Complete Deletion of a *POLG1* Allele in a Patient with Alpers Syndrome

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Abstract Mutations in the gene encoding the catalytic subunit of polymerase γ (*POLG1*) are a major cause of human mitochondrial disease. More than 150 different point mutations in the gene have been reported to be disease causing, resulting in a large range of clinical symptoms. Depending on the mutation or combination of mutations, disease onset can occur in early infancy or late in adult life. Here, we describe the use of multiplex ligation-dependent probe amplification (MLPA) analysis to detect deletions within *POLG1*, which could otherwise go undetected by solely sequencing of the gene. We present a case where an entire *POLG1* allele is deleted, with a known pathogenic mutation (W748S) on the remaining allele. The deletion was found in a boy with Alpers syndrome, presenting at 18 months of age with slightly retarded motor development, balance problems, and seizures. Administration of valproic acid (VPA) led to

rapidly progressive fatal liver failure in our patient, and we would like to highlight the need to carry out complete *POLG1* gene analysis before administration of VPA in cases of pediatric seizure disorders of unknown origin. Debut and severity of the disease in this patient was unique when compared to homozygous or heterozygous patients with the W748S mutation, leading to the conclusion that gene dosage plays a role in the clinical phenotype of this disease.

Introduction

The nuclear-encoded DNA polymerase γ (POLG) is the sole polymerase replicating mitochondrial DNA (mtDNA) (Clayton 1982). This holoenzyme is a heterotrimer made up of one catalytic subunit (POLG1) and two accessory subunits (POLG2) (Yakubovskaya et al. 2006). POLG1 consists of an exonuclease domain at its N-terminal and a polymerase domain at its C-terminal, which are separated by a linker region. The crystalline structure of POLG has revealed that this linker region is where interaction between POLG1 and POLG2 occurs leading to increased processivity and substrate binding (Lee et al. 2009).

Mutations in the gene for POLG1 are major causes of human mitochondrial disease. The first POLG1 mutation associated with disease was described in 2001 (Van Goethem et al. 2001). Since then, approximately 150 pathogenic mutations have been identified (<http://tools.niehs.nih.gov/polg>) and linked to a wide spectrum of clinical phenotypes, ranging from severe progressive neurodegenerative disorders in early childhood, to milder syndromes with onset in adult age. Some major groups of different clinical phenotypes are seen (1) myo-cerebro-hepatopathy spectrum disorders, (2) Alpers syndrome,

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(3) ataxia-neuropathy spectrum disorders, (4) myoclonus epilepsy-myopathy-sensory ataxia spectrum disorders, (5) autosomal dominant progressive external ophthalmoplegia (adPEO), and (6) autosomal recessive progressive external ophthalmoplegia (arPEO) (Wong et al. 2008). However, many additional symptoms have been reported and categorization of patients into specific clinical groups is not always straightforward.

Here, we report the finding of a complete deletion of one *POLG1* allele in a patient with Alpers syndrome and acute liver failure induced by treatment with VPA. The deletion comprises the entire *POLG1* gene and the adjacent *FANCI* gene (OMIM#611360). On the retained *POLG1* allele, we found the W748S mutation, previously reported to be associated with Alpers syndrome.

Patient and Methods

Patient

The patient, a boy, was the second child of healthy, nonconsanguineous parents of Swedish origin. An older brother is healthy. Pregnancy was uneventful and the patient was born at term.

The boy showed normal mental development, however, his motor development was delayed; he was able to roll over from 9 months of age and started to crawl on his knees at 18 months of age. By the age of 2 years, he was able to walk independently but his gait was wide based and unsteady, with frequent falls. Epileptic seizures, mainly absences, were observed from around 18 months of age.

Electroencephalography demonstrated both generalized spike and slow waves and focal sharp waves in frontal

regions. A computed tomography of the brain at 27 months of age showed normal findings. Routine blood parameters including liver function values were normal. Treatment with valproic acid (VPA) was started from 28 months of age with the patient responding well to treatment; there were no further seizures. Blood concentration of VPA was 700–870 $\mu\text{mol/L}$.

Three months later, the boy was hospitalized because of generalized fatigue and vomiting. Laboratory investigations revealed liver involvement with increased serum levels of transaminases, γ -glutamyl-transpeptidase, prothrombin-INR (international normalized ratio), and ammonia. He had elevated blood lactate 2.8–3.9 mmol/L (normal <2,3). Although VPA was stopped within a few days from admission, his liver disease progressed and he developed an acute, fulminant, and noncholestatic liver failure with severe coagulopathy. Liver biopsy could not be performed due to the tendency of bleeding.

