Two Novel *HOGA1* Splicing Mutations Identified in a Chinese Patient with Primary Hyperoxaluria Type 3

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Key Words

Primary hyperoxaluria type 3 · *HOGA1* · Splice mutation · Mini-gene assay

Abstract

Background: Twenty-six *HOGA1* mutations have been reported in primary hyperoxaluria (PH) type 3 (PH3) patients with c.700 + 5G>T accounting for about 50% of the total alleles. However, PH3 has never been described in Asians. *Methods:* A Chinese child with early-onset nephrolithiasis was suspected of having PH. We searched for *AGXT*, *GRHPR* and *HOGA1* gene mutations in this patient and his parents. All coding regions, including intron–exon boundaries, were analyzed using PCR followed by direct sequence analysis. *Results:* Two heterozygous mutations not previously described in the literature about *HOGA1* were identified (compound heterozygous). One mutation was a successive 2 bp substitution at the last nucleotide of exon 6 and at the first nucleotide of intron 6, respectively (c.834_834 + 1GG>TT), while the other one was a guanine to adenine substitution of the last nucleotide of exon 6 (c.834G>A). Direct sequencing analysis failed to find these mutations in 100 unrelated healthy subjects and the functional role on splicing of both variants found in this study was confirmed by a minigene assay based on the pSPL3 exon trapping vector. In addition, we found a SNP in this family (c.715G>A, p.V239I). There were no mutations detected in *AGXT* and *GRHPR*. *Conclusion:* Two novel *HOGA1* mutations were identified in association with PH3. This is the first description and investigation on mutant gene analysis of PH3 in an Asian.

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Introduction

Primary hyperoxalurias (PHs) are rare, autosomal recessive, monogenic disorders of glyoxylate metabolism that result in excessive endogenous oxalate synthesis and the formation of calcium oxalate kidney stones [1, 2]. Progressive renal inflammation and interstitial fibrosis from advanced nephrocalcinosis, recurrent urolithiasis and urinary tract infections can cause reduced renal func-

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Fig. 1. Abdomen CT of the proband: **a** bilateral renal calculi; **b** upper ureteral calculi; **c** bladder stones.

tion, systemic oxalate deposition and end-stage renal failure with time [3–5].

There are 3 types of PH. PH1 (MIM# 259900; gene *AGXT*, MIM# 604285), the most frequent and most severe PH, results from mutations in peroxisomal alanine: glyoxylate aminotransferase [6–9]. PH2 (MIM# 260000; gene *GRHPR*, MIM# 604296) is caused by deficient glyoxylate reductase/hydroxypyruvate reductase (GR/HPR) enzyme activity [10]. In general, PH2 shows a less severe phenotype with the absence of infantile oxalosis and ESRD occurring in about 20% of patients [11, 12]. PH type 3 (PH3, MIM# 613616) is a result of the enzymatic function of loss of 4-hydroxy-2-oxoglutarate aldolase (encoded by *HOGA1*, MIM# 613597) [13]. The 4-hydroxy-2-oxoglutarate aldolase is 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. Mutations in *HOGA1* cause the build-up of hydroxy-2-oxoglutarate aldolase, inhibiting GR/HPR function [14]. Compared to PH1 and PH2, PH3 might be the least severe form, with good preservation of kidney function in most patients. The typical clinical characteristic is early onset of recurrent urolithiasis, but less active stone formation later [11, 12, 15–17]. Both PH2 and PH3 may have similar prevalence of about 10% of total genetically characterized PH cases [17].

To date, more than 200 mutations have been described in PH patients, including 26 variants in PH3 with the 2 most common alleles (c.700 + 5G>T and p.E315del) accounting for more than 70% of the total [15–18]. However, PH3 patients among Asians have never been reported. In this article, we report a PH3 case carrying 2 compound heterozygous splicing mutations in *HOGA1*. The effect of these variants on the splicing process was investigated by minigene assays.

