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ORIGINAL ARTICLE

MUTATIONAL ANALYSIS OF *SLC12A3* GENE IN A JAPANESE GENERAL POPULATION OF NORTHERN JAPAN

Shoji Tsutaya[1, 2)], Kazuhiro Sugimoto[1)], Shigeyuki Nakaji[3)] and Minoru Yasujima[1)]

Abstract Gitelman's syndrome (GS) is considered to be a rare disorder. To assess the prevalence of GS in the Japanese general population and the relationships between mutations of related genes and blood pressure, we performed mutational analysis of the *SLC12A3* gene on 1,567 subjects from Aomori Prefecture in northern Japan. Three GS mutations in *SLC12A3*, identified in the subjects with hypokalemia in a preliminary study were assessed by the TaqMan PCR method. We detected T180K, L849H and R919C missense mutations in 40, 49 and 57 subjects, respectively. The overall frequency of GS mutations was 8.9%. The mutant allele frequency of T180K, L849H and R919C was 1.3, 1.6 and 1.9%, respectively. GS mutant allele frequency in the 1,567 Japanese was more than 4.8%. In addition, subjects with L849H mutation had significantly lower systolic blood pressure when compared to subjects with wild-type *SLC12A3* (121.8 ± 18.4 versus 127.8 ± 18.9 mmHg; $P < 0.05$). In conclusion, the present results suggest that the frequency of GS mutations is unexpectedly high, although GS is considered to be a rare disorder. The results also suggest that loss-of-function in the *SLC12A3* via the L849H mutation reduces blood pressure, thereby contributing to resistance to hypertension.

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Key words: *SLC12A3*; Gitelman's syndrome; genetics; epidemiology; blood pressure.

原　著

北日本の地域住民における *SLC12A3* 遺伝子変異検出頻度について

蔦谷昭司[1, 2)]　杉本一博[1)]　中路重之[3)]　保嶋　実[1)]

抄録　ギテルマン症候群の責任遺伝子である *SLC12A3* 遺伝子変異検出頻度とそれらの変異と血圧との関連性について検討した．青森県の地域住民1,567例を対象とし，T180K，L849H，R919C の 3 変異について TaqMan PCR 法で行った．それらの 3 変異は，以前の研究で低 K 血症を呈した対象者の *SLC12A3* 遺伝子解析により検出した．1,567例中140例(8.9%)で変異が検出され，ホモ型変異が 3 例，コンパウンドヘテロ型変異が 5 例，ヘテロ型変異が132例であった．また，3 変異の変異型アリルの検出頻度の合計は4.8%であったことから，本邦におけるギテルマン症候群の頻度は従来考えられていたより高い可能性が示唆された．L849H では変異アリルを有する群が対照群に比べ収縮期血圧が低値であったことから，*SLC12A3* 機能低下を介して血圧を減少させ高血圧に防御的に働く遺伝的因子と成り得る可能性が示唆された．

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[1)] Department of Laboratory Medicine, Hirosaki University Graduate School of Medicine
[2)] Department of Clinical Laboratory, Hirosaki University Hospital
[3)] Department of Social Medicine, Hirosaki University Graduate School of Medicine
Correspondence: S. Tsutaya
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[1)] 弘前大学大学院医学研究科臨床検査医学講座
[2)] 弘前大学医学部附属病院検査部
[3)] 弘前大学大学院医学研究科社会医学講座
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Introduction