Magnetic resonance imaging (MRI) of the brain showed abnormalities in thalamus and basal ganglia. Signs of atrophy were seen (Fig. 1). The clinical picture was consistent with Alpers syndrome. A muscle biopsy was performed, and DNA analysis of the *POLG1* gene was started. The boy deteriorated further and died at the age of 32 months, due to hepatic failure. Autopsy was not carried out.

Mitochondrial ATP Production, Respiratory Chain Enzyme Activities and Morphological Analysis

A muscle specimen was obtained from *Tibialis anterior* at 30 months of age. Mitochondria were isolated from muscle and mitochondrial ATP production rate and respiratory

Fig. 1 Magnetic resonance imaging of the brain performed at 32 months of age. **(a)** Axial T2-weighted images show symmetrical, bilateral, high signaling abnormalities and edema of thalamus and caput nucleus caudatus. Signal changes also present in globus pallidus

bilaterally, seen in other slices of the examination. Signs of frontal and temporal atrophy. Cerebellar atrophy also present, not included in this image. **(b)** Diffusion images show increased diffusion in thalamic regions

chain enzyme activities were determined as previously described (Wibom et al. 2002). In addition, standard techniques were applied for routine and enzyme histochemical stainings of cryostat sections and for electron microscopy (Larsson and Oldfors 2001).

Sequence Analysis of the *POLG1* Gene and Breakpoint

Total DNA was isolated from whole blood using QIAamp DNA mini kit (Qiagen). Exons 2 to 23 of *POLG1* were amplified by PCR using intronic primers. Sequencing reactions were carried out on the PCR products followed by sequencing on a 3130xl DNA sequencer (Applied Biosystems, Foster City, CA). For sequencing of the deletion breakpoint, a forward primer 5'-AAGGGCTGATGGCATCTC-3' was designed within the multiplex ligation dependent probe amplification (MLPA) probe C15igs7 and reverse 5'-AGAGAAAGTGTGTTGATGCC-3' within the MLPA probe C15igs6, as both probes were known to lie outside of the deletion (Table 1). The Expand Long Template PCR System (Roche, Mannheim, Germany) was used to amplify the junction breakpoint using these primers and successful amplicons were sequenced.

Multiplex Ligation Dependent Probe Amplification Analysis

MLPA analysis, using kit P010 (MRC Holland, Amsterdam, The Netherlands), for detection of deletions or duplications of each exon of the *POLG1* gene was performed according to the manufacturer's protocol, starting from 100 ng DNA. The PCR products were separated by capillary electrophoresis on an ABI 3130xl genetic analyzer. Trace data was analyzed using the GeneMapper v3.7 software, and the integrated peak heights were exported to an Excel spreadsheet (Microsoft) for further calculations. For each sample, the peak heights were first normalized to the average peak height of the control probes, followed by normalization to the average peak height of the control samples included in the run. The analysis was considered acceptable if the ratio for the internal control was between 0.8 and 1.2. Threshold values for deletions and duplications were set at 0.7 and 1.3, respectively.

Synthetic probes to narrow down the breakpoint regions were designed as previously described (Barbaro et al. 2008; Stern et al. 2004). Different probe combinations, together with the EK1 reagent kit (MRC Holland), were used for the MLPA reactions. Quantification analysis was performed as described above. Synthetic probe sequences, together with their results, are summarized in Table 1.

Results

Morphological studies of the muscle biopsy showed a slightly increased amount of fat in the muscle, but no other abnormalities. Mitochondrial ATP production rate and respiratory chain enzyme activities were normal.

Sequencing of all coding exons and exon/intron boundaries of the *POLG1* gene revealed that the patient was homozygous for the W748S mutation. The father was shown to be a heterozygote carrier, whilst the mother lacked the mutation completely. Interestingly, the mother was homozygous for an intronic change (c.2074-22T), which the patient had not inherited. This led us to believe that the patient had a deletion in one of his *POLG1* alleles, either a spontaneous deletion or one inherited from his mother.

