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ORIGINAL PAPER

Two novel genotypes of the thiazide-sensitive Na-Cl cotransporter (*SLC12A3*) gene in patients with Gitelman's syndrome

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Abstract Gitelman's syndrome is an autosomal recessive disorder marked by salt wasting and hypokalaemia resulting from loss-of-function mutations in the *SLC12A3* gene that codes for the thiazide-sensitive Na-Cl cotransporter. Gitelman's syndrome is usually distinguished from Bartter's syndrome by the presence of both hypomagnesaemia and hypocalciuria. Although recent advances in molecular genetics may make it possible to both diagnose and differentiate these diseases, the phenotypes sometimes overlap. Here we report two sporadic cases of Gitelman's syndrome and two novel genotypes of *SLC12A3*. Patient 1 was a compound heterozygote with a known missense mutation, L849H, and a novel mutation, R852H in exon 22. Patient 2 was homozygous for the missense mutation L849H. To our knowledge, this is the first report of a patient homozygous for 849H. Interestingly, both patients were affected with autoimmune thyroid disease. Patient 1 was affected with Hashimoto's disease, and Patient 2 was affected with Graves' disease. The symptoms of Patient 2 were more serious than those of Patient 1. Although the patients both carried the 849H allele (Patient 1 as a heterozygote and Patient 2 as a homozygous), their clinical symptoms differed. The difference in the clinical features may have been due both to phenotypic differences and the fact that Gitelman's syndrome is a complicated disorder.

Keywords Gitelman's syndrome · Missense mutation · Compound heterozygote · Homozygous · Graves' disease

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Introduction

Gitelman's syndrome (GS) was first described by Gitelman and colleagues in 1966 [1]. This syndrome has characteristics similar to those of Bartter's syndrome, such as salt wasting, hypokalaemia, metabolic alkalosis and normal blood pressure under hyperreninaemic hyperaldosteronism. GS is distinguished from Bartter's syndrome by the presence of hypomagnesaemia and hypocalciuria. However, these features also present in a subset of Bartter's syndrome patients [2–4]. And current pivotal treatments are potassium replacement for both syndromes. Therefore, there may be sufficient overlap between the two syndromes from clinical viewpoints. Advances in molecular genetics have clarified that GS are caused by renal tubule electrolyte transporter dysfunction. GS is an autosomal recessive disorder resulting from mutations in the gene (*SLC12A3*) encoding the thiazide-sensitive Na-Cl cotransporter (TSC) [5–8]. The human *SLC12A3* gene, which is located on chromosome 16, consists of 26 exons and encodes a protein that contains 12 putative transmembrane domains with long intracellular amino and carboxy termini.

To date, a number of different *SLC12A3* mutations have been reported, including missense, frame shift, nonsense,

and splice-site mutations. Missense mutations are the most common presented abnormality. The majority of these mutations have been collected in the Human Gene Mutation Database (HGMD, http://www.hgmd.cf.ac.uk/). In a recent study, the functional consequences of GS mutations were assessed by measuring tracer $^{22}Na+$ uptake in Xenopus oocytes or in Chinese hamster ovary (CHO) cells [9, 10]. The T180K variation was found to be simply a polymorphism, whereas the L849H mutation was found to be a loss-of-function mutation that appears to be responsible for GS [10].

It is known that thyroid status affects electrolyte balance in the kidney. Disturbances of thyroid function are accompanied by widespread alterations in renal haemodynamics and tubular handling of electrolytes [11, 12]. Munro et al. [13], studying exchangeable potassium and sodium, found that PTU or surgical therapy of hyperthyroidism caused an increase in body potassium but no consistent change in sodium content, and therapy of myxoedema with T_4 caused a loss of both sodium and potassium. Patients with thyroid disease may tend to develop the symptoms of GS. In this report, we describe two sporadic cases of Gitelman's syndrome whose patients were affected with autoimmune thyroid disease and two novel genotypes of *SLC12A3*.

Patients and methods

Case 1

Patient 1, a 40-year-old woman, was referred to our department for evaluation of hypokalaemia. She had complained of shoulder discomfort and numbness, and painful muscle cramps in her extremities. She had been treated for diabetes mellitus for several years. No other family member had a similar illness, and there was no history of parental consanguinity. The physical examinations were unremarkable except for a homogenous goiter. Her blood pressure was 136/86 mmHg, and her resting heart rate was 78 beats/min. Her body mass index (BMI) was 26.3 kg/m^2. She had neither muscle weakness nor paresthesia. The deep tendon reflexes were normal. The biochemical studies showed moderate hypokalaemia, normomagnesaemia and normotensive hyperreninaemia. Urinary calcium to creatinine ratio was 0.029. Arterial blood gas showed mild metabolic alkalosis. Thyroid function was normal, although anti-thyroperoxidase antibodies were present (Table 1). Due to these clinical and biochemical findings, we concluded that this was probably a case of GS and also diagnosed Hashimoto's disease. We performed genetic analyses of *SLC12A3* to confirm the diagnosis of GS.

