Reduced Urinary Excretion of Thiazide-Sensitive Na-Cl Cotransporter in Gitelman Syndrome: Preliminary Data

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Background: The relationship between *SLC12A3* mutations and actual sodium-chloride (Na-Cl) cotransporter (NCC) expression in patients with Gitelman syndrome (GS) was rarely evaluated. Detection of urinary thiazide-sensitive NCC was not tried in patients with GS.

Study Design: Case series.

Setting & Participants: 6 patients with GS and 1 patient with surreptitious vomiting.

Outcomes & Measurements: Renal clearance study, mutation analysis using reverse-transcription polymerase chain reaction and direct sequencing for the *SLC12A3* gene, and immunohistochemical staining for NCC, Na-K-2Cl-cotransporter, α1-subunit of Na^+,K^+-ATPase, and calbindin-D_{28K} of the renal biopsy specimens were performed. Membrane fractions of urine were obtained by using differential centrifugation and probed with antibodies against human NCC and aquaporin 2.

Results: Results of clearance studies were consistent with GS, showing decreased distal fractional chloride reabsorption with only furosemide. *SLC12A3* gene mutations were found in all patients with GS. Immunohistochemistry showed markedly decreased NCC expression in the distal convoluted tubule, whereas expression of other transporters remained intact. Urinary NCC excretion was markedly decreased in patients with GS, but not in the patient with surreptitious vomiting.

Limitations: Small number of patients and lack of mutation analysis of *CLCNKB*.

Conclusions: There were no relations between NCC expression and types of mutations. Detection of urinary NCC might be helpful for the differential diagnosis of GS.

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INDEX WORDS: Gitelman syndrome; sodium-chloride cotransporter; immunohistochemistry; mutation analysis; urinary sodium-chloride cotransporter.

Gitelman syndrome (GS) is an autosomal recessive renal tubular disorder characterized by hypokalemic metabolic alkalosis, hypomagnesemia, and hypocalciuria.[1] GS is caused by inactivating mutations in the *SLC12A3* gene encoding the thiazide-sensitive sodium-chloride (Na-Cl) cotransporter (NCC) of the distal convoluted tubule (DCT).[2,3] To date, more than 100 mutations in the *SLC12A3* gene were identified.[4] However, the relationship between mutations and the molecular mechanism of the disease was not yet identified.

Before the establishment of mutation analysis of the *SLC12A3* gene, the diagnosis of GS was dependent on clinical and biochemical features and was supplemented by results of clearance studies using thiazide and furosemide in sequence, which was cumbersome and had no established cutoff values.[5] Genotyping all patients with suspected GS is the best way to diagnose the disease, but is impractical because of laboratory unavailability in most hospitals at the present time.[6,7] Moreover, a genetic abnormality other than *SLC12A3* gene mutations may cause similar clinical features.[8] An unusual degree of phenotypic variation is not uncommon in patients with GS.[9-11] These phenotypic variations can be explained by the different mechanisms by which mutations reduce or abolish

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transporter activity[4,12] and might be dependent on the degree or pattern of NCC expression in the DCT. Therefore, mutation analysis of the *SLC12A3* gene might not be the ultimate diagnostic method for GS. For a definite diagnosis and understanding of the pathophysiologic mechanism of GS, it is necessary to investigate the actual expression of NCC in patients' renal tissues.

Recently, it was shown that apical renal sodium transporters could be detected in urine by means of immunoblot, and these urinary transporters might be used as a biomarker for a specific disease.[13,14] The aims of this study are to delineate the relationship between *SLC12A3* gene mutation and NCC expression in renal tissues from patients with GS and evaluate the potential role of urinary NCC as a biomarker for the differential diagnosis of GS. In the present study, we investigated relations among NCC protein expression in DCTs of renal tissue, urinary excretion of NCC, and *SLC12A3* gene mutations in patients with GS diagnosed by using clinical and biochemical characteristics.

