Genetic Variants of Thiazide-Sensitive NaCl-Cotransporter in Gitelman’s Syndrome and Primary Hypertension

Olle Melander, Marju Orho-Melander, Kristina Bengtsson, Ulf Lindblad, Lennart Råstam, Leif Groop, U. Lennart Hulthén

Abstract—Gitelman’s syndrome is an autosomal recessive disorder characterized by electrolyte disturbances and low blood pressure. The disease is caused by homozygous or compound heterozygous inactivating mutations in the thiazide-sensitive NaCl-cotransporter gene leading to reduced renal sodium reabsorption. We report 4 patients with Gitelman’s syndrome from southern Sweden, all in whom we identified compound heterozygous mutations in the thiazide-sensitive NaCl-cotransporter gene (Gly439Ser, Gly731Arg, Gly741Arg, Thr304Pro, and 2745insAGCA), of which the latter 2 have not been described before. We hypothesized that such mutations in their heterozygous form protect against primary hypertension in the general population and that the gene may also harbor activating mutations that increase the risk for primary hypertension. Accordingly, the gene was screened for mutations in 20 patients with primary hypertension and in 20 normotensive subjects by single-strand conformation polymorphism and direct DNA sequencing. The Arg904Gln, Gly264Ala, and C1420T variants, found in the mutation screening of subjects without Gitelman’s syndrome, were studied further. Population genotype frequencies were determined in 292 unrelated patients with primary hypertension and 264 unrelated normotensive subjects from southern Sweden. Gln904 homozygotes were overrepresented in hypertensive patients compared with normotensive subjects (5 of 292 versus 0 of 264; \(P = 0.03\)). In conclusion, we confirm that Gitelman’s syndrome is caused by mutations in the thiazide-sensitive NaCl-cotransporter gene. Our results further suggest that subjects homozygous for the Gln904 variant have an increased risk for development of primary hypertension. (Hypertension. 2000;36:389-394.)

Key Words: DNA ■ genes ■ sodium channels ■ hypertension, genetic

Primary hypertension is a multifactorial and polygenic disease with unknown cause.\(^1\) During the past years, the molecular genetic defects have been described for 3 monogenic forms of hypertension\(^2\,\,3\) and for 3 monogenic diseases characterized by salt wasting and low blood pressure.\(^4\,\,9\) All these genetic defects involve abnormal renal sodium reabsorption. The amiloride-sensitive epithelial sodium channel gene has been of particular interest as a model of genetic blood pressure regulation, since activating mutations in this gene cause sodium retention and hypertension (Liddle’s syndrome)\(^2,10\) whereas inactivating mutations cause salt wasting and hypotension (pseudohypoaldosteronism type 1).\(^9\)

Gitelman’s syndrome (the predominant subset of patients who were earlier referred to as having “Bartter’s syndrome”) is an autosomal recessive disease characterized by sodium wasting, low blood pressure, secondary hyperaldosteronism, hypokalemia, alkalosis, hypomagnesemia, and hypocalciuria.\(^7,11-13\) The patients may have fatigue, joint pains, and neuromuscular symptoms. These features closely resemble those of the adverse effects of thiazide diuretics. Gitelman’s syndrome has been shown to be caused by mutations in the gene coding for the thiazide sensitive NaCl-cotransporter (TSC),\(^7,14-17\) which is the target for the thiazide diuretic class of antihypertensive drugs. These mutations are believed to reduce the capability of the TSC to reabsorb salt in the distal renal tubules, where the cotransporter is specifically expressed.\(^7,18\)

