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Congenital chloride diarrhea needs to be distinguished from Bartter and Gitelman syndrome

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Key words

Pseudo-Bartter syndrome, Pseudo-Gitelman syndrome, Targeted sequencing, Next-generation sequencing, Congenital chloride diarrhea, *SLC26A3*

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Abstract

Pseudo-Bartter/Gitelman syndrome (p-BS/GS) encompasses a clinically heterogeneous group of inherited or acquired disorders similar to Bartter syndrome (BS) or Gitelman syndrome (GS), both renal salt-losing tubulopathies. Phenotypic overlap frequently occurs between p-BS/GS and BS/GS, which are difficult to diagnose based on their clinical presentation and require genetic tests for accurate diagnosis. In addition, p-BS/GS can occur as a result of other inherited diseases such as cystic fibrosis, autosomal dominant hypocalcemia, Dent disease or congenital chloride diarrhea (CCD). However, the detection of variants in genes other than known BS/GS-causing genes by conventional Sanger sequencing requires substantial time and resources. We studied 27 cases clinically diagnosed with BS/GS but with negative genetic tests for known BS/GS genes. We conducted targeted sequencing for 22 genes including genes responsible for tubulopathies and other inherited diseases manifesting with p-BS/GS symptoms. We detected *SLC26A3* gene variants responsible for CCD in two patients. In Patient 1, we found *SLC26A3* compound heterozygous variants: c.354delC; and c.1008insT. In Patient 2, we identified compound heterozygous variants: c.877G>A, p.(Glu293Lys); and c.1008insT. Our results suggest that a comprehensive genetic screening system using targeted sequencing is useful for the diagnosis of patients with p-BS/GS with alternative genetic origins.

Introduction

Bartter syndrome (BS) and Gitelman syndrome (GS) are autosomal recessive inherited salt-loss tubulopathies characterized by hypokalemic metabolic alkalosis with normal or low blood pressure despite hyperreninemia and hyperaldosteronemia. BS is reportedly caused by pathogenic variants in genes encoding renal tubular ion transporters or channels, leading directly or indirectly to loss of function [1-6]. Types I, II, IV, and IVb BS usually present during the neonatal period with relatively severe symptoms (antenatal BS), whereas type III BS (classic BS) and GS present during early childhood with milder symptoms. Moreover, variants in known disease-related genes have not been identified in about half of the patients with clinically diagnosed BS/GS [7]. This suggests that some clinical conditions may cause a BS-like disorder, or pseudo-BS/GS (p-BS/GS), associated with loss of sodium or chloride in the urine, stool, or vomitus, or with chloride-intake deficiency, resulting in clinical symptoms identical to those of BS/GS. It is difficult to clearly distinguish between p-BS/GS and BS/GS based on clinical findings owing to phenotypic overlap. Moreover, some other inherited diseases, such as cystic fibrosis [8], autosomal dominant hypocalcemia [9, 10], Dent disease [11] or congenital chloride diarrhea (CCD) [12] can also cause p-BS/GS symptoms. Despite the need for accurate genetic diagnosis in this heterogeneous group, the traditional strategy for genetic testing using single-gene Sanger sequencing lacks power for a comprehensive analysis and requires substantial time and resources to analyze many candidate genes.

77 Recently, next-generation sequencing (NGS) has become available for the diagnosis
78 of a number of disorders in clinical practice [12-16]. NGS can be used to analyze the whole
79 genome sequence or whole exome sequence (WES). NGS is useful not only to discover new
80 pathogenic genes for an unknown cause of genetic disease, but also to comprehensively
81 analyze known causative genes, simultaneously, by targeted sequencing. A recent study
82 demonstrated that a disease-related gene, *SLC26A3,* was identified by the application of
83 targeted sequencing in 5 of 39 patients with suspected BS but who did not have pathogenic
84 variants in known genes for this disease [12].
85 In this study, we studied 27 cases clinically diagnosed with BS/GS for whom genetic
86 tests for known BS/GS genes using Sanger sequencing were negative. We conducted targeted
87 sequencing for 22 genes including genes responsible for tubulopathies and other inherited
88 diseases manifesting as p-BS/GS.
89
90 **Methods**
91 ***Ethics***
92 All procedures were approved by the Institutional Review Board (IRB) of Kobe University
93 Graduate School of Medicine and in accordance with the Helsinki Declaration of 1975, as
94 revised in 2000 (IRB number: 301). Informed consent was obtained from all patients or their
95 parents.