Gitelman's syndrome (GS) is characterized by low blood pressure due to renal sodium wasting, hypokalemia, hypomagnesemia and hypocalciuria. GS is a variant of Bartter's syndrome, and is an inherited autosomal recessive renal tubular disorder. It is caused by inactivating mutations in the solute carrier family 12, member 3 (*SLC12A3*) gene that encodes the thiazide-sensitive Na-Cl cotransporter (TSC)[1]. The TSC is expressed in the distal convoluted tubule, and has been shown to reabsorb sodium chloride. The clinical features of GS are also observed in thiazide diuretic therapy, and reduced salt reabsorption in GS may be associated with resistance to hypertension. GS is relatively frequent, and more than 140 different, putative loss-of-function mutations in the *SLC12A3* gene have been identified to date[2]. The number of reported *SLC12A3* mutations in Japanese patients with GS has recently increased[3-8]. In addition, carriers of mutations causing GS have been reported to show reduced blood pressure[9-11]. These results suggest that heterozygous GS mutations may not be as innocuous as previously thought, and in fact, may contribute to resistance to hypertension. Tago et al.[12] reported that the frequency of GS mutations selected from previous studies conducted in Japan was unexpectedly high in the Japanese general population. In a preliminary study, we performed mutational analysis of the *SLC12A3* gene in 26 Japanese subjects with hypokalemia (serum potassium below 3.5mmol/l), screened from 1,407 subjects in Aomori Prefecture, northern Japan, and we identified three missense mutations, T180K, L849H, and R919C, in 2, 3, and 2 subjects, respectively.

The purpose of the present study was to perform mutational analysis of the *SLC12A3* gene in the Japanese general population of northern Japan, and to investigate the relationships between these mutations and blood pressure.

Subjects and Methods

Mutational analysis was performed in 1,567 subjects in Aomori Prefecture, northern Japan. All subjects participated in the Iwaki Health Promotion Project. This study was conducted after obtaining the approval of the Ethics Committee of Hirosaki University School of Medicine. In addition, before the study began, the objectives and requirements of the study were explained to all subjects and written informed consent was obtained. The characteristics of the subjects analyzed in the present study are summarized in Table 1. Hypertension was defined as systolic blood pressure greater than 140 mmHg and/or diastolic blood pressure greater than 90 mmHg, or receiving anti-hypertensive medication.

Genomic DNA was extracted from the peripheral blood of each subject using commercially available DNA extraction kits (NR-101; KURABO, Osaka, Japan), in accordance with the manufacturer's instructions. We selected three GS mutations, T180K, L849H and R919C, from our previous study in 26 Japanese subjects with hypokalemia (serum potassium below 3.5 mmol/l). Genotyping of these mutations was performed using the Custom Taqman® SNP Genotyping Assay (Applied Biosystems, Foster City, CA, USA) and the TaqMan PCR method.

Table 1 Characteristics of the study population

Number of subjects	1,567
Sex (male/female)	585/982
Age (years)	57.2 ± 14.0
BMI (kg/m^2)	23.1 ± 3.2
SBP (mmHg)	127.6 ± 18.9
DBP (mmHg)	75.3 ± 11.1
HTN (%)	42.0

Data are expressed as mean ± SD. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HTN, prevalence of hypertension.

Table 2 Primers and probes for mutational analysis of the *SLC12A3* gene

Mutation	Primers	Probes
T180K 539C→A	F5'-CAGTCCTGACCTGGATCATCATC-3' R5'-TGGAGAGGCCTGTGATGGA-3'	5'-Vic-TCGGTCACGGTGACC-MGB-3' 5'-Fam-TCGGTCAAGGTGACC-MGB-3'
L849H 2546T→A	F5'-CTCTGTCCTGAGTGTATTCTTGTCA-3' R5'-GCATTTGCTCCACCTCCTCTT-3'	5'-Vic-CTCATTCCCTATCTCCTTGG-MGB-3' 5'-Fam-CATTCCCTATCACCTTGG-MGB-3'
R919C 2755C→T	F5'-ACCAAGAGGTTTGAGGACATGATTG-3' R5'-GCCTCATCCTTGAAGCCATCA-3'	5'-Vic-CAGACGGAAGGGTG-MGB-3' 5'-Fam-TCAGACAGAAGGGTG-MGB-3'

Custom Taqman® SNP Genotyping Assay consisted of the primers and probes that are shown in Table 2. The reaction components for a single 20-μL reaction (using a 96-well plate) included sample genomic DNA, TaqMan® Genotyping Master Mix (Applied Biosystems), Custom TaqMan® SNP Genotyping Assay Mix (Applied Biosystems), and DNase-free water, in accordance with the manufacturer's instructions. PCR thermal cycling conditions were as follows: initial denaturing at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Thermal cycling was performed using a GeneAmp PCR System 9700 (Applied Biosystems). After PCR, plate read-out and allele discrimination were performed using an ABI Prism 7700 Sequence Detection System (Applied Biosystems).

