The Pharmacological Characteristics of Molecular-Based Inherited Salt-Losing Tubulopathies

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Context: Our understanding of inherited salt-losing tubulopathies has improved with recent advances in molecular genetics. However, the terminology of Bartter syndrome and Gitelman syndrome does not always accurately reflect their pathophysiological basis or clinical presentation, and some patients are difficult to diagnose from their clinical presentations.

Objective: In the present study, we conducted molecular analysis and diuretic tests for patients with inherited salt-losing tubulopathies to clarify the pharmacological characteristics of these disorders.

Patients: We detected mutations and subsequently conducted diuretic tests using furosemide and thiazide for 16 patients with salt-losing tubulopathies (two with *SLC12A1*; two with *KCNJ1*; nine with *CLCNKB*; and three with *SLC12A3*).

Results: Patients with *SLC12A1* mutations showed no response to furosemide, whereas those with *SLC12A3* mutations showed no response to thiazide. However, patients with *CLCNKB* mutations showed no response to thiazide and a normal response to furosemide, and those with *KCNJ1* mutations showed a good response to both diuretics. This study revealed the following characteristics of these disorders: 1) subjects with *CLCNKB* mutations showed one or more biochemical features of Gitelman syndrome (including hypomagnesemia, hypocalciuria, and fractional chloride excretion insensitivity to thiazide administration); and 2) subjects with *KCNJ1* mutations appeared to show normal fractional chloride excretion sensitivity to furosemide and thiazide administration.

Conclusions: These results indicate that these disorders are difficult to distinguish in some patients, even when using diuretic challenge. This clinical report provides important findings that can improve our understanding of inherited salt-losing tubulopathies and renal tubular physiology. **(*J Clin Endocrinol Metab* 95: E511–E518, 2010)**

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Abbreviations: BS, Bartter syndrome; ClC-Kb, Cl channel Kb; FECl, fractional excretion of Cl; FENa, fractional excretion of Na; GS, Gitelman syndrome; MLPA, multiplex ligation-dependent probe amplification; NCCT, Na–Cl cotransporter; NKCC2, Na–K–2Cl cotransporter; ROMK, renal outer medullary K channel; SLT, salt-losing tubulopathies.

Bartter syndrome (BS) and Gitelman syndrome (GS) are autosomal, recessive, inherited salt-losing tubulopathies (SLT) characterized by hypokalemic metabolic alkalosis with normal or low blood pressure, despite hyperreninemia and hyperaldosteronemia. Recent studies have shown that these diseases are caused by mutations in genes encoding ion transporters or channels, including subunits that lead directly (*i.e.* transporter/channel encoded) or indirectly (*i.e.* of another transporter/channel) to loss of function. The first gene is the *SLC12A1* gene, which encodes the apical furosemide-sensitive Na–K–2Cl cotransporter (NKCC2); the second is the *KCNJ1* gene, which encodes the apical renal outer medullary K channel (ROMK); and the third is the *CLCNKB* gene, which encodes the basolateral Cl channel Kb (ClC-Kb). Mutations in these genes lead to types I, II, and III BS, respectively (1–3). On the other hand, mutations in the *SLC12A3* gene, which encodes the apical thiazide-sensitive Na–Cl cotransporter (NCCT), are responsible for GS (4). In recent years, the co-occurrence of the most severe phenotype of antenatal BS and sensorineural deafness has been recognized, and the responsible gene has been identified as *BSND*, which encodes barttin, a subunit of ClC-Ka and ClC-Kb, and this phenotype is now known as type IV BS (5). A digenic inheritable disease with mutations in both the *CLCNKA* and *CLCNKB* genes has been classified as type IVb BS [and sometimes as type V (6)], which possesses the same phenotype as type IV BS (6–8). However, the terminology of BS and GS does not accurately reflect their pathophysiological basis or clinical presentation in some patients. For example, as in GS, type III BS patients usually show hypomagnesemia and hypocalciuria. Accordingly, it is difficult to distinguish these two diseases without molecular diagnosis.

