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ORIGINAL ARTICLE

Mutations in *HAO1* encoding glycolate oxidase cause isolated glycolic aciduria

Yaacov Frishberg,[1,2] Avraham Zeharia,[3] Roman Lyakhovetsky,[1] Ruth Bargal,[1] Ruth Belostotsky[1]

[1]Division of Pediatric Nephrology, Shaare Zedek Medical Center, Jerusalem, Israel
[2]The Hebrew University School of Medicine, Jerusalem, Israel
[3]Ambulatory Day Care Hospitalization Unit, Schneider Children's Medical Center of Israel, Petach Tikva, Israel

Correspondence to
Professor Yaacov Frishberg, Division of Pediatric Nephrology, Shaare Zedek Medical Center, P.O.Box 3235, Jerusalem 91031, Israel; yaacovf@ekmd.huji.ac.il

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ABSTRACT

Background The primary hyperoxalurias are a group of recessive kidney diseases, characterised by extensive accumulation of calcium oxalate that progressively coalesces into kidney stones. Oxalate overproduction is facilitated by perturbations in the metabolism of glyoxylate, the product of glycolate oxidation, and the immediate precursor of oxalate. Glycolic aciduria associated with hyperoxaluria is regarded as the hallmark of type 1 primary hyperoxaluria. The genetic basis of isolated glycolic aciduria is reported here.

Methods and results Two brothers, born to consanguineous healthy parents of Arab descent, were evaluated for psychomotor delay associated with triple-A-like syndrome (anisocoria, alacrima and achalasia). The proband showed markedly increased urinary glycolic acid excretion with normal excretion of oxalate, citrate and glycerate. Abdominal ultrasound showed normal-sized kidneys with normal echotexture. The genetic nature of triple-A-like syndrome in this kindred was found to be unrelated to this metabolic abnormality. Direct DNA sequencing of glycolate oxidase gene (*HAO1*) revealed a homozygous c.814-1G>C mutation in the invariant -1 position of intron 5 splice acceptor site. Since HAO1 is a liver-specific enzyme, the effect of this novel mutation on splicing was validated by an in vitro hybrid-minigene approach. We confirmed the appearance of an abnormal splice variant in cells transfected with mutant minigene vector.

Conclusions Our results pinpoint the expression of defective splice variant of glycolate oxidase as the cause of isolated asymptomatic glycolic aciduria. This observation contributes to the development of novel approaches, namely, substrate reduction, for the treatment of primary hyperoxaluria type I.

BACKGROUND

Glyoxylate is a two-carbon keto acid generated by intermediary metabolism, with hydroxyproline and glycolate as the best-known sources in humans (figure 1). The normal endpoint of glyoxylate metabolism is conversion to glycine in liver peroxisomes by alanine:glyoxylate aminotransferase (AGT). However, in the cytosol, glyoxylate can readily be oxidised by lactate dehydrogenase (LDH) to oxalate. In vertebrates, oxalate cannot be metabolised and is excreted in the urine. It can form calcium-oxalate crystals that are insoluble in body fluids and tend to precipitate in various tissues, primarily the kidneys. Since LDH is abundant in the hepatocyte cytosol, in order to prevent excessive oxalate synthesis, high concentrations of glyoxylate in the cytosol should be avoided. Perturbations in glyoxylate metabolism are associated with excessive oxalate synthesis, mainly in the liver, leading to hyperoxaluria.

The human liver-specific enzyme glycolate oxidase (HAO1; also known as GO, GOX1 and HAOX1) catalyses the oxidation of glycolate to glyoxylate within the peroxisomes of hepatocytes. HAO1, a member of the FMN-dependent α-hydroxy acid oxidase enzyme family,[1 2] is encoded by an 8 exons-spanning gene that is located on chromosome 20p12. Glycolate derived from vegetable food can readily cross the peroxisomal membrane where oxidation to glyoxylate takes place. The second and major source of glycolate is mitochondrial catabolism of hydroxyproline. Hydroxyproline is derived primarily from animal protein in diet and from the turnover of endogenous collagen, a central constituent of extracellular matrix (figure 1).

