Novel mutations in the thiazide-sensitive NaCl cotransporter gene in patients with Gitelman syndrome with predominant localization to the C-terminal domain

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Gitelman syndrome (familial hypokalemia-hypomagnesemia syndrome) is an autosomal recessive inherited renal disorder characterized by defective tubular reabsorption of magnesium and potassium. In this study a group of 18 unrelated and 2 related Gitelman patients, collected from six different countries have been screened for mutations in the human thiazide-sensitive sodium-chloride cotransporter (SLC12A3) gene. Fourteen novel SLC12A3 mutations are presented along with six mutations described earlier, and three neutral polymorphisms. Among the tested patients are two who carry a total of three heterozygous SLC12A3 mutations. Two-thirds of the total number of mutant SLC12A3 alleles are amino acid substitutions. Most SLC12A3 gene mutations, 14 out of a total of 20, are localized at the intracellular carboxy-terminal domain of the NCCT protein. The pathogenicity of individual SLC12A3 mutations is based upon their predicted effect on SLC12A3 protein, and segregation in family members. Evolutionary conservation of substituted amino acid residues and their frequency in control chromosomes is presented. Identical mutations have been found in Gitelman families from different geographical origin, suggesting ancient mutations originating from a common ancestor. As yet, we have not found any evidence for a possible genotype-phenotype correlation.

Gitelman syndrome (GS; MIM 263800) is an inherited renal tubular disorder, primarily characterized by hypokalemic metabolic alkalosis in combination with significant hypomagnesemia and low urinary calcium [1]. Patients with this disorder are often asymptomatic, with the exception of transient periods of weakness and tetany, sometimes accompanied by abdominal pain, vomiting and fever. The relatively mild clinical course of GS, with absence of polyuria and growth retardation, and the late age of onset usually allows the differentiation from the more severe hypokalemic tubulopathy, Bartter syndrome (BS; MIM 241200 and MIM 209930) [2, 3]. In addition, magnesium levels are normal or only slightly decreased in patients with BS and urinary calcium excretion is either normal (in classic BS) or largely increased (in the antenatal variant of BS). GS is clearly inherited as an autosomal recessive trait and the possibility of autosomal dominant inheritance in some families with GS, as suggested by Bettinelli et al [4], was recently dismissed by molecular genetic analysis showing that inheritance in these sort of families is in fact pseudo-dominant [5, 6].

Both the observation that the electrolyte disturbances in GS resemble the effects of chronic thiazide administration [7–9], as well as the results of clearance studies [10, 11] have pointed to a defect in distal thiazide-sensitive sodium-chloride transport in GS. This hypothesis has recently been substantiated by the demonstration that GS results from presumptive loss of function mutations in the SLC12A3 gene [5, 6, 12], which encodes the thiazide-sensitive sodium-chloride co-transporter (TSC, NCCT) which is located in the distal convoluted tubule [13].

The SLC12A3 gene belongs to the solute carrier protein family 12 (SLC12). In mammals, three distinct members (SLC12A1, SLC12A2 and SLC12A3) of these electroneutral Na-(K)-Cl cotransporters are known to share a common structure consisting of 12 transmembrane domains and intracellular amino- and carboxyterminal regions [14].
In this article, we report the identification of 20 SLC12A3 mutations, including 14 novel mutations, in a cohort of 20 GS patients from different geographical origins. The presumptive effect of these mutations on the function of the NCCT protein is discussed. Our findings confirm genetic homogeneity in GS and suggest the existence of several ancient mutations.

**METHODS**

**Gitelman syndrome families**

Seven index patients from six families with GS and 13 sporadic cases were collected from Pediatric Nephrology and adult Nephrology centers in the Netherlands, Germany, France, the United States and Canada. Patients 4, 5, 6, 12, 13, 15, and 20 are familial cases and the rest of the 20 index patients (Table 1) are sporadic cases. The family from patient 5 has been described in a previous report [5]. The same holds true for patients 12 and 13, who are affected siblings from a larger pseudodominant GS family. Patients 6 is the offspring of a consanguineous couple and has four affected sibs. This is the largest Dutch GS family known and has been presented in our previous study as well [5]. The sporadic cases 2, 7, and 10 have been described before [15]. The parents of patient 2 are first cousins.

