Mutational analyses of the ATP6V1B1 and ATP6V0A4 genes in patients with primary distal renal tubular acidosis

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ABSTRACT

Background. Mutations in the ATP6V1B1 and the ATP6V0A4 genes cause primary autosomal-recessive distal renal tubular acidosis (dRTA). Large deletions of either gene in patients with dRTA have not been described.

Methods. The ATP6V1B1 and ATP6V0A4 genes were directly sequenced in 11 Japanese patients with primary dRTA from nine unrelated kindreds. Large heterozygous deletions were analyzed by quantitative real-time polymerase chain reaction (PCR). The clinical features of the 11 patients were also investigated.

Results. Novel mutations in the ATP6V1B1 gene were identified in two kindreds, including frameshift, in-frame insertion and nonsense mutations. Large deletions in the ATP6V0A4 gene were identified in two kindreds. Exon 15 of ATP6V0A4 was not amplified in one patient, with a long PCR confirming compound heterozygous deletions of 3.7- and 6.9-kb nucleotides, including all of exon 15. Direct DNA sequencing revealed a heterozygous frameshift mutation in

ATP6V0A4 in another patient, with quantitative real-time PCR indicating that all exons up to exon 8 were deleted in one allele. Clinical investigation showed that four of the six patients with available clinical data presented with hyperammonemia at onset.

Conclusions. To our knowledge, these dRTA patients are the first to show large deletions involving one or more entire exons of the ATP6V0A4 gene. Quantitative PCR amplification may be useful in detecting heterozygous large deletions. These results expand the spectrum of mutations in the ATP6V0A4 and ATP6V1B1 genes associated with primary dRTA and provide insight into possible structure–function relationships.

INTRODUCTION

Primary distal renal tubular acidosis (dRTA) is a rare genetic disease caused by impaired excretion of hydrogen ions (H+) by intercalated cells in the collecting ducts [1, 2]. Its clinical
features include hyperchloremic acidosis with inappropriately alkaline urine, hypokalemia, hypercalciuria, nephrocalcinosis and nephrolithiasis. Affected infants present with polyuria, dehydration and failure to thrive [3]. Both autosomal-dominant and autosomal-recessive forms of dRTA have been described. Mutations in the ATP6V1B1 gene, which encodes the B1 subunit of the H+–ATPase, have been associated with autosomal-recessive dRTA accompanied by severe sensorineural hearing loss (SNHL), whereas mutations in the ATP6V0A4 gene, which encodes the a4 subunit of the H+–ATPase, have been associated with autosomal-recessive dRTA without SNHL [1, 4, 5]. Recent genetic analyses, however, have revealed that some individuals with mutations in the ATP6V0A4 gene also have early-onset severe SNHL [6, 7]. To expand knowledge of mutations in patients with dRTA, we analyzed the ATP6V1B1 and ATP6V0A4 genes in 11 Japanese patients from nine unrelated kindreds with dRTA.

MATERIALS AND METHODS

Patients

We analyzed 11 Japanese patients with dRTA belonging to nine unrelated kindreds; their clinical features and representative biochemical data are shown in Table 1. The diagnosis of dRTA was based on metabolic acidosis with a normal anion gap and overly high urinary pH, with or without nephrocalcinosis and SNHL. The parents of the patients in families 1 and 5 were consanguineous. Patients 1-1 and 1-2, and patients 7-1 and 7-2 were siblings. Detailed clinical data were not available for patient 5-1 at initial presentation, but she presented with full-blown symptoms of dRTA, including nephrocalcinosis and SNHL.

The study protocol was approved by the Ethics Committee of The University of Tokyo (Approval No. 2204). Informed consent was obtained from each patient and his or her parents.

Mutation analysis

Genomic DNA was extracted from the peripheral blood of the patients and their family members using QuickGene DNA whole-blood 5 kits (Fujiﬁlm). Thirteen and 20 pairs of oligonucleotide primers were generated to amplify all exons of the ATP6V1B1 and ATP6V0A4 genes, respectively (Supplemental Tables 1 and 2). Polymerase chain reactions (PCRs) were performed in 20 µL of solution containing AmpliTaq Gold 360 Master Mix (Applied Biosystems), ~30-ng genomic DNA and 10 pmol of each primer. The ampliﬁcation protocol consisted of an initial denaturation step at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at the temperatures shown in Supplemental Tables 1 and 2 for 30 s and elongation at 72°C for 30 s. PCR samples were subjected to bidirectional sequencing on an ABI 3130xl Genetic Analyzer (Applied Biosystems).