The primary hyperoxalurias (PH) are rare recessive disorders that represent glyoxylate metabolic derangements. Phenotypically they are characterised by recurrent formation of kidney stones and nephrocalcinosis, resulting in gradual decline of kidney function. Once renal function deteriorates, excessive oxalate can no longer be eliminated through the urine and thus calcium oxalate crystals precipitate in various tissues, leading to devastating multiorgan disease called systemic oxalosis. Three types of PH have been characterised phenotypically, genetically and biochemically. Primary hyperoxaluria type 1 (PH1; OMIM #259900) is the most common form of PH. This disorder is caused by the absence, deficiency or mistargeting of the liver peroxisomal AGT. Primary hyperoxaluria type 2 (PH2; OMIM 260000) results from deficiency of the enzyme glyoxylate reductase-hydroxypyruvate reductase (GRHPR). We recently posed the deficiency of the mitochondrial enzyme 4-hydroxy-2-oxoglutarate aldolase (HOGA1, formerly DHDPSL) as the primary cause of hyperoxaluria type 3 (PH3; OMIM 613616).[3]

Loss of AGT function causes glyoxylate accumulation within the peroxisome, its transportation into the cytosol and subsequent conversion into oxalate. Increase in glyoxylate concentration leads also to parallel augmentation of the cellular glycolate levels due to reduction by cytosolic GRHPR or HAO1 inhibition by its product. Therefore, the combination of hyperoxaluria and glycolic aciduria comprises the biochemical hallmark of PH1. Prior to our discovery of PH3 pathogenetic mechanism,

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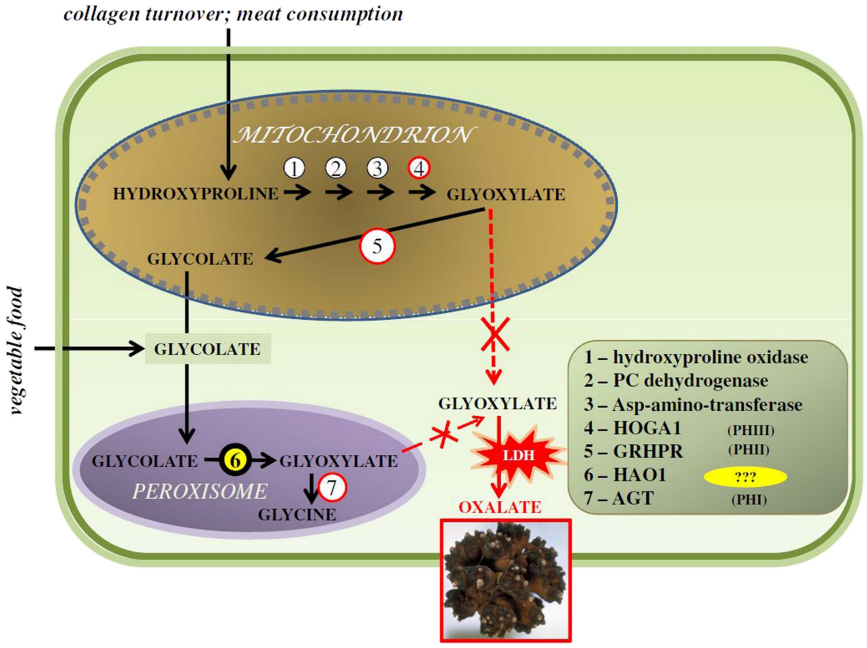

Figure 1 Outline of glyoxylate metabolism in human liver. Enzymes are designated by numbers (listed in the right corner). Enzymes associated with different forms of hyperoxaluria are designated with red circles. HAO1 is marked in yellow.

