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CLINICAL REPORT

A novel *CLCNKB* variant in a Chinese family with classic Bartter syndrome and prenatal genetic diagnosis

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Abstract

Background: Type III Bartter syndrome (BS), often known as classic Bartter syndrome is caused by variants in *CLCNKB* gene, which encoding the basolateral chloride channel protein ClC-Kb, and is characterized by renal salt wasting, hypokalemia, metabolic alkalosis, increased renin, and aldosterone levels.

Methods: A 2-year-old boy presented severe malnutrition, severe metabolic alkalosis and severe hypokalemia and was clinically diagnosed with BS. The trio exome sequencing (ES) was performed to discover the genetic cause of this patient, followed by validation using Sanger sequencing and quantitative polymerase chain reaction subsequently.

Results: The genetic analysis indicated that this patient with a compound heterozygous variants of *CLCNKB* gene including a novel nonsense variant c.876 T > A and a whole-gene deletion. The two variants were inherited from his parents, respectively. Subsequently, target sequencing of *CLCNKB* gene was performed for next pregnancy, and prenatal genetic diagnosis was provided for the family.

Conclusions: The results of current study identified the compound heterozygous variants in a patient with classic BS. The novel variant expands the spectrum of *CLCNKB* variants in BS. Our study also indicates that ES is an alternative tool to simultaneously detect single-nucleotide variants and copy-number variants.

KEYWORDS

classic Bartter syndrome, *CLCNKB*, exome sequencing, prenatal genetic diagnosis

1 | INTRODUCTION

Bartter syndrome (BS) is a rare autosomal recessive salt-losing tubulopathy, characterized by hypokalemic metabolic alkalosis, hyperreninemic hyperaldosteronism with normal-to-low blood pressure, and juxtaglomerular apparatus cell hyperplasia (Bartter et al., 1962; Hebert, 2003).

BS has been clinically classified into two types: antenatal BS (aBS) and classic BS (cBS). Compared with cBS, patients with aBS exhibit severe symptoms, such as

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maternal polyhydramnios, premature birth, and growth retardation (Brochard et al., 2009; Laghmani et al., 2016). Also, BS is categorized into five genetic subtypes (types I-V) based on the underlying variant genes:Type I BS (OMIM: 601678) is caused by variants in *SLC12A1* gene (OMIM: 600839), which encodes the sodium–potassium–chloride cotransporter NKCC2; Type II BS (OMIM: 241200) is caused by variants in *KCNJ1* gene (OMIM: 600359) which encodes the apical inwardly rectifying potassium channel ROMK; Type III BS (OMIM: 607364) is caused by variants in *CLCNKB* gene (OMIM: 602023), which encodes the basolateral chloride channel protein ClC-Kb; Type IVa BS (OMIM: 602522) is caused by variants in *BSND* gene (OMIM: 606412), which encodes barttin, an essential beta subunit for the chloride channels CLCNKA and CLCNKB. Type IVb BS is caused by simultaneous variants in both *CLCNKB* (OMIM: 602023) and *CLCNKA* (OMIM: 602024) genes. Type V BS (OMIM: 300971) is caused by variants in *MAGED2* gene (OMIM: 300470), which encodes melanoma associated antigen D2 (Al Shibli & Narchi, 2015; Hebert, 2003; Laghmani et al., 2016; Seyberth, 2008).

Type III BS is also known as cBS, caused by the pathogenic variants in *CLCNKB* gene, which mapped in chromosome 1p36.13 and encoding the basolateral chloride channel ClC-Kb. ClC-Kb is a vital member of the ClC chloride channel family, which plays a very important role in the trans-membrane transport of chloride in the renal tubules (Zelikovic et al., 2003). As a result, disease-causing variants could inactivate ClC-Kb, reducing the reabsorption of chloride. Subsequently sodium reabsorption would also be reduced. Due to the loss of water and sodium chloride, the renin–angiotensin–aldosterone system further activates and the loss of potassium aggravates (Andrini et al., 2015; Zelikovic et al., 2003).The phenotype of cBS is highly variable, including sporadical volume depetion and dehydration during early infancy, short stature, and polyuria during childhood or asymptomatic hypokalemia (Jeck et al., 2000; Konrad et al., 2000).

