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PYCR2 mutations cause a lethal syndrome of microcephaly and failure to thrive

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Abstract

Objective—A study was undertaken to characterize the clinical features of the newly described hypomyelinating leukodystrophy type 10 with microcephaly. This is an autosomal recessive disorder mapped to chromosome 1q42.12 due to mutations in *PYCR2* gene, encoding an enzyme involved in proline synthesis in mitochondria.

Methods—From several international clinics, eleven consanguineous families were identified with *PYCR2* mutations by whole exome or targeted sequencing, with detailed clinical and radiological phenotyping. Selective mutations from patients were tested for effect on protein function.

Results—The characteristic clinical presentation of patients with *PYCR2* mutations included failure to thrive, microcephaly, craniofacial dysmorphism, progressive psychomotor disability, hyperkinetic movements, and axial hypotonia with variable appendicular spasticity. Patients did not survive beyond the first decade of life. Brain magnetic resonance imaging (MRI) showed global brain atrophy and white matter T2 hyperintensities. Routine serum metabolic profiles were

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Authorship

MSZ, TS, MI, LS, IG, GA-S identified patients for study, H-JJ performed cell culture and western blotting, MSA-H performed Sanger confirmation, GB, IMV, ED organized clinical data and generated figures. MSZ and JGG supervised the project, and GB, IMV, MSZ and JGG contributed to the manuscript.

Potential Conflicts of Interest

Nothing to report.

unremarkable. Both nonsense and missense mutations were identified, which impaired protein multimerization.

Interpretation—*PYCR2*-related syndrome represents a clinically recognizable condition in which *PYCR2* mutations lead to protein dysfunction, not detectable on routine biochemical assessments. Mutations predict a poor outcome, probably as a result of impaired mitochondrial function.

Keywords

PYCR2; hypomyelinating leukodystrophy-10; brain atrophy; microcephaly; pyrroline-5-carboxylate reductase 2; proline metabolism

Introduction

Proline is a non-essential amino acid involved in protein biosynthesis and antioxidant reactions.^{1, 2} It is endogenously synthesized from glutamate *via* pyrroline-5-carboxylate synthase (P5CS), which converts glutamate to pyrroline-5-carboxylate (P5C). P5C can be converted to proline by one of three paralogues in mammals: *PYCR1*, *PYCR2*, and *PYCR1L*, linked to the group of rare *PYCR*-related disorders (Fig 1A). Defects of proline synthesis have major deleterious effects on health, leading to intellectual disability,^{2–4} skin and joint hyperelasticity, osteopenia,^{5, 6} and cataracts.⁷

The clinical and molecular phenotype of disorders of proline synthesis is currently under intensive investigation. To date, mutations only in *PYCR1* (Online Mendelian Inheritance in Man [OMIM] 616406) and *PYCR2* (OMIM 179035) have been associated with human disease. Homozygous mutations in *PYCR1* were reported in families presenting with cutis laxa and progeroid features.^{8,9} Fibroblasts from patients showed defective mitochondrial morphology, reduced mitochondrial membrane potential, and a fivefold increase in apoptosis upon oxidative stress.⁹ Proline levels in plasma were normal. Mutations in *PYCR2* were recently reported in two consanguineous families with microcephaly and cerebral hypomyelination.¹⁰ Similar to patients with *PYCR1* mutations,⁸ no evidence for proline depletion was found in patient serum, suggesting functional redundancy of multiple *PYCR* paralogues in proline production. *PYCR2*-related disorder has been classified as hypomyelinating leukodystrophy-10 (OMIM 616420) based on the report of these two families.

Here, we expand the clinical account of *PYCR2*-related disorders by presenting fourteen patients from eleven consanguineous families with homozygous *PYCR2* mutations. The clinical phenotype was remarkably consistent between patients, characterized by microcephaly (occipito-frontal circumference of –3 to –6 standard deviations below the mean), severe psychomotor delay, failure to thrive, facial dysmorphism, and white matter hyperintensities with global brain atrophy on MRI. Intellectual and motor functions worsened overtime, and none of the patients survived beyond the first decade of life. Mutations clustered in the dimerization domain of the protein, and several tested mutations impaired multimerization. We propose that *PYCR2*-related disorder is a clinically

recognizable lethal genetic leukoencephalopathy syndrome without biochemical alterations on standard clinical assays.