Quantitative real-time PCR amplification

The heterozygous large deletion in the ATP6V0A4 gene of patient 4-1 was analyzed by quantitative genomic real-time PCR using SYBR Green (Bio-Rad Laboratories, Hercules, CA) and the primer sequences shown in Supplemental Table 2. The standard curve method was used to calculate the target genome numbers in patient 4-1, with the target copy number normalized to normal human genomic DNA. Statistical significance was assessed using Student’s t-tests.

RESULTS

Clinical findings

A total of 11 patients from nine unrelated kindreds were enrolled in this study (Table 1). Consanguinity was noted in two families. Among the 10 patients with available data at onset, all but 1 (patient 2-1) presented with metabolic acidosis with a normal anion gap and abnormally high urinary pH. Although patient 2-1 had a near normal serum bicarbonate concentration, she presented with hypokalemia, hypercalciuria, growth retardation and bilateral SNHL. Supplementation with bicarbonate improved her growth. Urine anion gap was positive or near-zero in all patients with available data, which is consistent with reduced NH4+ excretion as seen in dRTA [8].

Serum creatinine concentrations in all patients were within normal range throughout the follow-up period. Growth retardation in infancy occurred in 9 of the 11 patients, with alkali therapy improving their growth to almost normal range. Of the two patients without growth retardation at onset, one (patient 7-2) was diagnosed with dRTA by screening tests before presenting with any symptoms because her older sister had been diagnosed with dRTA. The other patient with normal growth during infancy (patient 9-1) presented with severe weight loss, metabolic acidosis, abnormally high urinary pH and hypercalciuria at age 7 years.

Abdominal ultrasonography showed nephrocalcinosis in nine patients. Unilateral or bilateral SNHL, as determined by audiograms, were observed in four patients. Hypokalemia was observed in six of the nine patients with available data and hypercalciuria in seven of nine. The chart of an additional patient (patient 3-1) mentioned hypercalciuria, although precise data were not available.

Hyperammonemina was observed in four of the six patients with available data, improving in all four after correction of metabolic acidosis. Of the two patients without hyperammonemia, one (patient 4-1) had high-normal (60 µg/dL) serum ammonia concentration, whereas the other (patient 2-1) had normal serum ammonia, a finding probably associated with the absence in this patient of profound metabolic acidosis at onset.

Patient 7-2, a younger sister of patient 7-1, was diagnosed with dRTA 10 days after birth by screening blood and urine tests. Since then, she has been treated with citrate and her growth is normal. At age 8 months, she presented with transient metabolic acidosis resulting from insufficient supplementation with citrate. A higher dosage of citrate improved her symptoms. Of note, she is free from nephrocalcinosis at age 2 years. In contrast, her older sister, patient 7-1, presented with prominent nephrocalcinosis at age 1 month.
Table 1. Clinical features of the patients

