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Gitelman syndrome (GS) is a recessive salt-losing tubulopathy that is caused by mutations in the SLC12A3 gene that encodes the sodium-chloride co-transporter (NCC). GS is characterized by significant inter- and intrafamilial phenotype variability, with early onset and/or severe clinical manifestations in some patients. No correlations between the disease variability and the position/nature of SLC12A3 mutations have been investigated thus far. In this study, extensive mutational analyses of SLC12A3 were performed in 27 patients with GS, including genomic DNA sequencing, multiplex ligation-dependent probe amplification, cDNA analysis, and quantification of allele-specific transcripts, in parallel with functional analyses in Xenopus laevis oocytes and detailed phenotyping. Twenty-six SLC12A3 mutations were identified in 25 patients with GS, including eight novel (detection rate 80%). Transcript analysis demonstrated that splicing mutations of SLC12A3 lead to frameshifted mRNA subject to degradation by nonsense-mediated decay. Heterologous expression documented a novel class of NCC mutants with defective intrinsic transport activity. A subgroup of patients presented with early onset, growth retardation, and/or detrimental manifestations, confirming the potential severity of GS. The mutations that were associated with severe presentation were the combination at least for one allele of a missplicing resulting in a truncated transcript that was downregulated by nonsense-mediated decay or a nonfunctional, cell surface–absent mutant. The most recurrent mutation on the second allele was a newly described NCC mutant that affected the functional properties of the co-transporter. These data suggest that the nature/position of SLC12A3 mutation, combined with male gender, is a determinant factor in the severity of GS and provide new insights in the underlying pathogenic mechanisms of the disease.


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transcriptional profile, and the clinical manifestations have not been addressed.

In this study, we investigated the mutational spectrum for SLC12A3 in 27 patients who received a clinical and biochemical diagnosis of GS. For all single base substitutions at or close to ss consensus sequence, the effect on the pre-mRNA splicing was confirmed by cDNA analyses, and the proportion of the different transcripts that were generated by these mutations was analyzed. We documented a significant phenotype heterogeneity among the 25 patients who harbored SLC12A3 mutations, including a subgroup of nine patients with early-onset and severe manifestations. We further characterized biochemically and structurally a panel of missense mutants in Xenopus laevis oocytes and delineated their association with phenotypic information. These data provide the first evidence to suggest that the potential severity of GS is related to a combination of the nature and the functional consequence of the SLC12A3 mutations with male gender. They also give new insights into the functional domains of NCC and the underlying pathogenic mechanisms of GS.

Materials and Methods

Patient Recruitment and Clinical Evaluation

Twenty-seven patients who had a diagnosis of GS from 23 nonconsanguineous Belgian families were recruited through the Belgian Network for the Study of Gitelman Syndrome. All patients met the classical diagnostic criteria for GS (5,21,23). The patients were documented for renal and extrarenal phenotypic manifestations, and routine parameters were recorded at time of diagnosis. The patients with GS were classified as “severe” when they had at least two of the following criteria: Early onset of the disease, before age 12; growth delay (height below the third percentile, or height velocity below the tenth percentile) and/or final short stature (below the third percentile); detrimental neuromuscular symptoms (e.g., repeated episodes of palsy, hypotonia, tetany, detrimental cramps, blurred vision); cardiac arrhythmia related to prolonged QT; and symptomatic chondrocalcinosis. The ethical committee of the Université Catholique de Louvain Medical School approved the present study, and informed consent was obtained from participants and/or their parents.

Screening for Mutations in SLC12A3

Total DNA was extracted from blood peripheral leukocytes (Gentra Systems Puregene, Minneapolis, MN). Twenty-six primer pairs were designed to amplify by PCR the coding region and flanking intronic sequences of SLC12A3 gene (Supplementary Table 1). Thirty cycles of PCR were performed in the presence of 1.5 μM of 25 mM MgCl2 using AmpliTaq Gold (Perkin Elmer Applied Biosystems, Foster City, CA). The PCR products were directly sequenced with the BigDye terminator kit (Perkin Elmer Applied Biosystems). Sequence reactions were purified with MultiScreen product. The six mutant constructs were linearized with EcoRI, and cRNA transcripts were synthesized using T7 RNA polymerase (Promega, Madison, WI). Concentration of the cRNA was measured with a spectrophotometer, and the integrity was confirmed by RNA formaldehyde-agarose gel electrophoresis.

