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The enzyme 4-hydroxy-2-oxoglutarate aldolase is deficient in primary hyperoxaluria type 3

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Abstract

Background. Mutations in the 4-hydroxy-2-oxoglutarate aldolase (*HOGA1*) gene have been recently identified in patients with atypical primary hyperoxaluria (PH). However, it was not clearly established whether these mutations caused disease via loss of function or activation of the gene product.

Methods. Whole-gene sequencing of *HOGA1* was conducted in 28 unrelated patients with a high clinical suspicion of PH and in whom Types 1 and 2 had been excluded.

Results. Fifteen patients were homozygous or compound heterozygous for mutations in *HOGA1*. In total, seven different mutations were identified including three novel changes: a missense mutation, c.107C > T (p.Ala36Val), and two nonsense mutations c.117C > A (p.Tyr39X) and c.208C > T (p.Arg70X) as well as the previously documented c.860G > T (p.Gly297Val), c.907C > T (p.Arg303Cys) and in-frame c.944_946delAGG (p.Glu315del) mutations. The recurrent c.700 + 5G > T splice site mutation in intron 5 was most common with a frequency of 67%. Expression studies on hepatic messenger RNA demonstrated the pathogenicity of this mutation.

Conclusions. The detection of a patient with two novel nonsense mutations within exon 1 of the gene, c.117C > A (p.Tyr39X) and c.208C > T (p.Arg70X), provides definitive proof that PH Type 3 is due to deficiency of the 4-hydroxy-2-oxoglutarate aldolase enzyme.

Keywords: Glyoxylate; 4-hydroxy-2-oxoglutarate aldolase; kidney stone disease; oxalate; primary hyperoxaluria

Introduction

The primary hyperoxalurias (PHs) are inherited diseases characterized by excessive endogenous formation of oxalate, an end product of metabolism, which is excreted in the urine complexed with calcium. Calcium oxalate is poorly soluble and precipitates out in the urine leading to stones in the renal tract and progressive renal failure. Two forms of PH have been well characterized: PH1 is due to deficiency of alanine:glyoxylate aminotransferase (AGT) and PH2 is due to lack of glyoxylate reductase (GR) [1, 2]. There are a number of patients who have the phenotypic and biochemical features of PH but do not have either PH1 or PH2 as the cause of their disease [3, 4]. A third type of disease, PH3, (OMIM 613616) has recently been identified in some of these unclassified PH patients by the discovery of mutations in *HOGA1*, formerly called *DHDPSL* [5]. The authors of this paper identified six mutations: four missense, one in-frame deletion and one splice site mutation.

The *HOGA1* gene is comprised of seven exons and spans 27 kb of chromosome 10q24 (NCBI reference sequence NG_027922.1). The product of this gene, 4-hydroxy-2-oxoglutarate aldolase (HOGA; E.C. 4.1.3.16), is found predominantly in the liver and kidney [5]. Following the cloning of the *HOGA1* gene by Riedel *et al.* [6], definitive proof that human HOGA catalyses the synthesis of mitochondrial glyoxylate in the pathway of hydroxyproline metabolism has been established. This enzyme catalyses a reversible lyase reaction, whereby 4-hydroxy-2-oxoglutarate is cleaved to glyoxylate and pyruvate (Figure 1). Since glyoxylate is a known precursor of oxalate, Belostotsky *et al.* [5] hypothesized that mutations in the gene might lead to activation of the HOGA enzyme increasing the amount of intramitochondrial glyoxylate. However, a further 10 patients were recently described [7], including a patient who was compound heterozygote for a stop codon in exon 6 supporting a loss of function disease mechanism in PH3.

In this manuscript, we report the findings of DNA sequence analysis of *HOGA1* in a cohort of 28 unrelated atypical PH patients from the UK referred to the University College London Hospitals Primary Hyperoxaluria Diagnostic Service.