mutations in *HAO1* were investigated as a possible cause of hyperoxaluria in a group of patients who did not map to PH1 or PH2, but this was refuted.[4]

We here describe the first patient with loss-of-function homozygous mutation in *HAO1*, resulting in massive glycolic aciduria, likely to be asymptomatic. This observation concurs with the phenotype of *HAO1*-deficient mice that have glycolic aciduria but are otherwise healthy.[5]

METHODS

Patients

Two brothers, born to consanguineous healthy parents of Arab descent, were evaluated for psychomotor delay (figure 2). Subsequently, anisocoria and alacrima were noted in both, while only one of them developed achalasia as well (II/1). None manifested adrenal insufficiency, and they were diagnosed clinically as having triple-A-like syndrome. Three sisters were healthy. Urinary organic acid profile performed in the 8-year-old proband (II/5) showed persistent markedly increased urinary glycolic acid excretion (2000 mmol/mol creatinine; normal reference for age: 18–92). Further evaluation revealed normal urinary excretion of oxalate, citrate and glycerate. Serum amino acid profile was unremarkable with normal glycine levels (268 µmol/L; N:12-480). Abdominal ultrasound demonstrated normal-sized kidneys without nephrocalcinosis or nephrolithiasis.

The brother with the triple-A-like syndrome (II/1) and all three healthy sisters (II/2-II/4) had normal urinary glycolate excretion.

The study was approved by the Shaare Zedek Medical Center Helsinki Committee.

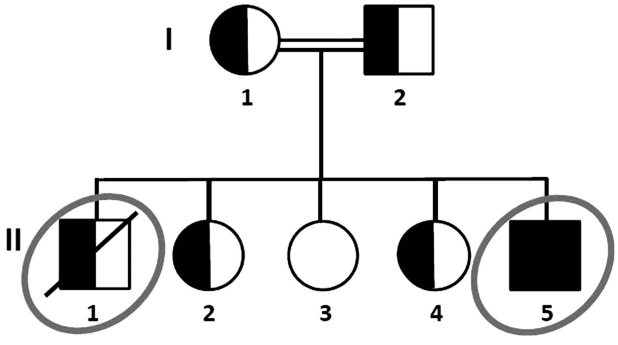

Figure 2 Pedigree of the study subject family. The filled symbols represent mutated *HAO1* alleles. Children with the triple-A-like syndrome are designated with grey ellipses.

Urinary organic acid concentrations

Urinary organic acid profile was performed by gas chromatography-mass spectrometry.

Mutation analysis

Direct sequencing of all exons and the exon–intron boundaries of genomic DNA was used for mutation screening in the *HAO1* gene.

HAO1 minigenes containing exon 5, a 5′ fragment of intron 5, a 3′ fragment of intron 5 through exon 7 and differential for c.814-1G>C mutation were generated by PCR and sequentially cloned into pEGFP-C2 (Clontech), resulting in pHAOex5-7WT and pHAOex5-7Mut vectors. Sequencing confirmed that the inserts differed only by the c.814-1G>C mutation. Chinese hamster ovary (CHO) cells were maintained in α-MEM supplemented with 5% fetal bovine serum, 50 U/mL penicillin and 50 µg/mL streptomycin. Cells were seeded in six-well plates (2×10^5 cells per well) and allowed to grow for 24 h (50–70% confluency) before transfection with 1 µg of pHAOex5–7WT or pHAOex5–7Mut minigene vectors using TranslT-LT1 transfection reagent (Mirus) in accordance with the manufacturer's instructions. One day after transfection, total DNA and RNA were prepared. The RNA was treated with DNase and reverse transcribed by using random hexamers per the instructions of the manufacturer (BioLab, Israel). DNA and cDNA encoding *HAO1* minigene fragments were amplified by PCR using primers corresponding to exon 5 and exon 7 and analysed on agarose gel. The products resulting from cDNA amplification were subjected to sequencing.