Expression Studies in Xenopus laevis Oocytes

Oocytes were isolated from Xenopus laevis and defolliculated by digestion at room temperature for 2 h with 2 mg/ml collagenase A (Boehringer Mannheim, Mannheim, Germany) as described (7). Injection of 10 ng of wild-type or mutant human NCC (hNCC) cRNA was performed 24 h after isolation. Immunoblotting. 22Na+ uptake assay, and immunocytochemistry were performed 2 d after injection as described previously (7). Isolation of total and plasma membranes was performed in 15 oocytes per condition. Protein samples were immunoblotted onto polyvinylidene difluoride membranes and successively incubated with 1:8000 dilution mouse anti-FLAG (Sigma, St. Louis, MO) and 1:2000 diluted sheep horseradish peroxidase conjugated to anti-mouse IgG (Sigma) antibodies, before visualization by enhanced chemiluminescence (Pierce, Havertown, PA). Twenty-four hours before the 22Na+ uptake assay, 15 oocytes per condition were transferred to a Cl-
free medium and next transferred to 500 μl of uptake medium that contained 1 μCi/ml 22Na+ for 2 h at room temperature. Ouabain and bumetanide were added to block the Na+-K+-ATPase and the Na+-K+-2Cl− co-transporter, respectively. Amiloride was added to block the Na+-H+ antiporter and Na+ channels. Next, washing in ice-cold uptake medium was performed five times to stop the uptake reaction. Each oocyte was solubilized in 200 μl of 10% (wt/vol) SDS, and radioactivity was counted in a liquid scintillation counter. For immunocytochemistry, the remaining vitelline membrane was removed 2 d after oocyte injection. Five to 10 peeled oocytes per condition were fixed at room temperature during incubation at 4°C with mouse anti-FLAG antibody (Sigma) diluted 1:200 followed by incubation at room temperature for 1 h with goat Alexa 488–conjugated anti-mouse IgG (Invitrogen, Eugene, OR) diluted 1:250.

Statistical Analyses

Results are expressed as means ± SEM. Comparisons between groups were made by unpaired t test or Fisher exact test as appropriate. The significance level was set at P < 0.05.

Results

Mutation Screening

Sequence analysis of the SLC12A3 gene of 23 probands (27 patients including four siblings) with a clinical diagnosis of GS revealed 26 different punctual mutations, including eight novel. These mutations include 22 missense, three ss, and one duplication (Table 1). All mutations co-segregated with the phenotype and were not detected in 220 control chromosomes. The novel variants are six missense mutations (R145C, S350L, T R145C 1 This study

Table 1. Mutations in SLC12A3 identified in the 25 patients with GSa

<table>
<thead>
<tr>
<th>Location</th>
<th>Nucleotide Changeb</th>
<th>Predicted Effect on Coding Sequencec</th>
<th>No. of Patients</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Missense</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ex 3</td>
<td>c.433C&gt;T</td>
<td>R145C</td>
<td>1</td>
<td>This study</td>
</tr>
<tr>
<td>ex 4</td>
<td>c.514T&gt;C</td>
<td>W172R</td>
<td>2</td>
<td>Syren et al., 2002 (6)</td>
</tr>
<tr>
<td>ex 5</td>
<td>c.644T&gt;C</td>
<td>L215P</td>
<td>1</td>
<td>Lemmink et al., 1998 (4)</td>
</tr>
<tr>
<td>ex 6</td>
<td>c.947G&gt;T</td>
<td>G316V</td>
<td>1</td>
<td>Syren et al., 2002 (6)</td>
</tr>
<tr>
<td>ex 8</td>
<td>c.1049C&gt;T</td>
<td>S350L</td>
<td>1</td>
<td>This study</td>
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<tr>
<td>ex 10</td>
<td>c.1195C&gt;T</td>
<td>R399C</td>
<td>4</td>
<td>Cruz et al., 2001 (5)</td>
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<td>ex 12</td>
<td>c.1452C&gt;G</td>
<td>C484W</td>
<td>1</td>
<td>This study</td>
</tr>
<tr>
<td>ex 12</td>
<td>c.1456G&gt;A</td>
<td>D486N</td>
<td>1</td>
<td>Simon et al., 1996 (2)</td>
</tr>
<tr>
<td>ex 12</td>
<td>c.1567G&gt;A</td>
<td>A523T, p.Cys482_Ala523&gt;LeufsX6</td>
<td>1</td>
<td>This study</td>
</tr>
<tr>
<td>ex 13</td>
<td>c.1664C&gt;T</td>
<td>S555L</td>
<td>3</td>
<td>Cruz et al., 2001 (5)</td>
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<td>ex 14</td>
<td>c.1763C&gt;T</td>
<td>A588V</td>
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<td>ex 15</td>
<td>c.1832A&gt;G</td>
<td>N611S</td>
<td>1</td>
<td>This study</td>
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<tr>
<td>ex 15</td>
<td>c.1868T&gt;C</td>
<td>L623P</td>
<td>1</td>
<td>Takeuchi et al., 1996 (3)</td>
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<tr>
<td>ex 15</td>
<td>c.1925G&gt;A</td>
<td>R642H</td>
<td>1</td>
<td>Lemmink et al., 1998 (4)</td>
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<tr>
<td>ex 16</td>
<td>c.1964G&gt;A</td>
<td>R655H</td>
<td>1</td>
<td>Simon et al., 1996 (2)</td>
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<td>ex 18</td>
<td>c.2166C&gt;G</td>
<td>G729V</td>
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<td>Cruz et al., 2001 (5)</td>
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<tr>
<td>ex 18</td>
<td>c.2221G&gt;A</td>
<td>G741R</td>
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<td>ex 22</td>
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<td>R852C</td>
<td>1</td>
<td>Lemmink et al., 1998 (4)</td>
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<tr>
<td>ex 24</td>
<td>c.2755C&gt;T</td>
<td>R919C</td>
<td>1</td>
<td>Lemmink et al., 1998 (4)</td>
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<td>ex 25</td>
<td>c.2903G&gt;A</td>
<td>R968Q</td>
<td>1</td>
<td>This study</td>
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<td><strong>Splice site</strong></td>
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<td></td>
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<td>ex 26</td>
<td>c.2938G&gt;A</td>
<td>G980R</td>
<td>1</td>
<td>De Jong et al., 2002 (7)</td>
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<tr>
<td>ex 26</td>
<td>c.2954G&gt;A</td>
<td>C985Y</td>
<td>1</td>
<td>Syren et al., 2002 (6)</td>
</tr>
<tr>
<td><strong>IVS7</strong></td>
<td>c.964+1G&gt;T</td>
<td>ex 7 skip, p.Ala285_Ala322&gt;ArgfsX48</td>
<td>1</td>
<td>This study</td>
</tr>
<tr>
<td><strong>IVS22</strong></td>
<td>c.2633+1G&gt;C</td>
<td>ex 22 skip, p.Gly841_Ala878&gt;GlyfsX6</td>
<td>1</td>
<td>Godefroid et al., 2006 (8)</td>
</tr>
<tr>
<td><strong>Duplication</strong></td>
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<tr>
<td><strong>IVS24</strong></td>
<td>c.2856+1G&gt;T</td>
<td>ex 24 skip, p.His907_Lys952&gt;HisfsX6</td>
<td>2</td>
<td>Simon et al., 1996 (2)</td>
</tr>
<tr>
<td>ex 23</td>
<td>c.2667dupA</td>
<td>p.Phe890llefsX3</td>
<td>1</td>
<td>This study</td>
</tr>
</tbody>
</table>

