ARTICLE

Novel findings in patients with primary hyperoxaluria type III and implications for advanced molecular testing strategies

Bodo B Beck^{*,1}, Anne Baasner^{1,16}, Anja Buescher^{2,16}, Sandra Habbig³, Nadine Reintjes¹, Markus J Kemper⁴, Przemyslaw Sikora⁵, Christoph Mache⁶, Martin Pohl⁷, Mirjam Stahl⁸, Burkhard Toenshoff⁸, Lars Pape⁹, Henry Fehrenbach¹⁰, Dorrit E Jacob¹¹, Bernd Grohe¹², Matthias T Wolf^{3,13}, Gudrun Nürnberg¹⁴, Gökhan Yigit¹, Eduardo C Salido^{15,16} and Bernd Hoppe^{3,16}

Identification of mutations in the *HOGA1* gene as the cause of autosomal recessive primary hyperoxaluria (PH) type III has revitalized research in the field of PH and related stone disease. In contrast to the well-characterized entities of PH type I and type II, the pathophysiology and prevalence of type III is largely unknown. In this study, we analyzed a large cohort of subjects previously tested negative for type I/II by complete *HOGA1* sequencing. Seven distinct mutations, among them four novel, were found in 15 patients. In patients of non-consanguineous European descent the previously reported c.700 + 5G > T splice-site mutation was predominant and represents a potential founder mutation, while in consanguineous families private homozygous mutations were identified throughout the gene. Furthermore, we identified a family where a homozygous mutation in *HOGA1* (p.P190L) segregated in two siblings with an additional *AGXT* mutation (p.D201E). The two girls exhibiting triallelic inheritance presented a more severe phenotype than their only mildly affected p.P190L homozygous father. *In silico* analysis of five mutations reveals that *HOGA1* deficiency is causing type III, yet reduced *HOGA1* expression or aberrant subcellular protein targeting is unlikely to be the responsible pathomechanism. Our results strongly suggest *HOGA1* as a major cause of PH, indicate a greater genetic heterogeneity of hyperoxaluria, and point to a favorable outcome of type III in the context of PH despite incomplete or absent biochemical remission. Multiallelic inheritance could have implications for genetic testing strategies and might represent an unrecognized mechanism for phenotype variability in PH.

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INTRODUCTION

At the moment three types of primary hyperoxaluria (PH; type I–III) can be accurately defined. In comparison with widespread idiopathic stone disease, they constitute rare autosomal recessive inborn errors of hepatic glyoxylate metabolism with excessive endogenous oxalate synthesis. High urinary oxalate excretion results in calcium-oxalate deposition within the renal parenchyma (nephrocalcinosis) and/or recurrent stone formation (urolithiasis), the clinical hallmarks of PH.¹

Type I PH (PHI, MIM# 259900; gene *AGXT*, MIM# 604285) is caused by deficient or absent activity of liver-specific alanine-glyoxylate-aminotransferase (AGT).^{2,3} It represents the most frequent and most severe PH phenotype with end-stage renal disease (ESRD) being the predictable outcome for the majority of adults. Moreover, ESRD within the first years of life, a condition termed infantile oxalosis,

occurs in a substantial subgroup of children representing 10–18% of the total cohort. In Europe and North America, PH I accounts for about 1% of childhood ESRD, whereas prevalence rates of up to 10% are reported from some countries in the Middle East and North Africa.^{4,5} With advanced renal insufficiency and failure to excrete the metabolic end product oxalic acid, the disease turns into a lethal multisystemic condition making renal replacement therapy and subsequent liver–kidney transplantation mandatory.^{6–10}

Type II PH (PHII, MIM# 260000; gene *GRHPR*, MIM# 604296) is a result of deficient glyoxylate reductase/hydroxypyruvate reductase (GRHPR) enzyme activity.¹¹ In general, PHII shows a milder course with the absence of infantile oxalosis and ESRD occurring in about 20% of patients. The subtype seems to be less frequent, with only 10 documented cases compared with 130 cases of PHI in Germany.

¹Institute of Human Genetics, University of Cologne, Cologne, Germany; ²Department of Pediatrics, University Children's Hospital Essen, Essen, Germany; ³Department of Pediatric and Adolescent Medicine, Division of Pediatric Nephrology University Hospital Cologne, Cologne, Germany; ⁴Department of Pediatrics, Division of Pediatric Nephrology, University of Hamburg, Hamburg, Germany; ⁵Department of Pediatric Nephrology, Medical University of Lublin, Lublin, Poland; ⁶Department of Pediatrics, Nephrology Unit, Medical University of Graz, Graz, Austria; ⁷Department of Pediatric and Adolescent Medicine, Division of Pediatric Nephrology, University of Freiburg, Freiburg, Germany; ⁸Department of Pediatrics, University Children's Hospital Heidelberg, Heidelberg, Germany; ⁹Department of Pediatric Nephrology, Hannover Medical School, Hannover, Germany; ¹⁰Department of Pediatric Nephrology Memmingen, Memmingen, Germany; ¹¹Earth System Science Research Centre and Department of Geosciences, Johannes Gutenberg University, Mainz, Germany; ¹²School of Dentistry, University of Western Ontario, London, Ontario, Canada; ¹³Pediatric Nephrology, Children's Medical Center of Dallas, University of Texas Southwestern Medical Center, Dallas, TX, USA; ¹⁴Cologne Center for Genomics, University of Cologne, Cologne, Germany; ¹⁵Center for Biomedical Research on Rare Diseases, Hospital Universitario Canarias, University La Laguna, Tenerife, Spain

*Correspondence: Dr BB Beck, Institute of Human Genetics, University of Cologne, Kerpener street 34, D-50931 Cologne, Germany. Tel: +49 221 478 86824; Fax: +49 221 478 86812; E-mail: bodo.beck@uk-koeln.de

¹⁶These authors contributed equally to this work.

