Novel Mutations in Thiazide-Sensitive Na-Cl Cotransporter Gene of Patients with Gitelman’s Syndrome

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Abstract. Gitelman’s syndrome (GS) is an autosomal recessive disorder characterized by metabolic alkalosis, hypokalemia, hypomagnesemia, and hypocalciuria. In this study, possible mutations in the TSC gene of six Japanese patients clinically diagnosed with GS were investigated. Twenty-six exon encoding TSC were amplified by PCR and then completely sequenced by the direct sequencing method. Patient A showed a missense mutation of Arg 642 to Cys on the paternal allele and a missense mutation of Val 578 to Met and a 2-bp deletion (nucleotide 2543–2544) on the maternal allele. This deletion results in a frameshift that alters codon 837 to encode a stop signal rather than phenylalanine, and it is predicted to lead to loss of the latter half of the intracellular carboxy terminus. In the second family, two affected sisters, patients B and C, had a homozygous missense mutation of Thr 180 to Lys. Both of their parents, who are consanguinely married, have a heterozygous Thr180Lys mutation. Patient D has a homozygous mutation Thr180Lys, which is the same as the second family. Haplotype analysis indicates that patients B and C are not related to patient D. In patients E and F, we could identify only one mutant allele; Ala569Glu and Leu849His, respectively. All of the mutations identified are novel except for the Arg642Cys mutation, which has been found in a Japanese GS patient. Although further in vitro study is required to prove that the mutations are responsible for GS, it is possible that Thr180Lys and Arg642Cys mutations might be common mutations in Japanese GS.

Materials and Methods
Gitelman’s Syndrome Patients

Six patients from five families with GS were collected from Keio University Hospital, Tokyo, and Ashikaga Red-Cross Hospital, Tochigi, Japan. The affected siblings B and C are offspring of a consanguineous couple, and patient D is also offspring of a consanguineous couple.

Representative biochemical data are shown in Table 1. Diagnostic criteria were as reported by Bettinelli et al. (9): hypomagnesemia (<0.65 mmol/L), hypokalemia (<3.6 mmol/L), and hypocalciuria (the molar ratio of urinary calcium to creatinine <0.10). Patients A, D, and E underwent renal clearance test using furosemide or thiazide. In all patients examined, urine volume and chloride clearance were increased after furosemide administration but not after thiazide administration. These findings confirmed the diagnosis of GS for patients A, D, and F (10). Informed consent was obtained from each participating subject.

Mutation Analysis

Genomic DNA was extracted from peripheral blood lymphocytes by a DNA extraction kit according to the manufacturer’s instructions (Stratagene, La Jolla, CA). Twenty-seven pairs of oligonucleotide primers were generated to amplify all 26 exons of the TSC gene according to data obtained from Simon et al. (4) and Mastroianni et al. (5). In the present study, M13 universal primer sequence (5’-GTA- AAACGACGCGCCAGT-3’) was added to each synthetic forward primer for direct sequencing. PCR was performed in 50 μl of solution containing 0.2 mM dNTP, 0.05 U/μl Taq polymerase, 1.5 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), approximately 50 ng genomic DNA, and 1 μM of each primer with thermodenature at 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, 35 cycles. The PCR products were separated on an agarose gel, isolated, and purified by a DNA...
purification kit (Bio 101, Vista, CA). Direct sequencing reaction using the purified PCR product was performed by a cycle sequencing method with the M13 universal primer. The resultant sample was then sequenced on an automated DNA sequencer (Perkin-Elmer-ABI, Foster City, CA).

**Haplotype Analysis**

Haplotypes of family members were defined using microsatellite markers D16S408 and D16S494 flanking the TSC locus. Microsatellite polymorphisms were amplified by PCR using forward primers labeled at the 5′9 end with fluorescent dye. PCR was conducted for 30 cycles with denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 45 s. Amplified products were separated on capillary gels run under denaturing conditions and analyzed by Genescan software (Perkin-Elmer-ABI).