METHODS

Subjects

Six patients with a diagnosis of GS by means of clinical features and biochemical parameters and 1 patient with surreptitious vomiting were enrolled in this study. The control patient with surreptitious vomiting flatly denied his vomiting or laxative use at first. Therefore, a misdiagnosis of GS was made despite low urinary chloride excretion in relation to urinary sodium excretion. During the follow-up period, he admitted that he vomited regularly and used laxatives for fear of obesity. A family history of salt craving, hypotension, nocturia, polyuria, or paralysis/paresthesia was unavailable for all patients. Clearance studies using diuretics, mutation analysis for the *SLC12A3* gene, and immunoblot for urinary NCC were performed. Western blot for urinary NCC also was performed in 8 healthy volunteers. Renal tissues from subjects were examined for NCC, Na-K-2Cl-cotransporter (NKCC2), α1-subunit of Na^+,K^+-ATPase, and calbindin-D_{28K} by using immunohistochemistry and compared with that from a patient who underwent surgical nephrectomy because of renal cell carcinoma.

All study participants gave their written informed consent before enrollment in the study. The study protocol was approved by the Institutional Review Board of Seoul National University Hospital.

Clearance Studies

Renal clearance studies were performed according to the protocol described elsewhere.[5] Patients drank water (20 mL/kg of body weight) after an overnight fast, followed by intravenous infusion of 0.45% saline solution at a rate of 40 mL/h. Urine volume and urine osmolality were checked every 20 minutes by using a cryoscopic osmometer (Osmomat 030-D; Gonotec, Berlin, Germany). Upon every voiding, patients drank additional water, in an amount equal to urine volume plus 20 mL. When urine flow reached its maximal level, plasma and urine samples were obtained. For the thiazide-loading test, 100 mg of hydrochlorothiazide was administered orally to patients. For the loop diuretic loading test, 40 mg of furosemide was administered intravenously to patients. Water-loading, thiazide-loading, and furosemide-loading tests were performed in sequence on the same day. Clearance data were obtained when urine flow reached maximal. Clearances were calculated as follows: maximal free-water clearance (CH_2O) = (1 − Uosm/Posm) × V; chloride clearance (CCl) = UCl × V/PCl; and distal fractional chloride reabsorption (DFCR) = CH_2O/(CH_2O + CCl), where Uosm is urine osmolality, Posm is plasma osmolality, V is urinary flow (milliliters per minute), UCl is urinary chloride concentration (milliequivalents per liter), and PCl is plasma chloride concentration (milliequivalents per liter).

Mutation Analysis

For *SLC12A3* gene analysis, both RNA and genomic DNA were isolated from peripheral-blood cells. RNA was reverse transcribed to complementary DNA, and 4 overlapping complementary DNA fragments covering most parts of the coding sequences of the *SLC12A3* gene were amplified by using nested polymerase chain reactions (PCRs) and directly sequenced. For exons 1, 2, and 3, which were not covered by this nested reverse-transcriptase (RT)-PCR, and for any fragments that failed to get good results by using nested RT-PCR, the corresponding exon sequences were analyzed by using genomic DNA PCR and direct sequencing. Mutations detected using nested RT-PCR also were confirmed through direct sequencing of the PCR product of the corresponding exon from genomic DNA. Sequences of primers used in the nested RT-PCR are as follows: for first-round PCR; sense 5′-AGCACCTTCTGCATGCG-CAC-3′ and antisense 5′-GATGGCGTAGGCCAGCAG-GTA-3′, sense 5′-GACCAGCTGTACCCACTGATC-3′ and antisense 5′-AGAGCTGTGGACAGGGATGTC-3′; for second-round PCR; sense 5′-CGTTGCATGCTCAA-CATTTG-3′ and antisense 5′-GTCCAGAAAATGGCCAT-GAG-3′, sense 5′-TTGTCCAGAACTTGGTGCCT-3′ and antisense 5′-GGCTCCTTGTTCTTGCCATA-3′, sense 5′-TACCCACTGATCGGCTTCTT-3′, and antisense 5′-GCA-TCATCTTGGACACGTTG-3′, sense 5′-TCCTCCATGATG-CCTTTGAG-3′ and antisense 5′-ACAGGGATGTCA-AAGCCTGG-3′.

