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Mutations in the Chloride Channel Gene, *CLCNKB*, Leading to a Mixed Bartter-Gitelman Phenotype

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ABSTRACT

Gitelman syndrome is an inherited renal disorder characterized by impaired NaCl reabsorption in the distal convoluted tubule and secondary hypokalemic alkalosis. In clinical practice, it is distinguished from other hypokalemic tubulopathies by the presence of both hypomagnesemia and normocalcemic hypocalciuria. To date, only mutations in a single gene encoding the thiazide-sensitive NaCl cotransporter have been found as the molecular basis of GS. We describe three unrelated patients presenting with the typical laboratory findings of GS. Mutational analysis in these patients revealed no abnormality in the *SLC12A3* gene. Instead, all patients were found to carry previously described mutations in the *CLCNKB* gene, which encodes the kidney-specific chloride channel ClC-Kb, raising the possibility of genetic heterogeneity. Review of the medical histories revealed manifestation of the disease within the first year of life in all cases. Clinical presentation included episodes of dehydration, weakness, and failure to thrive, much more suggestive of classic Bartter syndrome than of GS. The coexistence of hypomagnesemia and hypocalciuria was not present from the beginning. In the follow-up, however, a drop of both parameters below normal range was a consistent finding reflecting a transition from cBS to GS phenotype. The phenotypic overlap may indicate a physiologic cooperation of the apical thiazide-sensitive NaCl cotransporter and the basolateral chloride channel for salt reabsorption in the distal convoluted tubule. **(*Pediatr Res* 48: 754–758, 2000)**

Abbreviations

GS, Gitelman syndrome
cBS, classic Bartter syndrome
DCT, distal convoluted tubule
NCCT, thiazide-sensitive NaCl cotransporter
ClC-Kb, kidney-specific basolateral chloride channel
TAL, thick ascending limb of Henle's loop
HPS/aBS, hyperprostaglandin E/antenatal Bartter syndrome

GS (MIM 263800) is an inherited renal tubular disorder characterized by impaired conservation of potassium and magnesium (1). The mode of inheritance is autosomal-recessive. The first clinical presentation is usually observed during childhood or adolescence and may include transient episodes of weakness, paresthesia, and tetany. The course of disease is generally mild and some patients even remain asymptomatic (2, 3, 4). GS patients share several biochemical findings with the more severe cBS, such as hypokalemia, metabolic alkalosis, and elevated plasma aldosterone levels (5). However, the coexistence of hypomagnesemia and hypocalciuria is considered a pathognomonic laboratory finding in GS that allows the differentiation from cBS (6).

Both the observation that the electrolyte disturbances in GS resemble the effect of chronic thiazide administration (7, 8) and the results of clearance studies have pointed to a defect in the distal thiazide-sensitive sodium chloride transport (9). This hypothesis has been substantiated by the demonstration that GS is caused by mutations in the *SLC12A3* gene, which encodes the NCCT of the DCT (10). Meanwhile, more than 100 mutations throughout the NCCT have been described in GS patients, suggesting genetic homogeneity (4, 11, 12). Several mutations that cause GS have recently been found to be nonfunctional because the mutant NCCT is not processed normally, probably activating the "quality control" mechanisms of the endoplasmic reticulum (13).

The NCCT defect directly leads to NaCl wasting, activation of the renin-angiotensin system, increased aldosterone levels, and increased electrogenic sodium reabsorption *via* the epithelial sodium channel of the distal nephron in exchange for K^+ and H^+, accounting for hypokalemic alkalosis. The exact mechanisms underlying hypocalciuria and hypermagnesiuria are not fully elucidated yet, but there is enough evidence that both abnormalities are direct consequences of the primary

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defect. Both hypocalciuria and hypermagnesiuria are observed after administration of thiazide diuretics that inhibit the NCCT (7, 14). Moreover, an analogous dissociation of renal handling of Ca^{2+} and Mg^{2+} is observed in NCCT-deficient mice (15).