Materials and methods

Samples

Blood and DNA samples from patients with suspected PH were received for analysis by the University College London Hospitals Primary

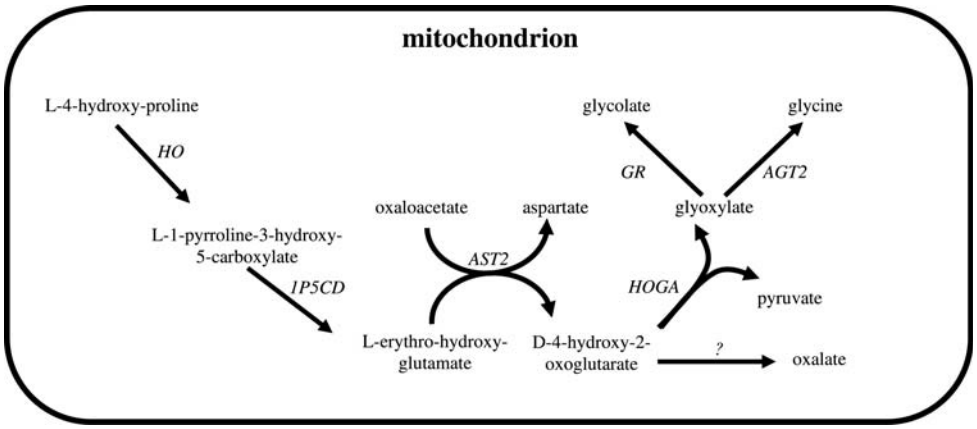

Fig. 1. Proposed model of the human mitochondrial hydroxyproline metabolic pathway and the site of action of HOGA. The source of oxalate is still not clear but may arise from degradation of 4-hydroxy-2-oxoglutarate. HO, hydroxyproline oxidase; 1P5CD, 1-pyrroline-5-carboxylate dehydrogenase; AST2, aspartate aminotransferase 2; HOGA, 4-hydroxy-2-oxoglutarate aldolase; AGT2, alanine glyoxylate aminotransferase 2.

Hyperoxaluria Diagnostic Service. Informed consent for DNA testing was obtained by the referring clinicians. PH1 and PH2 had been excluded by liver biopsy enzyme analysis ($n = 10$) and/or whole-gene DNA sequencing ($n = 22$).

DNA analysis

Genomic DNA was isolated from the peripheral blood leucocytes with the QIAamp DNA Blood Mini Kit (Qiagen, Crawley, UK) in accordance with the manufacturer's instructions. Intronic primers (Table 1) were designed based upon genomic reference sequence NG_027922.1. The exons and flanking intronic regions of the gene were amplified in three fragments by long-range polymerase chain reaction (PCR) using the Expand PCR system (Roche, Burgess Hill, UK) in accordance with the manufacturer's instructions. PCRs typically contained 100 ng of DNA, 350 μM NTPs and 1.75 mM $MgCl_2$ and were performed using a GeneAmp PCR system 2400 thermal cycler (Life Technologies, Paisley, UK) with primer annealing at 60°C. PCR products were purified using the QIAquick PCR purification kit (Qiagen) prior to cycle sequencing with suitably positioned internal primers and using a BigDye terminator ready reaction kit v. 3.1 (Life Technologies). Sequence analysis was carried out using an ABI 310 Genetic Analyser (Life Technologies). Numbering of mutations is based on complementary DNA (cDNA) reference sequence NM_138413.3, with nucleotide #1 denoting the first coding base. Nomenclature follows HUGO/HGVS recommendations (www.hgvs.org).

Analysis of splice site mutation

RNA was extracted from liver using the RiboPure kit (Life Technologies) according to the manufacturer's instructions. RNA (0.5–1 μg) was reverse transcribed using an Omniscript® reverse transcriptase (Qiagen) and cDNA was amplified using a forward primer in exon 2 and a reverse primer in the 3′-untranslated region (Table 1). This PCR reaction avoids the preferential amplification of an alternative splice variant that lacks exons 2–5 (Genbank NM_001134670.1). Products were sequenced with M13 universal primers, following TA cloning into the pCR®2.1-TOPO vector (Life Technologies).

