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ORIGINAL ARTICLE

Eleven novel *SLC12A1* variants and an exonic mutation cause exon skipping in Bartter syndrome type I

Yue Han[1,2] · Xiangzhong Zhao[2] · Sai Wang[1,2] · Cui Wang[1,2] · Dongxu Tian[3] · Yanhua Lang[4] · Irene Bottillo[5] · Xinsheng Wang[3] · Leping Shao[1,2]

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Abstract

Introduction Bartter syndrome type I (BS1) has been rarely reported in large groups. On the other hand, the phenomenon of exon skipping, in which exonic mutations result in abnormal splicing, has been reported to be associated with various diseases. Specifically, mutations that result in the disruption of exonic splicing enhancers (ESEs) and/or the creation of exonic splicing silencers (ESSs) can promote exon skipping. However, the aberrant exon skipping caused by an exonic variant in such splicing regulatory elements (SREs) sequences has never been reported in the causal gene of *SLC12A1* in BS1.

Methods We analyze the variants in nine Chinese families with BS1, including eight with antenatal BS (aBS) and one presenting as classical BS (cBS), by next-generation sequencing. Then we used bioinformatics programs to analyze all these variants found in this study and identify candidate mutations that may induce exon skipping. Furthermore, the effects of identified variants were classified according to the 2015 American College of Medical Genetics and Genomics (ACMG) standards and guidelines.

Results Fifteen different variants of *SLC12A1* gene were identified, including 11 novel ones. Two of the nine probands were homozygotes, the rest seven ones were compound heterozygotes. One candidate variant (c.1435C>G), not only significantly reduced ESEs scores but also markedly increased ESSs scores, were further investigated by mini-gene splicing assay, and found this single-nucleotide substitution causes abnormal splicing in vitro (exclusion of exon 11). Finally, among 15 variants, 9, 3, and 3 were classified as "pathogenic variants", "likely pathogenic variants", "variants with uncertain significance", respectively.

Conclusion These data would enrich the human gene mutation database (HGMD) and would provide valuable references to the genetic counseling and diagnosis of BS1 for Chinese population. Additionally, our results suggest that aberrant exon skipping is one previously unrecognized mechanism by which an exonic variant in *SLC12A1* can lead to BS1.

Keywords Bartter syndrome type I · *SLC12A1* gene · Exonic splicing enhancer · Exonic splicing silencers · Exon skipping

Introduction

Bartter syndrome (BS) is a heterogenic autosomal recessive disorder of salt reabsorption at the thick ascending limb

These authors contributed equally: Yue Han, Xiangzhong Zhao

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✉ Xinsheng Wang
qy_wangxs@126.com

✉ Leping Shao
lepingshao@163.com

[1] Department of Nephrology, The Affiliated Qingdao Municipal Hospital of Qingdao University, No.5 Donghai Middle Road, 266071 Qingdao, People's Republic of China

[2] Central Laboratory, The Affiliated Hospital of Qingdao University, 266003 Qingdao, People's Republic of China

[3] Department of Urology, The Affiliated Hospital of Qingdao University, 266003 Qingdao, People's Republic of China

[4] Department of Nursing, The Affiliated Hospital of Qingdao University, 266003 Qingdao, People's Republic of China

[5] Division of Medical Genetics, Department of Molecular Medicine, Sapienza University, San Camillo-Forlanini Hospital, Rome 00185, Italy

(TAL) of the loop of Henle, most presenting as polyuria and polydipsia, hypokalemic metabolic alkalosis, normotensive hyperreninemia, and hyperaldosteronism [1, 2]. Bartter syndrome type I (BS1) is caused by loss-of-function mutations of the *SLC12A1* gene, which encodes the furosemide-sensitive Na-K-Cl cotransporter (NKCC2) [3, 4]. Mainly based on phenotype, BS can be classified to classical BS (cBS) and antenatal BS (aBS). cBS presents during early childhood with mild symptoms, while aBS is characterized by life-threatening disorders arising in utero, where fetal polyuria causes polyhydramnios followed typically by premature delivery [5]. BS1 is typically classified as aBS, but late-onset manifestations and mild phenotypes have also been reported. So far, a total of 74 mutations of *SLC12A1* gene have been reported worldwide. Sixty-five variants of which were detected from patients with manifestations of BS, whereas the rest 9 ones found in researched members of the Framingham Heart Study were reported as being related to hypotension [6].

In addition, exonic point mutations are generally categorized into missense, silent, or nonsense mutations, and it is well known that point mutations that damage the authentic splice sites cause abnormal splicing. However, it was recently reported that certain point mutations regarded as missense alterations also induce the exclusion of an individual exon in various diseases [7]. The distinctive mechanism of "exon skipping" is closely linked to the disruption of the regulatory elements in genomic DNA. The correct recognition of the splice sites is precisely controlled. The inclusion of exons in mature mRNA depends on intrinsic regulatory sequences. Among these sequences, exonic splicing enhancers (ESEs) and/or exonic splicing silencers (ESSs), which are located at varying distances from the splice sites, help regulate normal splicing. The ESEs bind the serine/arginine-rich protein (SR) that enhances the recognition of the splice sites. Mutations within the ESEs exert an inhibitory effect on the binding of SR splicing factors (SRSFs) following a failure of correct splicing due to exon skipping in mature mRNA. On the contrary, the ESSs bind the heterogeneous nuclear ribonucleoprotein (hnRNP) that inhibits the recognition of the splice sites. And mutations that cause the creation of ESSs exert a stimulative effect on the binding of hnRNP. This "exon skipping due to ESE disruption and/or ESS creation" has been well investigated in certain neurologic and inherited pediatric disorders [8, 9].

Up to now, the majority of BS1 patients were reported in case reports. Half a year ago, our team reported 16 Chinese BS probans (including 14 cases of BS3, 1 BS1, and 1 BS4) and detected 11 novel variants of *CLCNKB* gene, as well as two novel mutations of *SLC12A1* gene [10]. Herein, we will describe additional eight Chinese BS1 families, identify their variants of causal genes, evaluate these variants by bioinformatics programs, then confirm candidate mutations that may induce exon skipping by a mini-gene splicing assay, and eventually, classify these sequence variants using the 2015 American College of Medical Genetics and Genomics (ACMG) standards and guidelines [11].