**Restriction Fragment Length Polymorphism**

Amplified DNA fragment containing the exon 4 was digested with Tsp45I (New England Biolabs, Beverly, MA) at 65°C for 12 h, and the sample was size-separated in a 10% denatured polyacrylamide gel. The PCR samples were from the GS family members and 50 unrelated healthy subjects.

**Allele-Specific Oligonucleotide Hybridization**

Exons 14, 15, and 21 of the human TSC gene were amplified from genomic DNA of the GS family members and 50 unrelated healthy subjects. The PCR products were blotted onto nylon filters with a Bio-Dot apparatus (Bio-Rad, Richmond, CA). The filters were prehybridized individually for 30 min at 55°C in 3 M tetramethylammonium chloride/50 mM Tris-HCl, pH 8.0/2 mM ethylenediaminetetra-acetic acid (EDTA)/0.1% sodium dodecyl sulfate/100 μg/ml denatured sonicated salmon sperm DNA/5X Denhardt’s solution. Synthetic 19-mer oligonucleotides, with the mutant or normal nucleotide in the central position, were 5′9 end-labeled by phosphorylation with [γ-32P]ATP and T4 polynucleotide kinase, and were added to the hybridization solution. Hybridization was continued at the same temperature for 1 h. Filters were washed twice in 2X saline-sodium phosphate-EDTA/0.1% sodium dodecyl sulfate for 10 min at room temperature. Then the filters were rinsed in 3 M tetramethylammonium chloride hybridization buffer minus carrier DNA and Denhardt’s solution, and washed again in this solution for 30 to 60 min at 60°C.

**Results**

Twenty-six exons of the TSC gene were amplified from the genomic DNA of GS patients by PCR, and then completely sequenced by direct sequencing. Sequence analysis of the TSC gene from patient A revealed three different mutations. One missense mutation was a heterozygous G to A base pair substitution at position 1738 in exon 14, which causes a Val to Met substitution at position 578 (Figure 1A). Val578 in TSC is conserved among various species. However, the substitution of Val to Met is not expected to result in any dramatic change in function of TSC, since Val and Met are classified in the same amino acid group. The second missense mutation is a heterozygous C to T base pair substitution at position 1930 in exon 15, which causes an Arg to Cys substitution at position 642 (Figure 1B). Arg 642 is well conserved in both TSC and bumetanide-sensitive cotransporter (BSC) between different species. The substitution of Arg to Cys is expected to cause a dramatic change in TSC protein function, since Cys introduces an SH residue. The third mutation is a heterozygous 2-bp (TT) deletion at 2543–2544 (Figure 1C). This deletion results in a frameshift that alters codon 837 to encode a stop signal rather than phenylalanine, and it is predicted to lead to loss of the latter half of the intracellular carboxy terminus. Genomic DNA was obtained from his mother and daughter, who did not show

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**Table 1. Biochemical data of patients with Gitelman’s syndrome**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Family and Patient Designation</th>
<th>GS Criteria</th>
</tr>
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<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>Age</td>
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<td>43</td>
</tr>
<tr>
<td>Na (mmol/L)</td>
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<td>K (mmol/L)</td>
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<td>Cl (mmol/L)</td>
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<td>96</td>
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<td>Mg (mmol/L)</td>
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</tr>
<tr>
<td>HCO₃ (mmol/L)</td>
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<td>33.0</td>
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<td>Urinary Ca/Cr (molar)</td>
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</tr>
<tr>
<td>Response to thiazide</td>
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<td>−</td>
</tr>
<tr>
<td>Response to furosemide</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mutation found in TSC gene</td>
<td>V578M b</td>
<td>T180K c</td>
</tr>
<tr>
<td></td>
<td>R642C b</td>
<td>T180K c</td>
</tr>
<tr>
<td></td>
<td>delTT b</td>
<td>A569E b</td>
</tr>
</tbody>
</table>

a GS, Gitelman’s syndrome; TSC, thiazide-sensitive Na-Cl cotransporter.

b Heterozygous mutation.

c Homozygous mutation.
any electrolyte disturbance in their laboratory data (their serum potassium levels were 4.1 mmol/L). Direct sequencing analysis of TSC genes of his mother and daughter revealed 2-bp deletion and Val578Met on the paternal allele of patient A. We investigated the prevalence of the mutations by an allele-specific oligonucleotide hybridization technique. No mutant allele containing the Val578Met, Arg642Cys, or delTT mutations was identified in 50 unrelated healthy subjects.