a Compound heterozygous, mild: c.[514T>C]+[1925G>A] (female), c.[644T>C]+[1049C>T] (female), c.[1195C>T]+[2554G>A] (female), c.[1868T>C]+[1456G>A] (male), c.[1925G>A]+[2221G>A] (male), c.[2186G>C]+[2554G>A] (male), c.[2856+1G>T]+[2938G>A] (female); compound heterozygous, severe: c.[433C>T]+[964+1G>T] (male), c.[514T>C]+[2221G>A] (male), c.[1664C>T]+[2221G>A] (male), c.[1763C>T]+[1657C>A] (male), c.[1664C>T]+[2633+1G>C] (female); simple heterozygous, mild: c.[2903G>A]+[?] (female), c.[1195C>T]+[?] (male), c.[2221G>A]+[?] (male), c.[2755C>T]+[?] (male); simple heterozygous, severe: c.[1925C>T]+[?] (male), c.[2856+1G>T]+[?] (male). No homozygous patients were identified. ex, exon; GS, Gitelman syndrome.

b The numbering of nucleotides starts at the first adenine of the translation initiation codon.

c The numbering of amino acids starts at the first methionine encoded by the translation initiation codon.
C484W, A523T, N611S, and R968Q) that substitute strongly conserved amino acids (data not shown); a ss mutation (c.964+1G>T) and a duplication of an adenine (c.2667dupA) that caused a frameshift that resulted in a stop codon at position 3 in exon 23 (p.Phe890IlefsX3; Figure 1). Heterozygosity was observed for all SLC12A3 mutations that were detected in this study. Two mutant alleles were detected in 15 affected individuals, whereas only one was identified in six individuals, and no mutation was identified in the remaining two patients (Table 1). MLPA was performed in the six patients who had GS and in whom only one mutant allele was found and in the two without detectable mutation. No alteration in the copy number of the 26 exons of SLC12A3 could be detected in any of the eight samples (data not shown).

The mutations were distributed throughout the SLC12A3 gene with the exception of exons 1, 2, 6, 9, 11, 17, and 19 to 21.

![Figure 1. Novel SLC12A3 mutations identified in patients with Gitelman syndrome (GS). Direct sequencing of PCR fragments of different exons for novel sodium-chloride co-transporter (NCC) mutations in control subjects (Ct) and patients with GS (Pt). The encoded amino acid sequence is indicated in three-letter format above the DNA sequence. Mutated bases are indicated above the normal base. Intronic bases are shown in lower case. For nomenclature, the numbering of nucleotide starts at the first adenine of the translation initiation codon.](image-url)
Exons 12 and 15 were the most frequent sites of mutation (six [23%] of 26 mutations). The most common mutations that caused GS were two missense, R399C and G741R (allele frequency eight [approximately 17%] of 46; Table 1). The missense S555L is the third recurrent mutation, with an allele frequency of approximately 6%, and was found in three patients. All other mutations were identified in only one or two patients.

