Allele-specific RT-PCR for the rapid detection of recurrent SLC12A3 mutations for Gitelman syndrome

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Recurrent mutations in the SLC12A3 gene responsible for autosomal recessive Gitelman syndrome (GS) are frequently reported, but the exact prevalence is unknown. The rapid detection of recurrent SLC12A3 mutations may help in the early diagnosis of GS. This study was aimed to investigate the prevalence of recurrent SLC12A3 mutations in a Taiwan cohort of GS families and develop a simple and rapid method to detect recurrent SLC12A3 mutations. One hundred and thirty independent Taiwan families with genetically confirmed GS were consecutively enrolled to define recurrent SLC12A3 mutations and determine their prevalence. Using TaqMan probe-based real-time polymerase chain reaction, we designed a mutation detection plate with all recurrent mutations. We validated this mutation detection plate and tested its feasibility in newly diagnosed GS patients. A total of 57 mutations in the SLC12A3 gene were identified and 22 including 2 deep intronic mutations were recurrent mutations consisting of 87.1% (242/278, 18 triple) of all allelic mutations. The recurrent mutation-based TaqMan assays were fully validated with excellent sensitivity and specificity in genetically diagnosed GS patients and healthy subjects. In clinical validation, recurrent mutations were recognized in 92.0% of allelic mutations from 12 GS patients within 4 h and all were confirmed by direct sequencing. Recurrent SLC12A3 mutations are very common in Taiwan GS patients and can be rapidly identified by this recurrent mutation-based SLC12A3 mutation plate.

INTRODUCTION

Gitelman syndrome (GS) is characterized by persistent renal salt wasting, chronic hypokalemic metabolic alkalosis with renal potassium (K+) wasting, hypomagnesemia with inappropriate renal magnesium (Mg2+) loss, and hypocalciuria. Clinical manifestations of GS vary dramatically from asymptomatic, fatigue, dizziness, numbness, paresthesia, joint pain (pseudogout and chondrocalcinosis), and neuromuscular weakness to paralysis or fatal arrhythmia1–4. Patients with GS were also found to carry an increased risk of the development of chronic kidney disease (CKD) and type 2 diabetes mellitus5.

GS is inherited as autosomal recessive renal tubulopathy caused by inactivating mutations in the SLC12A3 gene encoding thiazide-sensitive sodium chloride cotransporter (NCC) on the apical membrane of distal convoluted tubule6–7. Given a much higher prevalence of GS carrier (1–4%), GS may be the most common inherited salt-losing tubulopathy with an estimated prevalence of 1–16 in 40,000 (refs. 8–10). Although hypocalciuria and hypomagnesemia in the presence of persistent renal salt wasting are landmark findings for GS in the differential diagnosis of chronic hypokalemia, genetic testing is still the gold standard to confirm GS11–13.

The SLC12A3 gene on chromosome 16 is composed of 26 exons with >130 kb. To date, approximately 500 different mutations have been identified with a wide distribution along 26 exons and some introns of SLC12A3 (refs. 14–16). Direct Sanger sequencing has long been regarded as the gold standard to detect the genetic mutations for GS. However, large genomic rearrangement, large deletion, and deep intronic mutations that were not so rare in GS may not be detected by direct sequencing17. It has been described that recurrent SLC12A3 mutations were highly prevalent among unrelated GS families18–20. In a large French cohort of patients with GS, seven recurrent SLC12A3 mutations (p.A313V, p.G394fs (c.1180+1G>T), p.G741R, p.L559P, p.R861C, p.H916fs (c.2883+1G>T), and p.C994Y) contributed to 41.5% of all identified allelic mutations21. We collected a large cohort of GS families5,14 and found that recurrent SLC12A3 mutations occurred frequently with undefined prevalence. The rapid detection of these recurrent NCC mutations, albeit challenging, may provide an early genetic diagnosis of GS.

This study was aimed to define the prevalence of recurrent SLC12A3 mutations from 130 genetically confirmed GS families and design a recurrent mutation-based detection plate with validation using TaqMan probe-based real-time polymerase chain reaction (RT-PCR). In addition, we clinically evaluated the feasibility of the diagnostic plate in newly diagnosed GS patients. The results to be reported indicated that 22 recurrent mutations of the 57 identified SLC12A3 mutations comprised 87.1% of all allelic mutations. Using this simple recurrent mutation-based detection plate in 12 clinically diagnosed GS patients, 92.0% of allelic mutations were rapidly detected.

