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The genotypic and phenotypic spectrum of pyridoxine-dependent epilepsy due to mutations in *ALDH7A1*

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Abstract

Pyridoxine-dependent epilepsy is a disorder associated with severe seizures that may be caused by deficient activity of α -aminoacidic semialdehyde dehydrogenase, encoded by the *ALDH7A1* gene, with accumulation of α -aminoacidic semialdehyde and piperidine-6-carboxylic acid. The latter reacts with pyridoxal-phosphate, explaining the effective treatment with pyridoxine. We report the clinical phenotype of three patients, their mutations and those of 12 additional patients identified in our clinical molecular laboratory. There were six missense, one nonsense, and five splice-site mutations, and two small deletions. Mutations c.1217_1218delAT, I431F, IVS-1(+2)T>G, IVS-2(+1)G>A, and IVS-12(+1)G>A are novel. Some disease alleles were recurring: E399Q

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(eight times), G477R (six times), R82X (two times), and c.1217_1218delAT (two times). A systematic review of mutations from the literature indicates that missense mutations cluster around exons 14, 15, and 16. Nine mutations represent 61% of alleles. Molecular modeling of missense mutations allows classification into three groups: those that affect NAD⁺-binding or catalysis, those that affect the substrate binding site, and those that affect multimerization. There are three clinical phenotypes: patients with complete seizure control with pyridoxine and normal developmental outcome (group 1) including our first patient; patients with complete seizure control with pyridoxine but with developmental delay (group 2), including our other two patients; and patients with persistent seizures despite pyridoxine treatment and with developmental delay (group 3). There is preliminary evidence for a genotype-phenotype correlation with patients from group 1 having mutations with residual activity. There is evidence from patients with similar genotypes for nongenetic factors contributing to the phenotypic spectrum.

Introduction

Pyridoxine-dependent epilepsy (OMIM 266100) was first described clinically as a severe infantile seizure disorder that responded dramatically and persistently to treatment with pyridoxine (Hunt et al. 1954). Patients typically present in the neonatal or early infantile period with encephalopathy and persistent seizures that are refractory to treatment with various anticonvulsants (Baxter 2001). Some patients respond to a single intravenous dose of pyridoxine, whereas others require multiple doses of pyridoxine. Discontinuation of pyridoxine treatment is usually associated with a recurrence of seizures. Late onset forms have also been described (Baxter 2001). Most patients have mild to moderate developmental delay, with verbal IQ more affected than performance IQ (Baxter 2001; Rankin et al. 2007).

In the majority of patients with pyridoxine-dependent epilepsy, the disorder is caused by deficient enzyme activity of α -aminoadipic semialdehyde dehydrogenase (E.C. 1.2.1.31) with accumulation of α -aminoadipic semialdehyde and piperidine-6-carboxylic acid (Mills et al. 2006). The latter reacts with pyridoxal-phosphate depleting its level, thus explaining the link to treatment with pyridoxine. The enzyme is encoded by the *ALDH7A1* gene, also known as *Antiquitin-1*. A variety of mutations have been described in patients with pyridoxine-dependent epilepsy, but a comprehensive review of the mutation spectrum is lacking.

In this study, we report three patients with pyridoxine-dependent epilepsy presenting with variable phenotypes and responses to pyridoxine supplementation. We then compiled the mutation spectrum of these 3 patients and 12 additional patients identified in our clinical molecular laboratory based upon diagnostic testing, and compared the molecular data with previously published mutations in the *ALDH7A1* gene to review the genotypic spectrum of pyridoxine-dependent epilepsy.

Methods

Study population

Medical records of the patients with pyridoxine-dependent epilepsy followed at The Children's Hospital Denver Metabolic Clinic were reviewed after obtaining informed consent on an IRB-approved protocol. Mutations identified by sequence analysis of the *ALDH7A1* gene in clinical samples by the clinical molecular laboratory were reviewed based on an IRB-approved protocol.

Molecular studies

Exons and flanking intronic sequences of *ALDH7A1* (NM_001182.2, ENSG00000164904, GC05M125908) were amplified by PCR from genomic DNA followed by direct sequencing on an automated capillary sequencer (ABI@3730, Applied Biosystems, Foster City, CA) using the dye-terminator method. PCR was performed with intronic primers (see Appendix 1), which carried M-13 extensions [Fm-13(−20), 17-mer; 5'-GTAAAACGACGGCCAGT-3'; and m-13R(−26), 17-mer; 5'-CAGGAAACAGC-TATGAC-3'] used for the sequencing reaction. PCR protocol for all 18 exons of *ALDH7A1* was identical and made use of 50 pmol of primers. The PCR program included an initial DNA denaturing step at 95°C for 4 min; a step-down annealing temperature segment at 65 to 60°C decreasing by 1°C per cycle; repetitive cycles of denaturing at 95°C for 20 s, annealing at 60°C for 20 s, and synthesis at 72°C for 30 s; with a final elongation step of 4 min at 72°C after 35 cycles. All PCR experiments included a positive control of random DNA and a null control without DNA. DNA sequencing was performed in both directions for each exon of *ALDH7A1* with sequence analysis reaching at least 50–100 nucleotides into the flanking introns. Sequence files were analyzed by comparing patient sequences to the reference sequence utilizing CodonCode Aligner® (Codon-Code, Dedham, MA) software and subsequently hand-read twice to verify the identified sequence changes or polymorphisms. A total of 30 disease alleles were analyzed and compared to at least 100 healthy control alleles (for novel in/del, nonsense, and splice-site mutations) and 200 healthy control alleles for novel missense mutations or exonic sequence variations. Mutations are described according to the conventions of den Dunnen and Antonarakis (den Dunnen and Antonarakis 2000).

