Published in final edited form as:
Am J Kidney Dis. 2008 December ; 52(6): 1096–1103. doi:10.1053/j.ajkd.2008.07.041.

Phenotypic and Functional Analysis of Human *SLC26A6* Variants in Patients With Familial Hyperoxaluria and Calcium Oxalate Nephrolithiasis

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Abstract

Background—Urinary oxalate is a major risk factor for calcium oxalate stones. Marked hyperoxaluria arises from mutations in two separate loci, *AGXT* and *GRHPR*, the causes of primary hyperoxaluria (PH) types 1 and 2, respectively. Studies of null *Slc26a6 (−/−)* mice have revealed a phenotype of hyperoxaluria, hyperoxalemia and calcium oxalate urolithiasis, leading to the hypothesis that *SLC26A6* mutations may cause or modify hyperoxaluria in humans.

Study Design—Cross-sectional, case-control.

Setting & Participants—Cases were recruited from the International Primary Hyperoxaluria Registry. Control DNA samples were from a pool of adult subjects who identified themselves as being in good health.

Predictor—PH1, PH2, non-PH1/PH2 genotypes in cases.

Outcomes & Measures—Homozygosity or compound heterozygosity for *SLC26A6* variants. Functional expression of oxalate transport in *Xenopus* oocytes.

Results—A total of 80 PH1, 6 PH2, 8 non-PH1/PH2 and 96 control samples were available for *SLC26A6* screening. A rare variant, c.487C>T (p.Pro163Ser) was detected solely in one non-PH1/PH2 pedigree but this variant failed to segregate with hyperoxaluria, and functional studies of oxalate transport in *Xenopus oocytes* revealed no transport defect. No other rare variant was identified specifically in non-PH1/PH2. Six additional missense variants were detected in controls and in cases. Of these, c.616G>A (p.Val206Met) was most common (11%), and showed a 30% reduction in oxalate

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transport. To test p.Val206Met as a potential modifier of hyperoxaluria, we extended screening to PH1 and PH2. Heterozygosity for this variant did not affect plasma or urine oxalate in this population.

Limitations—We did not have a sufficient number of cases to determine whether homozygosity for p.Val206Met might significantly affect urine oxalate.

Conclusions—*SLC26A6* was effectively ruled out as the disease gene in this non-PH1/PH2 cohort. Taken together, our studies are the first to identify and characterize *SLC26A6* variants in hyperoxaluria. Phenotypic and functional analysis excluded a significant effect of identified variants on oxalate excretion.

Introduction

In industrialized nations, symptomatic urolithiasis has a life-time prevalence ranging from 5.9 to 12.5% in men and 3.7 to 5% in women (1–3) with the principal crystal component in the majority (70–80%) of these stones consisting of calcium oxalate monohydrate (whewellite) or dihydrate (weddellite) phases (4). Familial aggregation patterns and more recently, studies in twins, have suggested a high degree of heritability (5–7). Of the multiple metabolic abnormalities identified in these patients, even mild increases in the urinary excretion of oxalate, detected in about 20% (8), have been shown to significantly impact calcium oxalate supersaturation and consequently, calcium oxalate crystal mass and the risk of calcium oxalate stone formation (9).

Marked hyperoxaluria (> 1.0 mmol/1.73m^2/24 h) arises from mutations in 2 separate loci, *AGXT* and *GRHPR*, the causes of primary hyperoxaluria types 1 (PH1, OMIM [Online Mendelian Inheritance in Man] 259900) and 2 (PH2, OMIM 260000), respectively. To date, a third locus with large (monogenic) influence on urinary oxalate excretion is suspected, based on identification of several pedigrees with a phenotype similar to primary hyperoxaluria types 1 and 2 (marked and sustained hyperoxaluria, and calcium oxalate urolithiasis) in whom deficiencies of the hepatic enzymes (alanine-glyoxylate aminotransferase and glyoxylate reductase/hydroxypyruvate reductase) encoded by these genes (*AGXT* and *GRHPR*), have been excluded (10,11). Though the cause remains unknown, the term ‘non-PH1/PH2’ primary hyperoxaluria has been used to describe these families.

Notably, among recent findings in two separate lines of null *Slc26a6 (−/−)* mice are hyperoxaluria (12,13), with additional observations of hyperoxalemia and calcium oxalate urolithiasis in one of these null lines (12). A defect in intestinal oxalate secretion by chloride/oxalate exchange mediated by Slc26a6 has been demonstrated to account for the hyperoxaluria. In humans, the product of the *SLC26A6* gene, solute carrier family 26, member 6, is widely expressed in many tissues, with abundant expression in kidney, pancreas and colon (14–16). Although functional expression studies have shown that SLC26A6 can function in multiple modes of anion exchange involving chloride, formate, bicarbonate, sulfate, hydroxyl and oxalate as substrates (17–21), direct assessment of SLC26A6 transporter function in *vivo*, via generation of the null *Slc26a6 (−/−)* mice, has clearly underscored a key role in oxalate homeostasis. These studies have led to the hypothesis that *SLC26A6* mutations may cause or modify hyperoxaluria in humans (12).

To explore the possibility that sequence alterations in *SLC26A6* may impart large (monogenic) or small (polygenic) phenotypic effects, we screened families with non-PH1/PH2, PH1 and PH2 primary hyperoxaluria for *SLC26A6* variants. Because of the significant intra and inter-familial variability observed in PH1 and PH2 patients and the so far undefined genetic basis of non-PH1/PH2 primary hyperoxaluria, we deemed *SLC26A6* to represent an especially relevant and logical biological modifier or candidate gene in these phenotypes.

Methods

Study Design and Population

We recruited cases from the International Primary Hyperoxaluria Registry (IPHR) for participation. The IPHR is a secure, web-based international disease registry for patients with primary hyperoxaluria (types 1, 2 and non-PH1/PH2). All IPHR patients from the Mayo Clinic who consented to molecular research testing were included, and provided informed consent or assent, following approval by our Institutional Review Board. Control DNA samples were from a pool of adult subjects who identified themselves as being in good health. Because this was not a prospective study, specific clinical characteristics of the control group, other than healthy status, were not available. Both case and control populations were predominantly white. Genomic DNA was extracted from peripheral blood leukocytes using standard methods.