In this study, we reported clinical and molecular findings from a patient with cBS. The patient carried a novel nonsense variant (c.876T > A, p.Cys292*) combined with the entire gene deletion of *CLCNKB* inherited from his parents, respectively.

2 | MATERIALS AND METHODS

2.1 | Clinical summary

A 2-year-old boy presented a history of growth retardation and abnormal homeostasis. His mother had a history of polyhydramnios at 30^+ weeks of gestation, but no interventional prenatal genetic diagnosis (e.g., chromosome microarray analysis) was further performed during pregnancy. The boy was delivered at term, with a birth weight of 3500 g and length of 50 cm. However, he was diagnosed as "malnutrition" at the local hospital at the age of 2^+ months, manifesting mainly as milk-spitting and feeding difficulties. At the age around 7 months, he was transferred to pediatric department in West China Second University Hospital of Sichuan University, and blood tests demonstrated electrolyte disorders: Na^+ fluctuated between 124–129 mmol/L, K^+ fluctuated between 1.5 and 2.6 mmol/L, and Cl^- fluctuated between 76–83 mmol/L, with elevated arterial pH (7.59–7.67). Plasma aldosterone was markedly elevated (33.93 ng/dL), with normal results of metabolic-disease screening. No significant anomalies were uncovered through brain and chest CT, as well as echocardiography.

He was clinically diagnosed with BS, severe malnutrition, internal environment disorder: severe metabolic alkalosis, severe hypokalemia, hyponatremia, and hypochloremia. Oral spironolactone, indomethacin, and potassium supplements improved his serum electrolyte level (Table 1), without muscle weakness, polydipsia, and arrhythmia. At the age of 1 year old, he had two episodes of tetany after diarrhea and fever, respectively, with bilateral

TABLE 1 Blood gas level

Concentration age (months)	Sodium (135-145 mmol/L)	Potassium (3.5–5.5 mmol/L)	Calcium (1.09–1.30 mmol/L)	Chloride (96–108 mmol/L)	PH (7.35–7.45)
7	124–129	1.5–2.6	1.01–1.12	76–83	7.59–7.67
8	125–128	2.5–2.9	1.25–1.29	87–88	7.53–7.55
9	133	3.4	1.22	97	7.5
10	129	2.6–3.0	1.10–1.17	87–92	7.62
11	130	2.9	1.16	92	7.49
12	127	3.0	1.08	86	7.56
18	131	3.8	1.20	98	7.49
24	136	3.4	2.54	95	N/A

limb rigidity, staring gaze, circumoral cyanosis, and loss of consciousness. The electroencephalography was normal. Except fullness of the anterior fontanelle and slightly reduced density at local white matter of bilateral frontoparietal lobes, no remarkable abnormalities were detected on craniocerebral CT. Although continuous symptomatic treatment could maintain the stasis of internal environment, the subject was assessed as moderate to severe growth and psycho-motor retardation at local rehabilitation center. At the age of 2 years, his height was 80 cm (<3rd percentile), his weight was 10 kg (3rd percentile). He could merely speak double-syllable words and accomplish simple instructions, denial of communication resistance or stereotyped behaviors. According to the regular follow-up, he had no proteinuria or renal dysfunction.

His mother and father were non-consanguineous, and there was no family history of congenital malformations or genetic diseases. The pedigree of the family is shown in Figure 1. His mother was pregnant again, amniocentesis was performed at 23^{+} weeks of gestation. DNA extracted from the amniocytes was tested by massive parallel sequencing, targeting at genetic variants found by exome sequencing (ES) in the proband.