RESULTS

Identification of SLC12A3 mutations

As shown in Fig. 1, 57 different SLC12A3 mutations including 10 novel ones in 130 unrelated GS families were identified. The most common mutation type was a missense mutation (47.5%),
followed by frameshift (17.6%), splicing site mutations (17.3%), nonsense mutations (12.6%), and indels (5.0%). The majority of GS patients (112/130, 86.2%) were compound heterozygous. Eighteen GS families (13.8%) had triple mutations with p.T163M and p.R871H being exclusively on one allele at a much higher frequency (66.7%), suggestive of a founder effect (Table 1).

Prevalence of recurrent SLC12A3 mutations

As shown in Fig. 2, 22 recurrent SLC12A3 mutations were located in extra-cellular loops (7/22), intracellular loops (5/22), C-terminal domain (5/22), N-terminal domain (3/22), and rarely in transmembrane domains (2/22). A mutation (p.T60M) at the phosphorylation site of NCC regulated by STE20/SPS1-related proline/alanine-rich kinase/oxidative stress-responsive kinase-1 (SPAK/OSR1) was not only frequent in our cohort but also in Japan, Korea, and western countries. Three recurrent mutations with p.D486N, p.R642C, and c.506-1G>A were reported in many different geographic areas. Twelve recurrent mutations (p.R83Q, p.T613M, p.L215P, p.N442K, p.R642H, p.T649M, p.R871H, p.R959fs, c.602-16G>A, c.1670-191C>T, and c.2548+253C>T) have been reported in Japan. Three recurrent mutations (p.T649M, p.W844X, and c.602-16G>A) were detected in western countries. Mutation p.H90Y could be found in Korea.

Validation of recurrent mutation-based mutational detection plate

As shown in Fig. 3, the mutation detection plate employing TaqMan probe-based RT-PCR for 22 recurrent SLC12A3 mutations was created successfully with 100% accuracy. Every assay validated in samples from 130 GS patients and 50 healthy volunteers exhibited fairly high sensitivity (>99%) and specificity (>99%). One healthy volunteer harboring one recurrent mutation also confirmed by Sanger sequencing suggested a higher prevalence of GS carriers in Taiwan’s population.

Clinical validation in newly diagnosed GS patients

As shown in Table 2 and Supplementary Fig. 2, 12 newly diagnosed GS patients were included for the detection of recurrent SLC12A3 mutations. Ten (83.3%) of them were identified as biallelic recurrent mutations including one triple mutations. With direct sequencing, the remaining two GS patients with one recurrent mutation were confirmed to carry a novel splicing site mutation c.2285+2T>C (IVS18+2T>C) and p.D62G on the other allele, respectively. Overall, recurrent mutations were recognized in 92.0% (23/25) of allelic mutations.

DISCUSSION

In this study, we identified 57 distinct SLC12A3 mutations with 22 being recurrent in 130 unrelated genetically confirmed Taiwan GS families. The prevalence of these recurrent SLC12A3 mutations was

Table 1. Triple pathogenic SLC12A3 mutations in 18 unrelated GS families.

| Families | Mutation 1 | Mutation 2 | Mutation 3 |
|----------|------------|------------|------------|
| 01       | p.T163M    | p.R871H    | p.N426K    |
| 02       | p.T163M    | p.R871H    | p.R959fs   |
| 03       | p.T163M    | p.R871H    | c.2548+253C>T |
| 04       | p.T163M    | p.R871H    | c.2548+253C>T |
| 05       | p.T163M    | p.R871H    | c.2660+1G>A |
| 06       | p.T163M    | p.R871H    | p.K95N     |
| 07       | p.T163M    | p.R871H    | p.L425P    |
| 08       | p.T163M    | p.R871H    | p.R977X    |
| 09       | p.T163M    | p.R871H    | p.S178P    |
| 10       | p.T163M    | p.R871H    | p.S710X    |
| 11       | p.T163M    | p.R871H    | p.S710X    |
| 12       | p.T163M    | p.R871H    | p.S710X    |
| 13       | p.S710X    | c.1096-2A>G | c.1096-2A>G |
| 14       | p.S710X    | c.1670-191C>T | c.1670-191C>T |
| 15       | p.R83Q     | p.S710X    | p.W844X    |
| 16       | p.H90Y     | p.N640S    | p.S710X    |
| 17       | p.T60M     | p.T60M     | p.R959fs   |
| 18       | p.A13P     | p.T60M     | p.R83Q     |