Immunohistochemistry

Renal tissue from patients with GS and the patient who underwent radical nephrectomy was preserved in 2% periodate-lysine-paraformaldehyde solution. Fixed tissues were dehydrated and embedded in polyester wax, cut into 4-μm thick sections, and mounted on gelatin-coated glass slides. Sections were dewaxed with xylene and ethanol and treated with methanolic hydrogen peroxide for 30 minutes after rinsing with tap

water. Before incubation with primary antibodies, sections were incubated in phosphate-buffered saline containing 0.5% Triton X-100 (Bio-Rad, Hercules, CA) for 15 minutes for permeabilization. Sections then were blocked with 6% normal goat plasma (S-1000; Vector Laboratory, Burlingame, CA) for 15 minutes. Subsequently, sections were incubated overnight at 4°C with rabbit polyclonal antibodies to the human NCC,[15] NKCC2,[15] and Na^+,K^+-ATPase α1 subunit (Upstate Biotechnology, Lake Placid, NY) and with rabbit monoclonal antibodies to calbindin-D_{28k} (Swant, Bellinzona, Switzerland). After incubation, sections were rinsed with phosphate-buffered saline and incubated for 30 minutes in biotinylated goat antirabbit immunoglobulin G (BA-1000; Vector Laboratory) at room temperature. A peroxidase standard Vectastain ABC kit (PK-4000; Vector Laboratory) was added for 60 minutes at room temperature. Sections then were washed with phosphate-buffered saline and incubated in a 3,3′-diaminobenzidine substrate kit (SK-4100; Vector Laboratory). Hematoxylin staining was used as a counterstain. Slides were mounted with Canadian balsam.

Immunoblotting for NCC and Aquaporin 2 in Urine

Fresh morning urine from patients and 8 healthy volunteers (5 men, 3 women) was collected in a bottle that contained a collection solution (thymol, 2.5 mL of phenylmethylsulfonyl fluoride, 50 μL of leupeptin/50 mL of sample). After measuring the volume, the sample was spun at 1,300*g* for 15 minutes at 4°C to remove cells, nuclei, and large fragments. The supernatant was then transferred to six 8-mL high-speed tubes for ultracentrifuge (200,000*g* for 1 hour at 4°C). Urine creatinine was measured in this supernatant. The resultant pellets were suspended with 1 mL of isolation solution (0.25 mol/L of sucrose, 10 mmol/L of triethanolamine, 8.5 μmol/L of leupeptin, and 1 mmol/L of phenylmethylsulfonyl fluoride, pH 7.6) and pooled together. Ultracentrifugation was repeated with this sample under the conditions described previously. Pellets were resuspended with 0.4 mL of isolation solution. Samples were then stabilized by adding 0.1 mL of 5 × Laemmli sample buffer (sodium dodecyl sulfate, 3.75 g; glycerol; 15 mL; 1 mol/L of Tris, pH 6.8, 2.5 mL, bromophenol blue dab, ddH_2O to 50 mL), heated to 60°C for 10 minutes, and stored at −80°C until analysis. Just before being loaded for electrophoresis, samples were warmed at 37°C.

Two milligrams of urine creatinine equivalent of each sample was loaded into each lane of gel. Samples were electrophoresed in 8% (for NCC) and 12% (for aquaporin-2 [AQP2]) polyacrylamide-sodium dodecyl sulfate minigels using a Mini Protean III electrophoresis unit (Bio-Rad, Hercules, CA). For immunoblotting, proteins were transferred electrophoretically from gels to nitrocellulose membrane (Bio-Rad). After being blocked with 5% skim milk in 80 mmol/L of Na_2HPO_4, 20 mmol/L of NaH_2PO_4, 100 mmol/L of NaCl, and 0.1% Tween-20, pH 7.5 for 30 minutes, membranes were probed overnight at 4°C with 1:2,000 dilution of rabbit antihuman NCC antibody[15] and 1:1,000 antihuman AQP2 antibody.[16] Membranes were washed and incubated with goat antirabbit immunoglobulin G conjugated to horseradish peroxidase (Pierce, Rockford, IL) diluted to 1:3,000. Antigen-antibody reactions were visualized by using enhanced chemiluminescence (ECL RPN 2106; Amersham Pharmacia Biotech, Buckinghamshire, UK) before being exposed to X-ray film (Hyperfilm; Amersham Pharmacia Biotech).

RESULTS

Clinical and Biochemical Data

All subjects had hypokalemia, metabolic alkalosis, hypereninemia, and increased plasma aldosterone levels. All except patient B had hypocalciuria, and all except patients B and C had hypomagnesemia. Serum magnesium levels in patient B and C were 1.6 mg/dL (0.66 mmol/L) and 1.5 mg/dL (0.62 mmol/L), respectively; these were in the low-normal range. The magnesium level of the control patient (patient G) was within normal range, and urinary chloride excretion was decreased compared with urinary sodium excretion. The diagnosis of GS was favored by clinical features, patient age, the absence of overt polyuria, and the presence of hypocalciuria and/or hypomagnesemia in patients A to F (Table 1).