In the general population, it is likely that the individual blood pressure level is influenced by several different genetic variants, some of which increase and some of which lower blood pressure. Sodium sensitivity, that is, raised blood pressure after a sodium load, is believed to reflect decreased ability of the kidney to excrete sodium and could be an important factor in the development of primary hypertension in a subset of patients.\(^19\) Genetic variation in genes encoding proteins involved in renal sodium reabsorption are therefore likely to be of importance in determining the individual blood pressure level. Inheritance of one inactivating TSC mutation from each parent severely reduces sodium reabsorption and is required for the autosomal recessive Gitelman’s syndrome to manifest.\(^7\) Compared with the monogenic forms of hypertension,\(^2\,\,4\) Gitelman’s syndrome appears to be relatively com-
mon and, based on clinical features, the prevalence has been estimated to be \( \approx 19 \) per million in the city of Gothenburg in Sweden,\(^2\) suggesting a prevalence of heterozygous mutation carriers of \( \approx 0.9\%.\) However, the symptoms of the disease are generally mild and nonspecific, and the diagnostic workup is, in the majority of cases, initiated by incidental detection of hypokalemia.\(^2\) The prevalence of the disease and that of heterozygous carriers of Gitelman’s syndrome mutations are therefore most likely underestimated. We hypothesized that heterozygous carriers of inactivating TSC mutations may have moderately decreased renal sodium reabsorption, which protects them against development of hypertension. Furthermore, knowing that in the epithelial sodium channel gene both inactivating “hypotensive” mutations and activating “hypertensive” mutations have been described,\(^2,9,10\) we hypothesized that the TSC gene could also harbor activating mutations that would elevate blood pressure. The aims of this study were (1) to identify mutations in the TSC gene responsible for Gitelman’s syndrome in 4 patients from southern Sweden and to investigate if these mutations may protect against primary hypertension; (2) to identify variants in the TSC gene in individuals without Gitelman’s syndrome; and (3) to investigate whether such genetic variants influence the risk of developing primary hypertension.

Methods

Subjects and Phenotyping

Two siblings (patients 1 and 2) and 2 sporadic cases (patients 3 and 4) from southern Sweden were diagnosed with Gitelman’s syndrome at the Department of Endocrinology, Malmö University Hospital, based on the presence of hypokalemia (<3.5 mmol/L), hypomagnesemia (<0.70 mmol/L), hypocalciuria (<2.5 mmol/d), high plasma renin activity (>1.7 μg/L/hour in the supine position), and hyperaldosteronemia (>0.28 mmol/L in the supine position). All patients had fatigue, and one of them also had joint pain. In the mutation screening of the TSC gene in subjects without Gitelman’s syndrome, we included 20 unrelated patients (13 men and 7 women) with early onset (≤40 years of age) of primary hypertension, all of whom were receiving chronic pharmacological antihypertensive treatment. Their median (range) age was 58.5 (20.0 to 68.5) years, body mass index 26.4 (20.7 to 31.7) kg/m\(^2\), systolic blood pressure 160 (140 to 210) mm Hg, diastolic blood pressure 91.5 (80.0 to 113) mm Hg, and the age at onset of hypertension 36.5 (20.0 to 40.0) years. In addition, 20 healthy unrelated subjects (9 men and 11 women) who were ≥50 years of age and had a normal blood pressure (systolic and diastolic blood pressure of ≤140 mm Hg and ≤80 mm Hg, respectively) and who received no medication and had no family history of hypertension in first-degree relatives were included in the mutation screening. Their median (range) age was 61.8 (54.0 to 82.8) years, body mass index 24.8 (18.6 to 36.9) kg/m\(^2\), systolic blood pressure 124 (93.0 to 138) mm Hg, and diastolic blood pressure 72.5 (55.0 to 80.0) mm Hg. None of the 40 subjects had diabetes mellitus, kidney disease, or secondary hypertension. The reason for screening both hypertensive patients and normotensive subjects was to investigate whether such genetic variants influence the risk of developing primary hypertension.