Recently, Seyberth (6) proposed a new classification of BS and GS as one disease entity of inherited SLT and suggested that they should be classified into three groups of distal convoluted tubule disorders (NCCT and ClC-Kb), loop disorders (NKCC2 and ROMK), and compound disorders (barttin and ClC-Ka and -Kb) according to the location of these channels or transporters, including the β-subunit.

In this study, we conducted genetic and pharmacological typing of 16 patients with SLT.

Patients and Methods

This study was approved by the Institutional Review Board at Kobe University Graduate School of Medicine, and consent for the study was obtained from the patients or their parents.

Patients

We included a total of 16 patients with a diagnosis of antenatal BS, classical BS, or GS based on clinical findings and biochemical parameters. They were referred to our hospital for clinical evaluation or mutational analysis. Having undergone genetic diagnosis and diuretic tests, all 16 patients were included in this study. For patients II-1, II-2, and G-3, their parents are first cousins. Patients III-1 and -2, and III-6 and -7 are siblings. Although the blood relationship of the parents of patients III-6 and -7 was not clear, they came from the same local area.

Data collection

The patients' perinatal conditions and infantile stages were recorded. The lowest serum concentrations of potassium and magnesium were used for the present analysis.

Mutational analysis

Total DNA was extracted and purified from peripheral leukocytes in whole-blood samples using the standard phenol–chloroform extraction method. Specific exons of *SLC12A1*, *KCNJ1*, *CLCNKB*, and *SLC12A3* were amplified by PCR using previously described primer pairs (9, 10). The PCR-amplified products were then purified and subjected to sequencing. For *CLCNKB* analysis, we used semiquantitative PCR (7, 10) or multiplex ligation-dependent probe amplification (MLPA) with the SALSA P266-*CLCNKB* MLPA assay (MRC-Holland, Amsterdam, The Netherlands) to detect large heterozygous deletions. We did not conduct gene analysis of *CLCNKA* because sensorineural deafness was not detected in any of the subjects with *CLCNKB* mutations. SLT patients with homozygous or compound heterozygous mutations were included, whereas patients with only heterozygous mutations were excluded from this study.

Diuretic tests

All 16 patients were genetically diagnosed as BS or GS and underwent diuretic tests according to a protocol described elsewhere (11, 12). Patients drank water (10 ml/kg), followed by iv infusion of 0.6% saline solution at a rate of 10 ml/kg · h (maximum, 500 ml/h) to generate sufficient urinary flow. Two hours after starting infusion, either furosemide (20 mg iv) or trichlormethiazide (8 mg orally) was administered. Urine samples were collected every hour from spontaneous voiding, and blood samples every 2 h to measure Na, Cl, and creatinine as a marker for glomerular filtration rate. A simplified quantification of the diuretic effect was performed by calculating the maximal increases in urine excretion of Cl and Na over the mean basal levels to attain the test results. Electrolyte excretion was evaluated as fractional excretion (FECl and FENa) (11).

Results

Patient characteristics

Table 1 summarizes the patients' clinical features and laboratory data. All patients showed the clinical and laboratory findings typical of antenatal BS, classical BS, or GS, and some of the patients have been included in our

