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RESEARCH REPORT

5-Oxoprolinuria in Heterozygous Patients for 5-Oxoprolinase (*OPLAH*) Missense Changes

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Abstract The inherited 5-oxoprolinuria is primarily suggestive of genetic defects in two enzymes belonging to the gamma-glutamyl cycle in the glutathione (GSH) metabolism: the glutathione synthetase (GSS) and the 5-oxoprolinase (OPLAH). The GSS deficiency is the best characterized of the inborn errors of GSH metabolism, whereas the OPLAH deficiency is questioned whether it is a disorder or just a biochemical condition with no adverse clinical effects. Recently, the first human *OPLAH* mutation (p.H870Pfs) was reported in homozygosis in two siblings who suffered from 5-oxoprolinuria with a benign clinical course. We report two unrelated patients who manifested massive excretion of 5-oxoproline in urine. In both probands, the blood GSH levels were normal and no mutations were found in the *GSS* gene. The mutational screening of the *OPLAH* gene, which included the codified sequences, the intronic flanking sequences, the promoter sequence, and a genetic analysis in order to detect large deletions and/or duplications, showed that each patient only harbors one missense mutation in heterozygosis. The in silico analyses revealed that each one of these *OPLAH* mutations, p.S323R and p.V1089I, could alter the proper function of this homodimeric enzyme. In addition, clinical symptoms manifest in these two probands were not related to GSH cycle defects and, therefore, this study provides further evidence that oxoprolinuria may present as epiphenomenon in several pathological conditions and confound the final diagnosis.

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Introduction

There are several metabolic disturbances that are questioned if they only are a casual finding during biochemical screening of patients or have a direct clinical involvement. One of them is the 5-oxoprolinuria (pyroglutamic aciduria) whose etiology remains elusive in some circumstances. Several diseases and environmental conditions (special diets, drug metabolism and drug treatment, prematurity, malnutrition, some inborn errors of metabolism) have been associated with oxoprolinuria (Schwahn et al. 2005; Ruijter et al. 2006; Ristoff and Larsson 2007), although the hereditary 5-oxoprolinuria is primarily suggestive of genetic defects in two enzymes belonging to the gamma-glutamyl cycle in the glutathione (GSH) metabolism: the glutathione synthetase (GSS; EC 6.3.2.3) and the 5-oxoprolinase (OPLAH; EC 3.5.2.9).

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The glutathione synthetase deficiency (MIM 266130) is the best characterized and the most common of the inborn errors of GSH metabolism (Njalsson 2005; Ristoff and Larsson 2007). It presents with a wide spectrum of clinical signs. The most frequent hallmarks are 5-oxoprolinuria, metabolic acidosis and hemolytic anemia, and in severely affected patients, the central nervous system is affected. This deficiency is caused by mutations in the *GSS* gene and more than 30 mutations have been described which are transmitted in an autosomal recessive fashion (Al-Jishi et al. 1999; Dahl et al. 1997; Njalsson et al. 2003; Shi et al. 1996). Heterozygous carriers of *GSS* mutations are healthy and show an enzyme activity of 55% of the normal mean and normal levels of GSH (Njalsson et al. 2005). However, the 5-oxoprolinase deficiency (MIM 260005) is questioned whether it is a disorder or just a biochemical condition with no adverse clinical effects. To date, nine probands with 5-oxoprolinuria and a low activity of 5-oxoprolinase have been described worldwide (Almaghlouth et al. 2011; Bernier et al. 1996; Cohen et al. 1997; Henderson et al. 1993; Larsson et al. 1981; Mayatepek et al. 1995; Roesel et al. 1981). These patients lack a consistent clinical picture except for the 5-oxoprolinuria. Symptoms reported in individual patients include renal stone formation, mental retardation, neonatal hypoglycaemia, microcytic anemia, and microcephaly. The 5-oxoprolinase is encoded by the *OPLAH* gene, and mutations in this gene are expected to lead to a 5-oxoprolinase deficiency transmitted in an autosomal recessive manner. Recently, the first human *OPLAH* mutation (p.H870Pfs) was reported, which predicts a truncated protein. This change was identified in homozygosis in two siblings with a persistent increased 5-oxoproline excretion and with a benign clinical course (Almaghlouth et al. 2011).

