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Clinical Severity of Gitelman Syndrome Determined by Serum Magnesium

Lanping Jiang[a] Chen Chen[a, c] Tao Yuan[b] Yan Qin[a] Mingming Hu[b]
Xuemei Li[a] Xiaoping Xing[b] Xuewang Lee[a] Min Nie[b] Limeng Chen[a]

[a]Department of Nephrology and [b]Department of Endocrinology and Key Laboratory of Endocrinology, Ministry of Health, Peking Union Medical College Hospital, Peking Union Medical College, Chinese Academy of Medical Sciences, Beijing, and [c]State Key Laboratory of Medical Genetics, Department of Pediatrics, Xiangya Hospital, Central South University, Changsha, China

Key Words
Gitelman syndrome · Normomagnesemia · Hypomagnesemia · TRPM6

Abstract

Background/Aims: Normomagnesemia is considered atypical in Gitelman syndrome (GS). Here, we describe clinical, pathological and genetic characteristics in Chinese GS patients with or without hypomagnesemia in order to determine whether serum magnesium concentration indicates the severity of the disease. ***Methods:*** 7 normomagnesemic and 25 hypomagnesemic GS patients who were confirmed by direct sequencing of *SLC12A3* gene were included. Clinical manifestation and laboratory tests were documented. Supine and upright plasma renin activity, angiotensin II and aldosterone were determined by radioimmunoassay. Transient receptor potential channel melastatin subtype 6 (TRPM6) was detected by immunohistochemistry in paraffin-embedded renal biopsy sections of 12 GS patients. 14 patients with glomerular minor lesion served as controls. The distribution of the mutations on the predicted NCC protein was analyzed and compared between two subgroups. ***Results:*** Clinical manifestations, electrolyte abnormalities, metabolic alkalosis and renin-angiotensin-aldosterone system activation were found to be milder in normomagnesemic compared with the hypomagnesemic group. Compared with glomerular minor lesion controls, the TRPM6-positive area was significantly decreased in hypomagnesemic patients (4.96 ± 1.88 vs. 8.63 ± 2.67%) while it was near normal (7.82 ± 5.23%) in 2 normomagnesemic GS patients. A higher percentage of intracellular mutations was observed in normomagnesemic patients than hypomagnesemic patients (92.31 vs. 56.52%, p = 0.02). ***Conclusions:*** Normomagnesemia is not rare in GS. Serum magnesium may indicate the severity of GS.

Introduction

Gitelman syndrome (GS) (OMIM 263800) is an autosomal recessive renal tubular salt-wasting disorder characterized by hypokalemic metabolic alkalosis, hypomagnesemia and hypocalciuria, secondary renin-angiotensin-aldosterone system (RAAS) activation with normal blood pressure [1]. In most cases, GS results from loss-of-function mutations in the *SLC12A3* gene which consists of 26

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Limeng Chen and Min Nie
Peking Union Medical College Hospital
No. 1, Shuaifuyan, Wangfujing St
Beijing 100730 (China)
E-Mail chenlimeng@pumch.cn and nm_pumch@aliyun.com

exons and encodes the thiazide-sensitive NaCl co-transporter (NCC) protein (NM_000339.2; OMIM 600968) [2] of the distal convoluted tubule (DCT). A few cases have been reported in whom GS was caused by mutations of the CLCNKB gene encoding the kidney-specific chloride channel ClC-Kb [3, 4]. GS was formerly considered a subset of Bartter syndrome (BS) until the distinct genetic and molecular bases of these disorders were identified, because of some resemblance in clinical presentation, such as hypokalemic metabolic alkalosis, secondary RAAS activation and normotension [5]. Bartter syndrome is also an autosomal recessive hypokalemic metabolic alkalosis, but it derives from a mutation to the NKCC2 found in the thick ascending limb of the loop of Henle.

Urinary calcium excretion and hypomagnesemia of renal origin are typically used as laboratory tests to differentiate between GS and BS [6]. In a study of NCC-deficient mice, reduced expression of transient receptor potential channel melastatin subtype 6 (TRPM6) and early DCT atrophy have been proposed to be responsible for hypomagnesemia [7]. Nevertheless, a few GS cases with normomagnesemia were reported raising the question of the prognostic value of plasma magnesium concentration in GS [8–16]. To our knowledge there has been no systematic description of the differences in clinical, pathological and NCC mutations between normo- and hypomagnesemic GS patients. Thus, the main purpose of the study in 7 normomagnesemic and 25 hypomagnesemic GS patients was to assess whether serum magnesium concentration may be a useful predictor of the severity of their illness.

