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Original Article

Phenotypic and genotypic characteristics of children with Bartter syndrome

Serçin Güven[1], İbrahim Gökçe[1], Ceren Alavanda[2], Neslihan Çiçek[1], Ece Bodur Demirci[1], Mehtap Sak[1], Serim Pul[1], Özde Nisa Türkkan[1], Nurdan Yıldız[1], Pınar Ata[2], Harika Alpay[1]

[1]Division of Pediatric Nephrology, Department of Pediatrics and [2]Department of Medical Genetics, Marmara University Faculty of Medicine, İstanbul, Türkiye.

ABSTRACT

Introduction. Bartter syndrome (BS) is a group of autosomal-recessive tubular disorders and it is classified into five genetic subtypes. BS can also be classified by phenotype (antenatal, classic). Patients with mutations in the same gene can present different phenotypes. In the present study, target gene sequencing was performed to evaluate the genotype-phenotype relationship.

Methods. Biochemical, clinical and renal ultrasonography results were collected at presentation and the last clinic visit. Genetic analyses were performed. The findings of patients with classical BS (cBS) and antenatal BS (aBS) at presentation and the last visit were compared.

Results. Our study included 21 patients (12 female, 57.1%) from 20 families with BS. The median age at diagnosis was 8 months and the median follow-up period was 39 months. The most frequent complaint was growth failure. We have found 18 different types of mutations in four genes, including nine in the *CLCNKB* gene, seven in the *SLCA12A1* gene, one in the *KCNJ1* gene and one in the *BSND* gene. In ten patients, nine different types of CLCNKB gene mutations were detected, five of them were novel. Seven different mutations in the SLC12A1 gene were detected in eight patients, five of them were novel. Compared to patients with aBS and cBS, prematurity was significantly higher in the group with aBS. Nephrocalcinosis was present in only one patient with cBS, all the ten hypercalciuric patients with aBS had nephrocalcinosis at the time of diagnosis and the last visit. The mean height standard deviation score (SDS) of patients with aBS were significantly lower than the cBS group at the time of presentation. The mean weight SDS at the time of presentation was worse in patients with aBS than in patients with cBS. The mean plasma potassium and chloride concentrations were significantly lower in the patients with cBS at the time of diagnosis.

Conclusions. This investigation revealed the mutation characteristics and phenotype-genotype relationship of our patients and provided valuable data for genetic counseling.

Key words: Bartter syndrome, phenotype, genotype, mutation.

Bartter syndrome (BS) is a group of autosomal recessive tubular disorders characterized by hypochloremic, hypokalemic metabolic alkalosis with hyperreninemic hyperaldosteronism but normal blood pressure with a prevalence of 1 in 100,000.[1,2] BS is a heterogeneous disorder both clinically and genetically, that can be classified into five genetic subtypes (Suppl. Table I). Loss of function mutations in five genes: *SLC12A1*, *KCNJ1*, *CLCNKB*, *BSND* and *MAGE-D2* encoding proteins involved in ions transportation in the thick ascending limb of loop of Henle (TAL) and distal convoluted tubule (DCT) result in BS type I, type II,

✉ Serçin Güven
sercindr@yahoo.com

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type III, type IV and type V, respectively.[3] *SLC12A1* encodes the apical sodium-potassium-chloride cotransporter, NKCC2. *KCNJ1* gene encodes the apical voltage-dependent potassium channel, ROMK. *CLCNKB* gene encodes the basolateral chloride channel protein CIC-Kb. BSND gene encodes barttin, a β-subunit for CIC-Ka and CIC-Kb, expressed in the basolateral membrane of the TAL and cochlea. Loss of functions in alleles of both *CLCNKA* and *CLCNKB* are classified as BS type IVb, which results in a phenotype indistinguishable from that of BS type IVa. Recently, a novel transient form of aBS has been found to be caused by melanoma associated antigen-D2 (*MAGE-D2*) mutation, which is characterized by complete resolution of symptoms after birth, defined as BS type V[4] (Suppl. Table I). Gain of function mutation in the *CASR* gene encoding the basolateral calcium-sensing receptor, CASR, has been defined as BS type V in some reports.[5-8] But is nowadays classified as a Bartter like subform of familial hypocalcemia. BS type III is known as classical BS (cBS) whereas other types of BS are known as antenatal BS (aBS).

Typical findings of children with BS are growth retardation, polyuria, polydipsia, hypercalciuria and nephrocalcinosis. Different types of BS can be distinguished by different clinical manifestations. For example, type I BS presents with features of typical triangular facies, polyhydramnios, prematurity and hyperparathyroidism whereas patients with type II BS can have transient hyperkalemia. BS type III presents during early childhood with milder symptoms and generally without nephrocalcinosis, while type IV BS presents with sensorial deafness and mild hypochloremic alkalosis. Massive salt wasting and severe but transient hypochloremic metabolic alkalosis are characteristics of BS type V.[4,9] However, clinical findings may not always allow differentiation between subtypes, and even patients with mutations in the same gene may present different phenotypes.[10] For this reason, a gene-based classification is important for the definitive diagnosis.