Criteria for acceptance of the diagnosis of GS were: normal or slightly decreased concentrating ability, normal glomerular filtration rate, serum magnesium level less than 0.65 mmol/liter, serum potassium level less than 3.5 mmol/liter, and hypocalciuria (urinary calcium <2 mg/kg per day) [16].

Informed consent was obtained from each participating subject or the parents of the younger children.

**Mutation analysis**

Genomic DNA was extracted by a salting out procedure from peripheral blood lymphocytes [17] and used for PCR amplification of individual exons of the SLC12A3 gene. Twenty-six pairs of oligonucleotide primers were generated to amplify all 26 exons according to data obtained from Simon et al [12], and Mastroianni et al [6]. Exon 1 was amplified by using GIT024 as forward primer and GIT039 (5'-ATAGAGCTCATATGGGCAC-3') as reverse oligo. A different reverse primer was developed for exon 16 as well (GIT156; 5'-CATGCC TGG TCC CCT GTG CC-3'). Polymerase chain reactions (PCR) were performed in a 30 μl volume containing 100 ng genomic DNA, 50 ng primers, 5% DMSO, 1.5 μl 8.3 mM dNTPs, and 3 μl 10 × Taq buffer (100 mM Tris-HCl, pH 8.4, 100 mM (NH₄)₂SO₄, 100 mM KCl, 22.5 mM MgCl₂, 0.1% gelatin, 1% Triton X-100). PCR fragments were labeled by inclusion of 1 μCi of [α-32P]dCTP in the reaction mixture. Standard PCR conditions were performed using standard conditions with an initial denaturation step at 94°C for 5 minutes subsequently followed by 30 cycles with denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, and elongation at 72°C for 1 minute. Single Strand Conformation Polymorphism (SSCP) analysis [18] was performed by electrophoresis of amplified PCR fragments on 0.5 × MDE (FMC BioProducts, Rockland, ME, USA) gels prepared in 0.6 × TBE (54 mM Tris, 54 mM boric acid, 1.2 mM EDTA, pH 8.3) at two different electrophoresis conditions at 4°C temperature, one for 16 hours at 6W, and a second for 6 hours at 40W constant power.

The SLC12A3 exons resulting in SSCP shifts, were

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* Serum magnesium <0.65 mmol/liter
* Urinary calcium <2 mg/kg per day
* Serum potassium <3.5 mmol/liter
*ND is not determined.
reamplified for direct sequence analysis by PCR reactions with identical oligonucleotides as was used for PCR-SSCP analysis. Amplified DNA fragments were isolated from agarose gels, using the "freeze-squeeze" method and subsequently used as template in PCR cycle-sequencing reactions in the presence of dye-dideoxy terminators [19]. Reaction conditions were performed according to protocols supplied by the manufacturer (Perkin-Elmer-ABI, Foster City, CA, USA) and analyzed on an ABI 377 automated DNA sequencer (Perkin-Elmer-AB).

The segregation of mutations has been studied in members of the family of patients 6 (this study), 12 and 13 [5].

RESULTS

Clinical and biochemical data

The clinical and biochemical details of the index cases with GS are shown in Table 1. Four patients complained of serious joint pains due to chondrocalcinosis. Seven patients experienced severe fatigue interfering with daily activities. Eight patients had no symptoms of magnesium or potassium deficiency and GS was diagnosed when electrolyte measurements were performed for other reasons (abdominal pain, suspected appendicitis, mild fatigue, systematic preoperative work-up).

Differential diagnosis from BS in all patients was based on the presence of severe hypomagnesaemia, hypocalciuria, and urinary normal concentrating capacity.

