Unravelling 5-oxoprolinuria (Pyroglutamic aciduria) due to bi-allelic OPLAH mutations : 20 new mutations in 14 families

Unravelling 5-Oxoprolinuria (Pyroglutamic Aciduria) due to bi-allelic *OPLAH* Mutations: 20 new Mutations in 14 Families

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Abstract

Primary 5-oxoprolinuria (pyroglutamic aciduria) is caused by a genetic defect in the γ-glutamyl cycle, affecting either glutathione synthetase or 5-oxoprolinase. While several dozens of patients with glutathione synthetase deficiency have been reported, with hemolytic anemia representing the clinical key feature, 5-oxoprolinase deficiency due to *OPLAH* mutations is less frequent and so far has not attracted much attention. This has prompted us to investigate the clinical phenotype as well as the underlying genotype in patients from 14 families of various ethnic backgrounds who underwent diagnostic mutation analysis following the detection of 5-oxoprolinuria.

In all patients with 5-oxoprolinuria studied, bi-allelic mutations in *OPLAH* were indicated. An autosomal recessive mode of inheritance for 5-oxoprolinase deficiency is further supported by the identification of a single mutation in all 9/14 parent sample sets investigated (except for the father of one patient whose result suggests homozygosity), and the absence of 5-oxoprolinuria in all tested heterozygotes. It is remarkable, that all 20 mutations identified were novel and private to the respective families.

Clinical features were highly variable and in several sib pairs, did not segregate with 5-oxoprolinuria. Although a pathogenic role of 5-oxoprolinase deficiency remains possible, this is not supported by our findings. Additional patient ascertainment and long-term follow-up is needed to establish the benign nature of this inborn error of metabolism. It is important that all symptomatic patients with persistently elevated

levels of 5-oxoproline and no obvious explanation are investigated for the genetic etiology.

Key words: 5-oxoprolinuria; pyroglutamic aciduria; 5-oxoprolinase; γ-glutamyl cycle; glutathione synthetase

1. Introduction

5-oxoprolinuria, also called pyroglutamic aciduria, is characterized by the excretion of high amounts of 5-oxoproline, or pyroglutamic acid, in the urine, as detectable by organic acid analysis (Mayatepek and Jaeken, 2012). Primary 5-oxoprolinuria is due to an enzyme defect in the γ-glutamyl cycle, in which synthesis and degradation of glutathione takes place. The ubiquitous tripeptide glutathione (L-γ-glutamyl-L-cysteinylglycine) is known as an important antioxidant which is not only involved in various redox reactions, but also in the biosynthesis of DNA, proteins and leukotrienes, and in the metabolism of xenobiotics, and amino acid transport (Njålsson 2005; Blau and Dionisi-Vici, 2014). Among other functions, a regulatory role in cell proliferation and apoptosis has been reported for this molecule (Watson et al. 2003).

The primary metabolic defect of the γ-glutamyl cycle that results in 5-oxoprolinuria, affects either the enzyme glutathione synthetase or 5-oxoprolinase. While more than 70 patients are known with a genetic deficiency of glutathione synthetase, only a few patients with primary 5-oxoprolinase deficiency have been described in the literature (Mayatepek and Jaeken, 2012). So far, mutations in *OPLAH* have been reported in

only 7 patients with 5-oxoprolinuria; 4 identified with bi-allelic mutations and the remaining 3 with only a single (heterozygous) mutation (Almaghlouth et al., 2012; Calpena et al. 2013; Calpena et al. 2015; Li et al. 2015). The question whether 5-oxoprolinase deficiency due to *OPLAH* mutations causes only a biochemical phenotype, i.e. 5-oxoprolinuria, or also clinical symptoms has been debated since the report by Almaghlouth et al. (2012).

In view of the very limited information and the clinical heterogeneity of the few cases published, a more extensive assessment of the role of *OPLAH* mutations in patients with 5-oxoprolinuria is indicated. This prompted us to combine our experience with the diagnostic work-up of patients with 5-oxoprolinuria from 14 families of various origins, in order to provide a comprehensive overview on the molecular and clinical features of this inborn error of metabolism.

2. Patients and Methods

Analysis of urinary organic acids was performed as a diagnostic test according to standard procedures of the various commissioned (often local) diagnostic laboratories. While in the majority, the test was prompted by the clinical suspicion of an inborn error of metabolism (IEM), patient #3 was identified by the Québec newborn urine screening program. https://www.raredisorders.ca/content/uploads/Canada-NBS-status-updated-Sept.-3-2015.pdf). (This is a second newborn screen which is done by urine collected by a parent at newborn day of life 21. It covers a total of twelve amino acid disorders (including transport disorders of amino acids) , urea cycle disorders and organic aciduriasincluding 5-oxoprolinuria. After the urinary analysis revealed an elevated concentration of 5-oxoproline, further diagnostic steps were taken to identify the causal diagnosis.