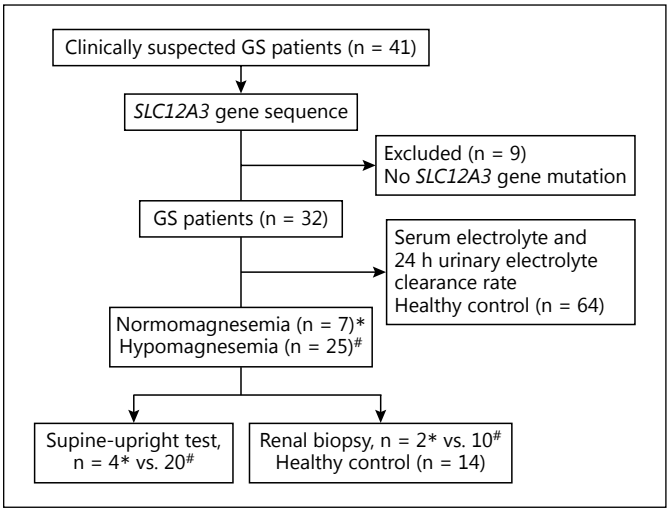

Fig. 1. Flow diagram of investigation of GS patients and healthy controls. DNA analysis was performed in 41 patients suspected of having GS. 32 of them showed mutations in the *SLC12A3* gene and were diagnosed as GS genetically. Among the 32 patients, 7 were defined as normomagnesemic since their minimal serum magnesium level was >0.7 mmol/l. Among these 7 patients, 4 underwent the supine-upright orthostatic test, while 2 received renal biopsy. Among the 25 hypomagnesemic GS patients, 20 were subjected to the supine-upright orthostatic test, while 10 received renal biopsy. Serum electrolyte levels and 24 h urine electrolyte clearance rates were obtained from 64 gender- and age-matched healthy volunteers. 14 isolated hematuria patients whose renal pathology was GML were selected as pathology control. * = Normomagnesemic GS patients; # = hypomagnesemic GS patients.

Subjects and Methods

The study protocol was approved by the Ethics Committee on Human Studies at Peking Union Medical College Hospital, Chinese Academy of Medical Sciences, Beijing, China.

Subjects

In 41 patients with clinical suspicion of GS (with the following clinical presentation: recurrent hypokalemia, metabolic alkalosis, RAAS activation, normotension, with or without hypomagnesemia and hypocalciuria) and complete clinical records, genomic DNA was isolated and purified from peripheral blood between 2004 and 2012 in Peking Union Medical College Hospital (fig. 1). 23 pairs of oligonucleotide primers were generated to amplify all 26 exons and flanking intronic regions of the *SLC12A3* gene according to data obtained from Shao et al. [17] and Fukuyama et al. [12], or designed by primer-5 based on the sequence of *SLC12A3* gene (online suppl. table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000360773). 32 patients from 29 non-consanguineous families were found to have mutated alleles in the *SLC12A3* gene and were therefore diagnosed as GS. In this study we defined normomagnesemia as any recorded serum total magnesium ≥0.7 mmol/l (serum magnesium parameter: 0.70–1.10 mmol/l). Among our 32 patients, minimal serum magnesium was <0.7 mmol/l in 25 patients. Serum magnesium was normal in 7 patients.

Clinical Presentations and Biochemical Characteristics

Before staying in our hospital, all GS patients but one were supplemented with potassium occasionally or regularly. There is 1 patient who had suffered drug-induced deafness at 1 year of age and was unable to express himself well. Hypokalemia was detected in the preoperative workup of cochlear implantation, thus we did not calculate his duration, precipitating factors and symptoms in table 2. Some of the hypomagnesemic GS patients took magnesium as well, but none of the normomagnesemic GS patients were supplemented with magnesium before. The clinical manifestation, serum and urinary biochemical indexes and ECG results were documented. The patients were on unrestricted diet or medicine during their stay in our hospital. The individual clinical data of 7 normomagnesemic GS patients are listed in table 1. Estimated glomerular filtration rate (eGFR) was calculated using the equation of the chronic kidney disease epidemiology collaboration (CKD-EPI) [18]. The average time patients spent in hospital is about 2 weeks. Generally, 24 h urinary electrolyte and simultaneous serum electrolytes were measured once at the outpatient setting, and at least

Table 1. Individual clinical data of 7 normomagnesemic GS patients

Patient	Sex	Onset age, years	Blood pressure, mm Hg	Minimal serum magnesium, 0.7–1.1 mmol/l	Minimal serum potassium, 3.5–5.5 mmol/l	Minimal serum sodium, 135–145 mmol/l	Minimal serum chloride, 96–111 mmol/l	Maximal pH, 7.35–7.45	PCO_2, 35–45 mm Hg	HCO_3^-, 22–26 mmol/l	ABE, –3 to 3 mmol/l	Mutation	Symptoms
1	m	30	120/90	0.81	2	139	96	7.472	38.8	27.6	4.2	R655H (homo)	muscle stiffness or pain, paresthesias, palpitation
2	m	19	106/73	1.05	1.5	134	88	7.520	48.0	36.6	11.2	T60M, R655H (co-homo)	fatigue, dizziness, muscle weakness, carpopedal spasm/ tetany, nocturia, polyuria, thirst
3	f	31	90/60	0.87	2.9	134	97	7.472	32.1	23.1	0.6	T60M (homo)	fatigue, dizziness, muscle weakness, arthralgia, palpitation
4	m	30	112/70	0.91	1.2	135	94	7.498	34.8	26.8	4.2	T60M (homo)	muscle weakness, muscle stiffness/tetany, polyuria, diarrhea, abdominal pain
5*	f	21	98/60	0.74	2.9	135	102	7.573	21.5	19.8	–0.3	Asn566Lys (het)	muscle weakness, palpitation
6	m	27	100/60	0.97	2.9	138	99	7.402	43.3	26.4	1.7	R913Q, c.1670-8c>T co-hetero	muscle weakness, paresthesias
7	m	17	120/78	0.85	1.9	140	102	7.412	38.8	23.9	–0.1	R913Q c.1670-8c>T co-hetero	muscle weakness, nocturia, polyuria, thirst, palpitations

homo = Homozygosity; hetero = heterozygosity; co-hetero = compound heterozygosity; co-homo = compound homozygosity. The serum magnesium, potassium, sodium and chloride levels are the minimal levels in the record, the maximal pH level is also the maximal in the record. * Patient 5 accompanied with recurrent ventricular arrhythmia and hyperventilation syndrome.

