athetosis, sensory neuropathy, ataxia, hearing deficit, ophthalmoplegia, intractable epilepsy and elevation of serum transaminases. The liver showed mtDNA depletion, whereas the muscle mtDNA was only slightly affected. Alpers–Huttenlocher syndrome has previously been associated with mutations of polymerase gamma, a replicative polymerase of mtDNA. We show here that recessive mutations of the close functional partner of the polymerase, the Twinkle helicase, can also manifest as early encephalopathy with liver involvement, a phenotype reminiscent of Alpers syndrome, and are a new genetic cause underlying tissue-specific mtDNA depletion.

Keywords: twinkle; IOSCA; Alpers; encephalopathy; mtDNA

Abbreviations: IOSCA = infantile onset spinocerebellar ataxia; PEO = progressive external ophthalmoplegia; MIRAS = mitochondrial recessive ataxia syndrome


Introduction

An increasing number of neuromuscular disorders are shown to be caused by defects in the nuclear-encoded proteins responsible for the maintenance of mitochondrial DNA (mtDNA). Especially, mutations in the mitochondrial DNA polymerase, polymerase γ (POLG), and replicative helicase Twinkle cause early and late onset neuromuscular phenotypes (Spelbrink et al., 2001; Van Goethem et al., 2001; Navaux and Nguyen, 2004; Van Goethem et al., 2004; Hakonen et al., 2005; Nikali et al., 2005; Horvath et al., 2006).

Dominant mutations in Twinkle cause pure progressive external ophthalmoplegia (PEO) and mitochondrial myopathy (OMIM #609286), but sometimes result in additional features, such as psychiatric symptoms, ataxia and parkinsonism (Suomalainen et al., 1997; Spelbrink et al., 2001; Hudson et al., 2005; Baloh et al., 2007). The disease is characterized by accumulation of multiple mtDNA deletions in the muscle, brain and the heart of the patients (Zeviani et al., 1989; Suomalainen et al., 1992, 1997). Most of the Twinkle PEO mutations cluster on the linker region of the protein—a domain thought to have a role in the subunit interactions of the hexameric helicase protein (Spelbrink et al., 2001). We have previously reported the sole cases of recessive Twinkle mutations in infantile onset spinocerebellar ataxia (IOSCA) (OMIM #271245). The main clinical features of IOSCA include muscle hypotonia, athetosis, ataxia, ophthalmoplegia, hearing deficit, sensory axonal neuropathy, female hypogonadism and epileptic encephalopathy (Koskinen et al., 1994; Nikali et al., 2005). In contrast to PEO patients, mtDNA deletions are not detected in the muscle of IOSCA patients.
Despite the early onset, IOSCA resembles the juvenile or adult onset mitochondrial recessive ataxia syndrome (MIRAS) caused by recessive POLG mutations (Van Goethem et al., 2004; Hakonen et al., 2005), and numerous POLG mutations underlie autosomal dominant or autosomal recessive PEO (Van Goethem et al., 2001; Horvath et al., 2006). However, only recessive POLG mutations have been described as an important cause of Alpers–Huttenlocher syndrome (OMIM #203700), an early onset disease characterized by psychomotor retardation, refractory epilepsy and liver failure with mtDNA depletion (Alpers, 1931; Naviaux and Nguyen, 2004; Davidzon et al., 2005; Ferrari et al., 2005; Horvath et al., 2006).

We report here that recessive Twinkle mutations can cause a severe early onsetencephalopathy with depletion of mtDNA and some signs of liver involvement, implicating that the gene encoding for Twinkle should be analysed in infantile hepatoencephalopathies that are not explained by POLG mutations. Furthermore, Twinkle mutations should be considered in diseases with mtDNA depletion.

Patients and methods
Patients 1 and 2 were 1/3 and 3/3 children of healthy non-consanguineous Finnish parents. The parents at examination did not have any neurological symptoms or ophthalmoplegia at the ages 36 and 40 years. The 2/3 son was at 8 years of age otherwise healthy but had a mild form of juvenile rheumatoid arthritis.

