Type I hyperprolinemia: genotype/phenotype correlations

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Complete List of Authors:	Guilmatre, Audrey; Inserm U614, IHU Legallic, Solenn; Inserm U614, IHU Steel, Gary; McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University School of Medicine Willis, Alecia; McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University School of Medicine Di Rosa, Gabriella; University Hospital, Department of Medical and Surgical Pediatrics Goldenberg, Alice; University Hospital, Department of Genetics DROUIN-GARRAUD, Valérie; University Hospital, Department of Genetics Guet, Agnès; Trousseau Hospital Mignot, Cyril; Trousseau Hospital Des Portes, Vincent; University Hospital Valayannopoulos, Vassili; Necker hospital, Department of Neuro-Metabolism Van Maldergem, Lionel; Regional Hospital Hoffman, Jodi; Tufts Medical Center Izzi, Claudia; Brescia University Espil-Taris, Caroline; University Hospital Orcesi, Simona; Fondation "Neurological Institute C. Mondino » Bonafé, Luisa; Lausanne university Le Galloudec, Eric; Beauvais Hospital Maurey, Hélène; Kremlin-Bicêtre Hospital Ioos, Christine; Raymond-Poincaré Hospital Afenjar, Alexandra; Pitié Salpêtrière Hospital Blanchet, Patricia; Arnaud de Villeneuve Hospital Echenne, Bernard; Gui de Chauliac Hospital ROUBERTIE, Agathe; CHU Hôpital, Service de neuropédiatrie Frebourg, Thierry; Inserm U614, IHU; University Hospital, Department of Genetics Valle, David; McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University School of Medicine Campion, Dominique; Inserm U614, IHU; CH Rouvray
Key Words:	PRODH, Type I hyperprolinemia, 22q11, POX enzymatic activity

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Audrey Guilmatre*(1), Solenn Legallic*(1), Gary Steel (2), Alecia Willis (2), Gabriella Di Rosa (3), Alice Goldenberg (4), Valérie Drouin-Garraud (4), Agnès Guet (5), Cyril Mignot (5), Vincent Des Portes (6), Vassili Valayannopoulos (7), Lionel Van Maldergem (8), Jodi D Hoffman (9), Claudia Izzi (10), Caroline Espil-Taris (11), Simona Orcesi (12), Luisa Bonafé (13), Eric Le Galloudec (14), Hélène Maurey (15), Christine Ioos (16), Alexandra Afenjar (17), Patricia Blanchet (18), Bernard Echenne (19), Agathe Roubertie (19), Thierry Frebourg (1,4), David Valle (2), Dominique Campion(1, 20).

* AG and SL have equally contributed to this work

(1) Inserm U614, IHU, Rouen, France

(2) McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore, USA

(3) Department of Medical and Surgical Pediatrics, University Hospital, Messina, Italy

(4) Department of Genetics, University Hospital, Rouen, France

(5) Trousseau Hospital, Paris, France

(6) University Hospital, Lyon, France

(7) Department of Neuro-metabolism, Necker Hospital, Paris, France

(8) Regional Hospital, Créteil, France

(9) Tufts Medical Center, Boston, MA, USA

(10) Brescia University, Brescia, Italy

(11) University Hospital, Bordeaux, France

(12) Fondation “Neurological Institute C. Mondino”, Pavia, Italy

(13) Lausanne, Switzerland

(14) Beauvais Hospital, Beauvais, France

(15) Kremlin-Bicêtre Hospital, Paris, France

(16) Raymond Poincaré Hospital, Garches, France

(17) Pitié Salpêtrière Hospital, Paris, France

(18) Arnaud de Villeneuve Hospital, Montpellier, France

(19) Gui de Chauliac Hospital, Montpellier, France

(20) CH Rouvray, Sotteville les Rouen, France

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Corresponding author: Dominique Campion, Inserm U614, Faculté de Médecine et Pharmacie, 22 boulevard Gambetta, 76000 Rouen, France (dominique.campion@univ-rouen.fr). Phone: +332-35-14-82-80. Fax: +332-35-14-82-37

Abstract

Type I hyperprolinemia (HPI) is an autosomal recessive disorder associated with cognitive and psychiatric troubles, caused by alterations of the Proline Dehydrogenase gene (*PRODH*) at 22q11. HPI results from *PRODH* deletion and/or missense mutations reducing proline oxidase (POX) activity. The goals of this study were first to measure in controls the frequency of *PRODH* variations described in HPI patients, second to assess the functional effect of *PRODH* mutations on POX activity and finally to establish genotype/enzymatic activity correlations in a new series of HPI patients. 8/14 variants occurred at polymorphic frequency in 114 controls. POX activity was determined for 6 novel mutations and 2 haplotypes. The c.1331G>A, p.G444D allele has a drastic effect whereas the c.23C>T, p.P8L allele and the c.[56C>A; 172G>A], p.[Q19P; A58T] haplotype result in a moderate decrease in activity. Among the 19 HPI patients, 10 had a predicted residual activity <50%. 8 out of 9 subjects with a predicted residual activity ≥50% bore at least one c.824C>A, p.T275N allele, which has no detrimental effect on activity but whose frequency in controls is only 3%. Our results suggest that *PRODH* mutations lead to a decreased POX activity or affect other biological parameters causing hyperprolinemia.