Subjects and methods

Research subjects

This study recruited nine BS1 probands from nine families who had been registered in our online registration system for kidney orphan disease (http://shenzanghanjianbing.com/) and hospitalized in our Nephrology Department of Qingdao University Affiliated Qingdao Municipal Hospital from June 2017 to January 2018. Among of them, one proband has been described in our previous report [10]. Five patients were male, four were female. Criteria for the diagnosis of aBS included polydipsia and polyuria, hypokalemia and hypochloremic metabolic alkalosis, evidence of renal salt wasting, activation of the renin–angiotensin–aldosterone system associated with normal to low blood pressure, nephrocalcinosis, maternal polyhydraminios, and premature delivery. Secondary BS or pseudo-BS due to long-term use of laxatives, diuretics, and cystic fibrosis was excluded.

Except for patient Z7 whose current age was 10 and a half year old, the average age of the other patients was 17 ± 14 months. All of the nine families were from Han Chinese population, and no parents were consanguineous. This study was approved by the ethics committee of Affiliated Qingdao Municipal Hospital of Qingdao University. Guardians or parents of the nine BS1 patients have signed informed consent.

High-throughput sequencing and bioinformatics analysis

Genomic DNA was extracted from peripheral blood of these probands and their family numbers by GenElute blood genomic DNA kit (Sigma, NA2010). High-throughput sequencing was used to analyze the exon regions and flanking intronic regions of seven genes (*SLC12A1*, *KCNJ1*, *CLCNKB*, *CLCNKA*, *BSND*, *CASR*, and *SLC12A3*) associated with BS and Gitelman syndrome. Reads that passed were then aligned to the human reference genome (UCSC hg19) using the Burrows Wheeler Aligner (University of California, Santa Cruz, CA, USA). The variant call file (VCF) containing these variants was annotated with Variant Effect Predictor v83 and the dbNSFP (Database for Non-synonymous SNPs' Functional Predictions) v3.1. After the selection process, we use the online software (SIFT, PolyPhen-2, and MutationTaster) to predict the pathogenicity of putative missense mutations.

Table 1 The basic information and laboratory results of the nine patients with BS1 at the first admission

Patient	Gender	Age[a] (weeks)	Age[b]	Birth weight (kg)	Laboratory results												
					Blood PH	CO_2CP (mmol L^{-1})	SNa (mmol L^{-1})	SK (mmol L^{-1})	SCl (mmol L^{-1})	SMg (mmol L^{-1})	ALD (ng L^{-1})	PRA (ng mL^{-1} h^{-1})	UK/UCr (mmol $mmol^{-1}$) (< 1.5)	UMg/UCr (mmol $mmol^{-1}$)	UCa/ UCr (mmol $mmol^{-1}$)	SCr (μmol L^{-1})	eGFR (mL min^{-1} per 1.73 m^2)
					(7.35–7.45)	(22–28)	(135–145)	(3.5–5.5)	(99–110)	(0.73–1.06)	(12.0–157.5)	(0.05–0.79)					
J1	F	29 + 2	19 mos	1.21	7.52	33.0	136.1	3.4	97.1	0.85	222.8	7.7	19.3	1.44 (0.4–2.2)	2.17 (0.09–2.2)	22.0 (14–30)	98.8 (39–114)
N2	F	40	36 mos	3.34	7.50	32.1	123.1	2.8	95.0	0.91	356.5	12.1	10.4	0.95 (0.07–0.34)	0.89 (0.02–0.50)	24.0 (23–63)	123.8 (89–165)
A3	F	28 + 4	2 mos	0.92	7.46	34.6	136.9	3.7	98.5	0.79	428.7	42.5	16.1	1.07 (0.4–2.2)	2.7 (0.09–2.2)	75.1 (37–81)	18.8 (17–60)
J4	M	32	17 mos	2.05	7.54	36.5	132.0	1.7	90.2	1.50	749.5	26.2	14.4	0.60 (0.4–2.2)	0.63 (0.09–2.2)	18 (14–30)	112.8 (30–86)
S5	F	30 + 4	5 mos	1.15	7.47	28.4	146.5	2.3	89.7	0.97	571.6	17.1	24.2	1.05 (0.4–2.2)	1.38 (0.09–2.2)	16.2 (15–33)	97.3 (30–86)
J6	M	29	60 mos	1.11	7.46	25.6	136.2	2.3	90.7	1.12	663.2	19.2	38.3	1.31 (0.07–3.4)	4.89 (0.02–0.50)	45 (23–63)	63.6 (89–165)
Z7	M	29	10.5 yrs	1.21	7.49	29.7	130.6	2.2	92.5	1.01	258.4	25.1	NA	NA	NA	68.5 (43–92)	68.8 (89–165)
H8	M	30 + 5	6 mos	1.40	7.51	31.0	130.0	3.7	91.0	1.05	234.6	30.7	24.7	0.84 (0.3–0.9)	0.75 (0.04–0.7)	20.7 (15–33)	99.8 (30–86)
J9	M	28	12 mos	2.00	7.47	30.4	134.5	3.0	95.6	0.94	651.8	19.7	NA	NA	NA	20.4 (14–30)	95.6 (30–86)

Figures in parentheses indicate normal ranges of the corresponding age [22, 23]. eGFR was calculated by Schwartz formula

mos months, *yrs* years, *M* male, *F* female, *J* Jiangsu province, *N* inner Mongolia autonomous region, *A* Anhui province, *S* Sichuan province, *Z* Zhejiang province, *H* Hebei province, *S* serum, *U* urine, *ALD* aldosterone, *PRA* plasma renin activity, *NA* not available, *BS1* Bartter syndrome type I, *eGFR* estimated glomerular filtration rate

[a]Age at the first admission

[b]Present age (corrected age)

Sanger sequencing verification

The potential candidate variant was validated by Sanger sequencing in patients and their family members. The suspected candidate mutation sites and its flanking regions were amplified by PCR and underwent direct Sanger Sequencing using an ABI Prism 3700 DNA Analyzer (Applied Biosystems, CA, USA).