TABLE 1. Clinical characteristics and laboratory data

Patient	Sex	Age (yr)	Onset age	Symptom	PH	GW	BW (g)	FT	NC	K (mEq/liter)	Mg (mg/dl)	BE (mmol/liter)	UCa/Cr (mg/mg)	eGFR (ml/min)	Renin (ng/ml · h)	Aldosterone	FENa (%)
SLC12A1																	
I-1	F	7.6	Antenatal	Polyhydramnios	+	29	890	+	+	2.6	1.8	1.2	0.67	92.8	9.7	2600 pg/ml	1.2
I-2	F	12.5	Antenatal	Polyhydramnios	+	35	1816	+	+	2.6	1.3	5	0.525	123.1	≥20	229 ng/dl	1.3
KCNJ1																	
II-1	F	12.2	7 d	Polyuria	+	33	1604	+	+	2.9	2.1	4.5	1.1	113.5	11	512 pg/ml	1.93
II-2	M	18	3 d	Hyperkalemia[a]	+	33	2090	+	+	3.1	1.9	5.1	0.8	104.1	≥20	184 ng/dl	1.92
CLCNKB																	
III-1	F	16	2 months	Failure to thrive	−	39	3570	+	−	1.2	1.4	6.3	<0.01	50	≥20	1400 pg/ml	2.4
III-2	M	8.5	1 month	Failure to thrive	−	39	3150	+	−	2.4	1.5	20.4	0.018	118	76.1	176.7 pg/ml	2.6
III-3	F	11.1	1 yr, 11 months	Chance exam	−	39	3780	+	−	2.5	1.9	13.1	0.004	115.1	45.4		1.3
III-4	F	11.2	1 month	Failure to thrive	+	40	3250	+	−	2.4	1.8	14.7	0.02	177.4	≥20	2500 pg/ml	1.44
III-5	F	9.4	Antenatal	Polyhydramnios	+	40	3840	+	−	2.7	1.9	11.9	0.14	141.5	≥20	450 pg/ml	0.85
III-6	F	24	4 months	Failure to thrive	−	40	3655	+	−	2.8	1.3	5.1	0.01	62.4			2.52
III-7	M	23	2 yr	Failure to thrive	−	41	3540	+	−	3.1	2.1	6.3	0.2	151.8	≥20		2.86
III-8	M	19	3 yr	Failure to thrive	+	39	3300	+	−	2.9	1.6	11.5	0.01	118.5	≥20	520 pg/ml	0.98
III-9	M	21.1	3 months	Failure to thrive	−	34	2900	+	+	1.7	2.4	4.8	0.044	99.9	≥20	447 pg/ml	1.79
SLC12A3																	
G-1	M	14	4 yr	Chance	−	40	2112	−	−	2.4	1.5		0.018	143.7	12.6		1.56
G-2	M	13.9	14 yr	Short stature	−	40	3000	−	−	2.8	1.8	3	0	150.9	≥20		0.96
G-3	M	3	1 yr	Chance	−	35	1338	−	−	2.3	1.4	5	0.021	113.4	≥20	976 pg/ml	0.35
Normal range										3.6–5.0	1.8–2.6	0			1.01 ± 0.14	<435 pg/ml 57–150 ng/dl	

PH, Polyhydramnios; GW, gestational week; BW, birth weight; FT, failure to thrive; NC, nephrocalcinosis; BE, base excess; UCa, urinary calcium; eGFR, estimated glomerular filtration rate; F, female; M, male.

[a] Hyperkalemia was transiently observed in the neonatal period, which is characteristic of type II BS (13).

TABLE 2. Mutations in the *SLC12A1*, *KCNJ1*, *CLCNKB*, or *SLC12A3* gene of all patients

Patient no.	Gene	Mutation	Amino acid change
I-1	*SLC12A1*	c.904C>T/c.538T>C	R302W/W936X
I-2	*SLC12A1*	c.577G>A/c.724 + 1G>A	G193R/exon5 skipping
II-1	*KCNJ1*	c.827G>A (homo)	S276N
II-2	*KCNJ1*	c.607delC (homo)	Frameshift
III-1	*CLCNKB*	Exon1-2 deletion/c.1830G>A	Exon1-2 deletion/W610X
III-2	*CLCNKB*	Exon1-2 deletion/c.1830G>A	Exon1-2 deletion/W610X
III-3	*CLCNKB*	c.1830G>A (homo)	W610X
III-4	*CLCNKB*	c.1830G>A (homo)	W610X
III-5	*CLCNKB*	c.1830G>A (homo)	W610X
III-6	*CLCNKB*	c.1172G>A (homo)	W391X
III-7	*CLCNKB*	c.1172G>A (homo)	W391X
III-8	*CLCNKB*	c.1830G>A (homo)	W610X
III-9	*CLCNKB*	c.226C>T/c.1830G>A	R76X/W610X
G-1	*SLC12A3*	c.539C>A/c.1732G>A	T180K/V578M
G-2	*SLC12A3*	c.1045C>T/c.1706C>T	P349S/A569V
G-3	*SLC12A3*	c.788 ins18bp (homo)	In frame insertion

previous reports (10, 13). All of the patients had low serum potassium levels and metabolic alkalosis.

