Mutations in SLC26A1 Cause Nephrolithiasis

Heon Yung Gee,1,2,10 Ikhyun Jun,1,3,10 Daniela A. Braun,2 Jennifer A. Lawson,2 Jan Halbritter,2,4 Shirlee Shril,3 Caleb P. Nelson,3 Weizhen Tan,2 Deborah Stein,2 Ari J. Wassner,6 Michael A. Ferguson,2 Zoran Gucev,7 John A. Sayer,6 Danko Milosevic,9 Michelle Baum,2 Velibor Tasic,7 Min Goo Lee,1,* and Friedhelm Hildebrandt2,7

Nephrolithiasis, a condition in which urinary supersaturation leads to stone formation in the urinary system, affects about 5%-10% of individuals worldwide at some point in their lifetime and results in significant medical costs and morbidity. To date, mutations in more than 30 genes have been described as being associated with nephrolithiasis, and these mutations explain about 15% of kidney stone cases, suggesting that additional nephrolithiasis-associated genes remain to be discovered. To identify additional genes whose mutations are linked to nephrolithiasis, we performed targeted next-generation sequencing of 18 hypothesized candidate genes in 348 unrelated individuals with kidney stones. We detected biallelic mutations in SLC26A1 (solute carrier family 26 member 1) in two unrelated individuals with calcium oxalate kidney stones. We show by immunofluorescence, immunoblotting, and glycosylation analysis that the variant protein mimicking p.Thr185Met has defects in protein folding or trafficking. In addition, by measuring anion exchange activity of SLC26A1, we demonstrate that all the identified mutations in SLC26A1 result in decreased transporter activity. Our data identify SLC26A1 mutations as causing a recessive Mendelian form of nephrolithiasis.

Nephrolithiasis (MIM: 167030) is a major health problem given that it affects up to 10% of the population in Western countries. Its prevalence has significantly increased among children over the last decades.1,2 This leads to significant medical cost due to expensive surgical interventions, progression to chronic kidney disease, and additional morbidity from renal colic and secondary urinary tract infection.3 The formation of kidney stones is multifactorial, resulting from an interaction of environmental, dietary, and genetic factors. Nephrolithiasis is genetically heterogenous, and mutations in at least 30 genes have been linked to this disorder.4,5 We recently demonstrated in a cohort of 166 adults and 106 children with nephrolithiasis or nephrocalcinosis that a monogenic cause can be detected in the surprisingly high percentage of 11.4% of adult cases and 20.8% of childhood-onset cases.6 In a follow-up study, we detected causative mutations in 1 of 30 linked genes in 16.7% of 143 individuals who manifested with nephrolithiasis or nephrocalcinosis before 18 years of age.7 Both studies thus demonstrated a high percentage of individuals with a monogenic mutation associated with kidney stone formation. However, genetic factors have been suggested to account for nearly 50% of nephrolithiasis cases,8,9 indicating that additional nephrolithiasis-associated genes remain to be discovered.

To identify additional genes mutated in nephrolithiasis, we generated a list of 18 hypothetical candidate genes and performed exon amplification with consecutive next-generation sequencing in a multicenter cohort of 348 individuals with nephrolithiasis or isolated nephrocalcinosis.10 The cohorts were previously described, and consisted of 201 children and 147 adults who had at least one urinary stone or nephrocalcinosis.5,7 For exclusion of known genetic causes of nephrolithiasis, 30 genes previously linked to nephrolithiasis were screened by exon sequencing in these individuals, but no causative mutations were detected. Written informed consent was obtained from all individuals enrolled in this study, and approval for human subject research was obtained from the institutional review boards at the Boston Children’s Hospital. The candidate genes screened in this study are listed in Table S1. We designed 210 target-specific primer pairs for coding exons and the adjacent splice site of 18 genes. Amplicon sizes were chosen to range from 250 to 300 base pairs. We achieved a median sequencing coverage of 198× per individual and 192× per amplicon. Only 9/350 (2.5%) individuals had a median coverage below 20.

