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SLC26A3 Gene Analysis in Patients with Bartter and Gitelman Syndromes and the Clinical Characteristics of Patients with Unidentified Mutations

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We analyzed the SLC26A3 gene in patients with a clinical diagnosis of Bartter and Gitelman syndromes in whom genetic diagnoses could not be determined. We also examined the genetic and clinical characteristics of patients for whom genetic proof could not be obtained. The present study included 10 patients. With regard to genetic characteristics, 1 patient harbored a heterozygous mutation in the SLC12A3 gene (c.2573T>A, p.L858H), which was also reported in a previous report. With regard to clinical characteristics, 3 patients had abnormalities that were identified incidentally during medical examinations and other illnesses and 1 patient had polyhydramnios. One case of nephrocalcinosis was also noted. Eight patients were of below average height. Although we analyzed the SLC26A3 gene in these 10 patients, none were found to have pathological mutations. Investigation of the outcomes of these cases showed that examination findings had normalized and medication was no longer necessary for 3 patients, whereas hypokalemia and metabolic alkalosis were observed in another patient only in the presence of acute disease. We concluded that few patients develop illnesses because of SLC26A3 mutations. Other disease-related genes may also be involved. Although hypokalemia and metabolic alkalosis are clinical characteristics of Bartter and Gitelman syndromes, many other conditions also present such symptoms, and thus, differential diagnosis is of paramount importance.

Bartter syndrome (BS) and Gitelman syndrome (GS) are congenital renal tubule dysfunctions characterized by hypokalemia and metabolic alkalosis (1, 14-19). With recent advancement in research on molecular genetics, the elucidation of disease-related genes in patients with BS, GS, and other congenital renal tubule dysfunctions has made rapid progress; as a result, these disease types have come to be classified and diagnosed primarily according to genotype rather than phenotype. However, even when a patient is clinically diagnosed with BS or GS, mutations in known disease-related genes are not observed. Moreover, sometimes, only heterozygous mutations can be identified, even in patients with recessive disorders (7). Although considerable improvements in methods for genetic analysis have made the performance of such analyses relatively easy, the cost of genetic testing remains high, both in terms of patient effort and finance. Therefore, although further elucidation of the clinical characteristics of BS and GS and the identification of

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patients who should undergo genetic testing are desirable, few indicators for such identification currently exist.

Recently, some patients with BS were shown to possess a mutated Cl^-/HCO_3^- exchanger protein which was encoded by *SLC26A3* gene (3) and this gene has been newly identified as a BS disease gene. Therefore, in this study, we analyzed the *SLC26A3* gene in patients who were clinically diagnosed with BS or GS, in whom mutations in known disease-related genes were not identified on genetic analysis. We also examined the genetic and clinical characteristics of patients for whom genetic proof could not be obtained.

MATERIALS AND METHODS

This study was conducted with the approval of the medical ethics committee of the Kobe University Graduate School of Medicine.

Patients

The study included 10 patients who were clinically diagnosed with BS or GS, in whom mutations in known disease-related genes. Genetic analyses were performed for all patients according to the BS/GS genetic analysis algorithm proposed by Nozu et al (8) (Fig 1).