with primary hypertension were diagnosed before the age of 60 years and were all receiving chronic pharmacological antihypertensive treatment. Subjects with diabetes mellitus, kidney disease, or secondary hypertension were excluded. The normotensive control subjects were selected as follows: (1) ≥40 years of age; (2) systolic and diastolic blood pressure of ≤150 mm Hg and ≤80 mm Hg, respectively; (3) no personal history of elevated blood pressure, diabetes mellitus, or any other chronic disease; (4) absence of medication; and (5) no family history of hypertension in first-degree relatives. The large association study material has been studied earlier\(^20\) and was thus selected before the mutation screening material. The mutation screening material was selected when the present study was designed, with stricter inclusion criteria. The two study materials thus represent independently selected random samples from the same pool of patients and control subjects attending or recruited through 5 outpatient clinics in southern Sweden. Twenty-nine individuals (13 patients and 16 normotensive subjects) in the mutation screening material had also been included in the large association study material. Methods for blood pressure and anthropometric measurements have been described earlier.\(^26\) Serum potassium concentrations were measured by standard biochemical methods. All study participants had given written informed consent, and the study was approved by the ethics committee of the Medical Faculty of Lund University.

Mutation Screening

Total genomic DNA was extracted from venous blood by standard methods.\(^21\) Mutation screening of the 26 exons of the TSC gene was performed with polymerase chain reaction (PCR) and single-strand conformation polymorphism techniques,\(^2,22\) with primers published by Simon et al\(^23\) except for exon 17, for which primers published by Mastroianni et al\(^24\) were used. Exon 1 was amplified in 2 fragments (exons 1A and 1B).\(^7\) PCRs were performed with 50 ng genomic DNA in a total volume of 20 μL containing 10 pmol of each primer, 2 mmol dNTPs, and 0.5 U Taq polymerase (Pharmacia Biotech) in either the PCR buffer recommended by the manufacturer (Pharmacia Biotech) (exons 1B, 2, 4, 6, 12, 14 to 15, 17 to 18, 20 to 21, and 24) or in 1×(NH\(_4\))\(_2\)SO\(_4\)-buffer (16 mmol/L (NH\(_4\))\(_2\)SO\(_4\); 67 mmol/L Tris pH 8.8; 0.01% Tween) (exons 1A, 3, 5, 7 to 11, 13, 16, 19, 22 to 23, and 25 to 26). Reactions were performed for 15.5 cycles (49°C for 30 seconds, 94°C for 1 minutes, and 72°C for 1 minutes). Reaction products were separated by polyacrylamide gel electrophoresis. Gels were run for 3 to 4 hours at 70 to 80 W and stained with silver nitrate. Further details are published by Mastroianni et al.\(^24\) In cases in which sequence differences or restriction fragment length polymorphism methods were created for the mutation screening, the same primers and conditions as in the mutation screening were used to confirm the presence of the exons containing the variants. PCR products were sequenced bidirectionally with the Thermo Sequenase II dye terminator cycle sequencing kit (Pharmacia Biotech) in an ABI PRISM 373 automated DNA Sequencer (Perkin Elmer).

Restriction Fragment Length Polymorphism

Restriction fragment length polymorphism methods were created for the Thr304Pro, Gly439Ser, Ala728Thr, Gly731Arg, Gly741Arg, 2745insAGCA, Arg904Gln, Gly264Ser, and C1420T variants to confirm them and to simplify their detection. The same primers and conditions as in the mutation screening were used to perform nonradioactive PCRs of the exons containing the variants, except for the Gly741Arg, Ala728Thr, and C1420T variants, for which the forward primers were replaced by (5′-AATTGGACCCCAACATTCTGTTGTT3′, (5′-ACCCCTATCCCCTGGCAGGGC) and (5′-CGGCTGCGATT-TCGGCAGC)3′, respectively, containing nucleotide mismatches (underlined) to create restriction enzyme cleavage sites. The Gly264Ser
TABLE 1. Variants Found in Patients With Gitelman's Syndrome (n=4)

