Original Article

Functional Confirmation of Gitelman's Syndrome Mutations in Japanese

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Gitelman's syndrome is an autosomal recessive inherited renal tubular disorder resulting from loss-of-function mutations in the thiazide-sensitive sodium chloride cotransporter gene (*SLC12A3*). We have previously reported that the combined allele frequency for the reported Gitelman's syndrome mutations is 0.0321. However, almost all of the reported Gitelman's syndrome mutations were from case reports without functional confirmation. In the present study, we assessed the functionality of the two most prevalent mutations in Japanese, T180K and L849H, using a mammalian cell expression system. Human *SLC12A3* cDNA was transiently expressed in Chinese hamster ovary (CHO) cells under the control of a cytomegalo virus (CMV) promoter. The T180K and L849H mutations were introduced by site-directed mutagenesis. The activity of the Na^+-Cl^- cotransporter was assessed by measuring tracer $^{22}Na^+$ uptake. While the T180K variation was just a polymorphism, the L849H mutation was confirmed to be a loss-of-function mutation and appears to be responsible for the Gitelman's syndrome. This observation may have very important clinical implications, since the allele frequency of this variation is 0.0126. (*Hypertens Res* 2005; 28: 805–809)

***Key Words*: *SLC12A3*, Gitelman's syndrome, blood pressure**

Introduction

Gitelman's syndrome (GS) is an autosomal recessive inherited renal tubular disorder resulting from loss-of-function mutations in the thiazide-sensitive sodium chloride cotransporter gene (*SLC12A3*) (*1*). The clinical features of GS resemble the effects of thiazide administration, and the reduced salt reabsorption seen in GS seems to be associated with resistance to hypertension (*2*).

In the previous study, we established a TaqMan system for 9 GS mutations reported in Japanese (T180K, A569V, L623P, R642C, L849H, A242V, F846X, R955Q, and an 18-bp insertion at 794–811), and investigated the frequencies of these mutations in the Suita Study. We found 56 subjects heterozygous with the 180K allele, 14 subjects heterozygous with the 569V allele, 1 subject with the 623P allele, 1 subject with the 642C allele, 47 subjects with the 849H allele, and 1 compound heterozygous subject with the 180K and 849H alleles, in total of 1,852 subjects (*3*). Thus, the combined allele frequency of the GS mutations was 0.0321. However, identification of these mutations is all based on case reports; functional analyses of the mutations have never been performed. The allele frequencies of the T180K and L849H mutations are 0.0151 and 0.0126, respectively, and these were the two most frequent mutations in our study population. The purpose of the present study was to determine whether these two GS mutations were really loss-of-function mutations. We also investigated phenotypes of subjects heterozygous with these GS mutations.

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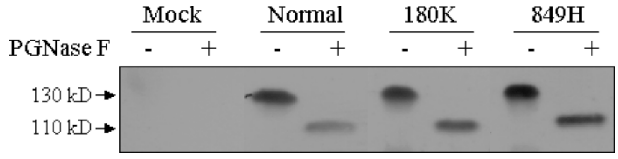

Fig. 1. *Western blot analysis of expressed mutants. Membrane fractions from Chinese hamster ovary cells transfected with empty (Mock), wild-type SLC12A3 (Normal), or mutant (180K and 849H) vector were treated with* N*-glycosidase F (PGNase F) and subjected to Western blotting with polyclonal antibodies that recognize the V5 tag as described in the Methods section.*