In this study, we collected clinical, laboratory and renal ultrasonography results from patients with BS. We aimed to detect new pathogenic mutations by target gene sequencing. We planned to evaluate the genotype-phenotype correlation, especially the effects on growth, in the presence of newly identified mutations. The presentation and follow-up findings of our patients with classical and antenatal BS were also compared.

Material and Methods

The study was approved by the Institutional Ethics Committee of Marmara University Hospital (Protocol number: 09.2021.156). A written informed consent was obtained from all individual participants older than 18 years of age and the parents of all children included in the study. This study included 21 patients from 20 families with BS who were followed up in the Pediatric Nephrology Department of Marmara University Hospital. All patients in this study were hypokalemic and hypochloremic and had metabolic alkalosis, high renin and aldosterone levels with normal blood pressure. We classified BS into four types (I-IV). Patients with secondary BS or pseudo-BS were excluded

Genetic analysis

After detailed pedigree analyses and written informed consents were obtained, all patients' and their parents' total genomic DNA was extracted from peripheral blood using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). *CLCNKB* (NM_000085), *CLCNKA* (NM_001042704), *BSND* (NM_057176), *SLC12A1* (NM_000338), *KCNJ11*(NM_000220) genes were sequenced using Sophia Nephropathies Solution (NES) kit via Next-Generation Sequencing (NGS) (Illumina Nextseq 500). Since the used kit (NES) did not contain the MAGE-D2 gene, this gene could not be analyzed in patients. Single nucleotide polymorphisms (SNPs) and Copy number variations (CNVs) were analyzed through Sophia-DDM-v4 platform. Variants with minor allele frequency

(MAF) <1% according to population studies [ESP, ExAC, 1000 Genome (1000G), and Genome aggregation database (gnomAD)] were filtered and retained variants were searched in the Human genome mutation database (HGMD), Clinvar and Varsome databases. Pathogenicity scores were predicted using Mutation taster, Provean, Polyphen, Human Splicing Finder (HSF) and Sorting Intolerant From Tolerant (SIFT) in silico tools. Segregation analyses for detected mutations were performed and exon deletions were confirmed via ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Patients and biochemical analysis

Biochemical results, presenting symptoms and medical treatments were recorded at presentation and the last visit (respectively). Hypomagnesemia was defined as a serum magnesium level below 1.7 mg/dl and hypokalemia as a serum potassium level below 3.5 mEq/L. Hypochloremia was defined as a serum chloride concentration below 98 mEq/L. Hypernatremia was defined as a serum sodium level greater than 145 mEq/L. Normocalciuria was defined with the normal ranges for the patient's age.[11] Estimated glomerular filtration rate (eGFR) was calculated using the modified Schwartz formula, with a k-value of 0.413 and with serum creatinine measured by enzymatic method.[12]

Classical BS and aBS and also patients with missense and truncating mutations were compared in terms of demographic, biochemical characteristics, anthropometric data, urine examinations and renal ultrasonography (US) results. Renal US was performed at presentation in all children and repeated in follow-up.

Assessment of growth

Body height, weight and body mass index (BMI) standard deviation scores (SDS) were recorded at the presentation and the last visit according to the anthropometric references in Turkish children.[13] Frequency of height SDS <-2 and BMI SDS <-2 were also recorded. An increase in SDS more than +1 SDS was defined as improvement; a decrease in SDS more than -1 SDS was defined as deterioration; cases in between were defined as stable.

Statistical analysis

All data were analyzed using the Statistical Packages for the Social Sciences (SPSS Inc., Chicago, IL, USA) 21.0 package. Categorical variables were expressed as numbers and percentages. The normality of distribution for continuous variables was confirmed by the Shapiro-Wilk test. Results are expressed as mean with standard deviation (mean ± SD) in case of normal distribution and median (IQR: Q1-Q3; min-max) in case of non-normal distribution. Comparisons of numerical variables between two independent groups were evaluated using t-test and Mann-Whitney U test in normal distribution and non-normal distribution, respectively. In related samples of groups, the paired samples t-test was used in case of normal distribution and Wilcoxon test in case of non-normal distribution. Chi-square test was used for comparison of the categorical data. A p-value of <0.05 was considered statistically significant.

Results

Genetic analysis results; known and novel mutations

The detected mutations determined by the molecular genetics studies are shown in Table I. In the entire cohort, a compound heterozygous mutation was detected in only one patient (P10), with a novel mutation affecting one allele and a known mutation affecting the other. Homozygous mutations were detected in all other patients. The most common mutated gene was *CLCNKB*. In ten patients, nine different types of *CLCNKB* (NM_000085) gene mutations were detected, five of them were novel (there were one compound heterozygous and four homozygous patients for novel mutations).

Table I. Eighteen variants with 10 novel variants (showed in bold) in Bartter syndrome-related genes.

