

Detection of a Transcript Abnormality in mRNA of the SLC12A3 Gene Extracted From Urinary Sediment Cells of a Patient With Gitelman's Syndrome

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ABSTRACT: To date, many mutations, including intronic nucleotide changes, in the *SLC12A3* gene encoding the thiazide-sensitive sodium-chloride cotransporter (NCCT) have been reported in Gitelman's syndrome (GS) patients. However, it has not been clarified whether intronic nucleotide changes affect mRNA content. Since mRNA analysis is possible only after obtaining renal biopsy specimens, no studies have been conducted to identify transcript abnormalities in GS. In the study reported here, we investigated such transcript abnormalities for the first time by using mRNA expressed in a patient's urinary sediment cells. Direct sequencing analysis of leukocyte DNA disclosed one known missense mutation (R399C) and one known nucleotide change of the splicing acceptor site of intron 13 (1670-1 g > t). mRNA extracted from the urinary sediment cells was analyzed by RT-PCR to determine the pathogenic role of the intron mutation. A fragment encompassing exon 13 to 15 was amplified as two products, one consisting of all three exons and the other lacking only exon 14 in its entirety. Our investigation was the first to demonstrate exon 14 skipping in an NCCT transcript in renal cells. This methodology thus constitutes a potential noninvasive analytical tool for every inherited kidney disease. (*Pediatr Res* 61: 502–505, 2007)

Gitelman's syndrome (GS, OMIM 263800) is an autosomal recessive renal disorder characterized by hypokalemia, hypomagnesemia, metabolic alkalosis, and hypocalciuria (1). Mild weakness, cramps, and general fatigue are clinically observable, but they are often so slight that patients with GS are not diagnosed until late childhood or even adulthood (2,3). Simon *et al.* demonstrated that mutations in the *SLC12A3* gene encoding the thiazide-sensitive sodium-chloride cotransporter (NCCT) are responsible for GS. The *SLC12A3* gene, which is located in chromosome 16 and comprises 26 exons, is a 1021-amino acid protein with 12 predicted transmembrane domains (4). The lack of a functional NCCT leads to a decrease in sodium and chloride

reabsorption in the distal convoluted tubule and an increase in solute delivery to the collecting duct. These changes result in blood volume reduction, activation of the renin-angiotensin-aldosterone system, and increased reabsorption of sodium and secretion of potassium and hydrogen ions in the collecting duct.

To date, more than 100 different mutations of the *SLC12A3* gene have been identified throughout the gene in patients with GS. Since a kidney biopsy is not usually performed for these patients, the majority of studies have dealt with mutational analyses of the *SLC12A3* gene by using genomic DNA obtained from peripheral blood leukocytes. Although nucleotide changes in the intronic sequence have been reported, the exact nucleotide pathogenic mechanism has not yet been identified. On the assumption that *SLC12A3* is expressed in urinary sediment cells, *SLC12A3* mRNA was investigated in a Japanese patient with GS to determine whether abnormal transcripts were present.

MATERIALS AND METHODS

Case report. A 14-y-old Japanese girl was referred to our institution for evaluation of hypokalemia. She was suffering from headaches, nausea and appetite loss. When she contracted acute tonsillitis at 7 y of age, her family doctor performed a blood examination and found that her serum potassium level was low, and since it remained low, the doctor diagnosed her with idiopathic hypokalemia. No medicine such as diuretics or laxatives had been prescribed. Upon admission, she was 139.3 cm tall (–2.5SD) and weighed 32.4 kg. Her blood pressure was 106/66 mm Hg and physical examination findings were normal. Laboratory results showed that serum potassium was 2.6 mEq/L (normal 3.5–4.7mEq/L), plasma renin activity was extremely elevated (23.7 ng/mL/h: normal 0.2–3.9 ng/mL/h) and aldosterone concentration was near the upper limit of the normal range (20.6 ng/dL: normal 3–21 ng/dL). Serum magnesium and urinary calcium levels were low. The main biochemical study findings are shown in Table 1. Her parents were noncon-sanguineous.

The Ethical Committee of Kobe University Graduate School of Medicine approved this study and consent for this study was obtained from the patient's parents.