Renal Clearance Study

Chloride clearance was increased in all subjects after furosemide loading. DFCR significantly decreased after furosemide administration. However, these 2 parameters were not affected significantly by hydrochlorothiazide administration (Table 2) in patients with GS. Although DFCR was slightly decreased in patients A, B, and F after hydrochlorothiazide administration, the decrease was less than that in the control patient. In the control patient, minimal urine osmolality was less than that of patients with GS, and change in DFCR after hydrochlorothiazide administration was greater than that of patients with GS. However, discrimination between patients with GS and the patient with surreptitious vomiting was not clear because of the unavailability of a cutoff value.

Mutation Analysis

Six different sequence variants in *SLC12A3* were detected in 6 patients (patients A to F). Missense point mutation was detected in patients A (p.Ser976Phe), B (p.Thr180Lys), and E (p.Asp848Asn and p.Ile988Thr). p.Gln95 del was found in patients C and F, and c.2666+1G del was identified in patient D. Only 1 mutant allele was detected in 3 of 6 patients (patients B, D, and

Table 1. Initial Presentation and Laboratory Data for Subjects

	Patients With Gitelman Syndrome						Control Patient	Reference Values
	A*	B	C	D	E*	F	G	
Age (y)	16	19	18	18	24	34	22	
Sex	F	M	M	M	F	M	M	
Serum								
Sodium (mEq/L)	135	136	142	137	135	133	143	135-145
Potassium (mEq/L)	2.8	3.2	2.9	3.0	1.9	2.9	3.0	3.5-5.5
Chloride (mEq/L)	93	90	97	100	94	90	91	98-110
Total carbon dioxide (mEq/L)	33	31	35	33	29	30	48	24-31
Calcium (mg/dL)	10.7	10.4	9.7	9.2	9.8	10.2	9.3	8.8-10.5
Phosphorus (mg/dL)	4.3	3.8	4.4	4.1	3.8	3.2	3.4	2.5-4.5
Magnesium (mEq/L)	1.4	1.6	1.5	1.4	1.1	1.4	1.8	1.5-2.5
Plasma renin activity (ng/mL/h)	32.8	117	11.0	43.8	14.7	10.4	7.8	1.0-2.5
Plasma aldosterone (ng/dL)	40.3	90.6	80.8	10.4	14.7	19.9	20.2	5.0-19.4
Urine								
Sodium (mEq/d)	210	172	186	180	209	218	125	
Potassium (mEq/d)	93	226	57	38	33	78	32	
Chloride (mEq/d)	211	363	228	154	170	181	65	
Calcium (mg/d)	8	96	63	19	14	17	69	70-180
Plasma pH	7.42	NC	7.50	7.60	7.54	7.47	NC	
Blood pressure (mm Hg)	110/80	120/70	90/60	110/60	110/70	110/70	110/70	

Note: Control patient is the patient with surreptitious vomiting. To convert serum sodium in mEq/L to mmol/L, multiply by 1; potassium in mEq/L to mmol/L, multiply by 1; chloride in mEq/L to mmol/L, multiply by 1; total carbon dioxide in mEq/L to mmol/L, multiply by 1; calcium in mg/dL to mmol/L, multiply by 0.2495; phosphate in mg/dL to mmol/L, multiply by 0.3229; magnesium in mg/dL to mmol/L, multiply by 0.4114; plasma renin activity in ng/mL/h to ng/(Lxs), multiply by 0.2778; plasma aldosterone in ng/dL to nmol/L, multiply by 0.02774.

Abbreviation: NC, not checked.

*Previously reported.[28]

F). p.Ser976Phe, p.Asp848Asn, and p.Ile988Thr were novel mutations for *SLC12A3*. No mutation in the *SLC12A3* gene was detected in the control patient.

