**Short Communication**

**Cryptic Exon Activation in *SLC12A3* in Gitelman Syndrome**

Kandai Nozu 1, Yoshimi Nozu1, Keita Nakanishi1, Takao Konomoto2, Tomoko Horinouchi1, Akemi Shono1, Naoya Morisada1, Shogo Minamikawa1, Tomohiko Yamamura1, Junya Fujimura1, Koichi Nakanishi3, Takeshi Ninchoji1, Hiroshi Kaito1, Ichiro Morioka1, Mariko Taniguchi-Ikeda1, Igor Vorechovsky4, and Kazumoto Iijima1

1Department of Pediatrics, Kobe University Graduate School of Medicine, Kobe, Japan; 2Department of Pediatrics, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan;

3Department of Pediatrics, Wakayama Medical University, Wakayama;

4University of Southampton Faculty of Medicine, Southampton, United Kingdom

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**Corresponding author:**

Kandai Nozu, M.D., Ph.D.

Department of Pediatrics,

Kobe University Graduate School of Medicine,

7-5-1 Kusunoki-cho, Chuo, Kobe, Hyogo 6500017, Japan.

Tel.: +81-78-382-6090; Fax: +81-78-382-6099

E-mail: nozu@med.kobe-u.ac.jp

**Abstract**

Gitelman syndrome (GS) is an autosomal recessive renal tubulopathy characterized by hypokalemic metabolic alkalosis with hypocalciuria and hypomagnesemia. GS clinical symptoms range from mild weakness to muscular cramps, paralysis or even sudden death as a result of cardiac arrhythmia. GS is caused by loss-of-function mutations in the *SLC12A3* gene, but molecular mechanisms underlying such a wide range of symptoms are poorly understood. Here we report cryptic exon activation in *SLC12A3* intron 12 in a clinically asymptomatic GS, resulting from an intronic mutation c.1669+297T>G that created a new acceptor splice site. The cryptic exon was sandwiched between the L3 transposon upstream and a mammalian interspersed repeat downstream, possibly contributing to inclusion of the cryptic exon in mature transcripts. The mutation was identified by targeted next-generation sequencing of candidate genes in GS patients with missing pathogenic *SLC12A3* alleles. Together, this work illustrates the power of next-generation sequencing to identify causal mutations in intronic regions in asymptomatic individuals at risk of developing potentially fatal disease complications, improving clinical management of these cases.

**Introduction**

Gitelman syndrome (GS, OMIM 263800) is one of the most common autosomal recessive kidney tubulopathies, with an estimated prevalence of 1:40,000 in Caucasians [1](#_ENREF_1), [2](#_ENREF_2). GS is characterized by hypokalemia, hypomagnesemia, metabolic alkalosis, and hypocalciuria, and usually manifests as mild weakness, cramps, and general fatigue. However, some GS cases are clinically asymptomatic or are not diagnosed until late childhood or adulthood[3](#_ENREF_3), but molecular mechanisms for the variable penetrance and age of onset are poorly understood.

GS is caused by mutations in the solute carrier family 12 member 3 gene (*SLC12A3*) [4](#_ENREF_4) that encodes the thiazide-sensitive sodium–chloride cotransporter NCCT [4](#_ENREF_4). The loss of NCCT function leads to a decrease in sodium and chloride reabsorption in the distal convoluted tubule, causing salt-wasting tubulopathy. To date, more than 400 different *SLC12A3* mutations have been identified in GS, however, as many as 20-41% patients were found only with a single pathogenic allele, suggesting that the mutation screening is unsatisfactory [3-8](#_ENREF_3). Although employing reverse transcription (RT)-PCR that may help identify deep intronic mutations resulting in RNA processing abnormalities [9](#_ENREF_9), [10](#_ENREF_10), this method may not identify all aberrant mRNAs. The reason for the large fraction of missing GS alleles is poorly understood.

Here, we report a case of latent GS caused by a partial cryptic exon activation in *SLC12A3* intron 12*.* Identification of the new exonwas facilitated by next-generation sequencing (NGS) followed by *in silico* analysis and RT-PCR validation of aberrant transcripts. The case highlights the importance of detecting intronic variants in low-penetrance genetic conditions at risk of potentially fatal complications, permitting more a focused management of affected families.

**Materials and Methods**

The proband was a 5-year-old girl diagnosed by chance with mild proteinuria (urinary protein/creatinine: 0.4 g/gCr), hypokalemia (2.7 mEq/L), hypomagnesemia (1.5 mg/dL), metabolic alkalosis (HCO3−: 26.7 mEq/L), and hypocalciuria (urinary calcium/creatinine: 0.005 mg/mg). The laboratory findings were indicative of GS, but she had not suffered any overt clinical symptoms.

All procedures were reviewed and approved by the Institutional Review Board of Kobe University School of Medicine. Informed consent was obtained from all patients or their parents.

NGS samples were prepared using a HaloPlex target enrichment system kit according to the manufacturer’s instructions (Agilent Technologies, Santa Clara, CA) to capture 12 genes (Supplemental Table 1), including *SLC12A3*, *CLCNKB*, *SLC12A1*, *KCNJ1*, and other genes associated with hypokalemia. Amplified target libraries were sequenced using MiSeq (Illumina, San Diego, CA), which was followed by variant analysis with SureCall (v. 3.0; Agilent Technologies). The *SLC12A3* reads were mapped to the human reference sequence NC\_000015.9 and NM\_000338.2. Exons were numbered according to a previous report[11](#_ENREF_11). Rare variants with a frequency less than 1% were analyzed using Human Splicing Finder v3.0 (<http://www.umd.be/HSF3/>).

