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Drug-resistant epilepsia and fulminant valproate liver toxicity. Alpers-Huttenlocher syndrome in two children confirmed *post mortem* by identification of p.W748S mutation in *POLG* gene

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: *POLG* (polymerase gamma) gene mutations lead to a variety of neurological disorders, including Alpers-Huttenlocher syndrome (AHS). The diagnostic triad of AHS is: resistant epilepsy, liver impairment triggered by sodium valproate (VA), and mitochondrial DNA depletion.

Material/Methods: A cohort of 28 children with mitochondrial encephalopathy and liver failure was qualified for retrospective study of mitochondrial DNA depletion and *POLG* mutations.

Results: The p.W748S *POLG* gene mutation was revealed in 2 children, the only ones in the cohort who fulfilled the AHS criteria. Depletion of mtDNA (16% of control value) was confirmed *post mortem* in available liver tissue and was not detected in the muscle. The disease started with drug-resistant seizures, failure to thrive and developmental regression at the ages of 7 and 18 months, respectively. Irreversible liver failure developed after VA administration. Co-existence of epilepsy, VA liver toxicity, lactic acidemia and muscle respiratory chain dysfunction led finally to the diagnosis of mitochondrial disorder (and AHS suspicion).

Conclusions: Our results confirm, for the first time, the occurrence of a pathology caused by *POLG* gene mutation(s) in the Polish population. *POLG* mutation screening and mtDNA depletion assessment should be included in differential diagnosis of drug-resistant epilepsy associated with a hepatopathy.

key words: **drug-resistant epilepsy • valproate liver toxicity • Alpers-Huttenlocher syndrome • *POLG* gene mutation**

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Background

Valproic acid (VA), a synthetic branch chain amino acid, is widely used as an anti-epileptic medication, and is considered relatively safe. Mild transient hypertransaminasemia may be observed during the first months of therapy. Mechanisms of liver damage are linked to the influence of VA on fatty acids beta oxidation (FAO), low CoA availability, impairment of oxidative phosphorylation (OXPHOS) and redox-oxidative stress (ROS) [1–4].

A retrospective epidemiological study in the United States uncovered a number of fatal side effects associated with VA therapy. Acute liver failure developed after VA administration in 1: 800 children with drug-resistant epilepsy under 2 years of age [5]. Misdiagnosis of valproic acid toxicity (and unsuccessful liver transplantation) was reported in children with Alpers-Huttenlocher syndrome OMIM (hepatocerebral degeneration, AHS) [6].

Characteristic brain changes of the syndrome were described for the first time in an autopsied infant [7] and later correlated with liver failure [8]. Alpers-Huttenlocher syndrome was comprehensively reviewed by Harding, who reported on 32 autopsied patients [9]. Gross neuropathological assessment usually reveals variably distributed cortical narrowing, granularity and discoloration. Cortical involvement, not always symmetrical, may be extensive, focal or minimal, with a striking predilection for the calcarine (visual) cortex, which may provide a valuable macroscopic diagnostic clue for AHS.

Microscopic examination of the cerebral cortex shows astrogliosis, spongiosis and variable degrees of neuronal loss. Advanced lesions show hypertrophy of astrocytes with pronounced vascularity leading over time to total loss of cortical architecture and formation of a ”gliomesodermal remnant”. Extracortical lesions are more variable and affect white matter, hippocamp, cerebellar cortex, amygdala, substantia nigra and dentate nuclei. Microscopic spongiosis, gliosis and neuronal loss of variable distribution and severity may be observed [9].

The liver is invariably affected. In most patients, microscopic lesions are similar, although non-specific, and consist of severe microvesicular fatty change, degeneration and necrosis, extensive bile duct proliferation/transformation and massive fibrosis, leading to considerable loss of organ architecture. Some patients show nodular regeneration; others show end-stage destruction and fibrosis [9].

Polymerase gamma (POLG) deficiency was established as the major cause of the disorder [31] and *POLG* gene mutations were identified in the majority of AHS patients [10–16]. The protein (catalytic subunit of DNA polymerase gamma) is essential for mtDNA replication and repair. Over 160 coding variations in the *POLG* gene have been identified and the various pathogeneities characterized [6,18–20].