Molecular modeling

Missense mutations in *ALDH7A1* were mapped and surface accessibilities determined using the UCSF Chimera package (version 1.4.1) from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco. All simulations were performed using Discovery Studio software (version 2.5.5; Accelrys, San Diego, CA). The crystallographic coordinates of the human aldehyde dehydrogenase 7A1 structure (PDB code: 2J6L) were obtained from the RCSB Protein Data Bank (<http://www.rcsb.org>). The mutant proteins were generated by substituting the missense amino acid residues. The ionizable residues were corrected for physiologic pH, and the potentials and charges of the complexes were corrected using CHARMM in all simulations. In all simulations the monomeric form of the protein was minimized using the conjugate gradient method (1,000 iterations) to a convergence of 0.001 kcal/mol. The minimized protein structures were viewed using Discovery Studio/Chimera to identify conformational changes in the protein structure. The hydropathy index is a calculated constant related to hydrophobicity or hydrophilicity of R groups. It describes the tendency of a specific residue to seek a hydrophobic (positive value) or aqueous environment (negative value) (Kyte and Doolittle 1982).

Case reports

Patient 1 is a 7-year-old girl. She was born at term via uncomplicated vaginal delivery and had a normal initial neonatal period. She had a fairly large head at birth (36.5 cm), and a CT of the brain was normal. Seizures started on day 9 of life and were treated unsuccessfully with phenobarbital and phenytoin. CSF glycine was 10 µM (normal 4–20 µM), and serum amino acids were normal. After receiving intravenous pyridoxine on day 13, seizures stopped and the electroencephalogram normalized. Pyridoxine treatment was continued, and all other antiepileptic treatment was discontinued. A temporary discontinuation in pyridoxine treatment resulted in resumption of seizures, which disappeared when pyridoxine

treatment was resumed. She has been seizure free except during periods of missing doses of pyridoxine. She was treated with 100 mg/day of pyridoxine. At age 5.4 years, her head circumference was 53.8 cm, the 97th percentile for age. At age 7 years she had macrocephaly (99th percentile for age), mild hypotonia, and mild myopia. She has had mild developmental delay with rolling over at age 8 months, and walking at age 18 months. She currently attends first grade and is in a normal classroom. Plasma pipelicolic acid was 5.4 μM (normal values for age 0.5–4.9 μM).

Patient 2 is a 21-year-old male. He was born at 42 weeks gestation, with Apgar scores 9 and 9 at 1 and 5 min, respectively. The initial neonatal period was uncomplicated until he started experiencing seizures on day 6. The recurrent seizures did not respond to treatment with phenobarbital, phenytoin, valproate, paraldehyde, and ACTH. Pyridoxine treatment with 50 mg daily given at age 6 months resulted in immediate seizure cessation and has been continued ever since. He has had no seizures since the start of pyridoxine treatment, except for a single febrile seizure at the age of 2 years. Persistent symptoms have been macrocephaly, strabismus, and developmental delay. He started walking at the age of 17 months and had his first words at age 3 years. Psychomotor evaluation at age 15 years with the WISC-II test showed a verbal IQ of 70, a performance IQ of 63, and a full scale IQ of 64. He has been independent in activities of daily living and maintains a job. Brain magnetic resonance imaging showed mild generalized volume loss and thin posterior ventricular white matter. His electroencephalogram was normal. Pipelicolic acid in serum was 6.08 $\mu\text{mol/L}$ (normal 0.7–2.5 $\mu\text{mol/L}$). Current treatment consists of pyridoxine 100 mg/day.

Patient 3 is a 2-year-old boy. The neonatal period of this patient has been described previously (Gallagher et al. 2009). He developed seizures in the first week of life, which responded to a combination of treatment with pyridoxine and folinic acid. Seizures recurred after discontinuation of both vitamins and only stopped with treatment with pyridoxine. His cerebrospinal fluid analysis for monoamine metabolites showed the two peaks previously considered characteristic for folinic acid-responsive seizures. CSF glycine was 9 μM (normal 4–20 μM) and threonine was 57 μM (normal 40–120 μM) with normal serum amino acids. CSF neurotransmitters were not deficient: homovanillic acid 1,489 nM (337–1,299 nM), 5-hydroxyindoleacetic acid 756 nM (control 208–1,159 nM), and 3-O-methyldopa 267 nM (control <300 nM). He has been treated with pyridoxine 15 mg $\text{kg}^{-1} \text{ day}^{-1}$ and folinic acid 2 mg $\text{kg}^{-1} \text{ day}^{-1}$. He has macrocephaly (98th percentile for age) and strabismus. He has been seizure free except for breakthrough seizures with fever. His initial electroencephalogram was unremarkable. Later studies have shown epileptic elements, but antiepileptic treatment was not started. His development was mildly delayed. At age 10 months he could roll, but not yet sit independently. At age 18 months he could sit and crawl. He started walking at age 21 months. At age 2 years he had only incomplete words, but better receptive language. His head circumference was 35 cm (12th percentile) at birth, and was 52.5 cm at age 2.1 year, above the 99th percentile. Brain imaging at 1 month of age revealed a large cisterna magna and a small right parietal hemorrhage.