A total of 80 PH1, 6 PH2, 8 non-PH1/PH2 and 96 control samples were available for *SLC26A6* screening. A diagnosis of primary hyperoxaluria (AGT deficiency, PH1; GRHPR deficiency, PH2) was confirmed by hepatic enzyme analysis in the proband or an affected sib (n=73 PH1, n=2 PH2), or by demonstration of 2 known causative mutations and supportive biochemical data (n=7 PH1, n=4 PH2). In the 8 non-PH1/PH2 patients, normal hepatic activities of AGT and GRHPR were documented in the proband or affected sib, and secondary causes of marked hyperoxaluria, including hyperabsorption of oxalate from the gastrointestinal tract, were excluded. The clinical features of 6 of these 8 non-PH1/PH2 patients have been published previously (10). Urine and plasma oxalate were measured by the oxalate oxidase method (22).

Genotyping

The entire coding sequence (21 exons) of *SLC26A6*, including exon-intron boundaries, was amplified and sequenced using the primer sequences and PCR reaction conditions established by Waldegger et al (15) using 96-well plates. All samples were sequenced in both directions using an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, CA) and chromatograms analyzed using both the 4.5 version of Sequencher Software (Gene Codes Corp, Ann Arbor, MI) and Mutation Surveyor v2.41 software from SoftGenetics, LLC. ‘SLC26A6’ and ‘Slc26a6’ denote the human and mouse orthologs, respectively, in keeping with the recommendations of the Human Genome Organization (HUGO) for genetic nomenclature. Nucleotide numbering refers to the cDNA sequence of human SLC26A6 splice isoform 1 (NM_022911.2), except that the +1 position corresponds to the “A” of the first ATG.

Statistical Analysis

The chi-square or Fisher’s exact test was used to test the association of genotype (homozygous wild, heterozygous, homozygous variant) with primary hyperoxaluria subtype (PH1, PH2, non-PH1/PH2) and controls. The rank-sum test was used to compare urine and plasma oxalate levels in those with versus those without specific *SLC26A6* variants. All tests were two-sided with alpha-level 0.05.

Oocyte Functional Expression Studies

Human *SLC26A6* (transcript variant 1) cDNA in pCMV6-XL4 was purchased from OriGene (OriGene, Rockville, MD). For expression studies, the coding sequence was amplified by PCR using primers designed to incorporate upstream and downstream BamHI and XbaI sites, respectively (forward primer: CAGGATCCACCATGGACTACCCATACGATGTTCCAGATTACGCTCTGCGGAGGC GA GACTAC; reverse primer: CATCTAGAGCAGACAAATCTTTATTCCTGAG). The forward primer also contains a sequence coding for the hemaglutinin (HA) tag (underlined),

which was inserted at one amino acid after the Kozak sequence. The product of the amplification reaction was digested with BamHI and XbaI and the hSLC26A6 coding region was ligated into the *Xenopus* expression plasmid pGH19 (23) and fully sequenced.

The missense mutations, p.Pro163Ser and p.Val206Met, were generated in human *SLC26A6* cDNA by using the QuikChange Site-Directed Mutagenesis technique (Stratagene, La Jolla, CA) in pGH19 containing full-length *SLC26A6* with 5'-HA tag. The cRNA of wild-type and both mutant *SLC26A6* cDNAs was expressed using the mMessage mMachine High Yield Capped RNA Transcription Kit (Ambion, Austin, TX).

The human *SLC26A6* cRNA of wild-type and mutant (p.Pro163Ser and p.Val206Met) was heterologously expressed in *Xenopus* oocytes as described previously (17). Oocytes were injected with 50 nl of water (control), wild-type SLC26A6 cRNA (25 ng), or *SLC26A6* cRNA (25 ng) containing the single base pair change resulting in either the p.Pro163Ser or p.Val206Met missense mutations. Oocytes were then incubated in ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5 mM Hepes, pH 7.5; supplemented with 5 mM sodium pyruvate and 50 units/ml penicillin-streptomycin) at 16° C for 48 hrs prior to transport studies. We then measured [^{14}C] oxalate uptake in the presence of an outward chloride gradient as a measure of transport function of SLC26A6 (17), since chloride-oxalate exchange is the favored mode of operation of SLC26A6 (12,17) and it is the mode most relevant to oxalate homeostasis (12,13). To assay chloride-oxalate exchange, oocytes were washed twice in 1 ml chloride-free buffer (98 mM potassium gluconate, 1.8 mM hemi-calcium-gluconate, 1 mM hemi-magnesium gluconate, 5 mM Tris-Hepes, pH 7.5) and then incubated in 500 μl of uptake solution (100 mM potassium-gluconate, 5 mM Tris, pH adjusted to 7.5 with Hepes) containing 20 μM [^{14}C]oxalate. After 30-min incubation at room temperature, external isotope was removed by washing the oocytes three times with 1 ml of ice-cold chloride-free buffer. Radioisotope content of each individual oocyte was measured by scintillation spectroscopy after solubilization in 0.2 ml of 10% SDS and addition of 3 ml scintillation fluid (Opti-fluor, Packard). Results shown in the bar graphs represent means +/− SE. In some cases the S.E. values are too small to be visible in the figures.

Yolk-free protein lysate from oocytes was prepared as described previously (24). In brief, oocytes were homogenized by pipetting in 20–40 μl (per oocyte) homogenization buffer (100 mM NaCl, 1% Triton X-100, 20 mM Tris-HCl, pH 7.6) containing a Complete Protease Inhibitor Cocktail tablet (Roche Biochemicals, Indianapolis, IN). After 5-min incubation on ice, the homogenate was centrifuged (15,000g, 4°C, 5-min), and the supernatant was retained for gel electrophoresis. Proteins were separated by SDS-PAGE using 4–15% polyacrylamide gels (Bio-Rad, CA), with subsequent electrotransfer to polyvinylidene difluoride (PVDF, Immobilon-P, Millipore).