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Until recently a group of patients displaying significant hyperoxaluria (typically ≥ 1.0 mmol/1.73 m² per day) with negative AGXT plus GRHPR mutational analysis, termed atypical PH, remained ill-defined.^{1,12}

In 2010, Belostotsky *et al*¹³ identified different mutations in the *HOGA1* gene (4-hydroxy-2-oxoglutarate aldolase, formerly known as *DHDPSL*; MIM# 613597) in some of their non-PHI/II patients causing PH type III (PHIII, MIM# 613616). *HOGA1* encodes a mitochondrial protein of 327 amino acids (35 kDa). The enzyme, expressed in liver and kidney, catalyzes the final step of mitochondrial hydroxyproline metabolism from 4-hydroxy-2-oxoglutarate to glyoxylate and pyruvate. Thus, an accumulation of the oxalate precursor glyoxylate could result in subsequent increased oxalate generation and therefore a gain-of-function mechanism was initially proposed by the authors, although activating mutations would be unusual in an autosomal recessive disorder.

Here, we present the results of complete *HOGA1* sequencing in a large cohort of non-PHI/II patients referred to us for suspected PH. These findings expand the spectrum of *HOGA1* mutations, revealing also a potential European founder mutation and the occurrence of triallelic PHIII in two individuals from a consanguineous family who were initially misclassified as having PHI. In addition, we provide further evidence that loss of *HOGA1* enzymatic function is the underlying pathology in PHIII. Furthermore, our clinical data points to characteristic features and the unique outcome associated with this subtype.

MATERIALS AND METHODS

DNA samples from 49 probands of 38 families including 5 consanguineous families with suspected PH referred to our institution were subjected to complete *HOGA1* sequencing. Genomic DNA was extracted from isolated peripheral blood lymphocytes by standard procedures. Only probands without identifiable causes of secondary (hyperabsorptive) hyperoxaluria were included in this study. Forty-two patients were previously tested negative for PHI/II by complete sequencing. Additionally we analyzed seven members from consanguineous family 12 with an established diagnosis of PHI in one individual but with two affected siblings, in whom only a single causative AGXT mutation could be identified on the maternal allele. Large deletions/insertions in the AGXT/GRHPR genes on the paternal allele were excluded using multiplex ligation-dependent probe amplification (MLPA kit P305-B1, MRC Holland, The Netherlands).

Data collection

Clinical information on each patient including age, gender, age at onset of symptoms, chief complaint, persistence of clinical symptoms, range and persistence of hyperoxaluria and hypercalciuria, renal function, type and number of surgical interventions, and imaging data were reviewed. All families except family 5 and family 24 reside in Germany. The ethnic background of the families is as follows: families 1–4, 6–8, 13–21, 23, 25–36 (German); 9, 24 (Turkish); 12, 31 (Lebanese); 22 (Austrian); 10 (Italian); 5 (Polish); 11, 37, 38 (Syrian). Informed written consent was obtained from all the patients and the participating family members. The study was approved by the institutional review board of the University of Cologne, Medical School.

Mutational analysis of the *HOGA1* gene

Mutational analysis was performed by complete exon (1–7) PCR of the *HOGA1* gene. Primer sequences for the exons and adjacent intron–exon borders were designed with the Exon Primer software (<http://ihg.helmholtz-muenchen.de/>). Primer sequences and PCR conditions are available on request. Purified PCR products were sequenced by BigDye terminator ready reaction kit v.1.1 (Life Technologies, Bleiswijk, The Netherlands), using a 3500 Genetic Analyzer (Applied Biosystems, Darmstadt, Germany). Resulting sequences were evaluated with the Sequence Pilot software (JSI Medical Systems GmbH, Kippenheim, Germany). All identified mutations were resequenced and segregation analysis was performed in all the available parents. The novel *HOGA1* mutations were checked in 100 healthy ethnically matched controls. The following databases were used to obtain genetic information: National

Center for Biotechnology Information (NCBI; <http://ncbi.nlm.nih.gov/>), UCSC Genome Bioinformatics (<http://genome.ucsc.edu/>), and 1000 Genomes (<http://1000genomes.org/>). Data on a freeze of about 5000 exomes made available by the NHLBI Exome Sequencing Project (ESP) were checked for the recurrence of any sequence variant identified by us utilizing the Exome Variant Server (<http://snp.gs.washington.edu/EVS/>). Missense variants were subjected to *in silico* analysis using the following tools: Mutation Taster (<http://neurocore.charite.de/MutationTaster/index.html>), PolyPhen-2 (Polymorphism Phenotyping v2, <http://genetics.bwh.harvard.edu/pph2/>), and SIFT (<http://sift.jcvi.org/>).^{14–17}

Automated genotyping and copy number analysis

The Affymetrix (Santa Clara, CA, USA) genome-wide Human SNP Array 6.0 utilizing more than 906 600 SNPs and more than 946 000 probes was used for the detection of copy number variations in patients 13, 14, and 15 (family 12). Quantitative data analysis was performed with GTC 3.0.1 (Affymetrix Genotyping Console; Affymetrix) using a reference file of 100 samples (ATLAS Biolabs GmbH, Berlin, Germany) at the following relevant positions: AGXT: chr2: 241 456 835–241 467 210; GRHPR: chr9: 37 412 707–37 426 986; *HOGA1*: chr10: 99 334 158–99 362 445 (all positions referring to NCBI Build 36.3).

Minigene assay/pSPL3 splicing assay

In vitro analysis of the frequent potential splice-site mutation c.700 + 5G > T was performed using the pSPL3 splicing assay. Fragments of the human *HOGA1* gene containing exon 5, flanked by 500 bp of upstream intronic sequence and 900 bp of downstream intronic sequence, were cloned into the splicing vector pSPL3 generating the plasmids pSPL3-DHDPSL (dihydrodipicolinate synthases)-Exon5-WT and pSPL3-DHDPSL-Exon5 + 5G > T. Plasmids were transfected into HEK 293T cells and mRNA was reverse transcribed.

Site-directed mutagenesis

The coding sequence of *HOGA1* cDNA was amplified from human liver cDNA, cloned into pCIneo vector (Promega, Madison, WI, USA), and its sequence was confirmed. Point mutations were introduced in the plasmid by PCR. The plasmids were sequenced to confirm the distinct mutations and the absence of off-target mutations in the constructs.