Subjects and Methods

Patient Material

We recruited subjects to this study as part of an assessment of children with neurodevelopmental disorders presenting to clinics in regions of the world displaying elevated rates of parental consanguinity. The total cohort includes over 5100 individual families and 9400 participants recruited between the period of 2004–2015 presenting with features of intellectual disability, autism-related conditions, microcephaly, structural brain disorders, epilepsy or neurodegeneration. The cohort was enriched for families with recessive pediatric brain disorders with homozygous mutations, due to documented consanguinity in over 80% of pedigrees and multiple affected members in 63% of families. General physical and neurological exams as well as evaluation of brain MRI or CT were carried out as part of the standard clinic evaluation. Pedigree analysis and blood sampling were pursued on all families, and subjects were selected for exome sequencing based upon a clinically defined neurodevelopmental genetic condition.

The study included eleven families, (family ID 1232, 1240, 2206, 2404, 2566, 2664, 2682, 2706, 3109, 3740 and 4592) with fourteen affected members with pediatric onset neurological features and autosomal recessive inheritance. Two families (2206 and 2404) had two diseased siblings each who presented identically to the ascertained probands, but were deceased prior to ascertainment. None of the families have been previously reported. All patients were examined by at least one of the authors and clinical details including videos were recorded at the time of each visit. The study was approved by the ethical committees of participating institutions, and all ascertainment was performed with informed consent.

Genotyping

Genomic DNA was extracted from blood leukocytes or saliva samples by standard methods from all genetically informative members wherever possible, to include parents, affected and unaffected offspring. The extracted DNA samples were tested for quality and family integrity with a panel of fluorescent genotyping markers using polymerase chain reaction (PCR) and analyzed using an ABI 3130xl automatic DNA Genetic Analyzer and ABI Peak Scanner Software v1.0 (Applied Biosystems, Foster City, CA).

Whole exome sequencing

Five hundred nanograms of genomic DNA were subjected to whole exome sequencing (WES), using Agilent (Santa Clara, CA) Sure Select Human All Exome 50 Mb reagents and the Illumina (San Diego, CA) HiSeq 2000 platform 150 bp paired-end sequencing, resulting in > 100X average exon coverage. Exomes were aligned to hg19 reference genome, and variants were identified using the GATK Toolkit,¹¹ annotated and prioritized using custom Python scripts.¹²

Sanger sequencing

Potential disease-causing variants identified from WES were validated and tested for segregation using fluorescent Sanger sequencing. Each member of the family on whom DNA was available was tested for zygosity. Allele frequencies were determined from the ExAC dataset and from an in-house 5000 WES library of geographically similar individuals. Sequences were analyzed with Sequencher 5.1 software (Gene Codes Corporation, Ann Arbor, MI).

Skin cell isolation and culture

Skin biopsies were performed on patients and unaffected available family members following informed consent. Mutant and control fibroblasts were generated from explants of dermal biopsies and tested negative for mycoplasma prior to study. Primary skin fibroblasts were cultured in MEM (GIBCO) supplemented with 20% FBS (Gemini) and penicillin as previously reported.¹³ All experiments were performed with fibroblasts at passage 5–8.

Cell transfection and co-immunoprecipitation

The wild-type human *PYCR1*, *2*, *L* were amplified from MGC fully sequenced cDNA clones (PYCR1, IMAGE:3505512; PYCR2, IMAGE:2901360; PYCRL, IMAGE:3533609) (GE Dharmacon, Lafayette, CO, 80026), cloned into either FLAG- or Myc/His-tagged mammalian expression vectors, and mutagenized to incorporate patient mutations. 293T cells (ATTC, Manassas, VA, 20110) were co-transfected with the FLAG- or Myc/His-tagged expression vectors using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA) and cell extracts were used for co-immunoprecipitation, performed with anti-Flag M2 affinity gel (Sigma, St. Louis, MO). Input and output samples were analyzed by Western blotting with primary antibodies against PYCR1 (Protein Tech, Rosemont, IL; 13108-1-AP), PYCR2 (Sigma, St. Louis, MO; HPA056873), PYCRL (Novus Biological, Littleton CO, H00065263-M01), FLAG (Sigma, St. Louis, MO; F7425), c-Myc (Santa Cruz Biotechnology, Dallas, TX, 75220; sc-40), 6X His (GeneTex, Irvine, CA, GTX30500) or α -tubulin (Sigma St. Louis, MO, T6074), detected by HRP-conjugated secondary antibodies and chemiluminescence (Thermo, Waltham, MA).