Subjects and Methods

Case Report

The proband was a 33-month-old male patient from healthy unrelated parents at full-term normal delivery and with a birth weight of 3.4 kg. His perinatal period was unremarkable. At around 10 months (0.8 year) of age, he was admitted to the Nanjing Children's Hospital because of 'slow urination'. Laboratory data showed leukocyte count to be 9.26×10^9/l, hemoglobin 129 g/l, blood urea nitrogen 1.89 mmol/l and SCr 21 μmol/l. Blood pH was 7.39, CO_2CP 26 mmol/l and serum electrolyte concentrations including K^+, Cl^- and Ca^{2+} were 5.23, 100.6 and 2.68 mmol/l, respectively. The concentration of Ca^{2+} in his urine was 50.5 mg/l, Cr 109 mg/l and the ratio of urinary calcium/creatinine was 0.46 mg/mg (normal values: ≤0.86 mg/mg at 0–7 months, 0.6 mg/mg at 7–18 months, and 0.42 mg/mg at 19 months to 6 years; or as a 24-hour urinary calcium excretion of ≤4 mg/kg/day in children older than 1 year [19]). His parathyroid hormone was 25.20 pg/ml (normal value: 12–88 pg/ml) and 25-hydroxy vitamin D was 65.9 nmol/l (reference ranges: deficiency, <25 nmol/l; insufficiency, 25–74 nmol/l; sufficiency, 75–250 nmol/l; latent intoxication, >250 nmol/l). Ultrasonography of the urinary system revealed suspected nephrolithiasis (up to 7 mm in diameter) in both kidneys and urethral calculus (6 mm in diameter). The infant underwent a cystoscope examination, which alleviated his dysuria by pushing the urethral calculus back into the bladder. Whereafter, the patient was transferred to the Peking University People's Hospital where an abdomen CT demonstrated his bilateral renal calculi, multiple bladder stones and upper ureteral calculi (fig. 1a–c). Under general anesthesia, the patient successively received percutaneous nephrostolithotomy of right kidney, combined with bladder lithotripsy, and of left kidney, respectively. Subsequent analysis revealed the predominant component of renal calcium stones from the operation as calcium oxalate monohydrate and calcium oxalate dihydrate. At the age of 27 months, this family visited our Department of Nephrology to make a definite diagnosis. Clinical characters of the proband including early onset of nephrolithiasis, abnormal increase in urinary excretion levels of oxalate (24-hour oxalate: 1.97 mmol/1.73 m², normal: <0.46 mmol/1.73 m²) and calcium (urine Ca/Cr ratio: 0.45) and decreased level of citrate (24-hour citrate: 329.90 mg/1.73 m², normal: 320–1,240 mg/1.73 m²) suggested the diagnosis of PH. His renal function was normal (GFR = 149.7 ml/min/1.73 m², estimated by Schwartz equation (GFR = 0.41 × height (in cm)/

Table 1. PCR primers for directed sequencing analysis of *HOGA1*

Exon	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product, bp
1	CCTGACCCTGGGAACACC	CTGCCTGTCTCCTGACTCCA	551
2, 3	TGGTTGAGCCACTGCATCCCA	TGGCAGTCCACCCGTTTCCTAT	777
4, 5	CCTGTGGGTGGGCAAGTCT	CCATCCTCCTTCAGCCAACAA	774
6	TTTGATGTAGCCAGCCAAGTGA	CAGTGGTGCTCGACTTTGTCC	461
7	CCCTGGGTGCCATAGAGTTGG	TCTCCGCTTCAGGCTGCAAGT	365

Table 2. PCR primers for directed sequencing analysis of *GRHPR*

Exon	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product, bp
1	GCTTCTGTACTGCCAGGTCC	CCAGAGAACTTTCTCCGAG	257
2	CATCAGAGGCCAGGATTCCCAG	GTATCGCAGGAAACGCCCTCAG	338
3	CAATAATAAGCGGTGTCCCCATG	GAATGGCCGAGGGATATGCAG	267
4	GGAAATGTCCCAGCAGTAGAC	CTCGAACTCCTAACCTCATGATC	283
5	GTTTATACCTTGGACCACAGTCA	CATGTGGGTCTTCTCAGCCTTC	282
6	TGGCCAGAAATGCTGGGTGGTGTC	GCCACCATTGAGAGAAAATACAGCC	253
7	GAGTTTCTGTTAGGGAGTCGG	CTCCAGATGGCAGCAGTG	301
8	GTAGGTTGTTCTGCCTCAAAGGT	CTCACACTAGCTCCACACCCG	302
9	GTGAAGGTGGGAGAGAAGGCAA	TGTCTGACAATACCAGGGCACG	327

SCr)). To confirm the diagnosis, genetic analysis was performed on the patient; his family members and healthy controls gave informed consent. The study protocol was approved by the Ethics Committee on Human Studies at the Affiliated Hospital of Qingdao University.