Mutation detection in the SLC12A3 gene

Human genomic DNAs from 13 sporadic Gitelman patients and 7 familial cases have been screened for mutations in the SLC12A3 gene by PCR-SSCP analysis of 26 exons that encode the complete coding region. Amplified DNA fragments resulting in SSCP band shifts were sequenced. A total of 20 different mutations have been identified consisting of 15 missense, 2 splice site, 1 deletion, 1 insertion, and 1 nonsense mutation (Table 2). Six of these mutations have also been found by others [6, 12]; the remaining 14 are novel molecular variants of the SLC12A3 gene. Remarkably, most SLC12A3 gene mutations, 14 out of a total of 20, are localized at the intracellular carboxy-terminal domain of the NCCT protein (Fig. 1 and Table 2).

In our total study group of 20 patients, seven patients carry homozygous SLC12A3 mutations (numbers 2, 4, 6, 8, 11, 13 and 20; Table 2) and seven are compound heterozygotes (numbers 3, 5, 6, 12, 14, 15 and 19; Table 2 and Fig. 2). Surprisingly, we have identified only one mutant SLC12A3 allele in 7 out of 20 patients (numbers 1, 7, 9, 10, 16, 17 and 18; Table 2). In one of these 7, a neutral polymorphism was detected as well (patient 16; Table 2). A total of three different silent mutations, Thr465Thr, Ser628Ser, and Ala714Ala (Table 2) was observed in some of our Gitelman patients. The two latter polymorphisms appeared to be present in 4 out of 50 and 3 out of 50 control alleles, respectively (data not shown).

Fifty control chromosomes were tested for the presence of the 15 missense mutations that have been identified in our study group. Thirteen of the 15 mutations appeared to be absent in these control chromosomes. The Arg904Gln and Arg919Cys mutations were each observed in 1 out of 50 control chromosomes (data not shown).

The SLC12A3 mutations identified in patient 12 and his relative patient 13 have been presented earlier [5]. Furthermore, patients 5 and 6 from two Dutch families were previously used for linkage analysis [5]. Patient 5 revealed three different heterozygous SLC12A3 mutations (Table 2): one pathogenic mutation is the intron 24 splice site mutation. The second presumed pathogenic mutation is the Leu215Pro substitution, which was not found in 50 control chromosomes after PCR-SSCP analysis of exon 5 (data not shown). In addition, the Leu215 amino acid residue is conserved in rat and winter flounder NCCT (Table 3). In contrast, Arg904 is present in the human NCCT protein only and not conserved through evolution in rat and winter flounder NCCT proteins (Table 3) and therefore the substitution of this arginine for a glutamic acid residue (the third mutation found in patient 5) is less likely to be pathogenic. An additional important argument against pathogenicity of the Arg904Gln substitution is the fact that this mutation was also found in 1 out of 50 control chromosomes.

One other patient (no. 12) was found to carry three different SLC12A3 mutations (Table 2). Patient 12 appeared to carry two missense mutations, Gly741Arg and Arg919Cys, in addition to a nonsense mutation. The Gly741 and Arg919 amino acid residues are strongly conserved through evolution (Table 3). The Gly741Arg mutation has not been identified in 50 control chromosomes, in contrast to the Arg919Cys mutation, which was observed in 1 out of 50 control chromosomes.

In patient 6 two different missense mutations, a heterozygous Ala226Thr and a homozygous Thr649Arg, were detected (Fig. 3 and Table 2). The parents, who are consanguineous, are both heterozygous for Thr649Arg while all affected children are homozygous for Thr649Arg (Fig. 3 and 4). Neither of these two mutations were detected in 50 control chromosomes after SSCP analysis of exon 5 and 16 (data not shown). Since both Ala226 and Thr649 amino acid residues are strongly conserved in homologous SLC12A1, SLC12A2 and SLC12A3 proteins from different species (Table 3), it is difficult to predict whether both or only one of the amino acid substitutions contributes to the clinical phenotype in these patients.

The Arg642His mutation found in patient 10 is caused by a guanine to adenine nucleotide substitution localized at the most 3’ end of exon 15 (Table 2). Because exon-intron boundaries are part of strongly conserved splice-site consensus sequences [26] we presume that this mutation may result in aberrant mRNA splicing instead of an amino acid
substitution. The splice site mutations in intron 18 and intron 24 (patients 5, 14, and 16) are changing conserved gt dinucleotide intron sequences that probably affect correct mRNA splicing. The deletion and insertion mutations identified in four patients caused a frameshift resulting in the introduction of a preliminary stop codon and result in truncated NCCT proteins. Shorter NCCT proteins are also predicted in patients carrying the Arg968Stop mutation (Table 2).