In silico splicing assay

In silico analyses by ESEfinder 3.0 (http://krainer01.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home) and HSF 3.1 software (http://www.umd.be/HSF3/HSF.shtml) were used to identify the putative ESEs/ESSs in wild and mutant DNA sequences. To analyze the potential effect of variants those are close to consensus 5′ donor or 3′ acceptor site on the recognition of splice site, in silico evaluation by software BDGP (http://www.fruitfly.org/seq_tools/splice.html) and Netgene2 (http://www.cbs.dtu.dk/services/NetGene2/) was performed.

Mini-gene constructions and expression

To confirm the probable splice mutation, in vitro analysis was performed using a mini-gene splicing assay based on the pSPL3 exon trapping vector as described previously [9, 12–14]. Fragments with the wild or mutant alleles containing exons of interest, flanked by upstream intronic sequence and downstream intronic sequence, were cloned into the splicing vector pSPL3 using specific primers linking the *Xho*I and *Nhe*I restriction enzyme sites (TGGAGC^TCGAG: *Xho*I; AATTTG^CTAGC: *Nhe*I). The ancestral and mutant type constructs were named pSPL3-W and pSPL3-M, respectively. All constructs were verified to contain the correct sequence by direct sequencing. Human epithelial kidney 293T (HEK 293T) cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin (100 U L^{-1}), and streptomycin (100 mg L^{-1}) at 37 °C in a 5% CO_2 atmosphere. One day before transfection, cells were transferred to six-well culture plate to grow to approximately 70–80% confluence in an antibiotic-free medium. Cells were then transfected with 4 μg plasmid DNA (pSPL3-W, pSPL3-M and empty pSPL3 control each) using OPTI-MEM® IMedium and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were harvested, total RNA was extracted after 24-h transfection with the RNAsimple Total RNA Kit (Tiangen, Beijing, China) and used for RT-PCR to confirm the splicing patterns. First-strand complementary DNA (cDNA) was synthesized from 2 to 3 μg of total RNA by random-primed reverse transcription with Superscript II Reverse

Table 2 Mutations identified in the *SLC12A1* gene of the nine patients with BS1

Patient	Gene	Allele1				Allele2			
		Exon	Nucleotide change	Amino-acid change	References	Exon	Nucleotide change	Amino-acid change	References
J1	*SlC12A1*	11	c.1435C>G	Splicing	[10]	12	c.1478delG	p.Gly493Alafs*53	[10]
N2	*SlC12A1*	11	c.1304C>T	p.Ala435Val	This study	23	c.2762G>T	p.Gly921Val	This study
A3	*SlC12A1*	22	c.2745G>A	p.Trp915*	This study	12	c.1522G>A	p.Ala508Thr	[16]
J4	*SlC12A1*	11	c.1388T>C	p.Leu463Ser	This study	12	c.1522_1533del12	p.Ala508_Ser511del	This study
S5	*SlC12A1*	6	c.731C>A	p.Ala244Asp	This study	18	c.2221A>T	p.Lys741*	This study
A6	*SlC12A1*	12	c.1479dupC	p.Leu495Profs*49	This study	12	c.1479dupC	p.Leu495Profs*49	This study
Z7	*SlC12A1*	21	c.2493_2494delAG	p.Arg833Ilefs*15	This study	3–8	c.(420 + 1_421-1)_(1087 + 1_1088-1)del	-	This study
H8	*SlC12A1*	7	c.887A>C	p.Asp296Ala	This study	11	c.1411C>T	p.Arg471*	[17]
J9	*SlC12A1*	11	c.1411C>T	p.Arg471*	[17]	11	c.1411C>T	p.Arg471*	[17]

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Transcriptase (Invitrogen Corporation, Carlsbad, CA). To evaluate the pattern of transcripts from the transfected minigenes, the following vector-specific primers were used for RT-PCR amplification: a forward primer SD6 (5′-TCTGAGTCACCTGGACAACC-3′) and a reverse primer SA2 (5′-ATCTCAGTGGTATTTGTGAGC-3′). The PCR amplification reaction was performed as follows: in 50 µL volume, 2 µl of cDNA, 5 µl of Expand High Fidelity buffer 3 (Roche, Mannheim, Germany), 1 µM of each primer, 0.8 µM dNTPs, and 2.6 U Expand High Fidelity enzyme mix (Roche, Mannheim, Germany) in a 9700 (Applied Biosystem, Foster City, CA, USA) thermal cycler. Thermal conditions were 30 cycles of 95 °C for 30 s, 58 °C for 30 s, and 68 °C for 1 min, preceded by 2 min at 95 °C, and followed by a final elongation step at 68 °C for 10 min. The PCR products were separated by electrophoresis on a 3% agarose gel and each band signal was quantified by Quantity One software (Bio-Rad, Richmond, CA). All transcripts were analyzed by sequencing.

Application of 2015 ACMG guidelines

The ACMG guidelines were applied to all variants identified in this study, and classification of pathogenic, likely pathogenic, variant of unknown significance (VUS), likely benign, or benign was assigned according to the published algorithm.

Results

Clinical manifestations and examination results

Nine unrelated Chinese patients were enrolled in this study (Table 1). Patient N2 was born at 40 weeks of gestation and weighted 3.34 kg, presenting as cBS. The other eight patients were diagnosed as aBS with premature delivery before 32 weeks of gestation (birth weight 1.60 ± 0.76 kg) as a result of polyhydramnios. The most common symptoms and signs was polyuria (9/9), followed by growth retardation (8/9).