Table 1. Primers used for amplification of *HOGA1* genomic and cDNA

PCR product	Forward primer	Reverse primer	Product size
Genomic DNA			
Exon 1	cctataggccttgcccctga	ggagcttcctgcctgtctcc	570 bp
Exons 2–6	tgaatcagggctacgtgtgc	gagactgagtggggcctggt	3.7 kb
Exon 7	ccaaaggaggcactcttcttcc	gaggtcaggggccgttagaa	584 bp
cDNA			
Exons 2–7	tgaccagcagtgagcgcctc	gatatcccctcagagccagccgttg	743 bp

Results

Of the 28 unrelated atypical patients, in whom DNA analysis was conducted, 15 were found to have mutations in *HOGA1*. A summary of genotype and the clinical and biochemical characteristics of these PH3 patients are presented in Table 2.

The c.700 + 5G > T splice site variant in intron 5 was found in 20 of 30 disease alleles (67% frequency). Eight patients were homozygous for this mutation and four patients were heterozygous. Sequencing of hepatic cDNA from a patient homozygous for the c.700 + 5G > T splice site mutation confirmed the pathological nature of this sequence change. The transcript contained 51 nucleotides of intron 5, consistent with activation of a downstream cryptic splice site (HGVS nomenclature, r.[700_701ins700 + 1_700 + 51; 700g > u]). This mutation would result in an in-frame insertion of 17 amino acids between amino acids 234 and 235 of the native protein sequence. All patients with this mutation were Caucasian and this may indicate a founder mutation in northern Europe.

The in-frame c.944_946delAGG (p.Glu315del) mutation was detected in a heterozygous state in two patients. Three missense mutations were also found: the previously documented c.860G > T (p.Gly297Val) and c.907C > T (p.Arg303Cys) mutations and a novel c.107C > T (p.Ala36Val) mutation in exon 1. While neither SIFT nor Polyphen 2 prediction programmes indicate that the p.Ala36Val mutation is pathological, this variant, which is at the N-terminal of the protein immediately following the mitochondrial cleavage site, was found in two symptomatic patients (10 and 14, Table 2), in trans with other disease causing mutations. Another patient was a compound heterozygote for two novel nonsense mutations within exon 1: c.117C > A (p.Tyr39X) and c.208C > T (p.Arg70X), which led to premature termination. The latter of these mutations was also detected in another patient, who was found to be compound heterozygote for this mutation and c.700 + 5G > T.

Table 2. Clinical and biochemical characteristics of PH3 patients[a]