In an individual of Macedonian descent (A3054-21) who is from non-consanguineous parents and clinically presented with acute renal failure due to bilateral obstructive calculi, nephrocalcinosis, and bilateral ureteropelvic junction obstruction (UPJ), we detected two compound-heterozygous mutations in SLC26A1 (solute carrier family 26 member 1 [GenBank: NM_022042.3] [MIM: 610130]) (Table 1, Figure 1, and Figure S1). This individual had acute renal failure due to ureteral obstruction with calculi at the
Table 1. Recessive SLC26A1 Mutations Detected in Individuals with Nephrolithiasis

<table>
<thead>
<tr>
<th>Individual</th>
<th>Sex</th>
<th>Ethnic Origin</th>
<th>Consanguinity</th>
<th>Amino Acid Change</th>
<th>Parental Nucleotide Change</th>
<th>Amino Acid Residue Conservation</th>
<th>dbSNP Frequency</th>
<th>ExAC Frequency</th>
<th>EVS Frequency</th>
<th>Stone Type</th>
<th>Age of Onset</th>
</tr>
</thead>
<tbody>
<tr>
<td>A3054-21</td>
<td>M</td>
<td>Macedonian</td>
<td>no</td>
<td>p.Thr185Met</td>
<td>c.554C&gt;T</td>
<td>0.996 DC</td>
<td>26/104,290</td>
<td>0/5/6,480</td>
<td>0/0/0</td>
<td>5 yr CaOx</td>
<td>0/0/0</td>
</tr>
<tr>
<td>B641-12</td>
<td>M</td>
<td>European</td>
<td>yes</td>
<td>p.Ala56Thr</td>
<td>c.166G&gt;A</td>
<td>1.000 DC</td>
<td>59/112,248</td>
<td>0/5/6,487</td>
<td>0/0/0</td>
<td>3 yr ND</td>
<td>0/0/0</td>
</tr>
</tbody>
</table>

Abbreviations are as follows: CaOx, calcium oxalate; DC, disease causing; het, heterozygous in affected individual; HOM, homozygous in affected individual; M, heterozygous mutation identified in mother; MAF, minor-allele frequency; Ml, male; MT, MutationTaster; NA, not applicable; ND, no data or DNA available; P, heterozygous mutation identified in father; PP2, PolyPhen-2 prediction score HumVar; yr, years.

Age of five. He had recurrent episodes of renal colic due to calcium oxalate nephrolithiasis and hypocitraturia and underwent surgery for UPJO. The analysis of 24 hr urine showed that this individual also had hyperoxaluria; otherwise, urine and blood chemistries were normal (Table S2). The two compound heterozygous missense mutations (c.554C>T, p.Thr185Met and c.1073C>T, p.Ser358Leu) in SLC26A1 are reported as SNPs in the dbSNP database; however, their minor allele frequencies are below 0.0006 and they never occurred in a homozygous state (Table 1). The amino acid residues affected by two missense mutations were conserved throughout evolution down to Danio rerio (Table 1 and Figure 1). Because this individual had UPJO, we therefore performed whole-exome sequencing (WES) and examined variants in genes associated with congenital anomalies of the kidney and urinary tract (CAKUT) or UPJO. However, we could not detect any additional pathogenic variants in these genes. In addition, we examined genomic structural abnormality by using WES data, but no structural variation was detected.

In a European-American boy (B641-12) who had nephrolithiasis and was born to consanguineous parents, we detected a homozygous missense mutation in SLC26A1 (Table 1, Figure 1, and Figure S1). The paternal grandfather of this individual also had nephrolithiasis. A kidney stone was initially found in this individual during a computerized tomography scan, which was performed for the evaluation of abdominal pain. In a 24 hr urine test, oxalate was within normal range (Table S2). Renal function of this individual was normal. The detected missense mutation (c.166G>A, p.Ala56Thr) is reported as a SNP in the dbSNP database; however, its minor allele frequency is 0.0002 and it never occurred in a homozygous state (Table 1). Murine SLC26A1 has Ala at the position corresponding to amino acid position 56 of human SLC26A1, whereas the protein from Xenopus tropicalis or Danio rerio has Val, which is also chemically related as a non-charged residue (Table 1 and Figure 1).