As can be seen from Table 1, all patients had lower than normal serum chloride concentration (89.7–98.5 mmol L^{-1}/93.4 ± 3.3 mmol L^{-1}), metabolic alkalosis (pH value of arterial blood 7.46–7.54; CO_2CP 25.6–36.5 mmol L^{-1}/31.4 ± 3.5 mmol L^{-1}), and elevated basal renin activity and aldosterone. Seven patients had hypokalemia (1.7–3.7 mmol L^{-1}/2.8 ± 0.7 mmol L^{-1}). Except for the two patients who had not undergone the test of urine electrolytes and urine creatinine at the first admission, the rest seven patients (7/7) had increased urinary potassium to creatinine ratio (21.1 ± 9.2 mmol $mmol^{-1}$), whereas four of them had elevated urinary calcium/creatinine ratio (4/7). No Gitelman-like syndrome (hypokalemia, hypomagnesemia, and hypocalciuria (urine calcium/creatinine < 0.1 mol mol^{-1})) was found.

Six (J1, N2, S5, J6, Z7, and J9) out of nine patients accepted urinary system ultrasound examination were detected with bilateral nephrocalcinosis.

Gene analysis

As shown in Table 2, 15 different mutations of *SLC12A1* gene were found in nine patients by high-throughput sequencing, including seven missense mutations (c.731C>A, p.Ala244Asp; c.887A>C, p.Asp296Ala; c.1304C>T, p.Ala435Val; c.1388T>C, p.Leu463Ser; c.1435C>G, p.Leu479Val; c.1522G>A, p.Ala508Thr; c.2762G>T, p.Gly921Val), three nonsense mutations (c.1411C>T, p.Arg471*; c.2745G>A, p.Trp915*; c.2221A>T, p.Lys741*), three small deletions

(c.1478delG, p.Gly493Alafs*53; c.1522_1533del12, p.Ala508_Ser511del; c.2493_2494delAG, p.Arg833Ilefs*15), one duplication (c.1479dupC, p.Leu495Profs*49), and one gross deletion (c.(420 + 1_421-1)_(1087 + 1_1088-1)del). Except for the four previously reported variations (c.1411C>T, p.Arg471*; c.1435C>G, p.Leu479Val; c.1478delG, p.Gly493Alafs*53; c.1522G>A, p.Ala508Thr) [10, 15, 16], we had found 11 additional novel mutations in this study. Both alleles of *SLC12A1* gene were detected mutations inherited from parents in each of the nine probands. Two of them were homozygotes, the other seven were compound heterozygotes.

The analysis of software BDGP on the variants (c.1304C>T and c.2762G>T) nearby the splicing sites showed no significant finding, whereas NetGene2 predicted that the mutation c.2762G>T would remove the acceptor site of the exon 23 of *SLC12A1* gene (Supplemental Table 1). A substantially negative impact on the regulation of splicing process through destruction of three or more ESEs motifs and creation of three or more ESSs motifs was predicted of mutation c.1435C>G by ESEfinder 3.0 and HSF 3.1. (Supplemental Table 2 and Fig. 1). Because of the potential significant impact of c.1435C>G on splicing and the contradictory prediction results of c.2762G>T from BDGP and NetGene2 analysis, both c.2762G>T and c.1435C>G were subject to following in vitro splicing assays, whereas c.1304C>T and the other variants identified in this study entered into next step of sequence variants interpretation according to 2015 ACMG guideline.

Functional analysis of the mutation of c.1435C>G and c.2762G>T

To define the transcript level effects of this mutation of c.1435C>G and c.2762G>T, we performed exon trapping using the pSPL3 plasmids. The fragments with the wild or mutant alleles involving exon 11 (152 bp) or exon 23 (212 bp) flanked by upstream intronic sequence (202 bp or 174 bp) and downstream intronic sequence (166 bp or 134 bp), were cloned into the splicing vector pSPL3 using specific primers (Primers for exon 11: forward, 5′-GAGTTGAAATGGCAGCCTGT-3′; reverse, 5′-CTGCCATGTAATGACTGTAT-3′; Primers for exon 23: forward, 5′- ACTGCATGAGAAG GTATATG-3′; reverse, 5′- TAAACACGAGGCCTATCCTG -3′) linking the *Xho*I and *Nhe*I restriction enzyme sites. The mini-gene splicing assays showed that both the empty pSPL3 control