At 1 year and 3 months, there was mild volume loss of white matter in the parietal-occipital lobes, thinning of the posterior corpus callosum, increased T2 and FLAIR signal in the frontal white matter, and a mega cisterna magna. Plasma pipelicolic acid was 14.4 μM (normal 0.1–5.3 μM).

Results

Patient 1 was identified with the common G477R missense mutation in exon 17 and the previously reported intronic IVS-16(+5)G>A splice-site mutation (see Table 1). Patient 2 was found with two known heterozygous mutations, R82X and c.750 G>A, which unmasks

a cryptic splice-site. Patient 3 has compound heterozygous missense mutations G83E and P403L.

All 15 clinical samples (including cases 1–3) showed two mutations in *Antiquitin-1*. They included six different missense mutations, one nonsense mutation, and five splice-site mutations (Table 1, Fig. 1). There were also two small deletions: c.419–422 delTCTT and c.1217_1218 delAT. The mutations c.1217_1218 delAT, I431F, IVS-1 (+2)T>G, IVS-2(+1)G>A, and IVS-12(+1)G>A are novel mutations not previously recorded. None of the splicing mutations identified in the patients were found in 114 healthy control alleles, and the missense mutation was not identified in 236 control alleles. Some mutations were recurrent: E399Q occurred eight times, G477R occurred six times, and R82X and Y406delAT each occurred twice. While the nonsense mutation and the small deletions causing a frame shift obviously have a deleterious effect, the interpretation of the novel missense mutation and splice-site mutations is more difficult. The splice-site mutations all affect highly conserved consensus splice donor nucleotides, and bioinformatics analysis with online splice-site finder software (NNSPLICE 0.9; Reese et al. 1997) predicted altered splicing for all of them.

The amino acid isoleucine in amino acid codon 431 is conserved throughout higher eukaryotes (*M. musculus*, *R. norvegicus*, *B. taurus*, *D. rerio*, *X. tropicalis*) and semi-conserved across multiple other species (leucine in *C. elegans*, *C. savignyi*). For further interpretation of the novel I431F missense mutation we modeled its impact on protein-folding and 3-D structure utilizing the Accelrys Discovery Studio software package. Minimizations performed on the mutated protein in silico did not reveal major conformational changes within the cofactor or substrate binding sites of the human ALDH7A1 monomer. Alterations were identified throughout the ALDH7A1 oligomerization domain and the hydrophobic dimer contact surfaces. It appears these changes are the result of the loss of a β -sheet secondary structure and loop projection (Fig. 2a). Interactions between the oligomerization domains of ALDH7A1 monomers are responsible for both dimer and tetramer assembly (Rodriguez-Zavala and Weiner 2002), indicating the I431F mutation may alter oligomerization.

The P403L mutant was also modeled using Discovery Studio software. The proline located at residue 403 is required for proper alignment of multiple residues that directly interact with the NAD⁺cofactor. Replacing the proline with a leucine removes the proline-induced bend in the peptide backbone that confers proper residue conformation within the cofactor binding site (Fig. 2b). The residues most impacted by these changes are E399 and F401. E399 stabilizes the nicotinamide ribose through hydrogen bonding. Conformational changes induced by leucine substitution at residue 403 would prevent hydrogen bond formation (Fig. 2b, right circle). F401 forms the side of the binding pocket and the aromatic ring stacks against the cofactor. In the mutant protein the ring projects into the binding pocket (Fig. 2b, left circle).

A systematic overview of all the mutations previously reported in the *ALDH7A1* gene in patients with PDE is shown in Fig. 1 and previously reported mutations are referenced in Table 1 (Mills et al. 2006;Plecko et al. 2007;Salomons et al. 2007;Kanno et al. 2007;Kluger et al. 2008;Kaczorowska et al. 2008;Striano et al. 2009;Bennett et al. 2009;Gallagher et al. 2009;Schmitt et al. 2010;Millet et al. 2010). There are 26 missense mutations, 13 of which cluster in exons 14, 15, and 16. There are 5 nonsense mutations, 11 small deletions or insertions, 9 splice mutations, and 1 cryptic splice enhancer. There are 9 common mutations, which in total represent 61% of the alleles (90 of 147 alleles). These include the following mutations in decreasing order of frequency: E399Q (48 alleles), R82X (12 alleles), c.750G>A (6 alleles), P403L (5 alleles), G477R (5 alleles), S430N (4 alleles), delT495-S499

(4 alleles), C450S (3 alleles), and G83E (3 alleles). The residues altered as a result of *ALDH7A1* missense mutations were mapped onto the human *ALDH7A1* crystal structure (Fig. 3a–b). Many of the mutations are surface accessible indicating they may alter either dimer or tetramer assembly. The surface accessible residues are highlighted in Fig. 3b. The effects of missense mutations on residue polarity, charge, and hydrophobic indices are listed in Table 2.