Data are expressed as means ± SD. Age, body mass index (BMI), systolic blood pressure (SBP) and diastolic blood pressure (DBP) were compared between subjects with GS mutations and subjects without GS mutations by unpaired t-test. Differences in the relationship between the *SLC12A3* mutation and hypertension were evaluated by χ^2 test. P values of less than 0.05 were considered to be statistically significant.

Results

Among the 1,567 subjects, we detected T180K, L849H and R919C missense mutations in 40, 49 and 57 subjects, respectively. Three subjects had homozygous mutations; one subjects with L849H and 2 subjects with R919C. Five subjects had compound heterozygous mutations; 2 subjects with T180K and R919C, one subject with T180K and R919C, and 2 subjects with L849H and R919C. One hundred thirty-two subjects had heterozygous mutations, and 1,427 subjects had no mutations (Figure 1). As shown in Table 3, the frequency of T180K genotypes was 97.4% for TT, 2.6% for TK and 0% for KK, respectively. The frequency of L849H genotypes was 96.9% for LL, 3.0% for LH and 0.1% for HH, respectively. The frequency of R919C genotypes was 96.4% for RR, 3.5% for RC and 0.1% for CC, respectively. The overall frequency of GS mutations was 8.9%, and the mutant allele frequencies for T180K, L849H and R919C were 1.3, 1.6 and 1.9%, respectively (Table 3). GS mutant allele frequency in the 1,567 Japanese subjects screened was more than 4.8%.

Furthermore, we investigated the relationships between *SLC12A3* mutations and blood pressure. The phenotypes associated with these mutations are summarized in Table 4. Subjects with L849H mutation had significantly lower systolic blood pressure when compared to subjects with wild-type *SLC12A3* (121.8 ± 18.4 versus 127.8 ± 18.9 mmHg; $P < 0.05$). On the other hand, blood pressure in subjects with GS mutations for T180K or L849H did not significantly differ from subjects without GS mutations (wild-type subjects). In addition, subjects with L849H mutation had significantly lower serum potassium levels than subjects with wild-type *SLC12A3* (3.97

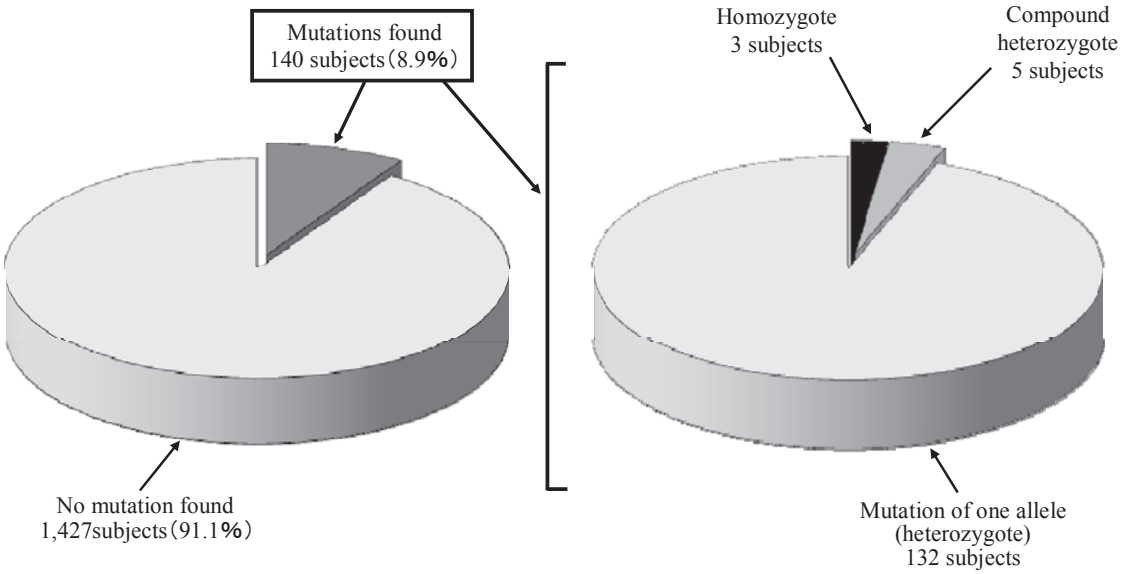

Figure 1 Mutation found in 140 subjects with 1,567 Japanese subjects.
Three subjects had homozygous mutations; one subjects with L849H, two subjects with R919C. Five subjects had compound heterozygous mutations; two subjects with T180K and R919C, one subject with T180K and R919C, and two subjects with L849H and R919C. One hundred thirty-two subjects had heterozygous mutations, and 1,427 subjects had no mutations.