Patient number	Age of onset	Presenting symptoms	Stone type	Urine oxalate μmol/24 h/1.73m^2 (normal < 460)	Urine oxalate/cr μmol/mmol	Plasma creatinine μmol/L (current age)	Genotype
1	2 Months	Bilateral stones	CaOx	n.a.	361–657 (98)	n.a. (6 years)	c.[700 + 5G > T][700 + 5G > T] p.[splice mut][splice mut]
2	3 Months	UTI, bilateral renal stones	94% CaOx	535–805	59–137 (98)	53 (9 years)	c.[700 + 5G > T][208C > T] p.[splice mut][Arg70X]
3	5 Months	UTI, bilateral renal stones	100% CaOx (34% COM)	750–880	53–657 (98)	25 (5 years)	c.[700 + 5G > T][700 + 5G > T] p.[splice mut][splice mut]
4	6 Months	Renal stones	100% CaOx	800–1200	n.a.	31 (2 years)	c.[700 + 5G > T][700 + 5G > T] p.[splice mut][splice mut]
5	1 Year	Haematuria, bilateral stones	CaOx and CaPhos	n.a.	204–283 (72)	44 (2 years)	c.[700 + 5G > T][700 + 5G > T] p.[splice mut][splice mut]
6	15 Months	Renal stones	n.a.	2800	n.a.	46 (5 years)	c.[700 + 5G > T] [944_946delAGG] p.[splice mut][Glu315del]
7	18 Months	UTI, renal stone	98% CaOx	938–1070	117–206 (72)	31 (2 years)	c.[700 + 5G > T][700 + 5G > T] p.[splice mut][splice mut]
8	2.5 Years	Haematuria, left renal and bladder stones	100% CaOx (68% COM)	257–899	75–230 (72)	43 (6 years)	c.[700 + 5G > T][907C > T] p.[splice mut][Arg303Cys]
9	9 Years	Haematuria, left renal stone	93% CaOx	716–2086	75–274 (71)	77 (13 years)	c.[117C > A][208C > T] p.[Tyr39X][Arg70X]
10	9 Years	Nephrocalcinosis, renal stones	CaOx	493–1138	29–114 (71)	70 (22 years)	c.[700 + 5G > T][107C > T] p.[splice mut][Ala36Val]
11	10 Years	Haematuria, bilateral stones	96% CaOx (82% COM)	1044–1422	67–99 (71)	68 (13 years)	c.[700 + 5G > T][700 + 5G > T] p.[splice mut][splice mut]
12	10 Years	Bilateral stones	CaOx	500–1400	41–70 (71)	105 (27 years)	c.[700 + 5G > T][700 + 5G > T] p.[splice mut][splice mut]
13	19 Years	Renal stones	100% CaOx (88% COM)	1260–1320	74–127	117 (54 years)	c.[860G > T][944_946delAGG] p.[Gly287Val][Glu315del]
14	20 Years	Renal stones	100% CaOx (24% COM)	723–848	45–50	88 (42 years)	c.[107C > T] [860G > T] p.[Ala36Val][Gly287Val]
15	33 Years	Loin ache, renal stones	CaOx	770–1359	61–88	94 (41 years)	c.[700 + 5G > T][700 + 5G > T] p.[splice mut][splice mut]

[a]For oxalate/creatinine ratio, upper limit of normal is 38 μmol/mmol, except where indicated otherwise. n.a., not available; CaOx, calcium oxalate; CaPhos, calcium phosphate; COM, calcium oxalate, monohydrate form; UTI, urinary tract infection.

In all cases, urinary oxalate excretion was raised (Table 2), overlapping with that observed in PH1 and PH2. Urinary glycolate was not routinely assayed but was measured in 12 patients and found to be elevated in 5 of them (Table 3) confirming that this metabolite should no longer be regarded as being specific for PH1. Urine calcium excretion was determined in 11 patients (Table 3) with levels observed above the reference range in 3 adult patients. None of the paediatric cases were found to have raised urine calcium. The age of disease onset in the PH3 patients was similar to that seen in PH1 and PH2 (Figure 2). Renal stones were the commonest presenting feature in all three forms of PH, with nephrocalcinosis appearing less frequently in PH3 (Table 4).

Table 3. Urine glycolate and urine calcium results of PH3 patients[a]

Patient number	Urine Ca/cr mmol/mmol (upper limit of normal)	Ca excretion mmol/24 h (normal < 9)	Urine glycolate/cr μmol/mmol (upper limit of normal)
1	n.a.		n.a.
2	0.08–0.37 (0.74)		21–37 (57)
3	0.59 (1.5)		27–72 (54)
4	n.a.		100 (54)
5	n.a.		18–216 (54)
6	n.a.		12 (57)
7	0.04 (0.74)		n.a.
8	0.7 (1.1)		48–74 (68)
9	0.37 (0.74)		6–24 (36)
10		10.7	59–120 (57)
11		6.2	30–49 (57)
12		2.8–14.3	25–32 (57)
13		8.8	13 (57)
14		7.3	n.a.
15		7.3–17.8	19–22 (57)

[a]n.a., not analysed. Age-related upper limits of normal shown in parentheses reflect the patients' ages at time of sampling.