Results

Identification of mutations in PYCR2

We sequenced two affected individuals in multiplex families, or one affected and both parents in simplex families. Analysis of family 1232 led to the identification of homozygous nonsense mutations in *PYCR2*, which then prompted a search of our exome database, identifying 6 additional families 1240, 2206, 2404, 2566, 2664 and 2682 with *PYCR2* mutations. Once the clinical features were clarified, additionally four families were identified with *PYCR2* mutations from clinics (2706, 3109, 3740, 4592) by direct Sanger sequencing. Only one family where presenting features matched the *PYCR2* phenotype was negative for *PYCR2* mutation, and this family is still under investigation. In total, eleven families displayed convincing homozygous mutations, and clinical features for all were uniformly similar. No other homozygous variants in *PYCR2* met criteria for causation

(<0.1% allele frequency, GERP score >4.5, homozygous in all affected sequenced) in any other families, irrespective of the clinical presentation. Mutations included introduction of a stop codon (p.Arg266Stop shared by five families), substitution of highly conserved amino acids (p. Cys232Gly, p.Arg199Trp shared by two families, or p.Val184Ala, p.Gly159Arg), or splice site mutation (3' splice site of intron 2) (Fig 4B). Direct questioning showed no evidence of shared ancestry for families sharing a common mutation. No correlation was detected between individual mutation with disease severity. No biases were detected in severity or age of onset based upon patient sex, and all mutations were fully penetrant without phenocopies within the family observed.

Clinical features of the affected individuals

Birth history—Clinical features of the 14 affected individuals are summarized in Table 1, and reported in detail in Supplementary Table 1. All families were of Egyptian origin, except for family 2706, which was Pakistani. Parental consanguinity was present in all families except family 1240 (Fig 1C). The study included 7 females and 7 males, and ages ranged from 4 months to 6 years. All patients were born full term without complications during pregnancy and delivery. Measurements at birth including weight and height were in the normal range. Head circumference at birth was available in 10 out of 14 patients and ranged from 1.8 to 3 standard deviations below mean (-SD) (see Supplementary Table 1).

Childhood presentation—Patients presented with profound psychomotor delay, microcephaly, truncal hypotonia with variable appendicular spasticity, and failure to thrive from the ages of 2 months to 1 year. All stagnated at the developmental level of a 4–5-month old infant. They were able to achieve some head control, visual tracking and could recognize family members and some were able to sit with support (patient 2682-III-1, 2682-III-2). None developed meaningful language or fine motor skills, and none were able to stand or walk independently. Primitive neonatal reflexes such as grasp and sucking reflex were seen in those more severely affected (patient 3740-III-2) even after ages 2–3 years. Seizures were seen in 57% of patients with onset usually before 1 year of age, consisting of focal myoclonic and generalized tonic-clonic seizures, controlled with anticonvulsant medications. Excessive vomiting of unknown etiology was seen in 29% of patients (patient 1232-III-1, 1232-IV-2, 1240-II-2 and 4592-III-3) with onset at about 1–2 months of age. Audiology evaluation, wherever available, showed bilateral mild to severe hearing loss. Serial clinical follow up demonstrated progression of symptoms in patients. Families 2206 and 2404 had two siblings each (not included in this study) with a similar clinical presentation and early death. Patients 1232-III-1, 1240-II-2, 2566-IV-2, 2664-III-1, 2682-III-1 died at or before age 6 years due to complications of pulmonary infection, fever of unknown origin and/ failure to thrive, and no patient survived beyond age 10 years (Fig 1B).

Examination—Although all individuals were born with average growth parameters, failure to thrive was noted by 1 year of age. Head circumference was less than 3–6 -SD; height was less than 2–5 -SD; and weight was less than 4–9 -SD. A characteristic craniofacial dysmorphism was noted in most of the patients, which included triangular facies, malar hypoplasia, large malformed ears with over-folded helices, upturned nose with bulbous tip, and a long smooth philtrum (Fig 2). Additional facial features of hypertrichosis

and full lips were noted in four patients. Bitemporal narrowing due to severe microcephaly was noted in less than half of the patients. Skeletal features of long thin fingers and toes were observed in four patients, while two had pectus carinatum. Family 1232 (patient 1232-III-1 and 1232-III-2) demonstrated a slightly different facial gestalt compared to the other patients including synorhysis, prominent glabella, short forehead, gum hyperplasia, arched narrow palate, and dental malocclusion.