once a week during the hospitalization. Reference values for biochemical parameters were from our data on the healthy general population on unrestricted diet. Control results for serum electrolyte and 24 h urinary electrolyte clearance rate were obtained from 64 gender- and age-matched healthy volunteers studied in 2012 in our laboratory. The onset age is defined as the first occurrence of hypokalemia, and the duration is defined as the time span between onset and admittance to our hospital. Polyuria was defined as the average of 24 h urine volume being >3 liters. Nocturia was defined as voiding at least twice during sleeping hours [19].

Supine-Upright Renin-Angiotensin-Aldosterone System Test

A supine-upright RAAS test was carried out in 4 normomagnesemic and 20 hypomagnesemic GS patients (in 1 of them we did not measure supine aldosterone levels) during the stay in our hospital. The protocol of the supine-upright test began after spironolactone was discontinued at least 2 weeks before the test. On the day of the test, patients were asked to lie supine for at least 4 h before collecting the fasting blood sample at 8 a.m. Patients were then asked to keep an upright position for at least 2 h with the second blood sample collected at 10 a.m. Plasma renin activity (PRA), angiotensin II (AngII) and aldosterone were determined by radioimmunoassay. As for a GS patient, RAAS activation was defined as any index of his/her supine or upright PRA, AngII and aldosterone being elevated.

TRPM6 Immunohistochemistry-Paraffin (IHC-P) Staining

Renal biopsies were obtained for assistant diagnostic purposes of 12 GS patients after signing the consent form. TRPM6 was detected by IHC-P in renal biopsy sections of 2 normomagnesemic and 10 hypomagnesemic GS patients. 14 patients with isolated hematuria whose renal pathology results indicated glomerular minor lesion (GML) were selected as controls. IHC-P was done on deparaffinized and rehydrated sections which were heated in a pressure cooker with 0.01 mol/l citrate buffer (pH 6.0) for 12 min for antigen retrieval and incubated with the primary antibody-TRPM6 (Abcam, USA) overnight at 4°C. After incubation with 0.3% H_2O_2 for 15 min, sections were incubated with the 1:500 HRP-conjugated anti-guinea pig IgG (ImmunoReagents, USA) for 1 h at 37°C. 3,3′-Diaminobenzidine (DAB) was used as a staining substrate. All section images were captured by a Nikon microscope (Eclipse 80i; Nikon, Japan) equipped with a digital photo camera (DS-U1; Nikon, Japan). For semiquantitative determination of the ratio of areas stained positive for TRPM6 and the section's total area, images were analyzed by Image-Pro Plus 6.0 (Media Cybernetics, USA), which allowed automatic calculation of the stained area and the section's total area. Staining and scoring were performed blindly on coded slides.

Table 2. Clinical features of GS patients with or without hypomagnesemia

	Normomagnesemic patients (n = 7)	Hypomagnesemic patients (n = 25)	p value
Basic information			
Age, years	29.6±6.7	31.5±13.2	0.6
Male, %	71.4%	72%	0.9
Onset age, years	25.0±5.9	23.1±13.3	0.7
Duration, months	46 (1–83)	84 (3–432)[#]	0.07
Precipitating factors			
Upper respiratory infection, %	2/7	6/24[#]	0.9
Physical activity, %	2/7	6/24	0.9
Symptom			
Fatigue	2/7	11/24	0.4
Dizziness	2/7	2/24	0.3
Muscle weakness, %	6/7	19/24	0.7
Carpopedal spasm/tetany, %	1/7	13/24	0.03
Muscle stiffness or pain, %	2/7	7/24	0.9
Arthralgia, %	1/7	3/24	0.9
Nocturia, %	2/7	12/24	0.3
Polyuria, %	2/7	4/24	0.5
Thirst, %	1/7	5/24	0.7
Abdominal pain, %	1/7	4/24	0.9
Diarrhea	1/7	4/24	0.9
Paresthesias	2/7	10/24	0.6
Palpitations, %	4/7	8/24	0.3
Others			
Albumin, g/l	44.4±3.7	45.4±3.0	0.5
SBP, mm Hg	100.9±21.1	107.6±9.0	0.4
DBP, mm Hg	75.9±14.9	70.3±7.8	0.4
BMI, kg/m^2	21.920±3.646	22.455±3.612	0.8
eGFR, ml/min/1.73 m^2	105.81±30.25	107.97±17.85	0.8
QTc, ms	436.4±51.2	451.7±45.1	0.5

SBP = Systolic blood pressure; DBP = diastolic blood pressure; BMI = body mass index; QTc = corrected QT interval. Results are given as positive percentage, mean ± SD or median. [#] The patient who was unable to express himself due to drug-induced deafness was excluded.