Table 1 Clinical and biochemical findings in two patients with Gitelman's syndrome

Clinical symptoms	Normal range	Patient 1 Shoulder discomfort numbness	Patient 2 Periodic paralysis thirst, diuresis
Serum electrolyte levels			
Na	136–148 mmol/l	141	144
K	3.6–5.0 mmol/l	3.3	1.7
Cl	98–109 mmol/l	98	96
Ca	8.8–10.8 mg/dl	9.5	8.9
Mg	1.7–2.3 mg/dl	1.8	1.5
Urinary electrolyte excretions			
Na	130–350 mEq/day	67.2	145.6
K	40–65 mEq/day	41.8	90
Urinary Ca/Cr	0.05–0.15	0.029	0.02
Urinary NAG	1.3–4.5 U/day	0.6	9.1
Urinary β2MG	5–300 µg/l	13	70
Plasma aldosterone	29.9–159 pg/ml	130	79
Plasma renin activity	0.3–2.9 ng/ml/h	13	22
TSH	0.34–3.80 µU/ml	2.02	0.01
Free T_3	2.0–3.8 pg/ml	3.66	24.58
Free T_4	0.8–1.5 ng/dl	1.41	>7.77
TPO-Ab	<0.3 U/ml	124	–
TRAb	<1.0 IU/l	–	17.9
BGA (room air)			
pH	7.35–7.45	7.458	7.506
HCO_3^-	22–26 mmol/l	29.3	35.4
BE	–2 to +2 mmol/l	5.2	12.2

NAG, N-acetyl-β-D-glucosaminidase; β2MG, β2-microglobulin; TSH, thyroid stimulating hormone; T_3, triiodothyronine; T_4, thyroxine; TPO-Ab, antithyroid peroxydase antibody; TRAb, TSH receptor antibody; BGA, Blood gas analysis; BE, Base excess

Case 2

Patient 2 was 28-year-old woman. For 1 year, she had suffered from transient paralysis of the lower extremities whenever she ran a fever. She was admitted to our hospital because of muscular weakness that progressed to paralysis involving all extremities. She also had complained of thirst and excessive urination. Relevant medical history included a diet at the age of 22 years that led to hospitalization with tetany due to hypocalcaemia at 23 years of age. She denied any form of self-medication, surreptitious diuretic and laxative abuse or persistent vomiting and diarrhoea at present. Her parents were not consanguineous. Her mother and a maternal grandmother had a history of hyperthyroidism. On physical examination, her blood pressure was 118/65 mmHg, her resting heart rate was 108 beats/min and BMI was 27.4 kg/m^2. The thyroid gland was enlarged.

Manual Muscle Testing [14] revealed grade 4 in her upper limbs and grade 2 in her lower limbs. The deep tendon reflex was present but decreased. Laboratory tests showed severe hypokalaemia, low thyroid stimulating hormone (TSH) levels, high free T_4 levels and the presence of anti-TSH receptor antibodies, which led to the diagnosis of Graves' disease. Furthermore, she had hypomagnesaemia, hypocalciuria and normocalcaemia. The plasma aldosterone concentration was normal, although plasma renin activity was elevated. The molar ratio of urinary calcium/creatinine was 0.02. Arterial blood gas showed metabolic alkalosis (Table 1). On the basis of these findings, the diagnosis of GS was made. Genetic analysis of the *SLC12A3* gene was performed to confirm diagnosis.

Mutation analysis

We performed *SLC12A3* gene mutation analysis for each patient. We also had the opportunity to study the parents of Patient 1. They did not have any apparent clinical symptoms. Informed consent was obtained from each individual according to a protocol approved by the Human Studies Committee of Nihon University. Blood samples were collected into tubes containing 50 mM EDTA-2Na, and genomic DNAs were extracted as described [15]. We positioned primers in introns at the 5′ and 3′ boundaries of *SLC12A3* to amplify exons for sequence analysis (Table 2). Exon 1 was amplified with two PCR reactions that produced two overlapping PCR products. PCR products were purified with ExoSAP-IT® reagent (GE Healthcare Life Sciences, Piscataway, NJ) including exonuclease I (Exo I) and shrimp alkaline phosphatase (SAP) and subjected to automated DNA sequencing analyses with fluorescence-labelled dideoxyterminators (BigDye Terminator Cycle Sequencing Kit, Applied Biosystems, Foster City, CA) according to the manufacturer's instructions (ABI PRISM 3700 Genetic Analyzer, Applied Biosystems) [16]. Sequencing was performed for both strands. Patient 1 was found to be a compound heterozygote with a single-base substitution at nucleotide 2552 (CTC-to-CAC, L849H) and a substitution at nucleotide 2561 (CGC-to-CAC, R852H) in