Expression of *HOGA1* in Cos and CHO cells

HOGA1 wild type and mutants were transfected in Cos and CHO cells using cationic lipids (Transfast, Promega), following the manufacturers guidelines. Expression was analyzed after 48 h, using an anti-human *HOGA1* antibody raised in rabbits immunized with recombinant human *HOGA1* protein. Mitotracker (Life technologies) was used to label mitochondria. For *HOGA1* immunolocalization, cells were fixed and permeabilized by standard methods, and incubated with 1:5000 dilution of rabbit anti-*HOGA1* serum in 3% BSA. Alexa-488 anti-rabbit IgG (Life technologies), diluted 1:600 in BSA–PBS, was used as secondary antibody and coverslips were analyzed by confocal microscopy. *HOGA1* expression was assessed in cell lysates from the same culture dish. In brief, cells were harvested in RIPA buffer (50 mM TrisHCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS), protein concentration of supernatants was measured by bicinchoninic assay (Sigma, St Louis, MO, USA), and 25 µg protein was separated on 10% acrylamide gels in tris-glycine buffer (SDS-PAGE). Anti-*HOGA1* antibody, diluted 1:10 000 to probe membranes for 2 h, and HRP-conjugated anti-rabbit IgG secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA), diluted 1:20 000 were used.

Urinary oxalate and calcium excretion, plasma oxalate (Pox) monitoring

Urinary oxalate and calcium excretion was measured by standard procedures. A daily urinary oxalate excretion of <0.50 mmol (<45 mg)/1.73 m² and a daily calcium excretion <0.1 mmol/kg (<4 mg/kg) body weight in children, respectively, <8 mmol/d in adults were considered normal for 24 h collected urine samples. In spot samples the age-related reference values for urinary oxalate and calcium:creatinine ratios were used.¹⁸ Pox concentration was determined as previously described by ion chromatography (normal range: 6.3 ± 1.3 µmol/l).¹⁹

RESULTS

HOGA1 analysis

Complete Sanger sequencing of *HOGA1* in 48 probands tested negative for PHI/II identified recessive mutations in 15 patients (31%) from 12 different families (Figures 1a and b). A synopsis of relevant clinical, biochemical and genetic data is given in Table 1 (positive cases) and Table 2 (negative cases).

The c.700+5G>T splice-site mutation was by far the most frequent pathogenic variant found in 14 of 30 disease alleles (allelic

frequency 47%). The mutation was exclusively detected in unrelated, non-consanguineous families from different parts of Germany ($n=7$) and Poland ($n=1$), with six patients bearing the mutation in homozygous state and only two patients showing compound heterozygosity for the splice site and a missense mutation, p.V74G (patient 8, novel) or p.R70P (patient 6, reported). Segregation analysis, proved all parents to be heterozygous carriers. Analysis of the c.700+5G>T mutation using a minigene assay to assess splicing *in vitro* showed activation of a new splice site 52 bases downstream from the

Figure 1 (a) *HOGA1* mutations identified in patients with PHIII. Electropherograms of the predominant splice-site mutation c.700+5G>T and the four novel *HOGA1* point mutations compared with the parental carriers (if available) or wild-type sequence. (b) Pedigree and corresponding electropherograms of kindred 12. Segregation of the c.569C>T (p.P190L) *HOGA1* mutation and the c.603C>A (p.D201E) *AGXT* mutation in a consanguineous kindred from Lebanon.

Table 1 Synopsis of genetic, clinical, and biochemical findings of patients with recessive PHIII mutations (white cells) and additional probands from family 12 (shaded cells, see Figure 1b for details)

P/F sex age (years) EO	HOGA1	AGXT	GRHPR	Onset (years)	Initial complaints	First imaging	UOx range	UCa range	Procedures (number)	Follow-up (years)	Clinical outcome	Imaging outcome	Biochemical outcome	cRF Screat (eGFR)	cPox ($\mu\text{mol/l}$)
1; 1 f	c.700 + 5G > T (splice-site)	Neg.	Neg.	0.1	UTI, rec UL	Bilat UL	0.68–1.95	1.70–4.87	UC (1) dLB (1)	12.4	CR (5)	Normal	HO 1.57	0.52 (120)	10.8
12.5 Ger	c.700 + 5G > T (splice-site)														
2; 2 m	c.700 + 5G > T (splice-site)	Neg.	Neg.	0.8	UTI, rec UL	Bilat UL	0.51–2.49	2.03–6.62	UC (1) PNCL (1) ESWL (16)	13.7	CR (12)	Normal (13.9)	HO 2.49 HC 5.79	0.59 (117)	10.1
14.5 Ger	c.700 + 5G > T (splice-site)														
3; 3 f	c.700 + 5G > T (splice-site)	Neg.	Neg.	3.8	rec UL	Unilat UL	0.98–2.01	1.33–3.19	ESWL (3)	2.2	CR (5.5)	Normal (6.0)	HO 2.01	0.46 (103)	ND
6.0 Ger	c.700 + 5G > T (splice-site)														
4; 4 f	c.700 + 5G > T (splice-site)	Neg.	Neg.	1.5	UTI, rec UL	Bilat UL	0.90–2.39	2.30–4.00	ESWL (13)	8.4	CR (4.5)	Normal	HO 0.90	0.39 (134)	ND
9.9 Ger	c.700 + 5G > T (splice-site)														
5; 5 m	c.700 + 5G > T (splice-site)	Neg.	Neg.	0.1	UTI, rec UL	Bilat UL	0.68–1.33	2.71–7.15	dLB (1) ESWL (2)	6.1	CR (2)	HEK	HO 0.68 HC 5.26	0.46 (103)	7.6
6.3 Pol	c.700 + 5G > T (splice-site)														
6; 6 f	c.209G > C (p.R70P)	Neg.	Neg.	0.75	UTI, rec UL	Unilat UL	0.11–2.54	0.90–4.30	dLB (1) PN (1) ESWL (1)	11.75	Ongoing UL	Unilat UL	ND	0.47 (120)	ND
12.5 Ger	c.700 + 5G > T (splice-site)														
7; 7 f	c.700 + 5G > T (splice-site)	Neg	Neg	0.1	rec UL	Unilat UL	270–376 ^d	0.4–0.69 ^d	None	0.9	CR (0.4)	Normal	HO 376 ^d	0.21 (100)	9
1	c.700 + 5G > T (splice-site)														
8; 8 m	c.221T > G (p.V74G)	Neg.	Neg.	0.3	UTI, rec UL	Bilat UL	763–639 ^d	0.23–0.62 ^d	PNCL (2) ESWL (2)	2.6	Ongoing UL	Unilat UL	HO 639 ^d	0.20 (144)	12.2
2.9 Ger	c.700 + 5G > T (splice-site)														
9; 9 ^c m	c.346C > T (p.Q116X)	Neg.	Neg.	1	rec UL	Bilat UL	0.79–1.99	1.61–8.90	PNCL (1) ESWL (1)	14.3	CR (10)	Normal (14)	HO 0.79	0.75 (90)	ND
15.4 Tur	c.346C > T (p.Q116X)														
10; 10 ^c m	c.733G > A (p.V245I)	Neg.	Neg.	1.8	rec UL	Bilat UL	0.78–1.64	3.53–7.48	UC (1) ESWL (1)	4.6	CR (4.6)	HEK	HO 1.64 HC 5.5	0.38 (121)	6.3
6.4 Ita	c.733G > A (p.V245I)														