On examination patients were awake and alert to the environment, and none were verbal. Nystagmus was noted in 21% of patients, commonly manifested as slow horizontal and symmetric eye movements in both directions. Truncal hypotonia with hyperreflexia was seen in most patients. Mild to severe appendicular spasticity was present in 57%. Muscle atrophy was evident in all except patient 1232-III-2. The majority of patients demonstrated hyperkinetic movements of upper and lower extremities, along with spontaneous mouth chewing and head titubation (supplementary video).

MRI features and biochemical investigation—Brain imaging (see Fig 3 and Supplementary Fig 1) was available in 13 of the 14 subjects. Six out of 14 patients underwent CT head imaging while others had MRI. Some patients showed severe cerebral atrophy (patient 2404-III-7, 3740-III-2) with ventriculomegaly, in contrast to other patients in which atrophy was less severe. Thin corpus callosum was seen in 61% of patients. Overall, cerebellum and brainstem were preserved except in patients 2706-III-3 and 2706-III-4. Metabolic profile including serum creatinine, BUN, CPK, ammonia, lactate, plasma amino acids and urine organic acids were normal in all patients. Urine amino acids were available in two families (2206 and 2664), which showed mildly elevated glutamate levels at 84 and 109 nmol/mg creatinine (reference level <76 nmol/mg creatinine) respectively, compared to unaffected carriers who had normal levels.

Effects of mutations on protein function

PYCR2 is expressed broadly, including dermal fibroblasts.¹⁴ To study the effect of patient mutations, we obtained skin biopsies, cultured primary skin fibroblasts, then collected whole cell lysates from cultures at early passage from affected individuals and carrier parents from three families, representing three different mutations (Fig 4). Western blot analysis using a c-terminal PYCR2 antibody demonstrated presence of the full-length protein associated with both missense alleles tested (p.199Arg/Trp and p.232 Cys/Gly), suggesting that the mutations did not appreciably destabilize the protein. As expected, less protein was detected from the parent carrier associated with the truncated allele tested (p.266Arg/Stop) due to the lack of the c-terminal antigen. No protein was detected in the affected individual. As a reference, we included primary skin fibroblasts from a family with *PYCR1* null mutation³, in which PYCR2 protein, but not PYCR1 protein, was detected (Fig 4A). We also noted essentially unchanged expression levels of PYCR1 in *PYCR2*-mutated samples, suggesting lack of expression compensation. Interestingly, we noted an upper shadow band on the PYCR1 blot, which, we concluded, likely represents non-specific binding to PYCR2 because it was absent in the homozygous truncated p.266Stop/Stop sample at the correct molecular weight. Furthermore, the two samples harboring the p.266Arg/Stop mutation also showed a band of lower molecular weight of lower intensity upon longer exposure, likely

corresponding to the truncated PYCR2 protein. We conclude that the missense mutations tested result in stable protein and that the p.266Arg/Stop mutation results in some retention of mutant truncated protein.

Since missense mutations tested did not disrupt stability of the protein, we tested whether mutations affect function. P5C-reductase family members function as multimers, and family members contain a conserved P5CR-dimerization domain.¹⁵ We thus introduced patient mutations (p.199Arg/Trp, p.232 Cys/Gly, p.266Arg/Stop) into a FLAG- or Myc/His-tagged mammalian expression vectors encoding PYCR2 and co-transfected 293T cells with differently tagged expression vectors for co-immunoprecipitation studies. We also included alanine substitutions (p.Glu221Ala, p.Ser233Ala, and p.Thr238Ala) of amino acids that were suggested to be crucial for dimerization based on PYCR1 structure.^{15, 16} PYCR1 and PYCRL, the two other paralogues, were also included to test dimerization. The cell lysates were subjected to immunoprecipitation using an anti-FLAG affinity gel and the eluted samples were analyzed by Western blot with antibodies against c-Myc or 6X His (Fig 4C). We found that all the expression vectors produced detectable protein of predicted molecular weight. Wild-type PYCR2 co-immunoprecipitated wild-type PYCR2, as did PYCR2 proteins with the tested alanine mutations, whereas PYCR2 with the patient missense or nonsense mutations showed reduced co-precipitation of wild-type protein. PYCR2 were also co-precipitated with PYCR1, but barely with PYCRL, whereas PYCR1 and PYCRL each co-precipitated with itself. We conclude that patient mutations can impair protein multimerization, and that there is potential promiscuity in the association between PYCR paralogue proteins.