Here we report two new unrelated patients who manifested a massive excretion of 5-oxoproline in urine. After an exhaustive genetic analysis, in each patient, only one heterozygous missense mutation was identified in the *OPLAH* gene, suggesting that only one mutation could alter the normal activity of this homodimeric enzyme. Moreover, clinical features in these two probands were not related supporting the 5-oxoprolinase deficiency is a benign biochemical condition.

Materials and Methods

Patients This study protocol was approved by the ethics committee of the Hospital Sant Joan de Déu. Written informed consent was obtained from parents of the patients.

Case 1 The patient is a 1.5-month-old girl (ID no. 943, family AR-141; Fig. 1a), the first child of healthy non-consanguineous parents from Indian origin, with an uneventful pregnancy and delivery. She was admitted in our hospital due to repeated episodes of choking (respiratory difficulties and perioral cyanosis shortly after breast feeding) and generalized tonic-clonic seizures. First baseline tests revealed hypocalcemia (1.4 mmol/L; control values: 2.1–2.7), increased phosphate values (2.8 mmol/L: control values 1.4–2.2), 25 hydroxyvitamin D deficiency (5.5 ng/mL: control values 12–62), increased PTH values (15.6 pmol/L: control values 0.5–5.5) normocytic normochromic anemia (hemoglobin: 8.5 g/dL: control values 9.0–13.0) with low plasma cobalamin (139 pmol/L: control values >198), normal plasma total homocysteine values (3.8 µmol/L: control values < 7.5), and metabolic acidosis. EEG and brain ultrasound were normal. General and neurological examinations were normal between episodes. The patient was treated with oral vitamin D supplementation and intramuscular vitamin B12 due to a suspicion of deficiency related to maternal vegetarian diet. The outcome was excellent with no other seizure recurrence, normalization of all altered biochemical parameters, and normal developmental delay at 1 year of age. Urine organic acids were analyzed three times, disclosing increased pyroglutamic acid excretion twice during decompensation (7,828 mmol/mol creatinine and 3,255 mmol/mol creatinine) which led us to the suspicion of pyroglutamic aciduria. These values were completely normalized at 1 year of age (9 mmol/mol creatinine; reference values <10). No causes of secondary increased excretion of pyroglutamic acid were detected including special diets, drugs (vigabatrin paracetamol and antibiotics), inborn errors of metabolism (urea cycle defects, cystinosis, tyrosinemia, and homocystinuria) and low glycine values secondary to malnutrition. Blood glutathione levels were normal (3.2 mmol/L; reference values: 1.5–3.1).

Case 2 An 8-year-old boy (ID no. 1037, family AR-152; Fig. 1a) diagnosed of Duchenne muscular dystrophy (DMD; MIM 310200) harboring a deletion that encompasses exons 46 to 51 at the *dystrophin* gene. Familiar antecedents were uneventful. Clinical presentation at the moment of exploration was classical, with muscular involvement and mental disability. No other symptoms or signs were present. Blood count (no anemia) and other routine laboratory parameters were normal besides increased serum creatine kinase activity. Urine organic acids were collected to study metabolomic profile by gas chromatography mass spectrometry, and we encountered a serendipitous finding of increased excretion of pyroglutamic acid (13,208 mmol/mol creatinine: reference values: <10). This huge excretion was further confirmed in a second urine sample (7,931 mmol/mol creatinine). In that moment, patient was under corticoid therapy (prednisone, 20 mg/day). No causes of secondary increased excretion of pyroglutamic acid were detected. Blood GSH values in were normal (2.45 mmol/L, reference values: 1.5–3.1).