In family 2, two sisters were diagnosed with GS. The parents are a consanguineous couple and did not show any electrolyte disturbance in their laboratory data (their serum potassium levels were 3.9 and 3.8 mmol/L). Direct sequence analysis revealed that the TSC gene of patient B has a homozygous C to A base pair substitution at position 545 in exon 4 (Figure 2A). This mutation causes a Thr to Lys substitution at position 180. Thr180 is only present in the human TSC protein and not conserved in rat or winter flounder TSC. The rest of the members of family 2 were analyzed by a PCR-restriction fragment length polymorphism (PCR-RFLP) method. The 166-bp fragments containing exon 4 of the TSC gene were amplified from DNA of the members of family 2, followed by digestion with Tsp45I. There were two Tsp45I sites in the normal control sequence, producing cleavage fragments of 100, 60, and 6 bp (Figure 2B, lane 5). The C to A transition abolishes one Tsp45I site. Tsp45I cleaves the amplified DNA fragment from the mutant allele into only two fragments of 100 and 66 bp. PCR-RFLP analysis shows cosegregation of the Thr180Lys mutation among the affected individuals in this family. The affected sisters are homozygous, and their parents are heterozygous for the Thr180Lys mutation (Figure 2B, lanes 1 to 4). Direct sequencing analysis confirmed that patient C has a homozygous Thr180Lys mutation and the parents have heterozygous Thr180Lys mutations.

Patient D is the offspring of a consanguineous couple. Direct sequencing analysis of TSC genes of his mother and daughter revealed 2-bp deletion and Val578Met on the paternal allele and Arg642Cys on the maternal allele of patient A. We investigated the prevalence of the mutations by an allele-specific oligonucleotide hybridization technique. No mutant allele containing the Val578Met, Arg642Cys, or delTT mutations was identified in 50 unrelated healthy subjects.

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sequencing analysis revealed that the TSC gene of patient D had the same homozygous Thr180Lys mutation as patients B and C. To examine whether families 2 and 3 are unrelated, we performed haplotype analysis using microsatellite markers D16S408 and D16S494, which flank the TSC locus (4). Patients B and C had a homozygous 3 to 1 haplotype, whereas patient D had a homozygous 2 to 6 haplotype for D16S408-D16S494 (Figure 3). This confirmed that the two families are unrelated. It is possible that Thr180Lys mutation might be common in Japanese GS patients. We investigated the prevalence of Thr180Lys mutations in the Japanese population by PCR-RFLP. PCR-amplified DNA fragments containing exon 4 from 50 unrelated healthy subjects were digested with Tsp45I. The heterozygous Thr180Lys mutation was observed in two of 50 healthy subjects. Their serum potassium levels were 3.9 and 3.8 mmol/L, and their serum magnesium levels were 1.15 and 0.78 mmol/L, respectively.

Patient E had been formerly diagnosed with Bartter’s syndrome. His urinary calcium excretion was relatively low (calcium:creatinine molar ratio 0.16), but did not fulfill Bettinelli’s criteria for Gitelman’s syndrome (calcium:creatinine molar ratio <0.10). Patient E underwent renal clearance test using furosemide or thiazide, which is useful in distinguishing between Bartter’s syndrome and GS (10). The urine volume and chloride clearance were increased after furosemide administration but not after thiazide administration. Patient E was diagnosed with GS. Family members did not complain of any symptoms suggesting GS. The serum potassium levels of his mother and children were within the normal range (Figure 4A). Direct sequencing analysis revealed that patient E has a heterozygous C to T base pair substitution at position 1712 in exon 14 (Figure 4B). This mutation causes an Ala to Glu substitution at amino acid 569. Ala569 is present in the human and rat TSC protein but is not conserved in winter flounder TSC protein. The substitution of Ala to Glu introduces an acidic side chain. We could not identify any mutation on another allele. We obtained DNA from his children. Direct sequencing analysis revealed that they did not carry the mutant allele. The screening using the allele-specific oligonucleotide