RESULTS

The proband had markedly augmented urinary glycolate levels, approximately 20-fold increase above the upper limit of normal for age, which persisted since infancy. His urinary oxalate concentrations were always normal and he has not developed kidney stones or nephrocalcinosis. The combination of glycolic aciduria with normal urinary oxalate excretion led us to assume that the patient has non-functional HAO1. Indeed, lack of activity of this enzyme, which catalyses the oxidation of glycolate to glyoxylate in hepatocytes, should lead to the accumulation of glycolate and prevent formation of glyoxylate—the sole precursor of oxalate. Direct sequencing of all exons and the exon–intron boundaries of *HAO1* demonstrated that patient II/5 is homozygous for c.814-1G>C mutation (figure 3). We surmised that this mutation should eliminate the splice acceptor site, thus skipping exon 6 and giving rise to an aberrant transcript. The resulting mRNA is predicted to encode a protein internally shortened by 53 amino acids (p.272_324del) with no frameshift and to be expressed at normal level. Both parents were found to be heterozygous for this mutation. His affected brother with triple-A-like syndrome who had normal urinary glycolate excretion and their three unaffected sisters were not carrying bi-allelic mutations in *HAO1*. The c.814-1G>C mutation represents an unknown variant and was not reported in the Database of Single Nucleotide Polymorphisms (dbSNP) and 1000 genome databases.

To confirm the predicted effect of the splice site mutation, we tried to amplify *HAO1* cDNA derived from RNA extracted from a blood sample collected from patient II/5. However, since the expression of this gene is restricted to hepatocytes, the amplification was unsuccessful.

To overcome this hurdle, we implemented the method of minigenes,[6] which allows investigating the consequences of splice site mutations in an in vitro system. Since intron 5 is very long (>9 kbp), exon 5 and the 5′ fragment of intron 5 of *HAO1* were fused in frame 3′ to the EGFP sequence in pEGFP-C2 vector, followed by fragment of the *HAO1* gene including 3′ portion of intron 5 with the branch point and exons 6 through 7, resulting in pHAOe5-7WT plasmid. pHAOe5-7Mut plasmid represents the same construct, but carrying the c.814-1G>C mutation (outlined in figure 3). Total DNA and cDNA preparations from CHO cells transiently transfected with these plasmids were amplified with a forward primer matching *EGFP-HAO1*—exon 5 junction and a reverse primer matching exon 7. The DNA product was 1053 bp in length in both lines, the cDNA product resulted from the spliced RNA product of