Immunohistochemistry

In the normal tissue, NCC immunohistochemistry showed distinct labeling along the apical membrane of the DCT (Fig 1-H1). However,

Table 2. Results of Renal Clearance Study of Subjects

	Patients With Gitelman Syndrome						Control Patient
	A*	B	C	D	E*	F	G
Minimal urine osmolality (mOsm/kg)	71	140	93	77	NA	98	53
Chloride clearance (mL/min/100 mL GFR)							
Basal	0.8	3.0	2.7	2.0	2.3†	2.2	1.0
Furosemide loading	8.6	11.6	19.7	25.2	11.6†	21.8	14.4
Thiazide loading	1.1	8.1	NA	2.6	1.9†	5.4	4.4
Distal fractional chloride reabsorption (%)							
Basal	86.1	48.2	62.6	85.2	65.0	65.7	85.7
Furosemide loading	9.7	14.7	6.5	35.3	14.0	21.7	10.8
Thiazide loading	81.4	39.4	NA	85.3	68.0	58.4	61.6

Note: Control patient is the patient with surreptitious vomiting. To convert GFR in mL/min to mL/s, multiply by 0.01667.

Abbreviations: GFR, glomerular filtration rate; NA, not available (due to missed data).

*Previously reported.[28]

†Milliliters per minute.

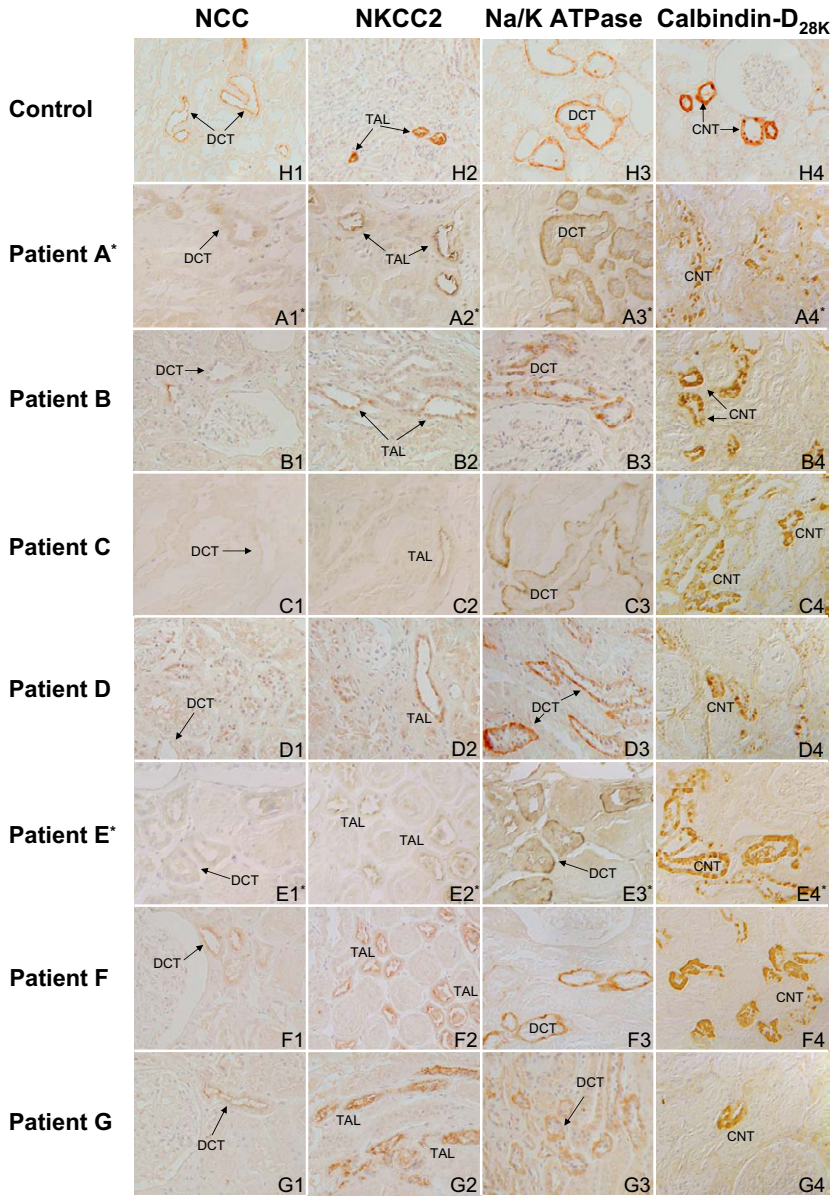

Figure 1. Immunohistochemistry of the thiazide-sensitive sodium-chloride (Na-Cl) cotransporter (NCC), thick ascending limb Na-K-2Cl cotransporter (NKCC2), Na^+, K^+-ATPase α1 subunit, and calbindin-D_{28K} in renal biopsy tissue from subjects and the healthy control. Although NCC immunohistochemistry in the healthy control showed (A1) distinct labeling along the apical membrane of the distal convoluted tubule (DCT), (A1 to F1) results of renal tissues from patients with Gitelman syndrome were devoid of intact NCC immunostaining (see text for details). Conversely, immunostaining for NKCC2 in the cortical thick ascending limb (TAL), Na^+,K^+-ATPase α1 subunit in the DCT, and calbindin-D_{28K} in the connecting tubule (CNT) were intact in both the control and patients. *Previously reported.[20]