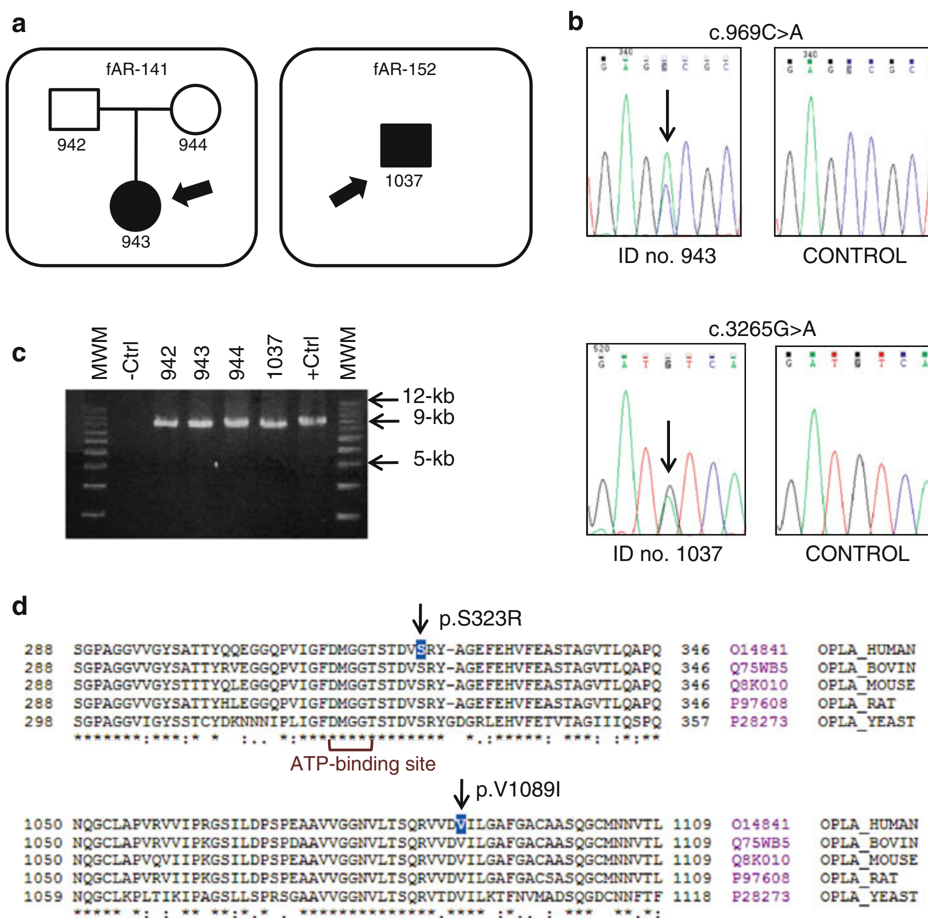

Fig. 1 Genetic findings. (**a**) Available DNAs from probands and their relatives. (**b**) Electrophoregrams show the identified mutations in proband ID no. 943 (c. 969C>A, p.S323R) and in proband ID no. 1037 (c.3265G>A, p.V1089I) in the *OPLAH* gene, together with the corresponding controls' electrophoregrams. (**c**) Electrophoresis gel shows the obtained band of 9,0-kb which corresponds to the amplification of the *OPLAH* gene. No differences were found in any of the analyzed samples ruling out large deletions or duplications (1st and 8th lanes: MWM, Molecular Weight Marker, 1-kb plus DNA ladder; 2nd lane: –Ctrl, PCR negative control; 3rd to 6th lanes: analyzed DNAs, 942, 943, and 944 from family AR-141, and 1037 from family AR-152; 7th lane: +Ctrl, DNA from a healthy subject). (**d**) Alignment of sequences of the OPLAH protein from human, bovin, mouse, rat, and yeast showing that both mutated residues are conserved amino acids. Identical amino acids in all of these sequences are indicated with an asterisk (*). The closest ATP-binding site to the p.S323R change is shown, which consists of the sequence DMGGT (from residue 324 to 328 in yeast)

Biochemical analyses Organic acids in urine were analyzed by gas chromatography/mass spectrometry (GC-MS) (Agilent Technologies Inc., Santa Clara, CA, USA) after extraction of the urine sample with ethylacetate and diethyleter and derivatization with *N*,*N*-bis(trimethylsilyl) trifluoroacetamide. The concentration of pyroglutamic acid was quantified by comparing the signal of m/z 156 obtained with standard solutions of the pure compound, using undecanoate as internal standard.

Genetic analyses A search for mutations was performed by Sanger sequencing of the PCR products of exons and their intronic flanking sequences in the *GSS* gene (NM_000178) as well as in the *OPLAH* gene (NM_017570.3) in an ABI Prism 3130xl autoanalyzer (Applied Biosystems, Foster City, CA, USA). The promoter sequence was also analyzed in the *OPLAH* gene.

With the aim to discard the possible existence of large deletions or insertions, we amplified all the codified exons and introns of the *OPLAH* gene (9,007 bp) in the two probands, the available relatives (ID no. 943's parents), and three control subjects. The PCR was performed using Long PCR Enzyme Mix (Mbi Fermentas, Glen. Burnie, MD, USA) and the following primers: forward 5′-GTGGGTCTCTCCCTCAGGAACC-3′, and reverse 5′-CTGCAGCTCCGAGTCTCAGTGTC-3′. The amplified product was resolved by 0.8 % agarose electrophoresis. To improve the resolution of this study, the PCR products were separately digested with two enzymes, *Bam*HI and *Xho*I, which cut four or three times respectively. The digested fragments were under 5,500 bp and they were resolved by 0.8 % agarose electrophoresis.