Since none of the patients had presented with anemia, which is a key feature of

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glutathione synthetase deficiency, molecular analysis of *OPLAH,* encoding the 5-oxoprolinase enzyme, was performed. Reference sequence was NM 017570.3. Genomic DNA was extracted from peripheral blood using standard methods. Exons and exon-intron boundaries of *OPLAH* from patient DNA samples (except for that of patient #7) were amplified by PCR and prepared for bidirectional sequencing on capillary Sanger sequencer machines. Analyses covered the region between exon 2 (with the start codon) and exon 27 (with the stop codon), including adjacent intronic regions. For confirmation, samples of parents and other relatives were studied for identified mutations where appropriate and feasible. Clinical information was obtained from medical records. According to them two patients (#4, #11) temporarily received anticonvulsive treatment, while vitamin treatment such as ricket-caries prophylaxis were reported in infancy only (patient #2).

For patient #7, singleton whole genome sequencing (WGS) was performed, using the Illumina HiSeq X Ten sequencer (Macrogen). The sequencing reads were aligned to the human reference genome version hg19 (coverage 30X) with bio-informatics analysis via a semi-automated bio-informatics pipeline, and subsequent Sanger confirmation of identified variants and segregation in the family.

For patients #7, #12, and #13 the investigations were covered by approvals by the appropriate institutional review boards (UBC IRB approval H12-00067; KFSHRC RAC#2121053). In all cases the physicians in charge of the patients obtained informed consent for the mutation analysis. Where appropriate, they have also obtained informed consent for publication. Predictions programs applied were phyloP (Pollard et al. 2010), PolyPhen2 (Adzhubei et al. 2013) and SIFT (Kumar et al. 2009).

3. Results

We present the results of *OPLAH* molecular analysis in individuals with 5-oxoprolinuria from 14 families of diverse ethnic backgrounds (Table 1). 20 different mutations in the *OPLAH* gene are reported, none of which have been described in 5-oxoprolinuria before. Fifteen of the mutations are missense, most affecting evolutionary conserved amino acids, predicted *in silico* to damage the 5-oxoprolinase enzyme.

The stop mutation identified in patient #7, the small deletions in patients #4, #10, and #14 are all expected to result in a modified, truncated protein of questionable stability and probable loss of function. The mutation p.Pro1267Argfs*31 identified in patient #8 should result in a slightly extended chain length and in the replacement of a set of semi-conserved amino acids. Using the frameshift classification path 4 according to Hu and Ng it is predicted to be damaging, with 0.783 confidence score (Hu and Ng, 2012).

In all patients with 5-oxoprolinuria, bi-allelic *OPLAH* mutations were identified; where parental samples were available for confirmation, the *trans* configuration was demonstrated (Table 1). In case of patient #6 our results suggest homozygosity for *OPLAH* mutation c.3307T>C (p.Cys1103Arg), but no parental samples were available and homozygosity could not be proven.

The autosomal recessive mode of inheritance of 5-oxoprolinase deficiency is further supported by confirmation of carrier status (i.e. a heterozygous mutation) in all

parental samples investigated. Exception is the healthy father of patient #13, who along with a healthy sibling, was homozygous for the same mutation (p.R768H) as the index; both turned out to have 5-oxoprolinuria. This pseudo-dominant inheritance is not infrequent in Saudi Arabia where consanguinity is very common (Alkuraya 2014). 5-oxoprolinuria was not identified in any of the tested relatives with only a heterozygous mutation in *OPLAH* (see Table 1 for family history for patients #7, #9, #11, #12).

In patient #7, WGS identified compound heterozygous variants in *OPLAH* and no other variants contributing to the phenotype. In patient #12 (again with 5-oxoprolinuria), WES and Sanger sequencing of *OPLAH* both revealed compound heterozygous mutations (p.Gly521Gly and p.Gly1208Arg). Her sister, with pronounced global developmental disability~~elay~~, but no oxoprolinuria, was a carrier for one of the mutations (p.Gly521Glu). WES of the index case did not reveal (likely) pathogenic variants in other genes to account for the global developmental d~~elay~~isability observed in both siblings. In patient #2, macrocephaly prompted additional analysis of *PTEN* which yielded a heterozygous novel mutation c452C>A (p.Ala151Asp), considered pathogenic for *PTEN*-associated disease; targeted Sanger analysis confirmed maternal inheritance.