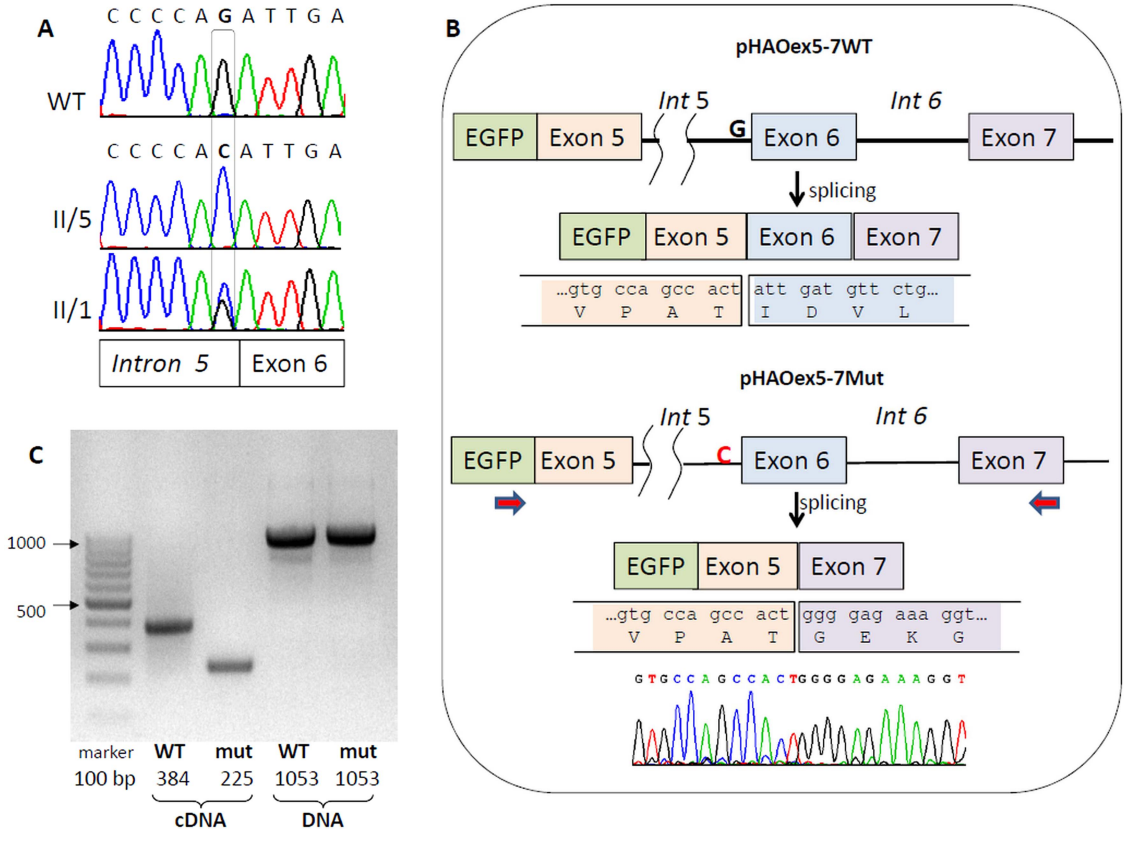

Figure 3 Detection of homozygous c.814-1G>C mutation in *HAO1* in proband II/5 and confirmation of its effect on splicing. (A) Electropherograms of 5′ boundary of *HAO1* exon 6 of control individual and cases II/5 and II/1; (B) schematic structure of the *HAO1* minigene reporter. A genomic segment of *HAO1* containing exons 5–7 (including 5′ and 3′ fragments of intron 5 and the entire intron 6) was subcloned downstream to *EGFP* in the pEGFP-C2 vector, resulting in pHAOex5-7WTand pHAOex5-7Mut plasmids differing in the c.814-1G>C mutation. The mutated variant was amplified from DNA of patient II/5. Chinese hamster ovary (CHO) cells were transfected with either the WT or Mut plasmid. cDNA from CHO cells carrying pHAOex5-7Mut was amplified with the primers shown by red arrows. Splicing of pHAOex5-7Mut minigene results in skipping exon 6. The electropherogram of this transcript is shown below; (C) PCR amplification of cDNA and DNA preparations from CHO cells transfected with ether pHAOex5-7WT or pHAOex5-7Mut plasmids with the primers shown by red arrows in (B). The calculated lengths of unspliced DNA and of spliced transcripts resulting from WT and Mut plasmids are displayed.

pHAOex5-7WT vector containing exons 5, 6 and 7 was 384 bp and the pHAOex5-7Mut product containing only exons 5 and 7 was 225 bp long (figure 3). Skipping of exon 6 in the mutation-bearing product was confirmed by sequencing.

DISCUSSION

We describe an 8-year-old child with extremely high urinary glycolate levels since infancy, without hyperoxaluria or kidney stone disease, due to homozygous loss-of-function mutations in *HAO1* encoding glycolate oxidase. This splice site-destroying mutation leads to production of shortened protein (p.272_324del). Since the tertiary structure of the HAO1 molecule has been described in detail,[7–9] we evaluated the consequences of skipping 53 residues encoded by exon 6. Deletion of α-helices 6, 7 and E as well as β-strands 7 and 8 should lead to destabilisation of the entire structure and particularly interfere with binding of the cofactor FMN.