Each identified change was also analyzed in more than 250 chromosomes from healthy individuals using DHPLC (Denaturing High Performance Liquid Chromatography; Transgenomic WAVE, Crewe, UK). We also investigated in silico the biological relevance of the mutated residues. Conservation of residues was analyzed by alignment of related sequences using the program BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al. 1990). Sequence-based predictions of the phenotypic consequences of mutation were assessed using the SIFT (http://sift.jcvi.org/) and the PolyPhen (http://genetics.bwh.harvard.edu/pph/) softwares.

Results

No mutations were detected in the *GSS* gene in any of the two probands. In patient ID no. 943, a novel change was detected in the *OPLAH* gene: c.969C>A (Fig. 1b), which is predicted to generate the novel p.S323R amino acid change. We also analyzed her parents, and the mother was a heterozygous carrier of the p.S323R change whereas the father did not harbor it. In the three available samples, we discarded the existence of large deletions or duplications in the *OPLAH* gene (Fig. 1c). The p.S323R was not detected in 128 control subjects. The residue S323 is an evolutionary conserved amino acid, invariant across more than 100 different species (Fig. 1D). Both SIFT and PolyPhen algorithms predicted that the p.S323R change would be probably damaging.

Patient ID no. 1037 was carrier of the *OPLAH* c.3265G>A change (Fig. 1b), which is predicted to produce the p.V1089I amino acid change. Large deletions and/or duplications of the *OPLAH* gene were ruled out in this patient (Fig. 1c). A search for the p.V1089I change was performed in 154 healthy individuals and this was observed as a heterozygous change in three individuals. In fact, this change is annotated as SNP rs185836803 in dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=185836803), although information about heterozygosity is not available. The residue V1089 is a relatively conserved amino acid (Fig. 1d). Results obtained from the in silico analysis were controversial: the SIFT software predicted that p.V1089I could be tolerated and the PolyPhen algorithm that this would be damaging.

Discussion

Several inborn errors of GSH metabolism are known, glutathione synthetase deficiency being the first cause of GSH cycle diseases (Shi et al. 1996). GSH takes part in multiple fundamental processes apart of being one of the most important antioxidants in the eukaryotic organism. Deficiency of glutathione synthetase could affect the central nervous system and other tissues and may cause neurological involvement and anemia, among other signs. Thus, the massive excretion of 5-oxoproline in our patients could be attributed to mutations in the *GSS* gene, although GSH values in blood were within normal limits and no mutations were identified in the *GSS* gene in any of our two probands, ruling out this diagnosis.

The deficiency of 5-oxoprolinase has been associated with a wide spectrum of clinical symptoms (Ristoff and Larsson 2007). To date only one homozygous mutation has been described in two siblings who show a benign clinical course but with a persistent increased excretion of 5-oxoproline (Almaghlouth et al. 2011). The proband ID no. 943 presented with an increased urinary excretion of 5-oxoproline at the beginning of the clinical picture, which spontaneously normalized at 1 year of age, together with a complete recovery of her clinical picture. In this case, we cannot rule out that low vitamin D and cobalamin levels could reflect a malnutrition status that could contribute to the pyroglutamic aciduria. Nevertheless, the presence of the reported mutation was probably necessary for triggering the pyroglutamic aciduria, since no hyperhomocysteinemia was revealed in this patient and plasma glycine values were always normal. The proband ID no. 1037 was a patient with a classical Duchenne clinical presentation, with no other signs that could be attributed to oxoprolinuria. In each of them, only one missense change in heterozygosis was identified in the *OPLAH* gene after analyzing the codified exons, their intronic flanking regions, and the promoter sequence, and discarding large deletions/insertions. Thus, another mutation in the *OPLAH* gene is improbable except located in a deep intronic region. Splicing mutations account for around 10 % of all reported mutations and deep intronic mutations represent less than 1 % of known splicing mutations (Cooper et al. 2010). All these data together suggest that these two probands seem to be carriers of only one defect in the *OPLAH* gene.