<table>
<thead>
<tr>
<th>Consanguinity</th>
<th>Age/sex</th>
<th>Age at onset</th>
<th>FTT</th>
<th>NC</th>
<th>SNHL</th>
<th>pH</th>
<th>HCO₃⁻ (19–23) mmol/L</th>
<th>Potassium (3.5–6.0) mEq/L</th>
<th>Serum ammonia (12–66) µg/dL</th>
<th>Urine pH</th>
<th>Urine anion gap, mEq/L</th>
<th>Urine calcium excretion (&lt;4 mg/kg/day, Ca/Cr &lt;0.21)</th>
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<td>Patients with mutations in the ATP6V1B1 gene</td>
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<tr>
<td>1-1</td>
<td>+</td>
<td>46 years/M</td>
<td>Infancy</td>
<td>+</td>
<td>+</td>
<td>+rt</td>
<td>7.230</td>
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<td>3.4</td>
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<td>4</td>
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<tr>
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<td>+</td>
<td>38 years/F</td>
<td>Infancy</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>7.215</td>
<td>12.4</td>
<td>1.8</td>
<td>NA</td>
<td>7.0</td>
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<td>2-1</td>
<td>–</td>
<td>1 year/F</td>
<td>9 months</td>
<td>+</td>
<td>+</td>
<td>+bil</td>
<td>7.325</td>
<td>19.6</td>
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<tr>
<td>3-1</td>
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<td>2 months</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>7.293</td>
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<td>1 month</td>
<td>+</td>
<td>+</td>
<td>+bil</td>
<td>NAᵇ</td>
<td>NAᵇ</td>
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<td>+</td>
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<td>–</td>
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<td>14.1</td>
<td>4.1</td>
<td>94</td>
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<tr>
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<td>1 month</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<td>11.2</td>
<td>4.1</td>
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<th>Age at onset</th>
<th>FTT</th>
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<th>Urine calcium excretion (&lt;4 mg/kg/day, Ca/Cr &lt;0.21)</th>
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<tr>
<td>7-2</td>
<td>−</td>
<td>3 years/F</td>
<td>10 days</td>
<td>−</td>
<td>−</td>
<td>7.354</td>
<td>14.1</td>
<td>3.8</td>
<td>144</td>
<td>8.0</td>
<td>28</td>
<td>Ca/Cr 0.27</td>
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<td>8-1</td>
<td>−</td>
<td>3 years/F</td>
<td>1 year 0 month</td>
<td>+</td>
<td>+</td>
<td>6.999</td>
<td>9.2</td>
<td>2.2</td>
<td>NA</td>
<td>7.5</td>
<td>−2</td>
<td>Ca/Cr 0.06</td>
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<tr>
<td>9-1</td>
<td>−</td>
<td>7 years/F</td>
<td>7 years</td>
<td>−</td>
<td>−</td>
<td>7.120</td>
<td>9.0</td>
<td>2.2</td>
<td>78</td>
<td>7.5</td>
<td>15</td>
<td>Ca/Cr 0.59</td>
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</table>

Normal values for infants are shown in parentheses.
FTT, failure to thrive; NC, nephrocalcinosis; SNHL, sensorineural hearing loss; rt, right; NA, not available; bil, bilateral; Ca/Cr, urine calcium to creatinine ratio (mg/mgCr).

aAlthough specific data were not available, hypercalciuria was noted in the chart.
bDetailed clinical data at initial presentation were not available in patient 5-1, but she presented with full-blown symptoms of dRTA, including nephrocalcinosis and SNHL.
cPatient 7-2 was diagnosed by screening tests when she had no symptoms.
dAlthough no growth retardation was noted in patient 9-1 in infancy, she presented with weight loss at the age of 7.
**Genetic analysis**

Three novel mutations in the \emph{ATP6V1B1} gene were identified in two of the nine kindreds (Figure 1). Patients 1-1 and 1-2 (family 1) presented with novel homozygous single nucleotide deletions (c.33delG) in exon 1, causing a frameshift that resulted in the premature termination of the protein at codon 19 (L19X) (Figure 1A). In addition, patient 2-1 presented with novel compound heterozygous mutations, consisting of an in-frame insertion (c.978_979insGCC or p.A326insA) and a nonsense mutation (c.1251C>G or p.Y417X). Genotyping of family members of patient 2-1 revealed that this patient had inherited the p.Y417X mutated allele from her mother (Figure 1B).

We observed novel aberrations of the \emph{ATP6V0A4} gene in two of the nine kindreds. In patient 3-1, the genomic PCR product of exon 15 was not amplified (Figure 2A), suggesting homozygous deletions that included exon 15. To confirm this finding, we performed long genomic PCR using primers for exons 14 (forward) and 16 (reverse), which amplified a 7.9-kb product derived from wild-type \emph{ATP6V0A4} allele in the healthy control (Figure 2B). In patient 3-1, however, these primers amplified two PCR products, of 4.2 and 1.0 kb, suggesting that deletions involving exon 15 were responsible for the aberrant \emph{ATP6V0A4} transcripts. Subsequent sequencing disclosed the presence of 3.7- and 6.9-kb deletions within introns 14 and 15, respectively, in each allele. These deletions caused a frameshift that resulted in premature termination of the protein at codon 536 (L536X) in exon 16 (Figure 2C).