In all patients with ~~persistent~~ 5-oxoprolinuria, bi-allelic *OPLAH* mutations were identified. As obvious from the data summarized in Table 1, clinical features of patients with bi-allelic *OPLAH* mutations cover a wide range. Some patients were

completely asymptomatic. They were diagnosed following urinary newborn screening in the Canadian Province of Québec, after a single disease episode or due to an affected relative (#3,5,6,7,8,13 and father and sibling of the latter), while others (#1,2,4,11,12,14) presented with psychomotor or developmental ~~delay~~ disability and/or failure to thrive, with or without other clinical abnormalities.

The five previously reported mutations in *OPLAH* and 19 of our 20 newly report mutations appear to be randomly distributed over the three functional domains of homodimeric 5-oxoprolinase enzyme (EC 3.5.2.9) (Figure 1). (The remaining mutation p.Pro1267Argfs*31 is located at the C-terminus of the protein and was also predicted to be damaging.)

4. Discussion

Secondary, transient 5-oxoprolinuria has been observed in patients with several metabolic disorders not directly affecting the γ-glutamyl cycle and in individuals receiving certain drugs and diets (Blau and Dionisi-Vici, 2014). Notably, (non-enzymatic) decomposition of urinary glutamine may also yield an increase in signals of 5-oxoproline (Kumar and Bachhawat, 2012; Blau and Dionisi-Vici, 2014). However, clinical and laboratory data on our patients, who presented with persistent 5-oxoprolinuria, provided no evidence for secondary transient 5-oxoprolinuria.

Two genetic defects in the γ-glutamyl cycle have been described as causes of persistent 5-oxoprolinuria. In glutathione synthetase deficiency, typical presentation

includes hemolytic anemia, metabolic acidosis and neurological symptoms. Milder forms of glutathione synthetase deficiency may present with isolated hemolytic anemia only (Mayatepek and Jaeken, 2012; Blau and Dionisi-Vici, 2014). The absence of such clinical features in the patients described here, raised suspicion for 5-oxoprolinase deficiency. Consequently, our investigations focused on *OPLAH,* which encodes 5-oxoprolinase.

It is remarkable that in all patients with 5-oxoprolinuria novel, private mutations were identified. All identified variants seem extremely rare and only one homozygote is described on ExAC for the p.G825R mutation (AF: 0.001343 on ExAC). Only one of the 20 *OPLAH* mutations identified in our families, p.Val1089Phe in patient #7, involved an amino acid residue in the 5-oxoprolinase protein previously associated with another substitution: p.Val1089Ile had been reported by Calpena et al. 2013 in a patient with 5-oxoprolinuria.

Our results indicate that bi-allelic mutations are required for functional oxoprolinase deficiency causing 5-oxoprolinuria. None of the tested heterozygotes presented with 5-oxoprolinuria. This raises the question whether the three reported oxoprolinuria patients (Calpena et al. (2013) and Li et al. (2015)) with a single missense mutation harbor a second, unidentified (e.g., intronic/cryptic/indel) sequence variant.

Several patients identified with bi-allelic *OPLAH* mutations have shown psychomotor or developmental ~~delay~~disability. These clinical features, however, are frequently the

indication for organic acid analysis. Therefore it appears plausible that by chance organic acid analysis will occasionally identify 5-oxoprolinuria and lead to further investigation which may identify a defect in *OPLAH*. In view of such ascertainment bias, a causal relationship between an *OPLAH* defect and a clinical phenotype cannot be confirmed.

Clinical features do not segregate with 5-oxoprolinuria. This is not only underlined by patient #13 and her family (see Table 1), but also by the sibling of patient #12 who suffers from a more severe ~~delay~~ disability than the index case, but carries a single mutation only and is free of 5-oxoprolinuria.

However, 5-oxoprolinuria patients #4, 6, and 11 presented with clinical features while their siblings without 5-oxoprolinuria were considered healthy. In the case of the brother of patient #9, the developmental disability~~elay~~ was at least less pronounced than in the index case with 5-oxoprolinuria.

In contrast, patient #3 never had any symptoms, but was identified via organic acid analysis within a newborn urinary screening program. In patient #7 with episodic torticollis at age 2 years, the organic acids had been ordered in error revealing 5-oxoprolinuria quite unexpectedly; 5 years later the torticollis had resolved and he is developmentally above average and asymptomatic.