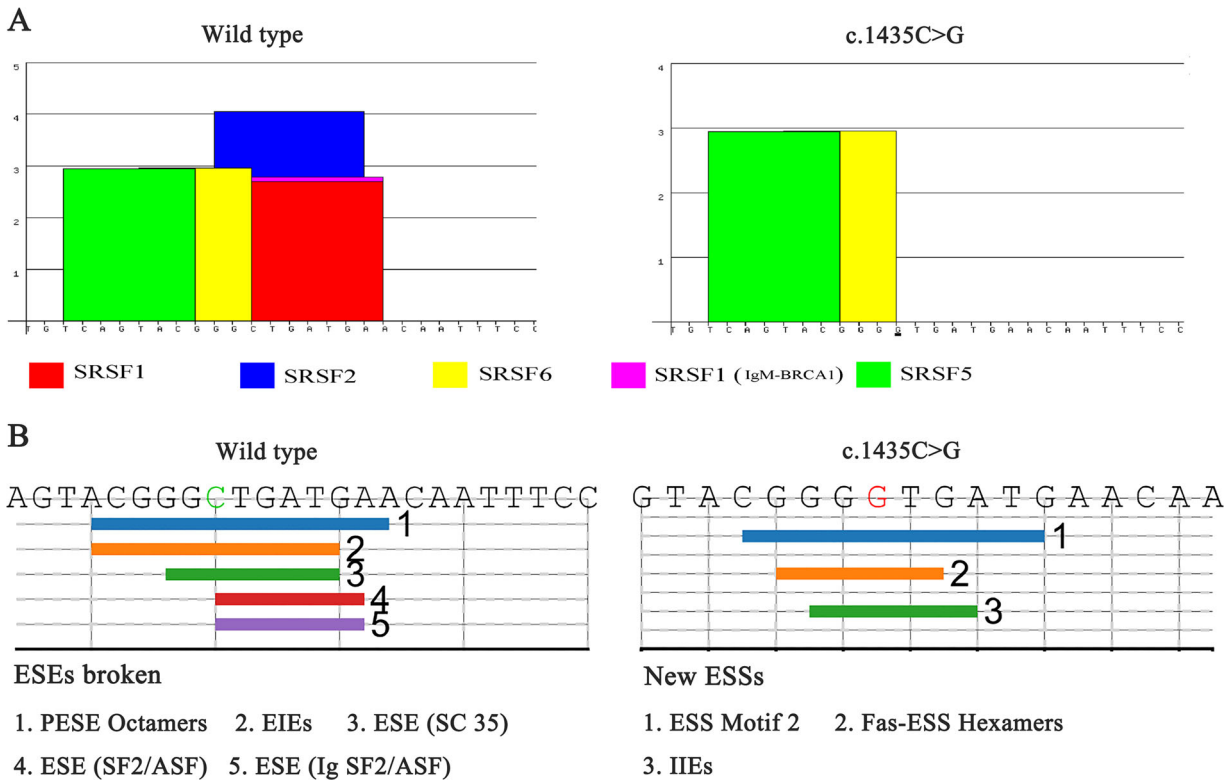

Fig. 1 In silico prediction of exonic splicing of mutation c.1435C>G in *SLC12A1*. **a** The potential exonic splicing enhancers (ESEs) sequences were identified by ESEfinder 3.0. The boxes are illustrated in red for SRSF1, blue for SRSF2, green for SRSF5, and yellow for SRSF6. The Y axis indicates the numerical score identical with the consensus motifs of each SRSF. Left, wild-type; Right, c.1435C>G. **b** ESEs and exonic splicing silencers (ESSs) motifs analysis of sequences harboring the variant c.1435C>G by Human Splicing Finder 3.1. Left, wild-type; Right, c.1435C>G. The broken splicing enhancer matrices and new splicing silencer matrices were displayed at the bottom, respectively

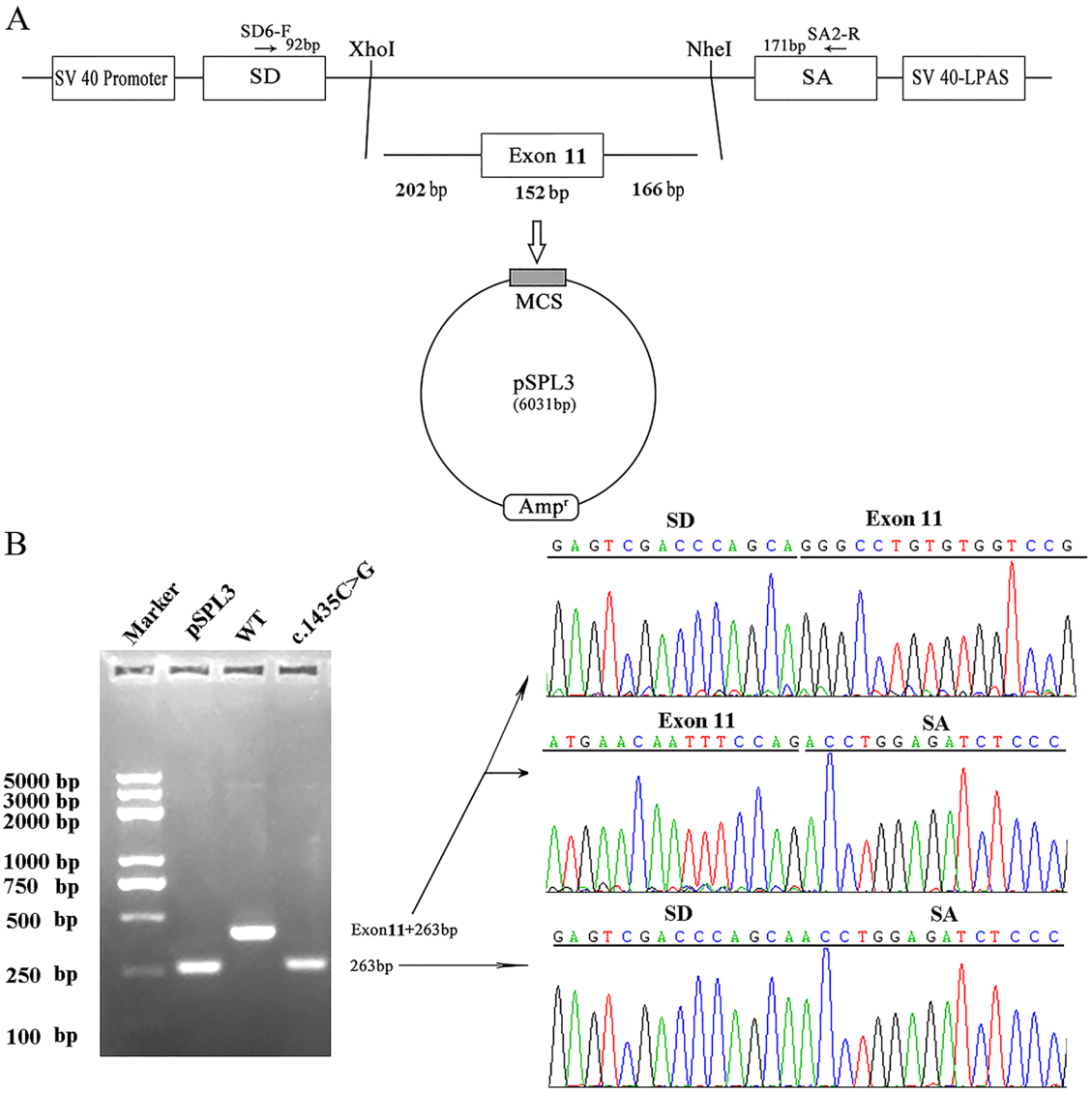

Fig. 2 The mini-gene splicing assay based on the pSPL3 exon trapping vector. **a** The pSPL3 vector contains two exons SD and SA, and a functional intron, with transcription beginning following the SV40 promoter and ending at the LPAS (late poly (A) signal). Wild pSPL3-W and mutant pSPL3-M plasmids containing 202 bp of intron 10, 152 bp of exon 11, and 166 bp of intron 11 were separately cloned into the *Xho*I and *Nhe*I cloning sites of the pSPL3 vector. **b** Agarose gel electrophoresis of RT-PCR products. SD6 and SA2 primers were designed for RT-PCR amplification of complementary DNA (cDNA) sequences generated by transfected 293T cells. Lane1: Marker; Lane2: empty vector (263 bp); Lane3: 415 bp (263 bp + 152 bp); Lane 4: 263 bp. MCS multiple cloning sites