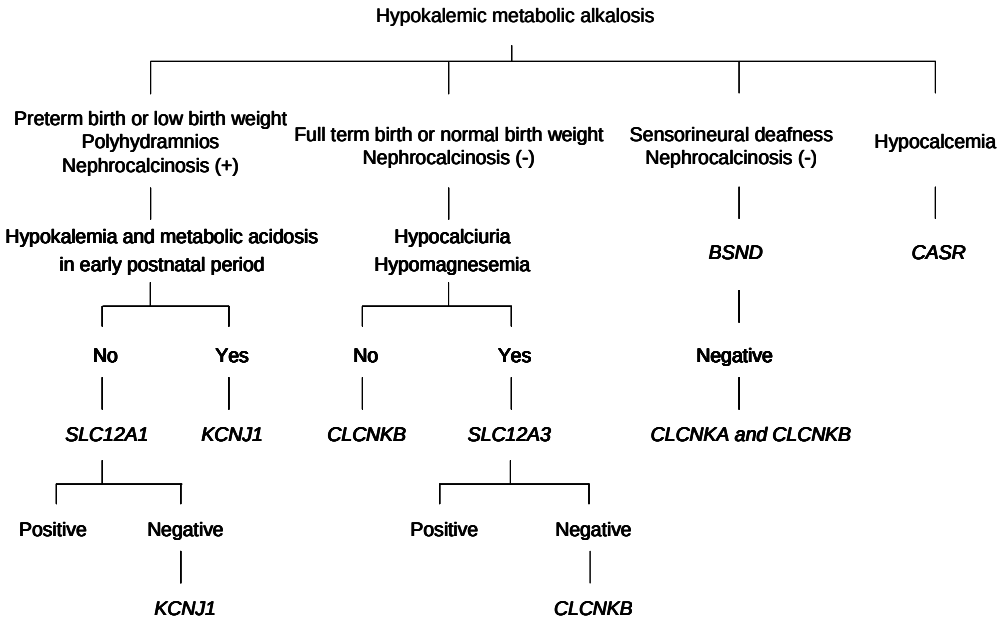

Figure 1. Algorithm of genetic analysis for Bartter and Gitelman syndrome

Patients were diagnosed with BS or GS by a clinician and fulfilled the following 3 diagnostic criteria: 1) hypokalemia (<3 mEq/L); 2) metabolic alkalosis; 3) no clear findings of anorexia nervosa, chronic diarrhea, vomiting, or long-term administration of laxatives or diuretics as determined by interviews or examinations (thus allowing for the elimination of pseudo-Bartter/Gitelman syndrome).

The patients' clinical data were collected using a clinical course questionnaire created by one of the authors (KN), and the clinicians were asked to provide the necessary details as per

the patients' medical records. In the event of unclear or imprecise descriptions, the responsible clinician was telephoned or e-mailed to verify the responses, and the necessary details were obtained.

Mutational Analysis

After first obtaining consent from the patient or the patient's guardian, genomic DNA was extracted from patients' peripheral blood lymphocytes; exons and exon-intron boundaries of the *SLC12A1*, *KCNJ1*, *CLCNKB*, *BSND*, *CASR*, and *SLC12A3* genes were amplified using polymerase chain reaction (PCR) (Figure 1). We analyzed the products using direct sequencing (primer designs for all genes can be provided if needed). One patient (Case 1) had a heterozygous mutation in the *SLC12A3* gene, which has been previously reported as a GS disease-causing mutation (c.2573T>A, p.L858H). Analysis using multiplex ligation-dependent probe amplification (MLPA) did not show extensive deletions; in other words, this mutation was not a compound heterozygous mutation with extensive deletions. MLPA was performed using the SALSA MLPA assay (MRC-Holland, Amsterdam, the Netherlands).

The *SLC26A3* gene was analyzed as follows. Genomic DNA was extracted from patients' peripheral blood lymphocytes using a Qiagen kit (Qiagen Inc., Chatsworth, CA, USA). After PCR amplification of exons and exon-intron boundaries of the *SLC26A3* gene (initial denaturation at 95°C for 5 minutes; followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 1 minute; and a final extension at 72°C for 5 minutes), we performed direct sequence analysis. Primers were designed according to those described by Choi et al (3). Direct sequencing was performed using an automatic DNA sequencer (Perkin-Elmer-ABI, Foster City, CA, USA).

Laboratory Evaluation

Estimated glomerular filtration rate (eGFR) was used as an indicator of renal function. The Schwartz formula was used for patients less than 18 years of age (11,13), whereas the Modification of Diet in Renal Disease (MDRD; modified for Japanese patients) formula (6) was used for patients of 18 years of age or more. For children, previous reports (5,12) were used as references to establish normal eGFR values.

RESULTS

Patient Background

Patient background data are shown in Table I. The patient group comprised 4 male and 6 female patients with a median age of 28 years (range, 0.3–55 years) at the time of genetic analysis. Three of the 10 patients had abnormalities identified incidentally during medical examinations and other illnesses. Polyhydramnios was observed in 1 patient, but none of the patients had a history of low birth weight or premature birth. Nephrocalcinosis was observed in 1 patient. The median SD value for height at the time of genetic analysis was -1.0 SD (-2–0.3 SD).

Examination findings are shown in Table II. The median serum potassium value was 2.7 mEq/L (1.7–2.9 mEq/L); all patients presented with hypokalemia and metabolic alkalosis. Decreased eGFR levels were observed in 3 patients (Cases 1, 3, and 6).