Fig. 1  Topology of human NCC with position of each mutation in this study. Amino acid exchanges of the mutations identified in this study are shown and their locations are indicated by arrows.
deep intronic mutations within introns 13 and 21, resulting in a pseudo-hotspots on SLC12A3. For samples with single or without detectable recurrent mutations, including deep intronic mutations, were accurately recognized with easily interpreted results. A prospective test of this mutation detection plate in 12 newly diagnosed GS patients rapidly identified >90% of allelic mutations. For samples with single or without detectable recurrent mutations, we performed a step-by-step approach and identified two additional non-recurrent SLC12A3 mutations on the other allele in two GS patients. Considering that the majority of recurrent mutations in our plate are recognized frequently in different geographic regions, we suggested that using the mutation detection plate as the primary screen for GS should improve the efficacy of GS diagnosis. Furthermore, this recurrent mutation-based detection approach may also be applied to other genetic diseases with a high prevalence of recurrent mutations such as hemophilia A, cystic fibrosis, and bilateral sensorineural hearing loss.

Fig. 2 Twenty-two recurrent mutations with different percentage of all 57 SLC12A3 mutations. Twenty two recurrent mutations comprise 87.1% of all allelic mutations, compared with 35 non-recurrent mutations responsible for only 12.9% of all allelic mutations.
This study has some limitations. First, rare, novel, and non-recurrent mutations could not be recognized by this recurrent mutation-based detection plate. To expand the application, more mutational spots should be added to enhance the diagnostic efficiency. Second, we did not compare the cost-effectiveness between this mutation detection plate and conventionally used sequencing or other genetic analyses.

In conclusion, recurrent mutations in the SLC12A3 gene are highly prevalent in Taiwan GS patients and can be quickly detected by this accurate recurrent mutation-based detection plate.
Table 2. Recurrent mutation-based detection in clinically diagnosed GS patients.

| No | Sex | Age (Year) | K+ (mmol/L) | Mg2+ (mg/dL) | UCa/Cr (mmol/mmol) | Allele 1 | Allele 2 |
|----|-----|------------|-------------|-------------|-------------------|---------|---------|
| 01 | F   | 63         | 2.9         | 1.3         | 0.03              | p.R959fs| p.R959fs|
| 02 | F   | 35         | 2.1         | 1.6         | 0.08              | p.R642C | p.T649M |
| 03 | F   | 24         | 3.0         | 1.7         | 0.02              | p.T60M  | p.N442K |
| 04 | M   | 36         | 1.7         | 1.6         | 0.06              | p.W844X| p.R959fs|
| 05 | M   | 24         | 3.2         | 1.7         | 0.08              | p.R83Q  | p.R871H |
| 06 | M   | 19         | 1.7         | 1.9         | 0.03              | p.T60M  | p.T60M  |
| 07 | F   | 32         | 2.8         | 1.4         | 0.02              | p.R83Q  | p.2285C|
|    |     |            |             |             |                   |         | -2T>C   |
| 08 | M   | 60         | 1.8         | 1.1         | 0.10              | p.R83Q  | p.R83Q  |
| 09 | M   | 22         | 2.8         | 1.3         | 0.07              | c.1670- | p.D486N |
|    |     |            |             |             |                   | 191C>T |         |
| 10 | F   | 26         | 2.7         | 1.2         | 0.03              | p.N442K | p.R959fs|
| 11 | F   | 28         | 2.4         | 1.3         | 0.14              | p.R959fs| p.D62G   |
| 12 | M   | 18         | 1.9         | 1.5         | 0.02              | p.T163M | p.D486N |
|    |     |            |             |             |                   |         | R871H   |

*The mutations detected by direct sequencing. The two bold values represent mutations which were initially not detected by recurrent mutation-based mutation detection plate because both of them were not included in the recurrent mutations defined in this study. Since only monoallelic mutation was identified in the two patients, other genetic tests were conducted and the two SLC12A3 mutations were identified.