2.2 | Exome sequencing

Genomic DNA of the family members was extracted from peripheral blood leukocytes using QIAamp DNA Blood Mini Kit (QIAGEN). To reveal the genetic causes for the proband, trio-ES was performed using genomic DNA from the family members (II-1, II-2, III-1).

Exome capture sequencing was performed using the NanoWES Human Exome V1 (Berry genomics) following the manufacturer's protocol. The DNA libraries after enrichment and purification were sequenced through Illunima NovaSeq6000 platform with 150-bp paired-end reads.

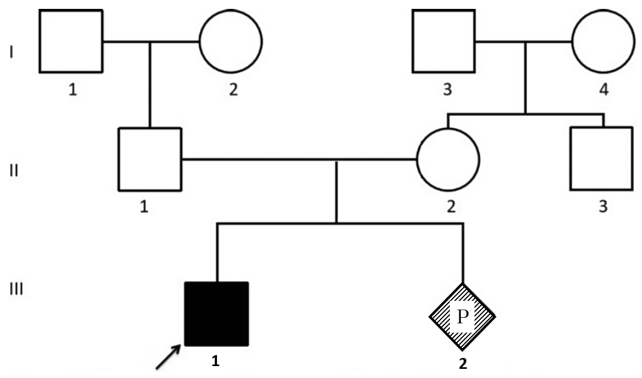

FIGURE 1 The pedigree of the present family. The proband is indicated by an arrow.

Next, Burrows-Wheeler Aligner software tool was used for aligning the sequencing reads with hg38/GRCh38. After that, local alignment and recalibration of base quality of the Burrows-Wheeler aligned reads was performed by the GATK Indel Realigner and the GATK Base Recalibrator, respectively (broadinstitute.org/). Then, single-nucleotide variants (SNVs) and small insertions or deletions (InDels) were identified by GATK Unified Genotyper (broadinstitute.org/). Finally, functional annotation was performed using ANNOVAR and the Enliven Variants Annotation Interpretation System (Berry genomics).

Public databases using for filtering consist of gnomAD (http://gnomad.broadinstitute.org/), 1000 Genomes Project (1000G) (http://browser.1000genomes.org), and etc. Pathogenicity of the detected SNVs were evaluated based on the scientific medical literature and disease databases, including OMIM (http://www.omim.org), PubMed (https://www.ncbi.nlm.nih.gov/pubmed/), ClinVar (http://www.ncbi.nlm.nih.gov/clinvar), and Human Gene Mutation Database (HGMD) (http://www.hgmd.org). The detailed process for identifying the variants is described previously (Yang, Xie, et al., 2021; Yang, Xu, et al., 2021).

2.3 | Sanger sequencing and quantitative polymerase chain reaction

Sanger sequencing was underwent in validating candidate variants on each independent gDNA sample (II-1, II-2, III-1). Polymerase chain reaction (PCR) amplification was performed using primer pairs (Table 2) designed to cover variants identified by ES.

Triplicate quantitative PCR for gDNA was performed using SYBR Green quantitative polymerase chain reaction (qPCR) Master Mix (Thermo Fisher Scientific, 00850445) on a RT-PCR System (Thermo Fisher Scientific, 7500 Real-Time PCR Systems). Delta CT value analysis method was performed to evaluate relative copy number of genome exon 10 and exon 18. Specific and internal control gene primer pairs were as well designed using Primer 3 software Version 0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0/) (Table 2). Sequencing of PCR products were conducted by ABI 3500 Genetic Analyzer (Thermo Fisher Scientific). Data were evaluated using the Chromas software (2.6.5).