Mutation Analysis

Genomic DNA was extracted from the peripheral blood of the patients and their family members by GenElute blood genomic DNA kit (Sigma, NA2010). Five and 9 pairs of oligonucleotide primers were generated to amplify all exons and flanking intronic regions of the *HOGA1* and *GRHPR* genes, respectively (tables 1, 2). The primer sequences of *AGXT* were designed according to previous studies [20]. PCRs were performed in 25 μl of solution containing 0.2 mM dNTP, 0.03 U/μl Taq polymerase (Takara EX Taq Hot start version, DRR006B), 2.0 mM $MgCl_2$, 2.5 μl 10 × PCR Mg^{2+}-free Buffer (Takara), approximately 30 ng genomic DNA and 1 mM of each primer. Gradient PCRs were performed with an initial denaturation step at 95°C for 5 min, subsequently followed by 33 cycles with denaturation at 95°C for 45 s, annealing at 56–66°C for 45 s and elongation at 72°C for 45 s. PCR samples were subjected to bidirectional sequencing. The sequence reactions were run on an ABI Prism 3700 DNA Analyzer (Applied Biosystems, Calif., USA).

Minigene Constructions and Expression

To confirm the probable splice mutation affecting an intron–exon junction site, in vitro analysis was performed using a minigene splicing assay based on the pSPL3 exon trapping vector [21]. Fragments with the wild or mutant alleles containing the exon of interest, flanked by upstream intronic sequence and downstream intronic sequence, were cloned into the splicing vector pSPL3 using specific primers linking the XhoI and NheI restriction enzyme sites (TGGAGC^TCGAG: XhoI; AATTTG^CTAGC: NheI). The ancestral and mutant type constructs were named pSPL3-W and pSPL3-M, respectively. All constructs were verified to contain the correct sequence by direct sequencing.

Human Embryonic Kidney 293 T cells were cultured in DMEM medium containing 10% FBS, 1% penicillin-streptomycin (Invitrogen, Calif., USA) at 37°C in a 5% CO_2 atmosphere. One day before transfection, cells were transferred to 6-well culture plate to grow to approximately 70–80% confluence in an antibiotic free medium. Cells were then transfected with 4 μg plasmid DNA (pSPL3-W, pSPL3-M and empty pSPL3-control each) using Lipofectamine 2000 (Invitrogen, Calif., USA), according to the manufacturer's instructions. Cells were harvested, total RNA was extracted from the Human Embryonic Kidney 293 T cells after 48 h of transfection using TRIzol reagent (Invitrogen, Calif., USA) and used for RT-PCR to confirm the splicing patterns. First-strand cDNA was synthesized from 1 μg of total RNA by random-primed reverse transcription using PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Japan). To evaluate the pattern of transcripts from the transfected minigenes, the following vector-specific primers were used for RT-PCR amplification: a forward primer SD6 (5′-TCTGAGTCACCTGGACAACC-3′) and a reverse primer SA2 (5′-ATCTCAGTGGTATTTGTGAGC-3′). The PCR amplification reaction was performed as follows: in 50 μl volume, 2 μl of cDNA, 10 μl of 5× PrimerSTAR Buffer (TaKaRa, Japan), 1 μM of each primer, 0.8 μM dNTPs and 0.5 ul PrimerSTAR HS DNA Polymerase (TaKaRa, Japan) in a 9700 (Applied Biosystem, Calif., USA) thermal cycler. Thermal conditions were 29 cycles of 98°C for 15 s, 58°C for 15 s and 72°C for 50 s, fol-