Mutational analysis

Table 2 shows the mutations in the *SLC12A1*, *KCNJ1*, *CLCNKB*, or *SLC12A3* genes of all patients. We detected homozygous or compound heterozygous mutations in all 16 patients (two with *SLC12A1*, two with *KCNJ1*, nine with *CLCNKB*, and three with *SLC12A3*). *CLCNKB* analysis showed that two of the nine patients possessed large heterozygous deletions that could be detected with semiquantitative PCR or MLPA. In addition, all *CLCNKB* mutations were nonsense, splice site, or deletion mutations, and none of them showed missense mutations. As previously reported, the W610X mutation in the *CLCNKB* gene, which was detected in seven of the nine patients, is the founder-effect mutation in the Japanese population (10). We confirmed all novel missense mutations in 200 control chromosomes to eliminate single nucleotide polymorphisms.

Diuretic tests for SLT

Renal clearance tests using furosemide and thiazide were performed in all patients with *SLC12A1*, *KCNJ1*, *CLCNKB*, and *SLC12A3* mutations. The results are shown in Tables 3 and 4 and Figs. 1 and 2. As expected,

TABLE 3. FECl results of diuretic tests

	Furosemide			Thiazide		
Patient	Pre (%)	Post (%)	Ratio	Pre (%)	Post (%)	Ratio
SLC12A1						
I-1	1	1.5	1.50	1	15.2	15.20
I-2	1.98	2	1.01	1.98	6.9	3.48
KCNJ1						
II-1	2.44	14	5.74	2.44	11.8	4.84
II-2	2.11	18.16	8.61	2.11	9.17	4.35
CLCNKB						
III-1	4.5	12.5	2.78	4.5	4.3	0.96
III-2	3.9	14.5	3.72	3.9	4.1	1.05
III-3	1.8	5.5	3.06	1.8	1.7	0.94
III-4	3.52	13.7	3.89	3.52	4.7	1.34
III-5	2.33	6.17	2.65	2.33	2.99	1.28
III-6	3.47	11.83	3.41	3.47	3.95	1.14
III-7	4.98	15.39	3.09	4.98	8.15	1.64
III-8	3.37	22	6.53	3.37	7.05	2.09
III-9	3.11	21	6.75	3.11	7.12	2.29
SLC12A3						
G-1	2.39	12.16	5.09	2.39	4.24	1.77
G-2	1.32	20	15.15	1.32	1.57	1.19
G-3	0.51	4.6	9.02	0.46	0.56	1.22
Control						
C-1	3.85	14.8	3.84	3.85	13.1	3.40
C-2	0.6	4.35	7.25	0.6	2.95	4.92

TABLE 4. FENa results of diuretic tests

	Furosemide			Thiazide		
Patient	**Pre (%)**	**Post (%)**	**Ratio**	**Pre (%)**	**Post (%)**	**Ratio**
SLC12A1						
I-1	1.2	1.57	1.31	1.2	15	12.50
I-2	1.3	1.7	1.31	1.3	4.45	3.42
KCNJ1						
II-1	1.93	8.44	4.37	1.93	7.08	3.67
II-2	1.92	11.96	6.23	1.92	6.54	3.41
CLCNKB						
III-1	2.4	8.2	3.42	2.4	2.2	0.92
III-2	2.6	10.4	4.00	2.6	3.4	1.31
III-3	1.3	4.4	3.38	1.3	1.2	0.92
III-4	1.44	8.4	5.83	1.44	2.62	1.82
III-5	0.85	3.9	4.59	0.85	1.37	1.61
III-6	2.52	8.64	3.43	2.52	3	1.19
III-7	2.86	9.47	3.31	2.86	5.27	1.84
III-8	0.98	13.2	13.47	0.98	3.2	3.27
III-9	1.79	13	7.26	1.79	3.75	2.09
SLC12A3						
G-1	1.56	8.14	5.22	1.56	2.74	1.76
G-2	0.96	11.9	12.40	1.57	2.75	1.75
G-3	0.35	2.54	7.26	0.52	0.36	0.69
Control						
C-1	2.76	9.01	3.26	2.76	7.71	2.79
C-2	0.42	3.7	8.81	0.42	2.37	5.64