We report on three unrelated patients in whom renal salt wasting coincides with hypomagnesemia and hypocalciuria. Surprisingly, all three children were found to carry mutations in the *CLCNKB* gene encoding the kidney-specific chloride channel ClC-Kb, raising the question of genetic heterogeneity in GS.

SUBJECTS AND METHODS

GS patients. The three patients were referred to our hospital for molecular analyses at the age of 3, 5, and 7 years. All of them were offspring of consanguineous parents. Diagnosis of GS was based on the following criteria described by Bettinelli *et al.* (3): (1) hypokalemia of renal origin, defined as plasma potassium <3.6 mM in the presence of inappropriately high potassium excretion (fractional excretion of potassium $>16\%$); (2) hypomagnesemia of renal origin, defined as plasma magnesium <0.65 mM in the presence of inappropriately high magnesium excretion (fractional excretion of magnesium $>4\%$); (3) hypocalciuria, defined as daily urinary calcium excretion <2 mg/kg, or urinary calcium/creatinine molar ratio ≤ 0.10. The laboratory data and medication at the time of referral to our hospital are indicated in Table 1. Patient C was reported previously (16).

Genetic analyses. Genomic DNA was extracted from peripheral blood lymphocytes by standard procedures. Haplotype construction was performed with six dinucleotide repeat markers for each loci 16q13 (*SLC12A3*) and 1p36 (*CLCNKB*) (Fig. 1) following protocols previously described (17). The resulting PCR fragments were sized on a 6% nondenaturating polyacrylamide gel in an ALF Express DNA Sequencer™ using the ALF Fragment Manager™ 1.0 software (Pharmacia Biotech, Uppsala, Sweden).

Both the *SLC12A3* gene and the *CLCNKB* gene were screened for mobility shifts by SSCA (18) using intronic primers previously published (10, 19). DNA segments yielding aberrant banding patterns were reamplified and subjected to direct sequencing on an ALF Express DNA Sequencer™ following the protocols provided by the manufacturer.

These studies were approved by the local ethics committee and informed consent was obtained from the parents.

RESULTS

Genotypes. Mutational screening of the whole coding region of the *SLC12A3* gene by SSCA did not reveal any abnormality in the three patients. To clarify whether the *SLC12A3* gene was involved in the disease at all, we subsequently performed haplotype analysis with microsatellites closely flanking the gene locus on chromosome 16q13 (Fig. 1). None of the patients revealed a region of homozygosity at this locus, confirming that the phenotype was not related to mutations in the *SLC12A3* gene. Instead, further analyses revealed haplotypes that were highly suggestive for cosegregation of the phenotype with chromosome 1p36 (Fig. 1). Because the *CLCNKB* gene encoding the kidney-specific chloride channel ClC-Kb localizes to this cytogenetic region, we subsequently screened our patients for *CLCNKB* mutations and, indeed, all three patients were found to carry pathogenic mutations in this gene. Patients A and B were shown to carry a homozygous deletion of the complete *CLCNKB* gene (Fig. 2*A*). Deletions of the entire gene were described previously (16, 19), and thought to be due to the close vicinity to the highly homologous *CLCNKA* gene, which might predispose to nonhomologous *crossing over*. In patient C, we identified a homozygous exchange of guanine for adenine at the consensus acceptor splice site of intron 7 (IVS7–2) (Fig. 2*B*). This mutation had already been found in an unrelated cBS patient (16), whereas it was not found in 100 control alleles.