SLC26A1 (also known as SAT1) was cloned as a sulfate transporter from liver. It was subsequently characterized as an anion exchanger that transports anions by mediating electroneutral sulfate-oxalate, sulfate-bicarbonate, or oxalate-bicarbonate exchange. The Slc26a1−/− mice exhibit hyposulfatemia, hypersulfaturia, calcium oxalate urolithiasis, and nephrocalcinosis in the setting of hyperoxalemia and hyperoxaluria. Sk26a1−/− mice also show infiltration of leukocytes around renal cortical vessels, suggestive of UPJO. In this regard, it is of note that one of the individuals with SLC26A1 mutations (A3054-21) had UPJO, which could be based on a pathogenesis similar to the one described in the murine model. In addition, Slc26a1−/− mice show enhanced drug-induced liver toxicity, which might reflect reduced availability of intracellular sulfate ion for drug conjugation reactions. These findings demonstrate that, in mammals, SLC26A1 plays a critical role in oxalate and sulfate homeostasis, which is thought to be associated with kidney stone disease.
However, to date, the contribution of genetic variants in \( \text{SLC26A1} \) to nephrolithiasis in human is unknown. Dawson et al. examined \( \text{SLC26A1} \) in 13 individuals with recurrent calcium oxalate urolithiasis, but they were unable to detect any biallelic mutations in \( \text{SLC26A1} \). The c.1667A>G, p.Gln556Arg variant, which was found in 6 out of 13 individuals examined, including one homozygous individual, is a known SNP (rs3706622), and its allele frequency is 0.6737 (26,773 homozygotes out of 79,535 individuals are reported in the Exome Aggregation Consortium [ExAC] Browser), suggesting that this SNP is probably benign, rather than disease causing.

SLC26A1 transporter is known to localize at the basolateral membrane of renal proximal tubular epithelial cells and enterocytes, as well as at the sinusoidal membrane of hepatocytes. We confirmed by immunofluorescence the localization of SLC26A1 in the rat kidney and intestine (Figure 2). We show that SLC26A1 is present in the basolateral membrane of peanut lectin-positive proximal tubules in the renal cortex and basolaterally in the AQP2-positive principle cells, as well as the AQP2-negative intercalated cells of collecting ducts in the medulla (Figures 2A and 2B). SLC26A1 also localizes to cytoplasm and basolateral membranes of the ileum and colon (Figure 2C and 2D).

To evaluate the impact of the identified human mutations on SLC26A1 function, we performed immunofluorescence in HEK293T cells upon overexpression of wild-type (WT) and mutant SLC26A1s. A mouse \( \text{Slc26a1} \) clone was purchased from Open Biosystems (clone accession no. BC025824), and mutant clones of \( \text{Slc26a1} \) were generated by PCR-based site-directed mutagenesis. The WT protein and the variant protein harboring p.Ala56Thr localized to plasma membranes (Figure 2E), whereas the p.Thr190Met variant (which corresponds to p.Thr185Met in human protein) failed to reach the plasma membrane and was trapped in the endoplasmic reticulum (ER) as shown by colocalization with an ER marker, BiP (Figure 2E). The SLC26A1 variant harboring p.Ser358Leu, which corresponds to p.Ser358Leu in human protein, reached the cell surface; however, a significant portion of protein was trapped in the ER, suggesting a processing defect (Figure 2E). The protein amounts of WT and variant SLC26A1s were analyzed in HEK293T cells upon overexpression of wild-type (WT) and mutant SLC26A1s (Figure 3). SLC26A1 undergoes the Golgi-mediated complex-glycosylation (band...
after an initial core-glycosylation at the ER (band B). In general, the Golgi-glycosylated forms of SLC26A1 are present at the cell surface. When we examined the protein amounts of variant proteins, we found them significantly reduced, as compared to WT proteins (Figures 3 A and 3D). SLC26A1 p.Thr190Met in particular was not processed to the fully glycosylated form (Figures 3 A and 3D), which suggests that the p.Thr190Met protein is degraded via the ER-associated protein degradation (ERAD) pathway and is consistent with the finding that the p.Thr190Met protein was not observed in the plasma membrane (Figure 2 E).