TRPM6 and NCC Immunofluorescence Double Staining

TRPM6 and NCC immunofluorescence double staining was done on frozen human kidney biopsy slides using the following protocol: After immersion in iced acetone for 5 min, the slides were blocked by 5% donkey serum for 1 h at room temperature, then incubated with primary antibody mixture (TRPM6-ab47017 and NCC-ab95302; Abcam, USA) overnight at 4°C. Secondary antibodies – DyLight-594-conjugated donkey anti-rabbit IgG (EarthOx, USA) and fluorescein-conjugated AffiniPure donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, USA) – were applied and incubated at 37°C for 1 h, then DAPI counterstaining was performed. The micrographs were taken by confocal laser microscopy (Leica, Germany).

Thiazide Test

The thiazide test was carried out in 5 patients and 20 healthy volunteers using the protocol referred to a previous study [20]: Discontinue spironolactone at least 1 week; stop potassium and magnesium supplementation for 1 day. After an overnight fast, the patient is kept recumbent for 4 h and is invited to drink 10 ml/kg b.w. water in 15 min before the test to facilitate spontaneous voiding, followed by 150 ml every hour until the end of the test. They remain supine throughout the test except for voiding. After two 30-min 'basal' clearances, hydrochlorothiazide (HCT, 50 mg orally) is administered and six additional 30-min clearances are performed. Blood samples are taken after 60 and 240 min, and urine is collected every 30 min by spontaneous voiding and analyzed for magnesium and creatinine. Magnesium excretion is evaluated as fractional excretion (FE; with creatinine as a GFR marker), with the formula: $FE_{Mg} = (U_{Mg}/S_{Mg}) \times (S_{Cr}/U_{Cr}) \times 100\%$, where S_{Cr} = serum creatinine and U_{Cr} = urinary creatinine.

Distribution of SLC12A3 Gene Mutations

SLC12A3 gene encodes for NCC, which is a 12-transmembrane protein. According to the relationship of the NCC protein to the cell membrane, mutation sites can be intracellular, extracellular and transmembranal. We calculated the mutation distribution of our GS patients as well as some previous Asian reports with normomagnesemic GS patients.

Statistical Analysis

Normally distributed variables were expressed as mean ± SD and compared using unpaired t tests between two groups and one-way ANOVA and LSD post hoc test for three groups. Nonparametric variables were expressed as median and compared using Kruskal-Wallis test. Mutation distribution differences were compared by Fisher's exact test. Spearman's correlation test was used to determine the relationship between biomarkers and the minimum of serum magnesium. Differences were considered significant when $p < 0.05$. All statistical analyses were performed with SPSS statistical software 17.0 (SPSS, Inc., Chicago, Ill., USA).

Results

Clinical Presentations and Biochemical Data

Individual clinical data and *SLC12A3* gene mutations of 7 normomagnesemic GS patients are listed in table 1. Reference values of biochemical parameters are also shown in table 1. All patients presented with normotensive hypokalemia. Milder hyponatremia and hypochloremia were found in 2 patients. As shown in table 2, the typical onset of the disease occurs in young adults, with a higher incidence in males. There were no differences between normo- and hypomagnesemic patients, except for carpopedal spasm/tetany which was much more frequently observed in the latter group.

As shown in table 3, compared with control group, GS patients manifested hypokalemia and a lower serum so-

Table 3. Normomagnesemic GS group presents milder electrolyte disorder and metabolic alkalosis than hypomagnesemic group

	Control (n = 64)	Norm GS patients (n = 7)	Hypo GS patients (n = 25)
Serum, mmol/l			
Potassium[a]	4.18±0.38[b, c]	3.36±0.88	3.04±0.38
Sodium[a]	139.9±2.8[c]	139.3±2.2	138.2±2.3
Chloride[a]	103.4±2.5[b, c]	99.8±2.3[d]	95.8±3.2
Magnesium[a]	0.878±0.076[c]	0.882±0.062[d]	0.534±0.157
Calcium	2.356±0.118	2.438±0.075	2.405±0.136
Phosphorus	1.240±0.240	1.175±0.239	1.273±0.197
Urinary electrolyte excretion, mmol/day			
Potassium[a]	47.18±18.89[b, c]	98.41±49.24	112.20±59.71
Sodium[a]	189.6±83.1[b, c]	268.3±92.4	259.8±130.0
Chloride[a]	235.0±100.4[b, c]	339.4±109.1	310.0±139.9
Magnesium[a]	8.633±4.663[c]	8.182±6.571	5.152±1.607
Calcium[a]	4.973±3.226[c]	3.314±2.512[d]	0.758±0.747
Phosphorus	20.56±10.80	19.15±6.15	18.58±11.51
Ca^{2+}/Cr[a]	0.138±0.090[c]	0.092±0.068	0.024±0.019
Arterial blood gas			
pH	–	7.477±0.061	7.474±0.021
HCO_3^-	–	26.31±5.26[e]	29.17±2.22
ABE	–	3.07±4.04[e]	5.42±1.92

Norm GS patients = Normomagnesemic GS patients; Hypo GS patients = hypomagnesemic GS patients; Ca^{2+}/Cr = weight ratio of urinary calcium to creatinine (mg/mg). [a] $p < 0.05$ one-way ANOVA test for three groups; [b] $p < 0.05$ LSD post hoc test for healthy controls vs. normomagnesemic GS patients; [c] $p < 0.05$ LSD post hoc test for healthy controls vs. hypomagnesemic GS patients; [d] $p < 0.05$ LSD post hoc test for normo- vs. hypomagnesemic GS patients; [e] $p < 0.05$ unpaired t test for normo- vs. hypomagnesemic GS patients.

dium and chloride level with a corresponding increase in their urinary excretion; meanwhile, there was higher serum calcium level accompanied by decreased excretion of calcium, while hypomagnesemia along with a reduced magnesium excretion presented in the majority of cases. Furthermore, normomagnesemic patients exhibited higher serum chloride and higher urinary calcium levels than hypomagnesemic patients.