96

97 ***Patients***

98 We studied 27 cases clinically diagnosed with BS/GS and for whom genetic tests for BS/GS

99 genes; *SLC12A1*, *KCNJ1*, *CLCNKB*, BSND and *SLC12A3*, using Sanger sequencing were

100 negative. Ten cases who were negative for *SLC26A3* variants examined by Sanger sequencing

101 were also included [17]. We conducted targeted sequencing for 22 genes, including genes

102 responsible for tubulopathies and other inherited diseases manifesting as p-BS/GS (Table 2).

103 Clinical information for all 27 patients is shown in Supplementary Table 1.

104

105 ***Preparation of the patients' DNA and NGS***

106 Genomic DNA samples were extracted from peripheral blood mononuclear cells using the

107 QuickGene whole blood kit S (Kurabo, Osaka, Japan). For NGS library preparation, we

108 designed and used a comprehensive diagnosis custom gene panel using the HaloPlex target

109 enrichment system kit (Agilent Technologies, Santa Clara, CA) according to the

110 manufacturer's instructions, including 22 known genes associated with inherited

111 tubulopathies and p-BS/GS (Table 2). Libraries were sequenced on a MiSeq platform

112 (Illumina, San Diego, CA, USA). The sequence data that were generated were analyzed using

113 SureCall software (Agilent Technologies, Santa Clara, CA). Variants were confirmed by

114 standard Sanger sequencing using a 3130 genetic analyzer (Thermo Fisher Scientific).

Results

We identified *SLC26A3* gene compound heterozygous variants responsible for congenital chloride diarrhea (CCD) in two cases. These variants were confirmed by Sanger sequencing (Figure 1). We did not detect any causative gene variants in the other 25 cases. Detailed clinical pictures for these two cases are as follows. Clinical data are shown in Table 1.

Patient 1

Patient 1 was a 6-month-old boy. He was born to unrelated parents at 37 weeks after a hydramniotic pregnancy, with a birth weight of 2500 g, and had no family history. He presented with polyuria from birth (after diagnosis, this was determined to have been watery diarrhea) and was admitted to a local hospital at 8 days of age because of poor sucking, 15% weight loss and jaundice. Although laboratory tests revealed severe hyponatremia, dehydration and hyperbilirubinemia and suspected BS/GS, further examinations to determine the cause were not conducted at that time. At 6 months of age, he was again admitted to the same local hospital with failure to thrive and suspected viral gastroenteritis (watery diarrhea). He received fluid replacement treatment for the correction of electrolytes and dehydration, followed by daily oral sodium chloride and potassium chloride. His symptoms resolved and electrolyte abnormality normalized. His clinical characteristics and laboratory test results are

shown in Table 1. He was clinically diagnosed with BS/GS based on the clinical presentation, including polyhydramnios, hypokalemia, metabolic alkalosis, hyperreninemia and hyperaldosteronemia. After confirming the absence of obvious acquired disorders, we performed genetic tests using Sanger sequencing based on the genetic analysis algorithm proposed by Peters et al. [18], but there were no variants in known genes responsible for BS/GS.

Patient 2

Patient 2 was a 7-year-old girl who was born to unrelated parents at 37 weeks after a hydramniotic pregnancy, with a birth weight of 3195 g, but with no family history. At 3 months of age, she was admitted to a local hospital owing to failure to thrive and acute gastroenteritis (watery diarrhea). She received fluid replacement treatment for the correction of electrolytes and dehydration. Her symptoms resolved and electrolyte abnormality normalized. At 6 months of age, in follow-up examination, she showed hyponatremia, hypokalemia, metabolic alkalosis, hyperreninemia and hyperaldosteronemia, and was clinically diagnosed with BS/GS. She started to receive daily treatment with oral sodium chloride, potassium chloride and an NSAID (non-steroid anti-inflammatory drug). However, genetic analysis was not conducted at that time. At the age of 7 years, she visited the local hospital for the purpose of a second opinion. Her clinical characteristics and laboratory test

results are shown in Table 1. After confirming the absence of apparent acquired disorders, we performed genetic tests using Sanger sequencing, but there were no pathogenic variants in known genes for BS/GS.