Table 1 (Continued)

P/F sex age (years) EO	HOGA1	AGXT	GRHPR	Onset (years)	Initial complaints	First imaging	UOx range	UCa range	Procedures (number)	Follow-up (years)	Clinical outcome	Imaging outcome	Biochemical outcome	cRF Screat (eGFR)	cPox ($\mu\text{mol/l}$)
11; 10 ^c f	c.733G>A (p.V245I)	Neg.	Neg.	2	rec UL	Bilat UL	0.97–1.50	2.90–3.62	None	4.4	abdominal/ loin pain	HEK	HO 1.15	0.36 (126)	6.5
6.4 Ita	c.733G>A (p.V245I)														
12; 11 ^c m	c.728C>A (p.A243D)	Neg.	Neg.	0.6	rec UL	Bilat UL	552 ^d	2 ^d	None	0.2	Ongoing UL	Bilat UL	HO 552 ^d	0.39 (ND)	ND
0.8 Syr	c.728C>A (p.A243D)														
13; 12 ^c m	c.569C>T (p.P190L)	Neg.	Neg.	2	UL	Bilat UL	0.52	9.7 ^e	UC (1)	34	CR	Normal	HC 9.7 ^e	wnl	ND
36.2 Leb	c.569C>T (p.P190L)														
14; 12 ^{b,c} f	c.569C>T (p.P190L)	c.603>A (p.D201E)	Neg.	0.3	UTI, rec UL	Bilat UL	0.60–3.20	2.15–6.60	dLB (1) ESWL (5)	9.5	CR (1)	HEK	HO 2.15 HC 5.15	0.73 (93)	ND
10.1 Leb	c.569C>T (p.P190L)	WT													
15; 12 ^{b,c} f	c.569C>T (p.P190L)	c.603C>A (p.D201E)	Neg.	0.6	rec UL	Bilat UL	1.06–2.01	0.70–3.74	UC (1)	3.6	CR (1)	HEK	HO 1.72 HC 4.30	0.57 (84)	ND
3.9 Leb	c.569C>T (p.P190L)	WT													
16/12 ^c f	c.569C>T (p.P190L)	c.603C>A (p.D201E)	Neg.	NA	Urinary gravel	ND	0.60	3.7	None	ND	CR	ND	HO 0.60	ND	ND
33 Leb	WT	WT													
17/12 ^c m	c.569C>T (p.P190L)	c.603C>A (p.D201E)	Neg.	NA	Urinary gravel	ND	ND	ND	None	NA	CR	ND	ND	ND	ND
51 Leb	WT	WT													
18/12 ^c m	Neg.	c.603C>A (p.D201E)	Neg.	3	rec UL	Bilat UL	0.75–2.10	ND	dLB (1) LKTx	18.4	LKTx (17.8)	NA	ND	1.52 CKD3	29 ^a
21.4 Leb		c.603C>A (p.D201E)													
19/12 ^c f	Neg.	c.603C>A (p.D201E)	Neg.	NA	Urinary gravel	ND	ND	ND	None	NA	CR	ND	ND	ND	ND
44 Leb		WT													

Abbreviations: P, patient/proband; F, family; Neg., negative; UTI, urinary tract infection; rec UL, recurrent urolithiasis; bilat, bilateral; unilat, unilateral; NA, not applicable; ND, no data; CR, complete remission; HO, Hyperoxaluria; HC, hypercalciuria; dLB, diagnostic liver biopsy; HEK, hyperchogenic kidneys; cRF, current renal function; Screat, serum creatinine (mg/dl); wnl, within normal limits, no exact value given in medical reports; eGFR, estimated GFR (ml/min per 1.73 m²); CKD, chronic kidney disease; cPox, current plasma oxalate concentration (Indicates oxalate excretion/plasma oxalate concentration measured at GFR below 40 ml/min per 1.73 m²); WT, wild type; EO, ethnic origin; Ger, Germany; Pol, Poland; Ita, Italy; Syr, Syria; Leb, Lebanon; ESWL, extracorporeal shock wave lithotripsy; PN, percutaneous nephrolithotomy; UC, ureteroscopic kidney stone removal; LKTx, status post liver kidney transplantation. ^aIndicates triallelic PHIII, novel HOGA1 mutations are depicted in bold letters. ^cConsanguinity. ^dOxalate excretion in oxalate (mmol); creatinine (mol) and calcium excretion given in calcium (mol); creatinine (mol). ^eCalcium excretion in mmol/d (adults). Outcome refers to clinical, imaging and urinary status on last follow-up in 2011, numbers in brackets state precise age in years if known. Oxalate excretion in mmol/1.73 m² and calcium excretion in mg/kg body weight per day, except.

