Polymerase $\gamma$ Gene POLG Determines the Risk of Sodium Valproate-Induced Liver Toxicity

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Sodium valproate (VPA) is widely used throughout the world to treat epilepsy, migraine, chronic headache, bipolar disorder, and as adjuvant chemotherapy. VPA toxicity is an uncommon but potentially fatal cause of idiosyncratic liver injury. Rare mutations in POLG, which codes for the mitochondrial DNA polymerase $\gamma$ (pol$\gamma$), cause Alpers-Huttenlocher syndrome (AHS). AHS is a neurometabolic disorder associated with an increased risk of developing fatal VPA hepatotoxicity. We therefore set out to determine whether common genetic variants in POLG explain why some otherwise healthy individuals develop VPA hepatotoxicity. We carried out a prospective study of subjects enrolled in the Drug Induced Liver Injury Network (DILIN) from 2004 to 2008 through five US centers. POLG was sequenced and the functional consequences of VPA and novel POLG variants were evaluated in primary human cell lines and the yeast model system Saccharomyces cerevisiae. Heterozygous genetic variation in POLG was strongly associated with VPA-induced liver toxicity (odds ratio = 23.6, 95% confidence interval [CI] = 8.4-65.8, $P = 5.1 \times 10^{-7}$). This was principally due to the p.Q1236H substitution which compromised pol$\gamma$ function in yeast. Therapeutic doses of VPA inhibited human cellular proliferation and high doses caused nonapoptotic cell death, which was not mediated through mitochondrial DNA depletion, mutation, or a defect of fatty acid metabolism.

Conclusion: These findings implicate impaired liver regeneration in VPA toxicity and show that prospective genetic testing of POLG will identify individuals at high risk of this potentially fatal consequence of treatment. (HEPATOLOGY 2010;52:1791-1796)

Over 1 in 37,000 subjects exposed to sodium valproate (valproic acid, VPA) develop idiosyncratic liver toxicity, with the risk reaching $\approx 1$ in 500 in young children on polytherapy. Increased awareness has contributed to a decline in fatal VPA-induced liver failure,2 but the worldwide use of VPA continues to increase through its use in other clinical contexts. In addition to its use as a first-line anticonvulsant, VPA is now in regular use for migraine, bipolar disorder, chronic headache, and as adjuvant chemotherapy. The prompt recognition of early symptoms and immediate discontinuation of the drug can prevent fulminant liver failure,2 but initial clinical clues are often mild and nonspecific, making it difficult to identify individuals before significant liver damage occurs. Liver biopsy characteristically reveals microvesicular steatosis, and occasionally severe hepatocellular necrosis.3 Fever, rash, lymphadenopathy, and/or peripheral eosinophilia are rarely present during VPA hepatotoxicity, consistent with a direct toxic

Abbreviations: AHS, Alpers-Huttenlocher syndrome; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; COX, cytochrome c oxidase; DILIN, Drug Induced Liver Injury Network; EtBr, ethidium bromide; POLG, polymerase $\gamma$; ULN, upper limit of normal; VPA, sodium valproate.

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effect of the drug, rather than an immune-mediated hypersensitivity reaction typical of other antiepileptic drugs.4

The recent description of mutations in mitochondrial DNA (mtDNA) polymerase γ (POLG) as a major cause of Alpers-Huttenlocher syndrome (AHS)5 provides a clue to the underlying mechanism of VPA hepatotoxicity. AHS is a rare childhood encephalopathy characterized by developmental delay and intractable epilepsy and liver disease.6,7 Most cases have homozygous or compound heterozygote mutations in POLG, and 1/3 of AHS patients develop liver failure within 3 months of exposure to VPA.8,9 This raises the possibility that a common genetic variation in POLG predisposes individuals to VPA-induced liver failure in the absence of a recognizable AHS-phenotype.