Patient 1 was born healthy after an uneventful pregnancy at full term. His birth weight was 3980 g and height 53 cm. His early psychomotor development was considered normal, but at 6 months of age the parents noticed peculiar eye movements and involuntary movements of the hands and face. At the age of 8 months he had slight muscle hypotonia, poor head control and temporary vomiting. At 13 months of age he had pronounced athetoid movements in his face, trunk and limbs. The deep tendon reflexes were absent and the eye fixation was poor. He was unable to sit or stand unsupported, and moved himself by rolling over or in a crawling like manner using the proximal arm muscles. The disease progressed rapidly: the patient lost his ability to crawl, and was never able to speak. Hearing deficit, restriction of eye movements, and sensory axonal neuropathy were detected. Brainsstem auditory evoked potentials (BAEP) were abnormal at the age of 18 months, and at the age of 30 months, the electro-neuromyography (ENMG) showed no responses of the sensory nerves, whereas the motor responses were within normal limits. At the age of 3.5 years he had prominent athetosis, ataxia, muscle hypotonia, poor head control, distal amyotrophy, extensor plantar responses and total ophthalmoplegia. At that time, the quadriceps femoris muscle biopsy sample showed slight atrophy of type II muscle fibres, marginal focal fibre-type grouping, and slight decrease of histochemical COX activity in some fibres, but no totally COX-negative fibres (Supplementary Material, Fig. S1). The sural nerve biopsy sample showed mild to moderate axonal neuropathy with pronounced loss of large myelinated fibres (Fig. 5F). The T2-weighted MRI images at the age of 3.5 years showed high signal intensity areas around the fourth ventricle, and in the region of the superior cerebellar peduncle, and the dentate nuclei (Fig. 1). In addition, symmetric cerebellar cortical atrophy was present in the MRI. During the follow-up the blood and cerebrospinal fluid (CSF) lactate levels were marginally elevated.

At the age of 4.5 years he died of his first epileptic attack: epilepsy partialis continua progressing to a therapy-resistant status epilepticus. The antiepileptic drugs used during these 2 weeks were phenytoin, barbiturates and benzodiazepines, but not valproate, which is known to be toxic in POLG-Alpers. The liver transaminases increased in the serum (aspartate aminotransferase or ASAT 988 units/l, and alanine aminotransferase or ALAT 1188 units/l, the reference values being 10–50 units/l), and the thromboplastin time decreased to 23–35% (reference values 70–130%), while alkaline phosphatase and bilirubin remained normal. The liver transaminases had also been repeatedly elevated, up to nearly 4 times the normal values at 12–25 months of age: ASAT varying between 64 and 184 U/l and ALAT between 80 and 186 U/l. At that time, the serum alfa-1-fetoprotein level had also been increased, and the lactate dehydrogenase levels mildly elevated, but the glutamyltransferase level and the thromboplastin time had been normal.

Patient 2 was born healthy after an uneventful pregnancy at full term. His development was considered normal during the first months of his life. He learned to roll over at the age of 3 months, but at the age of 5 months his parents noticed abnormal,
momentary upward eye movements. On examination at the age of 6 months he had muscle hypotonia, difficulties in eye fixation, a suspicion of ataxia and loss of deep tendon reflexes.

At the age of 8.5 months neurological examination revealed muscle hypotonia, loss of deep tendon reflexes, mild ataxia and atheildoid movements in the hands and face. The ophthalmologist noted jerky pursuit eye movements, momentary upward eye movements and nystagmus. The brain MRI (Fig. 1) and EEG were normal. Sensory axonal neuropathy was confirmed with the absence of sensory responses on the ENMG and loss of large myelinated axons on the sural nerve biopsy sample. The muscle biopsy sample showed some accumulation of lipids, as indicated by Oil red O staining (Supplementary Material, Fig. S1B). Some fibres showed a slight decrease in histochemical COX activity, but no totally COX-negative fibres (Supplementary Material, Fig. S1C), and COX activity in biochemical analysis was slightly decreased. CSF lactate was normal, and pyruvate low. The liver transaminases were mildly elevated in the serum at 8.5 months–1 year of age: ASAT increasing up to 147 units/l and ALAT up to 100 units/l. The creatine phosphokinase values remained normal.