Both mutations were detected in healthy subjects: p.V1089I in three controls and the p.S323R in the proband ID no. 943's mother. The clinical course of the two patients with known mutations in the *OPLAH* gene is largely benign (Almaghlouth et al. 2011), as confirmed in our first case. Concerning the second case, DMD is an X-linked neuromuscular disorder due to mutations in the gene encoding dystrophin, and therefore has a well-defined etiology. Patients diagnosed of DMD and also with oxoprolinuria have not been reported. We tested pyroglutamic acid excretion in urine in five further patients with DMD, but disclosed normal results (data not shown). Therefore, persistent oxoprolinuria in the proband ID no. 1037 does not seem to be related to the DMD. Two female siblings were reported with a severe neurological picture, but only

one of them manifested oxoprolinuria (Cohen et al. 1997). In that case, authors concluded that both siblings suffered from an unknown hereditary disease, unrelated to the 5-oxoprolinase deficiency observed in one of them. In the aggregate, our study supports the proposition that deficiency of 5-oxoprolinase is a benign condition. For this reason, mutations in the *OPLAH* gene could remain undiscovered since they probably would not lead to relevant clinical symptoms but may cause biochemical alterations that confound the final diagnosis.

This study reveals that heterozygous missense mutations in the *OPLAH* gene could cause oxoprolinuria. The in silico analyses show that both mutations could be pathological. For both changes, the sequence alignment revealed that the affected residues are conserved throughout evolution, being identical in mammals and yeast (Fig. 1d). At least one of the algorithms used to predict the possible pathogenicity revealed that each one of these mutations could be damaging. Little is known about the functionality of the human 5-oxoprolinase. In *Saccharomyces cerevisiae*, the *OXP1/YKL215c* encodes for an ATP-dependent 5-oxoprolinase (Kumar and Bachhawat 2010). This enzyme functions as a dimer and contains two distinct domains. The first domain, from residue 1 to 736, is the actin-like ATPase motif which contains three essential ATP-binding sites. The function of the second motif, from residue 745 to 1286, remains confused. Thus, the *OPLAH* p.S323R would be located in the first domain extremely close to one of the ATP-binding sites (Fig. 1d), suggesting that this change in the human 5-oxoprolinase is probably affecting the proper activity of the enzyme. Regarding the *OPLAH* p.V1089I, this change would be placed in the second domain whose function is still unknown. This change has been recently annotated as SNP rs185836803. Other SNPs have been annotated in the public databases associated with diseases. Thus, the overwhelming majority of cases of familial amyloid polyneuropathy (FAP; MIM 105210) result from the p.V30M substitution in the *TTR* gene, which is referred to as SNP rs28933979 (Benson 2001). The fact is that the *OPLAH* p.V1089I is the only detected mutation in our patient and he suffers from a persistent oxoprolinuria.

Unfortunately, we could not measure oxoprolinase activity since patients did not collaborate with this study and at present are not controlled in the hospital. However, the homodimeric structure of the enzyme could make possible a dominant negative effect of the mutations, although these are rare in metabolic diseases. However, some forms of hypophosphatasia (MIM 146300, 241500, 241510) and also of cortisone reductase deficiency (MIM 604931) can be transmitted in a dominant manner and in these cases the mutations can exhibit a dominant-negative effect by inhibiting the enzymatic activity of the heterodimer (Lawson et al. 2011; Lia-Baldini et al. 2001). These dominant-negative mutations are in the active site or in an area which probably affects the formation of functional dimers. A similar process could occur in heterozygous patients of 5-oxoprolinase deficiency. In some particular conditions, interactions between both identical molecules would not be properly made and would trigger a massive excretion of 5-oxoproline in urine. This anomalous biochemical condition would be normalized when the stressing conditions disappear, as in our proband ID no. 943. In other cases, the oxoprolinuria could persist, as in our proband ID no. 1037. Further studies are needed to clarify the possible mechanism causing this biochemical alteration in heterozygous individuals for *OPLAH* mutations. Independently, mutational analysis of the *OPLAH* gene may be advisable to elucidate causes of unknown massive pyroglutamic aciduria in order to understand better this biochemical alteration that may confound the final diagnosis.

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A Concise One-Sentence Take-Home Message

Oxoprolinuria, due to heterozygous mutations in the *5-oxoprolinase* gene, may present as epiphenomenon in several pathological conditions and confound the final diagnosis.

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Competing Interest

None declared.

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