Introduction

Type I hyperprolinemia (MIM# 239500) is characterized by an increase in plasma proline level in absence of urinary pyrroline-5-carboxylate (P5C) (typical of type II hyperprolinemia). It was initially described in 1962 as a nephropathy associated with cerebral dysfunction [Efron, 1965; Schafer et al., 1962]. The association with renal disease was fortuitous and was due to a recruitment bias since patients with nephropathy were preferentially screened by amino acid chromatography at this time. As a result of the analysis of new families with HPI ten years later, it was considered that hyperprolinemia was a benign condition without mental retardation or renal dysfunction [Mollica et al., 1976]. The gene associated with this condition, named *PRODH* (MIM# 606810), was mapped in 1997 on 22q11 [Campbell et al., 1997] and encodes for proline oxidase (POX), the first enzyme involved in proline catabolism. Its expression is limited to liver, brain and kidney. In 1999, a mouse model deficient for POX was described. These mice present behavioral problems, especially a sensory motor gating impairment, which is an endophenotype of schizophrenia [Gogos et al., 1999]. A few years later, [Humbertclaude et al., 2001]described a case with severe neurologic impairment and raised the question whether HPI really was a benign trait in humans, which opened up again the discussion.

In 2003, we described [Jacquet et al., 2003] a recurrent 350kb deletion encompassing the *PRODH* and *DGCR6* (MIM# 601279) genes, mediated by low copy repeats surrounding this region, which was subsequently found to be present in 1/250 individuals in the general population [Guilmatre et al., 2009; Ohtsuki et al., 2004]. Numerous missense mutations in the *PRODH* gene have also been described, most of them appearing recurrently because of a gene conversion between *PRODH* and a non-processed pseudogene located 1.4 Mb telomeric on chromosome 22 [Liu et al., 2002]. A functional characterization of POX enzymatic activity

was established demonstrating that some of these mutations had a severe impact (> 70% reduction) on its activity whereas others had a mild or moderate effect [Bender et al., 2005]. Further genetic studies in 3 patients with mental retardation, epilepsy and very high plasma proline level allowed us to establish that the severe form of HPI was a recessive disorder resulting from homozygous *PRODH/DGCR6* deletion or mutations with a drastic effect on enzymatic activity [Jacquet et al., 2003]. In accordance with the mouse model phenotype, hyperprolinemia was then shown to be a risk factor for schizoaffective disorder [Jacquet et al., 2005]. The link between hyperprolinemia and cognitive or psychiatric troubles was further strengthened by the study of patients with velo cardio facial syndrome (VCFS) that results from a 1.5-3Mb deletion on chromosome 22q11. Although all these patients are hemizygous for the *PRODH* gene, only 30-50% have elevated plasma proline level [Goodman et al., 2000; Raux et al., 2007], while 50% have mental retardation and 30% psychosis [Murphy et al., 1999]. We have shown that in VCFS increased plasma proline level (in association with the *COMT* (MIM# 116790) genotype) was a risk factor for psychosis and that prolinemia was inversely correlated to IQ [Raux et al., 2007]. Based on the functional predictions, this study also showed that a reduction of at least 50% of the enzymatic activity was required to produce mild to moderate hyperprolinemia while severe hyperprolinemia was generally associated with a near complete loss of enzymatic activity. In addition to these observations, *PRODH/DGCR6* deletion and mild hyperprolinemia were recently found at a high frequency in autistic subjects [Guilmatre et al., 2009].

Despite this large body of data, several points remain to be clarified. In particular, correlations between *PRODH* genotype and various levels of hyperprolinemia, ranging from mild to severe, remain poorly understood. In the present report, three issues were examined: first we measured in controls the allelic frequency of *PRODH* variations found in HPI patients, second we assessed the functional effect of previously uncharacterized mutations or haplotypes on

enzymatic activity, and third we attempted to establish correlations between genotype and enzymatic activity in a new series of HPI patients.

Material and methods

This study was approved by the Ethics Committee of Rouen University Hospital.