A total of six different mutations are found in more than one Gitelman family: The 2 bp insertion at the nucleotides 260-263 in three families, one from Canada, one from Germany and one from Italy; Gly741Arg in four families, one from Germany, two from the Netherlands, and one from the United States [1]. Arg209Trp is found in four Gitelman families, one from the United States, one from Portugal, and two from Italy. The intron 24 splice donor site mutation is in two families, one from the United States.
Fig. 1. Localization of SLC12A3 mutations on predicted NCCT protein. Predicted protein structure of NCCT protein with intracellular amino- and carboxy terminal domains [12] and transmembrane domains 1 to 12. Introns 18 and 24 do not indicate intracellular to transmembrane and extracellular to transmembrane domains because they are not part of protein encoding sequences.

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Fig. 2. DNA sequence analysis of two SLC12A3 missense mutations identified in exon 18 from patient 3 (Table 2). Amino acid residues are indicated and the corresponding codons are underlined. The coding sequence (3' to 5' end) is shown for A, B, and C which are reverse sequences. D shows forward sequences with coding sequence from 5' to 3' end. Arrows indicate heterozygous nucleotide substitutions (=N). (A) Control sequence with Gly741 codon (GGG) and Leu738 codon (5'-CTG-3'). (B) Patient 3 with two heterozygous missense mutations (Gly741Arg and Leu738Arg). (C) The mother carries the heterozygous Gly741Arg substitution, while the father carries the heterozygous Leu738Arg substitution (D).
and one from the Netherlands; Arg908Stop is in three families from the Netherlands; and Leu850Pro is found in two families, one from Germany, and one from Spain (Table 2).

**DISCUSSION**

The assumption that Gitelman syndrome is caused by a defect in the thiazide-sensitive sodium-chloride cotransporter in the renal distal tubule has recently been proven by the identification of several mutations in the SLC12A3 gene in patients with GS [5, 6, 12]. In the present study the specific involvement of this cotransporter in the etiology of this disorder is further substantiated by the finding of 20 mutations, including 14 new mutations, in a cohort of 20 GS patients from different geographical origin.

Fourteen of the 20 mutations identified in this study are located at the intracellular C-terminal end of the SLC12A3 protein. Although the SLC12A3 mutations reported in previous studies are distributed throughout the whole protein [6, 12], our finding is very remarkable, and may indicate that the carboxy-terminal end represents a hot spot for mutations. The localization of SLC12A3 gene mutations at the C-terminal domain may have a deleterious effect on the activity of the NCCT cotransporter. This domain contains three putative protein kinase phosphorylation sites, predicted at Thr908, Ser804, and Ser953 residues, and one potential phosphorylation site for cAMP-dependant protein kinase at Ser857 [22].

Although data about phosphorylation of the NCCT cotransporter are not yet available, it is well known that protein phosphorylation plays an important role in regulating activity of many ion transport systems. Thus, it is conceivable that structural alterations due to SLC12A3 mutations in the C-terminal domain interfere with phosphorylation of the NCCT protein and as such with its regulation. In addition, a protein disulfide isomerase, an enzyme known to participate in protein folding, has been identified that interacts with the carboxy-terminal end of the NCCT protein at the apical membrane of distal tubule cells [27].