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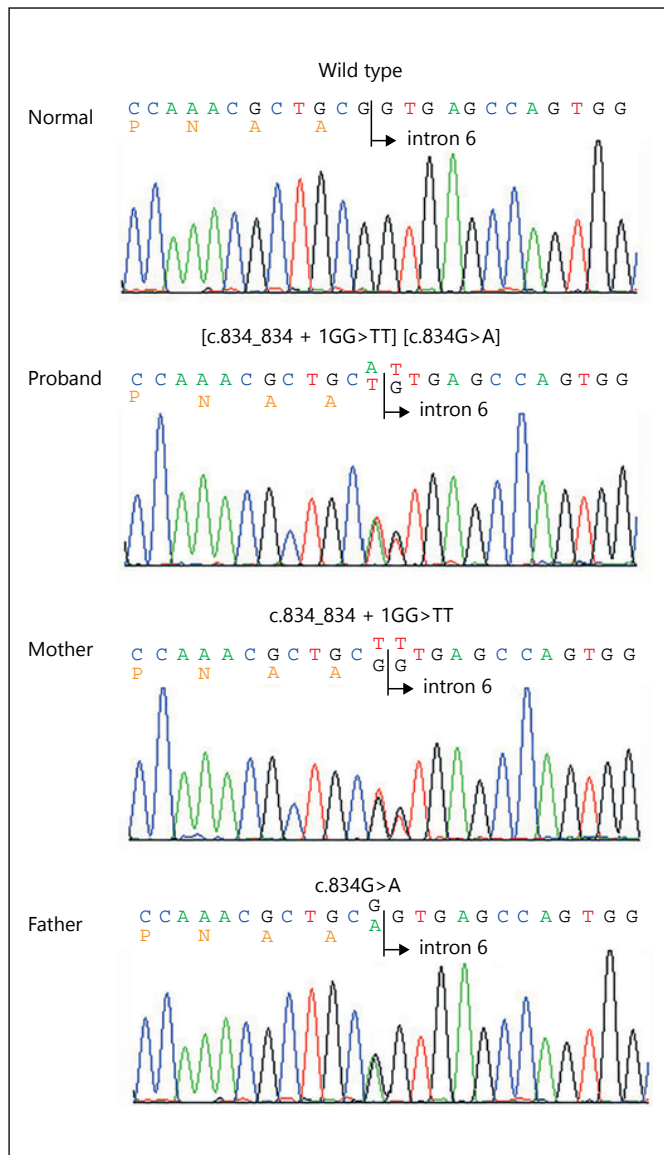
**Fig. 2.** *HOGA1* mutations identified in this Chinese family with PH3.

lowed by a final elongation step at 72°C for 10 min. The PCR products were separated by electrophoresis on a 2% agarose gel, and each band signal was quantified by Quantity One software (Bio-Rad, Richmond, Calif., USA). All transcripts were analyzed by sequencing.

Results

Mutational analysis in the patient showed compound heterozygosity for mutations in *HOGA1* (fig. 2). One mutation was a successive 2-nucleotide substitution at the last position of exon 6 and the first position of intron 6 respectively (c.834_834 + 1GG>TT), including the change of classical donor splicing site (GT→TT) combined with the alteration of its upstream close neighbor nucleotide, both sites may play an important role as splicing modulator. The BDGP splice prediction program (available at http://www.fruitfly.org/seq_tools/splice.html) was employed to test whether this mutation altered the splicing of the *HOGA1* transcript. That prediction resulted in the abolishment of the donor splice site (score cutoff 0.40). The second variant found in the patient was a guanine to adenine substitution of the last nucleotide of exon 6 (c.834G>A), which result in a synonymous mutation (p.Ala278Ala). However, this variant may also affect canonical splice donor nucleotide positioning by BDGP in silico analysis (the score decreases from 0.86 to 0.24). We inferred the haplotype's phase by analyzing the parents DNA. We found that the father carried the heterozygous c.834G>A variant, while the mother carried the heterozygous c.834_834 + 1GG>TT mutation. In addition, we found in the patient a missense mutation (c.715G>A, p.V239I) inherited from his father. PolyPhen-2 (available at http://genetics.bwh.arvard.edu/pph2) analysis predicted that this variant is 'benign'. This mutation is also listed in the NCBI dbSNP database. No mutation was detected in *AGXT* and *GRHPR*. Both the heterozygous c.834G>A and c.834_834 + 1GG>TT mutations were absent in 200 normal chromosomes, while the p.V239I was found in heterozygosity in 6 ones (6 of 200). So p.V239I is unlikely to be a disease-causing mutation, and it is more likely a benign SNP.