Controls

To establish the allelic frequency of *PRODH* (NM_016335) variants, we genotyped 114 healthy caucasian subjects originating from France (46 men, 68 females) previously included in a study aimed to establish the normal values of plasma proline in the adult population [Jacquet et al., 2005]. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

Patients with HPI

Blood samples from patients with HPI were sent to our laboratory from centers expert in the diagnosis of Autism or mental retardation in order to analyze the *PRODH* gene. All patients were examined by experienced clinicians and psychiatric diagnoses were made according to DSM IV criteria. Biological investigations included MRI imaging, karyotype, Fragile X testing and amino-acid chromatography. Plasma proline levels were determined by ion exchange chromatography after overnight fasting. After informed consent was obtained, DNA was extracted from peripheral lymphocytes. Patients as well as their parents were genotyped in order to obtain the phase of the mutations.

PRODH sequencing and QMPSF

PRODH exons were sequenced using PCR conditions and primers previously described [Jacquet et al., 2005; Raux et al., 2007]. In some subjects in which *PRODH* exon 7 could not

be amplified , due to indels in both flanking introns, the presence of the c.824C>A, p.T275N variation was assessed using the forward primer 5'TGCAGTTCTCAGAGGTGCTG 3' in combination with the previously described reverse primer.

PRODH deletions were characterized by quantitative multiplex analysis of short fluorescent fragments (QMPSF), as previously described [Jacquet et al., 2002].

Array CGH

DNA samples were analyzed using Human High-Resolution Discovery Microarray Kit 4x180K (Agilent Technologies, Santa Clara, California, USA), using standard recommended protocols. Non commercial genomic DNA pool of 10 control individuals was used as reference. Hybridization results were extracted with Feature extraction software and analyzed using Agilent's DNA-analytics software. The data were processed using ADM-2 algorithm, with the threshold set at 6.0 SD.

Functional assay for POX activity

We purchased restriction enzymes and buffers from New England Biolabs: Pfu-Turbo DNA polymerase from Stratagene and chemicals from Sigma. ^{14}C-proline was obtained from New England Nuclear and purified prior to use by ion-exchange chromatography on Dowex AG 50w-8x hydrogen form (BioRad) [Phang et al., 2001]. To assay green fluorescent protein (GFP) fluorescence, we used a Zeiss LSM 510 Meta confocal laser-scanning microscope. Functional test was realized as previously described [Bender et al., 2005]. Briefly, the *PRODH* ORF divided into three fragments previously cloned in the pBluescript KS vector (Stratagene) was used to perform the mutagenesis and the wild-type *PRODH* cDNA previously cloned into pTracer (Invitrogen) was used for expression analysis. We mutagenized the *Cla*I/*Bst*EII-modified *PRODH* cDNA in pBluescript KS by PCR using the QuikChange Mutagenesis Kit (Stratagene). Dependent on the position of the desired mutation, we subcloned either the mutagenized *Kpn*I/*Cla*I or mutagenized *Cla*I/*Bst*EII

fragment into the pTracer expression construct and resequenced the construct to verify the presence of the mutation and the integrity of the ligations. For all expression studies, we utilized a subclone (CHO-K1-C9) of CHO-K1 cells that lack endogenous POX activity [Valle et al., 1973]. For electroporation, we used 30 µg of the indicated plasmid DNA and 350 volts/ 400 Ω/960 µF in 300 µl of growth medium containing 1.25% DMSO and 5–7 × 10^6 cells. After 48 h, we harvested the cells by washing the monolayer with PBS and scraping them into cold PBS. The cells were collected by centrifugation at 480 x g and were resuspended in 0.1 M KPO_4 (pH 7.2). The cells and their organelles were disrupted by sonication for 1 min at a setting of 25% (Branson Sonifier 450 [Branson Ultrasonics]). Total protein was determined with the Pierce BCA protein assay (Pierce). POX-specific activity (nmol/prod/mg/hr) was assayed radioisotopically, as described elsewhere [Phang et al., 1975]. To account for variation in transfection efficiency, we expressed the data as a percentage of wild type (specific activity of mutant allele/transfection efficiency)/(specific activity of wild-type allele/transfection efficiency). For alleles with severe reduction in POX activity, we repeated the assay with the addition of 1 mM FAD. All samples were assayed in triplicate in independent transient transfection experiments.

Ex vivo splicing assay using a splicing reporter minigene

This assay was used to test the effect of the p.T275N variant in *PRODH* exon 7 splicing. This assay was realized in both Hela and ShSY5Y cells using the same protocol [Tournier et al., 2008], with DMRIE-C (Invitrogen) as the transfection reagent for the SH-SY5Y cells. Primer sequences are available upon request.