Discussion

The identification of the molecular and biochemical basis of PH Types 1 and 2 has helped to determine the metabolism of glyoxylate. However, there remains a number of patients with unexplained hyperoxaluric stone disease. From the findings presented in this manuscript, we can conclude that in PH3, there is deficient rather than overactive function of HOGA as was originally hypothesized [5]. Proof was established by finding two nonsense mutations in exon 1. These mutations which cause truncation at amino acid residues 39 and 70 reside close to the mitochondrial cleavage site at the N-terminal of the protein sequence and the resulting peptide would not be catalytically active [6]. We have additionally confirmed

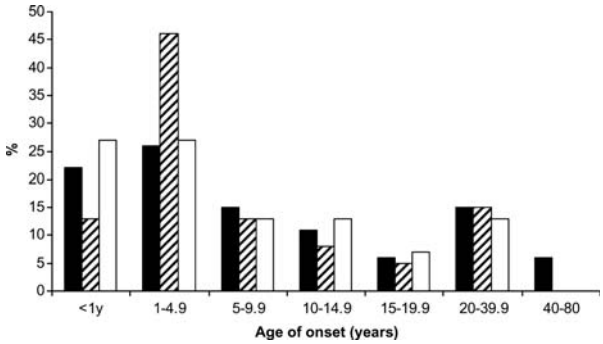

Fig. 2. Age of presentation of index cases with PH1 (solid colour), PH2 (hatched) and PH3 (white). For ease of comparison data are presented as per cent of total, where number of cases is 359, 39 and 15 for PH1, PH2 and PH3, respectively.

Table 4. Clinical features at presentation of index cases with PH1, PH2 and PH3 as reported by clinicians at time of specimen referral

	Type of PH		
	PH1 ($n = 359$)	PH2 ($n = 39$)	PH3 ($n = 15$)
Renal stones (%)	65	90	100
Nephrocalcinosis (%)	56	46	7
Renal failure (%)	39	21	0
Systemic oxalosis (%)	10	3	0
Total	359	39	15

that the effect of the potential splice site mutation c.700 + 5G > T, previously erroneously reported as c.701 + 4G > T [5], is activation of a cryptic splice site in intron 5 with the introduction of an additional 51 bp of intronic nucleotides into the expressed hepatic messenger RNA. While the liver and kidney are believed to be the prime sites of *HOGA1* expression, Monico *et al.* [7] also showed that a similarly mis-spliced product was obtained in immortalized leucocytes. It is not clear from their publication whether this represents ectopic expression following transformation or whether untransformed leucocytes could also be used. The effect of the novel missense mutation, p.Ala36Val, is not easy to confirm in the absence of an assay for the enzyme activity. This sequence change has not been described previously as a polymorphic variant and it occurs at the N-terminal of the protein immediately following the cleavage site of the mitochondrial targeting sequence. Both SIFT and Polyphen 2 suggest that the mutation is benign, but it was found in heterozygous form in two separate patients together with another disease causing mutation and with no other sequence changes. In the absence of parental studies, the mutations were assumed to be on separate alleles i.e. in trans.

The HOGA enzyme, encoded by the *HOGA1* gene, catalyses the last step of the mitochondrial hydroxyproline metabolic pathway. The reaction is fully reversible—a cleavage reaction in which 4-hydroxy-2-oxoglutarate is metabolized to glyoxylate and pyruvate, and a condensation reaction in which this reaction is reversed. Thus, glyoxylate is both a substrate and a product of the HOGA enzyme reaction. The equilibrium constants for the forward and reverse reactions, at least for the rat liver enzyme [8, 9], indicate that the former is the favoured reaction. These experiments were carried out in the presence of Tris which strongly binds to glyoxylate and would thus limit its availability as a substrate for the reverse (condensation) reaction. It is therefore not clear which reaction would be favoured within the mitochondrial environment. HOGA might have a protective effect metabolizing mitochondrial glyoxylate (analogous to the role of AGT in the peroxisomes and GR in the cytosol). Deficiency of HOGA could then cause the marked hyperoxaluria observed in PH3 by an accumulation of glyoxylate and metabolism of this substance to oxalate. The alternative is that accumulated 4-hydroxy-2-oxoglutarate, which is known to be relatively unstable [10], is metabolized either enzymatically or non-enzymatically to glyoxylate and/or oxalate. The direction of the HOGA enzyme reaction has significance for the treatment of PH3 since, if it is a forward (cleavage) reaction, reduction of dietary collagen could potentially reduce the amount of substrate and hence the amount of oxalate produced.