Surface-expressed proteins were biotinylated by a modification of the procedure of Hassan et al (24). Oocytes were washed five times in ND96 solution at 4°C and then incubated in the same solution containing the biotinylation reagent Sulfo-NHS-LC-biotin (2.2 mM; Pierce) at 4°C twice for 30 min. The oocytes were then incubated for 10 min in ND96 containing 5 mM glycine (to quench excess biotin) and subsequently washed twice for 10 min in ND96 solution at 4°C. The oocyte protein lysates were prepared as described as above. Oocyte lysates of equivalent amounts of protein were incubated 1 hour using a rotatory shaker with strepavidin-agarose beads at 4°C. The streptavidin-agarose beads were washed 3 times with oocyte lysate buffer. Biotinylated oocyte proteins were dissociated from the beads with SDS sample buffer (10% SDS, 2% beta-mercaptoethanol, 20% glycerol, 5 mM Tris-HCl, pH 6.8) containing 100 mM dithiothreitol. We performed immunoblotting using standard procedures described previously (25). The primary rabbit anti-HA was from Zymed Laboratories (Zymed, CA).

Results

The clinical characteristics of our study population are as listed in Table 1. Whole gene *SLC26A6* sequencing in cases with hyperoxaluria as well as controls revealed 7 coding sequence variants (Table 2). None of these 7 *SLC26A6* coding variants were detected in homozygosity or compound heterozygosity in any of our non-PH1/PH2 probands (Table 3). Six of these 7 coding variants were also detected in controls (Table 2), with c.616G>A (encoding a valine to methionine substitution at amino acid 206 [p.Val206Met]) occurring with the highest frequency (11%). Interestingly, we detected c.487C>T (encoding a proline to serine substitution at amino acid 163 [p.Pro163Ser]) solely in a non-PH1/PH2 family but the change did not segregate with the hyperoxaluria phenotype (Figure 1).

The function of c.616G>A (p.Val206Met) and of c.487C>T (p.Pro163Ser) was evaluated by functional expression studies in Xenopus oocytes. We measured chloride-oxalate exchange activity, as it is the favored mode of operation of SLC26A6 (12,17), and it is the mode whose absence leads to hyperoxaluria in null *Slc26a6 (−/−)* mice (12,13). Whereas p.Pro163Ser showed no detectable defect in oxalate transport (Figure 2A), the most common variant, p.Val206Met, showed a 30% reduction in oxalate transport activity compared to wild-type (Figure 2B). This reduction in oxalate transport activity by p.Val206Met was not attributable to a difference in total *SLC26A6* protein expression (Figure 3A), or in *SLC26A6* expressed on the plasma membrane as detected by surface biotinylation (Figure 3B).

p.Val206Met appeared to co-segregate with three intronic changes, forming a haplotype. These 3 intronic variants are IVS2–4C>A, IVS5+30C>T, and IVS9+32C>A, where numbering is based on either counting back from the 3’ end of the specified intervening sequence (negative number), or counting forward from the 5’ end of the specified intervening sequence (positive number). The c.673C>G (encoding a glutamine to glutamate substitution at amino acid 225 [p.Gln225Glu]) change w0061s detected on this haplotype background in one PH1 patient. c. 1906G>A (encoding an aspartate to asparagine substitution at amino acid 636 [p.Asp636Asn]) segregated with IVS18–51C>G in 2 PHI and 4 control patients in whom it was detected.

To test the hypothesis that p.Val206Met and potentially other functional *SLC26A6* missense variants may behave as hypomorphic (modifying) alleles that influence the degree of hyperoxaluria or hyperoxalemia in primary hyperoxaluria patients (PH1, PH2, non-PH1/PH2), we determined allelic and genotype frequencies of all coding variants (Table 2), and tested for correlations with urine and plasma oxalate (Figure 4).

Genotype frequencies did not differ significantly between primary hyperoxaluria subtype groups (PH1, PH2, nonPH1/PH2) and controls for any of the *SLC26A6* variants (Table 2). Moreover, we did not detect a statistically significant difference in plasma or urine oxalate in primary hyperoxaluria patients harboring *SLC26A6* sequence variants versus those without (Figure 4).

Discussion

In humans, urine oxalate excretion is a known risk factor for calcium oxalate stone formation (26–28). Recently, a *Slc26a6* knock-out mouse model was described with hyperoxaluria, hyperoxalemia, calcium oxalate urolithiasis, and diminished serosa-to-mucosa oxalate flux across intestine leading to increased net absorption of dietary oxalate (12). Consequently, we hypothesized that genetic variation in human *SLC26A6* might influence disease expression in patients with phenotypes characterized by hyperoxaluria and calcium oxalate urolithiasis.

To examine the possibility that rare functional *SLC26A6* sequence variants might account for the as yet undefined suspected monogenic basis of non-PH1/PH2 primary hyperoxaluria, we

sequenced the entire coding region and exon-intron boundaries of *SLC26A6* in 6 affected pedigrees. We did not identify any pathogenic alleles (Table 3), effectively excluding *SLC26A6* as the monogenic cause of the marked hyperoxaluria and calcium oxalate urolithiasis in our particular non-PH1/PH2 patient cohort. These findings are consistent with our previously published study of these patients, showing normal enteric oxalate absorption (10).

We were, however, very much intrigued by our detection of a functional missense variant (p.Val206Met) of relatively high frequency (11%) in human *SLC26A6*, in both cases and controls. This common polymorphism, first described by Waldegger et al (15), seemed a promising candidate modifier of hyperoxaluria and calcium oxalate urolithiasis phenotypes, given our *in vitro* functional analysis demonstrating a moderate defect in oxalate transport (30%), as also recently reported by Clark et al (29).