Phenotypes. At the time of genetic counseling, all three children presented with the biochemical findings consistent with the diagnosis of GS (Table 1). Review of the medical histories, however, revealed an early onset of the disease within the first year of life in all three cases. After normal pregnancy and birth at term all three infants suffered from weakness and failure to thrive beginning at the ages of 1 (A), 2 (B), and 11 (C) months. In addition, patients A and B experienced one episode of dehydration in the context of a

Table 1. *Important laboratory findings and medication in the three GS patients*

	Patient A	Patient B	Patient C	Normal range
K^+	3.5	3.4	3.3	3.6–4.5 (mmol/L)
HCO_3^-	30.1	31	31.5	23–27 (mmol/L)
Aldosterone	68.8	nd	34.2	5–13.4 (ng/dL)
Mg^{2+}	0.6	0.5	0.6	0.7–1.1 (mmol/L)
FeK	42	67	30	<16 (%)
FeMg	7.9	nd	6	<4 (%)
Urinary calcium excretion	0.57	0.9	0.62	2–4 (mg/kg/d)
Urinary calcium/creatinine ratio	0.07	0.10	0.06	0.2–0.6 (mol/mol)
Urine osmolality*	590	600	579	— (mmol/kg)
Creatinine clearance	86	124	126	80–120 (mL/min/1.7m^2)
Medication				
Potassium chloride	3.4	10	3	(mmol/kg/d)
Sodium chloride	—	4	—	(mmol/kg/d)
Magnesium glutamate	—	600	—	(μmol/kg/d)
Indomethacin	3	4	3	(mg/kg/d)

* Value for 24 h urine.

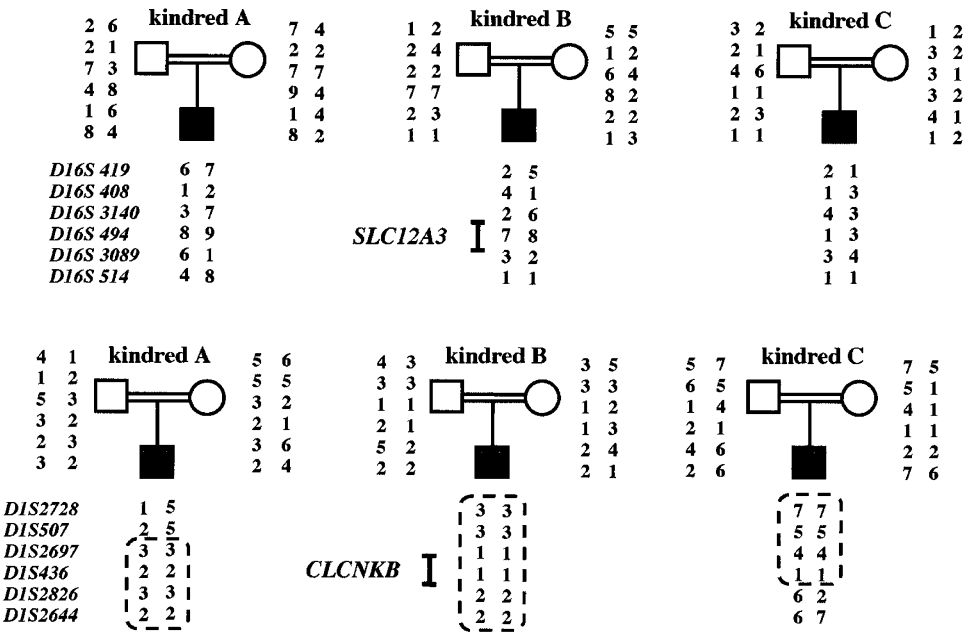

Figure 1. Pedigrees and haplotypes in three consanguineous GS/cBS kindreds. *Upper panel*: Lack of homozygosity for microsatellites linked to the *SLC12A3* locus on chromosome 16q13. *Lower panel*: Cosegregation of the phenotype with the *CLCNKB* locus on chromosome 1p36 indicated by homozygosity by descent.

gastrointestinal infection. The first laboratory examination consistently revealed plasma potassium levels below 3.0 mM, metabolic alkalosis, and hyperreninemic hyperaldosteronism. Plasma levels of magnesium were initially normal with the exception of patient C. Moreover, renal and systemic prostaglandin E formation was elevated in all three patients, which led to the introduction of indomethacin treatment in addition to potassium supplementation. Under this treatment, the patients were doing well. Urine osmolality, determined in 24-hour urine, ranged from 433 to 682 mmol/kg (median 588, $n = 12$) and from 289 to 727 mmol/kg (median 600, $n = 9$) in patients A and C, respectively. No further episodes of major electrolyte disturbances have occurred and physical development has been within normal range.