We also observed a novel heterozygous large deletion of the \emph{ATP6V0A4} gene in patient 4-1. This was accompanied by a novel heterozygous single nucleotide insertion (c.1185insC) causing a frameshift that resulted in premature termination of the protein at codon 429 (p.E429X) in exon 13, as shown by direct DNA sequencing (Figure 3A). The quantities of the PCR products up to exon 8 seemed to be reduced (data not shown), suggesting the presence of an interstitial heterozygous deletion of an \emph{ATP6V0A4} allele in patient 4-1. To determine the \emph{ATP6V0A4} gene copy numbers in this patient, we performed quantitative genomic PCR of exons 3, 5, 6, 7, 8, 9, 10 and 18 using the same amounts of genomic DNA (1 µg/tube) from the patient and a healthy control. We found that the signal intensities of exons 3, 5, 6, 7 and 8 were significantly reduced compared with those of exons 9, 10 and 18 (P < 0.05), indicating that the N-terminal region up to exon 8 was deleted in one of the \emph{ATP6V0A4} alleles of patient 4-1 (Figure 3B).

Given that large deletions including one or more entire exons were frequently identified, multiplex ligation-dependent probe amplification analyses for both the \emph{ATP6V0A4} and \emph{ATP6V1B1} genes were performed in the five patients without any alterations of these two genes. No abnormalities in relative copy numbers were detected in these patients, suggesting that

![Figures 1-3](https://example.com/figures.png)
FIGURE 2: Novel large interstitial deletions in the \textit{ATP6V0A4} gene in patient 3-1. (A) Genomic PCR product of exon 15 was absent, whereas DNA fragments of exons 14 and 16 were amplified in patient 3-1. (B) Long genomic PCR using primers for exon 14 (forward) and exon 16 (reverse) in patient 3-1. Two aberrant sizes of the PCR products, 4.2 and 1.0 kb, were observed in patient 3-1, while a 7.9-kb product was detected in the healthy control. (C) Schematic presentation of large deletions in the \textit{ATP6V0A4} gene in patient 3-1. Deletions of 3.7- and 6.9-kb nucleotides including whole exon 15 were confirmed by subsequent sequencing analysis.

FIGURE 3: Novel compound heterozygous mutations comprising an insertion and a large deletion in the \textit{ATP6V0A4} gene in patient 4-1. (A) Genomic sequences of a novel heterozygous mutation in patient 4-1 (upper panel) as well as normal DNA from a healthy control (lower panel) are shown. (B) Quantitative genomic PCR of the \textit{ATP6V0A4} gene in patient 4-1. The signal intensity of exons 3, 5, 6, 7 and 8 was significantly reduced as compared with exons 9, 10 and 18 in patient 4-1, while the signal intensity was not significantly reduced in any exons in healthy control. The signal intensity of each exon was adjusted so that the intensity score was 1 in exon 18. *P < 0.05. P-value was assessed by Student’s \textit{t}-test. ex: exon.
they carry no homozygous or heterozygous large deletions of these two genes (data not shown).

**Clinical features of the patients with mutations in the ATP6V1B1 and ATP6V0A4 genes**

All the patients with mutations in the ATP6V1B1 or ATP6V0A4 gene presented with growth retardation in infancy and nephrocalcinosis accompanied by hypercalcemia. Three patients from three unrelated families presented with early-onset SNHL. Of these, two had mutations in the ATP6V1B1 gene (patients 1-1 and 1-2), and the third had a truncating mutation in one allele of the ATP6V0A4 gene and a large deletion that included several exons in the other allele (patient 4-1). Patients 1-1 and 1-2 are siblings and possess the same mutation in the ATP6V1B1 gene. However, only the brother (patient 1-1) presented with early-onset unilateral SNHL, which was confirmed by audiogram at age 9 years, while his 38-year-old sister (patient 1-2) has had no audiographic evidence of SNHL to date.

**DISCUSSION**

We have identified three ATP6V1B1 mutations (one deletion, one insertion and one nonsense mutation) and four ATP6V0A4 mutations (one insertion and three large deletions involving one or more exons) in patients with primary dRTA. These findings expand the spectrum of mutations in the ATP6V0A4 and ATP6V1B1 genes associated with primary dRTA, and provide insight into possible structure-function relationships.

To date, about 30 mutations in the ATP6V1B1 gene and about 40 in the ATP6V0A4 gene have been identified, including missense, nonsense, frameshift and splicing site mutations [1, 3, 6, 7, 9–12]. Prior to this study, however, large interstitial deletions involving one or more exons in either gene had not been reported. Thus, to our knowledge, this is the first report of patients with large deletions in one or both alleles of the ATP6V0A4 gene. Homozygous large interstitial deletions can be revealed by the absence of PCR products of particular exons. Although heterozygous large deletions are much harder to detect, quantitative real-time genomic PCR can be useful when PCR products of particular exons show relatively weak bands.