To confirm whether the lower band of SLC26A1 in immunoblotting truly represented the ER form, we treated the protein samples of WT SLC26A1 and the p.Thr190Met variant with endoglycosidase H (Endo H) and peptide-N-glycosidase F (PNGase F) (Figures 3 B and 3E). The band size of Endo-H-treated WT sample did not change (band C), whereas the band size of the PNGase-F-treated sample decreased to that of the p.Thr190Met variant protein. On the other hand, the band size of the Endo-H-treated p.Thr190Met variant protein (band B) decreased to that of PNGase-F-treated WT or p.Thr190Met SLC26A1 (band A). The Endo-H-sensitive digestion of the p.Thr190Met protein suggests that this variant protein exists as an ER form (band B). We performed real-time PCR experiments to determine whether the reduced amounts of variant proteins were due to a reduced number of transcripts or to poor protein stability. We found that the number of transcripts of Slc26a1 was not significantly different in cells transfected with WT and mutant plasmids (Figure S2), indicating that mRNA of WT Slc26a1 and mRNAs harboring mutations are not different at the transcription level. These results also indicate that reduced amounts of the p.Ser363Leu variant protein is associated with deficient protein stability.

To verify functional abnormalities of SLC26A1 variant proteins, we measured the anion transporting activity of SLC26A1 by monitoring intracellular pH (pH$_i$) in HEK293T cells after transfection with Slc26a1 plasmids.
We measured pHᵢ with a pH-sensitive fluorescent probe, 2',7'-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxyethyl ester (BCECF-AM), as described previously. Previous studies have revealed that SLC26A1 can transport chloride, bicarbonate, oxalate, and sulfate. Although SLC26A1 also has HCO₃⁻/CO₃⁻-oxalate exchange activities, we instead analyzed SLC26A1-mediated SO₄²⁻/CO₃⁻ exchange activities because of a technical advantage allowing us to observe more robust pHᵢ changes in these activities. We first loaded HEK293T cells with HCO₃⁻/CO₂ by using endogenous Cl⁻-HCO₃⁻ exchange activities. Then, we analyzed the initial rate of pHᵢ reduction due to HCO₃⁻/CO₂-SO₄²⁻ exchange activities after challenging the cells with 25 mM SO₄²⁻. As shown in Figures 4A and 4B, cells expressing SLC26A1, but not mock-transfected cells, showed a strong HCO₃⁻-SO₄²⁻ exchange activity. Subsequently, we measured the transport activities of SLC26A1 variant proteins in the same manner. Compared to WT SLC26A1, all the variants showed decreased HCO₃⁻-SO₄²⁻ exchange activities (Figures 4B–4E), especially the folding defect mutant, p.Thr190Met, for which activity almost vanished (Figure 4D). The summaries of difference and slope of intracellular pH are given in Figure 4F.

Here, using a candidate gene approach, we demonstrate that mutations in SLC26A1 cause an autosomal-recessive form of calcium oxalate nephrolithiasis. Our functional studies indicate that all the identified mutations in SLC26A1 lead to decreased transporter activity. Hyperoxalemia in Slc26a1-/- mice was most likely caused by reduced intestinal secretion of oxalate, based on reduced oxalate transport in basolateral membrane vesicles from the distal ileum, cecum, and proximal colon, as well as decreased cecal oxalate content. Hyperoxalemia led to hyperoxaluria and calcium oxalate nephrolithiasis. Similarly, reduced intestinal secretion of oxalate, which resulted from decreased transporter activity, probably led to calcium oxalate stones in individuals with SLC26A1 mutations. Slc26a1-/- mice have increased liver sensitivity to acetaminophen. Administration of acetaminophen led to a 4-fold increase in serum alanine transaminase levels and extensive liver necrosis in Slc26a1-/- mice. Therefore, it will be of high clinical relevance to determine whether individuals withbiallelic mutations in SLC26A1 also exhibit drug-induced
hepatotoxicity. According to the findings in Slc26a1−/− mice, acetaminophen might be contraindicated in individuals with SLC26A1 mutations.

Supplemental Data

Supplemental Data include three figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2016.03.026.

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Web Resources

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PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/
UCSC Genome Browser, http://genome.ucsc.edu
References