Table 2 Synopsis of clinical and biochemical findings of patients tested negative for PHI–III with normal renal function (white cells) and with CKD/ESRD (shaded cells)

P/F	sex	age	AGXT/GRHPR/ HOGA1	Onset (years)	Initial complaints	First imaging	UOx range	UCa range	Procedures (number)	Follow-up (years)	Clinical outcome (years)	Imaging outcome (years)	Biochemical outcome	cRF Screat or CKD_stage	cPox ($\mu\text{mol/l}$)
20/13	f	8.8	Neg.	2.4	Abdominal pain	NC	0.68–1.82	3.62–12.80	None	6.4	CR (3)	NC II	HO 1.21 HC 4.75	0.47	8.41
21/14	m	9.4	Neg.	3.9	nausea rec UL	Unilat UL, NC I	0.56–0.74	3.67–5.62	ESWL (3) PNCL (3)	5.5	rec UL	UL NC I	HO 0.73 HC 5.30	0.36	2.53
22/15	m	15.8	Neg.	0.5	rec UL	Bilat UL	335 ^b	0.40 ^b	OSS (1)	0.5	rec UL	Bilat UL	HO 335 ^b	0.20	ND
23/16	m	15.8	Neg.	ND	Hematuria, UL	Bilat UL	0.57–1.0	0.79	None	5.6	CR (14)	Normal (14)	CR (14.5)	0.60	ND
24/17	m	17.7	Neg.	2.4	Hematuria,	Diffuse NC	0.81–1.36	0.36–0.71	None	7.1	hematuria	HEK	HO 0.87	0.34	1.20
25/18	f	9.5	Neg.	5.5	rec UTI	NC III	0.52–1.28	5.85–10.13	None	3	CR (5.8)	NC III	HC 7.19	0.4	ND
26/19	m	11.6	Neg.	0.5	Abdominal pain	NC II	0.96–1.62	3.95–7.25	dLB (1)	11.1	CR	NC II	HO 1.28	0.41	7.0
27/19	m	14.8	Neg.	3.8	IF	NC I	0.82–1.40	1.80–4.71	None	11	NA	NC I	HO 0.83	0.46	1.99
28/20	m	17	Neg.	12.6	Hematuria, UL	NC II UL	0.56–1.67	4.73–6.60	None	4.2	CR (12.9)	NC II	HC 4.73	0.83	2.74
29/21	m	30/22	Neg.	ND	rec UL	Bilat UL	0.60–1.10	ND	ND	ND	rec UL	ND	ND	wnl	ND
30/22	m	1.8	Neg.	0.4	UTI; UL	Bilat UL	0.72	4.27	None	1.4	CR (1.8)	NC	ND	wnl	ND
31/23	m	1.3	Neg.	0.4	UL	Bilat UL	0.5–2.61	4.12–14.27	ESWL (1)	0.75	CR (0.6)	CR (1.3)	HO 2.61 HC 4.53	0.25	17.3
32/24	f	13.9	Neg.	3.5	rec UL	Bilat UL	0.71–1.76	0.63–1.09	ESWL (2)	10.4	CR	Normal	ND	0.5	ND
33/25	f	15.4	Neg.	3.0	UTI, rec UL	Bilat UL	0.62–1.54	1.20–3.84	ESWL (1) PNCL (1)	12.4	CR	Normal	ND	0.6	ND
34/26	f	8.5	Neg.	5	Hematuria	NC I–II	0.85–1.26	1.70–2.80	None	3.5	CR	NC I–II	HO 1.15	0.6	51
35/27	m	18.5	Neg.	2	UTI, unilat UL	Unilat UL	0.41–1.79	2.12–8.05	None	17.0	CR	Normal (18)	HO 1.09 HC 8.05	0.92	ND
36/28	m	10.5	Neg.	3	Hematuria	NC II	0.39–1.09	4.44–7.43	None	7.5	CR	NC I	HO 0.68 HC 7.43	0.55	ND

Table 2 (Continued)

P/F sex age (years)	AGXT/GRHPR/HOGA1	Onset (years)	Initial complaints	First imaging	UOx range	UCa range	Procedures (number)	Follow-up (years)	Clinical outcome (years)	Imaging outcome (years)	Biochemical outcome	cRF Screat or CKD_stage	cPox ($\mu\text{mol/l}$)
37/29 f	Neg.	3.7	IF	NC II	0.71–1.98	5.03–9.96	None	1.5	CR	NC II	HO 1.58 HC 9.96	0.32	ND
5.1 38/29 m	Neg.	3.7	IF	NC II–III	0.54–2.47	7.9–15.40	None	1.5	CR	NC II	HO 1.53 HC 7.3	0.67	ND
5.1 39/30 f	Neg.	ND	UTI, UL	Bilat UL	0.62–1.20	8.60–12.20	ND	ND	ND	ND	HO 2.47 HC 7.90	Wnl	ND
1.1 40/31 ^c f	Neg.	0.3	rec UL	Bilat UL	401–562 ^b	0.48–1.23 ^b	None	3.1	CR	Normal	HO 106 ^b	0.31	ND
3.4 41/31 ^c f	Neg.	6	UL	Bilat UL	0.39	3.85	None	11.7	CR (7)	Normal	Normal	0.55	ND
17.7 42/31 ^c m	Neg.	3	rec UL	Bilat UL	0.60–1.10 ^a	ND	ESWL (3) PNCL (2) unilat nephrectomy	17	ESRD (33)	ND	NA	ESRD	21 ^a
39 43/32 m	Neg.	21	Hematuria, rec UL	Bilat UL	0.79–1.09	ND	UC (1)	8.0	rec UL CKD2	Bilat UL	HO 0.79	CKD2	ND
29 44/33 m	Neg.	27	rec UTI, rec UL NC III	Bilat UL NC III	0.65–0.96	2.72–7.04	ESWL (5) JJ stenting (4)	21	rec UL, rec UTI CKD3	Bilat UL NC III	HO 0.92	CKD3	12.49
58 45/34 m;	Neg.	10	rec UL	Bilat UL	ND	ND	ND	31	rec UL CKD2	Bilat UL	ND	CKD2	ND
41 46/35 m	Neg.	15	rec UL	Bilat UL	ND	ND	UC (2)	26	CKD 4	Atrophic kidneys	NA	CKD4	ND
47/36 m	Neg.	6	rec UL	ND	ND	ND	PNCL unilat nephrectomy	31	ESRD (25)	Atrophic kidney	NA	ESRD	ND
37 48/37 m	Neg.	ND	rec UL	ND	ND	ND	ND	ND	ESRD (45)	Atrophic kidneys	NA	ESRD	ND
49 49/38 m	Neg.	ND	rec UL	ND	ND	ND	ND	26	ESRD (23)	ND	NA	ESRD	ND