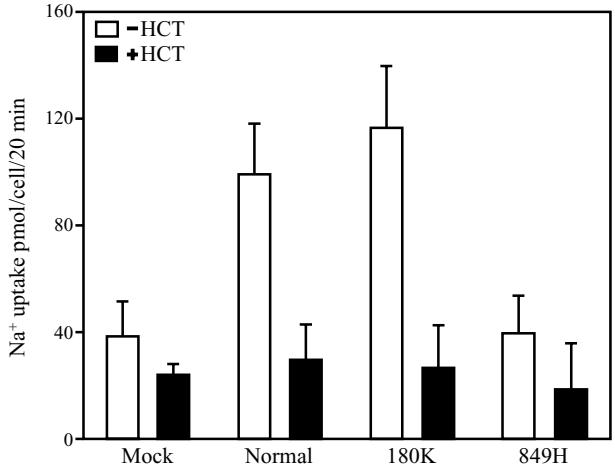

Fig. 2. *Thiazide-sensitive $^{22}Na^+$ uptake in Chinese hamster ovary cells (CHO). CHO cells were transiently transfected with empty (Mock), wild-type SLC12A3 (Normal), or mutant (180K and 849H) vector and analyzed for $^{22}Na^+$ uptake 36 h after transfection as described in the Methods section. Uptake was assessed in the absence (white bars) or presence (solid bars) of 100 μmol/l hydrochlorothiazide (HCT) in the uptake medium. Each bar represents the mean±SEM of four wells from three different experiments (*$n=12$*). The mean value of the $^{22}Na^+$ uptake in the presence of HCT was considered as a background value, and was subtracted from each value in the absence of HCT. One way ANOVA indicated that the values of the four groups were significantly different (*$p<0.0001$*). Subsequent Tukey-Kramer's HSD test indicated that the values of the 849H allele were significantly different from those of the normal allele (*$p<0.01$*).*

Methods

Construction of the SLC12A3 Expression Vector

Human *SLC12A3* cDNA was synthesized by reverse transcription–polymerase chain reaction (RT-PCR) amplification from a human kidney and was subcloned into the pcDNA-3.1/V5-His vector (Invitrogen, Carlsbad, USA). The V5-His epitope was fused to the carboxyl terminal of the human *SLC12A3* cDNA. The T180K and L849H mutations were introduced by using a Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, USA).

Cell Culture and Transient Transfection

Chinese hamster ovary (CHO) cells were grown in Ham's F-12 medium supplemented with 10% fetal calf serum, 100 unit/ml of penicillin, and 100 μg/ml streptomycin. Cells were transiently transfected with 1 μg of *SLC12A3* expression plasmids and 0.1 μg of a green fluorescent protein expression vector, phMGFP vector (Promega, Madison, USA), using Lipofectamine^{2000} (Invitrogen) according to the manufacturer's recommendations. Transgene expression was normalized from the fluorescence intensity of the green fluorescent protein.

$^{22}Na^+$ Uptake Assay

Functional analysis of the Na^+-Cl^- cotransporter was assessed by measuring tracer $^{22}Na^+$ uptake as described by De Jong *et al*. (*4*). At 36 h after transfection, cells were transferred to Cl^--free medium containing 5 mmol/l HEPES/Tris (pH 7.4), 96 mmol/l sodium gluconate, 2 mmol/l potassium gluconate, 1.8 mmol/l calcium gluconate, 1 mmol/l $Mg(NO_3)_2$, 2.5 mmol/l sodium pyruvate, and 5 μg/ml gentamycin and incubated for 3 h. Cells depleted of Cl^- were transferred to uptake medium containing 20 mmol/l HEPES/Tris (pH 7.4), 72 mmol/l *N*-methyl-D-glucosamine-HCl, 48 mmol/l NaCl, 5 mmol/l KCl, 2 mmol/l $Na_2H_2PO_4$, 1 mmol/l $CaCl_2$, 1 mmol/l $MgSO_4$, 0.5 mmol/l ouabain, 100 μmol/l amiloride, 100 μmol/l bumetanide, and 1 μCi/ml of $^{22}Na^+$ and incubated for 20 min at room temperature with or without 100 μmol/l hydrochlorothiazide. Under these conditions, $^{22}Na^+$ uptake increased nearly linearly between 5 and 30 min of incubation time. The uptake reaction was stopped by washing the cells six times with ice-cold uptake medium. After the cells were dissolved in 10% sodium dodecyl sulfate, tracer radioactivity was determined by a γ counter.