and the c.1435C>G mutant constructs gave rise to a 263-bp PCR fragment missing exon 11 of *SLC12A1* gene, whereas the wild-type yielded a RT-PCR product of 415 bp containing exon 11 (Figs 2a, b). Therefore, we determined via a combination of in silico and in vitro assays that the c.1435C>G caused exon 11 skipping in the *SLC12A1* transcripts. The complete skipping of exon 11 results in a 50 amino-acid deletion (residues 434–483) with a subsequent frame-shift from codon 435 and premature termination at position 492 in exon 13. However, the c.2762G>T did not show any effect on the correct splicing of exon 23 (data not shown). Thus, we confirmed that c.1435C>G was virtually a splicing mutation, and c.2762G>T was a missense variant.

ACMG classification

As shown in Table 3, according to the 2015 ACMG standards and guidelines, three nonsense variants and one large deletion can be classified as “pathogenic variants”. Among four small deletions/duplications, one variant c.1522_1533del12 (p.Ala508_Ser511del) can only be classified as “likely pathogenic variants” due to its in-frame characteristics, other three frame-shift ones can be classified

Table 3 Classification of sequence variants identified in this study

Variants	Nucleotide change	Mutation effect	In silico analysis			1000g	ExAc	Classification	Evidence
			SIFT	PolyPhen-2	MutationTaster				
1	c.1435C>G	Splicing	NA	NA	NA	0	0	Pathogenic	PVS1 + PM2 + PM3 + PP4
2	c.2745G>A	p.Trp915*	NA	NA	Disease causing	0	0	Pathogenic	PVS1 + PM2 + PM3 + PP4
3	c.1479dupC	p.Leu495Profs*49	NA	NA	Disease causing	0	0	Pathogenic	PVS1 + PM2 + PM3 + PP4
4	c.2493_2494delAG	p.Arg833Ilefs*15	NA	NA	Disease causing	0	0	Pathogenic	PVS1 + PM2 + PM3 + PP4
5	c.1411C>T	p.Arg471*	NA	NA	Disease causing	0	3*	Pathogenic	PVS1 + PM2 + PM3 + PP4
6	c.1478delG	p.Gly493Alafs*53	NA	NA	Disease causing	0	0	Pathogenic	PVS1 + PM2 + PM3 + PP4
7	c.2221A>T	p.Lys741*	NA	NA	Disease causing	0	0	Pathogenic	PVS1 + PM2 + PM3 + PP4
8	c.(420 + 1_421-1)_ (1087 + 1_1088-1) del	Multi-exon deletion	NA	NA	NA	0	0	Pathogenic	PVS1 + PM2 + PM3 + PP4
9	c.1522G>A	p.Ala508Thr	Damaging	Probably damaging	Disease causing	0	7*	Pathogenic	PS3 + PM2 + PM3 + PP3 + PP4
10	c.1522_1533del12	In-frame deletion	NA	NA	Polymorphism	0	0	Likely pathogenic	PM2 + PM3 + PM4 + PP4
11	c.887A>C	p.Asp296Ala	Damaging	Probably damaging	Disease causing	0	0	Likely pathogenic	PM2 + PM3 + PP3 + PP4
12	c.731C>A	p.Ala244Asp	Damaging	Probably damaging	Disease causing	0	0	Likely pathogenic	PM2 + PM3 + PP3 + PP4
13	c.1388T>C	p.Leu463Ser	Tolerated	Probably damaging	Disease causing	0	0	Uncertain significance	PM2 + PM3 + PP4
14	c.1304C>T	p.Ala435Val	Damaging	Benign	Disease causing	0	0	Uncertain significance	PM2 + PP3 + PP4
15	c.2762G>T	p.Gly921Val	Damaging	Probably damaging	Disease causing	0	0	Uncertain significance	PM2 + PP3 + PP4

NA Not applicable, *1000g* 1000 Genomes Project, *ExAc* Exome Aggregation Consortium carriers

*Number of heterozygous

as "pathogenic variants" as well. Regarding the variant c.1435C>G, it has been confirmed to lead to a null effect (exon 11 skipping and a frameshift) by the mini-gene splicing assay, thus it is also considered as "pathogenic variants". And the missense variant c.1522G>A (p.Ala508Thr) has been confirmed to be the "pathogenic variants" by in vitro study previously [5]. While both missense variants p.Asp296Ala and p.Ala244Asp can be classified as "likely pathogenic variants" because they are *in trans* with other pathogenic variants (nonsense variants), respectively. However, p.Leu463Ser, p.Ala435Val, and p.Gly921Val were classified as VUS mainly due to lack of sufficient information and/or evidence for it to be classified as likely pathogenic/pathogenic.

Treatment and follow-up

The present treatment regimen was shown in Table 4. Patient A3 refused any medication treatment, patient S5 only agreed to supplementary treatment of potassium chloride, patient N2 took indomethacin alone, and patient J1, J4, J6, Z7, H8, and J9 accepted combined treatment of indomethacin and potassium chloride. The adjustment of medication dosage was based on the degree of recovery from growth retardation and electrolyte disturbance. The mean follow-up period was 6 months (2–18 months).

The growth curves of these patients and the time of medication intervention were shown in Fig. 3. The patient Z7 is 10.5 years old now, and he has never suffered from growth retardation. At present, he is 140 cm tall and weighs 45 kg, which is appropriate for his age. On the contrary, both height and weight of the other eight patients were two standard deviations lower than the mean value. By treatment, concentration of plasma electrolytes could maintain within the normal ranges in all patients with the exception of patient J4 who still presented with mild hypochloremia. Patients treated with indomethacin (J1, N2, J4, J6, H8, and J6) acquired varying degrees of recovery in growth rate, especially for the patient N2, who expressed prominent restoration within a short course of treatment. But most patients have not achieved standard level of height or of body weight up to now. Patients who did not receive indomethacin treatment (A3, S5) showed no increase of growth rate. The estimated glomerular filtration rate (eGFR) of patient J6 and Z7 were below the normal range on the first admission, but did not markedly decline during the treatment and follow-up. The eGFR of patient S5 showed obvious decline from 97.3 mL min^{-1} per 1.73 m^2 to 52.5 mL min^{-1} per 1.73 m^2 in 6 months.