Abbreviations: P, patient/proband; F, family; m, male; f, female; Neg., negative; IF, incidental finding; UTI, urinary tract infection; rec UL, recurrent urolithiasis; bilat, bilateral; unilat, unilateral; NC, nephrocalcinosis grades I, II, III on ultrasound according to Dick; ND, no data; NA, not applicable; CR, complete remission; HO, Hyperoxaluria; HC, hypercalcaemia; dLB, diagnostic liver biopsy; HEK, hypercholesterolemia; cRF, current renal function; Screat, serum creatinine (mg/dl); wnl, within normal limits, no exact value known to us; CKD, chronic kidney disease; ESRD, end-stage renal disease (number in brackets states precise age in years if known); cPox, current plasma oxalate concentration (indicates oxalate excretion/plasma oxalate concentration measured at time of at GFR below 40 ml/min per 1.73 m²); ESWL, extracorporeal shock wave lithotripsy; PNCL, percutaneous nephrolithotomy; UC, ureteroscopic kidney stone removal; OSS, open stone surgery; JJ, double J ureteric stenting. Oxalate excretion in mmol/1.73 m² and calcium excretion in mg/kg body weight per day, except where ^bindicates oxalate excretion in oxalate (mmol)/creatinine (mol) and calcium excretion given in calcium (mol)/creatinine (mol). ^cConsanguinity. Outcome refers to clinical, imaging and urinary status on last follow-up in 2011, numbers in brackets states precise age in years if known.

Figure 2 pSPL3 splicing assay and sequencing result for the frequent splice-site mutation c.700 + 5G>T compared with wild-type cDNA. Fragments of the human *HOGA1* gene containing exon 5 were cloned into the splicing vector pSPL3 generating plasmids pSPL3-*HOGA1*-Exon5-WT and pSPL3-*HOGA1*-Exon5 + 5G>T. After transfection mRNA was extracted from cells and reverse transcribed cDNA was amplified using specific primers within flanking pSPL3 exons. The G>T sequence alteration on position + 5 cripples the wild-type donor site and activates an aberrant donor site on position + 52, which leads to the in-frame insertion of 51 nucleotides of intron 5 (17 amino acids) into the native protein. The interrupted black lines mark the spliced out intronic sequences. The blue box indicates *HOGA1* exon 5 while the blue lines represent the flanking 500 bp of upstream and 900 bp downstream intronic sequence of *HOGA1* exon 5. The gray boxes mark pSPL3 exons A and B with the adjacent black lines depicting the flanking intronic sequence of the pSPL3 exons. The red box represents the 51 bp insertion resulting from the c.700 + 5G>T splice-site mutation.

wild-type donor splice site. The insertion of 51 nucleotides of intron five would lead to an in-frame insertion of 17 amino acids to the native protein (Figure 2).

All other mutations identified were homozygous point mutations (p.P190L, p.A243D, and p.V245I) occurring in single consanguineous families, including a homozygous stop mutation in exon 3 (p.Q116X). Again, segregation analysis confirmed all parents to be heterozygous carriers (except for family 10 due to missing parental material), ruling out *de novo* mutations or large deletions on one paternal allele.

None of the novel point mutations were listed as SNPs in databases (dbSNP, 1000 Genome Project) nor could they be detected in 100 ethnically matched controls, and all were predicted to be disease causing by the Mutation Taster, Polyphen-2, and SIFT software. Multisequence alignment indicated strong evolutionary conservation of these positions and, most importantly, data on 5000 exomes available from the NHLBI ESP did not contain any distinct *HOGA1* mutation identified by us. To further categorize their functional consequences, constructs were introduced into Cos and CHO cells, and protein expression was analyzed by western blot and confocal microscopy. All missense mutants showed similar expression levels and regular mitochondrial localization while the p.Q116X was not expressed, as expected (Figures 3a and b).

In none of the eight PHI/II-negative patients with chronic kidney disease (CKD) or ESRD suffering from recurrent calcium oxalate stone disease we were able to confirm a diagnosis of PHIII (Table 2). Only a single missense *HOGA1* variant p.A148V (rs149896877) was identified in heterozygous state in patient 42 and his two offspring (patients 40, 41). This sequence change could also be found at low frequency (3/10758) on the exome variant server, it was predicted as benign, and the expression and subcellular mitochondrial localization *in vitro* were unremarkable (data not shown).

In a consanguineous Lebanese kindred (family 12; Figure 1b and Table 1, patients 13–19) we failed to establish a molecular diagnosis of PHI in two girls (patients 14, 15) presenting with typical features of infantile onset PH. A definite diagnosis of PHI had previously been made in another family member (patient 18) by liver biopsy (AGT activity 4.4 $\mu\text{mol/h/mg}$ protein, reference range 19.1–47.9) and had been reconfirmed by molecular testing revealing homozygosity for the AGXT p.D201E mutation.²⁰ Complete AGXT/GRHPR sequencing and MLPA performed in patients 14 and 15, however, identified the familial p.D201E mutation only in heterozygous state, segregating from the mother. Both girls were initially misclassified as having PHI, based on their severe phenotype in conjunction with results obtained on a liver biopsy from the older girl showing reduced liver AGT activity (15.8 $\mu\text{mol/h/mg}$ protein; a level also compatible with carrier status in an asymptomatic proband).

Subsequent *HOGA1* sequencing of the father (patient 13) and his two daughters, proved all three to be homozygous for the p.P190L mutation in exon 4, demonstrating triallelic inheritance, defined as the presence of a disease-modulating heterozygous AGXT mutation, in both offspring affected by PHIII. Onset of recurrent urolithiasis in the girls was 0.3 and 0.6 years compared with a single stone episode requiring surgery in their father at the age of two years. Now, at the age of 36 the father is clinically unremarkable apart from marked hypercalciuria (9.7 mmol/d), while his daughters demonstrate persistent substantial hyperoxaluria accompanied by intermittent hypercalciuria. Reanalysis of the *HOGA1* position with the Affymetrix SNP array 6.0 could not detect any copy number variations indicating a deletion of exon 4 on one paternal allele (data not shown). The mother (proband 16) carried heterozygous alterations in both genes (AGXT, *HOGA1*), and her past medical history stated urinary gravel, but she denied actual urolithiasis. Only a single collected 24 h urine