Genomic DNA analysis. Genomic DNA was isolated from peripheral blood leukocytes of the patient as well as normal control subjects using the Qiagen kit (Qiagen Inc., Chatsworth, CA, USA), according to the manufacturer's instructions. Twenty-six pairs of oligonucleotide primers were generated to amplify all 26 exons of the NCCT gene (4). Polymerase chain reaction (PCR) was performed with 20 μ L of a solution containing 0.2 mM dNTP, 0.05 U/ μ L *Taq* polymerase, 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), approximately 50 ng genomic DNA, and 1 μ M of each primer with a thermoprofile of 96°C for 1 min, 58°C for 1 min, and 72°C for 1 min, 35 cycles each. The PCR products were separated on an agarose gel, isolated, and

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Abbreviation: GS, Gitelman's syndrome

Table 1. Biochemical data of the GS patient

Parameter	Normal range	Value
Serum		
Creatinine (mg/dl)	0.5–1.3	0.6
Urea (mg/dl)	10.0–20.0	17.6
Sodium (mEq/l)	137–146	138
Potassium (mEq/l)	3.5–4.7	2.6
Chloride (mEq/l)	99–109	97
Magnesium (mg/dl)	1.7–2.3	1.4
pH	7.35–7.45	7.461
HCO ₃ (mmol/l)	21–29	32.2
BE (mmol/l)	0 ± 2	8.2
Renin Activity (ng/ml/hr)	0.2–3.9	23.7
Aldosterone (ng/dl)	3–21	20.6
Urine		
Calcium/Creatinine	0.1–0.3	0.03

purified with a DNA purification kit (Qiagen Japan, Tokyo). They were then analyzed, including every intron-exon boundary, by direct sequencing with a DNA sequencer (Perkin-Elmer-ABI, Foster City, CA, USA).

RNA expression analysis. Total RNA was extracted from urine sediments. The urine sediments were obtained by centrifugation at 1500 r.p.m. for 10 min from 50 mL of early morning urine. Microscopic examination of these sediments confirmed that there were sufficient renal tubular epithelial cells. RNA was isolated by using Trizol (Invitrogen, Carlsbad, CA, USA), and was then reverse-transcribed into cDNA by using random hexamers and the Superscript III kit (Invitrogen). cDNA was amplified by nested PCR with forward primers located in exon 10 or 13, and the reverse primer complementary to exon 15 (exon 10, first forward: CTCCAAGGGCTTCTCAGC; second forward: TCTGGACGAC-CATTTCCTACC; exon13, first forward: GTACCCACTGATCGGCTTCT; second forward: TTCTCTGTCTCTATGCC; exon 15, first reverse: TCCTCG-GCAATGACATCC; second reverse: GGGCGGTAGTCTTGTATGT). After 40 cycles of amplification, PCR products were separated on 2% agarose. In addition, PCR products were sequenced with a DNA sequencer (Perkin-Elmer-ABI, Foster City, CA, USA). We used the Human Kidney cDNA Library (Invitrogen) to obtain normal control kidney cDNA.

RESULTS

Direct sequencing of the PCR-amplified products disclosed two peaks of C and G at position 1195 in exon 10. This nucleotide change altered the 399th codon from arginine to cysteine (1195 C > T, R399C; Fig. 1A) showing a heterozygous missense mutation in exon 10. The second abnormality was double peaks of g and t, that is, a heterozygous g to t substitution at position –1 bp of the 3' acceptor splice site of intron 13 (1670-1 g > t; IVS 13-1 g > t in one allele; Fig. 1B). It was therefore hypothesized that exon 14 might have been skipped in the transcript.

To confirm the inactivation of the splice-acceptor site, mRNA extracted from urinary sediments was used to amplify a fragment of exons 13 to 15 by RT-PCR with a pair of primers recognizing exon 13 and 15. PCR products from the patient's sample showed two bands, one the same size as the control, and the other shorter (Fig. 1C). Sequencing of the shorter product disclosed complete absence of the exon 14 sequence: exon 13 was directly joined to exon 15 (Fig. 1D).

The best way to prove compound heterozygous mutation when a patient has two heterozygous mutations at the different position is to analyze the patient's parents. In our case, however, we could not conduct a genetic analysis of her parents because informed consent could not be obtained. To confirm the presence of these two mutations in each of the alleles, a fragment of exon 10 to 15 was also amplified by

RT-PCR and two PCR products were obtained, one the same size as the normal control, and the other shorter. Each of the two products was then separated and sequenced, enabling us to demonstrate that the normal size PCR product included a complete C to G nucleotide substitution at position 1195 in exon 10, and the shorter one completely lacked exon 14 without the C to G nucleotide change in exon 10 (Fig. 2). These results suggest that our patient is characterized by a compound heterozygous mutation in *SLC12A3*, and that these mutations had caused GS.

DISCUSSION

We identified two heterozygous mutations, 1195 C > T and 1670-1 g > t, in the genomic DNA from the leukocytes of a typical case of GS. These mutations are common as previously reported (4,5), and we supposed that these compound heterozygous mutations of NCCT had caused GS in our patient. To confirm this hypothesis, we amplified *SLC12A3* mRNA extracted from urinary sediments and confirmed beyond a doubt that exon 14 has been skipped and those two mutations exist on different alleles.