Table 4 The treatment regimen and the after-therapy laboratory results of the nine patients with BS1

Patient	Indomethacin (mg kg^{-1} d^{-1})	KCl (g kg^{-1} d^{-1})	SK (mmol L^{-1})	SCl (mmol L^{-1})	CO_2CP (mmol L^{-1})	eGFR (mL min^{-1} per 1.73 m^2)
J1	1.02	0.04	4.60	105.8	25	190.8
N2	1.19	0.00	3.91	102.6	27	123.2
A3	0.00	0.00	3.45	99.6	26	36.5
J4	1.19	0.11	4.44	95.0	25	153.2
S5	0.00	0.13	4.57	101.8	26	52.5
J6	1.41	0.25	3.85	101.5	24	65.0
Z7	0.42	0.22	3.50	106.2	26	67.7
H8	1.05	0.15	3.78	108.6	25	129.4
J9	1.02	0.38	3.52	99.0	27	118.7

eGFR was calculated by Schwartz formula

BS1 Bartter syndrome type I

Discussion

Since Simon et al. first discovered that BS could be caused by mutations in *SLC12A1* gene in 1996, only two huge group studies of BS1 have been reported by Vargas-Poussou R (13 families in 1998) and Puricelli E (10 cases in 2010) so far [3, 15, 17]. In our previous studies, we proposed that the incidence of BS3 was likely the highest among the subtypes of BS in Chinese population [10]. To date, we have recruited 41 BS cases, in which the number of BS3 patients still consumes the highest percentage (65.9%, 27/41). The second largest number of BS1 patients (22.0%, 9/41) suggesting that the incidence of BS1 was only second to BS3, whereas BS2 only represents 7.3% (3/41) of all BS patients. However, in a recent study from Legrand et al., the type 1, 2, and 3 BS accounts for 24%, 25%, and 19%, respectively, in a large multi-ethnic cohort (White 56%, North African 21%, Middle East 12%, and African 9%) [18]. This discrepancy may indicate differences in incidence between Asians and other ethnic groups, the more credible incidence of Asians concerning various type of BS depends on more investigations involved larger cohort of patients or prospective studies to confirm however.

Among those nine patients with BS1, a total of 15 different mutations of *SLC12A1* gene were identified in this study, including 11 novel ones, which would expand the mutation spectrum of *SLC12A1* gene in human gene mutation database (HGMD) from 74 mutations to 85 (HGMD Professional 2018.3). Except for two patients detected carrying homozygous mutation p.Leu495Profs*49 and p.Arg471*, the other seven patients were all detected with compound heterozygous mutations of *SLC12A1* gene.

Among the novel mutations in this study, the most common type was missense variant (5/11). As for the five novel missense variants, the assessment results of p.Ala244Asp and p.Asp296Ala using 2015 ACMG standards

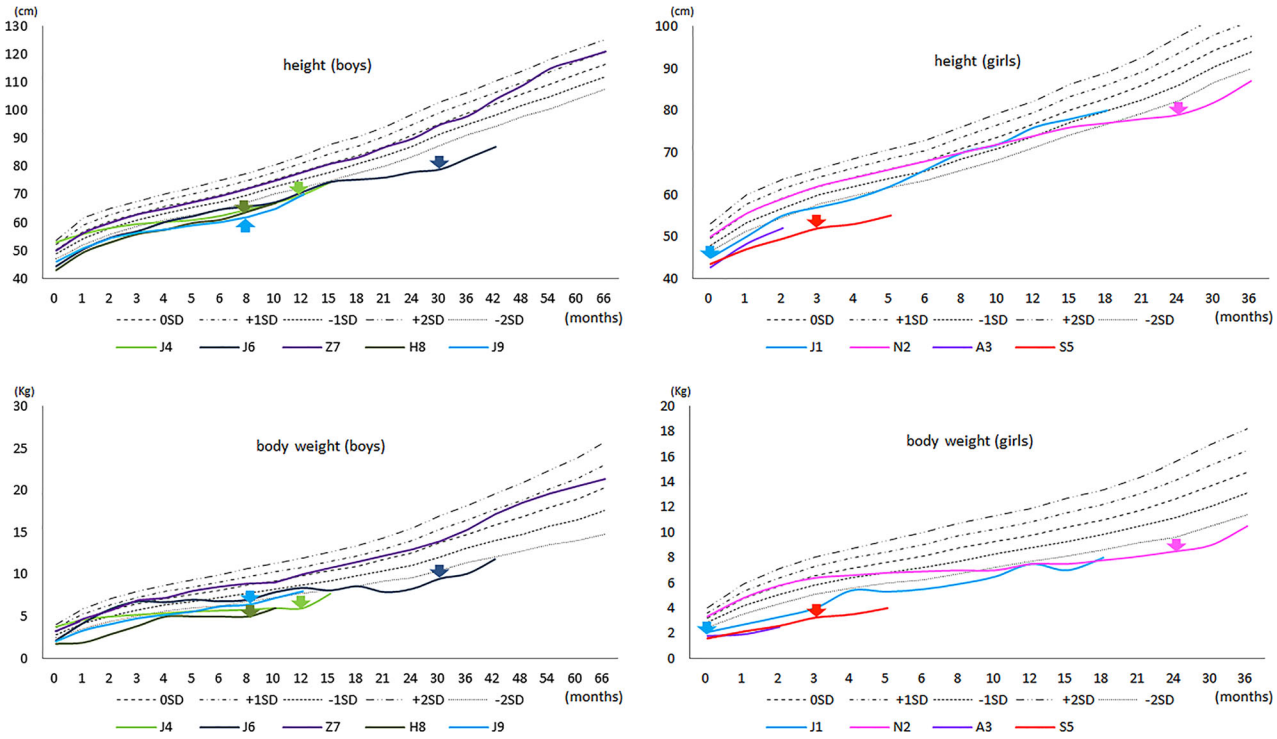

Fig. 3 The growth curve of the nine patients with BS1. Arrows point to the beginning of the treatment.SD standard deviation