Results

Frequency of *PRODH* variations in controls

The genotyping of 114 controls without psychiatric or neurological disorders (four of them having plasma proline level above the upper threshold, range 389-482 µmol/l) allowed us to determine in a control population the frequency of missense mutations that were present in HPI patients [Raux et al., 2007] (and this study) (Table 1). For each mutation, the distribution of genotypes followed the Hardy-Weinberg equilibrium. Among the 14 variations we analyzed, 8 occurred at a polymorphic frequency (> 0.01), two of them (c.56C>A, p.Q19P and c.553T>C, p.R185W) being very common with a minor allele frequency of 0.48 and 0.36, respectively.

Functional consequences of *PRODH* missense mutations

In addition to data already reported, that are summarized in Supp Table S1 [Bender et al., 2005], we describe here the results of functional analyses for a new series of *PRODH* missense mutations (c.23C>T, p.P8L ; c.32G>C, p.R11P ; c.88C>T, p.P30S ; c.172G>A, p.A58T ; p.T275N and c.1331G>A, p.G444D) and haplotypes (c.[56C>A; 172G>A], p.[Q19P; A58T] and c.[56C>A; 88C>T], p.[Q19P; P30S]) (Figure 1). We show that the p.G444D substitution has the most drastic effect resulting in only 27% of residual enzymatic activity. p.P8L and the haplotype p.[Q19P; A58T] have both moderate effect. However the other mutations have no effect on enzymatic activity or even a tendency to increase it. As previously described for other haplotypes [Bender et al., 2005] the POX activity encoded by the p.[Q19P; A58T] allele was dictated by the most severe missense mutation with no evidence of intra allelic complementation. By contrast, analysis of the p.[Q19P; P30S] haplotype showed that the p.P30S variation fully complemented the deleterious effect of the p.Q19P mutation.

Genotype/phenotype correlations in patients with type I hyperprolinemia

Clinical and demographic characteristics of a novel series of HPI patients whose *PRODH* genotype and plasma proline level have been determined are displayed in Table 2a. Most of these patients present with psychiatric and cognitive troubles and epilepsy. MRI findings were normal except for two patients who displayed agenesis of the corpus callosum, symmetrical temporal lobe hypoplasia, thalamus fusion and right opercular dysplasia, respectively. Dysmorphism was noted in 4 patients who were all negatively screened for DNA rearrangement by aCGH, as was patient 109 who had an abnormal MRI but without dysmorphism.

In this series of patients, abnormal plasma proline levels ranged from 1667 to 327 µmol/l with, for several subjects, considerable variation between two measures obtained at different time (Table 2b). Based on the results of functional assay, four subjects had a genotype predicted to result in a drastic loss of enzymatic activity. In accordance with these predictions, they all present with severe hyperprolinemia. Overall, only 10/19 patients had a predicted residual activity <50% which, according to our working hypothesis [Raux et al., 2007], is required to produce hyperprolinemia. Strikingly, 8 out of 9 subjects with a predicted residual enzymatic activity ≥50% bore at least one p.T275N allele, which has no effect on enzymatic activity but whose frequency in the control population is only 3%. Using an ex vivo splicing assay to further analyse the effect of this variant, we observed no effect on the splicing pattern of *PRODH* exon 7 (data not shown).

Discussion

All patients included in this report present cognitive impairment and/or behavioral problems. Epilepsy was also frequent. It should however be stressed that this study was not designed to

formally assess the clinical spectrum of HPI. Thus, although consistent with the view that HPI is associated with psychiatric and neurologic disabilities, our data may just reflect a recruitment bias since subjects presenting such conditions are more likely to be screened for inborn error of amino acid metabolism than normal subjects.

Nonspecific dysmorphic features were present in a subset of patients raising the question of a putative link between HPI and syndromic MR. In these patients, array CGH analysis failed to document the co-occurrence of another genetic abnormality that can explain these physical features. Thus, it remains unclear whether dysmorphism may be a component of the HPI syndrome or whether another as yet undetected genetic defect is present in this subset of patients.

Concerning the relationship between plasma proline and the predicted POX activity, a rather poor correlation was observed. However, as previously noted [Bender et al., 2005] our functional assay circumvent any effect these mutations might have on RNA splicing or protein stability. It is thus conceivable that several variations may be wrongly classified as benign on the basis of this sole test. In a previous report concerning VCFS patients we had already noted the unexplained enrichment of the c.1292G>A, p.R431H mutation in patients with high proline levels [Raux et al., 2007]. In the present report, the increased frequency of the functionally neutral p.T275N mutation in HPI patients is noteworthy, pointing out that it is indeed a probable pathologic variant. Since this variation does not modify the splicing pattern of exon 7, its biological effect remains elusive and it is most likely in linkage disequilibrium with another functional variant. Indeed, DNA variations in the regulatory regions or in introns of *PRODH* may influence transcription or splicing of the gene. A polymorphism located in the 5' region of *PRODH* and resulting in a 1.5-fold increase in promoter activity has been described [Hoogendoorn et al., 2004]. However, genotyping this polymorphism in our

patients did not reveal any relationship between the low transcriptional activity allele and HPI (data not shown). Characterization of further variations in these regulatory regions is thus required.