In Patient 1, we found compound heterozygous variants: c.354delC; and c.1008insT. Each parent was found to be heterozygous for one of these variants. In Patient 2, compound heterozygous variants were also identified: c.877G>A, p.Glu293Lys; and c.1008insT. p.Glu293Lys was a novel missense variant, and was predicted to be pathogenic by three variant prediction tools, Mutation Taster (http://www.mutationtaster.org/), PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/), and SIFT (http://sift.jcvi.org/). Each parent was found to be heterozygous for one of these variants.

Discussion

This study demonstrates a comprehensive genetic screening approach using NGS with a custom panel and revealed the causative gene variants in two p-BS/GS patients. We identified these rare pathogenic variants in *SLC26A3*, a gene which has been associated with CCD, a distinct inherited disease manifesting p-BS/GS symptoms. This finding suggests that NGS is useful for the genetic diagnosis of p-BS/GS. These two patients were misdiagnosed as having BS/GS because they presented with clinical symptoms identical to BS/GS, such as polyhydramnios, hypokalemic metabolic alkalosis, hyperreninemia, hyperaldosteronemia and

failure to thrive. It was not until the genetic test revealed them as having CCD that chronic diarrhea in these two cases was considered an important symptom for diagnosis of the underlying disease. In Patient 1 the chronic diarrhea was even initially misdiagnosed as polyuria with the watery stool mistaken for urine.

As previously reported, p-BS/GS may be caused by a wide variety of inherited conditions, including cystic fibrosis, autosomal dominant hypocalcemia, Dent disease or CCD, or acquired conditions, such as surreptitious diuretic use, laxative abuse, a chronic chloride deficient diet or cyclic vomiting [3]. Thus, identification of disease-causing disorders is essential for accurate diagnosis. In this study, we conducted comprehensive genetic screening for genes that can cause p-BS/GS including *CFTR*, *CASR*, *CLCN5*, *OCRL* and *SLC26A3* and detected pathogenic variants in *SLC26A3* in two cases (Table 2). We recently reported that acquired p-BS/GS was particularly common among adult women with lower body mass index (BMI) and estimated glomerular filtration rate (eGFR). These results suggested that age at diagnosis, sex, BMI, and eGFR should be taken into consideration for the differential diagnosis. Moreover, we found that patients with p-BS/GS had a significantly lower mean fractional excretion of sodium and chloride (FENa and FECl) than patients with BS/GS (FENa 0.32 ± 0.28% vs. 1.62 ± 0.79%, respectively; $P < 0.001$, FECl 0.44 ± 0.45% vs. 2.80 ± 1.44%, respectively; $P < 0.001$), because of sodium chloride loss into not urine but stool in p-BS/GS [7]. In the current two cases, both patients showed low levels of FENa and

FECl. The measurement of FENa and FECl may help to diagnose p-BS/GS caused by *SLC26A3* pathogenic variants.

CCD is a rare autosomal recessive disease that is characterized by persistent watery diarrhea with high fecal chloride from infancy, failure to thrive, hypochloremia, hypokalemia, hyponatremia and metabolic alkalosis. The *SLC26A3* gene encodes an intestinal $Cl^-/HCO3^-$ exchanger protein [19, 20]. Some previous reports suggested that CCD patients were easily misdiagnosed as BS/GS because of excessive loss of sodium chloride into the stool, resulting in a BS/GS-like phenotype [21]. Early and precise diagnosis improves the prognosis of CCD, and appropriate electrolyte treatment prevents significant morbidity or mortality [22, 23]. In the current study, two patients were not diagnosed with CCD, despite showing the symptoms of watery diarrhea and dehydration during admission in infancy. Patient 2 was not correctly diagnosed until 7 years of age. This case indicates that it is quite difficult for clinicians to make a precise diagnosis of this quite rare inherited disease based on the patient's limited clinical data. The reason these two cases were not accurately diagnosed with CCD was speculated to be because this disease is not widely recognized even by neonatologists. Comprehensive gene testing usually needs high cost and it should be avoided as much as possible. For that reason, it is necessary to remember CCD as a differential diagnosis for BS/GS.