renal tissues from patients with GS were devoid of intact NCC immunostaining in the DCT (Fig 1). In patient A, NCC immunostaining was absent in the apical membrane of the DCT and faint staining was observed diffusely in the cytoplasm (Fig 1-A1). In patient F and the patient with surreptitious vomiting (patient G), NCC immunostaining was moderately decreased along the apical membrane of the DCT (Fig 1-F1 and G1). Immunoreactivity to NCC in the apical membrane was equivocal in patients B, D, and E (Fig 1-B1, D1, and E1). In patient C, no discernible NCC immunostaining was found (Fig 1-C1). The pattern of abnormal NCC immunoreactivity in the DCT was not associated with *SLC12A3* genotype. Conversely, immunostaining for NKCC2, Na^+,K^+-ATPase α1 subunit, and calbindin-D_{28K} were relatively well preserved in the respective renal tubular segments (Fig 1).

Immunoblotting

Figure 2 shows urine immunoblot for NCC in healthy volunteers (Fig 2A) and subjects (Fig

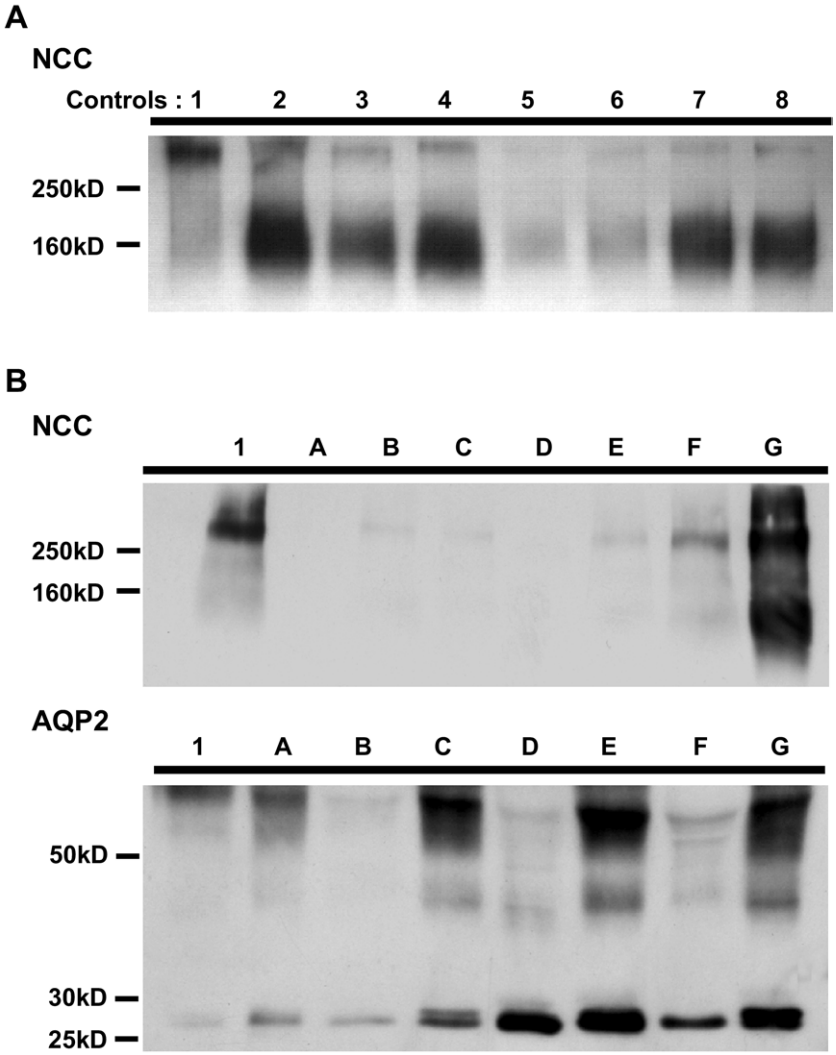

Figure 2. Urine immunoblot for sodium-chloride (NaCl) cotransporter (NCC) and aquaporin 2 (AQP2). (A) Urine from healthy volunteers showed positive NCC signals at 160 kd and higher molecular-weight range when probed with anti-NCC antibody. (B) Urine immunoblot for NCC and AQP2 in urine from subjects and 1 of the healthy volunteers who served as a positive control. Urine samples from patients A and D were negative for NCC, whereas patients B, C, E, and F showed weak to moderate NCC signals. Note the strong NCC signal in subject G (patient with surreptitious vomiting; see text for details). For AQP2, 29-kd and higher molecular-weight bands are seen clearly in all subjects, suggesting no abnormality in AQP2 expression in the collecting duct.

2B). Although the pattern was not uniform, lower and higher molecular-weight band signals were observed distinctly in healthy volunteers. These bands were considered to represent NCC signals, and the higher molecular-weight bands were believed to be protein complexes.[13,14] No NCC band signal was detectable in urine samples from patients A and D. The density of NCC bands observed in patients B, C, E, and F was markedly decreased in comparison to that of the healthy control. NCC band density in the control patient with surreptitious vomiting was much more intense than that of healthy volunteers. Although differences in band densities were seen among samples, urine AQP2 signal was clearly observed in all subjects (Fig 2B).

DISCUSSION

We described NCC expression in renal tissues and urinary NCC excretion in 6 patients with GS and 1 patient with surreptitious vomiting. NCC expression in the DCT was markedly decreased, and urinary NCC excretion was also markedly decreased in patients with GS.