MLPA analysis revealed that the entire *POLG1* gene was deleted on one allele in both the patient and the mother. By designing synthetic probes for MLPA, we could narrow down the breakpoint region and determine that the centromeric and telomeric breakpoints lie between probes c15igs7-c15igs13, and probes c15igs11-c15igs6, respectively (Fig 2). Following this, the breakpoint region was amplified by extra long PCR and sequenced, establishing that the deletion was almost 118 kb in length. The presence of a 4-bp microhomology region at the breakpoint junction indicates that the deletion was created by a nonhomologous end-joining mechanism. In addition to the deletion of *POLG1*, an adjacent gene, *FANCI*, was also completely deleted.

Discussion

Alpers syndrome is a disease in the severe end of the POLG disease spectrum, clinically characterized by intractable epilepsy, psychomotor regression, and hepatopathy, with or without liver failure (OMIM#203700). Additional symptoms such as ataxia, hypotonia, and cortical blindness are frequently seen (McFarland et al. 2008). Onset is usually in early childhood, although later forms exist (Uusimaa et al. 2008). Skeletal muscle is not always affected and a muscle biopsy often turns out to be normal in children with Alpers syndrome. Bicknese et al. described VPA induced liver toxicity in children with Alpers syndrome already in 1992. Since then, several cases have been reported, often with fatal outcome (Kayihan et al. 2000). The mechanism of VPA-induced liver failure in POLG disease is not clear; however, VPA is known to affect the mitochondrial respiratory chain function, among other ways by inhibition of mitochondrial fatty acid oxidation (Silva et al. 2008). Its effect on POLG and mtDNA replication has not been investigated. This case