To test the hypothesis that presence of the p.Val206Met variant might influence urine oxalate excretion or plasma oxalate concentration in patients with types 1 and 2 primary hyperoxaluria, we also screened for this variant in these population subsets. Heterozygosity for p.Val206Met did not appear to influence urine oxalate excretion in the primary hyperoxaluria patients tested (Figure 4). This is perhaps not surprising, since heterozygosity for this variant may be expected to result in only a 15% defect in transport, which might not be sufficient to alter oxalate homeostasis. And in our population of endogenous over-producers of oxalate, such an effect may be masked. Unfortunately, we did not have a sufficient number of patients to determine whether homozygosity for this variant might significantly affect oxalate excretion. It is important to note, however, that no other common frequency variants with potential to alter function were identified in *SLC26A6*. As such, the probability that heterozygosity for common *SLC26A6* variants is associated with hyperoxaluria and that it thus alters the risk of idiopathic calcium oxalate urolithiasis is low.

In conclusion, our studies are the first to identify and characterize *SLC26A6* variants in patients with hyperoxaluria. Phenotypic and functional analysis excluded a significant effect of these variants on oxalate excretion in this patient population. It is conceivable that the modest effect of a common frequency functional variant such as p.Val206Met may become discernible only in phenotypes characterized by lesser degrees of hyperoxaluria, and that genotype frequencies differ in other calcium oxalate stone formers. *SLC26A6* screening in such populations may thus prove informative. Additional members of the SLC26 transporter family, such as *SLC26A1*, have also been shown to transport oxalate (20,30), so future studies should also be directed at similarly evaluating these as potential candidate (non-PH1/PH2) or modifier (PH1, PH2) genes in hyperoxaluria.

Acknowledgements

We are grateful to our patients for their gracious participation.

Support: Research funding was received from the National Institutes of Health [grants DK 64865 (Dr Monico), DK 73354 (Dr Milliner) and DK 33793 (Dr Aronson)] and the Oxalosis and Hyperoxaluria Foundation (Dr Milliner).

Financial Disclosure: None.

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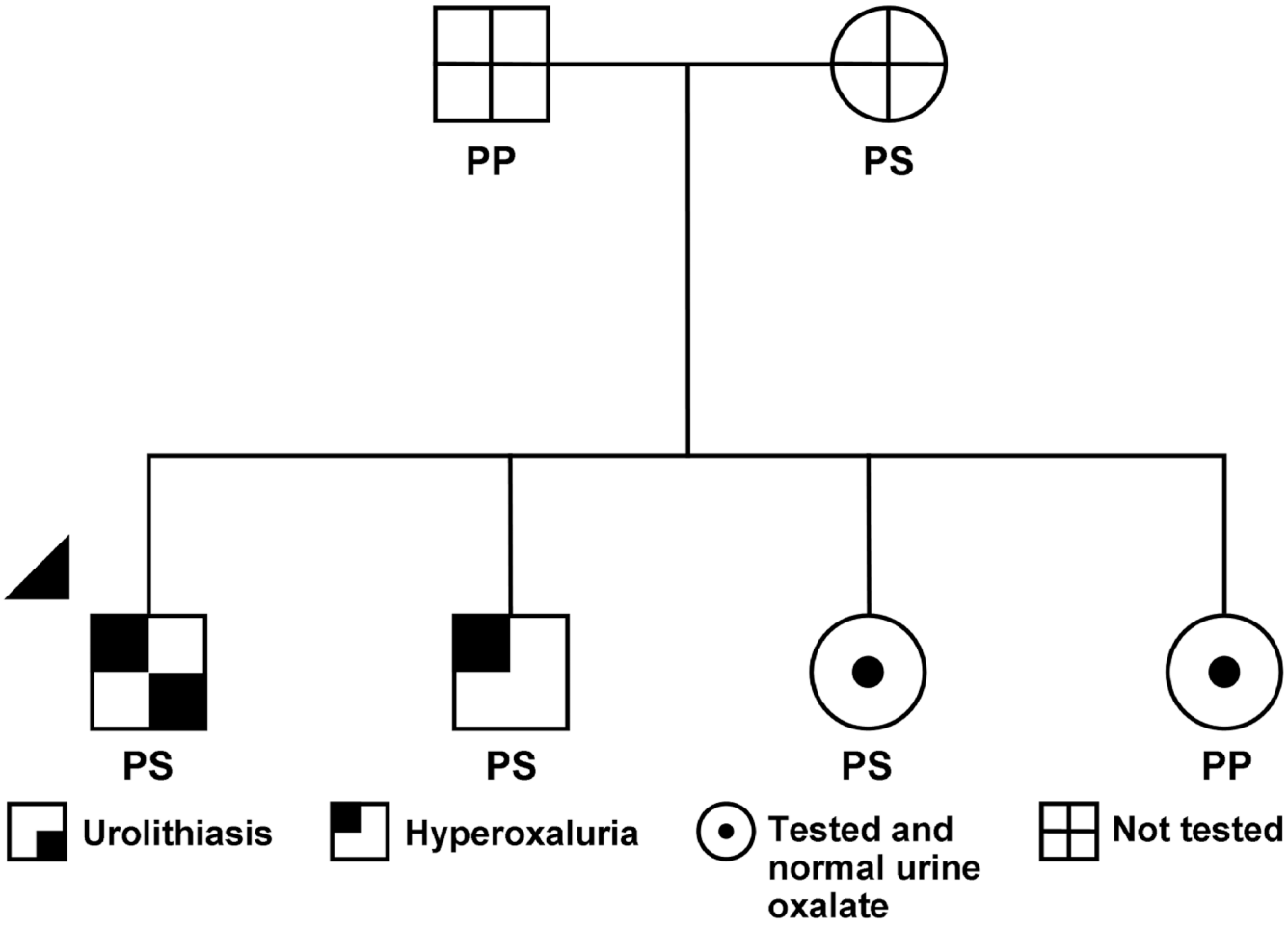


Figure 1. Segregation Analysis of the c.487C>T (p.Pro163Ser) Variant in Non-PH1/PH2 Pedigree # 6

The proband is indicated by the arrow. P, proline allele; S, serine allele.