As previously noted, plasma proline is subject to fluctuations over time in most patients, thus requiring, as a rule, repeated determinations**.** However, several patients bearing a potentially deleterious genotype had only mild hyperprolinemia on repeated measures. Since it seems unlikely that such a slight increase in proline level is sufficient to produce a detrimental effect, this result questions the value of plasma proline level determination as an estimate of CNS proline value. Because peripheral proline cannot gain access to the central nervous system (CNS) across the blood- brain barrier [Hawkins et al., 2006], blood and brain proline form two largely independent pools. Brain proline, the concentration of which in the cerebro spinal fluid (CSF) is low (4.2 µmol/l), is not subject to nutritional fluctuations. Its level depends on local synthesis from ornithine and glutamate and from degradation of collagen of the extra cellular matrix. In severe HPI [Efron, 1965; Humbertclaude et al., 2001] as well as in *PRODH* -/- mice [Gogos et al., 1999], CNS concentration of proline is elevated. Unfortunately, CSF data are not available for our patients.

Another point to be kept in mind when interpreting slight increases in plasma proline is that normative values of plasma proline are lower in children as compared with adults. Accordingly, in mice *PRODH* transcripts are expressed at higher level in the young brain as compared with the mature brain [Maynard et al., 2003]. Physiologically, the fine tuning of *PRODH* expression facilitates proline catabolism during periods in which most refinement and stabilization of synaptic connections occur. Therefore, it can be hypothesized that any genetic defect causing even a modest increase in proline levels during critical periods of development may have a dramatic effect on CNS functioning.

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References

Bender H, Almashanu S, Steel G, Hu C, Lin W, Willis A, Pulver A, Valle D. 2005. Functional consequences of prodh missense mutations. Am J Hum Genet 76:409-420.

Campbell HD, Webb GC, Young IG. 1997. A human homologue of the drosophila melanogaster sluggish-a (proline oxidase) gene maps to 22q11.2, and is a candidate gene for type-i hyperprolinaemia. Hum Genet 101:69-74.

Efron ML. 1965. Familial hyperprolinemia. report of a second case, associated with congenital renal malformations, hereditary hematuria and mild mental retardation, with demonstration of an enzyme defect. N Engl J Med 272:1243-1254.

Gogos JA, Santha M, Takacs Z, Beck KD, Luine V, Lucas LR, Nadler JV, Karayiorgou M. 1999. The gene encoding proline dehydrogenase modulates sensorimotor gating in mice. Nat Genet 21:434-439.

Goodman BK, Rutberg J, Lin WW, Pulver AE, Thomas GH. 2000. Hyperprolinaemia in patients with deletion (22)(q11.2) syndrome. J Inherit Metab Dis 23:847-848.

Guilmatre A, Dubourg C, Mosca A, Legallic S, Goldenberg A, Drouin-Garraud V, Layet V, Rosier A, Briault S, Bonnet-Brilhault F, Laumonnier F, Odent S, Le Vacon G, Joly-Helas G, David V, Bendavid C, Pinoit J, Henry C, Impallomeni C, Germano E, Tortorella G, Di Rosa G, Barthelemy C, Andres C, Faivre L, Frebourg T, Saugier Veber P, Campion D. 2009. Recurrent rearrangements in synaptic and neurodevelopmental genes and shared biologic pathways in schizophrenia, autism, and mental retardation. Arch Gen Psychiatry 66:947-956.

Hawkins RA, O'Kane RL, Simpson IA, Vina JR. 2006. Structure of the blood-brain barrier and its role in the transport of amino acids. J Nutr 136:218S-26S.

Hoogendoorn B, Coleman SL, Guy CA, Smith SK, O'Donovan MC, Buckland PR. 2004. Functional analysis of polymorphisms in the promoter regions of genes on 22q11. Hum Mutat 24:35-42.

Humbertclaude V, Rivier F, Roubertie A, Echenne B, Bellet H, Vallat C, Morin D. 2001. Is hyperprolinemia type i actually a benign trait? report of a case with severe neurologic involvement and vigabatrin intolerance. J Child Neurol 16:622-623.

Jacquet H, Berthelot J, Bonnemains C, Simard G, Saugier-Veber P, Raux G, Campion D, Bonneau D, Frebourg T. 2003. The severe form of type i hyperprolinaemia results from homozygous inactivation of the prodh gene. J Med Genet 40:e7.