Table 1 Probes used for MLPA analysis to narrow down the breakpoint

Probe name	Cytoband	Size	5' half probe ^a	3' half probe ^b	Results ^c
POLGex23UTR	15q26.1	93	CTTGCTAAGGCCAGCCATTTTTCAG	TAGCAGGACCTGCCAAGAAGATTCC	-
RLBP1ex1	15q26.1	96	GGGCGGTGAGAAAGTTCCTTATGACACAC	TAATCCCAACCTGCTGACCGGACCAC	+
POLGex1UTR	15q26.1	99	CAATCCACCTGCTCCTTGAGAAAGGGGAGG	TTGGCCTTTGGTTTCGCGCTTCCGCAAG	-
c15igs13	15q26.1	102	CCTCTTCTTCCAGGCCTTTGAGA	AACAGGGAACAGATTGGGAGAAAGAGCTAACAC	-
c15igs5	15q26.1	102	GTGAACACAGACGGATAAGATTGCTCCTTAG	TTAAGGAAGCCTGCACACAGGGTGAAAAAC	-
RHC'Gintr10	15q26.1	102	GCTTGGCAATAGCTGGGTCTGGGGCCTGAGT	GATGTGGCAAGAGAGGATGTTAGGGTGCC	+
c15igs9	15q26.1	108	CTGCCCTAGAGAATGCTCTCCCTTCTGCTA	TTCCTGTCTGTGCCAGCACAGTGGCTTGC	+
c15igs15	15q26.1	108	GCAGAAAGACTCAGTGGCTCCCAATGTCAA	CAAGGCCACTGAGGTATGACAGTGCCCAAGAGG	-
c15igs4	15q26.1	108	GAGCAAGCTCGTTTCTGGACTTCTCTCGAGT	GACCGAACACTTGTCTGCTGACACTTCTTCTCATG	+
c15igs7	15q26.1	111	CCACCTCTCCACAGAAAAGGGCTGAGATGGCAT	CTCAAAGGCCCAAGTTTGTCTGATATTATAGACCAG	+
FANCIex4	15q26.1	111	CTGCTCTGAGGAAGCTGGAACACTTAGGAGAC	GTAAGATATACACTTGTGTATCCAGTTGGTGAATC	-
c15igs6	15q26.1	114	CCACTTGGCAGGTGCCAGGCAATGTC AACACACTTCT	CTGTAGAGGTACACAGAAAGGCTCTGGGGTTGGTCC	+
c15igs3	15q26.1	114	GGGCTCTCTCTCTGTTCCATCACATATGAAGTC	TCTCACTTGCAAGCAAGAACAGCCCTGAAAACGTTGG	+
ACANex1	15q26.1	117	GCTGCTCGCCAGGTGTGTGGGACTGAA GTTCTTGGGA	GAAAGGAGTCCAACTCTTCAAGGTAACTGTCTCTTCC	+
c15igs8	15q26.1	117	GGTAAACCTCTGTCTCAAACCTTACCAAACCTGAA	TTTCTTCCAGGTGGAGTAAACAGGTTCCTCTTAACAAAC	-
ABHD2ex1	15q26.1	120	CTTATCCAGTAGCGTCTGGGTCCCTGGACTAAAGCCGTCAA	ATGTAAACCAATTGCTGAGGAGCTCTCCCTAAGAATCCCGCC	+
c15igs12	15q26.1	120	GTATGTCTGGCACACAGACAGACGTCTGTCTATTC	TTGTCCCCATCCCATTTTGTCCCTCCTTAGCTCCTGACCAG	-
c15igs14	15q26.1	120	CAAATATGGGATCATCCAAATACCCAGTATCTCTGTAACCTGA	AAGCTATCATTACGCTGAATATCATGGACATCCCTGAC	-
c15igs10	15q26.1	123	CCCAGTGAGATAAATTTCTTTTTCGCCCTCTGTACCCATTGT	ATCATTACAGAGTTCAGACCAAGAACTAGGAAACAGACTG	-
c15igs2	15q26.1	123	CCCCACACAGTGCTAGGGTCCCTCTCGAGTTTCTCAT	CTGCCCTCAGGTCACTTTCCACCCCTGATGCCCTTGGCTTGTCTG	+
Chr15-b88159461	15q26.1	123	GTGCTACTGCGCCTTCTGAACACTACGAGTCCCAGGTACCAAGA	AAGTTGAATTGCTGCTGTGGCGGGTCCGTTATCTGAC	+
c15igs1	15q26.1	126	GGCAGGAGAGTTCTTCCAGTGTGGCAATGAGATCCAGAC	TTGGGATCTCACATTCTCAGGGAGGCCAGTCTGAGGCCCTTG	+
c15igs11	15q26.1	126	CTTTCTCTGGCAGTGCCAGCCTGTGGATACAGCTGGAATGAC	TTTGACATAGTATCTCTCACTCTGGGCTCTCTGCCAGTGAC	-
Control probes					
GABRA4(pilot)	4p12	84	CAGCCTGTTGTCTATAACCATCG	AGCAAACTGTCCAGGATGCG	+
RELN2 (control)	4q13.3	87	CAGCATTACGGAATGAAGGTCA	CCACAAGAGTGGCTTCAACAAC	+
CLDN16	3q28	105	GACACAAGGGTGTAA AATGCACG	TTTCAGGGTGTGTTTGCATATGATTATATCAATCAGTATG	+
RB1ex23	13q14.2	129	GTCACCAATACCTCACATTCTCGAAGCCCTTACAAAGTTTCTCT	AGTTTCACTTACGGATTCTCTGGAGGGAACATCTATATTTCCACC	+
SRY135	Yp11.31	135	CAGTGCAAAAGGAAGGAGAGCTTCTCCGGAGAGCGGGAATTTCT	CTTGACAGCTGAGCTGTAATCATCGTGTGTAATACGCTTAACATAG	+

^aThe 5' half-probes are preceded by the universal tag sequence GGGTTCCCTAAGGGTTGGA^bThe 3' half-probes are preceded by the universal tag sequence TCTAGATTGGATCTTGTCTGGCAC and are phosphorylated at the 5' end^cPlus and minus signs indicate non-deleted and deleted regions, respectively

Fig. 2 Fine mapping of the *POLG1* locus deletion using MLPA. (a) Representation from the UCSC database (March 2006 assembly) of the *POLG1* and *FANCI* loci on 15q26.1. Probes used to narrow down

the deletion are represented by red vertical lines and the dark green horizontal line is showing the span of the deleted DNA. (b) Deletion junction alignment with the 4-bp microhomology boxed

further emphasizes the importance of considering the possibility of *POLG* disease and liberally performing *POLG1* DNA testing in pediatric seizure disorders of unknown etiology, especially if a psychomotor regression is also seen.

The reported deletion also comprised the adjacent *FANCI* gene. Mutations in *FANCI* cause Fanconis anemia, an autosomal recessive disorder affecting all bone marrow elements and associated with cardiac, renal, and limb malformations. Our patient had no symptoms of Fanconis anemia.