Metabolic alkalosis is another common clinical manifestation of GS patients (table 2). Arterial blood pH was >7.45 in 87.1% of GS patients (normal reference 7.35–7.45). Normomagnesemic patients displayed milder metabolic alkalosis with a significantly lower bicarbonate concentration (HCO_3^-) and actual base excess (ABE) level than hypomagnesemic patients. Additionally, the minimum serum magnesium was positively correlated with urinary calcium clearance rate (data not shown).

Supine-Upright RAAS Test

Significant activation of RAAS was observed in GS patients even when serum potassium levels were close to normal. 19 out of 24 GS patients (79.17%) exhibited upright AngII activation, while 5 out of 24 GS patients (20.83%) showed an upright PRA increase. An increase of plasma aldosterone with upright posture was seen in 8 out of 24 GS patients (33.33%). A higher percentage of RAAS activation was found in the hypomagnesemia group (100%) than the normomagnesemic group (50%). Supine and upright PRA were significantly lower in the normomagnesemic group than the hypomagnesemic group (fig. 2). No significant differences of AngII and aldosterone level were observed between the two groups.

TRPM6 Expression Level

The expression of TRPM6 in renal biopsy sections is shown in figure 3. TRPM6 staining was much weaker and less extensive in the hypomagnesemic group than in controls, while there was no significant difference between normomagnesemic GS patient and controls. A semiquantitative analysis of the percentage of TRPM6-positive areas revealed a reduction of TRPM6 staining in hypomagnesemic GS patients compared to GML controls (4.96 ± 1.88 vs. 8.63 ± 2.67%, $p = 0.001$). In 2 normomagnesemic GS patients the percentage of the TRPM6-positive area was comparable to GML controls (7.82 ± 5.23%).

Mutation Analysis of SLC12A3 Gene

We detected 30 *SLC12A3* gene mutations in 32 GS patients, including 23 missense mutations, 4 frameshift mutations, 1 nonsense mutation, and 2 already known disease-causing intronic mutations [21]. All of the mutations found in normomagnesemic GS patients are missense mutations except for one intronic mutation.

The extronic mutation distribution of hypomagnesemia is significantly different from the normomagnesemic group (table 4). In our study, 7 normomagnesemia patients carried 13 mutated alleles of which 12 were intracellular, 1 transmembranal, and none extracellular. In contrast, 25 hypomagnesemia patients harbored 46 mutated alleles with 26 distributed intracellularly, 15 located within transmembrane regions, and 5 extracellularly. Thus, a significantly higher percentage of intracellular mutations was observed in normomagnesemic patients than hypomagnesemic patients (92.31 vs. 56.52%, $p = 0.02$).

Furthermore, the other studies of Asian normomagnesemic GS patients were enrolled, their mutation distributions of *SLC12A3* gene on the predicted protein were cal-