Clinical diagnosis of AHS depends on the co-existence of resistant epilepsy [21], hepatopathy (frequently triggered by VA), mitochondrial DNA depletion [22,23], and *POLG* gene mutation identification. At the metabolic level, the condition demonstrates OXPHOS dysfunction, and is included in the group of mitochondrial disorders [24].

The diagnosis of mitochondrial disorder was established in 2 Polish patients with epileptic encephalopathy who developed fatal liver failure shortly after valproic acid administration. The p.W748S *POLG* gene mutation was identified in both patients *post mortem,* finally confirming an AHS suspicion.

Material and Methods

A cohort of 28 children with mitochondrial encephalopathy and liver failure was qualified for retrospective *POLG* mutations screening. Availability of the patient's frozen tissue sample was the only inclusion criteria. The samples were used for assessment of mtDNA/nDNA ratio and/or DNA isolation. One of the patients was included in the reported Polish-Czech cohort of COX deficiency [25].

The search for *POLG* gene mutations revealed 2 patients bearing the p.W748S mutation. Clinical, biochemical and morphological documentation of the patients was re-analyzed in detail.

The study protocol was approved by the Bioethics Commission at the Children's Memorial Health Institute.

Patient 1

This female patient, born in 2002, was first admitted to our hospital at the age of 12 months. She was the second child born to a non-consanguineous couple. Her older brother was healthy. Pregnancy, delivery and neonatal period were uneventful. The girl was born with Apgar score 10, and birth weight 3800 g. At the age of 2 months she had a short episode of diarrhea with mild hypertansaminasemia (50–80 U/l). Her psychomotor development was normal up to the age of 7 months.

At the age of 7 months, she developed focal status epilepticus involving her left limbs, followed by postictal hemiparesis. Partial and generalized seizures recurred, progressing commonly to status epilepticus, and were often followed by postictal palsy. Laboratory investigations revealed elevated blood lactate concentration (4.1, 4.09 mmol/l., control <2.0 mmol), increased excretion of lactate in urine, and high alanine concentration in plasma (548.2 umol/l). Several antiepileptic drugs were implemented, including sodium valproate, but satisfactory seizure control was not achieved.

Upon initial admission to our institute, the child was drowsy and anxious. Significant psychomotor delay was noted – the child could not sit and raise her head. Muscle tone was decreased and tendon reflexes were very weak or absent. Her EEG recording showed diffuse, irregular slowing of the background and focal delta activity over the left fronto-temporal region. The delta activity correlated with clinical clonic seizures recorded continuously at the same time. In subsequent EEG examination, progressing deterioration was noted. Brain CT scans showed diffuse atrophy.

Liver failure developed during observation, with hypertransaminasemia (GGTP 164 u/l, AspAT 351 u/l, AlAT 303 u/l), severe clotting abnormalities, jaundice (bilirubin concentration increased from 1 mg% to 11.3 mg%), and ascites. Metabolic workup revealed relatively low ceruloplasmin level (13 mg%), and a slight increase in alfa-fetoprotein

concentration (115 IU). Triglyceride accumulation was found by liver biopsy (by thin layer chromatography). Tyrosinemia type I was excluded by the absence of succinylacetone excretion. Organic acids profile showed nonspecific dicarboxylic aciduria (C6-C10), hydroxyisovaleric aciduria and ketonuria. Biotinidase activity was normal.

Mitochondrial disorder with Alpers-Huttenlocher syndrome phenotype was established, and additional CMV infection was suspected depending on positive IgM test. Liver transplantation was decided against because of the clearly poor prognosis. The girl died within 3 months from the onset.

Autopsy of the patient was refused by the parents. Liver and muscle biopsies were performed after death, with the shortest acceptable delay of 2 hours.

Patient 2

The girl was born in 2004 with weight 4470 g and Apgar score 8/9/10, and developed normally. At the age of 18 months she presented with recurrent complex partial seizures. Transaminases were not increased at that point. Electroencephalography showed generalized epileptic discharges. MRI examination performed at the age of 20 months revealed delayed myelination in the occipital and parietal regions. Epilepsy was diagnosed and valproic acid treatment was started. No psychomotor delay was observed at that time.