During follow-up, all three patients developed hypomagnesemia and urinary calcium excretion below 1 mg/kg/d (Fig. 3). Patient B received permanent magnesium supplements from 2 y of age onward, whereas patient C required magnesium supplementation only in the initial phase. Persistence of both hypomagnesemia and hypocalciuria after withdrawal of indomethacin could be demonstrated in patients A and C. When indomethacin was discontinued in patient B, plasma magnesium concentration was 0.5 mM only. Concurrent urinary calcium was not available.

DISCUSSION

The syndrome of familial hypokalemia-hypomagnesemia or GS is thought to represent a genetically homogeneous renal disorder. Mutations in a single gene, *SLC12A3*, have been consistently found in nearly all GS families studied (4, 10–12). Only a few investigators had conceded the fact that in certain GS patients no mutant *SLC12A3* allele had been identified (12, 20). It was likely that these rare unsolved cases might reflect the limited sensitivity of the screening methods. Secondly, the pathogenic mutations might have been located in the noncoding region of *SLC12A3*, such as the promotor region, which were generally not included in the mutation analyses.

However, the three kindreds with *CLCNKB* mutations described in this report raise the question of genetic heterogeneity in GS. Although our patients did not present with the typical GS phenotype, they reproducibly shared both hypomagnesemia and hypocalciuria, which is thought to be a pathognomonic pattern of GS (6). The additional findings, such as manifestation during the first year of life, volume depletion, failure to thrive, and hyperprostaglandinuria are certainly much more compatible with cBS than the GS phenotype (21, 22). Indeed, mutations in the *CLCNKB* gene, which were originally found to be related to a very variable phenotype (19), have subsequently been described as the major molecular defect of cBS (16). In this regard, the clinical presentation of our three patients may be best described as a mixed Bartter-Gitelman phenotype. So far, the present data do not contradict the concept of genetic homogeneity of GS, but may stimulate the search of mutation in more typical GS patients with no *SLC12A3* mutation.

The phenotypic overlap between GS and cBS is not a novel observation. Recently, the concomitant occurrence of GS and cBS in the same family has been reported (23). One child presented a phenotype of renal salt wasting with isolated hypokalemic alkalosis, and a sibling additionally exhibited hypomagnesemia and hypocalciuria. Moreover, a controversial discussion lasted over nearly three decades about how to distinguish these tubular disorders (24, 25). Hypomagnesemia is by definition a feature of GS (1), but it is also present in approximately one-third of the patients with Bartter syndrome (6), although very low levels are unusual in the latter. Even the two patients described in Bartter's original report (5) presented with tetany, which is typically observed in GS, most likely due