Discussion

The clinical presentations of the three patients reported here are typical for pyridoxine-dependent epilepsy caused by α -aminoadipic semialdehyde dehydrogenase deficiency. Mild developmental delay, mild hypotonia, mild posterior corpus callosum thinning and posterior white matter volume loss on brain magnetic resonance imaging, and most characteristically a seizure disorder responsive to pyridoxine in part or in whole have been reported in most patients. It is remarkable that all three patients reported here have macrocephaly, without a tendency towards the previously reported feature of hydrocephalus (Baxter 2001).

The clinical entity of pyridoxine-dependent epilepsy has allowed the identification of associated genetic disorders (Fig. 4). Most commonly patients are affected by α -aminoadipic semialdehyde dehydrogenase deficiency, although other disorders not linked to this gene also exist (Bennett et al. 2005; Kabakus et al. 2008). Following the recognition of mutations in the *ALDH7A1* gene, it is now possible to describe the clinical phenotype associated with this specific disorder. The outcome of these patients can be divided in three categories. Many patients with α -aminoadipic semialdehyde dehydrogenase deficiency respond with near complete seizure control to treatment with pyridoxine alone, with perhaps only recurrence of seizures with fever, as is present in our patients. Forty-seven such cases were presented in a recent report from the North American Registry of pyridoxine-dependent epilepsy (groups 1 and 2 in Gospe 2002). A subset of these patients, which we call group 1, have normal developmental outcome, including our patient 1 (8 cases in Basura et al. 2009; case C2 in Mills et al. 2006; 7/28 cases in Haenggli et al. 1991; 1 case in Kluger et al. 2008; patient 2 in Striano et al. 2009; 3/11 patients in Been et al. 2005; 1/6 cases in RamachandranNair and Parameswaran 2005; 2/9 patients with elevated pipercolic acid in Plecko et al. 2005; and 1 case in Millet et al. 2010). In contrast, the majority of patients with complete seizure control have mild to moderate developmental delays; these we call group 2, which includes patients 2 and 3 reported here. Some patients though have a worse prognosis; these we call group 3. While the seizure disorder is greatly improved with pyridoxine, the patients still develop seizures that require additional anticonvulsant medications. In some, the seizures are controlled with additional anticonvulsant therapy (8/47 cases in group 3 according to Basura et al. 2009), whereas other patients have seizures that are not completely under control with anticonvulsant therapy (another 8/47 cases according to Basura et al. 2009; Nicolai et al. 2006). Patients with persistent seizures tend to have worse developmental outcome (Basura et al. 2009); they always have developmental delays and usually have abnormal findings on brain MRI, such as seen in patient 3 reported here.

It will be important to study whether these differences in outcome are related to the genetic basis of the condition as well as to nongenetic factors. Clearly the underlying gene causing the pyridoxine-dependent epilepsy is important, as patients with pyridoxine-responsive encephalopathy that present with hypsarrhythmia (EEG), do not have mutations in *ALDH7A1*, and have normal biochemical parameters such as pipercolic acid (Bennett et al. 2009; Kanno et al. 2007, patient 5) tend to have an excellent outcome with normal development (Fig. 4). Thus, this condition should be distinguished from α -aminoadipic semialdehyde dehydrogenase deficiency. Within α -aminoadipic semialdehyde

dehydrogenase deficiency, formal data relating genotype to phenotype are currently not available, but preliminary evidence suggests that the severity of the mutation may be contributory, while additional factors are also likely contributory. Patients with a normal developmental outcome have had mutations that may be associated with residual enzyme activity such as the mutation c.1512delG in patient C2 in Mills et al. 2006, the mutation T297R (in addition to R82X) in patient K3019 in Bennett et al. 2009, and the “leaky” splicing mutation IVS-16(+5)G>A (in addition to R82X) in patient 2 in Striano et al. 2009. It is interesting that our patient 1 with normal developmental outcome has this same splice mutation. The residual activity of the mutation F410L reported in a patient with normal developmental outcome is not known (Millet et al. 2010). In contrast, a patient homozygous for the A171V mutation, which on expression had no residual activity (Mills et al. 2006, patient G) was reported as having low-normal cognitive development with dysfunction in expressive language (Schmitt et al. 2010, group 2).