Fig. 3 A schematic presentation of TaqMan probe-based RT-PCR and interpretation of allelic discrimination plot. a The probe is labeled with a 3′ quencher (a dark QSY or a non-fluorescent minor groove binder (MGB) quencher) and a 5′ fluorescent reporter dye (ABY, JUN, FAM, or VIC). During the standard procedure of RT-PCR, the TaqMan polymerase with 5′-3′ nuclease activity will release the fluorescent reporter which will be detected in each cycle. b Example of the output of the allelic discrimination software and interpretation of allelic discrimination plot based on the signal measurement of VIC and FAM fluorescent reporter dye. c Interpretation of the results from our p.D486N and p.T60M assays on validation. The target recurrent single-nucleotide variants were inserted into vector pUC57 as positive control for each assay as well as two no-template controls as negative control. As shown with p.T60M, six blue plots represented one positive control and five homozygous samples. The number of green plots and red plots indicated the number of samples carrying one (heterozygous) or no (wild type) allelic mutation p.T60M.

Detection of pathogenic mutations in SLC12A3

Using genomic DNA and/or cDNA from blood leukocytes (National Center for Biotechnology Information No. NM-000339), a series of meticulous genetic analyses of SLC12A3 were performed. Genetically confirmed GS was defined as recognition of inactivating SLC12A3 mutations in both alleles. Sanger sequencing was performed first with primers pairs that were designed for exons and introns based on previous studies. For those with no biallelic mutations, denatured high-performance liquid chromatography, direct sequencing, multiplex ligation-dependent probe amplification for large deletions, cDNA analysis via RT-PCR for splicing site and deep intronic mutations, and targeted sequencing panel for all genes relevant in the differential diagnosis of GS were performed in order as previously reported.

To confirm the pathogenicity of the identified nucleotide variants, we excluded single-nucleotide variants with a frequency of >1% at first using a publicly available database (HapMap, dbsNP150 (http://www.ncbi.nlm.nih.gov/projects/SNP/), the 1,000 Genomes Project (http://www.1000genomes.org), the Genome Aggregation Database (gnomAD, https://gnomad.broadinstitute.org), and the Exome Aggregation Consortium (ExAC database). Pathogenicity score then was evaluated via in silico computational methods using SIFT, PolyPhen2, LRT, Mutation Taster, Mutation Accessor, FATHMM, M-CAP, CADD, and GERP. Furthermore, the pathogenicity of all candidate variants was annotated based on American College of Medical Genetics and Genomics (ACMG) standards and guidelines.

Definition of recurrent SLC12A3 mutations and prevalence

Recurrent SLC12A3 mutation was defined as the reappearance of the same mutation in two or more unrelated families. Triple mutations were defined as two different mutations in one allele and one mutation in another allele. If two or more siblings from one family carried the same mutations, "one occurrence" of each mutation was calculated to prevent the over-estimation of prevalence. Accordingly, the prevalence of recurrent mutations was determined as the total number of recurrent mutations divided by the total number of all allelic mutations in 130 “families” rather than patients.