![Figure 3. Pedigrees and haplotypes of families 2 and 3. Affected individual is indicated by a filled symbol. Males and females are indicated by squares and circles, respectively. Allele numbers for markers D16S408 and D16S494 are indicated below the pedigree symbols.](image)

![Figure 4. (A) Pedigree of family 4. Affected individual is indicated by filled symbols. Males and females are indicated by squares and circles, respectively. Serum potassium, magnesium, and bicarbonate concentrations are given below the pedigree symbols. ND, not determined. (B) Identification of TSC mutation in patient E by direct sequencing analysis. DNA chromatograms from a control individual (top) and patient E (bottom) are shown. The patient E has a heterozygous transition (C→T) at nucleotide 1712 located in exon 14, resulting in an Ala to Glu substitution at amino acid 569.](image)
hybridization technique did not identify the Ala569Glu mutation in any of 50 unrelated healthy subjects.

In family 5, patient F and his twin brother and his sister have been diagnosed with GS. The serum potassium levels of his parents were within the normal range (Figure 5A). Consanguinity was not identified in family 5. Only genomic DNA from patient F was available. Sequence analysis revealed that patient F has a heterozygous T to A base pair substitution at position 2579 in exon 22 (Figure 5B). This mutation causes a Leu to His substitution at position 849. Leu849 is well conserved in TSC and BSC from different species. The substitution of Leu to His introduces a basic chain. We could not identify any mutation on another allele. The screening using a direct sequencing technique did not identify the Leu849His mutation in any of 50 unrelated healthy subjects.

Discussion

We have identified six TSC gene mutations in Japanese GS patients, including five missense mutations and one frameshift mutation. Five of the mutations are novel variants of the TSC gene, and one mutation has been found previously in a Japanese GS patient.

In patient A, the TSC gene had three different mutations: Arg642Cys on the paternal allele, and a 2-bp deletion and Val578Met on the maternal allele. Yahata et al. reported a homozygous mutation of Arg642 to Cys in a Japanese GS patient (11). Lemmink et al. also reported a homozygous mutation of Arg642 to His in a German GS patient (8). In addition, Arg642 is strongly conserved in TSC and BSC from different species. Arg642 might play a critical role in these transporters. On the other allele, the 2-bp deletion is more likely to be pathogenic than the Val578Met mutation. The 2-bp deletion results in a frameshift that alters codon 837 to encode a stop signal rather than phenylalanine, and it is predicted to lead to loss of the latter half of the intracellular carboxy terminus. This region contains a putative cAMP-dependent protein kinase phosphorylation site, a putative protein kinase C phosphorylation site, and two putative casein kinase phosphorylation sites (4).

The two affected sisters, patients B and C, had a homozygous missense mutation of Thr180 to Lys. Their consanguineous parents have a heterozygous Thr180Lys mutation, but their laboratory data are within the normal range. The Thr180Lys mutation completely segregates with the disease phenotype. Surprisingly, we also found the homozygous Thr180Lys mutation in the TSC gene of patient D. The family of patients B and C came from Ishikawa Prefecture, and the family of patient D came from Hiroshima Prefecture. Haplotype analysis using microsatellite markers D16S408 and D16S494 flanking the TSC locus demonstrated that the families are unrelated. It is possible that the Thr180Lys mutation is a common mutation for Japanese patients with GS.