There is increasing evidence for a phenotypic overlap between GS and Bartter syndrome[8,10] and for relatively common phenotypic variations in patients with GS.[9,17,18] These variations could be explained in part by differences in expression and function of NCC protein according to the underlying mutations.[4,12,19,20] Six different sequence variations in *SLC12A3* were detected in 6

patients (patients A to F). All 6 sequence variants were not detected in 75 control Korean subjects (150 alleles), except for p.Thr180Lys, which was detected in 1 control subject. We could not examine the frequencies of these variants in family members of probands.

Because we could not test functional effects of the 4 missense mutants by using a heterologous expression system, we analyzed biochemical effects of the 4 mutations by using 2 computer programs, SIFT and PolyPhen.[21,22] Both the SIFT and Polyphen programs suggested that p.Ser976Phe may affect protein function. The Polyphen program predicts both p.Asp848Asn and p.Ile988Thr to be "possibly damaging," whereas the SIFT program predicts this substitution would be tolerated by the NCC protein. On the basis of the available information, we cannot rule out the possibility that these variants contribute to disease susceptibility. A p.Thr180Lys substitution was reported first as a possibly pathogenic mutation.[7] However, a later functional study showed that this variation was a polymorphism.[23] Considering that the patient with this mutation (patient B) had clinical and biochemical features compatible with GS, it is possible that we did not find mutations that could not be detected by using current mutation analysis methods. The intronic mutation in patient D can cause abnormal splicing and should be pathogenic. However, we cannot predict the possible effect of p.Gln95del on protein function at the moment.

Up to 40% of patients are found to have heterozygous mutations in the *SLC12A3* gene, rather than compound heterozygous or homozygous mutations.[4,24] Only 1 mutant allele was identified in 3 of the 6 patients, compatible with previous reports.[4,24] Several explanations for this deficit of detection are possible. Because of the technical limitation of mutation analysis, only exon and exon-boundary mutations could be detected, and mutations within promoter regions, regulatory 5′-untranslated regions, 3′-polyadenylated tails, and deep introns could not be assessed. Another gene, such as *CLCNKB*, may be involved in GS. Unavailability of mutation analysis of *CLCNKB* is one of the limitations of this study.

There could be 5 mechanisms by which mutations reduce or abolish transporter activity.[4,12,19,20,25,26] A mutation could: (1) impair protein synthesis,[25] (2) impair protein processing,[12,20] (3) interfere with insertion of an otherwise functional protein into the plasma membrane,[12,19] (4) modify functional properties of the cotransporter,[26] and (5) accelerate protein removal or degradation. All mechanisms except the fifth were already mentioned in GS.