To define effects of these 2 novel variants at the transcript level, we performed minigene studies using the pSPL3 plasmids. According to the above description, in 'Methods', the fragments with the wild or mutant alleles involving exon 6 (134 bp), flanked by upstream intronic sequence (388 bp) and downstream intronic sequence (438 bp), were cloned into the splicing vector pSPL3 using specific primers (forward, 5′-TTCCCATCCGTCCTACCCT-3′; reverse, 5′-CCTGCCTTCTAACTCCTTTGC-3′) linking the XhoI and NheI restriction enzyme sites (fig. 3a). The minigene assays showed that both the empty pSPL3 control and c.834_834 + 1GG>TT mutant constructs gave rise to a 263 bp PCR fragment missing exon 6 of *HOGA1* gene, while the wild-type gave rise a 397 bp PCR product containing exon 6. However, the c.834G>A mutant construct yielded 2 lengths of RT-PCR products corresponding to 263 and 397 bp PCR fragments, respectively (the gradation ratio of 2 bands is about 2:1), which was determined by sequencing analysis (fig. 3b).
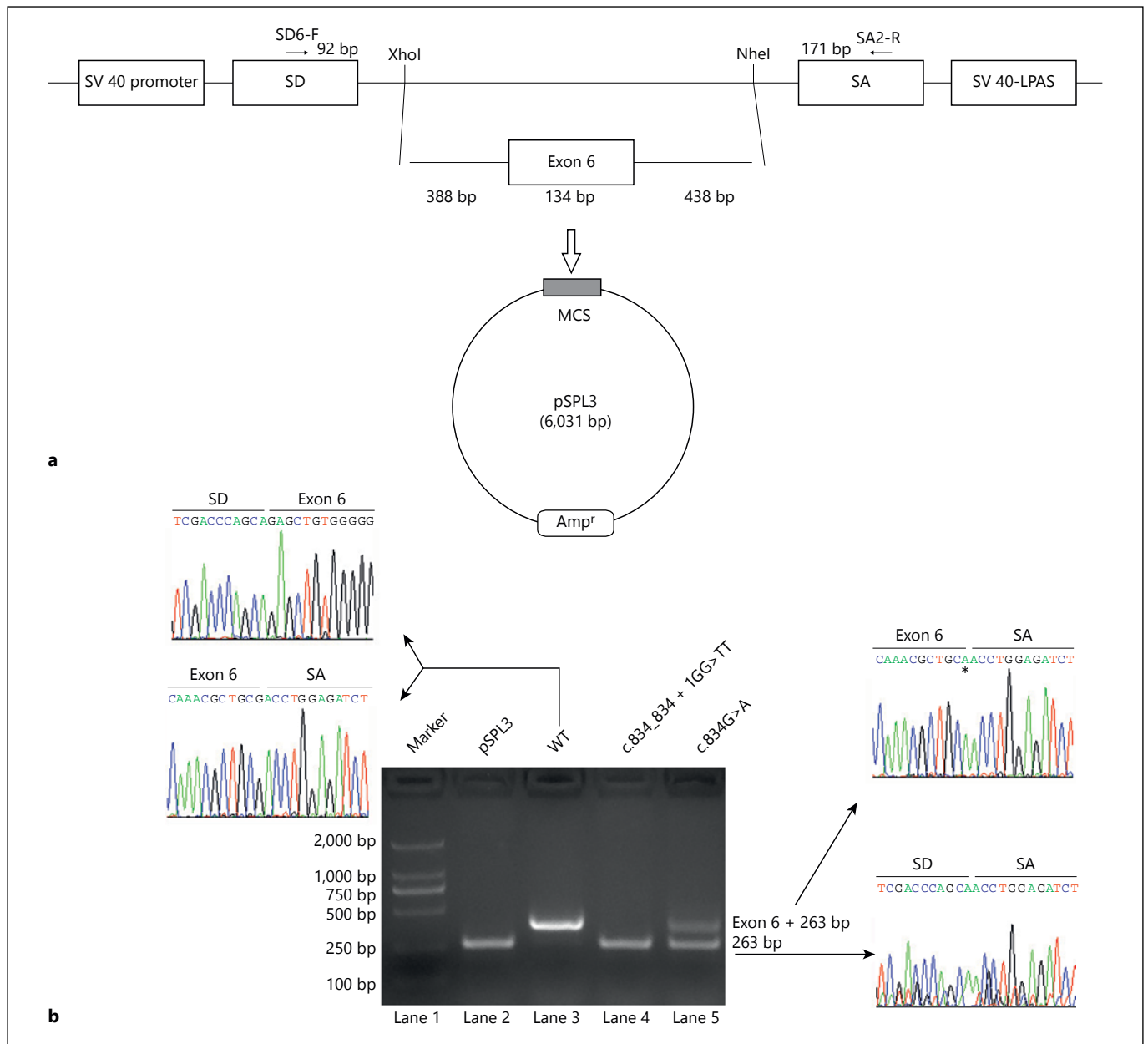
Fig. 3. The minigene splicing assay based on the pSPL3 exon trapping vector. **a** The pSPL3 vector contains 2 exons SD and SA, and a functional intron, with transcription beginning following the SV40 promoter and ending at the LPAS. Wild pSPL3-W and mutant pSPL3-M plasmids containing 388 bp of intron 5, 134 bp of exon 6 and 438 bp of intron 6 were separately cloned into the XhoI and NheI cloning sites of the pSPL3 vector. **b** Agarose gel electrophoresis of RT-PCR products. SD6 and SA2 primers were designed for RT-PCR amplification of cDNA sequences generated by transfected 293 T cells. Lane 1: marker; Lane 2: empty vector (263 bp); Lane 3: 397 bp (263 + 134 bp); Lane 4: 263 bp; Lane 5: 263 and 397 bp (263 r+ 134 bp). MCS = Multiple cloning sites; LPAS = late poly(A) signal.