Previous reports have described the possible existence of unidentified inherited

disorders in patients with p-BS/GS and the existence of new loci other than those in genes already identified for these phenotypes [3, 24]. In fact, a novel causative gene for transient antenatal BS, *MAGED2,* was identified quite recently [25]. We recently reported 56 % of p-BS/GS patients had apparent acquired underlying causes of hypokalemia and metabolic alkalosis, including excessive diuretic or laxative abuse, or anorexia. On the other hand, no clear underlying causes were identified in the remaining 44% of p-BS/GS patients despite detailed interviews. This suggests some of those p-BS/GS patients might be caused by inherited causes other than BS/GS [7]. In this study, we conducted targeted sequencing for 27 cases from those who were suspected to have inherited diseases.

Identification of defects in other genes may significantly improve our understanding of the underlying mechanisms of salt homeostasis. NGS is a promising tool which is expected to allow the genetic characterization of these undiagnosed cases and to allow for the detection of unidentified pathogenic variants. Choi et al. [12] recently identified pathogenic *SLC26A3* variants responsible for CCD using NGS in 5 of 39 p-BS patients with no pathogenic variants in known genes for BS. A recent publication by Mori et al. also reported that they designed a NGS custom panel for major inherited kidney diseases and applied the panel to 73 patients clinically diagnosed with some type of inherited kidney diseases, allowing a fast, easy, and comprehensive diagnosis regardless of the disease type [15].

NGS is a highly relevant technology for use in the diagnosis of BS/GS and it has

largely replaced single-gene Sanger sequencing. Moreover, early assessment and classification of BS/GS and p-BS/GS are becoming increasingly important because of the clinical and genetic heterogeneity underlying p-BS/GS resulting from many different inherited disorders. This technology will lead to improvements in our understanding of the causative disorder and will provide better assessment of prognosis, detection of complications in organs other than the kidneys, better treatment choice, carrier diagnosis and genetic counseling. These multiple advantages may significantly contribute to improving the patient's life.

In conclusion, our results suggest that comprehensive analysis using NGS with targeted sequencing is useful for detecting genetic mutations in some cases with p-BS/GS.

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Conflict of interest

All the authors have declared no competing interest.

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330 **Figure legends**

331 **Fig. 1**

332 Results of genetic analysis confirmed by Sanger sequencing

333 A. Patient 1: Genetic analysis revealed *SLC26A3* compound heterozygous variants:

334 c.354delC(Top); and c.1008insT(Bottom). The former variant was in exon 4 of the maternal

335 allele, leading to an out-of-frame product. The latter was in exon 9 of the paternal allele,

336 leading to an out-of-frame product.

337 B. Patient 2: Genetic analysis showed *SLC26A3* compound heterozygous variants: c.877G>A,

338 p.(Glu293Lys); and c.1008insT. The former variant was in exon 7 of the maternal allele, and

339 was identified as a pathogenic novel missense variant by three variant prediction tools. The

340 latter was in exon 9 of the paternal allele, resulting in an out-of-frame product.

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345 **Table 1. Clinical characteristics and results of genetic diagnosis in patients**

Parameter	Patient 1	Patient 2
Sex	Male	Female
Age at analysis	6 months old	7 years old
Age at diagnosis of BS	6 months old	6 months old
Treatment	oral sodium chloride, potassium chloride	oral sodium chloride, potassium chloride, NSAID
Body weight (kg)	5.9 (-2.3SD)	21.7 (-0.5SD)
Body height (cm)	61 (-2.8SD)	119.7 (-0.4SD)
BMI (kg/m2)	15.9	15.1
Blood pH level	7.491	7.383
Blood HCO_3^- level (mEq/l)	39.4	28.1
Serum Na^+ level (mEq/l)	133	137
Serum K^+ level (mEq/l)	2.3	2.5
Serum Cl^- level (mEq/l)	74	102
Serum Mg^{2+} level (mg/dl)	2.7	1.7
Serum creatinine (mg/dl)	0.27	0.31
Estimated GFR (ml/min/1.73m2)	58.4	129.1
Plasma renin activity (ng/ml/hr)	170	5.5
Plasma aldosterone level (pg/ml)	928	9.8
Urinary Ca^{2+}/creatinine ratio (mg/mg)	0.01	0.15
FENa (%)	0.17	0.46
FECl (%)	0.09	0.36
Echogram	Normal	Normal
Responsible gene	*SLC26A3*	*SLC26A3*
Mutation	c.1008insT/c.354delC	c.1008insT/c.877G>A

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Table 2. Gene list for targeted sequencing in this study