Five months later, she was admitted to the hospital again due to vomiting and progressive liver failure symptoms. Her seizure control was not satisfactory. EEG recording performed at that point showed slow activity and almost continuous spike waves over the central, parietal, and occipital regions of both hemispheres. Metabolic testing revealed high tyrosine and methionine levels (125 and 848 umol/l, respectively), normal iron concentration (108 ug%), and slightly abnormal transferrin glycosylation pattern (15.2%, 12.8% and 12.2%, control value <7.6%), assessed as secondary to liver failure.

Liver transplantation from the mother was considered. After administration of prednisone and gancyclovir, a clinical remission appeared. The girl was discharged home for some weeks before planned MRS brain imaging. The patient was lost from our further observation and died at the local hospital.

Methods

Real-Time PCR quantification

Total DNA from patient's liver and muscle samples was extracted using QIAamp DNA Mini Kit and protocol (Qiagen Inc.). DNA concentration was determined by using a microtiter plate reader/spectrophotometer (Perkin Elmer), and DNA was diluted in ddH$_2$O for mtDNA and nDNA amplification. Quantification of the mtDNA copy number was performed using real-time PCR amplification on Light Cycler (Roche Diagnostics) and Light Cycler FastStart DNA Master SYBR green I (Roche Diagnostics) following the instructions of the manufacturer.

Standard DNA curves for quantization of the products were used. Both mitochondrial (16S rDNA) and nuclear (β-globin gene) target sequences were PCR amplified. The primers used to amplify the mtDNA were as follows: forward, 5'- CGA AAG GAC AAG AGA AAT AAG G, and reverse, 5'-CTG TAA AGT TTT AAG TTT TAT GCG. Total DNA quantity was corrected by simultaneous measurement of the amount of β-globin gene, using oligonucleotides: 5'-CAA CTT CAT CCA CGT TCA CC-3' and 5'-GAA GAG CCA AGG ACA GGT AC-3' as primers. The PCR products were purified with Clean-UP kit (A&A Biotechnology) and were subjected to precise estimation of DNA concentration. Serial dilutions were made from products, and PCR reactions were performed to construct the standard curve for mitochondrial and nuclear DNAs. Standard curves were generated using 5 10-fold serial-dilutions (10–100 000 copies) of the 152 bp PCR product of mtDNA, and 268 bp PCR product of nDNA. The PCR conditions were as follows: 95°C for 10 minutes and 45 cycles at 95°C for 6 sec, 53°C for 6 sec, 72°C for 4 sec, and a final extension step at 72°C for 7 minutes, for 16S rDNA, and 95°C for 10 minutes followed by 45 cycles at 95°C for 4 sec, 56°C for 4 sec, 72°C for 12 sec, and a final extension step at 72°C for 7 minutes, for the β-globin gene. The standard curves were saved as external standard curves and were later used to quantify the mtDNA and nuclear DNA after each run. Samples were run in duplicate. PCR products of mtDNA and nDNA were quantified by using the corresponding external standard.

During creation of standard curves, amplifications of external standards were performed with the same primers and conditions as those used for further patient's mtDNA and nDNA amplifications. Conditions of the mtDNA and nDNA amplifications were adjusted in order to assess the same efficiency of both reactions. The threshold cycle or CT value within the linear exponential phase was used to construct the standard curve and to measure the original copy number of DNA template.

PCR reactions were set up according to the manufacturer's recommendations, with final amounts and/or concentrations: ready Master Mix (containing FastStart Taq DNA Polymerase, reaction buffer, MgCl2, SYBR Green I dye and dNTP mix) 1 µl, 0.5 µM of each primer, 1 µl of the extracted DNA (10 ng/µl) or 1 µl of Standard and water to the final volume of 10 µl. The reactions were performed under the following conditions: for mtDNA amplification: initial denaturing at 95°C for 10 minutes and 40 cycles at 95°C for 6 sec, 53°C for 6 sec, 72°C for 4 sec; for nDNA: 95°C for 10 minutes and 50 cycles at 95°C for 6 sec, 60°C for 5 sec. The SYBR Green fluorescence was read at the end of each extension step for mtDNA amplification and annealing step for nDNA amplification. A melting curve was systematically analyzed in order to check for the absence of contamination and quality of amplification. Real-time PCR was performed in triplicate for each amplicon.