<table>
<thead>
<tr>
<th>Mutation at Nucleotide</th>
<th>Predicted Effect on Protein</th>
<th>Patient</th>
</tr>
</thead>
<tbody>
<tr>
<td>935A→C (exon 7)</td>
<td>Thr304Pro</td>
<td>1, 2</td>
</tr>
<tr>
<td>1340G→A (exon 10)</td>
<td>Gly439Ser</td>
<td>3</td>
</tr>
<tr>
<td>2207G→A (exon 18)</td>
<td>Ala728Thr*</td>
<td>1, 2</td>
</tr>
<tr>
<td>2216G→A (exon 18)</td>
<td>Gly731Arg</td>
<td>1, 2</td>
</tr>
<tr>
<td>2246G→A (exon 18)</td>
<td>Gly741Arg</td>
<td>3, 4</td>
</tr>
<tr>
<td>2745insAGCA</td>
<td>frameshift introducing a premature stop codon</td>
<td>4</td>
</tr>
<tr>
<td>(exon 23)</td>
<td>6 codons downstream</td>
<td></td>
</tr>
</tbody>
</table>

*Represents a common polymorphism, not regarded as functional (see Results section).

The genotype frequency distributions of the Arg904Gln, Gly264Ala, and C1420T variants were similar to the 261 normotensive control subjects.

TABLE 2. Variants Found in Mutation Screening of Hypertensive (n=20) and Normotensive (n=20) Individuals Without Gitelman’s Syndrome (n=40)

<table>
<thead>
<tr>
<th>Mutation at Nucleotide</th>
<th>Predicted Effect on Protein</th>
<th>HT (n)</th>
<th>NT (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>816G→C (exon 6)</td>
<td>Gly264Ala</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2402G→A (exon 20)</td>
<td>Val793Met</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>2736G→A (exon 23)</td>
<td>Arg904Gln</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>391A→G (exon 2)</td>
<td>Ala122Ala</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>1420C→T (exon 11)</td>
<td>Thr465Thr</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>1909G→A (exon 15)</td>
<td>Ser628Ser</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>2167C→T (exon 17)</td>
<td>Ala714Ala</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>2686A→T (exon 23)</td>
<td>Arg887Arg</td>
<td>10</td>
<td>17</td>
</tr>
</tbody>
</table>

*HT indicates hypertensive; NT, normotensive.

Statistics

A BMDP statistical package (Biomedical Data Processing, version 1.1) was used for the statistical analyses. Frequency differences were calculated with χ² test or Fisher’s exact test where appropriate. Differences in continuous variables were calculated with a t test and ANOVA or Mann-Whitney and Kruskal-Wallis tests where appropriate. Data are given as mean±SD if nothing else is mentioned.

Results

Mutation Screening of TSC Gene in Patients With Gitelman’s Syndrome

We identified compound heterozygous mutations in all 4 patients with Gitelman’s syndrome (Table 1). Two of these, Thr304Pro and 2745insAGCA, have not been reported before. The insertion mutation 2745insAGCA is due to duplication of the last 4 nucleotides of exon 23 (AGCA), which introduces a frameshift after codon 906Glu. This frameshift leads to a premature stop codon 7 codons downstream (Glu906→Glu907Gln908Ala909His910Gln911Glu912Val913Stop). The missense mutations Thr304Pro, Gly439Ser, Gly731Arg, and Gly741Arg all change amino acids, which are strictly conserved through evolution.18 Patients 1 and 2 with the Thr304Pro and Gly731Arg mutations were also carriers of an Ala728Thr variant (Table 1), which has been reported earlier in patients with Gitelman’s syndrome.7,14 This variant is not conserved through evolution18 and represents a common polymorphism in Swedes (see below).

Screening for Gitelman’s Syndrome Mutations in Hypertensive Patients and Normotensive Control Subjects

In contrast to the Thr304Pro, Gly439Ser, Gly731Arg, Gly741Arg, and 2745insAGCA mutations, the Ala728Thr variant was found to be relatively common in both the 292 patients with primary hypertension (Ala728 Ala, n=282; Ala728Thr, n=8; and Thr728Thr, n=2) and in the 264 normotensive control subjects (Ala728 Ala, n=259; Ala728Thr, n=4; and Thr728Thr, n=1) (P=0.54). The 3 subjects homozygous for the Thr728 allele all had normal serum potassium values (3.9, 4.1, and 4.2 mmol/L), and none of them had symptoms of Gitelman’s syndrome.