patients with *SLC12A1* mutations showed no response to furosemide and a good response to thiazide, whereas those with the *SLC12A3* mutations showed no response to thiazide and a good response to furosemide. However, patients with *KCNJ1* mutations showed a good response to both diuretics, similar to the response by normal controls, whereas patients with *CLCNKB* mutations showed no response to thiazide and a normal but weaker response to furosemide compared with that in patients with *SLC12A3* mutations.

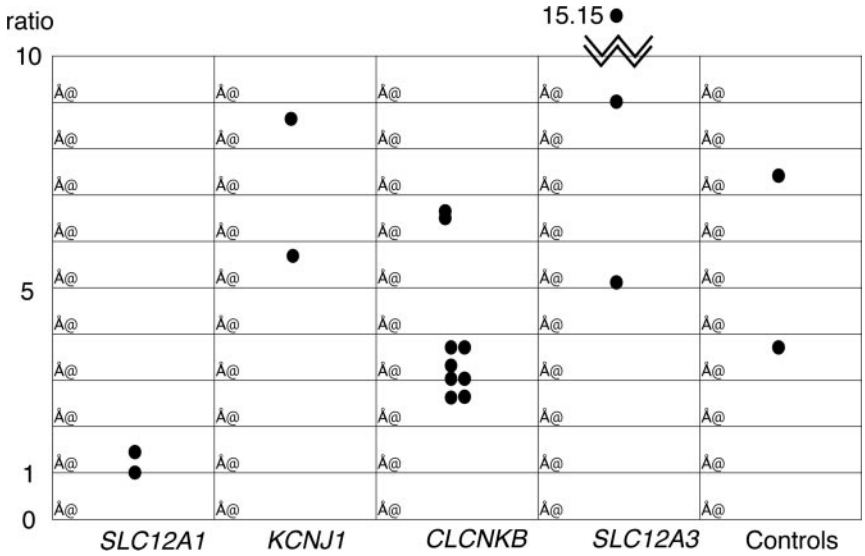

FIG. 1. Furosemide test results in the patient groups. The fractional excretion of Cl (FECl) ratio was calculated as the value after testing divided by the value before testing. Values of 1 indicate no changes of FECl after the administration of furosemide.

Discussion

In the present study, we conducted diuretic tests using furosemide and thiazide for 16 patients with SLT. All patients were genetically diagnosed with types I–III BS or GS. As expected, patients with *SLC12A1* mutations showed no response to furosemide and a good response to thiazide, whereas those with the *SLC12A3* mutations showed no response to thiazide and a good response to furosemide. These findings for BS and GS patients have been reported elsewhere (11, 12, 14–16). However, patients with the *KCNJ1* mutations showed a good response to both diuretics, and those with *CLCNKB* mutations showed no response to thiazide and a normal response to furosemide. These results indicate that defects in ROMK as a result of *KCNJ1* mutations are unlikely to affect NKCC2 and NCCT functions, and that defects in ClC-Kb as a result of *CLCNKB* mutations cause secondary dysfunction of NCCT but not NKCC2.

It seems reasonable to believe that the lack of a response to furosemide is due to dysfunctions in NKCC2. In fact, several studies have already identified antenatal BS patients with no response to furosemide, although most of the patients were not genetically diagnosed

