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# Mutations in antiquitin in individuals with pyridoxine-dependent seizures

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**We show here that children with pyridoxine-dependent seizures (PDS) have mutations in the *ALDH7A1* gene, which encodes antiquitin; these mutations abolish the activity of antiquitin as a $\Delta^1$-piperideine-6-carboxylate (P6C)–α-aminoadipic semialdehyde (α-AASA) dehydrogenase. The accumulating P6C inactivates pyridoxal 5′-phosphate (PLP) by forming a Knoevenagel condensation product. Measurement of urinary α-AASA provides a simple way of confirming the diagnosis of PDS and *ALDH7A1* gene analysis provides a means for prenatal diagnosis.**

PLP is an essential cofactor for many enzymatic reactions, including those involved in neurotransmitter metabolism. Individuals with PDS need an unusually large daily intake of the PLP precursor pyridoxine to prevent fits; this is the basis of the diagnosis[1]. However, while it is known that PDS maps to 5q31 (ref. 2), the mechanism of pyridoxine dependency in PDS has not been identified. Elevated levels of pipecolic acid (**Fig. 1**) in plasma and cerebrospinal fluid have been reported in individuals with PDS[3]. This is not caused by a defective PLP-dependent reaction in the catabolism of pipecolic acid because the only PLP-dependent enzyme involved in catabolism of pipecolic acid is α-aminoadipic acid (α-AAA) transaminase, and α-AAA is not increased. A clue to the pathogenesis of PDS came from observations of a child with hyperprolinemia type II, a defect in L-$\Delta^1$-pyrroline-5-carboxylate (P5C) dehydrogenase leading to accumulation of P5C (**Fig. 1**). The accumulating P5C reacts with PLP by Knoevenagel condensation[4]; depletion of PLP provides an explanation for the observed seizures in hyperprolinemia type II. Comparison of the structure of P5C with that of the metabolite after pipecolic acid in the lysine catabolism pathway, P6C (**Fig. 1**), suggested that P6C probably also reacts with PLP by Knoevenagel condensation. Conversion of P6C to α-AAA in *Streptomyces clavuligerus* requires a 'P6C dehydrogenase' and its gene has been sequenced[5]. P6C is the cyclic Schiff base of α-AASA; in solution they are in equilibrium[6] and the P6C dehydrogenase is probably an α-AASA dehydrogenase (**Fig. 1**).

A BLAST search using the sequence FAAVGTAGQRCTTLRRL (which is highly conserved between the P6C dehydrogenase from *S. clavuligerus* and related dehydrogenases[5]) identified the human gene *ALDH7A1* (ref. 7), which encodes antiquitin. It mapped to 5q31, the locus for PDS[2]. Antiquitin has aldehyde dehydrogenase (ALDH) activity[8], but its physiological substrate has remained uncertain.

Sequencing of *ALDH7A1* from 13 individuals with PDS (**Supplementary Methods**, **Supplementary Table 1** and **Supplementary Table 2** online) revealed homozygous and compound heterozygous mutations (**Fig. 2a–i** and **Supplementary Fig. 1**, **Supplementary Fig. 2** and **Supplementary Table 3** online). In all eight families,