Table I. Background data of the patients

Case	Age (years)	Sex	Chief complaint	Polyhydramnios	Nephrocalcinosis	Height (SD)	Candidate responsible genes analyzed
1	55	M	Limb weakness	NA	-	0.3	*CASR, CLCNKB, SLC12A3*
2	0.3	M	Loss of consciousness	-	-	-1.5	*SLC12A1, KCNJ1, CLCNKB, BSND, SLC12A3*
3	23	M	Abnormal laboratory examination	NA	-	-0.8	*CLCNKB, SLC12A3*
4	55	M	Upper limb numbness	-	-	-0.6	*SLC12A1, KCNJ1, CLCNKB, CASR, SLC12A3*
5	35	F	Abnormal laboratory examination	NA	-	-0.1	*CLCNKB, SLC12A3*
6	35	F	Limb weakness	-	-	-1.5	*CLCNKB, SLC12A3*
7	0.3	F	Poor sucking	-	-	-2.0	*CLCNKB, SLC12A3*
8	0.4	F	Enlarged head	+	-	-1.0	*SLC12A1, KCNJ1, CLCNKB, SLC12A3*
9	33	F	Polyposia and polyuria	-	-	NA	*SLC12A1, KCNJ1, CLCNKB, SLC12A3*
10	13	F	Numbness in all limbs	-	+	-1.0	*CLCNKB, SLC12A3*

NA : not available

Table II. Patient examination findings

Case	Serum K (mEq/L)	Serum Mg (mg/dL)	BUN (mg/dL)	Cr (mg/dL)	eGFR (ml/min/1.73m²)	HCO3 (mEq/L)	Urine Ca/Cr (mg/mg · Cr)
1	1.7	1.8	19.5	1.62	36.2	25.5	0.15
2	2.9	2.3	3.5	0.23	84.0	31.7	0.06
3	2.9	1.4	20	1.15	58.5	29.6	0.04
4	1.9	2.1	8.2	0.62	103	29.4	0.03
5	2.9	1.8	18	0.57	95.6	27.3	0.02
6	2.4	2.3	12	1.23	40	39.9	0.04
7	2.6	1.9	12.6	0.25	74	48.2	0.06
8	2.9	2.1	7	0.15	120	31.3	0.18
9	2.7	2.4	NA	NA	NA	26.5	0.02
10	2.0	2.0	13.2	0.52	113	21	0.58

NA : not available eGFR : estimated glomerular filtration rate BUN : blood urea nitrogen

SLC26A3 Gene Analysis

The *SLC26A3* gene was analyzed in all 10 patients. A c.1299G>A homozygous mutation was observed in 2 patients (Cases 2 and 5); a c.1299G>A heterozygous mutation was observed in 2 patients (Cases 6 and 9); a c.2130C>T heterozygous mutation was observed in 1 patients (Case 3); and a c.1661G>A heterozygous mutation was observed in 2 patients (Case 5) (Fig. 2). However, as per the single nucleotide polymorphisms listed in the Ensembl Database (http://asia.ensembl.org/index.html), these were not classified as disease-causing mutations.

Figure 2. Results of genetic analysis
A c.1299G>A homozygous mutation was observed in Cases 2 and 5; a c.1299G>A heterozygous mutation was observed in Cases 6 and 9; a c.2130C>T heterozygous mutation was observed in Case 3; and a c.1661G>A heterozygous mutation was observed in Case 5.

Case Outcomes

Clinicians were contacted again regarding patient outcomes. Examination findings had normalized and medication was no longer necessary in 3 patients (Cases 3, 7, and 10). Hypokalemia and metabolic alkalosis were observed in another patient (Case 8) only in the presence of acute disease; otherwise, medication was not required and examination findings were normal. Hypokalemia and metabolic alkalosis persisted in all the other patients; the condition of these patients was clinically consistent with BS and GS.

DISCUSSION

In this study, we examined the presence of mutations in a new candidate gene (*SLC26A3*) in patients who were clinically diagnosed with BS or GS and did not harbor mutations in known disease-related genes; we also examined the clinical findings of patients who did not have any genetic mutations. Genetic analysis was the most useful method for establishing definitive diagnoses of BS and GS; however, although mutation identification rates improved

dramatically, the disease-causing gene mutation could not be identified in some cases. We conducted this study assuming that a mutation in the *SLC26A3* gene may be involved in such cases, but *SLC26A3* mutations were not identified in any of the 10 patients included in this study.