Igarashi *et al.* were the first to demonstrate transcript abnormalities by extracting mRNA from urinary sediment cells of patients with Dent's disease (6). This method proved to be very useful for analyzing mRNA expression in renal tubular cells in various kidney diseases, but no further studies have been reported since then and our study is the first to use this method for GS.

The mere detection of intronic mutations is not adequate for an accurate analysis of inherited diseases, however. It is especially important to identify transcript abnormalities in every target organ as well as to prove the presence of two heterozygous nucleotide changes as a compound heterozygous mutation. Renal biopsy specimens can be used as one source for analyzing mRNA in inherited kidney diseases, but renal biopsy is invasive and not needed for some kidney diseases. Urinary sediments, on the other hand, contain cells derived from the kidney, and genetic analysis using those cells constitutes an entirely noninvasive, simple method for the diagnosis of inherited kidney diseases. Furthermore, measurement of urinary mRNA expression has recently gained attention as a potential noninvasive tool for the monitoring of certain kidney disease activities. Some studies have demonstrated that mRNA analysis in the urine may serve as a noninvasive tool to assess intrarenal damage in chronic kidney disease (CKD) or lupus nephritis activity (7,8). Genetic analysis using mRNA in urinary sediment cells can also be used for all other inherited kidney diseases including renal tubular disorders. We therefore conducted RT-PCR using urinary sediment cells to confirm the presence of both transcript abnormalities in the target organ cells and of a compound heterozygous mutation in our patient.

In addition, we used RT-PCR to prove that two mutations in exon 10 and intron 13 exist in different alleles. However, it was not possible to prove this by using genomic DNA because the two mutations are more than 3000 base pairs (bp) apart, while in the case of cDNA, the distance is only about 700 bp and it is easy to amplify the fragment using RT-PCR. In this