One of the compound heterozygous mutations in patient 2-1 was an in-frame insertion mutation (c.978_979insGCC/p. A326insA), the functional consequence of which should be addressed. A326 and T327 are highly conserved among species. Three-dimensional structural analysis shows that these amino acids are located near the hydrophobic residues at the interface between subunits A and B, which is critical for the catalytic activity [13].

In five of the nine kindreds, we observed no mutations in the ATP6V1B1 and ATP6V0A4 genes. In contrast, 80% of patients with dRTA were found to have a mutation in either gene [5, 14]. The low mutation frequency of these genes in our patients may be due to our small sample size and/or to differences in ethnicity, suggesting that dRTA is genetically heterogeneous. The clinical diagnosis seemed accurate in all of our patients, as all had metabolic acidosis with a normal anion gap and abnormally high urinary pH, accompanied by either nephrocalcinosis or hypercalcemia. Although hypokalemia was not noted at onset in three patients, it developed later in their clinical courses. H+-ATPase is composed of at least 13 different subunits, including the B1 and a4 subunits. The expression of the isoforms C2, G3 and d2, which are encoded by the ATP6V1C2, ATP6V1G3 and ATP6V0D2 genes, respectively, is restricted to the kidneys and a few other tissues [2, 15]. Although these genes were assessed as candidate causative genes in patients with recessive dRTA, no mutations were identified [15].

Our findings have important clinical implications. Administration of citrate salts is recommended for the prevention of nephrocalcinosis in dRTA, because they correct hypercalcemia and hypocitraturia [16]. Patient 7-2 was diagnosed with dRTA at age 10 days by screening blood samples, because her older sister, patient 7-1, had previously been diagnosed with dRTA. Alkali therapy was instituted immediately after diagnosis in patient 7-2, and this patient has shown no evidence of nephrocalcinosis at age 2 years. In contrast, her older sister, patient 7-1, presented with marked nephrocalcinosis at age 1 month. This difference in clinical manifestation between these two sisters, who are theorized to carry the same genetic abnormalities, suggests that nephrocalcinosis may be prevented by alkali therapy. As for early-onset SNHL, it was present in four patients, two with mutations in ATP6V1B1, one with a mutation in ATP6V0A4, and one without any mutations in either gene. This finding and the fact that only the brother (patient 1-1) in kindred one presented with unilateral SNHL confirm that intrafamilial variations in clinical manifestations can occur and that some individuals with mutations in the ATP6V0A4 gene also have early-onset SNHL [6, 7]. The mechanism of intrafamilial variations in hearing disabilities remains unclear. Although the younger sister (patient 1-2) was diagnosed with dRTA earlier than her older brother (patient 1-1), earlier initiation of alkali therapy is reported to have no effect on hearing loss [17]. In regard to laterality in SNHL (patient 1-1), asymmetrical progression of hearing disabilities has been previously described, the mechanism of which is also unknown [18]. We found that four of six patients with available data presented with hyperammonemia. To our knowledge, a high frequency of hyperammonemia in patients with dRTA has not been previously described, although a few patients were found to have hyperammonemia secondary to dRTA [19–21]. Hyperammonemia may result from an imbalance between increased synthesis and reduced excretion of ammonia. Chronic metabolic acidosis and hypokalemia can increase renal ammoniagenesis. However, in our cases, hypokalemia was noted in only one of four patients presenting with hyperammonemia. The precise mechanism by which hyperammonemia develops in dRTA remains to be determined.

In conclusion, we have described patients with dRTA and large interstitial deletions involving one or more exons in the ATP6V0A4 gene. Long genomic PCR as well as quantitative genomic PCR analyses can be useful in detecting large homozygous or heterozygous deletions. Clinically, nephrocalcinosis may be prevented by early and adequate alkali therapy.
Hyperammonemia is a frequent finding at onset in dRTA, suggesting that the latter condition be included as a miscellaneous cause of hyperammonemia.

SUPPLEMENTARY DATA

Supplementary data are available online at http://ndt.oxfordjournals.org.

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CONFLICT OF INTEREST STATEMENT

None declared.

REFERENCES


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