Table 2 Primers used for sequencing analysis of the *SLC12A3* gene

Target regions	Forward primers	Reverse primers
Exon1–1	5′-GATCCTGGCCCCTCCCTG	5′-TGCTGTTGGCATAGTGCTCA
Exon1–2	5′-CCACCAGCTGCCTATGACA	5′-CGAGGTCACACAGCAGGAAG
Exon2	5′-GAGACGCCGTCCCTAGCACC	5′-TGGACATCACGCACCACCCA
Exon3	5′-GGTGTCACCCAGGTGGCCTC	5′-GGCAAGCTGGGAAGAATGGG
Exon4	5′-GGCTCCTCCCTTGGGAAATG	5′-GACCCACGAGAGGAGGGCCT
Exon5	5′-ACCGACTCATCTGGTTTCAT	5′-GATCCCTCTACCCAGGGTCC
Exon6	5′-GGTGTTGCAGCCTGGCCCAT	5′-ACGTGACCACCTCCATGTCC
Exon7	5′-GGCTTCCCAGAGAGGTAGAA	5′-GTCCCCAGAGCCATGGTCAG
Exon8	5′-GGTCAAGCCCTCCAGGTGAG	5′-TAGCCCCTGTGCAGTGCCAG
Exon9	5′-CCTTCAGGACCCTGCTAT	5′-GACACTGCAGGGTGGAGGCC
Exon 10	5′-CAGAGTAAGGAGGGAAGGCA	5′-CCACTGTGTCTGGTGGGTCA
Exon 11	5′-CAGCCCTCACCGTGGAGTCC	5′-CCCACCCCCTGTCATCTCGA
Exon 12	5′-GGAAGTGGCAGGTCCCAGCC	5′-CAGGAGGCCAGGCCCTGTGA
Exon 13	5′-AGTTGCCCAACAGGCTGTCC	5′-CCATGCCCCAGTTCCTCCTG
Exon 14	5′-CGACTGCCAGGCATGCCCAC	5′-CCGCCTGCATGGCTACCCTG
Exon 15	5′-CTGGTTTCCTCTAGTGATTC	5′-TCACTGGCCCTGGGGTCCCA
Exon 16	5′-CTCTCCTGATGGCTCCTGCC	5′-TGCTGGGTTTACAGGCATGAG
Exon 17	5′-GAGGGTGAAGGCAGCTGGTG	5′-GCCACCAAGCCGTAAGTCCT
Exon 18	5′-GATCACCAACTCTGCCCCTC	5′-ATGGCCCAAATTAACAGACC
Exon 19	5′-AGTGGGAGCTGGGGGAGAAG	5′-CTAGAACTTTCTGGGAGTGG
Exon 20	5′-ACGGTGCCCTCAGACAAGGAG	5′-GAGTGCCCTGAGCTCTGAGTG
Exon 21	5′-GCGCGGCGCTGGCTCTGC	5′-CCGGGCAGGAGGGCTGATCC
Exon 22	5′-ATTCTTGTCATGACTCACGG	5′-TGGAGCTAAGATGACACTGG
Exon 23	5′-CAGAGCAAGACGCTGTCTCA	5′-TCTCCAGGCACACAGTTGGC
Exon 24	5′-CTCAGCCGGCCTCAACCCAC	5′-CCCTGACCCAGTGATGTGTC
Exon 25	5′-GGTGAAGGATTGAGTGACCT	5′-CACCTGACTCTGGACAGACT
Exon 26	5′-CTTTGCCCATAGGGAGGAAG	5′-GAGCTGTGGACAGGGATGTC

exon 22. Familial linkage analyse confirmed that 849H was the paternal allele and 852H was the maternal allele. Patient 2 was homozygous for the L849H mutationin (Fig. 1).

Clinical course

Patient 1 was treated with potassium supplementation (potassium chloride 32 mEq/day). After 2 years of follow-up, her neuromuscular symptoms were almost absent. Patient 2 was treated with thiamazole 30 mg/day for Graves' disease and potassium supplementation (same dose as Patient 1) for Gitelman's syndrome. At 1 year of follow-up, her thyroid function was normal, and when potassium supplementation was ceased, the neuromuscular symptoms did not reappear. Although hypocalciuria persisted in both patients, serum electrolytes (magnesium and potassium) were normal.