Analysis of Splicing Mutations

DNA analysis revealed three different point mutations that occurred in the invariant motif of the 5' donor ss: c.2633+1G>C, c.2856+1G>T, and the novel c.964+1G>T (Table 1). Two missense mutations, A523T and R642H, that resulted from guanine-to-adenine transitions at the last nucleotide upstream of the 5' donor ss of exons 12 and 15, respectively, were also predicted to affect the splicing. Using Automated Splice Site Analyses (24), mutations that affected the consensus 5'donor ss in intron 7 (c.964+1G>T), intron 22 (c.2633+1G>C), and intron 24 (c.2856+1G>T) were predicted to abolish the splicing (ΔRI -7.8, ΔRI -12.8, ΔRI -7.8), whereas both missense A523T and R642H were predicted to affect splicing without activation of a cryptic site (ΔRI -1.1).

Reverse Transcriptase–PCR and cDNA Sequencing. cDNA sequencing was performed to confirm the splicing predictions (Figure 2). The cDNA from the two siblings who carried the heterozygous c.964+1G>T yielded no full-length product but a shorter transcript characterized by skipping of exon 7. At the protein level, this change resulted in a new reading frame that ended in a stop codon at position 48 in exon 9 (p.Ala285_Ala322>ArgfsX48). Analysis of the sibling patients who carried the mutation c.1567G>A revealed an aberrantly spliced transcript that lacked exon 12, in addition to correctly spliced transcript with the predicted missense mutation, A523T. This skipping resulted in a frameshift and the introduction of a stop codon at position 6 of exon 13 (p.Cys482_Ala523>LeufsX6). Analysis of the patient who carried the mutation c.1925G>A revealed the presence of one aberrant splice transcript that contained an insertion of a 104-bp intronic

Figure 2. Schematic overview of SLC12A3 and representation of abnormal splicing in patients with GS. The structure of SLC12A3 and its 26 exons is shown on top. Dotted boxes enclose the abnormally assembled exons after RNA splicing. For nomenclature, the numbering of nucleotide starts at the first adenine of the translation initiation codon; numbering of amino acids starts at the first methionine encoded by the translation initiation codon. Consequences of five splicing mutations on the RNA splicing are illustrated. For each mutation, a scheme that includes the correct splice transcript and the aberrant product(s) is shown. Triangles mark the mutated residues; mutant threonine at position 523 and histidine at position 642 are also indicated.
sequence between exon 15 and exon 16 (r.1926_1926ins104bp), responsible for a new reading frame that ended in a stop codon at position 2 (p.Arg642ArgfsX2). In addition, a correctly spliced transcript with the predicted missense R642H was observed. The presence of the splicing mutation c.2633+1G>C in one patient caused the occurrence of an abnormal transcript that lacked the entire exon 22 in addition to the normal-length transcript. As a consequence, a new reading frame that ended in a stop codon at position 6 in exon 22 was created (p.Gly841_Alal878>GlyfsX6). Analysis of the cDNA from two patients who carried c.2856+1G>T showed the skipping of the entire exon 24 in the abnormally spliced transcript with the introduction of a stop codon as result of the frameshift at position 6 of exon 25 (p.His907_Lys952>HisfsX6).

**Relative Quantification of Mutant Transcript Levels.** To assess whether NMD operates for truncated mutant SLC12A3 transcripts, we determined the percentage of expression of each aberrantly spliced transcript versus the correctly spliced product (taken as 100%). The expression of frameshifted transcripts was significantly reduced when compared with the correctly spliced products: 58 and 46% for the two siblings who carried the c.964+1G>T mutation; 13% for both siblings with c.1567G>A; 53% for c.2633+1G>C; 32% for the patient who carried the mutation c.1925G>A; and 9 and 13%, respectively, for the two patients from different families with c.2856+1G>T mutation. Of note, the proportions were homogeneous among the five truncating mutations for which transcript levels could be assessed in more than one patient. These results suggest that NMD is triggered by the incorrectly spliced products of SLC12A3.

**Phenotype Analysis**

On the basis of their clinical features, the 25 patients who harbored mutations in SLC12A3 were classified either in a severe (at least two criteria of severity) or mild (classical GS features, no criteria of severity) subgroup (Table 2). There were eight male patients for only one female patient in the severe features, no criteria of severity) subgroup (Table 2). There were severe (at least two criteria of severity) or mild (classical GS