**Results**

Conventional mutation screening of *SLC12A3* in the proband revealed only a single pathogenic allele on the maternal chromosome (c. 2927C>T, p.Ser976Phe), which was previously reported in two patients [12](#_ENREF_12), [13](#_ENREF_13). We next used the HaloPlex target enrichment system analysis of candidate genes for tubulopathies/pseudo-GS, including *SLC12A3*. NGS detected a total of 88 heterozygous intronic *SLC12A3* variants (Figure 1A). Analysis of variants with a Minor Allele Frequency (MAF) of less than 1% using the Human Splicing Finder (v 3.0) software [14](#_ENREF_14) revealed a single heterozygous variant predicted to create a new 3′ splice site, c.1567+297T>G, located in intron 12. This change had a potential of activating a cryptic exon with a high-score cryptic 5′ splice site further downstream (Supplemental Figures 1A,B). Validation of the putative cryptic exons using RT-PCR with primers in exons 12 and 13 revealed a 108-bp insertion of the new coding sequence in the patient’s *SLC12A3* mRNA (Figures 1C, D), confirming the *in silico* prediction. The new exon was sandwiched between the L3 transposon and a mammalian interspersed repeat located upstream and downstream, respectively (Fig. 1C,D and Supplemental Figure 2 and 3). The presence of each mutation was confirmed in parental samples (Figure 1E). These results indicated that the GS proband was a compound heterozygote for *SLC12A3* mutations, one resulting in amino acid substitution S976F in the C-terminal domain of NCCT and the other in protein truncation through cryptic exon activation.

We then employed NGS in two more GS cases with previously reported deep intronic splicing mutations. In Case 1, NGS detected a total of 30 heterozygous intronic *SLC12A3* variants, including mutation c.1670-191C>T, which created a new 5′ splice site (Supplemental Figures 4A,B,C) and activated the cryptic exon, as reported [10](#_ENREF_10). In Case 2, NGS detected a total of 27 heterozygous intronic *SLC12A3* variants, including variant c.2548+253C>T, which creates a new 3′ splice site (Supplemental Figures 5A,B, C) [9](#_ENREF_9).

**Discussion**

Previously, we reported two deep intronic *SLC12A3* mutations, both of which were hotspots for Japanese and Taiwanese GS patients, suggesting that these cases might be observed more frequently [9](#_ENREF_9), [10](#_ENREF_10). In this report, we used NGS to detect missing alleles in a suspected GS. Our results clearly demonstrate that NGS can facilitate their identification in asymptomatic cases that may develop potentially life-threatening complications later in life. As an example, arrhythmia as a result of hypokalemia can develop in GS children as young as 6 years of age[15](#_ENREF_15). To confirm the utility of this method, NGS successfully identified known variants in two other GS cases possessing deep intronic variants that affected splicing.

Interestingly, the novel cryptic exon was activated in a region closely flanked by a long interspersed repeat upstream and a mammalian interspersed repeat (MIR) downstream (Supplemental Figure 3). MIRs have a propensity to exonize by a single mutation, and have very high exonization levels compared with other transposable elements [16](#_ENREF_16), although it remains to be seen if this element facilitated usage of the new 3’ splice site.

Although NGS is still more expensive compared to conventional direct sequencing, NGS of DNA samples may complement RT-PCR approaches and facilitate identification of aberrant transcripts missed by conventional techniques. Definite genetic diagnosis of GS will permit better management of patients and improve their quality of life.

In conclusion, we identified a novel *SLC12A3* allele in GS that activates a cryptic exon flanked by interspersed repeats deep in intron 12. Our study illustrates the power of NGS to fully define the mutation pattern in GS and also improve our understanding of the phenotypic variability of this condition. This approach can be used more widely to identify individuals at risk of fatal complications in many other genetic disorders, providing a better support for their clinical management.

**Conflict of Interest:** The authors have nothing to disclose.

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**Figure legends**

**Figure 1. Cryptic exon activation in *SLC12A3* in GS.** A. Flow chart illustrates the step-wise identification of the intronic mutation in our proband. MAF, minor allele frequency. B. Genomic sequence of *SLC12A3* intron 12 in the proband. Mutation c.1567+297T>G was detected by next-generation sequencing and confirmed by Sanger sequencing. C. Transcript analysis by RT-PCR. Left panel: S, size marker with fragment sizes shown to the left; C, control; P, patient. The fraction of aberrant transcripts was ~12% of the total signal from polyadenylated RNAs. Right panel: Direct sequencing of the larger transcript showed a 108-bp insertion between exons 12 and 13. D. Schematics of the cryptic exon activation. Exons are shown as boxes, introns as lines, aberrant transcripts as dotted lines. Transposed elements are schematically shown by green rectangles and correspond to alignments in Supplemental Figure 3. E, Pedigree of the GS family. The proband is denoted by an arrow. Each GS allele was inherited from the parents.