Immunoblotting

The CHO cells were harvested, suspended, and broken by sonication in ice-cold buffer (0.32 mol/l sucrose, 5 mmol/l Tris-HCl, pH 7.5, and 2 mmol/l EDTA). The cell lysate was centrifuged at $3,000 \times g$ for 10 min. Then the supernatant was centrifuged at $100,000 \times g$ for 1 h. The pellet was resuspended in buffer containing 5 mmol/l Tris-HCl, pH 7.5, and 2 mmol/l EDTA and used as the membrane fraction. The membrane fraction was subjected to digestion with 0.25 unit of *N*-glycosidase F (Roche Molecular Biochemicals, Mannheim,

Table 1. Characteristics According to Genotypes

	T180K			L849H		
	TT	TK	*p*	LL	LH	*p*
N	3,510	106		3,534	82	
Age	64.7±11.3	65.2±10.4	ns	64.7±11.3	62.6±10.6	0.09
BMI (kg/m^2)	22.8±3.1	22.7±3.0	ns	22.8±3.1	22.5±3.1	ns
SBP (mmHg)	129.3±19.7	132.1±19.6	ns	129.4±19.6	128.2±18.2	ns
DBP (mmHg)	77.7±10.2	77.6±10.1	ns	77.8±10.2	77.2±9.7	ns
ResSBP (mmHg)	−0.1±19.4	2.4±17.6	ns	0.0±17.6	0.6±16.8	ns
ResDBP (mmHg)	0.0±9.7	0.1±10.0	ns	0.0±9.7	−0.3±9.2	ns
HTN (%)	29.7	32.1	ns	29.9	29.3	ns
K (mmol/l)	4.3±0.3	4.3±0.3	ns	4.3±0.3	4.2±0.2	0.03
Mg (mg/dl)	2.5±0.4	2.5±0.4	ns	2.5±0.4	2.5±0.4	ns
U-pH	6.0±0.8	6.1±0.9	ns	6.0±0.8	6.2±0.9	0.06

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; ResSBP, residuals of SBP adjusting for age and BMI; ResDBP, residuals of DBP adjusting for age and BMI; HTN, prevalence of hypertension; K, serum potassium level; Mg, serum Mg level; U-pH, urine pH. Values are expressed as mean±SD.

Germany) for 12 h at 37°C. The reaction was terminated with 6 mmol/l Tris-HCl (pH 6.8), 1% SDS, 1 mmol/l DTT, and boiling for 3 min. Samples were separated by SDS-PAGE and electroblotted onto a PVDF membrane. The protein blot was blocked with 5% nonfat dry milk in Tris-buffered saline (10 mmol/l Tris-HCl, pH 7.5, 150 mmol/l NaCl) at room temperature for 2 h. The blots were then probed with anti-V5 antibody (Invitrogen) diluted 1:2,000 in Tris-buffered saline for 2 h at room temperature, washed five times with Tris-buffered saline, probed with horseradish peroxidase-conjugated secondary antibody in blocking buffer, and washed as before. The signals were detected using an enhanced chemiluminescence Western blotting kit (Amersham Biosciences, Uppsala, Sweden).

Study Population

The selection criteria and design of the Suita Study have been reported previously (*5*–*8*). The genotypes of the T180K and L849H mutations were determined by the TaqMan™ method in 3616 consecutive subjects enrolled between April 2002 and March 2004, who gave their informed consent for genetic analysis. The ethics committee of the National Cardiovascular Center approved the study protocol.

Statistical Analysis

Values are expressed as the mean±SD or the mean±SEM. All statistical analyses were performed with the JMP statistical package (SAS Institute, Inc., Cary, USA). Residuals of blood pressure values were calculated by adjusting for age and body mass index (BMI). Residuals represent the difference between the actual blood pressure value for each observation and the value predicted on the basis of age and BMI. For the $^{22}Na^+$ uptake assay, one way ANOVA followed by Tukey-Kramer's HSD test ($p<0.01$) was applied.

Results

Expression and Maturation of SLC12A3 in Transiently Transfected CHO Cells

To assess the expression of wild-type and mutant SLC12A3 protein transfected in CHO cells, membrane fractions were subjected to Western blotting with polyclonal antibodies that recognize the V5 tag. Bands of approximately 130 kD appeared in the wild-type and the 180K and 849H mutants (Fig. 1). The SLC12A3 cotransporter is a glycoprotein. The *N*-linked glycosylation of membrane proteins may play a role in protein folding and membrane targeting (*9*). The effect of glycosylation on the functional activity of SLC12A3 has been described previously (*10*). For determination of the maturation of SLC12A3, membrane fractions were treated with *N*-glycosidase F (PGNase F), which cleaves all types of *N*-glycans. Bands of 130 kD nearly disappeared after treatment with PGNase F, and digested proteins with a molecular size of approximately 100 kD migrated (Fig. 1). These data demonstrate that the wild-type and mutant SLC12A3 cotransporters are expressed as glycosylated proteins on the cell surface of CHO cells.