Discussion

Expansion of the clinical phenotype

In this study we identified fourteen individuals with mutations in the *PYCR2* gene, expanded and refined the clinical presentation, and tested the effects of mutations in primary patient cells and in heterologous cells. Key clinical features noted in all patients included an early presentation of neurodevelopmental delay within the first year of life, secondary microcephaly which became pronounced with age given the progressive neurodegenerative nature of the disease¹⁷ and characteristic triangular facies, malar hypoplasia, large malformed ears, and upturned bulbous nose (Supplementary table1). Similar minor dysmorphisms were observed in previously reported patients.¹⁰ We appreciated some variability in facial features, likely due to the varying range of facial muscular atrophy and age of evaluation. Skeletal features of arachnodactyly, long toes, and pectus carinatum observed in four patients were not previously reported. Besides severe muscle wasting, limb hypertonía, brisk reflexes, inability to ambulate, and hyperkinetic extremity movements aided in making the diagnosis.¹⁰

Although this condition has been classified as a hypomyelinating leukodystrophy, we found that the imaging features were variable ranging from subtle white matter T2 hyperintensities to progressive brain atrophy. Hypomyelinating leukodystrophies (HLDs) represent a genetically heterogeneous group of neurological disorders characterized by impaired myelin formation in the brain. To date, eleven different genetic forms of HLD have been described

with mutations in genes involved in myelin formation and oligodendrocyte maturation.¹⁸ A new term “genetic leukoencephalopathy” (gLE) was recently coined by Global Leukodystrophy Initiative (GLIA) Consortium to define inherited disorders which are in fact primary neuronal diseases with secondary hypomyelination, such as Cockayne syndrome, *AGCI*-related disorders or other genetic conditions with major systemic manifestations,¹⁹ and one could consider that *PYCR2* could be included in this group. The clinical features of early onset intellectual disability, microcephaly, epilepsy, and brain atrophy suggest primary neuronal dysfunction with secondary deficit in myelination, in contrast to most patients with HLD, often presenting with cerebellar ataxia, axial hypotonia, spasticity, and nystagmus, without severe diffuse cerebral atrophy.¹⁸

During the study course, about half of the cohort of *PYCR2*-mutated patients succumbed from failure to thrive and complications from pulmonary infections, and there were no living survivors beyond the age of ten years. No specific cause of death has been ascribed that would suggest other organ involvement. In the previous report, none of the patients died and the longest living survivor was 11 years 6 months old.¹⁰ Whether the difference in mortality in our cohort reflect differences in the ascertained patient population, the standard of medical care, the opportunistic infection, or outlier effects remains to be determined.

The Spectrum of PYCR related disorders

The pivotal role of PYCR proteins in proline metabolism, the proximity to the urea and citric acid cycles, and the clinical overlap between *PYCR1* and *PYCR2* mutations underscore the potential myriad of biochemical defects that may result from PYCR-family gene mutations. *PYCR1*-related cutis laxa and progeroid syndrome presents with the core features of intrauterine growth retardation, wrinkled skin, joint hyperlaxity, and a typical progeroid gestalt.²⁰ Some of the facial features such as triangular face, bulbous upturned nose and long philtrum mirror *PYCR2*-facies, but cutis laxa and progeroid features are missing. Furthermore, *PYCR1* mutations usually associate with milder intellectual disability and can have normal head circumference. Rare associations with *PYCR1* mutations are corneal clouding, cataracts, strabismus, contractures, inguinal hernia, and athetoid movements.^{9, 20} The abnormal movements described as “stereotypic dystonic and jerky arm movements” are similar to the hyperkinetic movements seen in our cohort. We postulate that *PYCR1* and *PYCR2*-related disorders are likely a continuum of the same neurodevelopmental disorder due to defect in mitochondrial proline synthesis, wherein cutaneous findings are prominent with *PYCR1* mutations, and patients with *PYCR2* mutations have a severe neurological phenotype. While PYCRL shares 45% amino acid similarity to the other two isoforms, mutations in its gene has not been associated with human disease. On the other hand, *ALDH18A1*, encoding the upstream P5CS protein, is mutated in autosomal recessive cutis laxa type IIIA²¹ and the autosomal dominant (AD) form of progeroid de Barsy-like cutis laxa syndrome,²² suggesting that PYCR1 and P5CS have a more important role in skin function than *PYCR2*.⁶ Another rare finding seen in one-third of our patients was excessive vomiting, which has been only reported in a 12-year-old male with autosomal dominant *ALDH18A1* cutis laxa phenotype.²³