DNA analysis

DNA extracted from skeletal muscle and from liver samples was used as a template to amplify the selected regions of the *POLG* gene, and two most frequently occurring mutations were assessed as described [26]. We used, as reference for POLG nucleotide positions, the cDNA sequence corresponding to GenBank ID NM_002693.1. Sequence analysis was performed on PCR products previously purified by ExoSAP-IT treatment (USB Corp.), using the BigDye terminator Ready Reaction Kit v.3 on a 3730 Genetic Analyzer

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Table 1. Clinical characteristics of two children with W748S *POLG* gene mutation in relation to the Nguyen and Naviaux' criteria of Alpers-Huttenlocher syndrome [14].

Nguyen and Naviaux' criteria	Patient 1	Patient 2
Major criteria (triad)		
Refractory, mixed type seizures that often included a focal component	Yes	Yes
Psychomotor regression, often episodic, triggered by recurrent infection	Yes	Yes
Hepatopathy with or without acute liver failure sometimes triggered by valproic acid (but not dose-dependent)	Yes	Yes
Additional criteria		
MRS: ↓N-acetylaspartate, ↑lactate	ND	ND
MRI, CT: Cerebral volume loss; Central>cortical	CT: progressive cerebral atrophy	MRI (20 mo): decreased myelinisation of parietal and occipital white matter neurons
EEG: multifocal paroxysmal activity with high amplitude slow waves (200–1000 uV, 0.75–3 Hz)	Yes	Yes
Cortical blindness or optic atrophy	Yes	ND
Visual evoked potential with normal ERG	ND	ND
Liver or muscle mtDNA depletion (<35% of control)	Liver 16% Muscle 135%	Liver ND Muscle 220%
Deficient POLG enzymatic activity (<10% of control value)	ND	ND
↑CSF and blood lactate	CSF 20.3 mg% (borderline) blood 34.7 mg%	ND
Muscle COX deficit or combined RC defect (<20% of control)	Normal COX activity (also Table 2)	Isolated COX deficit
Positive family history (affected Alpers' sibling)	Negative	Negative
Wilson disease parameters	Ceruloplasmin 13 mg% Cu_u 13 ug/l (normal <50)	Ceruloplasmin 22 mg% Cu_s 58, 101mg% Cu_u 9 ug/l (normal <50)

ND – not determined; CSF – cerebrospinal fluid; MRS - brain proton magnetic resonance spectroscopy; MRI – magnetic resonance imaging; CT – computer tomography; EEG – electroencephalogram; ERG – electroretinogram; mtDNA – mitochondrial DNA; POLG – polymerase gamma; COX – cytochrome oxidase; Cu_u – copper excretion in urine.

Automatic Sequencer (Applied Biosystems). Sequencing data were analyzed using the ChromasLite2.01 software.

Morphological and histochemical study

Skeletal muscle samples obtained during open surgical biopsy of the vastus lateralis was snap frozen in isopentane cooled with liquid nitrogen. Myopathology panel of stains and reactions of frozen sections comprised: hematoxylin and eosin; modified Gomori trichrome; oil red O; succinate dehydrogenase; NADH dehydrogenase; cytochrome c oxidase; acid phosphatase; and myosin ATP-ase at pH 4,3/4,6/9,4.

Liver core needle biopsy fixed in 4% buffered formalin was processed routinely for paraffin sections stained with: hematoxylin and eosin, periodic acid Shiff (PAS), PAS after diastase digestion, AZAN for collagen fibers, and silver impregnation for reticulin fibers.