None of the 292 patients with primary hypertension carried any of the Thr304Pro, Gly439Ser, Gly731Arg, Gly741Arg, or 2745insAGCA mutations, whereas 3 (1.1%) of the 264 normotensive control subjects were heterozygous for 2 of these mutations (Gly741Arg, n=2, and Gly439 Ser, n=1) (P=0.11). There was no significant difference in systolic blood pressure (125±11.0 mm Hg versus 125±12.9 mm Hg; P=0.99), diastolic blood pressure (70.7±7.0 mm Hg versus 71.6±7.1 mm Hg; P=0.66), or serum potassium concentrations (4.1±0.56 mmol/L versus 4.2±0.25 mmol/L; P=0.59) between the 3 heterozygous mutation carriers compared with the other 261 normotensive control subjects.

Mutation Screening of TSC Gene in Subjects Without Gitelman’s Syndrome

In the 20 patients with early onset of primary hypertension and the 20 normotensive subjects who were screened for TSC mutations, we found 5 silent polymorphisms and 3 variants leading to amino acid substitutions (Table 2). We focused our further studies in the large study population on 3 variants: (1) The Arg904Gln variant was selected because it was common and changes the amino acid sequence of the TSC. (2) The Gly264Ala variant was selected because it changes a conserved amino acid just like the identified Gitelman’s syndrome missense mutations, which most likely are functional. This suggested that the Gly264 allele could be of importance for normal TSC function and that the Ala264 allele could alter TSC function. (3) We screened for the C1420T variant because the T1420 allele was present in 10 of 20 patients with primary hypertension but in only 1 of 20 normotensive subjects participating in the mutation screening (P=0.001) (Table 2).

Screening of Arg904Gln, Gly264Ala, and C1420T Variants in Hypertensive Patients and Normotensive Control Subjects

The genotype frequency distributions of the Arg904Gln, Gly264Ala, and C1420T variants (Table 3) were similar to...
those expected from the allele frequencies according to the Hardy-Weinberg equilibrium. The genotype frequency distribution of the Arg904Gln variant differed between the patients with primary hypertension and the normotensive control subjects ($P=0.05$) (Table 3). Five of the patients with primary hypertension (1.7%) were homozygous for the Gln904 allele, whereas no Gln904Gln homozygotes could be found among the normotensive control subjects ($P=0.03$), whereas the frequency of the T1420 allele was significantly higher in patients with primary hypertension than in control subjects (Table 3). The genotype frequency distribution of the Gly264Ala variant did not differ significantly between the patients with primary hypertension and the normotensive control subjects (Table 3). The Arg904Gln and C1420T variants were not in linkage disequilibrium (data not shown). If the 29 patients with primary hypertension and the normotensive control subjects (Table 3), although the 5 Gln904Gln homozygotes had slightly higher serum potassium concentrations ($P=0.14$) than the other patients with primary hypertension (Table 3).

**Discussion**

We have identified compound heterozygous mutations in the TSC gene, 2 of which have not been reported before (Thr304Pro and 2745insAGCA), in 4 patients with Gitelman’s syndrome (Table 1). The 4-bp insertion at the end of exon 23 (2745insAGCA) causes a frameshift leading to a premature stop codon in exon 24. This truncation of a large portion of the C-terminal part of the protein corresponding to exons 24 to 26 is likely to interfere with the function of the protein as 2 potential protein kinase phosphorylation sites, predicted at Thr908 and Ser953,18 are destroyed. Amino acid sequence alignment of the human, rat, and winter flounder TSC as well as of the related bumetanide-sensitive Na-K-2Cl cotransporter from human, rat, rabbit, and shark has shown that the amino acid residues corresponding to the Thr304, Gly439, Gly731, and Gly741 of the human TSC are strictly conserved through evolution,18 indicating that these amino acid residues are essential for the function of the protein. The substitutions of these residues in our patients with Gitelman’s syndrome (Table 1) are therefore likely to be pathogenic, although definite proof would require functional studies. The 2 siblings with Gitelman’s syndrome (patients 1 and 2) carried 3 different variants (Table 1). Of them, the Ala728Thr variant, which has been described in patients with Gitelman’s syndrome before,7,14 may not be pathogenic for the following 2 reasons: (1) Unlike all the other missense mutations in the patients with Gitelman’s syndrome (Table 1), the Ala728 Thr amino acid residue is not conserved through evolution.18 (2)
Three of the 556 individuals screened for the Ala728Thr variant were homozygous for the 728Thr allele but had no symptoms of Gitelman’s syndrome, and all had normal serum potassium values.