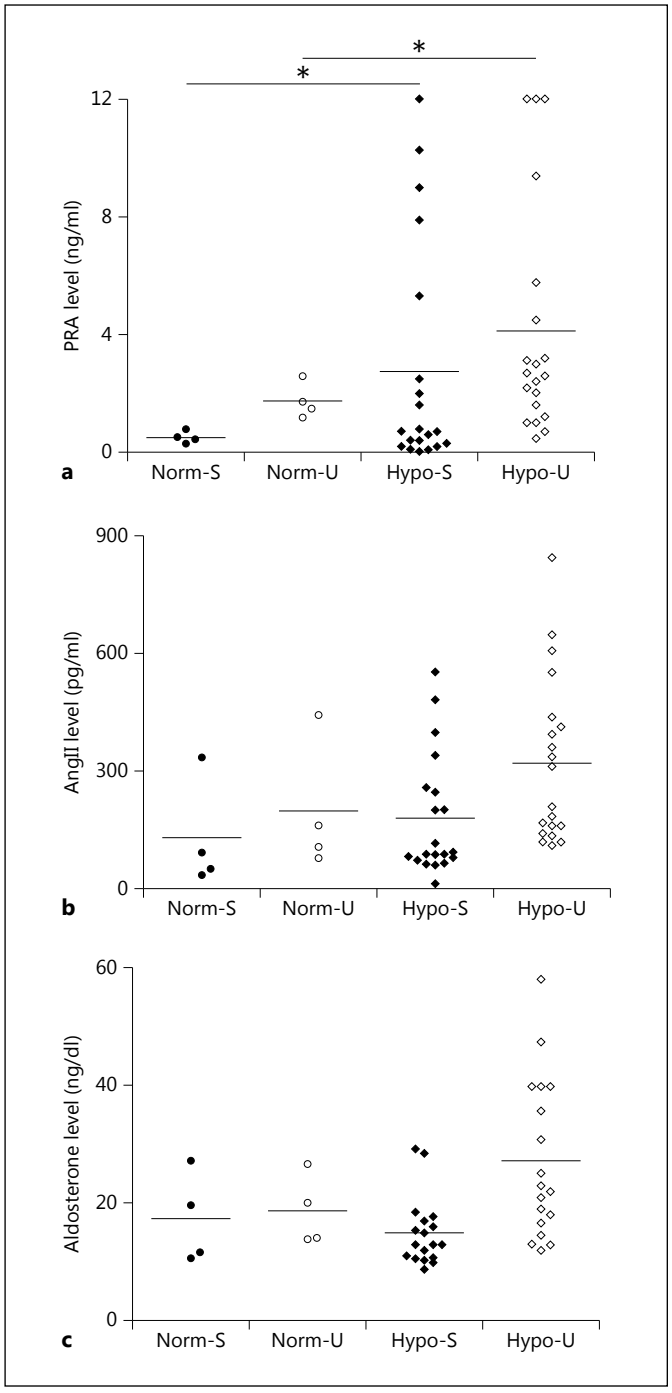

Fig. 2. Postural RAAS activation in normo- vs. hypomagnesemic GS patients. The supine-upright orthostatic test was performed in 4 normomagnesemic and 20 hypomagnesemic GS patients. **a** Supine and upright PRA of the normomagnesemic group is much lower than the hypomagnesemic group. **b**, **c** No statistically significant difference was observed within or between two subgroups in AngII and aldosterone levels. * $p < 0.05$. Norm-S = Supine of normomagnesemic GS patients; Norm-U = upright of normomagnesemic GS patients; Hypo-S = supine of hypomagnesemic GS patients; Hypo-U = upright of hypomagnesemic GS patients.

culated. 33 mutated alleles were found in 18 normomagnesemic GS patients, 29 of these 33 alleles were distributed intracellularly. 100 mutated alleles were detected 54 hypomagnesemic patients, 67 of 100 alleles were distributed intracellularly. The percentage of intracellular mutations of normomagnesemic GS patients is also significantly higher than hypomagnesemic patients (87.88 vs. 67%, $p = 0.02$).

Discussion

The symptoms of GS, first reported by Gitelman et al. [22] in 1966, were thought to typically include hypokalemia, metabolic alkalosis, hypomagnesemia, and hypocalciuria. However, there are some previous reports in which normomagnesemia has been observed in GS patients [8–16]. In the present study, we show that (1) normomagnesemia is not rare in GS, and that GS patients with normal magnesium exhibit milder clinical manifestations than hypomagnesemic patients; (2) a reduction of TRPM6 expressions was found in renal biopsy sections of hypomagnesemic GS patients, and (3) in normomagnesemic GS patients, a greater number of mutations are found in the intracellular regions of the protein than in hypomagnesemic patients.

Tosi et al. [10] have reported that serum-ionized magnesium may reduce in GS patients despite normal total magnesium. Nevertheless, whether total magnesium and ionized magnesium can be dissociated was controversial [23]. In our patient cohort, normomagnesemic GS patients manifested a lower frequency of carpopedal spasm or tetany, and milder electrolyte disorders, metabolic alkalosis and RAAS activation than hypomagnesemic patients, results that are in agreement with a previous publication [9]. Besides, carpopedal spasm or tetany was believed to attribute to hypomagnesemia [24]. It is believed that inactivating mutations of NCC impair NaCl reabsorption in the DCT resulting in increases of sodium delivery to the collecting duct and of urinary Na excretion. The mild volume depletion is presumably the cause of the stimulation of the renin-angiotensin-aldosterone pathway [25]. Raised aldosterone levels increase sodium reabsorption in the cortical collecting duct via the epithelial sodium channel [26] and maintain salt homeostasis at the expense of an increased secretion of potassium and hydrogen ions, resulting in an attendant hypokalemia with metabolic alkalosis [27]. In this study, 2/4 normomagnesemic and 20/20 hypomagnesemic GS patients exhibited RAAS activation. The lower renin levels observed