In rats, hydroxyproline loading increased the urinary excretion of oxalate, which was significantly reduced when mitochondrial AGT1 was induced by glucagon [11]. This finding suggests an important role for mitochondrial AGT in glyoxylate detoxification. Since AGT1 is solely peroxisomal in humans, other mechanisms must exist for mitochondrial glyoxylate detoxification and candidate enzymes include AGT2 and GR (Figure 1). In normal subjects, loading doses of gelatine, a collagen source, were shown to produce a slight increase in the output of urine hydroxyproline and oxalate, but they had a greater effect on glycolate excretion [12]. This observation suggests that glycolate, rather than oxalate, is the major metabolite arising from hydroxyproline breakdown in humans and suggests an important role for mitochondrial and/or cytosolic GR in the detoxification of mitochondrial glyoxylate by its reduction to glycolate.

The phenotype of PH3 clearly overlaps that of PH1 and PH2 since the levels of urinary oxalate concentration and the age of onset of the disease are similar (Figure 2). Renal stones were present in all the PH3 patients at presentation, with nephrocalcinosis in only one patient. However, nephrocalcinosis, previously considered to be an uncommon presenting feature of PH2 [13, 14], was present in 46% of PH2 patients diagnosed by the University College London Hospitals laboratory. Urine glycolate was raised in some, but not all, of the PH3 cases we analysed. Thus, urine oxalate and glycolate cannot discriminate reliably among the different types of PH. Increased urinary calcium was not a consistent feature of PH3 in this study, contrasting with previous reports [7, 15], and was only observed in adult patients.

In the PH3 patients described previously, 50% presented with stones before 5 years of age [5, 7, 15] an observation consistent with the findings reported here. Of particular note in this manuscript are the three patients who did not present until adulthood, a finding which suggests that PH3 may be a milder form of the disease.

Audit of our diagnostic database with > 700 patients with clinical symptoms suggestive of PH has shown that PH1 is the most common disorder (~63% cases) and that PH2 and PH3 account for ~15 and 9%, respectively. For a diagnostic service, it makes sense to look for PH1 first and then, if *AGXT* gene sequencing is negative, look for the common mutations causing PH2 and PH3 before going on to sequence the remainder of these genes.

The frequency of the recurrent c.700 + 5G > T mutation was 67%, which is substantially higher than the frequency of 24% observed in the cases reported previously [7]. The high frequency of this mutation is such that it could be used as a first-line test for the diagnosis of PH3. This mutation causes the loss of an EcoNI site and could therefore easily be screened for by restriction digest analysis if desired. We now offer a Step 1 test for the diagnosis of PH3 that involves sequencing exons 5 and 7 to detect the c.700 + 5G > T mutation as well as the c.944_946delAGG (p. Glu315del) mutation that is prevalent in, but not restricted to, patients of Ashkenazi Jewish descent [5]. In those patients in whom the Step 1 test is negative, the remaining exons of the gene are sequenced in the Step 2 test.

Of 28 unrelated patients analysed in our study, only 15 were found to have mutations in the *HOGA1* gene, suggesting that there are other as yet undetermined defects in glyoxylate metabolism. Previous studies have already ruled out mutations in the *HAO1* [15] and *SLC26A6* genes [16]. Other mitochondrial glyoxylate metabolizing enzymes could be implicated in atypical PH and since they will also likely be relevant to the pathogenic mechanisms of PH3, attention should now be focussed on these enzymes and their role in human glyoxylate metabolism.

Conflict of interest statement. None declared.

(See related article by Hoppe. The enzyme 4-hydroxy-2-oxoglutarate aldolase is deficient in primary hyperoxaluria type III. *Nephrol Dial Transplant* 2012; 27: 3024–3026.)

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