**Patients and Methods**

**Participants.** Patients with suspected VPA hepatotoxicity were enrolled in the Drug Induced Liver Injury Network (DILIN) from 2004 to 2008 through five US centers involved at that time: North Carolina at Chapel Hill, the Universities of Connecticut, Michigan, Indiana, and California at San Francisco, and the coordinating center at Duke Clinical Research Institute.3 All had one of the following on presentation: jaundice or serum bilirubin >2.5 mg/dL and elevation in alanine aminotransferase (ALT), aspartate aminotransferase (AST), or alkaline phosphatase (ALP); no jaundice and serum bilirubin <2.5 mg/dL, but elevations in ALT or AST (>5-fold more than the upper limit of normal [ULN]) or elevations in ALP (>2×ULN; Table 1). Laboratory and clinical data were captured by the site investigator who crafted a clinical narrative describing the outcome. A committee of three experienced hepatologists then reviewed the cases, blind to the results of the study, and ranked the likelihood of causality on a scale of 1 (definite) to 5 (unlikely), as described.3 The study was conducted with local ethical and Institutional Review Board approval in accordance with the Declaration of Helsinki.

**Molecular Genetic Analysis.** POLG exons and flanking intronic regions (BC050559) were forward and reverse sequenced (Applied Biosciences Big Dye 3.1, ABI3100). Cellular mtDNA levels were measured

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**Table 1. Clinical and Genetic Data for the 17 Patients With Suspected Valproate-Induced Liver Injury**

<table>
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<th>ID No.</th>
<th>Age (Years)</th>
<th>Race/Ethnicity</th>
<th>Seizures</th>
<th>Bipolar Disorder</th>
<th>Epilepsy</th>
<th>Severe Headaches</th>
<th>Days from Drug Start to Onset of Hepatotoxicity</th>
<th>POLG change</th>
<th>Causality Assessment</th>
<th>Peak ALT (U/L)</th>
<th>Peak Bilirubin (mg/dL)</th>
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Causality assessment key: 2, highly likely (75%-95% likely); 3, probable (50%-75% likely); 4, possible (25%-50% likely); or 5, unlikely (5%-25% likely). ALT, serum alanine transferase; INR, international normalized coagulation ratio; n.a., not available.

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Additional supporting information may be found in the online version of this article.
(MTND1) relative to the nuclear-encoded B2M (AC025270) by real-time polymerase chain reaction (PCR) (iQ Sybr Green, BioRad ICycler, CA). MtDNA deletions were detected by long-range PCR.

**Functional Studies in Mammalian Cell Models.** Human hepatocyte cell lines from patients with POLG variants are not available. Given the direct toxic effect of VPA on skeletal muscle, we studied human primary myoblasts and myotubes from a p.Q1236H heterozygote, and a compound heterozygous for p.A467T/p.K1191N with AHS with local ethical approval (not DILIN subjects). Muscle cell culture was carried out as described. Both cell types were treated with VPA (2, 10, 50, 100 mM) for up to 10 days. To induce mtDNA depletion mimicking the depletion seen in AHS due to POLG mutations, myoblasts were treated with ethidium bromide (EtBr 50 ng/mL) for up to 10 days and myotubes with 300 µM Didanosine (Sigma) or 300 µM Stavudine (Sigma) for 3 days prior to and 6 days during differentiation. Trypan blue-negative (viable) cells were counted using a Mod-Fuchs hemocytometer. Apoptosis was determined using the Roche Apoptosis ladder kit. Cytochrome c oxidase (COX) activity was evaluated histochemically on day 10, and intermediary metabolites of fatty acid β-oxidation were analyzed by tandem mass spectrometry in culture media collected at days 0, 5, and 10. All cell culture studies were done in triplicate (Fig. 2A).

**Functional Studies in Yeast.** MIPI-human POLG chimera (MIPIC allele) was constructed through substitution of nucleotides 2911-2964 of MIPI wild-type (wt) allele with nucleotides 3658-3709 of POLG encoding sequence. p.Q1236H was introduced by site-specific mutagenesis. Frequency of petite mutants and of erythromycin resistant (EryR) mutants were measured as described.