2.4 | Prenatal genetic diagnosis

The mother of the proband was then pregnant later, and opted to undergo prenatal genetic diagnosis of the variants identified in the proband. Amniocentesis was performed at 23^{+} weeks of gestational. ES was performed on the DNA

TABLE 2 Primers used in the current study

Primers	Forward	Reversed
CLCNKB SNV (c.876T > A)	5′TGACCTGTGTTGAGCAAGAA3′	5′ACTTGTAGGTGGTGTTAGG3′
CLCNKB exon 10	5′TCTGTCAGCGAATCTTCTT3′	5′ACTTGTAGGTGGTGTTAGG3′
CLCNKB exon 18	5′CGTCTTATGCTGCTTCCT3′	5′CCTGAGTGGTTAAGTCGTT3′
β-actin	5′CTGGCACCACACCTTCTACAATG3′	5′CCTCGTAGATGGGCACAGTGTG3′

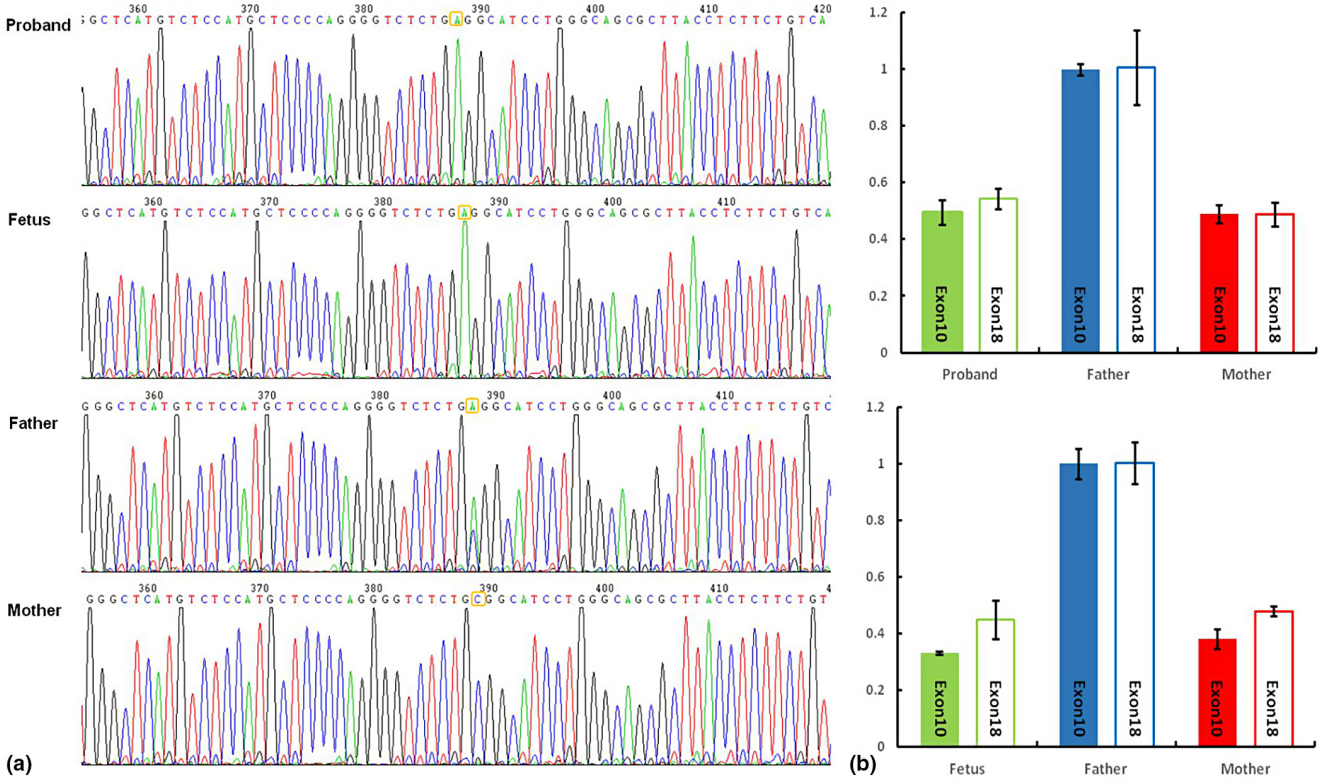

FIGURE 2 Validation and gene dosage effect of variants in *CLCNKB*. (a) Sanger sequencing confirmed variant (c.876T > A) in this family. (b) qPCR showed significant decrease of CLCNKB expression (exon 10 and exon 18) in the proband, the fetus and their mother. All the values are means ± SEM from three independent experiments, and statistical analysis was performed by one-way ANOVA.

extracted from amniocytes of *CLCNKB* gene and variants were validated by Sanger sequencing as well as qPCR.