The *SLC26A3* gene is expressed in the ileum, codes for Cl^-/HCO_3^- exchangers, and is a known disease-causing gene for congenital chloride diarrhea. Choi et al (9) reported that of 39 patients clinically diagnosed with BS, 5 showed extensive deletions involving the *SLC26A3* gene on exome analysis; in fact, homozygous mutations were detected in all 5 patients on direct sequencing analysis. In the present study, we evaluated patients with clinically diagnosed BS or GS. We analyzed the known disease-related genes and examined the *SLC26A3* gene; however, we could not determine the responsible mutation. This finding suggests that *SLC26A3* mutations may be an infrequent cause of BS or GS.

A heterozygous mutation of the *SLC12A3* gene was observed in 1 patient in the present study, in whom hypokalemia and metabolic alkalosis persisted; hyperreninemia, a typical finding of GS, was also observed at the time of genetic analysis. Therefore, we used MLPA to determine the presence of extensive deletions, but we found none. We could not make a definitive genetic diagnosis of GS because GS is a recessive disorder; however, on the basis of a previous report of a similar case of GS in which the patient harbored only a heterozygous mutation, loss of function due to a heterozygous mutation is conceivable. Furthermore, we performed only a genomic DNA analysis; the presence of abnormal splicing is also possible.

With regard to the clinical characteristics of the patients in the present study, declining renal function was observed in 3 patients. As previously stated, one of these patients harbored a heterozygous mutation of the *SLC12A3* gene. BS commonly progresses to renal failure, whereas GS advances to mild renal dysfunction or even renal failure (2,4). All 3 BS and GS patients with declining renal function in this study were adults. Although declining renal function is consistent with a diagnosis of BS and GS, patients with pseudo-BS/GS may also present with this condition. Therefore, because hypokalemia and metabolic alkalosis comorbid with renal dysfunction is common to BS/GS and pseudo-BS/GS, careful judgment is necessary regarding these diagnoses and the applicability of genetic analysis.

The following are possible reasons for our inability to identify the disease-related genes in the patients of this study. First, examination findings had normalized and medication had become unnecessary in 3 patients. The clinical symptoms of BS and GS do not normally remit spontaneously; rather, they persist throughout a patient's life. In the establishment of diagnostic methods in molecular genetics research, the timing of the performance of genetic analysis for such cases must be considered. Hypokalemia and metabolic alkalosis, which are clinical characteristics of BS and GS, are relatively common in other illnesses as well. Second, hypokalemia and metabolic alkalosis were observed in 1 patient only in the presence of acute illness. Although the course of this case was atypical, the progression of BS and GS is completely unknown. It is quite possible that this was a case of BS or GS with a mild phenotype. The patient was 0.4 years of age at the time of analysis. The patient presented with polyhydramnios and developed BS/GS during infancy; accordingly, we analyzed the *SLC12A1*, *KCNJ1*, *CLCNKB*, and *SLA12A3* genes, but found no mutations. We were able to observe the clinical course for only a relatively short period of time, and therefore, further follow-up could be necessary.

No gene mutations were observed in the remaining 6 cases, regardless of examination findings or diseases courses consistent with typical BS or GS. Genes other than those previously identified may be involved, including *SLC26A3*.

This study has several limitations. The first limitation is that the *SLC26A3* gene was analyzed only using Sanger's method with genomic DNA; extensive deletion and abnormal splicing were not considered at all. However, because BS and GS are recessive disorders, it is extremely unlikely that they would encompass only those mutations that were not detected by genomic DNA analysis using Sanger's method. The second limitation is that we did not analyze all known disease-related genes in all cases. However, the genetic analysis strategy (9, 10) proposed in this study yielded a higher mutation detection rate than that reported in earlier studies. Finally, only a small number of cases were included in this study.

In conclusion, this study analyzed the *SLC26A3* gene in patients who were clinically diagnosed with BS or GS, but no *SLC26A3* mutations were identified. Thus, cases of BS or GS with *SLC26A3* mutations may be very rare. Although hypokalemia and metabolic alkalosis are clinical characteristics of BS and GS, many other conditions also present such symptoms, and thus, differential diagnosis is of paramount importance. In the future, it will be necessary to evaluate more cases and conduct further examinations, including those for the identification of new disease-related genes.

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