We speculated that Gitelman’s syndrome is underdiagnosed and that heterozygous carriers of such mutations are relatively common in the population and could be protected from primary hypertension. The mutations that we regarded as pathogenic in the patients with Gitelman’s syndrome were rare in our study population. They were not found in patients with primary hypertension, as compared with a frequency of 1.1% of heterozygous carriers in normotensive control subjects; however, the difference was not statistically significant (P = 0.11). Several mutations have been described in the TSC gene in patients with Gitelman’s syndrome.7–14–17 In our large study population from southern Sweden, these were found in 4 patients with Gitelman’s syndrome (Table 1) and in the 40 subjects without Gitelman’s syndrome (Table 2) who were screened for mutations in the TSC gene. Apparently a larger number of study subjects and a broader spectrum of Gitelman’s syndrome mutations need to be studied to be able to convincingly show if heterozygotes for such mutations are protected from primary hypertension.

It is known that activating mutations of the amiloride-sensitive epithelial sodium channel gene cause hypertension (Liddle’s syndrome).2,10 whereas inactivating mutations in the same gene cause hypotension (pseudohypoaldosteronism type 1).9 An activating variant of the TSC gene was expected to give rise to the phenotype opposite to that of Gitelman’s syndrome, with hypertension and relatively high serum potassium concentrations. The finding of 5 subjects homozygous for the Gln904 allele of the TSC gene in the patients with primary hypertension as compared with none in the normotensive control subjects (P = 0.03) (Table 3) suggests that the Arg904Gln variant may represent such an activating variant, which contributes to elevated blood pressure.

There was no significant difference in the number of Arg904Gln heterozygotes between the patients with primary hypertension and the normotensive control subjects (Table 3), suggesting that 2 Gln904 alleles are required to increase the risk of hypertension. However, because more than 1 variant was tested for association with hypertension, some caution in the interpretation of the data is warranted.

Interestingly, Gordon’s syndrome (pseudohypoaldosteronism type II) displays a phenotype of inheritable hyperkalemic hypertension with acidosis that is reversible by thiazides,23–24 thus being the opposite of the Gitelman’s syndrome phenotype. However, activating mutations of the TSC gene as the cause of Gordon’s syndrome has not been supported by linkage studies, which have instead mapped the disease to 2 other chromosomal loci, 1q31–42 and 17p11–q21.23 Thiazides could be expected to be more effective in lowering blood pressure in subjects homozygous for the Gln904 allele. However, none of the 37 patients treated with thiazides was homozygous for the Gln904 allele. Among the 37 patients taking thiazides, there was no difference in systolic (158 ± 16 versus 159 ± 16 mm Hg; P = 0.82) or diastolic (85.0 ± 10.4 versus 87.6 ± 13.6; P = 0.56) blood pressure between carriers of the Arg904Arg (n = 29) and Arg904Gln (n = 8) genotypes.

In conclusion, we report 5 mutations in the TSC gene that most likely cause Gitelman’s syndrome (Gly439Ser, Gly731Arg, Gly741Arg, Thr304Pro, and 2745insAGCA), of which the latter two have not been described before. Furthermore, we provide data suggesting that individuals homozygous for the Gln904 allele of the TSC gene may be at increased risk for primary hypertension.

Acknowledgments

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References


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