At 11 months of age the patient had severe muscular hypotonia, ataxia and athetosis. As a consequence of frequent vomiting, dysphagia and weight loss, a percutaneous endoscopic gastrostomy (PEG) tube was inserted at the age of 22 months. At the age of 25 months he had his first attack of epilepsy partialis continua progressing to a refractory generalized convulsive status epilepticus. The EEG showed focal irritation with generalization. Since the first status epilepticus, the EEG remained abnormal, and in spite of antiepileptic medication he has continued having epileptic seizures—both myoclonic jerks and epilepsy partialis continua progressing to a status epilepticus. His antiepileptic remedy included phenobarbital, carbamazepine and benzodiazepines, but not valproate. At the present age of 4 years the patient has severe muscle hypotonia and neuropathy, and is unable to move himself or catch a toy. The severe neuropathy resulted in autonomous dysfunction: obstruction, difficulties in micturition, and dry eye syndrome. He has total ophthalmoparesis, severe dysphagia and no reaction to speech.

**Autopsy and neuropathological examination of patient 1**

A general autopsy including a neuropathological investigation was performed ~18 h post-mortem according to standard procedures. The parenchymal organs were sampled for histological examination. The paraffin-embedded samples from the general autopsy were sectioned at 6–8 μm and stained using haematoxylin–eosin (HE). In addition, the liver sample was stained with periodic acid–Schiff (PAS), including also diastase-PAS.

The brain and spinal cord were dissected after fixation in formalin. Paraffin-embedded samples were sectioned at 6–8 μm and stained using the following methods: HE and luxol fast blue-cresyl violet (LFB). Selected samples were studied by immunohistochemistry using monoclonal mouse antibodies to non-phosphorylated neurofilaments (SMI-311, Sternberger Monoclonals, Baltimore, MD, USA; diluted 1:2500).

**DNA extraction, DNA analyses, tissue lysates**

The vastus lateralis muscle of Patient 1 was biopsied at the age of 3 years and 8 months, and that of Patient 2 at the age of 8.5 months. The samples were snap-frozen in liquid nitrogen and stored at −80°C. The total muscle DNA was extracted using standard DNA extraction protocols. The genomic DNA of the parents was extracted from total peripheral venous blood using QIAamp DNA Blood Maxi Kit (Qiagen) according to the manufacturer’s recommendations. In order to exclude recessive POLG1 mutations as the underlying gene defect in the two siblings, we performed a DNA marker analysis of two informative polymorphic dinucleotide markers flanking both sides of the POLG1 gene, as previously described (Hakonen et al., 2005): the markers used were D15S276 (202 kb centromeric to POLG1) and D15S542 (83 kb telomeric to POLG1). We then sequenced the five exons and intron–exon boundaries of the gene PEO1 (GenBank NM_021830.3, the numbering starting from ATG), encoding Twinkle helicase, from our proband (Patient 1) using standard PCR amplification and sequencing methods. Family members were screened for the mutations found in the proband. In order to confirm the pathogenicity of the A318T mutation, we also screened for this mutation in 120 Finnish chromosomes. In addition, we used paraffin-embedded formalin-fixed tissue samples from post-mortem liver, kidney and brain of patient 1 (samples taken ~18 h post-mortem, formalin-fixed 8 years ago), as well as control liver samples, for mtDNA quantification, as follows: thin sections of paraffin-samples were deparaffinized by xylene and lyophilized, followed by proteinase K (0.05 μg/μl) treatment at 55°C for 1 h in the lysis buffer (1× standard PCR buffer, 0.45% Triton-X, 0.45% Tween 20). The proteinase was inactivated for 10 min at 95°C, and 4 μl of the lysis was used for amplification reactions. As control samples we used paraffin-embedded formalin-fixed post-mortem liver samples of five individuals of 2–6 years, with the following diagnoses: Finnish congenital nephrosis, Langerhans cell histiocytosis, meningococcal sepsis, acute lymphoblast leukemia and tetralogy of Fallot. The samples were taken 1–3 days post-mortem, and were formalin-fixed 1–4 years ago.