Jacquet H, Demily C, Houy E, Hecketsweiler B, Bou J, Raux G, Lerond J, Allio G, Haouzir S, Tillaux A, Bellegou C, Fouldrin G, Delamillieure P, Menard JF, Dollfus S, D'Amato T, Petit M, Thibaut F, Frebourg T, Campion D. 2005. Hyperprolinemia is a risk factor for schizoaffective disorder. Mol Psychiatry 10:479-485.

Jacquet H, Raux G, Thibaut F, Hecketsweiler B, Houy E, Demilly C, Haouzir S, Allio G, Fouldrin G, Drouin V, Bou J, Petit M, Campion D, Frebourg T. 2002. Prodh mutations and hyperprolinemia in a subset of schizophrenic patients. Hum Mol Genet 11:2243-2249.

Liu H, Abecasis GR, Heath SC, Knowles A, Demars S, Chen Y, Roos JL, Rapoport JL, Gogos JA, Karayiorgou M. 2002. Genetic variation in the 22q11 locus and susceptibility to schizophrenia. Proc Natl Acad Sci U S A 99:16859-16864.

Maynard TM, Haskell GT, Peters AZ, Sikich L, Lieberman JA, LaMantia A. 2003. A comprehensive analysis of 22q11 gene expression in the developing and adult brain. Proc Natl Acad Sci U S A 100:14433-14438.

Mollica F, Pavone L. 1976. Hyperprolinaemia: a disease which does not need treatment?. Acta Paediatr Scand 65:206-208.

Murphy KC, Jones LA, Owen MJ. 1999. High rates of schizophrenia in adults with velo-cardio-facial syndrome. Arch Gen Psychiatry 56:940-945.

Ohtsuki T, Tanaka S, Ishiguro H, Noguchi E, Arinami T, Tanabe E, Yara K, Okubo T, Takahashi S, Matsuura M, Sakai T, Muto M, Kojima T, Matsushima E, Toru M, Inada T. 2004. Failure to find association between prodh deletion and schizophrenia. Schizophr Res 67:111-113.

Phang JM, Downing SJ, Valle DL, Kowaloff EM. 1975. A radioisotopic assay for proline oxidase activity. J Lab Clin Med 85:312-317.

Phang JM, Hu C, Valle D. 2001. Disorders of proline and hydroxyproline metabolism. In: Scriver CR, Beaudet AL, Sly, WS VD, editors. The metabolic and molecular bases of inherited disease. McGraw Hill, New York. 1821-1838.

Raux G, Bumsel E, Hecketsweiler B, van Amelsvoort T, Zinkstok J, Manouvrier-Hanu S, Fantini C, Breviere GM, Di Rosa G, Pustorino G, Vogels A, Swillen A, Legallic S, Bou J, Opolczynski G, Drouin-Garraud V, Lemarchand M, Philip N, Gerard-Desplanches A, Carlier M, Philippe A, Nolen MC, Heron D, Sarda P, Lacombe D, Coizet C, Alembik Y, Layet V, Afenjar A, Hannequin D, Demily C, Petit M, Thibaut F, Frebourg T, Campion D. 2007. Involvement of hyperprolinemia in cognitive and psychiatric features of the 22q11 deletion syndrome. Hum Mol Genet 16:83-91.

Schafer IA, Scriver CR, Efron ML. 1962. Familial hyperprolinemia, cerebral dysfunction and renal anomalies occuring in a family with hereditary nephropathy and deafness. N Engl J Med 267:51-60.

Tournier I, Vezain M, Martins A, Charbonnier F, Baert-Desurmont S, Olschwang S, Wang Q, Buisine MP, Soret J, Tazi J, Frebourg T, Tosi M. 2008. A large fraction of unclassified variants of the mismatch repair genes mlh1 and msh2 is associated with splicing defects. Hum Mutat 29:1412-1424.

Valle D, Downing SJ, Phang JM. 1973. Proline inhibition of pyrroline-5-carboxylate reductase: differences in enzymes obtained from animal and tissue culture sources. Biochem Biophys Res Commun 54:1418-1424.

Figure and table legends

Table 1: Frequency of missense mutations in controls

PRODH (NM_016335)

Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

Table 2: Phenotypic and genotypic features of patients with Type I hyperprolinemia

a: clinical features

F: female; M: male; age (years); #: born from consanguineous parents; * corpus callosum agenesis, symmetrical temporal lobe hypoplasia, thalamus fusion; ** right opercular dysplasia

MRI: Magnetic Resonance Imaging; aCGH: array Comparative Genomic Hybridization

AF: autistic features; AP: atypical psychosis; At: ataxia; Au: autism; D: dyslexia; E: epilepsy; LD: learning disorder; MR: mental retardation; PE: pharmacoresistant epilepsy; PMD: Psychomotor delay.