The location of missense mutations in the gene can be informative regarding the functionality of the protein product. Figure 1 gives the locations of all the missense mutations combined from the literature and our own series. A high number of missense mutations cluster in exons 14, 15, and 16. In contrast, only four missense mutations are located in the first half of the gene consisting of exons 1 through 8. Splicing mutations are located across the gene as are various nonsense mutations, both premature stop mutations, and small insertions and deletions. The frequency of larger deletions comprising whole exons has not yet been studied. Several recurring mutations have been noted. Nine common mutations comprise 61% (90/147) of the reported disease alleles. A strategy of first analyzing a panel of recurring mutations would be possible prior to sequencing the entire gene. However, given that these recurring mutations are located throughout the gene, sequencing of the whole coding sequence remains a cost effective strategy.

Molecular modeling using the human ALDH7A1 crystal structure indicates that missense mutations in *ALDH7A1* can be divided into three categories. The first category consists of mutations that affect NAD⁺ cofactor binding or catalysis. Mutations that alter the substrate binding pocket make up the second category. The last category contains mutations that do not appear to affect cofactor or substrate binding or enzyme catalysis but potentially disrupt dimer or tetramer assembly. The mutations are expected to have different effects on enzyme activity. Missense mutations altering cofactor binding and catalysis are predicted to have the most significant impact on enzyme activity. The E399Q, E399D, and P403L mutations fall into this category. Previous studies have shown that the E399Q mutant is inactive (Mills et al. 2006). The proline located at residue 403 appears to be required for proper alignment of many residues that directly interact with the cofactor (Fig. 2b). Replacing this residue with a leucine would remove the proline-induced bend in the protein backbone that confers proper residue conformation within the cofactor binding site. Other mutations such as A171V and T297K change the shape of the substrate pocket. The exact effect on substrate binding and specificity for these mutations has yet to be determined. Similar to other aldehyde dehydrogenase enzymes such as succinic semialdehyde dehydrogenase ALDH5A1 (Murphy et al. 2003), the ALDH7A1 enzyme has catalytic activity on several different compounds (Brocker et al. 2010), and it is interesting to postulate that such substitutions could alter how the enzyme metabolizes a specific compound without affecting or abolishing the activity towards other substrates. Overexpression studies will be needed to provide definitive proof of the mutations on the activity of the enzyme towards its substrates (Mills et al. 2006).

A number of other missense mutations, such as I431F, do not appear to alter the cofactor or substrate binding site conformations. Instead, these mutations change regions responsible for dimer and tetramer assembly. For example, substitution of an arginine for glycine at residue 477 would have a significant effect on dimer formation. The residue lies between the three-

stranded anti-parallel β -sheets that comprise a large section of the oligomerization domains facilitating dimer assembly. Dimer formation is highly dependent on the interaction between these two hydrophobic surfaces (Rodriguez-Zavala and Weiner 2001). An arginine would introduce a very large positive charge between these two regions and significantly change the hydrophobicity as reflected in the associated hydropathy indices for glycine and arginine, which are -0.4 and -4.5 , respectively (Table 2). The G83E mutation is another mutation that would have an effect on the quaternary structure, but instead of hindering dimer formation, we expect it will have a negative effect on tetramer formation. A highly conserved arginine (R82) is found immediately N-terminal to G83. In ALDH proteins, this arginine facilitates tetramer assembly through salt-bridge formation with a conserved serine located within the adjacent dimer. The interaction between R82 and S499 within an opposing monomer is supported by the human ALDH7A1 crystal structure. The G83E mutant would place a negatively charged glutamate in very close proximity to R82 and most likely hinder or possibly abolish the R82-S499 salt bridge, thus deleteriously affecting multimer formation and enzyme activity. Studies have shown that replacing the conserved arginine (R84) found in ALDH1 with glutamine significantly disrupted tetramer assembly and reduced enzyme activity by 70% (Rodriguez-Zavala and Weiner 2001). The fact that patients heterozygous for any of these mutations do not exhibit the phenotype associated with pyridoxine-dependent epilepsy suggests the possibility that these inactive subunits do not act as a dominant negative and may be able to form functional multimeric protein when coupled to enzymatically active monomers.

Additional nongenetic factors that have been proposed to contribute to the neurodevelopmental outcome include age at onset of seizures with seizure onset >1 year portending a better prognosis, early institution of treatment, and higher pyridoxine dosing (Baxter 2001; Gospe 2002). Although no relation between the time of diagnosis or start of treatment and cognitive outcome was found in a series of 29 patients (Haenggli et al. 1991; Gospe 1998), prenatal treatment is still considered a possible beneficial factor. Analysis of the outcome of patients homozygous for the same common severe mutation E399Q can reflect this impact. Some patients with this mutation can have substantial developmental delay and seizures that are not completely controlled with pyridoxine (group 3; e.g., Bennett et al. 2009, patient K3020), whereas two other patients homozygous for this same mutation but treated prenatally with a high dose of pyridoxine had a normal developmental outcome (Bok et al. 2010) (group 1). Yet, prenatal treatment with pyridoxine alone does not completely protect against developmental delay (Rankin et al. 2007) reflecting additional complexity beyond prenatal treatment alone.