We investigated the frequency of Thr180Lys mutations in the general Japanese population. The homozygous Thr180Lys mutation was observed in two of 50 healthy subjects. Although the true prevalence of GS is unknown, the prevalence of heterozygotes based on phenotypic expression is approximately 1% in the Swedish and Italian populations (4,12). If this estimation is applied to the Japanese population, the prevalence of the Thr180Lys mutation in healthy subjects is too high. To prove that the Thr180Lys mutation is responsible for GS, additional in vitro studies are required. Studies are under way.

Figure 5. (A) Pedigree of family 5. Affected individuals are indicated by filled symbols. Males and females are indicated by squares and circles, respectively. Serum potassium, magnesium, and bicarbonate concentrations are given below the pedigree symbols. ND, not determined. (B) Identification of TSC mutation in patient F by direct sequencing analysis. DNA chromatograms from a control individual (top) and patient F (bottom) are shown. The patient F has a heterozygous transition (T→A) at nucleotide 2579 located in exon 22, resulting in a Leu to His substitution at amino acid 849.
to measure the effect of individual mutations on sodium-chloride transport in a functional expression system.

In patients E and F, we identified only one mutant allele. Because their parents lack the GS phenotype, heterozygosity for the Ala569 Glu or Leu849His mutation is considered insufficient to cause the phenotype. Patients E and F must have an unidentified gene abnormality on the other allele in addition to the Ala569 Glu or Leu849His mutation. In previous studies, only one mutant allele was detected in five of 11 (4), five of 12 (5), and seven of 20 patients (8). There are several explanations to justify this failure to identify both mutant alleles in patients E and F. First, mutations may be present in gene-regulating fragments such as promoter or enhancer segments, intron sequences, or 5′ and 3′ noncoding regions, which have not yet been screened for mutations. Second, large heterozygous TSC gene deletions, such as have been found in the CLCNKB gene of patients with Bartter’s syndrome (13), will not be identified by mutation detection techniques based on analysis of individual exons. Third, it is possible that there is a concurrent heterozygous mutation in a gene other than the TSC gene, particularly in one of the three genes known to cause Bartter’s syndrome: NKCC2, ROMK, and CLCNKB. Patients E and F have an intermediate phenotype between Bartter’s and Gitelman’s syndrome. Urinary calcium excretion was mildly depressed in patient E but did not fulfill Betinelli’s criteria. In patient F, serum magnesium was only mildly depressed. The two patients with an intermediate phenotype suggest the possibility of compound heterozygotes for the TSC and Bartter genes.

All of the mutations we identified were novel except for Arg642Cys, which had been found in a Japanese GS patient. To date, Arg955Gln, Leu623Pro, and Arg642Cys mutations have been reported in Japanese GS patients (4,6,11). All of them are homozygous mutations. Together with the earlier studies and this report, the Arg642Cys and Thr180Lys mutations were found in at least two unrelated families. It is possible that the Arg642Cys and Thr180Lys mutations are common mutations in Japanese GS patients.

Acknowledgments
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References

This article can be accessed in its entirety on the Internet at http://www.lww.com/JASN along with related UpToDate topics.
Errata

The article by Monkawa et al., “Novel Mutation in Thiazide-Sensitive Na-Cl Cotransporter Gene of Patients with Gitelman’s Syndrome” (J Am Soc Nephrol 11: 65–70, 2000) contained an error in the amino acid substitution caused by a mutation. In patient E, the C-T amino acid change caused the mutation Ala569Val, rather than Ala569Glu. As a result, the following corrections should be noted: (1) In the Abstract, column 2, line 9, “Ala569Glu” should read “Ala569Val.” (2) In Table 1, “A569E” should read “A569V.” (3) In the Results section, p. 68, column 2, lines 22 and 25, “Ala to Glu” should read “Ala to Val”; p. 69, column 1, line 1, and p. 70, column 1, lines 5 and 7, “Ala569Glu” should read “Ala569Val.” (4) In Figure 4 legend, “Ala to Glu” should read “Ala to Val.” The authors regret any inconvenience caused by this error.