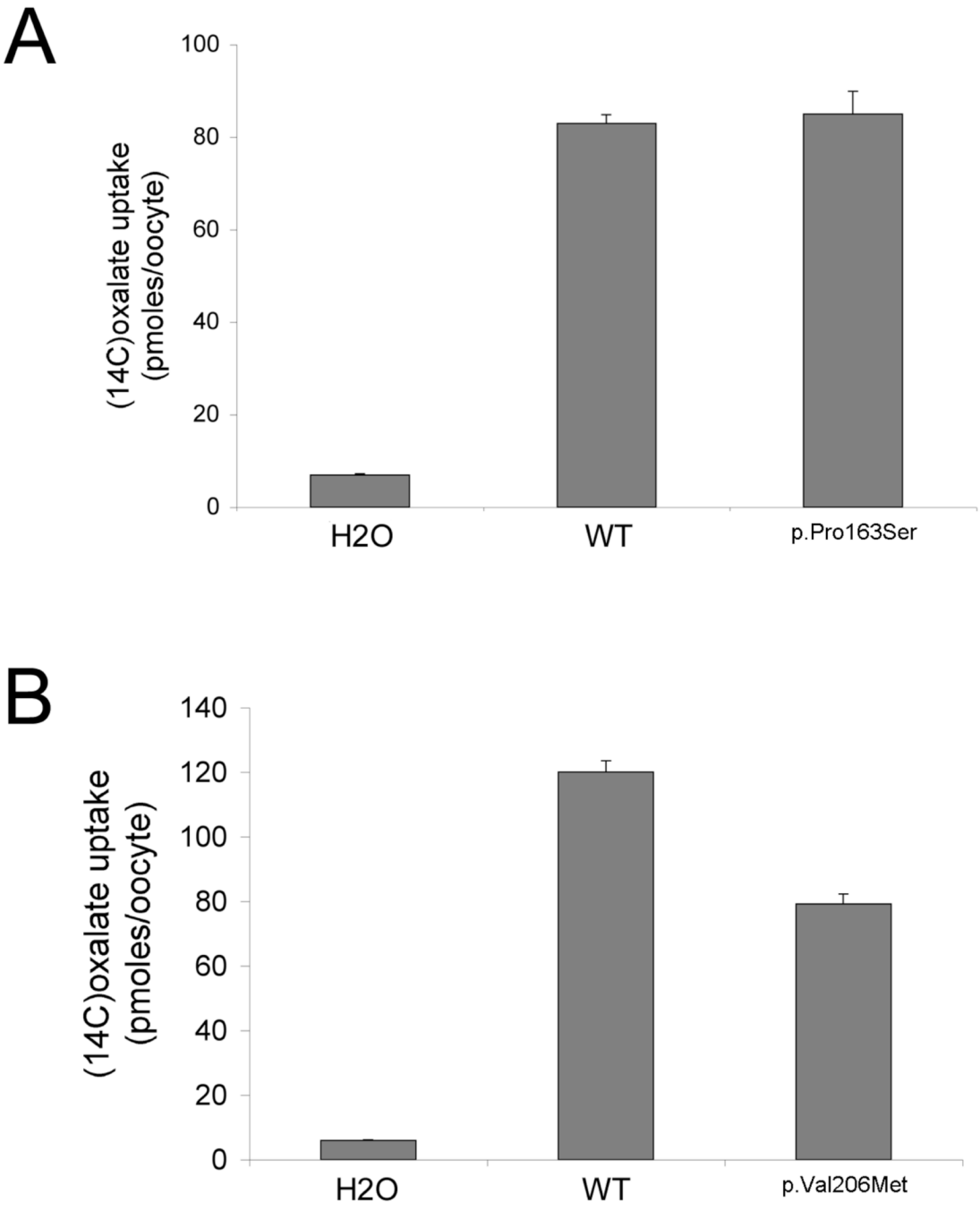


Figure 2. 2A–2B Oxalate transport activity of c.487C>T (p.Pro163Ser) (A) and c.616G>A (p.Val206Met) (B) sequence variants of SLC26A6 expressed in Xenopus oocytes

Oxalate transport activity of oocytes injected with cRNA encoding wild-type (WT) and variant SLC26A6 is compared to water-injected control oocytes. Data represent means ± SE for n = 20–24 oocytes in each group (A), or 50–60 oocytes in each group (B). The difference between wild-type and p.Val206Met transport activity (B) was highly significant ($P < 10^{-13}$ by Student's t-test).