The pathogenesis of this condition still has not been clearly elucidated. The interaction of piperidine-6-carboxylate with pyridoxal-phosphate through a Knoevenagel reaction forming a complex, and the clinical response of treatment with pyridoxine has resulted in the suggestion of a central pyridoxine deficiency state due to inactivation of central pyridoxal-phosphate (Mills et al. 2006; Plecko and Stöckler 2009). Low levels of pyridoxal-phosphate in the frontal and occipital cortex were previously measured post mortem only in one patient (Lott et al. 1978). In patients with pyridox(am) ine phosphate oxidase (PNPO) deficiency (OMIM 610090), a clear deficiency of central pyridoxal-phosphate exists, as documented by low levels in CSF and an increase in metabolites reflecting impaired activities of pyridoxal-phosphate-dependent enzymes such as disturbances in monoamines, threonine, and glycine (Mills et al. 2005; Hoffmann et al. 2007). Similar findings were hypothesized in patients with pyridoxine-dependent seizures (Plecko and Stöckler 2009). In contrast, in our patients with α -aminoadipic semialdehyde dehydrogenase deficiency, we did not find increases in threonine or glycine in serum or CSF, and no deficiencies in the monoamines homovanillic acid and 5-hydroxyindolacetic acid, or an increase in 3-O-methyldopa in CSF or in vanillic acid in urine. Similar differences between the two conditions have been remarked

on before (Hoffmann et al. 2007 and K. Hyland, personal communication). Thus, the pathophysiology is most likely more complex than a simple central deficiency of pyridoxal-phosphate and will require studies of patients and animal models. One possibility could be a deficiency limited to a region or a specific cell type in the brain. Direct toxicity of α -amino adipic semialdehyde binding to proteins or abnormalities in GABA metabolism have been proposed (Bok et al. 2010; Gospe 2002). The activity of α -aminoadipic semialdehyde dehydrogenase on other substrates such as betaine aldehyde and *trans*-2-nonenal opens the possibility of other contributing toxic substances (Brocker et al. 2010). Such a more complex pathophysiology can explain the only partial protection offered by pyridoxine treatment and the difficulty in establishing an easy genotype to phenotype correlation. In addition, the rarity of the condition limiting the number of patients for study and the often compound heterozygous nature of the mutations make studies of genotype-phenotype correlation difficult.

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Appendix

Table 3 presents the primer sequences used for the sequencing reaction.

Fig. 1.

Overview of all mutations identified in *ALDH7A1* (*Antiquitin-1*). All mutations identified from the literature (Mills et al. 2006; Plecko et al. 2007; Salomons et al. 2007; Kanno et al. 2007; Kluger et al. 2008; Kaczorowska et al. 2008; Striano et al. 2009; Bennett et al. 2009; Gallagher et al. 2009; Schmitt et al. 2010; Millet et al. 2010) and in this study are shown. Mutations recurring more than twice are shown in *red*. Mutations are organized as missense, nonsense, or splice mutations and deletions or insertions. The splice-site mutations are IVS1+2T>G is c.108+2T>G; IVS2+1G>A is c.162+1G>A; IVS3+2T>A is c.228+2T>A; IVS5+5G>A is c.433+5G>A; IVS5-1G>C is c.434-1G>C; IVS6del-1-3 is c.567del-1-3; IVS9+3-+ 6delAAGT is c.787+3+6delAAGT; IVS12+1G>A is c.1009+1G>A; IVS16+5G>A is c.1405+5G>A (nomenclature according to www.hgvs.org/mutnomen). The large intron 2 is drawn at half size. The 5' and 3' noncoding regions are drawn in *light color*

Fig. 2.

Effect of I431F and P403L mutations on ALDH7A1 protein structure. The I431F (**a**) and P403L (**b**) mutants were created in silico using Accelrys Discovery Studio software. Loop refinement was then performed followed by residue optimization and free energy minimizations. The mutant (*yellow*) and wild type (*white*) structures are superimposed to highlight differences in secondary and tertiary structure. The I431F mutation resulted in loss of β -sheet secondary structure and buckling of a loop into the hydrophobic surface required for monomer dimerization (**a**). The loss of a bend in the peptide backbone as a result of the P403L mutation causes conformational changes that negatively affect cofactor binding (**b**) including loss of hydrogen bonding (*right circle*) and steric interference (*left circle*)

Fig. 3. Mapped locations of missense mutations associated with pyridoxine-dependent epilepsy on the human ALDH7A1 (Antiquitin-1) protein structure. **a** Ribbon structure of human ALDH7A1 with PDE-affected residues highlighted in *red*. Bound NAD⁺ cofactor shown in *yellow*. **b** Molecular surface depiction with accessible mutant residues highlighted in *red*. Residues surrounding the substrate binding pocket are highlighted in *green*. Bound NAD⁺ is again shown in *yellow*. *Asterisks* indicate that the residue is surface accessible but not visible on given structures either because they are found within a binding pocket (ASN167 and ALA171) or hidden behind other structural features (GLY255 and GLY263)

Fig. 4. Overview of pyridoxine dependent/responsive seizure subtypes. Possible mutations associated with group 1 include T297R, IVS-16(+5)G>A, and c.1512delG