### Results

**Genetic Variation in POLG Is Common in Patients with VPA Hepatotoxicity.** POLG substitutions were identified in 8 of the 17 patients with suspect VPA-induced hepatotoxicity (Fig. 1A). One harbored compound heterozygous mutations: c.1399G>A/p.A467T, predicted to change alanine to threonine in the linker region of the protein (p.A467T); and c.911T>G predicted to alter a conserved leucine to an arginine residue in the exonuclease region of poly( Fig. 1b), previously reported in AHS. This patient was prescribed VPA for unexplained seizures and was known to have a peripheral neuropathy and clumsiness. With hindsight, these features were the first stage of the AHS, although this was not obvious on clinical presentation. This patient required a liver transplant following his initial exposure to VPA and then developed intractable seizures leading to an early death, highlighting the importance of identifying patients at risk of VPA hepatotoxicity before commencing treatment.

The remaining seven (41%) had a single heterozygous POLG substitution. Five harbored c.3708G>T, predicted to alter a glutamine in the polymerase domain (p.Q1236H, Fig. 1C), and two harbored c.3428A>G, predicted to alter a glutamic acid in the polymerase domain (p.E1143G, Fig. 1D). Both the frequency of p.Q1236H (P = 1.9 x 10⁻⁴) and the combined frequency of p.Q1236H and p.E1143G (P = 5.1 x 10⁻⁷) were significantly greater than in ethnically matched population controls (n = 968 alleles), giving a combined odds ratio (OR) = 23.6 (95%
The confidence interval (CI) = 8.4-65.8 (Supporting Information Table S1). The strongest association was in patients where VPA-induced liver toxicity was highly likely (≥1 variants in 4/6, or 66%), and likely (4/8, or 50%) compared to unlikely (0/2 or 0%).

**Functional Consequences of the p.Q1236H Substitution.** The functional effects of p.Q1236H have not been previously studied. We therefore constructed a Polg-Mip1 chimera (Mip1C) in the model system yeast *Saccharomyces cerevisiae* in which 971-988 amino acids of Mip1 were substituted with the corresponding 1220-1237 amino acids of pol (Fig. 2B). This was mutagenized to introduce the substitution p.Q1236H. The *mip1C^Q1236H* strain showed a ≈1.5-fold increase in petite frequency (18.0% [±1.3] versus 12.4% [±1.6]) (Fig. 2C), indicating extended mtDNA mutability; and a 2-fold increase of EryR mutant frequency, indicating increased mtDNA point mutability, (19.7 × 10^{-8} [±2.0] versus 10.9 × 10^{-8} [±1.2]). p.Q1236H is therefore highly likely to alter human polγ function. However, treatment with sublethal concentrations of VPA (1, 2, 5, 8, and 10 mM) did not alter the yeast phenotype. The functional effects of p.E1143G have been previously described both in yeast and in vitro.14,15 In yeast, a 2-fold increase of extended mutability was observed in a strain expressing the mutant version of Mip1.14 In vitro, purified polγ harboring the p.E1143G mutation showed slightly increased catalytic efficiency and intrinsic stability, but also a reduced thermostability.15 The phenotype of both substitutions is mild, explaining why these alleles are common throughout the world (p.Q1236H ≤8.6%, and p.E1143G ≤4%). p.Q1236H and p.E1143G may only be disadvantageous in specific contexts, such as exposure to VPA.

**No Evidence of a Secondary mtDNA Defect in Whole Blood.** Given the role of POLG in mtDNA replication we looked for evidence of a qualitative or quantitative defect of mtDNA in whole-blood cellular mtDNA because liver tissue was not available from the affected individuals. No mtDNA deletions were detected by long-range PCR and the mtDNA content was no different to age-matched controls (83.9 copies/cell, standard deviation [SD] 58.8; versus 85.8, SD 28.3; Supporting Information Fig. 1A).