Thiazide-Sensitive $^{22}Na^+$ Uptake in Transiently Transfected CHO Cells

The functionality of the SLC12A3 cotransporter expressed in CHO cells was determined by measuring thiazide-sensitive $^{22}Na^+$ uptake. Figure 2 shows that $^{22}Na^+$ uptake was significantly increased in cells expressing the wild-type SLC12A3 cotransporter as compared with the corresponding mock-transfected cells. Importantly, hydrochlorothiazide (100

μmol/l) reduced $^{22}Na^{+}$ uptake to the levels observed in mock-transfected cells. Then, we investigated the $^{22}Na^{+}$ uptake of the mutant SLC12A3 proteins. As shown in Fig. 2, the $^{22}Na^{+}$ uptake of the 180K mutant SLC12A3 protein was not decreased as compared with that of the normal type. However, the $^{22}Na^{+}$ uptake of the 849H mutant SLC12A3 protein was significantly reduced ($p<0.01$, one-way ANOVA followed by Tukey-Kramer's HSD test, compared to the normal allele SLC12A3 protein) to almost none, although the mutant protein was expressed at the cell surface (Fig. 1). Thus, it was confirmed that the L849H mutation is a loss-of-function mutation.

Characteristics of Heterozygous Subjects with the L849H Allele

Once the L849H mutation was confirmed as a loss-of-function mutation, we reassessed the characteristics of subjects with the L849H allele. Of the 3,616 subjects enrolled in this study, 82 subjects were heterozygous with the L849H allele. The allele frequency is 0.0113. Table 1 shows the characteristics of these heterozygous subjects. The serum potassium levels were slightly lower in the heterozygous subjects, and a tendency of metabolic alkalosis (high urine pH) was also observed in the heterozygous subjects. However, serum magnesium levels and blood pressure levels were not lower in the heterozygous subjects than in the wild-type subjects. Thus, heterozygous subjects with the 849H allele did not seem to exhibit mild GS features.

Discussion

We have previously reported that the combined allele frequency for the reported GS mutations is 0.0321 (*3*). However, almost all of the reported GS mutations were from case reports without functional confirmation (*2*, *3*). It is possible that the reported mutations were simply polymorphisms and the true mutations were in linkage with the polymorphisms in the reported pedigree. Without functional confirmation, we cannot neglect this hypothesis. Thus, in the present study, we assessed the functionality of the two most prevalent mutations, T180K and L849H, which are suspected to be responsible for GS.

Although the CHO cell is not a tubular cell that naturally expresses the SLC12A3 cotransporter, we chose this cell line for its high efficiency in transfection and its lack of intrinsic activity of the cotransporter. Previously, the transporter activity has been assessed using a Xenopus oocyte system. However, the Xenopus oocyte is not a mammalian cell and may have a different protein processing system. As shown in the present study, the activity of the normal type SLC12A3 cotransporter was reliably assessed in the CHO system.

The present study suggests that the T180K variation is simply a polymorphism and may not be responsible for GS. Moreover, the T180K polymorphism resides in the second transmembrane domain, and the polymorphism does not seem to influence the hydropathy plot of the protein (data not shown). There has been only one study in which the T180K polymorphism (mutation) was reported to be responsible for GS (*11*). The affected families were collected at Tokyo or Tochigi (not specified in the paper). An unknown mutation in linkage disequilibrium with this polymorphism may be responsible for GS. This might be confirmed by re-sequencing the entire *SLC12A3* (including the promoter and intronic regions) in the affected families.