+ hypertelorism, telecanthus, high palate, ligamentar hyperlaxity; ++ frontal bossing, thin lips, syndactyly 2 rd-3rd toes; +++ high forehead, frontal bossing, micrognathism, thin upper lip, short nose, short stature , microcephaly -2.5 SD; ++++ short nose with anteverted nostrils, prominent ridging of the metopic suture, long and flat philtrum and thin upper lip, small chin, hypertelorism, strabismus.

b: molecular features

PRODH (NM_016335); WT: wild type; del: deletion

Fasting abnormal plasma proline values: age >18 years: males > 377 µmol/l, females > 316 µmol/l; 5 years>age <18 years: > 270 µmol/l; age < 5 years: > 235 µmol/l**.**

PREA: predicted residual enzymatic activity/normal value.

Drastic mutations are in bold, mutations with moderate effect are in normal character and mutations without effect are in italics. The c.824C>A, p.T275N mutation is underlined.

Figure 1: proline oxidase activity of *PRODH* alleles

PRODH (NM_016335)

The thin vertical lines indicate standard deviation.

WT: wild type

Supp Table S1: proline oxidase activity of previously tested *PRODH* alleles [Bender et al., 2005] *PRODH* (NM_016335)

Table 1: Frequency of missense mutations in controls

PRODH (NM_016335)

Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

Variant	Exon	Genotypes (%)			Alleles (%)	
c.23C>T, p.P8L	2	PP (100)	PL (0)	LL (0)	P (100)	L (0)
c.32G>C, p.R11P	2	RR (100)	RP (0)	PP (0)	R (100)	P (0)
c. 56C>A, p.Q19P, rs2008720	2	QQ (26)	QP (51,5)	PP (22,5)	Q (52)	P (48)
c.88C>T, p.P30S, rs3815655	2	PP (82)	PS (18)	SS (0)	P (91)	S (9)
c.172G>A, p.A58T	2	AA (95)	AT (4)	TT (1)	A (97)	T (3)
c.553T>C, p.R185W, rs4819756	5	RR (43)	RW (41)	WW (16)	R (63,5)	W (36,5)
c.824C>A, p.T275N, rs5747933	7	TT (93)	TN (7)	NN (0)	T (97)	N (3)
c.1217C>T, p.P406L, rs3970555	11	PP (97)	PL (3)	LL (0)	P (98,5)	L (1,5)
c.1279G>A, p.V427M, rs2238731	12	VV (100)	VM (0)	MM (0)	V (100)	M (0)
c.1292G>A, p.R431H, rs2904552	12	RR (78)	RH (18)	HH (4)	R (87)	H (13)
c.1322T>C, p.L441P, rs2904551	12	LL (98)	LP (2)	PP (0)	L (99)	P (1)
c.1331G>A, p.G444D	12	GG (100)	GD (0)	DD (0)	G (100)	D (0)
c.1357C>T, p.R453C, rs3970559	12	RR (96)	RC (4)	CC (0)	R (98)	C (2)
c.1397C>T, p.T466M, rs2870984	12	TT (99)	TM (1)	MM (0)	T (99,5)	M (0,5)

Table 2: Phenotypic and genotypic features of patients with Type I hyperprolinemia

a: clinical features

F: female; M: male; age (years); #: born from consanguineous parents; * corpus callosum agenesis, symmetrical temporal lobe hypoplasia, thalamus fusion; ** right opercular dysplasia

MRI: Magnetic Resonance Imaging; aCGH: array Comparative Genomic Hybridization

AF: autistic features; AP: atypical psychosis; At: ataxia; Au: autism; D: dyslexia; E: epilepsy; LD: learning disorder; MR: mental retardation; PE: pharmacoresistant epilepsy; PMD: Psychomotor delay.

+ hypertelorism, telecanthus, high palate, ligamentar hyperlaxity; ++ frontal bossing, thin lips, syndactyly 2 rd-3rd toes; +++ high forehead, frontal bossing, micrognathism, thin upper lip, short nose, short stature , microcephaly -2.5 SD; ++++ short nose with anteverted nostrils, prominent ridging of the metopic suture, long and flat philtrum and thin upper lip, small chin, hypertelorism, strabismus.

b: molecular features

PRODH (NM_016335); WT: wild type; del: deletion

Fasting abnormal plasma proline values: age >18 years: males > 377 µmol/l, females > 316 µmol/l; 5 years>age <18 years: > 270 µmol/l; age < 5 years: > 235 µmol/l**.**

PREA: predicted residual enzymatic activity/normal value.

Drastic mutations are in bold, mutations with moderate effect are in normal character and mutations without effect are in italics. The c.824C>A, p.T275N mutation is underlined.