Development of recurrent mutation-based mutation detection plate

To create a recurrent mutation-based detection plate, SLC12A3 mutations that fulfilled the definition of recurrent mutation were obtained. Primers and TaqMan probes were designed by Primer Express software (Applied Biosystems, USA). Two types of dual-labeled probes were applied for RT-PCR in this study. The QSY probes incorporate a dark 3′ QSY quencher and a 5′ fluorescent reporter dye (ABY or JUN). The minor groove binder (MGB) probes contain a non-fluorescent quencher (NFQ) attached to a 3′ MGB moiety and a fluorescent reporter dye (FAM or VIC) incorporated into the 5′ end. Each mutation detection assay included a pair of primers and two allele-specific probes with different reporter dyes corresponding to mutant and wild-type alleles, respectively. Table 3 shows the primer and probe sequences. Using a 96-well plate, all TaqMan probe-based RT-PCR experiment was performed in a 10 μL reaction mixture consisting of 5 μL 2x TaqMan master mix buffer (Life Technologies), 300 nM of each primer, 300 nM of probes, and 100 ng DNA template. The preparation of the mixtures was carried out on E0Mate, an Automated Liquid Handling & Pipetting System. The TaqMan probe-based RT-PCR was done in QuantStudio 5 Real-Time PCR System (Thermo Fisher) with the following thermal cycle program: 60 °C for 30 s followed by 95 °C for 5 min and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Real-time data were collected during 40 cycles of...
| Nucleotide | Protein | Mutation type | Forward primer (sense) | Reverse primer (antisense) | wild probe (Allele 1) | Mutant probe (Allele 2) |
|------------|---------|---------------|------------------------|----------------------------|----------------------|------------------------|
| c.179C>T   | p.T60M  | Missense      | GCATGGCGCACTTTGGG      | TGCTGGGCACTATTGCTATAT      | CTTTGACCTAAACACTGAGT | CACCTTGAGCTACAACAAGGCT |
| c.248G>A   | p.R83Q  | Missense      | CGCCACCTTTGGCTACACAC   | CTTCTGAGAGCACTTTGCTG      | CTGAGGGCTACCTAGCTC   | CACCTTGAGCTACAACAAGGCT |
| c.268G>A   | p.H90Y  | Missense      | TGGAGGGGGAGGAGATTCTTC  | CACTCTTGACCTGACTC         | CTTCTGAGCTACCTAGCTC  | CACCTTGAGCTACAACAAGGCT |
| c.485G>T   | p.T163M | Missense      | AGAGAGAGAGAGAGACTG     | CACTCTTGACCTGACTC         | CTTCTGAGCTACCTAGCTC  | CACCTTGAGCTACAACAAGGCT |
| c.496-497delGCinsCT| p.A166L | Missense      | CGAGATTTGTTGCTACTCATG  | CACTCTTGACCTGACTC         | CTTCTGAGCTACCTAGCTC  | CACCTTGAGCTACAACAAGGCT |
| c.508-1G>A | IVS3    | Exon skipping | GAAATGCTGGCACTTTGGG    | CTTCTGAGCTACCTAGCTC       | CTTCTGAGCTACCTAGCTC  | CACCTTGAGCTACAACAAGGCT |
| c.602-16G>A| IVS4    | Exon skipping | CACGCAACCCGACTCTCAG    | CTTCTGAGCTACCTAGCTC       | CTTCTGAGCTACCTAGCTC  | CACCTTGAGCTACAACAAGGCT |
| c.644T>C   | p.R83Q  | Missense      | CGCCACCTTTGGCTACACAC   | CTTCTGAGAGCACTTTGCTG      | CTGAGGGCTACCTAGCTC   | CACCTTGAGCTACAACAAGGCT |
| c.734T>C   | p.L245P | Missense      | CAGAGAGAGAGAGAGACTG     | CACTCTTGACCTGACTC         | CTTCTGAGCTACCTAGCTC  | CACCTTGAGCTACAACAAGGCT |
| c.965-1G>A + c.965-977 | ggcacaatttttt | Exon7-8 skipping | CTTCTGAGCTGGCTACAGGG     | CTTCTGAGCTACCTAGCTC       | CTTCTGAGCTACCTAGCTC  | CACCTTGAGCTACAACAAGGCT |
| c.1297C>G  | p.N442K | Missense      | AGAGAGAGAGAGAGACTG     | CACTCTTGACCTGACTC         | CTTCTGAGCTACCTAGCTC  | CACCTTGAGCTACAACAAGGCT |
| c.1456G>A  | p.D486N | Missense      | TGGAGGGGGAGGAGATTCTTC  | CACTCTTGACCTGACTC         | CTTCTGAGCTACCTAGCTC  | CACCTTGAGCTACAACAAGGCT |
| c.1670-191C>T | IVS13 as-191C>T | 90 bp pseudoeoxon | TGATGCTGGCTGGAGAGG      | CTTCTGAGCTACCTAGCTC       | CTTCTGAGCTACCTAGCTC  | CACCTTGAGCTACAACAAGGCT |
| c.1919A>G  | p.N640S | Missense      | CTTGAGGTCACTCTACTC     | CTTCTGAGCTACCTAGCTC       | CTTCTGAGCTACCTAGCTC  | CACCTTGAGCTACAACAAGGCT |
| c.1924C>T  | p.R642C | Missense      | GACAGAGAGAGAGAGACTG     | CACTCTTGACCTGACTC         | CTTCTGAGCTACCTAGCTC  | CACCTTGAGCTACAACAAGGCT |
| c.1925G>A  | p.R642H | Missense      | GACAGAGAGAGAGAGACTG     | CACTCTTGACCTGACTC         | CTTCTGAGCTACCTAGCTC  | CACCTTGAGCTACAACAAGGCT |
| c.1946C>T  | p.T649M | Missense      | GACAGAGAGAGAGAGACTG     | CACTCTTGACCTGACTC         | CTTCTGAGCTACCTAGCTC  | CACCTTGAGCTACAACAAGGCT |
| c.2129C>A  | p.S710X | Nononsense    | GACAGAGAGAGAGAGACTG     | CACTCTTGACCTGACTC         | CTTCTGAGCTACCTAGCTC  | CACCTTGAGCTACAACAAGGCT |
| c.2532G>A  | p.W844X | Nononsense    | GACAGAGAGAGAGAGACTG     | CACTCTTGACCTGACTC         | CTTCTGAGCTACCTAGCTC  | CACCTTGAGCTACAACAAGGCT |
| c.2548-253C>T | IVS21 ds-253C>T | 238 bp pseudoeoxon | GACAGAGAGAGAGAGACTG     | CACTCTTGACCTGACTC         | CTTCTGAGCTACCTAGCTC  | CACCTTGAGCTACAACAAGGCT |
| c.2612G>A  | p.R871H | Missense      | GACAGAGAGAGAGAGACTG     | CACTCTTGACCTGACTC         | CTTCTGAGCTACCTAGCTC  | CACCTTGAGCTACAACAAGGCT |
| c.2875-2876delAG | p.R959fs | Frameshift    | GACAGAGAGAGAGAGACTG     | CACTCTTGACCTGACTC         | CTTCTGAGCTACCTAGCTC  | CACCTTGAGCTACAACAAGGCT |
amplification and were analyzed using the TaqMan Genotypeer software v.1.3 (Life Technologies).