On the other hand, the L849H mutation has been revealed to be a loss-of-function mutation and appears to be responsible for GS. Moreover, this mutation has been reported to be responsible for GS in at least three reports (*11*–*13*). Monkawa *et al*. (*11*) reported that 1 of their 6 subjects with GS (from Tokyo and Tochigi) was heterozygous for this mutation, Fukuyama *et al*. (*12*) reported that 1 of their 7 GS subjects (from Okinawa) was heterozygous for it, and Maki *et al*. (*13*) reported that 2 of their 8 GS subjects (from Akita) showed compound heterozygosity for the mutation. This reproducibility further strengthens the hypothesis that the L849H mutation is responsible for GS. The L849H mutation resides in the carboxyl-terminal cytoplasmic region of the SLC12A3 protein. Although the function of this domain remains to be clarified, four GS mutations (2543–2544 delTT, L849H, R955Q, R1008X) have been reported in this domain in Japanese. We have also confirmed that the R1008X mutation is really a loss-of-function mutation (data not shown). Thus, this carboxyl-terminal cytoplasmic domain seems to play important roles in the functions of SLC12A3, although the precise roles remain to be determined (*14*).

The subjects heterozygous with the 849H allele did not seem to exhibit mild GS features. However, it is possible that those heterozygous subjects may mask GS features by compensating for their tubular derangement by increasing their sodium uptake, as reported by Cruz *et al*. (*2*), and may exhibit GS features under a low sodium diet or diuretics administration. This awaits further investigation.

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References

1. Simon DB, Nelson-Williams C, Bia MJ, *et al*: Gitelman's variant of Bartter's syndrome, inherited hypokalaemic alkalosis, is caused by mutations in the thiazide-sensitive Na-Cl cotransporter. *Nat Genet* 1996; **12**: 24–30.
2. Cruz DN, Simon DB, Nelson-Williams C, *et al*: Mutations in the Na-Cl cotransporter reduce blood pressure in human. *Hypertension* 2001; **37**: 1458–1464.
3. Tago N, Kokubo Y, Inamoto N, *et al*: A high prevalence of Gitelman's syndrome mutations in Japanese. *Hypertens Res* 2004; **27**: 327–331.

4. De Jong JC, Willems PH, Goossens M, *et al*: Effects of chemical chaperones on partially retarded NaCl cotransporter mutants associated with Gitelman's syndrome in a mouse cortical collecting duct cell line. *Nephrol Dial Transplant* 2004; **19**: 1069–1076.
5. Mannami T, Baba S, Ogata J: Potential of carotid enlargement as a useful indicator affected by high blood pressure in a large general population of a Japanese city: the Suita study. *Stroke* 2000; **31**: 2958–2965.
6. Ono K, Iwanaga Y, Mannami T, *et al*: Epidemiological evidence of an association between *SLC6A2* gene polymorphism and hypertension. *Hypertens Res* 2003; **26**: 685–689.
7. Iwai N, Katsuya T, Mannami T, *et al*: Association between SAH, an acyl-CoA synthetase gene, and hypertriglyceridemia, obesity, and hypertension. *Circulation* 2002; **105**: 41–47.
8. Shioji K, Kokubo Y, Mannami T, *et al*: Association between hypertension and the α-adducin, β1-adrenoreceptor and G-protein β3 subunit genes in the Japanese population; the Suita Study. *Hypertens Res* 2004; **27**: 31–37.
9. Freeze HH: Human disorders in *N*-glycosylation and animal models. *Biochim Biophys Acta* 2002; **1573**: 388–393.
10. De Jong JC, Van Der Vliet WA, Van Den Heuvel LP, *et al*: Functional expression of mutations in the human NaCl cotransporter: evidence for impaired routing mechanisms in Gitelman's syndrome. *J Am Soc Nephrol* 2002; **13**: 1442–1448.
11. Monkawa T, Kurihara I, Kobayashi K, Hayashi M, Saruta T: Novel mutations in thiazide-sensitive Na-Cl cotransporter gene of patients with Gitelman's syndrome. *J Am Soc Nephrol* 2000; **11**: 65–70.
12. Fukuyama S, Okudaira S, Yamazato S, Yamazato M, Ohta T: Analysis of renal tubular electrolyte transporter genes in seven patients with hypokalemic metabolic alkarosis. *Kidney Int* 2003; **64**: 808–816.
13. Maki N, Komatsuda A, Wakui H, *et al*: Four novel mutations in the thiazide-sensitive Na-Cl co-transporter gene in Japanese patients with Gitelman's syndrome. *Nephrol Dial Transplant* 2004; **19**: 1761–1766.
14. Gamba G: Molecular physiology and pathophysiology of electroneutral cation-chroride cotransporters. *Physiol Rev* 2005; **85**: 423–493.