**Mitochondrial DNA analysis**

Long-range PCR analysis of muscle mtDNA was performed as previously described (Van Goethem et al., 2004), except for a minor modification in the forward primer. We amplified a 8.3 kb region of mtDNA at nucleotide positions 8232–16496 using Expand Long Template PCR System (Roche), with buffer system 1, 10 ng of total muscle DNA and primers 5′-TAAAAATCTTTTGAAATAGGGCCCGTATTTACC-3′ and 5′-CGGATACGTTCCACTTAGCTACCCTCCAAAGTG-3′. The analysis was performed also with DNA amounts of 5 and 20 ng for the patient samples (data not shown), to confirm the correct amplification of potential mtDNA deletions. The PCR amplifications were done with an annealing temperature of 63°C, either with an extension time of 8 or 3 min—the shorter extension time selectively amplifying mtDNA fragments containing deletions. As positive controls we used muscle DNA samples from a patient with multiple mtDNA deletions and a Kearns–Sayre patient with a single mtDNA deletion. The muscle mtDNA of patient 1 and patient 2 was sequenced by first amplifying mtDNA in two partially overlapping fragments of ~9 kb using Phusion High Fidelity DNA polymerase in HF buffer (Finzymes), and subsequently the PCR fragments were sequenced using standard sequencing methods.

Mitochondrial DNA was quantified from the muscle, liver and brain DNA using real-time quantitative PCR (Q-PCR) with TaqMan probes. A portion of cytochrome B gene (CytB) was used as the mitochondrial gene target, and a portion of a single copy gene amyloid
precursor protein (APP) as the nuclear gene target. These targets were amplified in the same PCR reaction tube, using TaqMan Universal PCR Master Mix (Applied Biosystems), 25 ng of total DNA, and the following primers and TaqMan probes (Applied Biosystems): 5'-GGCTGTGAGTTTCGCTCAACATC-3', 5'-AAGATGTCGATGTTGATCATGCTACCC-3', and 6-FAM-CACCCAGGCCCTCAACC GCCCT-TAMRA for CytB and 5'-TGTTGGCTCTCCAGAGGTC TA-3', 5'-CAGTTCTGGATGTCACCTGCG-3', and VIC-CCTGAA CTGCAGATCACCAATGTGGTAG-TAMRA for APP. The PCR reactions were performed in duplicate or triplicate and the mean values of the measurements were used for the analyses. The analyses were performed with ABI PRISM 7000 Sequence Detection System. Our method was first validated by generating a standard curve using known copy numbers of standard plasmids containing the CytB or APP gene targets, and subsequently, relative quantity of mtDNA to APP was measured as previously described (Livak and Schmittgen, 2001). The quantification of mtDNA was reproducible and the patients’ results were compared to age-matched controls.

**Statistical analysis**
Statistical significance of the results of the liver mtDNA quantification in three different Q-PCR experiments was assessed by using the two-tailed, unpaired Student’s t-test.

**Multiple sequence alignment**
Multiple sequence alignment was performed with a ClustalW program (available at http://www.ebi.ac.uk/clustalw/), with default settings and the following sequences: NP_068602.2 (Homo sapiens), NP_722491.2 (Mus musculus), and NP_508973.2 (Caenorhabditis elegans).