**Cellular Effects of Sodium Valproate.** Following treatment for 10 days with therapeutically relevant doses of VPA (2 and 10 mM) no significant decrease in mtDNA content was observed (Fig. 3A), nor detectable mtDNA deletions (Supporting Information Fig. 1b) despite the observed cell death. Treatment of control and patient myoblasts with the highest tolerated doses of VPA (50 and 100 mM) still showed no depletion of mtDNA but compromised cell proliferation, with extensive cellular ballooning, vacuolization, and detachment within 3 days of treatment (Supporting Information Fig. 2). The presence of mtDNA deletions was not investigated in these cells due to the short culture...
period, making the appearance of deletions highly unlikely. By contrast, EtBr-treated cells grown in parallel showed the expected decrease in mtDNA content after 10 days but no defect of cellular proliferation and no evidence of cell death (Fig. 3B). There was no evidence of apoptosis in any of the cell lines after 10 days of treatment. Multiple mtDNA deletions were not detected in any of the cell pellets, there were no differences in COX activity observed, and β-oxidation metabolites remained within normal limits (Supporting Information Table 2). We therefore extended our studies to postmitotic myotubes, which more closely model mtDNA depletion in vivo. MetDNA levels were significantly lower in AHS and Q1236H myotubes than in controls (Fig. 3C). To determine whether mtDNA depletion itself predisposes to further mtDNA loss after VPA exposure, we depleted the myotubes with didanosine and stavudine, which induce less severe myotube mtDNA depletion than EtBr. MetDNA depletion levels in Q1236H myotubes were less than in controls, and similar to the AHS cell lines, but there was no further decrease in mtDNA content with the addition of 10 mM VPA (Fig. 3C).

Discussion

VPA is a branched medium chain fatty acid known to inhibit mitochondrial β-oxidation, possibly through the microsomal production of toxic metabolites including 4-ene-VPA, or cytosolic and mitochondrial CoA sequestration effects. However, we saw no evidence of a β-oxidation defect, making this mechanism unlikely in this context. We also saw no evidence of a secondary mtDNA defect, despite the VPA dose-related growth inhibition and cell death. By contrast, treating identical cell lines with EtBr, didanosine, or stavudine caused profound but recoverable mtDNA depletion without cell death. VPA toxicity is therefore unlikely to be mediated through a direct effect on mtDNA, explaining why we did not observe a COX defect in VPA-treated cells. Although it is possible that these aspects are specifically deranged in the liver, we observed the morphological characteristics of VPA hepatotoxicity in human myoblasts, implicating the same mechanism in our in vitro model. Moreover, an elevated serum creatine kinase in patients with VPA toxicity points to a similar direct toxic effect on skeletal muscle.

Unlike mature skeletal muscle and brain, the liver can proliferate in response to damage, and there is clear evidence of hepatocyte proliferation in patients with AHS. We have shown that treatment with 2 and 10 mM VPA impairs cellular proliferation in vitro, and that p.Q1236H increases mtDNA mutability in yeast and may decrease mtDNA copy number in myotubes. The yeast system is a proven method to study the effects on mtDNA of both strong and weak POLG mutations, such as p.E1143G, whose effects are very mild and cannot easily be observed in higher eukaryotes. After a limited number of cell divisions most yeast mitochondria are homoplasmic, as the heteroplasmic state is always transient in S. cerevisiae.
Therefore, conditions that cause increased mtDNA mutability, even at a low extent, lead to an increase of respiratory deficient cells (i.e., petite mutants) after only a few generations. For the p.Q1236H mutation we observed a small but significant increase in extended mtDNA mutability determined as an increase in petite frequency. Observation of this effect in a yeast model predicts that a similar effect would occur in human cells, resulting in mtDNA copy number reduction, as observed in myotubes harboring this mutation. This raises the possibility that both mechanisms independently compromise the regenerative capacity of the liver, thus inhibiting the endogenous capacity for liver repair in response to an external insult. For VPA, this could be through the inhibition of histone deacetylases, which regulate gene expression by relaxing chromatin structure and facilitating access to DNA by the transcriptional machinery.19

In this study, over 50% (8/14) of patients with proba-
ble VPA hepatotoxicity had heterozygous POLG substi-
tutions associated with >20-fold increased risk of VPA-
induced liver injury, seven of whom harbored previously
described single nucleotide polymorphisms; p.Q1236H
and p.E1143G. Here we show that p.Q1236H is not
phenotypically neutral, with histidine at position 1236
increasing both mtDNA deletion frequency and point
mutability frequency in yeast. However, the phenotype
of both substitutions is mild, explaining why these alleles
are common throughout the world (p.Q1236H \( \leq 8.6\% \),
and p.E1143G \( \leq 4\% \)). This suggests p.Q1236H and
p.E1143G are only disadvantageous in specific contexts,
such as exposure to VPA. Screening for functional
POLG substitutions will minimize the risk of fulminant
liver failure in patients exposed to VPA.

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