Among the SLC12A3 mutations identified in our GS cohort are six variants that are likely to have a deleterious effect on the function of the SLC12A3 protein. Thus, the nucleotide insertions (patients 1 and 2; Table 2) and deletion (patient 17) and the nonsense mutation (no. 12, 13, 15, and 20) will cause premature termination of translation and result in truncated SLC12A3 transporters. Two different splice donor site mutations (patients 5, 14, and 16) alter splice site consensus sequences, and will probably lead to shorter SLC12A3 mRNAs due to exon skipping. Similarly, the guanine to an adenine nucleotide substitution is likely to affect correct splicing (patient 10; Table 2). The majority of the mutations detected in GS cohort, however, are amino acid substitutions. Our data together with the results of previous studies [5, 6, 12] indicate that 44 out of a total of 66 identified mutant SLC12A3 alleles are missense mutations. These mutations are inferred to be pathogenic when they substitute amino acids, which in view of their conservation through evolution are presumed to be of functional importance. All but one (Arg904Gln) of the 15 missense mutations detected in our GS cohort are substitutions of strongly conserved into non-conservative amino acids.
amino acids and were not found in 50 control chromosomes. Therefore, it is likely that the majority of these missense mutations are indeed harmful mutations and not innocuous polymorphisms. However, to definitely prove that these amino acid substitutions can indeed result in impairment or loss of NCCT function, studies are underway measuring the effect of individual mutations on sodium-chloride transport in a functional expression system, for example, measuring chloride uptake after microinjection of normal and mutant SLC12A3 cRNA into *Xenopus laevis* oocytes. In two patients (5 and 12; Table 2), three different mutations instead of two were found. In these patients one of the three mutations causes truncation of the protein and the other two are missense mutations, which are also possible pathogenic variants in two cases (Leu215Pro and Gly741Arg; Table 2) because they have not been identified in healthy controls and result in the substitution of an amino acid that is strongly conserved through evolution. The presence of the three mutations identified in these patients needs to be tested in their respective family...
members in order to establish the inheritance of the respective mutations, and to deduce which of these mutations, or may be all three, contribute to the phenotype. In this respect the finding of three distinct mutations in the muscle chloride channel gene CLCN1, all predicted to be disease-causing mutations, in a single myotonia congenita family, is interesting [28].

By definition, patients with an autosomal recessive disorder should inherit one mutant allele from each parent. In 40% of GS patients from our study cohort, however, we have identified only one mutant allele. This finding corroborates with two previous studies reporting the detection of only one mutant allele in 5 out of 12 and 5 out of 11 kindreds, respectively [6, 12]. There are several explanations to justify this failure to identify both mutant alleles in all GS patients. Firstly, although we have performed SSCP analysis for each SLC12A3 exon under two different conditions to increase detection sensitivity, it is known that mutations can be missed by PCR-SSCP analysis. Secondly, mutations may be present in gene regulating fragments such as promoter or enhancer segments, intron sequences or 5’ and 3’ non-coding regions, which have not yet been screened for mutations. Thirdly, large heterozygous SLC12A3 gene deletions will not be identified by mutation detection techniques based on analysis of individual exons. Therefore we plan to test Gitelman patients with only one mutant SLC12A3 allele for large gene rearrangements by Southern blot analysis or Pulsed Field gel electrophoresis.

Two patients with only one mutant SLC12A3 allele appeared to carry nothing else but a silent mutation on the second allele. Recently, a silent fibrillin-1 gene mutation in a patient with Marfan syndrome has been shown to induce exon skipping [29]. In the light of this intriguing finding, it will be interesting to determine whether silent SLC12A3 mutations can affect normal SLC12A3 function as well. Therefore, the three neutral polymorphisms identified in our study cohort will be subjected to further investigation. Functional effects of the so-called “normal” polymorphisms have been described for the β-subunit of the amiloride-sensitive sodium channel involved in Liddle syndrome [30] and for tyrosinase enzyme (TYR gene) activity in ocular albinism [31].

Five of the 20 mutations detected in our study were present in Gitelman patients from different geographical origins, indicating that these mutations may be ancient variants frequently occurring in GS. To determine whether

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**Fig. 4. PCR-SSCP analysis of exon 16 in control, patient 6 and family members.** Pedigree has been drawn with full symbols indicating affected persons, and open symbols with point are carriers. Arrows A indicate single-stranded DNA fragments in control and arrows B indicate shifted single-stranded DNA fragments. Carriers (heterozygous Thr649Arg mutation) show both A and B fragments (I-1, I-2, and II-2) while affected persons (homozygous Thr649Arg) reveal only B fragments (II-1, II-3, II-4, II-5, and II-7).
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