3 | RESULTS

3.1 | Novel *CLCNKB* variant in a Chinese family with cBS

To elucidate the underlying genetic etiology of cBS for this family, trio-ES was performed. The results indicated that a novel nonsense variant c.876T > A (p.Cys292*, NM_000085.5) of the *CLCNKB* gene was identified in the proband and inherited from his father. The presence of this variant was further validated by Sanger sequencing (Figure 2a). This nonsense was detected in the proband and his father but not in his mother. The novel heterozygous variant p.Cys292* in the exon 10 of *CLCNKB*, resulting in a change in cysteine acid to a premature stop codon at amino acid position 292 (p.Cys292*), has not been reported in any public database, such as HGMD, ClinVar or GnomAD databases. A synonymous variant (c.876T > C, p.Cys292Cys, NM_000085.5) also was detected in his father and mother. However, the C allele is common in the general population of the same nucleotide sequence, which is also a benign synonymous substitution (BA1, BP7) (https://gnomad-sg.org/region/1-16049824-16049824?dataset=gnomad_r3).

The results of trio-ES indicated that the proband also had a heterozygous deletion of the whole *CLCNKB* gene and inherited from his mother. This deletion was further confirmed by qPCR analysis on specific exons (Figure 2b). The qPCR analysis results showed that compared with the father, the proband and his mother had relative half-hold

copy for exon 10, exon 18, which indicated that the heterozygous deletion was detected in the proband and his mother but not in his father.

Together, the proband had both variants including a paternal-inherited nonsense variant c.876T>A in *CLCNKB* gene and a maternal-inherited entire gene deletion of the same gene. According to the criteria of ACMG, the nonsense variant on was categorized as pathogenic variant (PVS1, PM2, PP4) and the entire gene deletion was categorized as pathogenic variant (PVS1, PM3, PP4).

3.2 | Prenatal genetic diagnosis

The prenatal genetic diagnosis targeting *CLCNKB* gene using amniocytes showed that the fetal genotype was compound heterozygous as well, carrying both the nonsense variant c.876T>A and an entire *CLCNKB* gene deletion. Validation was performed using Sanger sequencing and qPCR subsequently (Figure 2). No abnormal findings were uncovered through routine antenatal care till amniocentesis.

4 | DISCUSSION

In our study, we identified a compound heterozygous pathogenesis for the cBS proband: a novel nonsense variant c.876T>A and an entire gene deletion. The pathogenic variants in the *CLCNKB* gene are the molecular basis for cBS (Simon et al., 1997). The clinical manifestation of *CLCNKB* disorder is a highly variable, overlapping with either aBS or Gitelman syndrome (GS) (Zelikovic et al., 2003). GS is a milder disease frequently associated with hypomagnesemia and hypocalciuria, while hypomagnesemia and hypocalciuria are not always present. GS is caused by dysfunction of *SLC12A3* (Ma et al., 2016). Thus, the genetic analysis can more effectively differentiate GS and BS.