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Subgroup of Patients with GS</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Severe (n = 9)</td>
<td>Mild (n = 16)</td>
</tr>
<tr>
<td>Gender ratio</td>
<td>1 F/8 M</td>
<td>13 F/3 M</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>101 ± 3</td>
<td>108 ± 6</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>56 ± 3</td>
<td>70 ± 4</td>
</tr>
<tr>
<td>pNa⁺ (mEq/L; NV 135 to 145)</td>
<td>138 ± 0.6</td>
<td>138 ± 0.6</td>
</tr>
<tr>
<td>pK⁺ (mEq/L; NV 3.5 to 5)</td>
<td>2.4 ± 0.2</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td>pHCO₃⁻ (mEq/L; NV 22 to 29)</td>
<td>31.5 ± 0.9</td>
<td>31.7 ± 0.8</td>
</tr>
<tr>
<td>pMg²⁺ (mEq/L; NV 1.45 to 1.9)</td>
<td>1.3 ± 0.1</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>uNa⁺ (mEq/L)</td>
<td>135 ± 18</td>
<td>97 ± 14</td>
</tr>
<tr>
<td>uK⁺ (mEq/L)</td>
<td>76 ± 14</td>
<td>61 ± 7.0</td>
</tr>
<tr>
<td>uNa⁺/K⁺</td>
<td>2.8 ± 0.7</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>uCa²⁺/creatinine (mg/mg; NV &lt;0.15)</td>
<td>0.033 ± 0.005</td>
<td>0.070 ± 0.016</td>
</tr>
</tbody>
</table>

aF, female; M, male; NV, normal value; p, plasma; u, urine.

Table 2. Clinical and laboratory parameters in the two subgroups of patients who had GS and harbored SLC12A3 mutations
A similar pattern was observed in oocytes that expressed the mutants R145C, A588V, S555L, and N611S, whereas the 110-kD band was detected only in mutants G316V and R399C. Immunoblotting of plasma membrane extracts showed the presence of mutants R145C, A588V, S555L, and N611S at the cell surface, with variable level of expression as compared with wild-type. In contrast, G316V and R399C mutants were not detected in the plasma membrane fraction (Figure 4B, bottom).

The functionality of mutant NCC co-transporters was investigated using thiazide-sensitive $^{22}\text{Na}^+$ uptake. The G316V, R399C, S555L, and A588V mutants showed the same level of activity as noninjected oocytes, whereas mutants R145C and N611S showed a rate of $^{22}\text{Na}^+$ uptake of 35 and 53%, respectively, compared with oocytes that expressed the wild-type protein (Figure 4C). Immunocytochemistry confirmed the results that were obtained by immunoblotting of the plasma membrane extracts (Figure 4D). Antibodies against FLAG-
epitope demonstrated typical plasma membrane staining in oocytes that expressed wild-type hNCC. By contrast, intracellular staining was observed exclusively in oocytes that expressed nonfunctional mutants such as G316V, whereas functional mutants such as N611S showed positive staining at both the plasma membrane and the cytoplasm. The mutants S555L and A588V, which did not exhibit a significant level of $^{22}\text{Na}^+$ uptake, showed a positive intracellular and plasma membrane staining. These results indicate that among the six mutations tested, G316V and R399C are not complex glycosylated, absent from the plasma membrane, and nonfunctional. The mutants S555L and A588V exhibit complex glycosylation and presence in both the plasma membrane and the cytoplasm, contrasting with a lack of intrinsic activity and thereby representing a novel class of NCC mutants that were not previously identified in GS.

Genotype–Phenotype Correlations

On the basis of the clinical, genetic, molecular, and functional data described, we investigated potential genotype–phenotype correlations in this cohort of patients with GS. When considering the nine patients who harbored a severe phenotype, we noticed that six of them carried a splice mutation that led to a truncated NCC transcript that was downregulated by NMD (Figure 2, Table 3). The prevalence of splicing mutations in the severe GS subgroup was significantly higher than in the classical, mild GS subgroup (six of nine versus two of 16, respectively; $P < 0.01$) and in the HGMD database (10 of 113; $P = 0.0001$). Five of these patients were compound heterozygous for the splice defect and a missense mutation that affected either

![Figure 4. Functional studies of missense mutations in SLC12A3. (A) Position of the selected mutations in the predicted topology of human NCC (hNCC). Glycosylation sites are shown as branches. The localization of each mutation is represented by a triangle. (B) Immunoblots of total membrane (top) and plasma membrane (bottom) extracts that were isolated from Xenopus oocytes, either noninjected (NI) or injected with 10 ng of wild-type (WT) or mutant hNCC cRNA. (C) $^{22}\text{Na}^+$ uptake in oocytes that expressed WT or mutant hNCC. Uptake values are shown as percentage of the WT, where 100% represents an uptake of 22.5 pmol/oocyte per 2 h. **$P < 0.05$, ***$P < 0.001$ in relation to the uptake of NI oocytes. (D) Representative immunocytochemistry of oocytes injected with WT or mutant hNCC: Plasma membrane staining in an oocyte injected with WT hNCC (a); NI oocyte with only background staining (b); oocyte expressing nonfunctional G316V mutant with intracellular staining only (a similar pattern was observed for R399C; c); oocyte injected with functional N611S mutation that presented both plasma membrane and intracellular staining (a similar pattern was found for R145C; d); nonfunctional S555L-expressing oocyte showing both plasma membrane and intracellular staining (a similar pattern was observed for A588V; e).](image)
ion transport activity (S555L, A588V) or routing to the cell surface of a functional mutation (R145C) rather than resulting in a direct impact on the glycosylation. Only one mutant allele (c.2856+1G>T) was found in the remaining patient. The remaining three patients with a severe phenotype were male and had at least a nonfunctional mutant, absent from the cell surface (G741R and R399C) in one allele. The most recurrent mutation on the second allele among the group of severely affected patients was a newly described NCC mutant, suggesting a defect in the intrinsic activity of the co-transporter. No patient harbored a severe phenotype. Although limited by the relatively small number of severe cases, these data suggest that the combination of male gender with the presence of an allele of a splice defect that results in a truncated transcript or, less frequently, a nonfunctional intracellularly retained mutation could explain the clinical severity of GS.