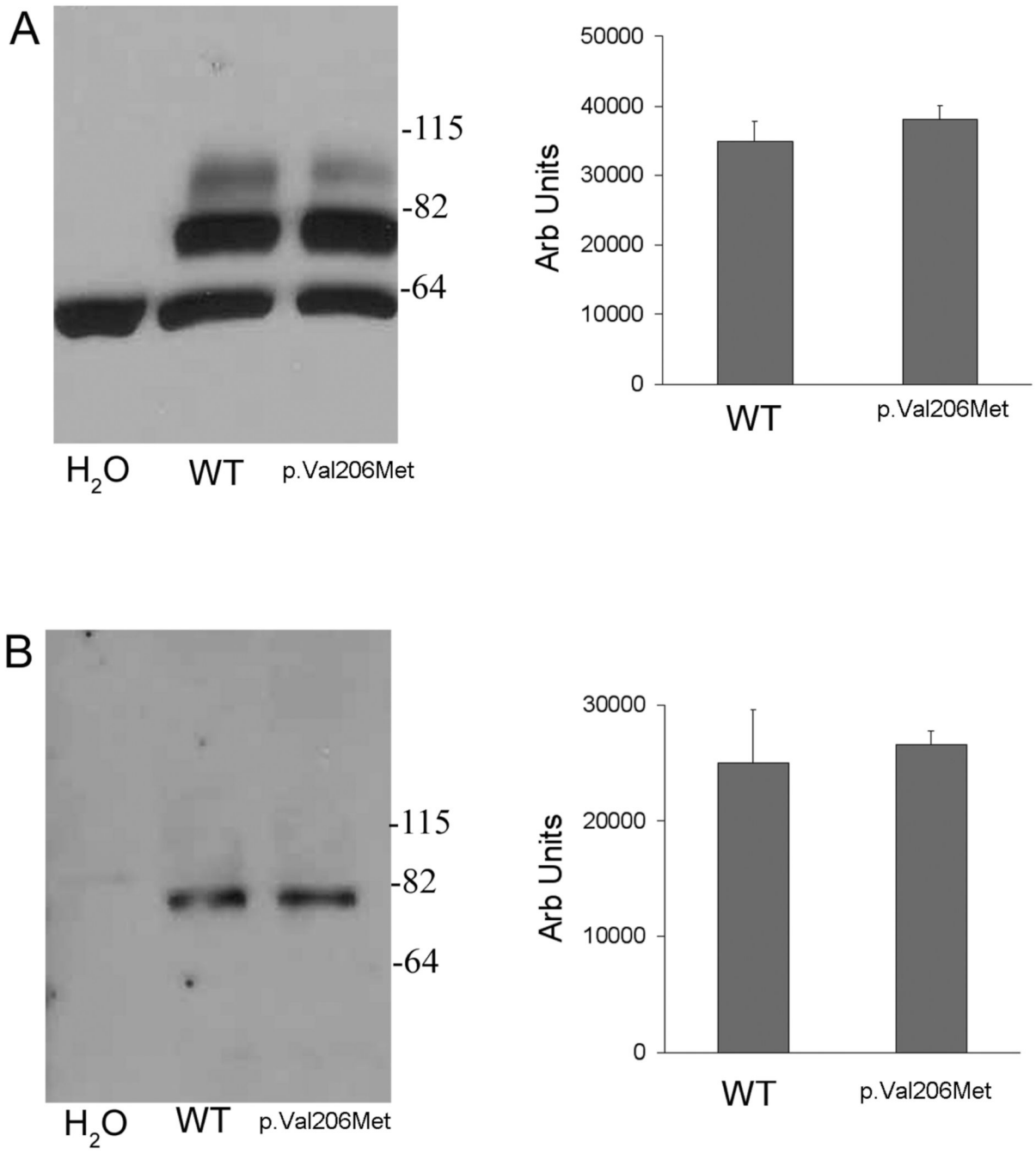
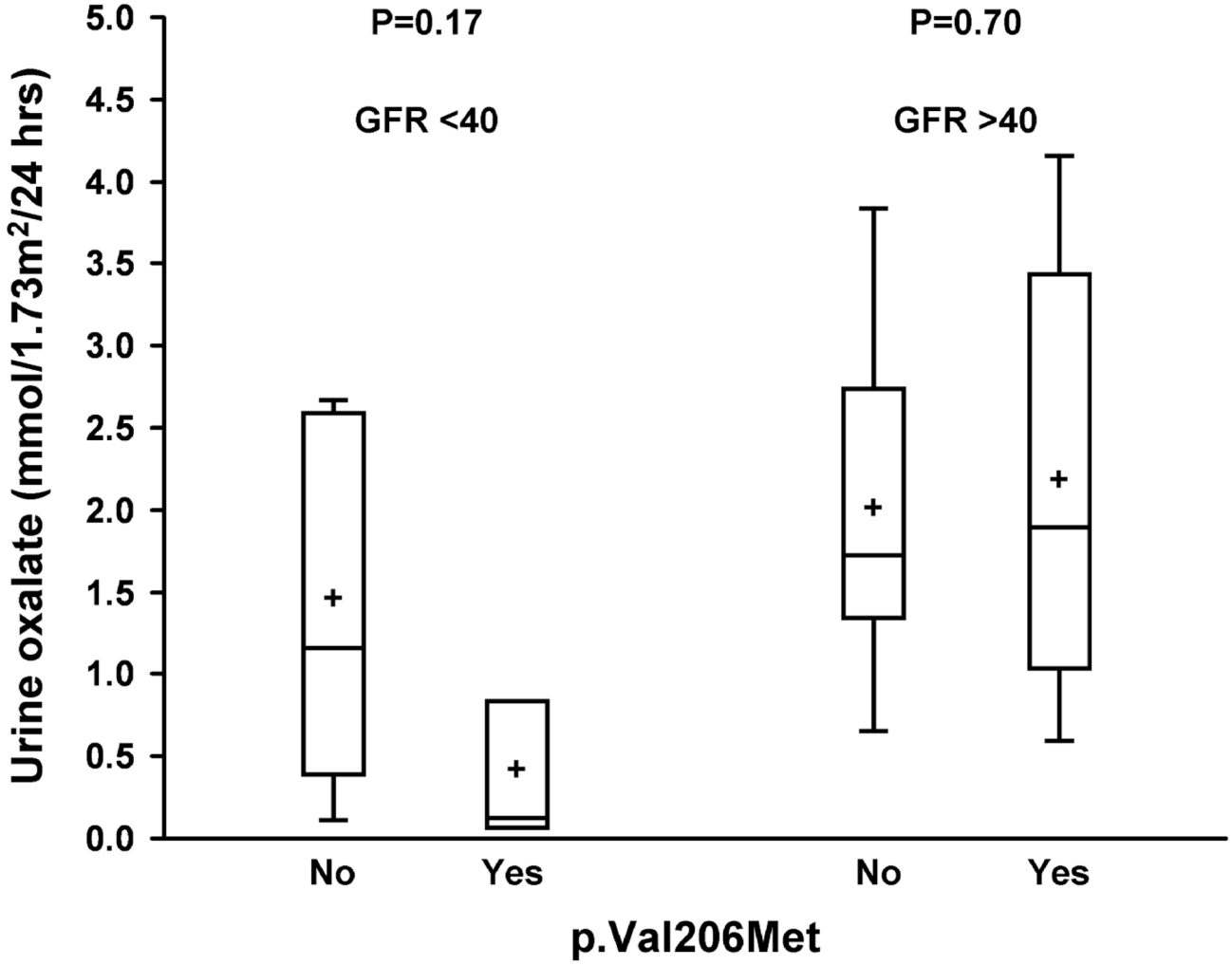