PYCR2 genotype-phenotype correlation and metabolic profiling

PYCR2 mutations detected in this study were mostly clustered around the dimerization domain. The variant c.796G>A (p.Arg266*) was the most common mutation, seen in 4 out of 12 families, whereas the c.595G>A (p.Arg199Trp) mutation was present in two families. The number of families sharing mutations suggests common founder alleles in these populations. Three of the mutations were unique in these families, including a predicted splice site mutation in the family from Pakistan, and none overlaps with published variants. In the current study, truncating mutations in *PYCR2* led to reduced protein levels in fibroblasts. The tested missense mutations showed intact protein levels but failure to multimerize, presumably a requirement for enzymatic activity based upon other studies.^{15, 24} Of note, two of our most severely affected patients (2404-III-7, 3740-III-3) with severe brain atrophy shared a common mutation, p.Arg199Trp, which could indicate a more severe effect on protein function, perhaps interfering with function of other PYCR family proteins through dominant negative activities.

PYCR1 and PYCR2 are proposed for biosynthesis of proline from glutamate via P5C in the mitochondrial milieu, whereas *PYCR1* is proposed for biosynthesis of proline from ornithine in the cytosol.²⁵ The reduction of P5C to proline utilizes cofactors NADH/NADPH, hence the isoforms are responsible for shuttling redox equivalents and maintaining an oxidative balance.^{26, 27} Increasing evidence suggests a correlation between NAD⁺ consuming enzymes with neuronal injury mediated via apoptosis-inducing factor (AIF) and ATP depletion.²⁸ PYCR isozymes have also been demonstrated to co-localize with the stress response protein, ribonucleotide reductase small subunit B (RRM2B) to prevent cellular injury from oxidative stress.²⁹ Recently, a role for *PYCR2* in cellular apoptosis was suggested in melanoma cells, as *PYCR2* knockdown led to decreased proliferative capacity and activation of AMPK/mTOR-induced autophagy.¹⁹ *PYCR2* deficiency led to increased apoptosis under oxidative stress when cells with homozygous frameshift mutations were treated with H₂O₂, and knockdown of *PYCR2* ortholog, *pycr1b*, in a zebrafish model resulted in smaller head/brain, shorter body, down-tilted tail, and hypomotility.⁴

The role of glutamate in neuronal toxicity has also been extensively studied,³⁰ and could very well be a part of neurological dysfunction cascade due to *PYCR2* mutations. In this study we did not detect any significant change in plasma amino acids of affected individuals tested. Slightly elevated level of glutamate in urine was noted in patient 2206-III-4 and 2664-III-1, which could potentially reflect the upstream accumulation of glutamate, but will require additional studies to confirm. A clear characterization of metabolic derangements in PYCR-related disorders is still lacking and reports to date fail to find evidence of systemic biochemical derangement.^{10, 20}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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FIGURE 1. Overview of proline metabolism and individuals with *PCYR2* mutations

A. Pyrroline-5-carboxylate reductase 2 (*PCYR2*) functions in the mitochondria with the paralogue *PYCR1* for conversion of pyrroline-5-carboxylate (P5C) to proline. P5C can also be converted to proline in the cytoplasm, mediated by *PYCR*-like (*PYCRL*). P5C can alternatively be used as a substrate for ornithine synthesis, which can enter the urea cycle, or can be interconverted to glutamate, mediated by pyrroline-5-carboxylate dehydrogenase (*P5CDH*). Glutamate can then be used as a substrate for the citric acid cycle (CAC) or can be converted back to P5C by pyrroline-5-carboxylate synthetase (*P5CS*). B. Bar diagram depicting cumulative survival of the 18 subjects, including 14 study patients, as well as 4 deceased siblings with similar clinical presentation at the time of ascertainment. The white bars represent the living and grey bars represent the deceased individuals at any given age

interval. By age 8 years, 9 individuals (5 study patients and 4 siblings) had died; there are no living survivors beyond 10 years of age. C. Pedigree diagrams showing eleven families, with a total of eighteen individuals who displayed features of neurodevelopmental delay, fourteen children were recruited to the study and found to have homozygous mutations in *PYCR2*. All pedigrees but 1240 had documented parental consanguinity (double bar), either first-cousin once removed (2566) or first cousin (all others). Generation number and individual family ID's are labeled, arrow: probands in each family with *PYCR2* mutation, affecteds: filled symbols, squares: males, circles: females, hashed: deceased, triangle: miscarriage.