The proband and his brother had in addition a triple-A-like syndrome characterised by anisocoria, alacrima and in one of them, also achalasia. Homozygous mutations in *GMPPA* (c.1000A>C, p.Thr334Pro), encoding guanosine diphosphate (GDP)-mannose pyrophosphorylase A, were recently found to be responsible for the triple-A-like syndrome in this kindred.[10] It is unlikely that either of the proband's symptoms are attributable to his genetic variant in *HAO1* as his brother had a similar phenotype without glycolic aciduria or homozygous mutations in *HAO1*. Also, none of their three healthy sisters had glycolic aciduria or bi-allelic mutations in this gene. Glycolic aciduria was not mentioned among the symptoms of the remaining 11 patients suffering from triple-A-like syndrome due to mutations in *GMPPA*. Although the precise pathogenetic mechanism underlying this type of triple-A-like syndrome remains elusive, it is suggested that this is an N-glycosylation disorder and that GMPPA may have a regulatory role in this process. These two genes are physically distant: *GMPPA* is on chromosome 2 and *HAO1*—on chromosome 20. Taken together, it is most likely that HAO1 deficiency in this patient is asymptomatic and is unrelated to his triple-A-like syndrome. Our observation concurs with the genetically manipulated *HAO1*-deficient mice model that display glycolic aciduria but are otherwise healthy.[5]

Glycolic aciduria is usually associated with hyperoxaluria, reflecting a disorder of glyoxylate metabolism, namely, primary hyperoxaluria. This combination has been regarded as the biochemical hallmark of PH1, although a portion of patients with PH3 were also found to have similar characteristics.[11 12] Currently, the only curative treatment of PH1 is preemptive liver transplantation, and once end-stage renal disease develops, a combined liver and kidney transplantation is indicated.[13] This therapeutic strategy carries major risks and is not readily available to many patients around the world. Several attempts have been made to develop better therapeutic strategies for this devastating disease. Among them was substrate reduction therapy (SRT), namely, an attempt to decrease oxalate production by decreasing cellular concentrations of its precursor, glyoxylate. One of the proposed treatments relies on reactivity of the carbonyl group of glyoxylate with the free sulfhydryl group of cysteine. It has been suggested that this approach has a significant potential because of the proximity of glyoxylate to the terminal step in oxalate synthesis. The cysteine precursor, (L)-2-oxothiazolidine-4-carboxylate (OTZ), was used as the therapeutic agent because of its lower toxicity. Although OTZ has been shown to decrease urinary oxalate concentration in the rat model of hyperoxaluria, a small study in patients with PH1 failed to replicate these findings.[14] Subsequently, it was suggested that the most appealing target should be to develop specific inhibitors of HAO1, encoding glycolate oxidase, as this is a non-essential metabolic pathway leading to glyoxylate.[5] HAO1 inhibitors are currently being developed as potential treatment for primary hyperoxaluria type 1.[8 15] However, the consequences of depletion of activity of this enzyme in humans remain elusive. Preliminary results show that *HAO1*-deficient mice have glycolic aciduria but otherwise are asymptomatic and that knocking out this gene in PH1 mice results in marked reduction in urinary oxalate excretion.[5] Our observation that a loss-of-function mutation in *HAO1* results in isolated but asymptomatic glycolic aciduria should encourage researches to further pursue this approach.

Contributors YF and AZ initiated the study. Molecular studies were done by RBe, RL and RBa. Clinical phenotyping was done by AZ and YF. The manuscript was written by YF and RBe with contributions and comments from all authors.

Competing interests None.

Patient consent Obtained.

Ethics approval The Shaare Zedek Medical Center Helsinky Committee.

Provenance and peer review Not commissioned; externally peer reviewed.

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Notes