Small tissue blocks for transmission electron microscopy were fixed in 2.5% cold glutaraldehyde for 1 hour, washed in cacodylate buffer, postfixed in 1% osmium tetroxide, dehydrated in graded alcohols and embedded in Epon 812. Ultrathin sections were counterstained with uranyl acetate and lead citrate and examined in a JEOL 1200EX electron microscope.

The parents of Patient 1 made their agreement to perform *post mortem* study limited to liver and muscle biopsies, only. The specimens were taken 2 hours after death.

Results

The p.W748S *POLG* gene mutation was revealed in 2 children, the only ones in the cohort of 28 who fulfilled the AHS criteria (Tables 1 and 2).

Table 2. Histopathological and spectrophotometric data of two patients with AHS.

	Patient 1	Patient 2
Liver		
Sample	Obtained post mortem (2 hours)	Core needle biopsy at the age of 2 years and 3 mo.
(1) steatosis	Severe mixed macro- and microvesicular	Mild microvesicular
(2) biliary system and cholestasis	Massive neocholangiolar proliferation. Mild cholestasis.	Bile ductular proliferation, mainly periportal. Moderate cholestasis.
(3) hepatocyte dropout or necrosis	Massive hepatocyte damage and necrosis	Severe degree of necrosis with remaining hepatocytic nodules
(4) collapse of liver cell plates	Total	Severe
(5) parenchymal disarray or disorganization of the normal lobular architecture	Total effacement of liver architecture with scant remaining severly damaged hepatocytes	Severe micronodular cirrhosis
(6) regenerative nodules	Not present	Scant, dominated and surrouded by fibrosis
(7) onkocytic change in scattered hepatocytes not affected by steatosis	Yes	Yes
(8) mononuclear inflammatory infiltrate	Mild, diffuse	Moderate portal and in fibrous septa
Muscle		
Sample (*m.vastus lateralis*)	Obtained post mortem (2 hours)	Open surgical biopsy at the age of 2 years and 3 mo.
Lipid accumulation	Severe	Mild
Fibre disproportion	Not present	Not present
Fibre type predominance	Not present	Type I (70–80%)
Cytochrome oxidase activity (histochemistry)	Diffuse deficit (*post mortem*)	Normal
Hypercontracted fibers	No	Scant
Conclusion	OXPHOS dysfunction	Non-specific changes
Spectrophotometry (muscle homogenate)		
– Complex I (CS%, normal >6.5)	6.5	9.5
– Complex II (CS% normal >6.5)	ND	24.2
– Complex II+III (CS%, normal >5.5)	3.5	14.7
– Complex III (CS%, normal >40.0)	239.7	271.0
– Complex IV (CS%, normal >10.0)	5.8	25.8
– Citric synthase (CS, normal 95–180 umol/min/mg protein)	184.1	122.2

* (1) – (8) typical findings in AHS liver (according to[15]).

Depletion of mtDNA (16% of control value) was found in available liver tissue of Patient 1, and was not detected in the muscle biopsy in both patients. Respiratory chain assessed in the muscle by histochemical and spectrophotometric methods showed abnormalities in 1 case (Patient 1, Table 2).

Both affected girls were born uneventfully and developed normally during the first several months of life. The onset of the disease was unexpected, with drug-resistant seizures at late infancy (7 mo) or early childhood (18 mo). Failure to thrive and mental retardation quickly progressed, but periods of slowing disease course were observed in both patients.

Irreversible liver failure developed after VA administration in both cases, and led within a few months to the critical stage.

Wilson disease was transiently considered in both cases due to relatively low ceruloplasmin levels (Table 1), but finally excluded.

Liver histopathology of both patients is shown in Table 2. Brain tissue was not available for histopathological verification of AHS diagnosis.

Co-existence of epilepsy, VA liver toxicity, lactic acidemia and muscle respiratory chain dysfunction finally led to established mitochondrial pathology. AHS suspicion was tested by molecular investigation after death of the patients.

Discussion

Mutation p.W748S in *POLG* gene was identified in 2 out of 28 studied patients with mitochondrial encephalohepatopathy. In both patients, we were not able to find a second mutation by sequencing of fragments of *POLG* gene coding regions, which has also been experienced by other researchers [27–29]. The p.W748S mutation was described for the first time in Finnish adults with autosomal recessive ataxic syndrome [30,31]. The mutation is localized within a block of 6 amino acids forming beta-sheet in the spacer region of mitochondrial polymerase gamma. Carriers of the mutation are asymptomatic [32].