	Genes	Diseases
1	SLC12A1 NM_000338.2	Type I Bartter syndrome
2	KCNJ1 NM_000220.4	Type II Bartter syndrome
3	CLCNKB NM_000085.4	Type III Bartter syndrome, hypomagnesemia
4	BSND NM_057176.2	Type IV Bartter syndrome
5	CLCNKA NM_004070.3	Type IVb Bartter syndrome
6	SLC12A3 NM_000339.2	Gitelman syndrome, hypomagnesemia
7	CASR NM_000388.3	Type V Bartter syndrome, hypomagnesemia
8	MAGED2 NM_177433.2	Transient antenatal Bartter syndrome
9	CFTR NM_000492.3	Cystic fibrosis
10	CLCN5 NM_000084.4	Type I Dent disease
11	OCRL NM_000276.3	Type II Dent disease
12	SLC26A3 NM_000111.2	Congenital chloride diarrhea
13	KCNJ10 NM_002241.4	EAST syndrome
14	CLDN16 NM_006580.3	Hypomagnesemia
15	CLDN19 NM_148960.2	Hypomagnesemia
16	FXYD2 NM_001680.4	Hypomagnesemia
17	EGF NM_001963.4	Hypomagnesemia
18	TRPM6 NM_017662.4	Hypomagnesemia
19	KCNA1 NM_000217.2	Hypomagnesemia
20	CNNM2 NM_017649.4	Hypomagnesemia
21	HNF1B NM_000458.3	Hypomagnesemia, CAKUT, ADTKD
22	SLC41A3	Hypomagnesemia (mice)

CAKUT, congenital anomalies of the kidney and urinary tract

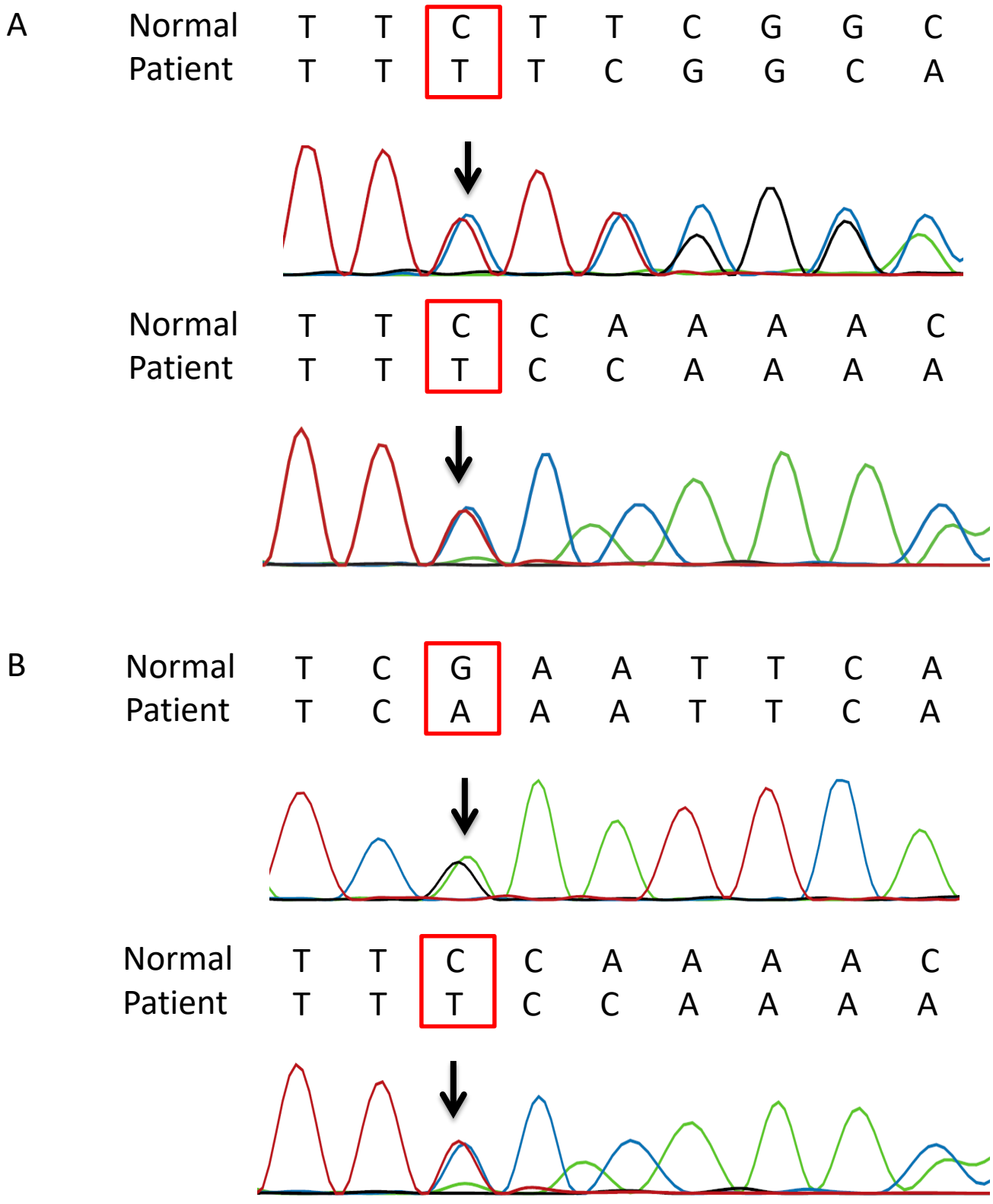
ADTKD, autosomal dominant tubulo-interstitial kidney disease