**Discussion**

In this study, a total of 26 different mutations in the SLC12A3 gene (including eight novel) were identified in a series of 25 patients with GS, including 22 missenses, three ss, and one duplication. All of the missense mutations substitute amino acids that are strongly conserved through evolution. Five mutations that were predicted to alter the normal RNA splicing were confirmed by cDNA analysis. Analysis of the transcription profiles demonstrated that all of the spliceing mutations trigger NMD. Functional characterization of missense mutations demonstrated the existence of a novel class of NCC mutants, characterized by a lack of intrinsic activity despite normal glycosylation and plasma membrane expression. A high prevalence of splicing mutations were detected in the subgroup of patients who harbored a severe phenotype. Although limited by the relatively small number of severe cases, these data suggest that the combination of male gender with the presence of one allele of a splice defect that results in a truncated transcript or, less frequently, a nonfunctional intracellularly retained mutation could explain the clinical severity of GS.

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**Table 3. Clinical presentation, genotype, functional defect, and splice products in the nine severely affected patients**

<table>
<thead>
<tr>
<th>Gender ID</th>
<th>Age at Onset</th>
<th>Phenotype at Presentation</th>
<th>Genotype (Class of Functional Defect)</th>
<th>Allele 1</th>
<th>Allele 2</th>
<th>Aberrant Splice Products (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M GS01.1</td>
<td>10 yr</td>
<td>Growth and pubertal delay, severe hypotonia, weakness, dysesthesia</td>
<td>c.964+1G&gt;T (Ex 7 skip. p.Ala285_Ala322&gt;ArgfsX48)</td>
<td>c.433C&gt;T, p.(R145C) Defective insertion, functional activity</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>M GS01.2</td>
<td>9 yr</td>
<td>Growth and pubertal delay, asthenia, convulsion with cardiac arrest, prolonged QT</td>
<td>c.964+1G&gt;T (Ex 7 skip. p.Ala285_Ala322&gt;ArgfsX48)</td>
<td>c.433C&gt;T, p.(R145C) Defective insertion, functional activity</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>F GS02.1</td>
<td>12 yr</td>
<td>Growth delay, short stature, cramps, dysesthesia, tetany, pseudotumor cerebri</td>
<td>c.2633+1G&gt;C (Ex 22 skip.</td>
<td>c.1664C&gt;T, p.(S555L) Defective intrinsic activity</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>M GS03.1</td>
<td>17.5 yr</td>
<td>Growth and pubertal delay, invalidating cramps, dysesthesia, nocturia, major salt craving</td>
<td>c.1567G&gt;A (A523T, p.Cys482_Ala523&gt;LeufsX6)</td>
<td>c.1763C&gt;T, p.(A588V) Defective intrinsic activity</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>M GS03.2</td>
<td>16 yr</td>
<td>Growth and pubertal delay, nocturia, repeated cramps and dysesthesia</td>
<td>c.1567G&gt;A (A523T, p.Cys482_Ala523&gt;LeufsX6)</td>
<td>c.1763C&gt;T, p.(A588V) Defective intrinsic activity</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>M GS04.1</td>
<td>9 yr</td>
<td>Growth delay, severe episodes of tetany and palsy, cramps, asthenia, polyuria, nocturia</td>
<td>c.2856+1G&gt;T (Ex 24 skip. p.His907_lys952&gt;HisfsX6)</td>
<td>?</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>M GS05.1</td>
<td>21 yr</td>
<td>Severe neuromuscular symptoms, chondrocalcinosis, profound hypomagnesemia, tetany, paralysis</td>
<td>c.2221G&gt;A, p.(G741R) Defective processing, non-functional</td>
<td>c.1664C&gt;T, p.(S555L) Defective intrinsic activity</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>M GS06.1</td>
<td>21 mo</td>
<td>Growth delay, hypotonia</td>
<td>c.2221G&gt;A, p.(G741R) Defective processing, non-functional</td>
<td>c.514T&gt;C, p.(W172R)ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>M GS07.1</td>
<td>18 mo</td>
<td>Growth and pubertal delay, hypotonia, dysesthesia</td>
<td>c.1195C&gt;T, p.(R399C) Defective processing, non-functional</td>
<td>?</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

*ND, not determined (for missense mutations); ?, unidentified mutation at the second allele.

bBased on Sabath et al. (31).

Relative quantification as percentage of the correct splice product from the other allele.