Table 2a

Subject	Sex	Age	Diagnosis	MRI	Dysmorphism	a-CGH
101	F	13	MR, Au, PE, At	normal	no	
102	M	3	MR, E	normal	yes +	normal
103	M	4	MR, PE, At	normal	no	
104	F	2	PMD, PE, At	normal	yes ++	normal
105	M	7	MR, Au	normal	no	
106	M	4	PE	normal	no	
107	F	14	LD	normal	no	
108	F	4	MR, E	normal	yes +++	normal
109	M	3	MR, E	abnormal*	no	normal
110	M	9	MR, E, , At	normal	no	
111	M	7	AF, E	normal	no	
112	M	9	LD, D	normal	no	
113	M	13	E	normal	no	
114	M	12	MR, E	abnormal **	yes ++++	normal
115	F	10	MR, E	normal	no	
116	M	31	AF, E	normal	no	
117	F	11	Au	normal	no	
116	M	14	MR, AP	normal	no	
119	F	9	LD	normal	no	

Table 2b

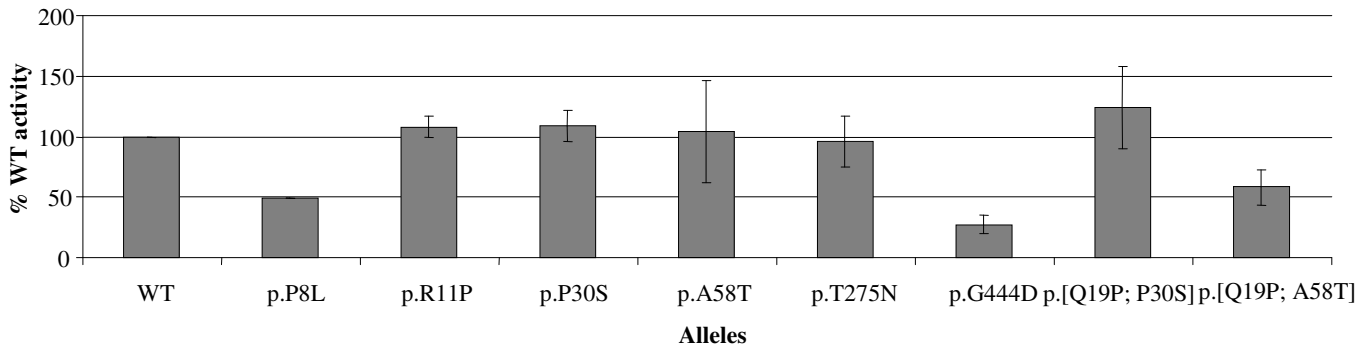
Subject	Plasma proline (µmol/l)	Genotype	PREA (%)
101	637-1667	**p.R453C/p.T466M**+p.R431H	15
102	1200	**p.T466M+p.R453C/p.T466M+p.R453C**	2
103	1084	**p.L441P/p.L441P+**p.R185W+p.Q19P	0
104	600-1000	**p.R453C**+p.V427M**+**p.R431H+*p.T275N*/*p.T275N*	55
105	599-899	**del/***p.T275N*	50
106	879	**p.R453C**/p.Q19P+*p.T275N*	35
107	692-878	**del/del**	0
108	826-862	**p.L441P/**p.Q19P+p.R185W	30
109	414-804	**p.T466M+p.R453C/**p.Q19P	30
110	781	p.R431H+*p.T275N*/*p.T275N*	80
111	450-700	**p.L441P+***p.T275N*/*WT*	50
112	679	**p.T466M+p.R453C+**p.R185W+p.Q19P+p.P30S/p.R431H	30
113	605	**p.T466M+**p.Q19P+p.R185W/p.R431H	40
114	574-847	**p.L441P+p.R453C**+p.R431H+p.R185W/*p.T275N*	50
115	395-534	p.Q19P+*p.T275N*/p.V427M+*p.T275N*	50
116	345-500	p.R431H/*p.T275N*	80
117	488	**del**/p.V427M+*p.T275N*	25
118	400	p.Q19P+*p.P30S*/p.R431H	60
119	243-327	**p.R453C**/*p.T275N*	55

Figure 1: proline oxidase activity of *PRODH* alleles

PRODH (NM_016335)

The thin vertical lines indicate standard deviation.

WT: wild type



Supp Table S1: proline oxidase activity of previously tested *PRODH* alleles [Bender et al., 2005] *PRODH* (NM_016335)

Alleles	%WT activity
p.Q19P	65
p.A167V	65
p.R185W	55
p.R185Q	100
p.L289M	85
p.P406L	12
p.D426N	40
p.V427M	45
p.R431H	65
p.L441P	0
p.R453C	8
p.A455S	90
p.T466M	18
p.A472T	72
p.Q521E	25
p.Q521R	122
p.[L441P; R453C]	1
p.[V427M; R431H; L441P]	8
p.[R453C; T466M; A472T]	3