Supplementary Table 1 Clinical characteristics of all patients included in this study

Patient ID	Gender	Age at diagnosis (years old)	Age at present (years old)	BMI (kg/m2)	eGFR ml/min/1.73m2	Clinical symptoms	Serum K (mEq/L)	Serum Mg (mEq/L)	HCO3- (mmol/L)
A14	Male	1	12	18	53.4	Epilepsy	2.8	1.5	27.6
B26	Female	0.7	3	-	117.1	None	2.1	1.9	29.2
B36	Female	17	18	24.3	82	None	2.4	1.7	25
B40	Female	antenatal	7	13.5	184	Polyhydramnios, Polyurea	2.2	2.3	26
B44	Male	36	36	25.6	85	None	2.2	2.1	25.8
B70	Female	27	27	22.5	111	None	2.7	1.9	25.3
B71	Male	1	1	-	91.2	Failure to thrive	2.4	2.6	47.2
B80	Male	0.6	0.7	-	-	Failure to thrive	3.2	2	34.9
B83	Female	49	49	20.9	65	Depression	3.4	1.1	27.3
B90	Female	26	56	20.8	27	None	2.1	1.5	29.6
B118	Male	0.5	34	29.2	91	Failure to thrive, Tetany	2.9	1.7	30.6
B120	Female	20	38	16.6	53	None	2.2	1.9	38.2
B121	Male	42	53	28.6	89	Fatigue, Tetany	2.7	1.5	32.7
B125	Male	27	42	25.1	108	None	3.7	1.8	26.6
B141	Female	30	48	18.5	59	Cramp	2.2	1.9	38.9
B145	Female	20	44	22.7	75	None	3.2	2.2	25.2
B152	Male	28	28	19.2	82	None	2.5	1	31.1
B154	Female	33	34	17.9	91	None	2.3	1.9	33.7
B162	Male	33	38	24.3	51	None	3.1	3.2	32.8
B164	Male	0.3	0.5	-	-	Failure to thrive	3.6	2.1	32.1
B169	Male	7	7	24.8	141.1	None	2.4	1.8	32.7
B177	Female	42	42	22.6	120	Fatigue	1.9	1.1	34.6
B188	Female	1	3	-	131.1	Failure to thrive	1.7	2.7	42.5
B190	Female	38	38	18.3	80	Fatigue, Cramp	3	2	18.6
B195	Female	2	2	-	141.4	Muscle weakness	1.8	1.9.	29.2
Patient 1	Male	0.5	7	15.9	58.4	Failure to thrive	2.3	2.7	39.4
Patient 2	Female	0.5	1	-	129.1	Failure to thrive	3.7	1.7	28.1

BMI: Body mass index
eGFR: estimated glomerular filtration rate

Figure 1


A
Normal T T C T T C G G C
Patient T T T T C G G C A
Normal T T C C A A A A C
Patient T T T C C A A A A
B
Normal T C G A A T T C A
Patient T C A A A T T C A
Normal T T C C A A A A C
Patient T T T C C A A A A