Figure 3. 3A–3B Levels of expression of total (A) and surface biotinylated (B) SLC26A6 in oocytes injected with cRNA encoding wild-type and p.Val206Met mutant transporters

Expression of HA-tagged transporters was assessed by immunoblotting. Representative Western blots are shown. In addition, band density was quantified with NIH Scion Image software for 4 similar experiments, and the mean results are expressed as arbitrary units. Data represent means ± SE.



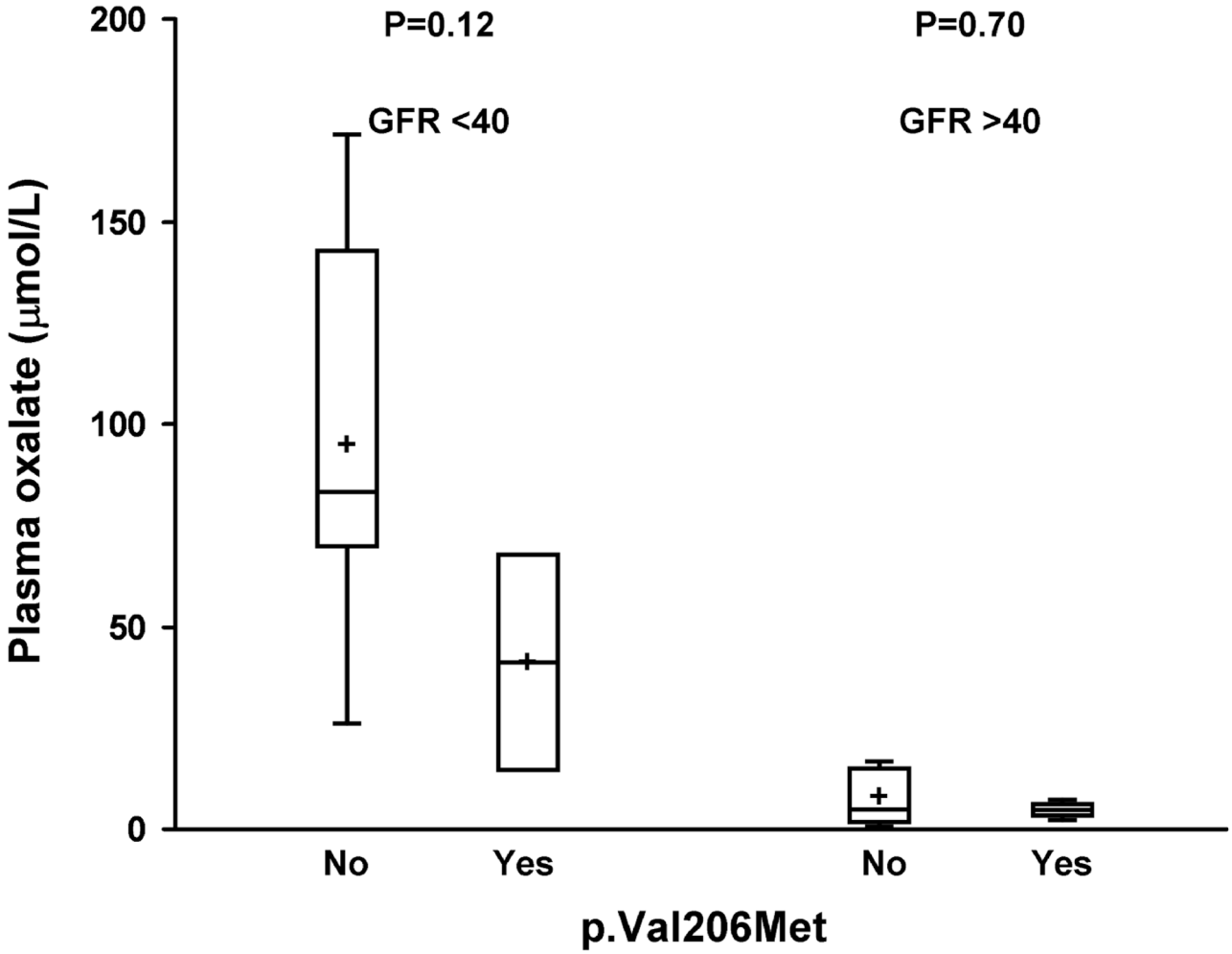


Figure 4. Plasma and Urine Oxalate in Primary Hyperoxaluria Patients (PH1, PH2, nonPH1/PH2) With and Without the p.Val206Met *SLC26A6* Variant

Rank-sums are given above the graphs. For Uox, only measurements at diagnosis were included (n= 9 for GFR<40, n=38 for GFR>40). For Pox, any available value was included (n=12 for GFR<40, n=15 for GFR>40).

Table 1

Clinical Characteristics of the Study Population

* Clinical Parameters	Cases			Controls
	PH1	PH2	nonPH1/PH2	
Sex (% M/F)	54/46	83/17	86/14	43/57
Age @ Diagnosis (years)	17 ± 17	21 ± 15	3.0 ± 2.8	47 ± 20
Urine Oxalate @ Diagnosis (mmol/1.73m^2/24 h)	2.1 ± 1.2	1.2 ± 0.6	1.4 ± 0.7	NA
GFR @ Diagnosis (ml/min/1.73m^2)	76 ± 47	114 ± 66	115 ± 29	NA
Duration of Follow-Up (years)	12 ± 10	27 ± 16	13 ± 6	NA
Total Stone Events/Year (spontaneous passage and procedures)	0.7 ± 1.3	0.1 ± 0.1	0.3 ± 0.2	NA
Predominant Stone Composition	COM	COD	Mixed	NA

* mean ± SD. COM=calcium oxalate monohydrate, COD=calcium oxalate dihydrate. Mixed=COM, COD, and/or calcium phosphate. NA=not available or not applicable.