While such cases and the heterogeneity of the clinical presentations advocate against

clinical consequences of 5-oxoprolinuria caused by 5-oxoprolinase deficiency due to *OPLAH* mutations, a clinically pathogenic role of 5-oxoprolinase deficiency remains possible. Except for patient #11 and the homozygous father of patient #13, the patients described here, are still very young. Since the four previously reported patients who were homozygous or compound-heterozygous for *OPLAH* mutations (Almaghlout et al. 2012; Calpena et al. 2015; Li et al. 2015) were also children, it needs to be considered that relevant/specific clinical features may become obvious later in life only. An example of an IEM which has been considered a non-disease for years, until a highly characteristic clinical (and neuroradiologic) pattern was identified in adulthood, is 3-methylglutaconyl-coenzyme A hydratase deficiency (methylglutaconic aciduria type 1) due to mutations in the *AUH* gene (Wortmann et al. 2010). Therefore, a role of the *OPLAH* mutations in the pathogenesis of the clinical features observed in some of 5-oxoprolinuria patients cannot be excluded.

To distinguish disease from non-disease is an important issue. For instance, millions of children were screened for histidinemia in the newborn screening programs of Massachusetts and New York and dozens were treated with low-histidine diets for what is today considered a benign disorder not requiring any dietary therapy (Brosco et al. 2010). Just very recently Shepard et al. (2015) conducted a study to determine the cause of the highly variable clinical phenotype seen in 3-methylcrotonyl-CoA carboxylase (MCC) deficiency, detected through newborn screening. In several cases, WES revealed rare mutations in other disease genes responsible for the clinical phenotypes. This underlines that the most obvious metabolic defect,

detectable by well-established biomarkers, is not necessarily the cause of the clinical phenotype.

Given the lack of evidence for a clear clinical phenotype for 5-oxoprolinuria caused by *OPLAH* mutations, we suggest evaluation for alternate genetic causes in all oxoprolinuria patients with clinical signs or symptoms. Investigations might comprise WGS or WES. In a WES-based study of 41 non-consanguineous families with an unexplained intellectual developmental disorder, more than 10% were diagnosed with 2 different genetic defects (van Karnebeek, personal communication). Further long-term follow-up of all patients for evolution of symptoms seems warranted.

The persistent 5-oxoprolinuria in all the patients studied reflects the functional consequences of the *OPLAH* mutations affecting the 5-oxoprolinase sequence/ stability. Biological roles of 5-oxoproline other than its role in the γ-glutamyl cycle have received little attention so far. 5-oxoproline has been identified as low-affinity substrate of the monocarboxylate transporter 1 (MCT1), which is encoded by *SLC16A1* and primarily known as a transporter for lactate and ketone bodies (Sasaki et al., 2015). Recently, humans with genetic MCT1 deficiency have been identified, (van Hasselt et al. 2015; Balasubramaniam et al., 2015), but no 5-oxoprolinuria has been reported in any of them.

5. Conclusions

This paper expands our understanding of 5-oxoprolinase deficiency. Bi-allelic

mutations in *OPLAH* cause 5-oxoprolinuria but appear not to cause clinical symptoms. However, additional patient ascertainment, with testing for other genetic defects combined with long-term follow-up, is needed to establish clinical symptomatology or the benign nature of this disorder. It is important that *OPLAH* molecular analysis will be performed in all patients with persistently elevated levels of 5-oxoproline without anemia (and especially, if normal erythrocyte glutathione levels/ glutathione synthetase data are available). If at all possible, identified cases (variants and phenotypes) should be made publicly available into public databases such as ClinVar or LOVD.

Conflict of interest

Jörn Oliver Sass, Corinne Gemperle-Britschgi, Maja Tarailo-Graovac, Nisha Patel, Melanie Walter, Albena Jordanova, Majid Alfadhel, Ivo Barić, Mahmut Coker, Aynur Damli-Huber, Eissa Ali Faqeih, Nuria García Segarra, Michael T. Geraghty, Bjørn Magne Jåtun, Sema Kalkan Ucar, Merten Kriewitz, Markus Rauchenzauner, Ivailo Tournev, Claudia Till, Bryan Sayson, Daniel Beumer, Cynthia Xin Ye, Lin-Hua Zhang, Colin Ross, Hilary Vallance, Fowzan S. Alkuraya, and Clara D.M. van Karnebeek declare that they have no conflict of interest.

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Figure legend:

Figure 1: 5-oxoprolinase (EC 3.5.2.9) protein and corresponding distribution of the amino acid changes identified in patients with 5-oxoprolinuria (pyroglutamic aciduria). The 20 variants depicted in black are new variants identified in this study, while the variants depicted in grey have been previously reported (Almaghlouth et al., 2012; Calpena et al. 2013; Calpena et al. 2015; Li et al. 2015). The Pfam domains are depicted in purple, blue and orange.