Discussion

We describe here two cases of normotensive hypokalaemia associated with GS. This diagnosis was confirmed by genetic analysis of these patients. This is the first report of the R852H variant of *SLC12A3* and the first report of a patient homozygous for the 849H allele. Lemmink et al. [17] reported that most *SLC12A3* gene mutations are localized in the intracellular carboxy-terminal domain of the TSC protein. The L849H and R852H mutations are located in the carboxyl-terminal cytoplasmic region of the TSC protein. The L849H mutation was found to underlie GS in three different studies of Japanese patients [18–20]. Moreover, Naraba et al. [10] investigated the functional consequences of *SLC12A3* mutations by measuring tracer

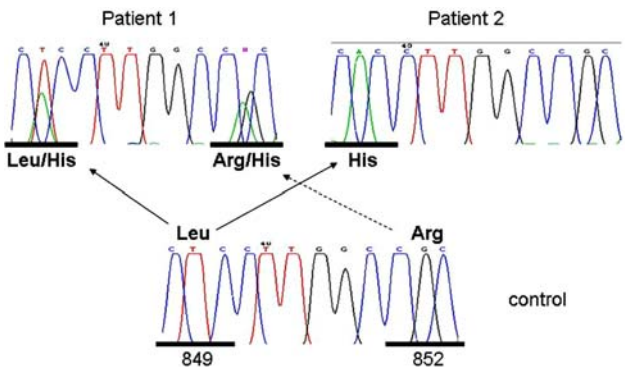

Fig. 1 Sequence analysis of the *SLC12A3* gene. In Patient 1, we found a heterozygous transition (T-to-A) at nucleotide 2552 in exon 22, resulting in a Leu-to-His substitution at amino acid 849, and a heterozygous transition (G-to-A) at nucleotide 2561 in exon 22, resulting in an Arg-to-His substitution at amino acid 852. Patient 2 is homozygous for the T-to-A transition in exon 22

^{22}Na+ uptake in CHO cells. They found that the L849H mutation is a loss-of-function mutation. We did not assess renal clearance of chloride, but Naraba et al. [10] indicated that these two cases are GS powerfully.

In contrast to Bartter's syndrome, GS is usually found in older children and young adults during routine investigation because the patients generally have mild symptoms, including cramps and fatigue, at presentation. However, in the present study, Patient 2, who had Graves' disease, had suffered from hypokalaemic periodic paralysis. Thyrotoxic periodic paralysis occurs mainly in Asian men [21, 22]. For a paralytic symptom, GS may be failed to notice as hypokalaemic periodic paralysis, such as thyrotoxic periodic paralysis or familial periodic paralysis, due to acute shift of K^+ into cells. Fortunately, Patient 2 had the typical clinical characteristics of GS, including hypokalaemia, hypocalciuria, hypomagnesaemia and increased renin levels.

It has been reported that the increased electrolyte excretion in the kidney due to excess thyroid hormone may have exacerbated the clinical features of GS [12]. This is because hypokalaemia and hypomagnesaemia were not observed after treatment of hyperthyroidism in Patient 2. It is possible that the symptoms of GS appear prominent when exposed to pathophysiologocal force to induce electrolyte imbalance such as hyperthyroidism. Symptoms of Patient 1 was mild because she diagnosed with Hashimoto's disease, but her thyroid function was normal.

Several reports showed that hypokalaemic nephropathy caused by long-term hypokalaemia could cause end-stage renal failure [23–25]. Patient 2 showed increase of urinary NAG, and we supposed she had tubular disorder. To prevent hypokalaemia-induced nephropathy, aggressive correction of hypokalaemia should be attempted.

Our patients with GS did not always have hypomagnesaemia. According to Maki et al. [20], two of seven patients with GS did not have hypomagnesaemia. Lin et al. [26] reported that four of 20 patients with GS did not have hypomagnesaemia. Recently, genetic studies have identified ''transient receptor potential (melastatin) 6'' (TRPM6) as the first component involved directly in epithelial magnesium reabsorption [27, 28]. Nijenhuis et al. [29] demonstrated that TSC inactivation was possibly associated with *Trpm6* downregulation. However, unknown magnesium regulators may compensate renal magnesium wasting caused by TSC defect.

In a recent study from Japan, genetic analysis revealed that the overall frequency of heterozygous GS mutations was 3.21%, which was higher than expected [30]. Confirmation of a diagnosis of GS with PCR/sequencing of *SLC12A3* is a useful tool for patients with hypokalaemia. And this genetic diagnosis of GS which precisely distinguishes GS from Bartter's syndrome may contribute to prove the disease prognosis and to develop appropriate therapy.

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