GFR in ml/min/1.73m2 may be converted to ml/s/1.73 m2 by multiplying by 0.01667.

Table 2

Allelic and Genotype Frequencies For Coding *SLC26A6* Variants Detected in Unrelated Primary Hyperoxaluria (PH1, PH2, non-PH1/PH2) Probands Compared to Controls.

SLC26A6 Sequence Variant	Population Tested	Allelic Frequency (%)		Genotype Frequency (%)		
		Wild-type	Variant	Homozygous Wild-type	Heterozygous	Homozygous Variant
c.67G>C (p.Asp23His) p=0.6	PH1	150/150 (100)	0/150 (0)	75 (100)	0	0
	PH2	12/12 (100)	0/12 (0)	6 (100)	0	0
	Non-PH1/PH 2	12/12 (100)	0/12 (0)	6 (100)	0	0
	Controls	188/190 (99)	2/190 (1)	93 (98)	2 (2)	0
c.616G>A (p.Val206Met) P=0.9	PH1	142/160 (89)	18/160 (11)	63 (79)	16 (20)	1 (1)
	PH2	12/12 (100)	0/12 (0)	6 (100)	0	0
	Non-PH1/PH2	11/12 (92)	1/12 (8)	5 (83)	1 (17)	0
	Controls	171/192 (89)	21/192 (11)	77 (80)	17 (18)	2 (2)
c.487C>T (p.Pro163Ser) p=0.06	PH1	158/158 (100)	0/158 (0)	79 (100)	0	0
	PH2	12/12 (100)	0/12 (0)	6 (100)	0	0
	Non-PH1/PH2	11/12 (92)	1/12 (8)	5 (83)	1 (17)	0
	Controls	192/192 (100)	0/192 (0)	96 (100)	0	0
c.673C>G(p.Gln225Glu) P=0.5	PH1	159/160 (99)	1/160 (<1)	79 (99)	1 (1)	0
	PH2	12/12 (100)	0/12 (0)	6 (100)	0	0
	Non-PH1/PH2	12/12 (100)	0/12 (0)	6 (100)	0	0
	Controls	192/192 (100)	0/192 (0)	96 (100)	0	0
c.1191C>A(p.Pro397Pro) P=1.0	PH1	158/158 (100)	0/158 (0)	79 (100)	0	0
	PH2	12/12 (100)	0/12 (0)	6 (100)	0	0
	Non-PH1/PH2	12/12 (100)	0/12 (0)	6 (100)	0	0
	Controls	191/192 (99)	1/192 (<1)	95 (99)	1 (1)	0
c.1818C>T (p.Gly606Gly) P=1.0	PH1	160/160 (100)	0/160 (0)	80 (100)	0	0
	PH2	12/12 (100)	0/12 (0)	6 (100)	0	0
	Non-PH1/PH2	12/12 (100)	0/12 (0)	6 (100)	0	0
	Controls	191/192 (99)	1/192 (<1)	95 (99)	1 (1)	0
c.1906G>A (p.Asp636Asn) P=0.8	PH1	156/158 (99)	2/158 (1)	77 (97)	2 (3)	0
	PH2	12/12 (100)	0/12 (0)	6 (100)	0	0
	Non-PH1/PH2	12/12 (100)	0/12 (0)	6 (100)	0	0

SLC26A6 Sequence Variant	Population Tested	Allelic Frequency (%)		Genotype Frequency (%)		
		Wild-type	Variant	Homozygous Wild-type	Heterozygous	Homozygous Variant
	Controls	188/192 (98)	4/192 (2)	92 (96)	4 (4)	0

Because not all PCR reactions were successful, the number of reported alleles (n) and genotypes varies slightly. Genotype frequencies did not differ significantly (see p-values for each variant) between primary hyperoxaluria subtypes (PH1, PH2, non-PH1/PH2) and controls.

Sequence variants are designated with respect to reference sequence NM_022911.2, except that numbering is based on the A of the ATG initiation codon being nucleotide 1, ie, c.67G>C (p.Asp23His) refers to a G-to-C change at nucleotide 67 in the coding sequence, with a predicted amino acid change of an aspartate to a histidine at amino acid 23.

Table 3

SLC26A6 Sequence Variants in Non-PH1/PH2 Pedigrees

Affected sibs are included.

Pedigree					
1	**2**	**3**	**4**	**5***	**6***
IVS10–17G>C c. *75G>A (3’ UTR)	c.616 G>A (p.Val206Met) IVS2−4C>A IVS9+32C>A IVS5+30C>T	None	None	None	c.487C>T (p.Pro163Ser)

* Pedigrees 5 and 6 each had 2 affected individuals, but there was no difference between in *SLC26A6* sequence variants between each pair.

Sequence variants are designated with respect to reference sequence NM_022911.2, except that numbering is based on the A of the ATG initiation codon being nucleotide 1, ie, c.616G>A (p.Val206Met) refers to a G-to-A change at nucleotide 616 in the coding sequence, with a predicted amino acid change of a valine to a methionine at amino acid 206.

In pedigree 1, the c. *G>A variant denotes a sequence change 75 nucleotides downstream of the termination codon (occurring in the 3’ untranslated region [UTR]).

Where sequence variants are given with respect to intervening sequences (pedigrees 1 and 2), the number of the intervening sequence (IVS; ie, an intron) is given, as well as the distance from the end of the IVS. A negative number is based on counting back from the 3’ end of an IVS; a positive number is based on counting forward from the 5’ end of an IVS.