**Results**

**DNA sequence analysis**
The phenotype of our patients resembled those associated with POLG mutations, and therefore we first excluded recessive POLG1 mutations as the causative gene defect by DNA marker analysis. The two siblings had inherited different POLG1 gene alleles from their parents: the dinucleotide marker D15S276 resulted in genotypes 1/2 and 1/3, and marker D15S542 resulted in genotypes 1/1 and 1/2, for patient 1 and patient 2, respectively. This excluded POLG1 as a shared disease allele in the siblings. We next analysed the PEO1 gene encoding for Twinkle, prompted by the similar clinical features in our patients as compared to IOSCA patients. By DNA sequencing, we identified two compound heterozygous nucleotide changes in PEO1 gene in both affected siblings (Fig. 2): c. 1523 A>G, resulting in a tyrosine > cysteine change at amino acid (aa) 508 of the polypeptide (Y508C) and c.952 G>A, resulting in a change of a tyrosine for an alanine at aa 318 (A318T). The mother was a heterozygous carrier of the Y508C mutation, and the father a carrier of the A318T mutation. The new variant c.952G>A was not a known polymorphism (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=56652), and was not present in 120 Finnish chromosomes screened.

The Y508C mutation has previously been described underlying recessive mitochondrial ataxia, IOSCA (Nikali et al., 2005).

**Mitochondrial DNA analysis**
The results of the long-range PCR analysis are shown in Fig. 3. The amplification of the 8.3 kb mtDNA fragment showed no deletions in the muscle of the two patients. Even when selectively trying to amplify shorter mtDNA fragments with a reduced extension time, no deletions were detected, although these were readily detected in a positive multiple mtDNA deletion control. The sequencing of muscle mtDNA confirmed that both patients shared the same wildtype mtDNA background of haplogroup K (GenBank DQ489512 and AC_000021.2). Figure 4 shows the results of mtDNA quantification assay. The mtDNA amount was significantly reduced in the liver of Patient 1 (7–13%, P = 0.025), when compared to the average of the age-matched control liver samples, whereas the muscle showed close to equal amount of mtDNA, when compared to age-matched muscle controls. The mtDNA quantity in each of our post-mortem paraffin-embedded age-matched liver controls varied in the three different Q-PCR experiments with different liver lysates, but the mtDNA amount in the liver of patient 1 was consistently significantly lower than in the control liver sample with the lowest mtDNA amount. The brain showed low mtDNA amounts, comparable to those in the patient’s liver, and the kidney showed 4–8-fold higher amounts than the patient’s liver, but suitable kidney and brain age-matched paraffin-embedded
control-samples were not available, leaving these results suggestive for mtDNA depletion in the brain. In patient 2, the skeletal muscle showed only slightly reduced mtDNA amount (42% of controls).

**Autopsy and neuropathological examination of Patient 1**

At the general autopsy, the most remarkable finding was small stature. The height was $-2.5$ SD and the weight $-27\%$. The gross findings and the weight of the parenchymal organs were normal, as well as the findings in the light microscopical examination, except for some glycogen loss and few small centrilobular lipid vacuoles in the liver (Supplementary Material, Fig. S1D). These mild histological changes were not, however, enough to alone establish a diagnostic finding of a hepatopathy.

At autopsy the brain was oedematous, a logical consequence of prolonged terminal status epilepticus with associated hypotonia and ischaemia. The histological examination revealed severe, quite acute ischaemic changes throughout the cerebral cortex (Fig. 5A), basal ganglia and thalami. In the brainstem, the pontine tegmentum (Fig. 5B) displayed acute ischaemic changes, as well as congestive capillary changes. In addition to the acute ischaemic changes, the cerebellar cortex showed a presumably pre-existing patchy dropout of Purkinje cells, but a preserved granular cell layer (Fig. 5C). The dentate nucleus was subtotally neuron-depleted and gliotic (Fig. 5D), consistent with a pre-existing pathology. In the spinal cord,
the posterior columns, as well as the posterior spinocerebellar tracts, were quite severely atrophic (Fig. 5E), and the posterior spinal nerve roots abnormally thin. Also the dorsal nucleus (Clarke’s column) showed neuronal loss, but anterior horn motoneurons and the pyramidal tracts seemed preserved.