Functional studies by de Jong et al. (7).
The mutation detection rate is this series of patients with GS is approximately 80%. Only one mutant allele has been identified in affected individuals from six families (26%), compatible with the 40% of simple heterozygosity previously reported (26). Among them, four were patients who presented in adulthood with mild symptoms but all of the classic biologic manifestations of GS, indicating that they are indeed affected by GS and not only carriers. The GS is recessively inherited, with simple heterozygous relatives being clinically and metabolically asymptomatic, although increased urinary sodium excretion reflecting higher sodium intake has been mentioned in one study that investigated a large Amish kindred (27). The high proportion of single heterozygosity among affected patients is likely due to a failure to identify the mutation in the other allele. Furthermore, mutations were not detected in two patients who met the classical diagnosis criteria of GS. Explanations for this detection failure include large genomic rearrangements or mutations in regulatory fragments or deeper intrinsic sequences that are not routinely screened. Alternatively, the expression of the NCC co-transporter may be influenced by epigenetic modifications and/or silent polymorphisms that could interfere with its function. Also, other genes that code for functionally related channels or transporters that are located in the distal nephron may be involved (26). The possibility of large deletions in our series was excluded by using MLPA (28), because no difference in the copy numbers of the 26 target exons was detected in the six simple heterozygous and the two patients who were negative for the mutation screening.

The majority (22 [85%] of 26) of mutations that were detected in this series are missense, as described previously (4,6). Fewer than one third of the mutations that are associated with GS and collected in the HGMD include nonsense, frameshift, and ss defects and gene rearrangements that are likely to have a loss-of-function effect on the co-transporter. These changes predict premature translation stop codons with likely loss through the mRNA surveillance pathway, NMD, which allows cells to degrade mRNA that contain premature translation stop codons if they are located at a position more than 50 to 54 nucleotides upstream of a splicing-generated exon–exon junction (29). We provide the first evidence that SLC12A3 splice defects that lead to frameshifted mRNA trigger the NMD process. The preferential degradation of each aberrantly spliced product has been documented, with mutant transcript levels ranging from 9 to 58% compared with the normal transcript, supporting that NMD operates with variable efficiency (30). The demonstration that the NMD surveillance mechanism operates on SLC12A3 splice mutations suggests the residual translation of mutated mRNA that will lead to truncated co-transporters. In turn, these abnormal transcripts could partially explain disease variability (Table 4).

Functional studies of mutant channels/transporters in the Xenopus laevis oocyte heterologous expression system have suggested that at least five different mechanisms can account for the reduced or abolished transport activity: (i) impaired protein synthesis; (ii) impaired protein processing to the cell surface; (iii) impaired insertion of an otherwise functional protein into the plasma membrane; (iv) impaired functional properties, with defective intrinsic activity; and (v) accelerating protein removal or degradation (31,32). De Jong et al. (7) investigated eight mutant hNCC cDNA and distinguished two different classes of mutations: Improperly glycosylated proteins retained in the cytosol and normally glycosylated, functional mutants that are partly impaired in their routing to the cell surface, which suggested that the NCC processing defect represents the underlying mechanism in GS.

We used the Xenopus heterologous expression system to investigate in detail six mutations that were detected in severely affected patients and/or clustering on residues within the central hydrophobic domain of NCC. On the basis of the glycosylation pattern, plasma membrane expression, and \(^{22}\text{Na}\) uptake, we identified a novel class of NCC mutation that is exemplified by A588V and S555L (Figure 4). These mutants are fully glycosylated and expressed in the plasma membrane, with partial retention inside the cell. However, they do not show intrinsic activity when present at the plasma membrane. This reduced activity is not due to defective protein synthesis, because the total expression level of all mutant proteins was similar to wild-type NCC expression. Therefore, our results suggest that A588V and S555L belong to a novel class of NCC mutants that affect the functional properties of the co-transporters. Of note, Sabath et al. (31) recently investigated the functional properties of the mouse A585V cDNA, which corresponds to the pathogenic human mutation A588V identified here. They showed that A585V generates a mutant protein that exhibits mature glycosylation bands but an activity that represents only approximately 6% of the wild type. In that work, 25 ng of cRNA was

<table>
<thead>
<tr>
<th>Pathogenic Mechanisma</th>
<th>SLC12A3 Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Missplicing resulting in a truncated transcript downregulated by nonsense-mediated decay; impaired protein synthesis</td>
<td>c.964+1G&gt;T, A523T, c.2856+1G&gt;T, c.2667dupA, R642H, c.2633+1G&gt;C</td>
</tr>
<tr>
<td>Defective protein processing, no functionality</td>
<td>G316V, R399C, G741Rb</td>
</tr>
<tr>
<td>Defective protein insertion, functionality</td>
<td>R145C, L215Pb, N611S, G980Rb, C985Yb</td>
</tr>
<tr>
<td>Defective intrinsic activity</td>
<td>S555L, A588V</td>
</tr>
</tbody>
</table>

aBased on Sabath et al. (31).
bMissense mutations previously studied by de Jong et al. (7).
injected (versus 10 ng injected in our study), and the Km values to assess the affinity of the mutant A585V for ions Na\(^+\) and Cl\(^-\) were too low to be detectable. We therefore can presume that mouse A585V, corresponding to human A588V, is indeed a nonfunctional mutation, likely the result of a defect on its intrinsic activity rather of a processing defect. The remaining four mutations that were investigated here belong to previously described nonfunctional (G316V and R399C) and partly functional (R145C and N611S) types of mutants.