and guidelines suggested pathogenic. The other three novel variants p.Ala435Val, p. Leu463Ser, and p.Gly921Val were all classified as VUS. The novel in-frame deletion mutation p.Ala508_Ser511del in this study deleted four amino acids from 508 to 511, which are located in an intracellular loop between the eighth and ninth transmembrane domain. The mutation p.Ala508Thr at the same site had been expressed on the *Xenopus laevis* oocyte system and was found to result in lower than normal expression level and nonfunctional NKCC2 by Starremans et al. [5]. The high conservation of 508–511 amino-acid residues and the surrounding sequences also indicates that it might be an important functional domain of NKCC2 (Supplemental Fig. 1). And apparently, except for the newly found five missense and one in-frame deletion variants, all the other novel ones were severe pathogenic mutations. The two novel nonsense variants, p.Lys741* and p.Trp915*, might produce truncated proteins different in length or lead to nonsense-mediated mRNA decay. Another novel small deletion c.2493_2494delAG (p.Arg833Ilefs*15) and duplication c.1479dupC (p.Leu495Profs*49) could be causative in a similar way of c.1478delG (p.Gly493Alafs*53), which has been described in our previous study [6]. And the gross deletion c. (420 + 1_421-1)_(1087 + 1_1088-1)del was thought to lose a great deal of genetic information from the exon 3 to exon 8 in *SLC12A1*.

Through splicing prediction of NetGene2, it was demonstrated the possible weakened recognition of 3′ acceptor splicing site of the novel variant c.2762G>T. However, the mini-gene splicing assay confirmed it is an authentic missense variant (p.Gly921Val). In addition, as can be seen from Fig. 1, we found the exonic mutation c.1435C>G, which was formerly categorized as missense mutation, perhaps lead to splicing errors through influencing related ESEs and/or ESSs motifs by the evaluation of ESEfinder 3.0 and HSF 3.1. Furthermore, we demonstrated that the exonic mutation of c.1435C>G disturbed the normal splicing in vitro (Fig. 2). Usually, a series of juxtaposed sequence motifs such as ESEs and ESSs act in a combinatorial manner to regulate exon usage. They enhance or silence the use of adjacent splice sites by recruiting different protein factors [9]. As shown in Fig. 1, this single-exonic nucleotide change not only brings about destruction of many kinds of ESEs, but also creates a variety of ESSs, leading to a prominent decrease in the ratio of numbers of ESEs to ESSs; thus, the total strength of recognition and usage of adjacent splice site markedly reduced. Additionally, the ESEs/ESSs effectively contribute to the exons with the authentic splice sites displaying reduced homology with the consensus sequences [9]. We therefore assessed the homology of all of the splice sites in the *SLC12A1* gene to identify the vulnerable exons using the BDGP. These analyses revealed that exon 11 had a weak 5′ donor site (Supplemental Table 3).

In several previous studies, mild phenotypes of BS1 have been reported [19–21], and some variants found from these

patients have been verified to have residual function of NKCC2 through functional studies. In this study, it is noteworthy that patient N2 harboring the variants p. Ala435Val and p.Gly921Val showed a phenotype of cBS. She was born at 40 weeks of gestation through spontaneous labor with birth weight of 3.34 kg, and she had neither suffered from maternal polyhydramnios nor underwent premature birth as the other aBS patients. Patient J4 carrying p. Leu463Ser and p.Ala508_Ser511del was born at 32 weeks gestation with birth weight of 2.05 kg, which proved to be the second largest number of gestation weeks and birth weight in this study. Meanwhile, he expressed good response to medical treatment with a rapid recovery of growth rate and restoring laboratory indicators in a short period of treatment. The milder phenotype of N2 and J4 might be related to milder genotype with pathogenic variants but possessing residual function. But the pathogenicity of these variants still requires confirmation through functional expression study. Surprisingly, the patient Z7, a boy detected with a huge deletion and a frame-shift mutation, showed normal growth development without any treatment until the age of 10, and his body weight and height are still within the normal range up to now. To account for the discrepancy between genotype and phenotype of patient Z7, we need to take consideration of potential environmental effects, diet habits or even some undiscovered modifying genes such as underlying genes taking a role in reabsorption of salt and water.

The recovery of growth in this study was not significant as in previous studies based on similar treatment regimen [17]. Till the end point of follow-up, more than half of patients failed to acquire great improvement in growth and development, that was to say, the height and body weight of those patients were still two standard deviations lesser than according average levels. Two main possible reasons for unsatisfied recovery were as follows. First, the follow-up period was still shorter (6.0 ± 6.4 months) compared with investigation from Puricelli et al. with an average follow-up of 11 years. Second, inadequate drug dosage as a result of short observation period and non-compliance of drug application in infant patients could be responsible for unexpected recovery. Taking the factors above into consideration, we are hopeful that patients in this research will obtain better growth and developmental tendency on the basis of appropriate dosage of medication during the long-term follow-up in the future.

The incidence of nephrocalcinosis in this study was high (6/9), which was consistent with classical characteristic of BS1. Compared with BS3, we observed that the hypokalemia was easier to correct in BS1. Of which the lower incidence of hypokalemia, the less medicine dosage and the higher serum potassium concentration all supported this characteristic.

In conclusion, these findings reveal that exonic mutations caused exon skipping in the *SLC12A1* transcript by the combination of in silico and in vitro assays. The concept of exon skipping due to ESEs disruption and/or ESSs creation provides an alternative approach to the functional analysis of the missense mutations responsible for BS1. Additionally, the present study had found 15 mutations, including 11 novel ones, which would enrich HGMD and would provide valuable references to the genetic counseling and diagnosis of BS1 for Chinese population.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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