The association between Alpers syndrome and *POLG1* mutations is well established (Longley et al. 2005). The genotype can vary, but most cases of Alpers syndrome carry either of the two linker mutations A467T or W748S, in compound heterozygous with another *POLG1* mutation (Nguyen et al. 2005). Recent crystallization of *POLG* has shown that the W748S mutation is located in a globular subdomain of the linker region, which contacts the primer-template DNA complex to promote intrinsic processivity of *POLG* (Lee et al. 2009; Falkenberg and Larsson 2009).

In our patient cohort, we have diagnosed four children compound heterozygous for the W748S mutation. They all had early onset of symptoms, including intractable epilepsy, and died before 16 months of life. In addition, we have diagnosed three patients who are homozygous for the W748S mutation. The first patient had mild cognitive problems, ataxia, and nystagmus with the onset of epilepsy at 16 years of age. VPA treatment was started and within a few months she developed a rapidly progressing liver disease and died 2 months later. The second patient is a girl with mild mental retardation, attention deficit, motor difficulties, and nystagmus. Epilepsy started at 11 years of

age and is successfully treated with lamotrigine and topiramate with no progress in her disease. The younger brother of this girl has a slight developmental retardation but no other symptoms at his current age of 3 years. These observations are consistent with previous reports that patients homozygous for the W748S mutation have a milder disease with onset in later childhood or even adult age (Uusimaa et al. 2008). The patient we report here has one deleted *POLG1* allele and the W748S mutation on the retained *POLG1* allele. In contrast to previously described patients homozygous for the W748S mutation, the patient of this report had a relatively early onset of disease at 18 months of age. At the same time, he did not show the typical severe phenotype seen in patients who are compound heterozygous for the W748S and another *POLG1* mutation. These findings suggest that gene dosage affects the phenotypic outcome. This is in agreement with two previous reports of the occurrence of Alpers syndrome in patients with a monoallelic expression of A467T due to nonsense-mediated decay of the transcripts from the corresponding allele (Chan et al. 2005, 2009). The patients in these reports had a much earlier onset of disease compared with homozygous A467T patients and interestingly also died of liver failure despite not receiving valproate. As a result, the authors suggested that gene dosage plays a key role in the relatively late onset of symptoms in homozygous A467T patients, while the disease outcome of compound heterozygotes with the A467T mutation is steered by the mutation on the corresponding allele. Hence, we support the hypothesis that *POLG1* gene dosage is an important determinant of the phenotype in *POLG* disease. Consistent with this hypothesis, we have previously reported that mice with heterozygous *POLG1*

knockout are viable but have a moderate reduction of mtDNA copy number (Hance et al. 2005).

Depletion of mtDNA can be observed in both muscle and liver of Alpers patients (Naviaux et al. 1999); however, no significant change in mtDNA level was observed in the muscle of the patient described here. Unfortunately, no liver tissue was available for analysis as neither a liver biopsy nor autopsy was performed. In a similar report where two siblings carrying W748S in *trans* with a stop mutation (Y1210X), normal mtDNA copy numbers were also observed along with normal activities of the respiratory chain complexes in muscle (Ferrari et al. 2005). The lack of muscle involvement in all three patients may be due to the rapid disease progression.

This case once again illustrates the effect gene dosage plays on the clinical outcome in Alpers syndrome. Since not all disease-causing mutations are detected by DNA sequencing, a comprehensive investigation of the *POLG1* gene should include methods capable of detecting copy-number changes and intragenic deletions.

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Synopsis

Monoallelic expression of *POLG1* with W748S leads to a more severe Alpers disease compared to patients homozygous for the W748S mutation, hence, *POLG1* gene dosage appears to be an important determinant of the phenotype in POLG disease.

Author Contributions

Karin Naess: Designing the study, interpretation of data, and writing of article

Michela Barbaro: Analysis and interpretation of data, drafting of article

Helene Bruhn: Analysis and interpretation of data

Rolf Wibom: Analysis and interpretation of data

Inger Nennesmo: Analysis and interpretation of data

Ulrika von Döbeln: Contributed to study design, drafting of article

Nils-Göran Larsson: Revising article critically for important intellectual content

Antal Nemeth: Collection and interpretation of data, drafting of article

Nicole Lesko: Designing the study, analysis and interpretation of data, and writing of article

Guarantor

Nicole Lesko

The authors declare no conflict of interest.

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The study was approved by the regional ethics committee in Stockholm, Sweden.

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