Some missense mutations show partial function, whereas some mutations in *SLC12A3* lead to complete loss of expression and cotransporter activity.[12,20,25] Immunoreactivity to NCC at the apical membrane of the DCT was not observed (patients A and C) or decreased (patients B, D, E, and F). From these immunohistochemistry results, we could speculate that the second or third mechanism might be the cause of decreased NCC expression. Discrepancies between results of urine immunoblot and immunohistochemistry in patients A and C could exclude the possibility of the first mechanism in our patients. However, functional analysis of the expressed mutants using a heterologous expression system should be required to confirm the mechanisms by which mutations regulate transporter activity and/or expression.

Serum magnesium levels were not decreased in 2 of the 6 patients (patients B and C), and 24-hour urinary calcium excretion was not decreased in 1 patient (patient B). Hypocalciuria and hypomagnesemia are not always prerequisite to GS and might change during the life cycle of a given patient.[17,27] Moreover, the 2 patients were being administered potassium and magnesium supplements at the time of study, which could influence results. Except for these biochemical abnormalities, we could not discriminate the phenotypic difference in patients according to mutations or NCC expression in renal tissue. We also could not find a relation between type of mutation and NCC expression in tissues. This result might originate from the limits of this study. First, the number of patients was too small to establish meaningful discrimination. Second, our inclusion criteria were limited to only patients who had typical clinical features of GS. In addition, only 2 of the 6 patients had a homozygous mutation in this study. Patients with a homozygous mutation would be more suitable for investigating the relationships between phenotype and a specific NCC mutation.

The differential diagnosis of GS depends on clinical features of the patient, a detailed account of the patient's history, measurement of serum and urine electrolytes, detection of diuretics in urine, and clearance studies. However, phenotype variability, patient denial of medication history, and the cumbersomeness of a clearance study sometimes make the differential diagnosis confusing. Despite its physiological background, there are no standard protocol and cutoff values for abnormal results in a renal clearance study. Although the patient with surreptitious vomiting showed a different pattern of response to hydrochlorothiazide administration, the difference was not impressive enough to interpret the result confidently. A simple, noninvasive, and reliable diagnostic method is required.

Although it recently was suggested that analysis of urinary membrane fractions might provide noninvasive information about the pathophysiological state of renal tubules,[13,14] no report investigating abnormalities in urinary transporters in a specific patient population was made. Preliminary studies were done to determine the amount of loading samples sufficient for clear NCC bands in healthy volunteers. Two milligrams of urine creatinine equivalent was enough to show NCC bands in all healthy volunteers (data not shown), and the presence of NCC in normal urine was ascertained in urine from healthy volunteers by means of immunoblot (Fig 2A). Bands in the higher molecular-weight range was believed to represent NCC multimers, which might be formed through the formation of multivesicular bodies, subsequent fusion with the apical plasma membrane, and excreted into the urinary space by the process of exocytosis.[14] Urinary NCC excretion was markedly decreased in patients with GS, which discriminates patients from healthy controls and a patient with surreptitious vomiting. Urine AQP2 signals in patients with GS could represent that all patients were in a volume-depleted condition, and at least AQP2 expression is regulated normally in the collecting duct.[16]

Urinary NCC band density in patient G was very intense. Considering that urinary chloride excretion was low, minimal urine osmolality was low, DFCR was decreased after hydrochlorothiazide administration, and no mutation of *SLC12A3* was detected, a diagnosis other than GS was suspected. However, phenotype variability of GS, the patient's denial of vomiting, no reference values for the clearance study, and limitation in the method of mutation analysis made the diagnosis confusing. Urinary NCC was the most distinguishing finding that discriminated this patient from the other subjects.

Results of urine immunoblot were similar to those of immunohistochemistry, but did not exactly represent NCC expression in the DCT. These discrepancies might be caused by: (1) the possibility that analysis of immunohistochemistry on biopsy samples is subject to technical errors and observational variations, and (2) different sensitivities of the 2 tests. We confirmed that urinary NCC excretion was markedly decreased, but the regulatory mechanism for its urinary excretion is yet to be investigated.

In conclusion, NCC protein expression in the luminal surface of the DCT from patients with GS was decreased, and no relation between NCC expression and type of mutation in the *SLC12A3* gene was found. Analysis of urinary NCC might be a promising tool for the differential diagnosis of GS. Additional studies with a large number of more defined patients to evaluate the clinical usefulness of urinary NCC excretion are necessary.

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