Validation of all recurrent mutations
To test the function of each mutation detection assay in this recurrent mutation-based mutation detection plate, a blind study was conducted for each assay. DNA samples from all 130 unrelated GS families were included with concealed genotypes and plated in duplicates on the 96-well plates, using 100 ng DNA of each sample as a template in the above-described RT-PCR assay. The genotypes of each sample were uncovered for comparison at the end of the blind study to evaluate the accuracy. Besides, target recurrent mutations were inserted into vector pUC57 as a positive control for each assay as well as two no-template controls as negative controls. When referring to the sensitivity and specificity, each TaqMan assay was accessed in DNA samples including GS patients with or without specific mutations and healthy subjects.

Clinical validation in newly diagnosed GS patients
Patients newly diagnosed as GS over a year were prospectively enrolled to validate this mutation detection plate with Sanger sequencing confirmation. Vector pUC57 carrying homozygous and heterozygous target recurrent mutations was applied concomitantly as a positive control. DNA samples from three healthy subjects and vector pUC57 with wild-type SLC12A3 were also used as wild-type control as well as one no-template control as a negative control (Supplementary Fig. 1). If the detection plate fails to make a definite diagnosis, further genetic analyses as mentioned above were performed.

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

DATA AVAILABILITY
Data generated or analyzed during this study are included in this published article and its supplementary information files.

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AUTHOR CONTRIBUTIONS

S.-H.L. and M.-T.Y. designed the study and drafted the paper; S.-S.Y. and M.-T.Y. carried out experiments; S.-H.L., J.-D.T., C.-J.C. and M.-T.Y. collected and analyzed data; C.-C.S. and M.-H.T. made the figures; S.-H.L., C.-J.C., Y.-J.H. and M.-T.Y. revised the paper; all authors reviewed the results and approved the final manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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