Table 3 Genotype and allele frequencies of the *SLC12A3* mutations

Mutation	Genotypes, n (%)			Alleles, %	
T180K	TT 1,527 (97.4)	TK 40 (2.6)	KK 0 (0.0)	T 98.7	K 1.3
L849H	LL 1,518 (96.9)	LH 48 (3.0)	HH 1 (0.1)	L 98.4	H 1.6
R919C	RR 1,510 (96.4)	RC 55 (3.5)	CC 2 (0.1)	R 98.1	C 1.9

± 0.40 versus 4.11 ± 0.36 mmol/l; $P < 0.05$), and subjects with GS mutations had significantly lower serum potassium levels than subjects with wild-type (4.03 ± 0.39 versus 4.11 ± 0.36 mmol/l; $P < 0.05$). No significant associations were seen between the prevalence of hypertension and *SLC12A3* mutations.

Discussion

In the present study, we clearly found that the frequency of GS mutations is unexpectedly high in some Japanese populations, although GS is considered to be a rare disorder. We selected three GS mutations, T180K, L849H and R919C, from a preliminary study in 26 Japanese subjects with hypokalemia; these mutations have been reported previously[13, 14]. Genotyping of these mutations was performed using the TaqMan PCR method in the present study. The TaqMan PCR method is widely used in the genotyping assay, and the T180K, L849H and R919C genotypes were confirmed by direct sequencing method. We detected mutations in *SLC12A3* in 140 of the 1,567 subjects; thus, the overall frequency of GS mutations was 8.9%, and the GS mutant allele frequency was more than 4.8%. In a similar study, Tago et al.[12]

Table 4 *SLC12A3* mutations and laboratory data

	T180K	L849H	R919C	GS mutations	Wild-type
Number of subjects	40	49	56	140	1427
Sex (male/female)	13/27	19/30	13/43	44/96	541/886
Age (years)	59.2 ± 11.0	55.9 ± 15.0	58.8 ± 11.2	58.1 ± 12.6	57.1 ± 14.2
BMI (kg/m^2)	22.6 ± 2.9	23.0 ± 2.7	23.7 ± 3.3	23.1 ± 2.9	23.2 ± 3.2
SBP (mmHg)	126.2 ± 21.0	121.8 ± 18.4*	127.7 ± 17.9	125.6 ± 19.1	127.8 ± 18.9
DBP (mmHg)	75.2 ± 11.2	75.6 ± 11.1	76.4 ± 10.9	75.8 ± 11.0	75.2 ± 11.1
HTN (%)	45.0	32.7	42.9	40.7	42.1
Serum sodium (mmol/l)	141.6 ± 1.6	141.5 ± 1.9	141.4 ± 1.6	141.5 ± 1.7	141.5 ± 1.9
Serum potassium (mmol/l)	4.01 ± 0.42	3.97 ± 0.40*	4.10 ± 0.37	4.03 ± 0.39*	4.11 ± 0.36

Data are expressed as mean ± SD. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HTN, prevalence of hypertension. GS mutations: subjects with GS mutations are combined into one group. *P* values were calculated against subjects without GS mutations (wild-type). *P values were significant ($P<0.05$).

reported that the frequency of GS mutations in the general Japanese population is higher than previously suspected. They selected nine GS mutations from previous studies performed in Japanese, and estimated that the prevalence of asymptomatic subjects with heterozygous GS mutations in the general Japanese population was 6.4%, and that the GS mutant allele frequency in the 1,852 Japanese subjects studied was more than 3.2%, while the prevalence of patients with GS was 0.1%, with almost no cognation. In addition, similarly to the present study, they found that the most common GS mutations were T180K and L849H, and that mutant allele frequencies were 1.5 and 1.3%, respectively.