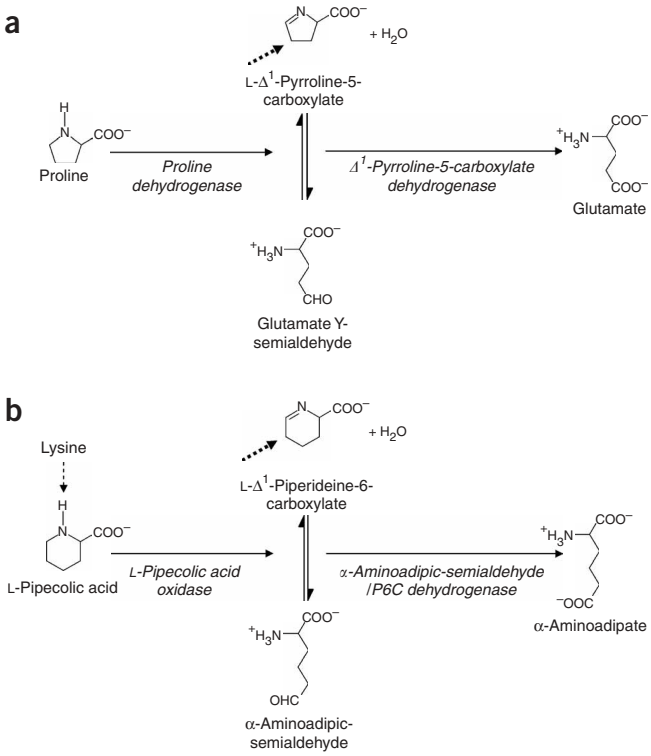


**Figure 1** Catabolism of (**a**) proline and (**b**) L-pipecolic acid showing the structures of $\Delta^1$-pyrroline-5-carboxylate (P5C) and $\Delta^1$-piperideine-6-carboxylate (P6C). The dotted arrow indicates the activated methylene group that we propose can react with the carbonyl group of PLP, shown by another group[4] for P5C.

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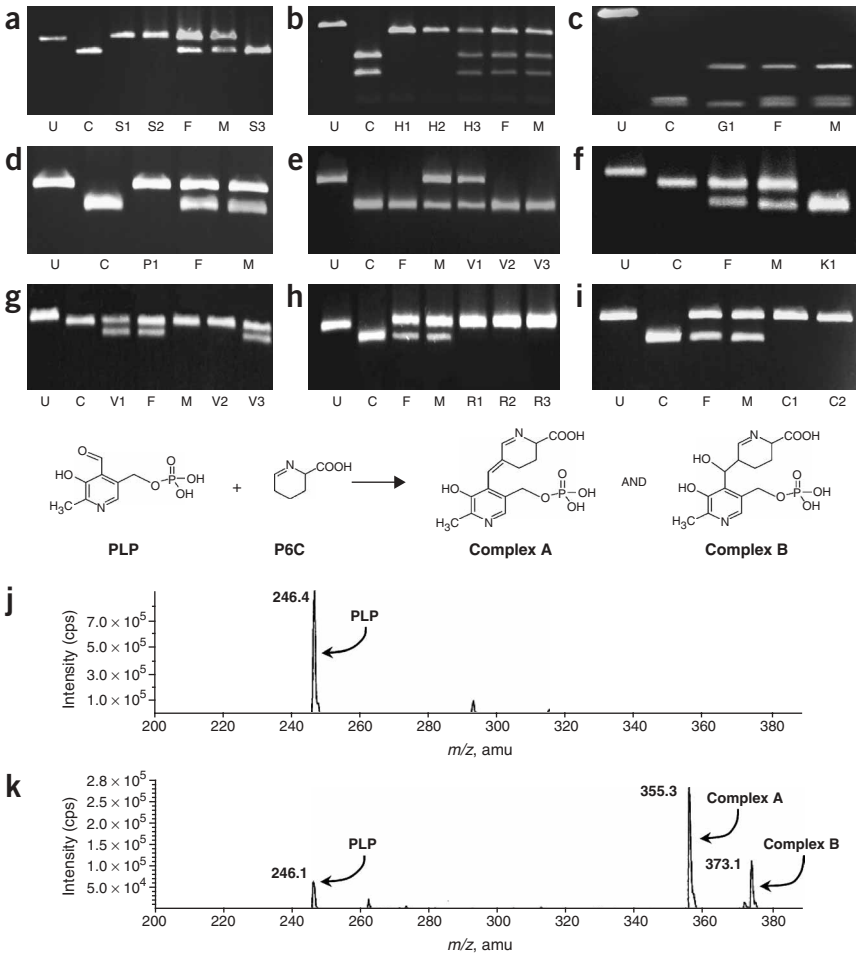