According to the HGMD (http://www.hgmd.cf.ac.uk), more than 196 variants have been reported in *CLCNKB* gene, including 103 missense or nonsense variants, 22 splice site variants, 24 small deletions, 39 gross deletions, 5 small insertions, and 3 complex rearrangements. Previous studies indicated that the entire gene deletion were of the highest allele frequency (Han et al., 2017, 2020). Heterozygous whole-or partial-gene deletions related to recessive inherited disease genes play a vital role in an individual's recessive carrier status (Boone et al., 2013), and also directly lead to disease by introducing compound heterozygous state where a deletion on one chromosome homolog coexisted with a loss of function or hypomorphic SNV allele on the other homolog elsewhere (Charng et al., 2016; Kremer et al., 2016; Lalani et al., 2016; Stray-Pedersen et al., 2017; Wu et al., 2015). Herein, our ES result revealed that a maternal heterozygous deletion and a paternal nonsense variant in *CLCNKB* gene in our patient, which has been considered to contribute to BS. WES has been widely used as a first-tier diagnostic tool to identify genetic causes of many suspicious Mendelian disorders. WES could detect different types of variants including SNVs, indels and copy-number variants (CNVs) in the meantime. However, due to the false positive rates of the CNVs identified from WES, data are highly recommended to be validated by an orthogonal methods, such as multiplex ligation-dependent probe amplification, SNP-array, CNV-seq, or qPCR (Ellingford et al., 2017; Kerkhof et al., 2017; Rajagopalan et al., 2020). Thus, qPCR was performed for validation for the family in our study.

Type III BS is characterized by salt reabsorption defect in the thick ascending limb of the Henle loop (Seyberth & Schlingmann, 2011). The main treatment is potassium supplement to reduce potassium loss, correct hypokalemia, and metabolic alkalosis. In addition, the use of prostaglandin synthesis inhibitors, such as NSAIDs, is an alternative therapy (Friis et al., 2005; Jensen et al., 1996; Verberckmoes et al., 1976; Wu et al., 2020). Nonetheless, there is no curative therapy for cBS up till now. Previous study reported that certain BS type III patients exhibited pathological proteiuria and kidney dysfunction even under treatment (Bettinelli et al., 2007). Moreover, the clinical characterization of cBS are highly heterogeneous, including polyuria, polydipsia, dehydration, developmental retardation, renal dysfunction, even sudden death in certain cases. Therefore, the prenatal genetic diagnosis is significantly necessary. In this study, target sequencing of these variants was performed for the fetus when the proband's mother was pregnant again. Unfortunately, both pathogenic variants identified in the proband were also detected in amniocytes, providing valuable prognosis information for this family. This couple decided to terminate the pregnancy after genetic counseling.

Of note, type IVb BS is caused by simultaneous variants in both the *CLCNKB* and *CLCNKA* genes (Nozu et al., 2008; Schlingmann et al., 2004), which contribute to a severe form of BS and sensorineural deafness. Hence, in view of the digenic inheritance of BS, these two genes should be considered to be sequenced simultaneously in the patients with severe BS manifestations. In addition, the type IVb BS and other aBS also result in severe maternal polyhydramnios during the pregnancy, fetal ES, which has been more widely used during pregnancy, could be recommended to identify gene variants related to BS.

To sum up, in the present study, compound heterozygous variants in *CLCNKB* gene were discovered for a cBS

patient using ES, consisting of a novel nonsense variant (c. 876T>A)and a whole gene deletion, which result in biallelic loss-of-function of this gene. The novel variant expands the spectrum of *CLCNKB* variants in BS. Subsequently, our outcomes provide important information for subsequent genetic counseling and prenatal genetic diagnosis of this family. Furthermore, the present study indicates that ES is an effective tool to uncover the genetic etiology for Mendelian disorders, which could simultaneously detect different types of variants.

AUTHOR CONTRIBUTIONS

QYZ and MY designed the study experiments. QYZ and XX collected the data and conducted the clinical evaluations. QQX, YT, and HBX performed PCR-seq. QYZ and MY wrote the article. MY, SLL, and HW supervised the study experiments. All authors revised and approved the article.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICAL COMPLIANCE

Experiment on human subjects was approved by the Ethical Review Board of West China Second University Hospital, Sichuan University.

CONSENT TO PARTICIPATE

Informed consent for participation to this study was obtained from the parents of the patients.

PATIENT CONSENT FOR PUBLICATION

Publication of data was informed consent to the parents of the patients involved in this study.

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