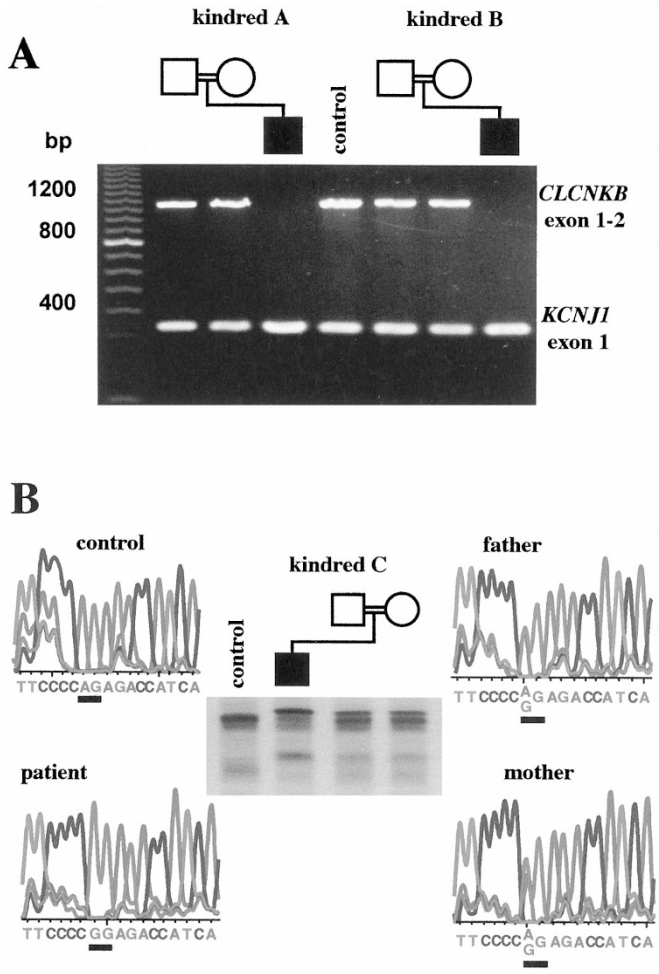

Figure 2. Molecular basis in the three patients with GS/cBS phenotype.(*A*) Patients A and B bear a deletion of the whole coding region of the *CLCNKB* gene. All 19 exons were amplified from genomic DNA and separated on agarose gels. The picture shows the deletion of a *CLCNKB* segment spanning exon 1 and 2. Coamplification of an unrelated gene segment (*KCNJ1*) was used for internal control. (*B*) Single strand conformation polymorphism analysis (SSCA) of exon 8 of the *CLCNKB* gene revealed an abnormal conformer in kindred C. Direct sequencing revealed a homozygous exchange from adenine to guanine affecting the 3'consensus splice site of intron 7.

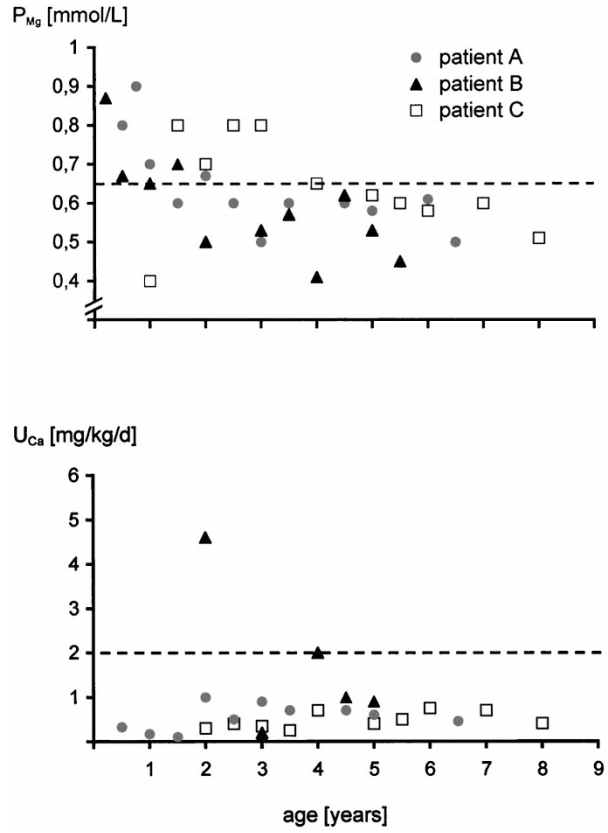

Figure 3. Follow-up of plasma magnesium levels (P_{Mg}) and urinary calcium excretion (U_{Ca}) in three patients with *CLCNKB* mutations. The *dotted line* indicates the lower normal level.

to low plasma magnesium levels. Instead, renal handling of calcium was found to be a more specific parameter to distinguish between GS and cBS (6).

After a large number of individuals affected by *SLC12A3* mutations had been described, it became apparent that the phenotypic consequences are rather heterogeneous. The age at clinical presentation was highly variable, ranging from 6 mo to 60 y of age (12). Bettinelli *et al.* reported failure to thrive (6) and growth retardation (26) in a subset of GS patients. In our hospital, we are following several GS patients carrying *SLC12A3* mutations, who consistently showed moderate hyperprostaglandinuria and who significantly benefited from indomethacin therapy (27).