Discussion

The molecular mechanisms of PH1 and PH2 were identified in 1988 and 1999, respectively [7, 10]. However, the association of PH3 and mutations in *HOGA1* gene was not established until 2010 [13]. So far, 26 variants have been described in the literature for this gene, including 2 splicing variants, 2 deletions, 3 nonsense mutations and 19 missense alterations (online suppl. table 1, see www.karger.com/doi/10.1159/000439232) [15–18, 22,

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23]. Up to now, all the above-described PH3 mutations came from the population of European Americans.

Preliminary mutation analysis of *HOGA1* gene in this family showed that the proband was a compound heterozygote of 2 different mutations. The minigene splicing assay using the pSPL3 plasmids confirmed c.834_834 + 1GG>TT was a spicing mutation causing a complete skipping of exon 6, resulting in a frame shift from codon 235 and premature termination at position 268 in exon 7. Interestingly, the mutation c.834G>A led to the expression of 2 transcripts of which the majority of splice product was missing exon 6, the minority was not. These results demonstrated that the last nucleotide (c.834G) of exon 6 in *HOGA1* gene was also an important controller of splicing, and the change of guanine to adenine at this position led to a significant decrease in the ability of splice site (donor site) recognition. However, we have to point out the limitation of this study in which only a small part of DNA fragment flanking exon 6 was cloned and analyzed in vitro compared with the large fragment with a 9520-bp intron 6. Therefore, we cannot exclude the probability of multiple exons skipping or activation of a cryptic splice site in deep intron 6 not investigated in this study under the circumstance of having a weaker splice site due to the mutation c.834G>A. Unfortunately, it was not possible to analyze the patient's RNA for confirming the in vitro results because we cannot obtain it. In spite of this, we have enough convincing reasons to think of c.834G>A as a virtual splicing mutation. Meanwhile, we should notice that the partial effect of mutation c.834G>A on splicing might lead to a mixed population of transcripts with the abnormal splicing and normal full length transcripts, which gives rise to a normal protein because the c.834G>A mutation is a synonymous mutation (p.Ala278Ala) in vivo, as seen in our study. Therefore, there may be some residual HOGA activity in the body of this patient, although the possibility is small. This study expanded the spectrum of mutations in the *HOGA1* gene associated with PH3 from 26 to 28 ones.

The association of clinical and biochemical characteristics, as well as the results of molecular analysis, suggested the diagnosis of PH3 in this case. However, the proband presented higher levels of urinary oxalate and lower urinary citrate, compared with the most previously described PH3 patients. This discrepancy may be associated with a more severe phenotype (with 2 splicing mutations) in this patient. And that, in turn, may suggest c.834G>A is also a severe splicing mutation. Hence, the evolution and prognosis of the patient deserve close follow-up.

According to the very recent study by Hopp et al. [17], the overall carrier frequency of PH is approximately 1:70, and the inferred prevalence is approximately 1:58,000. Surprisingly, the carrier frequency of PH3 is higher than PH1; however, PH3 is 6-fold less common in currently clinical populations. The difference between the expected and observed prevalence for PH3 may be due to the underdiagnosis of this disease, which has overall milder phenotypes and is much less likely than PH1 to result in ESRD [17]. Additionally, Monico et al. [18] found that some *HOGA1* carriers present with mild hyperoxaluria or idiopathic urinary stone disease, they suppose this may be related to haploinsufficiency. Therefore, *HOGA1* gene analysis of idiopathic calcium oxalate urolithiasis populations and other patients with PH phenocopies, but no known mutations, is likely to be informative regarding undiagnosed PH3 cases.

In summary, we identified 2 splicing mutations of *HOGA1* gene in a Chinese patient with PH3. This is the first report of a PH3 patient among Asians. Further investigations in this population, especially cases with PH phenocopies, such as unexplained hyperoxaluria, early onset of nephrolithiasis or nephrocalcinosis and familial urolithiasis, are highly needed.

Disclosure Statement

The authors have declared that no competing interests exist.

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