We also found that subjects with L849H mutation had significantly lower systolic blood pressure than subjects with wild-type *SLC12A3*, although blood pressure in subjects with GS mutations for T180K or R919C were not significantly different from subjects without GS mutations. Tago et al.[12] and Hsu et al.[15] reported no significant associations between blood pressure and *SLC12A3* mutations. However, Cruz et al.[9] reported that younger (under 18 years) subjects heterozygous for GS mutations had lower blood pressure levels. Previous studies suggested that GS-heterozygous subjects have significantly lower blood pressure when compared with matched control subjects lacking *SLC12A3* mutations in Amish[9], Swedish[10] and Framingham Heart Study subjects[11]. The reasons for the discrepancy are uncertain; however, they might be due to the difference in study population employed. The Asian population studies suggest a lack of association between *SLC12A3* mutation and blood pressure. Variations in GS mutations may also be involved. Monkawa et al.[13] studied T180K homozygotes diagnosed with GS by renal clearance test using furosemide (positive response) or thiazyde (negative response), and found the T180K mutation to be a loss-of-function mutation in the *SLC12A3* gene. Naraba et al.[16] also reported that the L849H mutation was a loss-of-function mutation using a mammalian cell expression system (Chinese hamster ovary cells) by measuring tracer $^{22}Na^{+}$ uptake, and that the mutation is responsible for GS. Furthermore, Lemmink et al.[14] studied the R919C mutation identified in patients with GS using the PCR-SSCP method, and found that structural alterations due to *SLC12A3* mutations (involving R919C) in the C-terminal domain may interfere with phosphorylation of the TSC protein, thereby affecting its regulation. Further studies are necessary in order to evaluate the effects of individual mutations on sodium-chloride transport in a functional expression system. No significant associations were seen between

the prevalence of hypertension and *SLC12A3* mutations in the present study, thus suggesting that these mutations do not contribute to resistance to hypertension. On the other hand, substitution of Arg904 to Gln in the *SLC12A3* gene reportedly predisposes Swedish[17] and Japanese[18] individuals to essential hypertension. This substitution is considered to be a gene polymorphism that may not in itself cause GS. Further studies are therefore necessary to determine the relationship between *SLC12A3* mutations and blood pressure or hypertension.

In the present study, subjects with L849H mutation had significantly lower serum potassium levels than subjects with wild-type *SLC12A3*, and subjects with GS mutations had significantly lower serum potassium levels than subjects without GS mutations. This suggests that L849H is a cause of loss-of-function mutation in the TSC.

The *SLC12A3* gene encodes the TSC, which is expressed in the distal convoluted tubule, and has been shown to reabsorb sodium chloride. Inactivating mutations of the *SLC12A3* gene lead to the clinical features of GS, and reduced salt reabsorption in GS may be associated with resistance to hypertension. A possible clinical implication is that subjects with heterozygous GS mutations may be susceptible to the side effects of thiazide diuretics. Subjects with heterozygous GS mutations may be prone to dehydration due to salt loss, such as through sunstroke or diarrhea. As the number of subjects with GS mutations is thought to be 1 in 11.2 subjects, which is higher than suspected, screening for GS mutations before diuretic treatment may be clinically beneficial. Tanaka et al[19]. reported that the *SLC12A3* gene is a candidate for conferring susceptibility to diabetic nephropathy, based on a genome-wide case-control association study using gene-based SNPs. Furthermore, Nicolet-Barousse et al.[20] reported that inactivation of the *SLC12A3* gene leads to an increase in bone mineral density in patients with GS. Further studies are needed to clarify the possible associations between *SLC12A3* genes and common diseases.

In conclusion, the present study clearly showed that the allele frequency of these GS mutations was 4.8% in a Japanese general population. These results also suggest that the allele frequency of GS mutations is higher than expected, although GS is considered to be a rare disorder, and that loss-of-function in the *SLC12A3* via the L849H mutation reduces blood pressure, thereby contributing to resistance to hypertension.

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