Taken together, there exists some variability of the phenotypes induced by mutations in either *SLC12A3* or *CLCNKB* (19, 16) leading in part to overlapping clinical features. This overlap makes it more difficult to clearly separate GS from cBS.

Moreover, the phenotypic similarities lead to the hypothesis that there might be a close functional relationship between the two disease-related proteins, NCCT and ClC-Kb, in transepithelial electrolyte transport. This hypothesis could provide us with the answer to the question of how defective ClC-Kb could lead to concomitant occurrence of hypomagnesemia and hypocalciuria.

The physiologic role of ClC-Kb and its functional cooperation with the various transport systems of the renal tubule has not yet been fully established. In the rat, the ClC-Kb homologue seems to be broadly expressed in the distal parts of the renal tubule (28). Transcripts of the chloride channel gene were found in tubular epithelial cells from medullary and cortical TAL, distal tubule, and collecting duct. ClC-Kb is polarized to the basolateral membrane and is most likely responsible for the chloride efflux from the cell into the blood stream.

A defect of the basolateral chloride channel is expected to result in an impaired chloride reabsorption in the distal nephron. The phenotypic consequences of impaired salt reabsorption exclusively in the TAL are demonstrable in HPS/aBS (27). Characteristics of this tubular disorder are saliuretic polyuria, isosthenuria, and hypercalciuria (29). Plasma magnesium levels are generally normal. The phenotype in our patients as well as in the majority of individuals affected by *CLCNKB* mutations clearly did not correspond to a TAL disorder (16). Affected individuals maintain their urinary concentrating capacity at least to a certain extent and display

normal or, as in our cases, even low urinary calcium excretion. Therefore, ClC-Kb apparently does not play an irreplaceable role in transcellular chloride reabsorption in the TAL. Indeed, Greger *et al.* (30) proposed the coexistence of chloride conductance and electroneutral K-Cl symport through the basolateral membrane of TAL epithelial cells. This suggestion has been substantiated by demonstration that the K-Cl cotransporter hKCC1 is expressed in this part of the nephron and may target to the basolateral membrane (31).

The dissociation of renal calcium and magnesium handling with hypocalciuria and hypermagnesiuria in our three patients points to an impaired salt reabsorption more distal from the TAL. Luminal NaCl reabsorption into the distal convoluted tubular epithelial cells is mediated by the NCCT.

Defective function of the NCCT results in a reduction of the intracellular sodium concentration probably followed by an activation of the basolateral Na-Ca exchanger. The Na-Ca exchanger might establish a gradient (low intracellular Ca^{2+}) that is responsible for increased calcium uptake from the tubular lumen to the cell causing hypocalciuria (32). The coincident hypermagnesiuria remains to be explained. It has been hypothesized that the basis of diminished magnesium absorption in the DCT is due to aldosterone-induced hypokalemia secondary to salt and volume depletion (33). Alternatively, the inhibition of an apical Na-Mg exchanger through low intracellular sodium concentration has been proposed (34). Therefore, the mechanisms underlying hypocalciuria and hypermagnesiuria appear to be directly related to impaired sodium uptake in the DCT. The hypomagnesemic-hypocalciuric phenotype of our patients further suggests that not only mutations in NCCT but also secondary inhibition of NCCT by intracellular chloride ion accumulation as a consequence of defective ClC-Kb could abolish transepithelial salt reabsorption in DCT. This observation provides further evidence that ClC-Kb channels play an important physiologic role in this part of the nephron.

In summary, there is no simple genotype-phenotype correlation in patients with *CLCNKB* mutations. The phenotypic heterogeneity probably reflects the wide distribution of ClC-Kb in the distal nephron. The potential for activating alternative routes for basolateral chloride secretion in the particular nephron segment might be crucial for the individual clinical presentation: the classic Bartter phenotype or the disorder of the DCT.

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