**Discussion**

We report here compound recessive mutations of the mitochondrial replicative helicase Twinkle in early onset encephalopathy with intractable epilepsy, liver involvement and mtDNA depletion. Dominant Twinkle mutations are
known to cause mitochondrial myopathy (Spelbrink et al., 2001), and the only known recessive phenotype has been a disease of Finnish disease inheritance, infantile onset spino-cerebellar ataxia, IOSCA (Nikali et al., 2005). Our present report expands the phenotypic spectrum of Twinkle mutations to severe early encephalopathies clinically resembling Alpers–Huttenlocher syndrome (Alpers, 1931). This syndrome has previously been associated with mutations in the mitochondrial polymerase gamma, a functional partner of Twinkle at the mtDNA replication fork (Naviaux and Nguyen, 2004).

The Y508C mutation, found in the current report as compound heterozygous, has been previously reported as homozygous in IOSCA (Nikali et al., 2005). The siblings in the present report developed their first symptoms by the age of 6 months, whereas the age of onset in IOSCA is on the average only at 14 months. The clinical features closely resembled IOSCA, with ophthalmoplegia, muscle hypotonia, athetosis, hearing loss, sensory axonal neuropathy and ataxia. Intractable epilepsy, often leading to fatal status epilepticus, is also common in IOSCA, but not before teenage or early adulthood (Lönqvist et al., 1998), whereas in the current siblings the epilepsy manifested already during the first years of life. The MRI changes were also evident at an early age in our patients, in contrast to IOSCA patients developing cerebellar white matter changes at teenage, or later during the course of the disease. The early onset and the rapid course of this disease with liver involvement distinguish the phenotype of the two siblings from IOSCA.

The new phenotype of the present report shares several clinical features with the POLG-associated Alpers–Huttenlocher syndrome, since both are characterized by rapidly progressive psychomotor retardation, intractable epilepsy, and liver involvement, with an onset of the disease in early life (Naviaux and Nguyen, 2004; Davidzon et al., 2005; Ferrari et al., 2005; Horvath et al., 2006; Tzoulis et al., 2006). The neuropathological findings of our patient, including atrophy of the posterior spinal cord and the cerebellum, resembled those in IOSCA (Lönqvist et al., 1998). Severe cortical ischaemic changes were a likely consequence of the lengthy terminal phase with status epilepticus and brain oedema. Our patients showed elevated liver transaminases in the serum, but the liver histology of the deceased patient could not establish a definitive diagnosis of hepatopathy, and the severe histological findings often seen in Alpers syndrome were absent (Huttenlocher et al., 1976; Naviaux and Nguyen, 2004; Davidzon et al., 2005; Ferrari et al., 2005). Alpers hepatopathy often manifests acutely after valproate treatment for epileptic seizures, but hepatopathic changes have also been reported prior to the exposure to valproate (Ferrari et al., 2005). In an IOSCA patient, we have observed valproate-induced hepatopathy, manifesting with icterus and vomiting, as well as an increase in the serum concentrations of transaminases, alkaline phosphatase, and bilirubin, and a decrease in albumin, and recovery after discontinuation of valproate treatment (Lönqvist, unpublished data). Our present patients never received valproate, indicating that liver dysfunction can be an inherent part of Twinkle-associated disorders.