Two of our patients (both girls) sufficiently fulfilled clinical criteria of AHS proposed in the literature (Table 1). According to this scheme [14], a minimum 3 symptoms are necessary for establishing the diagnosis: refractory mix-type epilepsy, mental retardation progressed in stepwise fashion, and hepatopathy. Additionally, patients should show 3 of 8 liver histopathological features (Table 2), or 2 of 11 neuropathological features.

Liver histopathological lesions of our patients (Table 2) were consistent with the typical spectrum of AHS. A quite similar pattern of liver damage was observed by us in children with mitochondrial DNA depletion resulting from DGUOK deficiency [33]. Unfortunately, in both cases, the brain was not available for study.

Our results confirm that normal mtDNA/nDNA ratio in muscle (as observed in our patients), and normal respiratory chain function (as in Patient 2) cannot exclude the diagnosis of AHS associated with *POLG* gene mutations [34]. The reported data are not consistent, probably due to differences in tissue damage progress [29].

In Patient 2, we observed a mild elevation of carbohydrate-deficient transferrin independently from fructose ingestion. A congenital disorder of glycosylation (CDG) may clinically resemble a mitochondrial disorder and mask it at the differential diagnosis of patients with progressive multiorgan pathology of unknown cause [35]. Because we also observed a slightly positive CDG test result in the other depleted (DGUOK deficient) patient [33], our finding should be followed up on in the future to assess its significance.

Identification *post mortem* of p.W748S mutation in *POLG* gene in our patients with progressive hepatocerebral mitochondrial disorder was in agreement with the diagnosis of AHS. However, because the disorder is inherited according to autosomal recessive trait, a search for the second mutation and genotyping of the parents as obligatory carriers would help in genetic counseling.

Recently, a high allele frequency of p.A467T mutation was found in populations in Germany, UK, and Sweden [36]. This led to the conclusion that a considerable number of mitochondrial disorders may originate from *POLG* gene changes. A wide, unfolding [37] clinical spectrum of *POLG1* disease is being reported, not only AHS. Mutations in *POLG* gene were found in progressive external ophthalmoplegia (autosomal or dominant), sensory atactic neuropathy, dysarthria and ophthalmoparesis, Parkinsonism, male infertility, and premature ovarian failure [2,38–41]. The mutation p.W748S found in our AHS patients in heterozygous status is a frequent founder mutation in the Finnish population (1:125 controls) and is responsible for mitochondrial recessive ataxia (MIRAS) in Scandinavia [42], but not in other part of Europe [43]. The p.W748S frequently co-existed with p.E1143G polymorphism, which may be its modulator [44] (p.E1143G change was not identified in our cases).

Many AHS patients have received liver transplants when they suddenly developed VA toxicity and fatal liver failure [45]. Liver transplantation was planned for 1 of our patients. Outcome of such patients is irreversibly poor due to development of severe neurological damage. This should be always considered before surgery.

Ketogenic diet was recently proposed as an alternative anti-epileptic therapy for AHS patients [46]. It is not clear to what extent the VA avoidance may change (improve) a liver impairment outcome and the final prognosis in AHS. The natural history of AHS in affected siblings treated and untreated with VA [11] demonstrates that VA restriction may improve short-term prognosis but does not influence poor long-term outcome.

Conclusions

Our results confirm for the first time the occurrence of *POLG* gene mutation(s) pathology in the Polish population. *POLG* mutation screening should be included in differential diagnosis of drug-resistant epilepsy [47–49], different neuropathological syndromes, progressive hepatopathy, Wilson disease, and also, in our experience, in protein glycosylation abnormalities.

Common mutations carrier frequency in the Polish population is not yet assessed, but may be high, as in the neighboring countries studied. Knowledge of the *POLG* population background would help to assess the level of potential risk of valproate use in neurological episodes of unknown etiology.

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