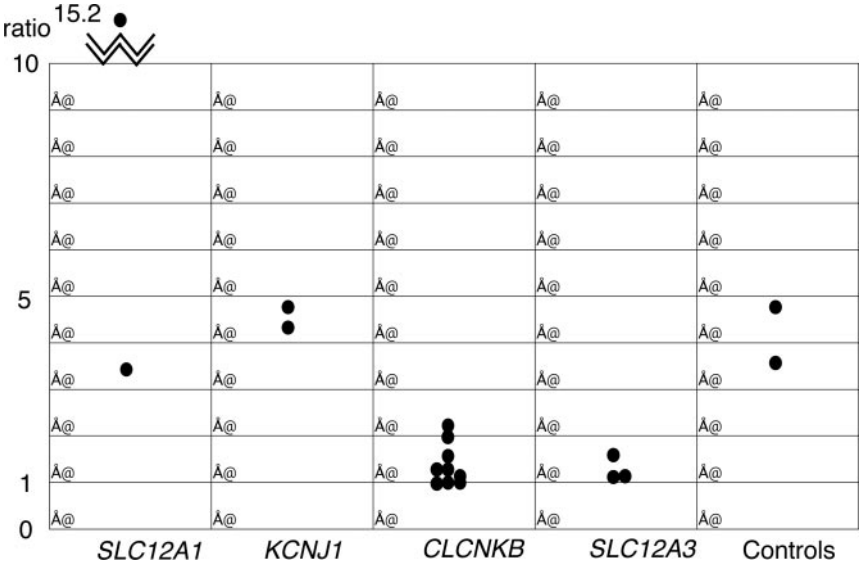

FIG. 2. Thiazide test results in the patient groups. The FECl ratio was calculated as the value after testing divided by the value before testing. Values of 1 indicate no changes of FECl after the administration of thiazide.

(14). On the other hand, loss of function of ROMK is thought to manifest as a secondary loop of Henle dysfunction, which may lead to insensitivity to furosemide. Recently, Seyberth (6), in his review article based on unpublished data, reported an antenatal BS patient who showed no response to furosemide and was later found to possess a *KCNJ1* mutation. However, no original studies have been published about the use of diuretic tests for the genetic diagnosis of type II BS, making this the first such report. We cannot explain why Seyberth's case showed no response to furosemide. It is possible that, in our patients, the secondary dysfunctions in NKCC2 are lessened with age. Recent findings indicate that these patients have borderline low serum potassium levels (usually 3.6 and 3.4 mEq/liter, respectively), despite marked hypokalemia when they were younger. Patients with ROMK defects usually show slightly higher serum potassium levels than those with NKCC2, ClC-Kb, or NCCT (17, 18). This suggests that ROMK defects may be manifested as milder NKCC2 dysfunction and that NKCC2 dysfunction may lessen with aging. In our study, two patients with *KCNJ1* mutations underwent diuretic tests after their serum potassium levels had spontaneously elevated without any treatment. This may explain why furosemide and thiazide affected NKCC2 and NCCT, respectively, in both patients. Therefore, there is a need for further investigation of patients with *KCNJ1* mutations, including diuretic tests.

Phenotype overlapping frequently occurs in patients with *CLCNKB* and *SLC12A3* mutations (18–20). Moreover, *CLCNKB* mutations typically show the classical BS phenotype but can also show antenatal BS or GS phenotypes (21). In our study, three patients with *CLCNKB* mutations presented with polyhydramnios and one presented with nephrocalcinosis, which is characteristic of antenatal BS, whereas four showed hypomagnesemia and seven showed low urine Ca excretion. ClC-Kb is not only expressed in the loop of Henle but is also likely to be found in distal tubules and is physiologically related to NCCT (6). This clinical overlap suggests that malfunctioning of ClC-Kb may interfere with the intracellular Cl concentration and may cause secondary dysfunction of NKCC2 and NCCT. However, Colussi *et al.* (11) reported that two type III BS patients showed a good response to thiazide. They concluded that the thiazide test can accurately distinguish GS from type III BS. On the other hand, all nine type III BS patients in our study who were subjected to diuretic tests showed a poor response to thiazide and a normal response to furosemide. We cannot find an explanation for these contradictory results. All of our patients possessed truncating mutations in the *CLCNKB* gene; however, of those reported by Colussi *et al.* (11), one possessed a homozygous missense mutation (P126L), and another possessed a homozygous exon 6 deletion. Missense mutations and in-frame deletion mutations (deletions in which the deleted base number is in a multiple of three) usually show milder phenotypes than truncating mutations in human inherited disorders. Exon 6 of the *CLCNKB* gene consists of 78 base pairs, and its deletion is an in-frame deletion mutation. There is also a possibility that this missense mutation and in-frame deletion mutation may maintain the function of ClC-Kb and showed a thiazide response. In fact, the changes in FECl in the thiazide tests were lower for *CLCNKB* mutations than those for *SLC12A3* mutations in the study by Colussi *et al.* (11). On the other hand, we revealed that ClC-Kb defects may lead to secondary NCCT dysfunction rather than NKCC2. These findings provide a clear reason why type III BS patients show combined characteristics of antenatal BS, classical BS, and GS. However, our study also has a limitation in that all patients with type III BS showed mild to severe hypocalciuria because the highest urinary Ca/Cr ratio was 0.2. It is unclear and we cannot determine whether type III BS patients with hypercalciuria will respond to furosemide during diuretic tests. Clearly, further studies are necessary to answer this question.