**Figure 2** Mutations in *ALDH7A1* and formation of complexes between PLP and P6C. Restriction digest tests. F, father; M, mother; U, undigested normal; C, digested normal; nv, not visible. (**a**) c.228+2t→a; digestion of exon 3 with *Dde*I. C, 121 bp (visible), 31 bp (nv). c.228+2t→a abolishes restriction site. Family S: parents, heterozygous; S1, S2 (both affected), homozygous; S3 (unaffected), no mutation. (**b**) c.434-1g→c; digestion of exon 6 with *Dde*I. C, 204 bp and 133 bp (both visible), 58 bp (nv). c.434-1g→c abolishes a restriction site: mutant allele, 337 bp (visible), 58 bp (nv). Family H: parents, H3 (all unaffected), heterozygous; H1, H2 (both affected), homozygous. (**c**) A171V; digestion of exon 6 with *Bts*I. C, 173 bp and 167 bp (both visible), 55 bp (nv). A171V abolishes a restriction site: mutant allele, 228 bp and 167 bp. Family G: parents, (unaffected) heterozygous; G1, (affected) homozygous. (**d**) E399Q; digestion of exon 14 with *Bsm*AI. Mutation abolishes restriction site. C, 150 bp and 142 bp (not resolved). Family P: parents, (unaffected) heterozygous; P1, (affected) homozygous. (**e**) E399Q confirmed as in **d**. Family V: mother (unaffected), V1 (affected), heterozygous; father and unaffected siblings (V2, V3), no mutation. (**f**) R82X; digestion of exon 4 with *Dde*I. C, 144 bp (visible), 11 bp (nv). Mutant allele, 125 bp (visible), 19 bp and 11 bp (nv). Family K: parents, (unaffected) heterozygous; K1, (affected) homozygous. (**g**) R82X confirmed as in **f**. Family V: father (unaffected), V1 (affected), V3 (unaffected), heterozygous; Mother, V2 (unaffected), no mutation. (**h**) Y380X; digestion of exon 14 with *Sfc*I. C, 192 bp (visible), 24 bp (nv). Y380X abolishes restriction site. Family R: parents, heterozygous; R1, R2, R3, (affected) homozygous. (**i**) c.1512delG; digestion of exon 18 with *Hae*III. C, 156 bp and 144 bp (not resolved). c.1512delG abolishes restriction site. Family C: parents, heterozygous; C1, C2, (affected) homozygous. Inactivation of PLP by P6C and the formation of complexes A and B was shown by liquid chromatography–tandem mass spectrometry operating in the *m/z* of –97 precursor-ion scanning mode. (**j**) Analysis of mixture at time 0 showing presence of PLP (*m/z*, –246.4). (**k**) Analysis of mixture after 24 h showing the presence of complex A (*m/z*, –355.3) and complex B (–373.1), 2 compounds that are the Knoevenagel products of PLP and P6C.

parents were shown to be heterozygotes; none of the mutations was detected in ≥60 control chromosomes (**Fig. 2a–i**).

Homozygous splice site mutations detected in family S members S1 and S2 (coding DNA 228+2t→a; c.228+2t→a) and family H members H1 and H2 (c.434-1g→c) are predicted to cause exon skipping (**Supplementary Table 4** online). c.434-1g→c will lead to skipping of exon 6 and, if translated, the protein would lack residues 145–189 and thus Gly158, Asn167 and Pro169 (found in >95% of ALDHs[9]). Asn167 is thought to stabilize the substrate's carbonyl oxygen during catalysis[9].

The missense mutation E399Q (family P member P1, homozygote; family V member V1, compound heterozygote for E399Q and R82X) affects a glutamate residue highly conserved in antiquitins (**Supplementary Fig. 2** online) and the human ALDH superfamily. In class 2 ALDHs, Glu399 interacts with the ribose moiety of NAD; mutation to glutamine converts the rate-limiting step from deacylation to hydride transfer[10]. Family G member G1 is homozygous for A171V. This mutation affects an alanine residue that is highly conserved across species (**Supplementary Fig. 2** online).