We found a novel compound heterozygous variant changing a hydrophilic threonine at aa 318 of the Twinkle polypeptide, in combination with the Y508C. Several facts support the pathogenic role of this variant: (a) the variant was not seen in 120 Finnish chromosomes and has not been reported as a polymorphism; (b) the amino acid is highly conserved, being an alanine in the mouse, fruit fly and C. elegans (Fig. 2); (c) the variant segregated with the disease in the pedigree, since both patients were compound heterozygous for both A318T and Y508C; (d) a previously reported mutation in the adjacent amino acid, K319E, manifested as a multisystemic disease with PEO, sensory ataxic neuropathy, dementia and refractory epilepsy (Hudson et al., 2005)—a phenotype sharing features with that of our patients. However, another mutation affecting the same amino acid, K319T, was associated to late-onset adPEO without other neurological or psychiatric features (Deschauer et al., 2003). Finally, (e) the variant occurred as compound heterozygous with a known recessive IOSCA-mutation, Y508C, and the resulting phenotype is IOSCA-like, but more severe. In our present pedigree, the father carrying the A318T showed no signs of ophthalmoplegia on examination at the age of 40 years, suggesting that the mutation either is truly recessive, or, if dominant, manifests in late adulthood.

The alanine 318 is located in a moderately conserved region of Twinkle, upstream of the linker region of the protein. In the T7 helicase, this region corresponds to the primase domain, but no signs of primase activity have been found in the human Twinkle (Spelbrink et al., 2001). The function of this domain may be involved in the subunit interactions of the hexameric protein, but this remains to be studied. The Y508C mutation is located in the helicase domain of Twinkle, close to the Walker B motif. Whether the two mutations are in close proximity in 3D structure of the monomer, or in the interfaces of the two adjacent subunits of the hexamer, remains to be studied. However, the more severe phenotype in the present siblings than in Y508C homozygotes suggests that the two mutations have synergistic detrimental effects to the helicase function.

In our patients, sensitive long-range PCR analysis did not reveal any mtDNA deletions, but mtDNA depletion was detected in the liver and possibly in the brain, indicating severely compromised Twinkle function in those tissues. MtDNA deletions are typical for the muscle of adults with dominant Twinkle mutations and are thought to accumulate by age (Suomalainen et al., 1997; Spelbrink et al., 2001; Deschauer et al., 2003; Hudson et al., 2005). The absence of deletions in the muscle of our patients could be accounted
for by their young age, or may reflect the specificity of CNS involvement. Furthermore, also patients with POLG-associated ataxia syndromes or IOSCA show no or low amounts of mtDNA deletions in the muscle (Van Goethem et al., 2004; Nikali et al., 2005). Depletion of mtDNA has been reported in the liver (Naviaux and Nguyen, 2004; Davidzon et al., 2005; Ferrari et al., 2005), muscle (Naviaux and Nguyen, 2004) and frontal cortex (Ferrari et al., 2005) of patients with POLG mutations and Alpers syndrome. Our patients show similar findings. Muscle mtDNA amount was only mildly reduced or normal in our patients, which agrees with the close-to-normal activity of cytochrome c oxidase in their muscle. Why dominant mutations of Twinkle manifest mainly late in life in the muscle, and recessive mutations in the brain and the liver, is intriguing, and subject to further studies.

Our report adds Twinkle to the list of proteins, the defects of which can cause mtDNA depletion. This list includes proteins involved in mitochondrial nucleoside pool regulation, as well as polymerase gamma, and the MPV17 protein with no known function (Mandel et al., 2001; Saada et al., 2001; Naviaux and Nguyen, 2004; Elpeleg et al., 2005; Spiazziola et al., 2006; Bourdon et al., 2007). We and collaborators have shown in cell culture that inhibition of Twinkle by RNAi reduces rapidly mtDNA copy number (Tyynismaa et al., 2004), but previously no disease phenotypes have linked Twinkle to tissue-specific mtDNA depletion. Our findings show that recessive Twinkle mutations can cause a phenotype reminiscent of POLG-associated Alpers syndrome, and should be considered in patients with hepatoneuropathies of unknown cause, as well as in patients with mtDNA depletion.

**Supplementary material**

Supplementary material is available at Brain online.

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