Figure 3 (a) Subcellular localization of HOGA1 protein in transfected Cos cells. Top panel: confocal microscopy of Cos cells transfected with wild-type human *HOGA1* cDNA in pCIneo vector showed the typical filamentous network of mitochondria after incubation with rabbit anti-human HOGA1 antibody and secondary Alexa-488-conjugated anti-rabbit antibody (green fluorescence, left image), which largely coincide with red signal from the mitochondria, visualized using MitoTracker Red (red fluorescence, central image), as can be demonstrated by merging both confocal images (yellow signal, right image). Bottom panel: confocal microscopy of Cos cells transfected with human *HOGA1* variants showed mitochondrial subcellular localization after incubation with rabbit anti-human HOGA1 antibody, like above (green fluorescence). The pattern coincided in all cases with MitoTracker labeled mitochondria (data not shown). (b) Western blot of HOGA1 protein in transfected Cos cells. Top panel: 25 μ g protein from Cos cells transfected with either wild-type (wt) or mutant HOGA1 cDNAs were electrophoresed in denaturing acrylamide gels, transferred to nitrocellulose and probed with rabbit antibody raised against recombinant human HOGA1. A band slightly above the expected 35.2 kDa of the HOGA1 monomer is observed in all lanes except mutant p.Q116X and untransfected Cos cells (cos). Molecular weight markers of 35 and 42 kDa are shown to the left (arrows). Ten μ g mouse liver protein (Lv) were loaded on the right-most lane as a positive control. Bottom panel: loading control showing similar amounts of protein per lane when the filter was re-probed with mouse anti-actin antibody (except for the liver control; last lane to the right, Lv; where the signal could only be seen upon longer exposures of the film (data not shown)).

sample from her was available and displayed normal excretion parameters apart from a moderately elevated urinary oxalate of 0.6 mmol/1.73 m² per day. Rechecking segregation of the p.P190L mutation in additional family members yielded that the father (proband 19) of the PHI index case also carried the *HOGA1* and the *AGXT* mutation in heterozygous state. He denied urolithiasis and objected repeat urinary analysis and ultrasound examination, but his medical records repeatedly mentioned the spontaneous passage of gravel-like material.

Out of the 23 parents being potential *HOGA1* carriers the mothers from families 2 and 5 reported a single episode of urolithiasis

associated with recurrent urinary tract infections during adulthood, while medical history of all other parents was unremarkable.

Outcome

Looking at the phenotype of our PHIII cohort, most patients show an early onset and rather fulminant initial clinical course resembling PHI in some aspects. Median onset was 0.75 years (range 0.1–3.8 years; 1.05 ± 1.02 years mean $\pm$ SD) compared with 2.0 years in 130 fully genotyped cases of the German PHI cohort (7.34 ± 13.11 mean $\pm$ SD). Severity of the condition was also reflected by a high rate of interventional urological procedures (12 out of 15 patients) and

documented liver biopsy in four children at a time when definite diagnosis relied mostly on determination of enzymatic activity. Recurrent urolithiasis and urinary tract infections were the leading symptoms in all PHIII patients, yet severe medullary or diffuse nephrocalcinosis on initial or follow-up ultrasound examination could only be noted among the non-PHIII group (Table 2). The range of oxalate excretion observed in PHIII was highly variable (from within normal limits to severe hyperoxaluria), but largely overlapping with the range seen in PHI and PHII. Next to persistent hyperoxaluria, temporary hypercalciuria of variable severity (range 4.87–8.90 mg/kg body weight/day) was noted in 9 out of 15 patients (60%), a phenomenon not observed in any of our PHI/II patients. On the other hand, the combination of hyperoxaluria plus intermittent hypercalciuria could also be found in comparable frequency among individuals tested negative for PHIII (Table 2). Of the 15 patients with recessive *HOGA1* mutations complete clinical remission was noted in 11 (out of 15) patients (73%) at a median age of 4.5 years (range 0.4–12.0 years). Clinical remission was paralleled by complete normalization of ultrasound findings in seven of them, and only minor residual kidney hyperechogenicity remaining in another five patients. Only three patients exhibited ongoing urolithiasis at the age of 0.8, 2.9, and 12.5 years, respectively, while a fourth patient still complained about loin pains at the age of 6.4 years in the absence of detectable calculi on serial ultrasound examination.

In contrast to overt clinical and radiological remission, persistence of hyperoxaluria was observed in 13 patients, with severe hyperoxaluria above 1 mmol/1.73 m² per day detectable in seven patients. Hypercalciuria in conjunction with hyperoxaluria occurred in three patients, while isolated severe hypercalciuria (9.7 mmol/d) was noted in the adult patient on last follow-up. After a median follow-up time of 6.1 years (range 0.2–34 years) mean estimated GFR was 112 ± 18 ml/min per 1.73 m² (mean ± SD), and no patient showed apparent impairment of renal function or has progressed to ESRD.

DISCUSSION

Clinical diagnosis of PH is based on the hallmarks of recurrent urolithiasis and/or nephrocalcinosis commonly presenting in childhood in combination with the biochemical finding of massively elevated urinary oxalate excretion in the absence of secondary causes. As clinical features especially in infants may be hard to distinguish between the three PH types and prognosis is largely depending on the subtype, a correct diagnosis is of paramount importance. Although the absolute amount of oxalate excretion allows some discrimination, with PHI patients showing the highest and PHIII the lowest oxalate levels,²¹ the large overlap between the types and intra-individual variation of oxalate excretion makes biochemical subtyping an impractical and erroneous approach. With PHIII detected in 31% of formerly unclassified PH patients, this entity is making up for about 10% of all proven cases and is currently the second most frequent PH type in Germany.

In this paper, we describe four novel mutations and 15 new PHIII cases from 12 families including first triallelic PHIII cases, where next to a homozygous *HOGA1* mutation a potentially disease-modulating *AGXT* mutation could be identified. With the detection of a novel homozygous stop mutation in exon 3 and identification of a frequent homozygous *HOGA1* splice-site mutation c.700 + 5G > T, initially reported in heterozygous state as c.701 + 4G > T,¹³ we provide further proof that loss-of-function is likely the underlying mechanism in PHIII. Employing a minigene assay, we were able to detect activation of a cryptic splice-site 51 bp downstream of the wild-type site leading to the introduction of 17 codons in-frame.

While this manuscript was being prepared, Monico *et al*¹⁹ have reported similar findings regarding the c.700 + 5G > T splice-site mutation by utilizing EBV-transformed patient lymphocytes. The high frequency of the c.700 + 5G > T splice-site mutation among unrelated families from central Europe suggests a founder mutation in our population – an important information in terms of rational genetic testing. The in-frame deletion c.944_946delAGG frequently observed in individuals from Ashkenazi Jewish descent could not be found in our population.