Table 1

Spectrum of mutations in *ALDH7A1* (*Antiquitin-1*) in the study cohort

Subject	Age/gender	Ethnicity	Mutation 1	Mutation 2	Mutation: novel/reported ^a
Case 1	5 years/F	C	G477R: c.1429 G>C	IVS-16(+5) G>A	Bennett, Striano
Case 2	22 years/M	C	R82X: c.244 C>T	c.750G>A ^b	Mills, Salomons
Case 3	3 months/M	A-A	G83E: c.248 G>A	P403L: c.1208 C>T	Gallagher, Kanno
Patient 4	16 years/M	H	E399Q: c.1195 G>C	G477R: c.1429 G>C	Mills, Bennett
Patient 5	9 months/F	A	IVS-12(+1) G>A	IVS-12(+1) G>A	Novel, novel
Patient 6	13 years/M	C	R82X: c.244 C>T	I431F: c.1291 A>T	Mills, novel
Patient 7	15 years/M	H	E399Q: c.1195 G>C	G477R: c.1429 G>C	Mills, Bennett
Patient 8	27 years/M	Oth	c.1217_1218 delAT	c.1217_1218 delAT	Novel, novel
Patient 9	11 months/F	C	E399Q: c.1195 G>C	G477R: c.1429 G>C	Mills, Bennett
Patient 10	9 years/M	H	E399Q: c.1195 G>C	G477R: c.1429 G>C	Mills, Bennett
Patient 11	Unk/Unk	Unk	E399Q: c.1195 G>C	E399Q: c.1195 G>C	Mills, Mills
Patient 12	5 months/F	C	IVS-1(+2) G>T	N420K: c.1260 T>A	Novel, Bennett
Patient 13	1 months/M	Unk	c.419_422 delTCTT	E399Q: c.1195 G>C	Gallagher, Mills
Patient 14	13 years/F	H	E399Q: c.1195 G>C	G477R: c.1429 G>C	Mills, Bennett
Patient 15	16 years/F	Unk	IVS-2(+1) G>A	IVS-2(+1) G>A	Novel, novel

M Male, F female, Unk unknown, A-A African American, A Asian, C Caucasian, H Hispanic, Oth other, Unk unknown

^aReferences: Gallagher = Gallagher et al. 2009; Kanno = Kanno et al. 2007; Mills = Mills et al. 2006; Salomons = Salomons et al. 2007; Striano = Striano et al. 2009

^bThis mutation introduces a cryptic new splice site (Salomons et al. 2007)

Table 2Effect on residue properties of *ALDH7A1* missense mutations that cause pyridoxine-dependent epilepsy

	Missense mutation	Effect on polarity/charge (at pH 7.4)	Hydropathy index ^a	Surface accessible ^b
G83E ^c	Glycine to glutamate	Nonpolar/neutral to polar/negative (acidic)	−0.4 to −3.5	Yes
G138V	Glycine to valine	Nonpolar/neutral to nonpolar/neutral	−0.4 to +4.2	Yes
N167S	Asparagine to serine	Polar/neutral to polar/neutral	−3.5 to −0.8	Yes
A171V	Alanine to valine	Nonpolar/neutral to nonpolar/neutral	+1.8 to +4.2	Yes
G174V	Glycine to valine	Nonpolar/neutral to nonpolar/neutral	−0.4 to +4.2	No
G255D	Glycine to aspartate	Nonpolar/neutral to polar/negative (acidic)	−0.4 to −3.5	Yes
G263E	Glycine to glutamate	Nonpolar/neutral to polar/negative (acidic)	−0.4 to −3.5	Yes
N273I	Asparagine to isoleucine	Polar/neutral to nonpolar/neutral	−3.5 to +4.5	No
T297K	Threonine to lysine	Polar/neutral to polar/positive (basic)	−0.7 to −3.9	Yes
T297R	Threonine to arginine	Polar/neutral to polar/positive (basic)	−0.7 to −3.5	Yes
R307Q	Arginine to glutamine	Polar/positive (basic) to polar/neutral	−4.5 to −3.5	No
V367G	Valine to glycine	Nonpolar/neutral to nonpolar/neutral	+4.2 to −0.4	Yes
G378R	Glycine to arginine	Nonpolar/neutral to polar/positive (basic)	−0.4 to −4.5	Yes
E399Q ^c	Glutamate to glutamine	Polar/negative (acidic) to polar/neutral	−3.5 to −3.5	Yes
E399D	Glutamate to aspartate	Polar/negative (acidic) to polar/negative (acidic)	−3.5 to −3.5	Yes
P403L ^c	Proline to leucine	Nonpolar/neutral to nonpolar/neutral	+1.6 to +3.8	No
F410L	Phenylalanine to leucine	Nonpolar/neutral to nonpolar/neutral	+2.8 to +3.8	No
N420K	Asparagine to lysine	Polar/neutral to polar/positive (basic)	−3.5 to −3.9	No
N421K	Asparagine to lysine	Polar/neutral to polar/positive (basic)	−3.5 to −3.9	Yes
Q425R	Glutamine to arginine	Polar/neutral to polar/positive (basic)	−3.5 to −4.5	Yes
S430N ^c	Serine to asparagine	Polar/neutral to polar/neutral	−0.8 to −3.5	No
I431F ^d	Isoleucine to phenylalanine	Nonpolar/neutral to nonpolar/neutral	+4.5 to +2.8	No
D449N	Aspartate to asparagine	Polar/negative (acidic) to polar/neutral	−3.5 to −3.5	Yes
C450S ^c	Cysteine to serine	Polar/neutral to polar/neutral	+2.5 to −0.8	Yes
G466R	Glycine to arginine	Nonpolar/neutral to polar/positive (basic)	−0.4 to −4.5	Yes
G477R ^c	Glycine to arginine	Nonpolar/neutral to polar/positive (basic)	−0.4 to −4.5	Yes