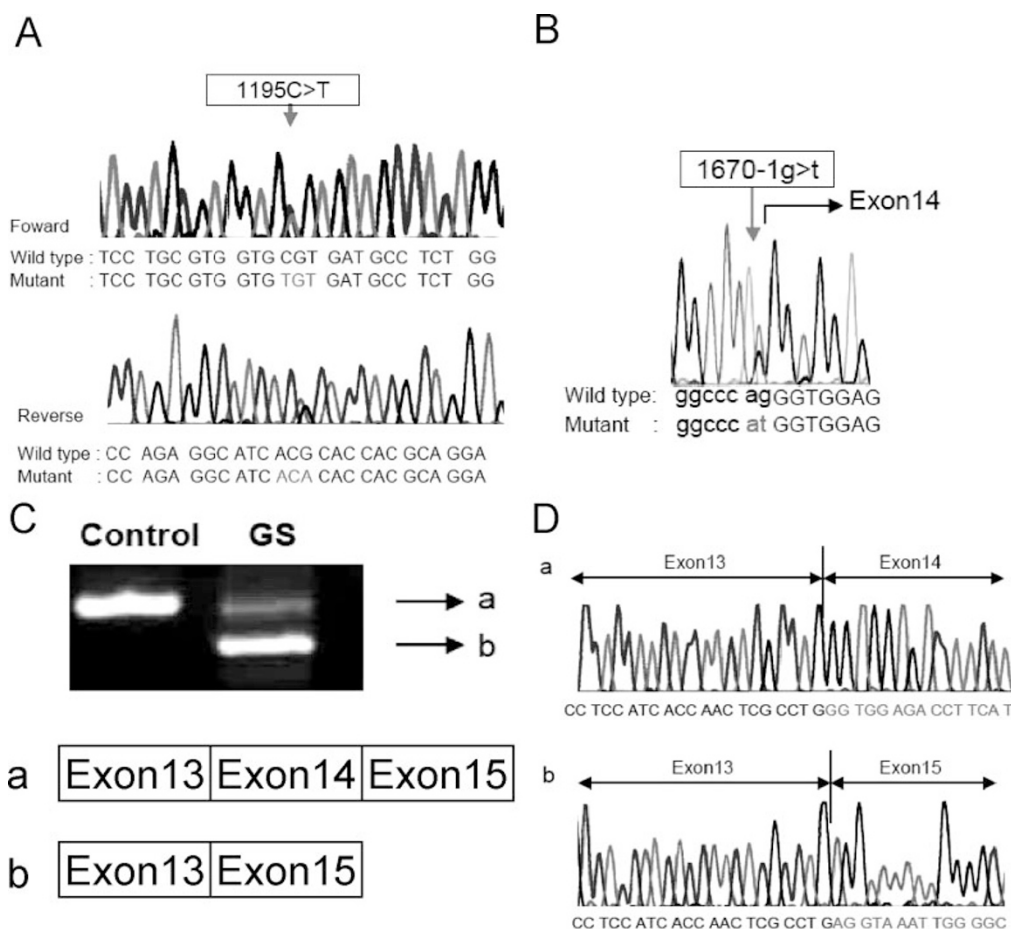


Figure 1. Nucleotide changes and a transcript abnormality in *SLC12A3* gene. (A) DNA chromatograms in forward (top) and reverse (bottom) frames of the patient. A heterozygous transition (C > T) at nucleotide 1195 located in exon 10 resulted in an arginine to cysteine substitution at amino acid 399 (1195 C > T, R399C). (B) Another nucleotide change identified in the GS patient by direct sequencing analysis. A heterozygous transition (g > t) at position -1 bp of the acceptor splice site of intron 13 (1670-1 g > t) led to the supposition that exon 14 might have been skipped in the transcript. (C) Electrophoresis of cDNA after PCR. PCR using the forward primer located in exon 13 and the reverse one located in exon 15. Control sample (left) clearly shows a single band. The sample from the GS patient (right) shows two bands, one the same size as the control sample (a), and the other smaller (b). (D) Fragments of the sequence of the sense strand of cDNA from the patient. Normal product (a) shows a normal sequence. The smaller product (b), however, shows that exon 13 is followed by exon 15, so that exon 14 has been skipped. The same result was found when the antisense strand was sequenced.

respect, the approach using mRNA for genetic analysis offers many more benefits.

In genetic analysis using RT-PCR, however, the problem is sometimes encountered that the nonsense-mediated decay (NMD) phenomenon interferes with detection of nonsense mutations in RT-PCR (9,10). The NMD pathway is an mRNA surveillance system that typically degrades transcripts con-

taining premature termination codons (PTCs) to prevent translation of unnecessary or aberrant transcripts. Failure to eliminate these mRNAs with PTCs may result in the synthesis of abnormal proteins that can be toxic to cells through dominant-negative or gain-of-function effects. In this study, we confirmed by means of RT-PCR that exon 14 was skipped in mRNA. However, exon 14 of the *SLC12A3* gene possesses 156bp and this exon skipping creates an in-frame mutation, so that NMD does not work in this case. It is possible that frame-shift exon skipping mutations cannot be detected with this method, however, some frame-shift mutations could be detected with RT-PCR methods as previously reported (11,12,13). Riancho *et al.* detected exon 9 skipping in the *SLC12A3* gene by means of RT-PCR using mRNA from peripheral leukocytes. Exon 9 extends over 85bp so that this exon skipping causes a frame shift (11). We previously reported the detection in Alport syndrome patients (12) or muscular dystrophy patients (13) of various types of nonsense mutations and frame-shift mutations in RT-PCR using mRNA from peripheral leukocytes or muscle. We have also con-

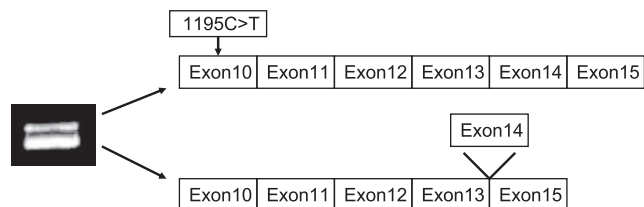


Figure 2. Confirmation of compound heterozygous mutation. Electrophoresis of cDNA from a GS patient after PCR using the forward primer located in exon 10 and the reverse one located in exon 15. Two bands are seen, both sequenced by direct sequencing. A transition (C > T) at nucleotide 1195 located in exon 10 (1195 C > T) is seen in the larger product, and the smaller one completely lacks exon 14. These results confirm that the patient has a compound heterozygous mutation in the *SLC12A3* gene.

ducted a genetic analysis of a type III Bartter syndrome case using the method we described in this report, and identified the mutation type as nonsense mutation (W610X) in the *CLCNKB* gene. This result demonstrated that the nonsense mutation could be detected in the transcript (data not shown). We should therefore keep in mind the possibility that the use of this technique may sometimes be affected by NMD, in that frame-shift exon skipping mutations may be missed when using RT-PCR.

In conclusion, we report the successful use of urinary sediment cells for the identification of a NCCT transcript abnormality in a GS patient. This mutation was found to eliminate a consensus splicing acceptor site, resulting in exon 14 skipping in the mRNA from the urinary sediment cells. Although this mutation is common, as previously reported, the functional test for the missense mutation and exon skipping of NCCT was not conducted, so that in this case it could not be determined whether these mutations necessarily lead to the disease. However, the most important finding of our study is that this transcript abnormality could be identified with such a noninvasive method. The analysis of mRNA in urinary sediment cells can be used for all other kidney diseases, not only for monitoring disease activities as previously reported, but also for genetic analysis to detect transcript abnormalities in kidney cells and this methodology may serve as a potential noninvasive analytical tool.

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