The urinary Ca/Cr ratio of patients included in the study by Simon *et al.* (1) was much higher than that of our patients, showing a striking dichotomy. Although there is

no clear explanation for this dichotomy, one possibility is a correlation between the genotype and phenotype. Seven of nine patients included in our study had a W610X nonsense mutation in the *CLCNKB* gene in at least one allele, and this mutation is the founder-effect mutation in the Japanese population (10). Two patients with this mutation in one allele have been reported previously, and both showed hypocalciuria (19). This suggests that this W610X mutation may be related to hypocalciuria. The other two patients in our report are siblings and possessed a W391X homozygous mutation. To our knowledge, this is a novel mutation and has not been described until now. However, this mutation may also lead to hypocalciuria. Further studies are needed to clarify the mechanisms underlying hypocalciuria in patients with type III BS, including the genotype–phenotype correlation.

Recently, Seyberth (6) suggested a new classification of SLT with three subtypes: distal convoluted tubule dysfunction caused by a defect in NCCT or ClC-Kb, loop dysfunction resulting from a defect in NKCC2 or ROMK, and combined dysfunction following a defect in barttin, the β-subunit of ClC-Ka and ClC-Kb, or digenic defects in ClC-Ka and ClC-Kb. We largely agree with this new classification. Classical typing of antenatal BS, classical BS, and GS no longer shows any merit or clinical convenience. Our study demonstrated that the diuretic response differed completely among types I–III BS. Furthermore, ClC-Kb defects showed secondary dysfunction of NCCT rather than NKCC2. Among patients with ClC-Kb defects, some show antenatal BS, some show classical BS, and others show the GS phenotype (18–21). Thus, in our opinion, the classification proposed by Seyberth (6) holds substantial merit and is worthy of further discussion. Because genetic testing is still unavailable to the majority of clinicians who treat these disorders, we believe that any new classification should accurately describe the clinical aspects and the underlying molecular etiology. Although these disorders can often be distinguished clinically, there are some patients in which the distinction is not entirely clear. We believe that the data presented here will help to clarify the diagnosis on clinical grounds, without the need to rely on genetic testing. However, our study has confirmed the substantial overlap between the diuretic responses observed in *CLCNKB* and *SLC12A3* patients, which are often the most difficult to distinguish clinically, so genetic testing may still be required.

In conclusion, we conducted diuretic tests for patients with genetically diagnosed SLT. As expected, patients with an NKCC2 defect showed no response to furosemide, and those with an NCCT defect showed no response to thiazide. However, patients with a ROMK defect showed good responses to furosemide and thiazide, and those with a ClC-Kb defect showed no response to thiazide but a normal response to furosemide. These results indicate that BS and GS should be combined into one disease category. A clear argument for abandoning the use of the confusing classification of antenatal BS, classical BS, and GS is that it cannot address the question as to whether patients with *CLCNKB* mutations showing hypomagnesemia and hypocalciuria should be diagnosed as GS or BS, and whether patients with *CLCNKB* mutations without symptoms in the neonatal period, except for polyhydramnios, should be diagnosed as antenatal BS or classical BS. We strongly believe that this clinical report provides important findings that can improve our understanding of renal tubular physiology, including SLT.

Acknowledgments

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