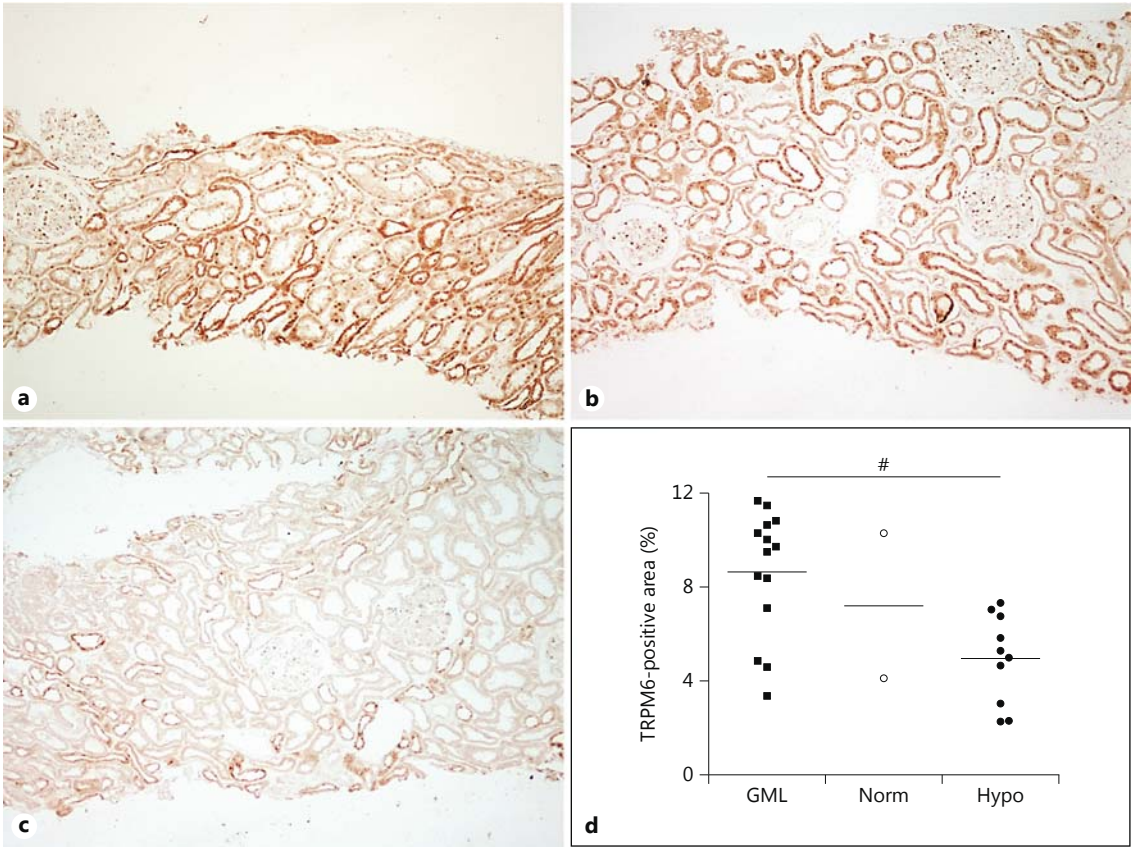

Fig. 3. TRPM6 expression in kidney biopsies of GS patients and controls. **a–c** TRPM6 staining in a GML patient (**a**), normomagnesemic GS patient (**b**), and hypomagnesemic GS patient (**c**). **d** Semiquantitative analysis of the percentage of TRPM6-positive areas indicates that hypomagnesemic GS patients had significantly less TRPM6 expression than GML patients. # p = 0.001. Magnification × 100.

Table 4. Gender and location of *SLC12A3* mutants on predicted protein in Asian GS patients with or without hypomagnesemia

Country, reference	Gender				Mutation location			
	norm-M	norm-F	hypo-M	hypo-F	norm-I	norm-O	hypo-I	hypo-O
Japan [7]	2	0	2	1	4	0	4	1
Korea [10]	1	0	0	1	0	2	0	1
Japan [11]	0	2	2	2	4	0	7	1
Japan [12]	1	1	4	1	2	1	7	3
China [13]	3	1	12	4	7	0	23	7
China, this study	5	2	18	7	12	1	26	20
Total	12	6	38	16	29	4	67	33

norm-M = Male normomagnesemic GS patients; norm-F = female normomagnesemic GS patients; hypo-M = male hypomagnesemic GS patients; hypo-F = female hypomagnesemic GS patients; norm-I = normomagnesemic GS patients' mutations distributed intracellularly; norm-O = normomagnesemic GS patients' mutations distributed transmembranally or extracellularly; hypo-I = hypomagnesemic GS patients' mutations distributed intracellularly; hypo-O = hypomagnesemic GS patients' mutations distributed transmembranally or extracellularly.