^aHydropathy index is a calculated constant related to hydrophobicity or hydrophilicity of R groups. It describes the tendency of a specific residue to seek a hydrophobic (positive value) or aqueous environment (negative value) (Kyte and Doolittle 1982)

^bAs determined by Chimera (version 1.4.1) molecular modeling software

^cMutations that have been identified more than two times

^dIndicates novel mutation identified during this study

Table 3

PCR primer sequences

Exon 1	ANT 1 For	5'M-13 for-CCCTGTAGCACTCCCATTGT-3'
	ANT 1 Rev	5'M-13 rev-TGACGTCGATTCTGCATAGC-3'
Exon 2	ANT 2 For	5'M-13 for-TCAGAAATGAAAGACAACCTCTG-3'
	ANT 2 Rev	5'M-13 rev-AGCCTGCACAACTCCTTGT-3'
Exon 3	ANT 3 For	5'M-13 for-CCTGTTTTACCGGGTTCTAGC-3'
	ANT 3 Rev	5'M-13 rev-ACAGTATCACAGCCCCAAG-3'
Exon 4	ANT 4 For	5'M-13 for-GCCTGGCCATATCACAGTTTT-3'
	ANT 4 Rev	5'M-13 rev-ATGATGAAACCCCATGTCTACTTTA-3'
Exon 5	ANT 5 For	5'M-13 for-CATGTTTTGCTTCCCCCTT-3'
	ANT 5 Rev	5'M-13 rev-TTGCACAGTCAATAGCCAGA-3'
Exon 6	ANT 6 For	5'M-13 for-TATCCCATGGCTGTGTAGCA-3'
	ANT 6 Rev	5'M-13 rev-GCTGAGTTCGCACCATTACA-3'
Exon 7	ANT 7 For	5'M-13 for-AAAGACACCCAGCTGAAGGA-3'
	ANT 7 Rev	5'M-13 rev-ATGACATGGCACTGAAAGCA-3'
Exon 8	ANT 8 For	5'M-13 for-AGTGGGCTGAAAAGCAAGA-3'
	ANT 8 Rev	5'M-13 rev-CCTCTGGGCAATTAAGACA-3'
Exon 9	ANT 9 For	5'M-13 for-TCATGAAGACCTTCCCTTGC-3'
	ANT 9 Rev	5'M-13 rev-GGAAAAGGTTGAGGGGAAA-3'
Exon 10	ANT 10 For	5'M-13 for-GGCTGTGTAGCAGTGTGCAG-3'
	ANT 10 Rev	5'M-13 rev-CAGGTTCTAGATCATCCAGGT-3'
Exon 11	ANT 11 For	5'M-13 for-GAAAGTGGCCTGACCAACAT-3'
	ANT 11 Rev	5'M-13 rev-GCCAGCCACATCTAGAGAGC-3'
Exon 12	ANT 12 For	5'M-13 for-AAAAAGACATGGCTTATGATTTTATTC-3'
	ANT 12 Rev	5'M-13 rev-AACCTGCTTCATGTGCCTTC-3'
Exon 13	ANT 13 For	5'M-13 for-ATGCCATAAAGGGCAAAATG-3'
	ANT 13 Rev	5'M-13 rev-TTTTCCAATATGCCCAGAGC-3'
Exon 14	ANT 14 For	5'M-13 for-ATCCTCTGACCCCAAGTCCT-3'
	ANT 14 Rev	5'M-13 rev-TCCAGTGAATTTAATCCACCA-3'
Exon 15/16	ANT 15/16 For	5'M-13 for-TTAGGGGAAAAATCCCAAAAT-3'
	ANT 15/16 Rev	5'M-13 rev-GAGGAGATGACGCAGGACTC-3'
Exon 17	ANT 17 For	5'M-13 for-TTGCAGGGGAGATATTGTGG-3'
	ANT 17 Rev	5'M-13 rev-CACTGCACAAAGACAGCACA-3'
Exon 18	ANT 18 For	5'M-13 for-TGGGCATGAAAATCTTCTGTT-3'
	ANT 18 Rev	5'M-13 rev-TGGCTATGTTGTAACAATTTTATTTG-3'