The nonsense codons (family K member K1, homozygous for R82X; family R members R1, R2 and R3, homozygous for Y380X) probably cause nonsense-mediated mRNA decay[11]. If translated, both mutations would produce proteins truncated at the C terminal unlikely to form active homotetramers. The R82X protein would lack residues 82–511, including the catalytic thiol Cys302 (highly conserved throughout the ALDHs). The Y380X mutation would produce a protein lacking residues 381–511.

The single base deletion (c.1512delG) (family C members C1 and C2, homozygous) probably affects enzyme activity as the frameshift changes the last 7 amino acid residues and extends the C terminus by 10 residues (**Supplementary Fig. 2** online). Antiquitin is a homotetramer[10], like other class 1 and 2 ALDHs. Mutant ALDH1 enzymes with 5 or 17 extra residues at the C terminus showed normal oligomerization, but reduced activity[12]. A change in the last seven residues of the normal sequence probably has additional effects on oligomerization or activity.

Transfection of CHO cells with wild-type *ALDH7A1* produced α-AASA dehydrogenase activity of 55 ± 15 μmol/min/mg protein (mean ± s.e.m.; $n = 3$; **Supplementary Methods** online). Transfection with *ALDH7A1* containing the mutation encoding Y380X, resulted in α-AASA dehydrogenase activity that was 1.8% of wild-type. *ALDH7A1* encoding E399Q, A171V, R82X and c.1512delG produced

undetectable α-AASA dehydrogenase activity. This supports our hypothesis that the pathogenesis of PDS involves inactivation of PLP by P6C and that P6C accumulates in antiquitin-deficient individuals because antiquitin is a P6C–α-AASA dehydrogenase.

Measurement of α-AASA in children with PDS and eight controls by liquid chromatography–tandem mass spectrometry (**Supplementary Methods** online) showed that the concentration of α-AASA in cerebrospinal fluid was 1–28 μmol/l (in controls, <0.1 μmol/l), 1.5–4.6 μmol/l in plasma (in controls, <0.2 μmol/l) and 7.5–168 mmol/mol creatinine in urine (in controls, <1 mmol/mol).

Our hypothesis required that P6C undergo Knoevenagel condensation with PLP; this was studied using tandem mass spectrometry (**Supplementary Methods** online). After incubation of P6C with PLP, two adducts with mass-to-charge ratio (*m*/*z*) of –355 and –373 appeared and, concomitantly, the PLP signal (*m*/*z*, –246) decreased (**Fig. 2j**,**k**). Incubation of PLP with the urine of an individual with PDS produced identical adducts. Formation of these compounds matches the Knoevenagel condensation of PLP and P5C (which has a molecular weight of 14 AMU less than P6C and produces compounds with *m*/*z* of –341 and –359)[4]. Knoevenagel condensation of PLP and P6C can be expected to inactivate PLP.

In conclusion, human antiquitin is the α-AASA dehydrogenase in the pipecolic acid pathway of lysine catabolism. Deficiency of antiquitin causes seizures because accumulating P6C condenses with PLP and inactivates this enzyme cofactor, which is essential for normal metabolism of neurotransmitters. Our subjects had 'classical PDS' (seizures commencing within 4 weeks of birth). Classical PDS is probably rare[1]; however, other types of pyridoxine responsiveness may not be so rare: 5% of children with intractable epilepsy may show a good response to pyridoxine[13]. It is tempting to speculate that such children have 'mild' mutations in *ALDH7A1*.

Measurement of α-AASA may facilitate rapid diagnosis of PDS, but neonatal status epilepticus is an emergency; if the 'electroclinical' features suggest PDS[14], pyridoxine should be given. Measurements of α-AASA and *ALDH7A1* analysis could be used to avoid the potentially dangerous withdrawal of pyridoxine currently recommended for definitive diagnosis of PDS. *ALDH7A1* analysis could also be used for prenatal diagnosis of PDS; although seizures are often fully controlled by pyridoxine, normal psychomotor development cannot be guaranteed.

*Note: Supplementary information is available on the Nature Medicine website.*

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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