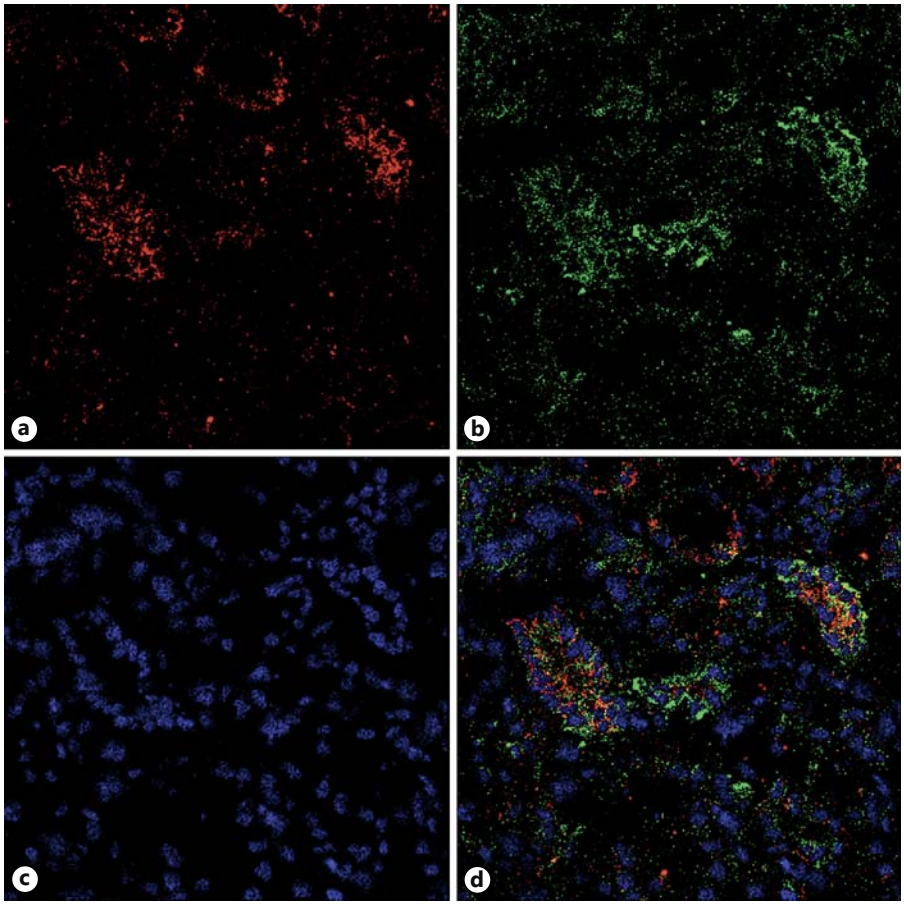

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Fig. 4. NCC and TRPM6 co-localize in frozen human kidney biopsy slide, confocal microscopy. Immunolabelling of TRPM6, NCC and DAPI in renal medulla: (**a**) red = NCC, (**b**) green = TRPM6, (**c**) blue = nuclear (DAPI), and (**d**) overlapped (TRPM6-ab47017 and NCC-ab95302; Abcam, USA).

in the normomagnesemic GS group may explain the less extent of metabolic alkalosis of normomagnesemic GS patients.

Since the clinical manifestation differences between the two groups were related to serum magnesium, we assume that the normo- and hypomagnesemic variants of GS reflect differences in the determinants of magnesium handling. Magnesium is the second-most abundant intracellular cation and plays an essential role in a wide variety of biological activities, such as co-factor in many enzymatic reactions. Serum magnesium concentration (0.70–1.10 mmol/l) is regulated principally by renal excretion which tightly matches intestinal absorption. Although the DCT only reabsorbs 5–10% [28] of the filtered magnesium through mediation of TRPM6, it is the last segment to absorb this ion and therefore it determines the final urinary magnesium concentration. It has been suggested recently that loss of TRPM6 may explain the magnesium abnormality of GS patients [8, 15]. Chronic administration of HCT and NCC gene deletion was associated with a reduction in TRPM6 expression that is perhaps related to the atrophy and shortening of the early DCT found in NCC-deficient mice [29]. Based on extensive studies, it has been suggested that TRPM6 downregulation might represent a general mechanism involved in the pathogenesis of hypomagnesemia accompanying NCC dysfunction [7]. The results of the present study confirm that TRPM6 expression was markedly reduced in renal sections of 10 hypomagnesemic GS patients while TRPM6 expression in 2 normomagnesemic GS patients was comparable with controls. The TRPM6 antibody used in this study has been shown to be specific in previous experiments in human tissue [26]. Furthermore, double staining with NCC and TRPM6 antibodies on frozen human kidney biopsy slides showed co-localization of these two proteins (fig. 4). Progressive loss of TRPM6 during NCC dysfunction over a prolonged time period could explain why GS patients begin to have symptoms including hypomagnesemia only in adolescence or adulthood [30]. In this study, the duration of the disease in normomagnesemic GS patients

tended to be shorter than in hypomagnesemic patients although the difference did not quite reach the 5% significance level ($p = 0.07$; table 1). Besides, in our study of 5 GS patients and 20 healthy controls who carried out the thiazide test as the protocol reported by Colussi et al. [20], we observed that fractional excretion of magnesium (FEMg) was significantly increased (2.4 times of baseline) in healthy controls after orally administrated 50 mg HCT, while the GS patients' FEMg was unchanged (data not shown). The different reaction to HCT between GS patients and healthy controls revealed that NCC dysfunction hampered magnesium reabsorption.

Different domains of the NCC protein have been found to carry different functions. For example, phosphorylation of conserved threonine residues at the N-terminus of NCC can result in trafficking of NCC to the cell membrane and increased co-transporter activity [31]. A single residue in transmembrane domain 11 – S575 – was found to be mainly responsible for the different metolazone affinities between rat and flounder NCC protein [32]. In this study, the percentage of mutated alleles distributed intracellularly was greater in normo- than hypomagnesemic patients. In table 4 we summarized the location of *SLC12A3* mutations on the predicted protein in another group of Asian GS patients with or without normomagnesemia and where a similar phenomenon (87.88 vs. 67%, $p = 0.02$) was observed [8, 11–14]. Therefore, we speculate that the mutational distribution might influence the extent of NCC protein dysfunction and the TRPM6 loss, and could manifest itself in serum magnesium level. To our knowledge, there is no systematic study of the relationship between the distribution of NCC mutations and serum magnesium levels. Further studies of the structure-function relationship of NCC are needed to predict the effect of defined gene mutations.

Previous results have suggested that gender may influence the serum magnesium level of GS patients. Lin et al. [15] reported two families with molecularly proven GS in which male patients were normomagnesemic whereas female patients with the same mutations were hypomagnesemic. However, in the present study and in other Asian patients, no significant difference between genders were observed among GS patients with or without hypomagnesemia (table 4), indicating that gender may not be a universal determinant of magnesium levels in GS.

In summary, we conclude that hypomagnesemia is not always reliable in the diagnosis of GS. Furthermore, serum magnesium may be an indicator of the severity of the disease in GS patients. TRPM6 loss might be responsible for the hypomagnesemia of GS patients. Finally, our data raise the possibility that the molecular distribution of the NCC mutations might influence serum magnesium levels, a suggestion that might be investigated in further studies.

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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