Our results extend the range of pathogenic mechanisms that underline GS by identifying NCC mutations that result in a defective intrinsic transport activity, a mechanism that has not been shown before to underline GS (Table 4). This pathogenic mechanism is not exclusive for NCC mutations. Recent studies have identified mutations that functionally impair other members of the electroneural co-transporter family, such as SLC12A1 and SLC12A6, without affecting their targeting to the plasma membrane (33,34). Similarly, some mutations in the CF gene alter the conduction of the Cl\(^-\) channel CFTR, without affecting its targeting (35). Of note, most of these peculiar CF mutations are located within the membrane-spanning domains of CFTR (36,37), and both mutations that were identified here are located within the central hydrophobic domain of NCC: S555L lies at the end of TM10, whereas A588V is within TM12. It has been suggested that the central domain of NCC determines ion translocation and thiazide-binding specificity (38,39), which could provide a basis for the pathogenic role of these missense mutations.

The transcriptional and functional analyses of SLC12A3 mutations, combined with clinical characterization, give new insights into the disease variability in GS. In agreement with reports that suggested that GS is more severe than generally anticipated (5,21,22), our series identified a subgroup of nine patients with a severe phenotype including childhood presentation, growth delay and/or pubertal delay, and severe clinical manifestations. Although this subgroup was relatively small, the phenotype was remarkably consistent (Table 3, Figure 3, Supplementary Table 2). Comparison between these severe cases and the more classical cases (Table 2) revealed significant differences in BP control and extent of hypocalcuria, as well as a trend for lower kalemia and increased urinary Na\(^-\). Strikingly, there were eight male patients for only one female patient in the severe subgroup, whereas an inverse gender ratio was found in the mild subgroup. These data confirm and extend previous studies that reported that female patients with GS show milder symptoms than do male patients (40), which could be explained by a positive effect of estrogens. Several lines of evidence have shown that estrogens affect a variety of mediators in renal tissues (e.g., by stimulating the transcription and/or activity of nitric oxide synthase isoforms, interacting with components of the renin-angiotensin system [41]). In turn, such estrogen-mediated mechanisms could play a role in the association of male gender with faster progression in various types of renal disease (42). Furthermore, estrogens seem to have a direct effect on the expression of NCC and the structure of the DCT: In ovariectomized rats, estradiol replacement restored DCT ultrastructure and NCC expression in the apical membrane of the tubule (43).

Of note, such a positive effect could also affect prepubertal children, which is important when considering that five patients in the severe group had a prepubertal onset (age <10 yr). Highly sensitive assays have detected very low concentrations of estradiol in children before puberty, with significantly lower levels (between three- and eight-fold) being observed in prepubertal boys compared with girls at the same age (44). These data suggest that the hypothalamic-pituitary-gonadal axis is functionally active and plays a biologic role during childhood, with children being extremely sensitive to sex steroid actions (45). One therefore could speculate that the lower levels of estradiol in prepubertal boys could play a role in the severe phenotype that is associated with some mutations of NCC, whereas a higher level of estrogens in prepubertal girls might be sufficient to attenuate the manifestations.

Mutation analysis also yielded important information. The SLC12A3 mutations that were associated with a severe presentation in this cohort were at least the combination for one allele of a missense mutation that resulted in a nonfunctional intracellular retained protein or, even more frequent, a missplicing that resulted in a short transcript that was downregulated by NMD. Such splicing defects were detected in two third of the severely affected patients, whereas their prevalence in the general population of patients with GS is <10%. It must be noted that the phenotype of heterozygotes who carry relatively NMD-resistant transcripts (40 to 60%) does not seem to be more severe than in patients who carry sensitive NMD transcripts (<15%). This seeming lack of dominant negative effect may reflect the inefficiency with which frameshifted mRNA are effectively translated. When we turn on the second allele of the patients with a severe phenotype, the more recurrent mutation was the novel NCC mutant, affecting the intrinsic functional properties of the co-transporter. Taken together, these data suggest that the nature/position of the harbored SLC12A3 mutation, combined with male gender, can explain the clinical severity in a subset of patients with GS. Accordingly, a remarkably similar phenotype was noted in two pairs of severely affected male siblings (c.[433C>T][964+1G>T] and c.[1763C>T][1567G>A]), whereas a clinical discrepancy was observed between one severely affected brother (c.[1664C>T][221G>A]) and his sister, who presented a much milder phenotype. The gender effect may also explain why a female patient who is compound heterozygous for a truncated mutation and a nonfunctional, intracellually retained substitution (c.[2667dupA][947G>T])—predicted to be severe—had a mild clinical and biochemical presentation.

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Disclosures
None.

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