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FIGURE 2. 'Triangular' facial appearance of individuals with *PYCR2* mutations

Typical triangular faces associated with bulbous nasal tip, malar hypoplasia and prominent low set ears were observed in most cases. Pedigree number and individual ID with corresponding homozygous mutation are shown.

FIGURE 3. Brain imaging of patients with *PYCR2* mutations

CT head or MRI brain imaging of 13 affected individuals are illustrated, patient 1232-III-1 (A, age 12 months), 1232-III-2 (B, Q, age 10 months), 1240-II-2 (C, N, age 21 months), 2404-III-7 (D, O, age 3 years), 2566-IV-2 (E, age 3 years), 2664-III-1 (F, R, age 18 months), 2682-III-1 (G, age 12 months), 2682-III-2 (H, age 12 months), 2706-III-3 (I, age 2 years), 2706-III-4 (J, age 9 months), 3109-III-3 (K, P, age 18 months), 3740-III-2 (L, S, age 2 years), 4592-III-3 (M, T, age 1 year). Axial T1-weighted brain MRI (A–M) demonstrated global brain atrophy, thin corpus callosum, mild cortical atrophy or severe brain atrophy (white arrows in E and L), as well as ventriculomegaly (white arrow in D). Axial T2-weighted (N–P) and midline sagittal T1-weighted (Q–T) brain MRI demonstrated hypomyelination with reduced white matter volume (most notable in N, shown by black arrows), basal ganglia, pons, and cerebellum were either mildly reduced in size or were within normal limits.

FIGURE 4. Altered protein levels and impaired dimerization correlate with patient mutations in *PYCR2*

A. Protein expression in skin fibroblasts from patients (affected) or parent (carrier) with corresponding mutations, using C-terminal PYCR2-specific, PYCR1 (lower with longer exposure), or tubulin antibodies. Parent showed reduced and affected showed absent PYCR2 protein level with p.266R/Stop mutation. Last lane is a lysate of skin fibroblasts from a patient with *PYCR1* null mutation, with absent PYCR1 and little PYCR2. Truncated protein detected in p.266 Stop/Stop lane with longer exposure was evident in the carrier. No compensatory increase in PYCR1 was detected in patient cells. B. PYCR2 protein domain structure and location of identified mutations. Putative phosphorylation and acetylation are indicated. C. Impaired multimerization of PYCR2 with the dimerization domain patient

mutations. 293T cells were co-transfected with expression vectors encoding FLAG-tagged PYCR2 (wild-type or mutants) or Myc/His-tagged wild-type PYCR2. Cell lysates were subject to co-immunoprecipitation with an anti-FLAG gel. Patient mutations (p.R199W, p.C232G, or truncating p.1–265) highlighted with grey, and additional mutations predicted to impair multimerization (p.E221A, p.S233A, p.T238A), wild-type PYCR1 and PYCRL were also included. The top four blots show protein levels in whole cell lysates (input); the bottom three show protein levels in elutants after anti-FLAG immunoprecipitation (output).

TABLE 1Summarized clinical features of patients with *PYCR2* mutations

Clinical finding	% of patients (of 14)
<i>Microcephaly</i>	100% (14/14)
<i>Failure to thrive</i>	100% (14/14)
<i>Excessive vomiting</i>	29% (4/14)
Facial findings	
<i>Typical facies: malar hypoplasia, upturned bulbous nose, low set prominent ears</i>	100% (14/14)
<i>Triangular face</i>	86% (12/14)
Neurological findings	
<i>Global developmental delay</i>	100% (14/14)
<i>Intellectual disability</i>	100% (14/14)
<i>Upper motor neuron signs</i>	100% (14/14)
<i>Ataxia or absent gait</i>	100% (14/14)
<i>Hyperkinetic movements</i>	100% (14/14)
<i>Muscle atrophy</i>	93% (13/14)
<i>Seizures</i>	57% (8/14)
<i>Spasticity</i>	57% (8/14)
<i>Nystagmus</i>	21% (3/14)
Brain imaging findings (of 13 patients)	
<i>Cortical atrophy</i>	100% (13/13)
<i>Thin corpus callosum</i>	61% (8/13)