Western blot analysis of all novel PHIII missense mutations showed stable expression and regular mitochondrial subcellular localization in transfected Cos and CHO cells. These changes, predicted to be pathogenic by algorithms based on sequence conservation and protein structure, could affect the catalytic activity. The structure of human *HOGA1* protein has been recently published,²² defining key residues of the β -strand 5, part of the active site such as Lys196, which forms a Schiff base with pyruvate, and Ser198, involved in the positioning of the substrate. Thus, the mutation p.P190L, located in the adjacent turn and involving a proline, is likely to disrupt the active site. Missense mutations p.A243D and p.V245I are located at the last β -strand of the (α/β)₈ domain and have the potential to disrupt the hydrophobic core of the barrel and thereby affect protein folding, tetramer formation or alter the distribution of *HOGA1* quaternary structure isoforms. The p.V74G mutation is located close to the G76-X-X-G-E80 pyruvate-binding motif and may affect substrate binding. On the other hand, the conservative change p.A148V, located at the beginning of the α -helix 4, does not affect residues located near the active site or involved in the dimer interphase. Its benign nature is further supported by bioinformatics predictions and its presence in the exome databases. There might be subtle differences in expression levels that could not be detected by our *in vitro* experiments. More information could potentially be obtained from immunohistochemistry or confocal microscopy performed on the kidney and the liver sections in the future, as some patients did undergo biopsy for diagnostic purpose. Differences in the efficiency of *HOGA1* mitochondrial import and leader peptide processing constitute a potential third pathomechanism apart from affecting enzymatic activity and oligomeric state. Close examination of western blot banding seems to reveal two bands with a very small difference in size. The lower one possibly resembles the mature form, after removing the mitochondrial targeting sequence, and some mutations seem to produce stronger unprocessed *HOGA* fractions than others. This finding could be explored by performing *in vitro* transcription–translation and mitochondrial import experiments.

Catabolism of 4-hydroxyproline derived from endogenous (collagen turnover) and dietary sources has been shown to result in significant glyoxylate and oxalate generation, which makes this pathway an interesting tool for understanding the full metabolic spectrum of PH including the so-called gut and kidney axis in hyperoxaluria.^{23–25} However, the precise molecular mechanisms that result in hyperoxaluria as a consequence of defective 4-hydroxyproline catabolism due to *HOGA1* mutations remain unknown, as much as the pathogenesis and the significance of hypercalciuria. Riedel and colleagues²² demonstrated that in contrast to related bacterial enzymes termed DHDPS human *HOGA1* favours forward cleavage of 4-hydroxy-2-oxo-glutarate to glyoxylate and pyruvate.²⁶ Glyoxylate is a highly reactive molecule that is metabolized, in a compartment-dependent manner by either GR (cytosol, mitochondria) to glycolate, by alanine-glyoxylate-aminotransferase (AGT; peroxisome) to glycine or by cytosolic lactate dehydrogenase to oxalate. Mitochondrial buildup of 4-hydroxy-2-oxoglutarate caused by *HOGA1* blockade and subsequent

leakage to the cytosol where a non-specific pyruvate aldolase would catalyze the formation of glyoxylate has been hypothesized.²¹

This study underscores the favorable outcome of PHIII in the context of PH in accordance with previous findings.^{13,21} Moreover, we show that clinical remission occurs in PHIII not because of biochemical remission but despite persisting hyperoxaluria (and to a lesser degree hypercalciuria) in the majority of patients. For the lack of a sufficient number of adults with long-term follow-up and potential sequelae arising from recurrent urolithiasis, we should still be cautious to call PHIII a benign condition. However, the absence of *HOGA1* mutations in our series of unclassified calcium oxalate urolithiasis with CKD/ESRD is reassuring. A self-limiting clinical course in PHIII might be one reason why the number of adult probands identified is so low. It would be intriguing to speculate on age-dependent changes in metabolism and/or diet accounting for the high rate of clinical remission seen with this type, a hypothesis that is challenged by the persistence of hyperoxaluria. In summary, early onset of calcium oxalate urolithiasis that becomes more and more quiescent with age, the presence of intermittent hypercalciuria, and the absence of marked nephrocalcinosis and renal insufficiency all provide valuable clinical clues to a diagnosis of PHIII.

We here report the occurrence of mutations in two genes *HOGA1* and *AGXT* involved in glyoxylate metabolism in a single consanguineous family with several affected members. The two patients with triallelic mutations presented with the highest and most constant oxalate excretion among our cohort and clearly demonstrated a more severe PHIII phenotype compared with the (p.P190L homozygous) father.

Interestingly, both heterozygous *AGXT/HOGA1* carriers (patients 16, 17) had appointments for urinary gravel, but denied any episodes of stone passage. In the absence of confirmed urolithiasis, with sparse or even lacking biochemical and imaging information we classified them as unclear cases. Digenic inheritance of PH remains unknown. The association of heterozygous PHIII mutations to idiopathic calcium oxalate urolithiasis reported by Monico *et al*²¹ presents an exciting hypothesis, in particular in conjunction with the observations from this family, although the low rate of urolithiasis observed in our parental cohort may question this conclusion.²⁷

Our findings are of particular relevance as mutation screening is usually halted as soon as causative mutations have been identified in a first gene. In the light of our working hypothesis that in some cases the PH phenotype may be modulated by mutations in other genes affecting glyoxylate metabolism, complete sequencing of all known causative genes should be considered, notably in those patients who lack a second causative mutation or those with an atypical presentation. This would not only prevent misclassification, but furthermore allow the uncovering of multiallelic inheritance, which may constitute one of the long-sought mechanisms for inter- and intra-familial phenotype variability in PH.

Obviously, much can be gained from a thorough investigation; nevertheless, the specific etiology of PH remains to be elucidated in a significant proportion of patients. Other genes involved in glyoxylate metabolism are promising first candidates. Unraveling the precise molecular and biochemical pathogenesis behind PHIII, a disorder where hyperoxaluria is present from birth, might have therapeutic implications even for the treatment of the more severe PH types. So far, we have thought that the time and the severity of hyperoxaluria in the latter types directly correlate with the clinical course, a concept that now might need some rethinking.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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