Patients	Clinical diagnosis	Gene	Status	Mutation	Position	Type of mutation	Reference
Type III+	Classical BS	*CLCNKB*	Homozygous	c.371C>T (p.Prol24Leu)	Exon 5	Missense	Simon et al., 1997
Type III+	Classical BS	*CLCNKB*	Homozygous	c.371C>T (p.Prol24Leu)	Exon 5	Missense	Simon et al., 1997
Type III	Classical BS	*CLCNKB*	Homozygous	**c.867-2delA**	Intron 8	Splice-site	Novel
Type III	Classical BS	*CLCNKB*	Homozygous	Exon 2-20 deletion	Exon 2-20	Gross deletion	Simon et al., 1997
Type III	Classical BS	*CLCNKB*	Homozygous	**c.499-2insG**	Intron 4	Splice-site	Novel
Type III+	Classical BS	*CLCNKB*	Homozygous	c.910C>T (p.Arg304*)	Exon 10	Nonsense	Messa et al., 2020
Type III+	Classical BS	*CLCNKB*	Homozygous	c.910C>T (p.Arg304*)	Exon 10	Nonsense	Messa et al., 2020
Type III	Classical BS	*CLCNKB*	Homozygous	**c.1930-2A>C**	Intron 18	Splice-site	Novel
Type III	Classical BS	*CLCNKB*	Homozygous	**c.499G>A (p.Gly167Ser)**	Exon 5	Missense	Novel
Type III	Classical BS	*CLCNKB*	Compound heterozygous	**c.66G>A (p.Trp22*)**	Exon 2	Nonsense	Novel
				c.865G>C (p.Gly289Arg)	Exon 9	Missense	Sahbani et al., 2020
Type IV	Antenatal BS	*BSND*	Homozygous	Exon 2-4 deletion	Exon 2-4	Gross deletion	Bircan et al., 2009
Type II++	Antenatal BS	*KCNJ1*	Homozygous	c.365T>A (p.Val122Glu)	Exon 5	Missense	Károlyi et al., 1997
Type II++	Antenatal BS	*KCNJ1*	Homozygous	c.365T>A (p.Val122Glu)	Exon 5	Missense	Károlyi et al., 1997
Type I	Antenatal BS	*SLC12A1*	Homozygous	c.596G>A (p.Arg199His)	Exon 4	Missense	Acar et al., 2019
Type I	Antenatal BS	*SLC12A1*	Homozygous	**c.2572C>T (p.Arg858*)**	Exon 21	Nonsense	Novel
Type I	Antenatal BS	*SLC12A1*	Homozygous	**c.1034C>A (p.Thr345Asn)**	Exon 8	Missense	Novel
Type I	Antenatal BS	*SLC12A1*	Homozygous	**c.2584A>T (p.Lys862*)**	Exon 21	Nonsense	Novel
Type I	Antenatal BS	*SLC12A1*	Homozygous	**c.2276G>A (p.Gly759Asp)**	Exon 18	Missense	Novel
Type I	Antenatal BS	*SLC12A1*	Homozygous	c.348dupT (p.Asn117*)	Exon 2	Nonsense	Adachi et al., 2007
Type I++	Antenatal BS	*SLC12A1*	Homozygous	**c.2485+5G>A**	Intron 20	Splice-site	Novel
Type I++	Antenatal BS	*SLC12A1*	Homozygous	**c.2485+5G>A**	Intron 20	Splice-site	Novel

+: unrelated patients, ++: related patients

Novel mutations were one nonsense (c.66G>A, p.Trp22*), one missense (c.499G>A, p.Gly167Ser) and three splice-site (c.867-2delA; c.499-2insG; c.1930-2A>C) mutations (Fig 1). Recurrent mutations were whole gene deletion (exon 2-20) and missense c.865G>C (p.Gly289Arg) mutation detected in one patient each, and missense c.371C>T (p.Prol24Leu) mutation and nonsense c.910C>T (p.Arg304*) mutation detected in two patients each. Seven different mutations in the *SLC12A1* (NM_000338) gene were detected in eight patients. Two missense (c.1034C>A, p.Thr34Asn; c.2276G>A, p.Gly759Asp) mutations, two nonsense (c.2572C>T, p.Arg858*; c.2584A>T, p.Lys862*) mutations and one splice-site (c.2485+5G>A) mutation were novel (Fig 2). *KCNJ1* (NM_000220) mutation (c.365T>A,p.Val122Glu) was detected in two patients who were related. One known gross deletion (exon 2-4 deletion) in the *BSND* (NM_057176) gene was detected in one patient. In the entire cohort, 9 (9/21) missense non-truncating mutations and 12 (12/21) truncating mutations were detected.

Patients' characteristics and biochemical analysis

Our study included 21 patients (12 female, 57.1%) from 20 families with BS. Fourteen consanguineous marriages were found in 20 families. The patients' demographic findings, biochemical parameters, urinary Ca/Cr ratios and renal US results at the time of presentation are summarized in Supp. Table II. The median age at diagnosis was 8 months (IQR: 4-18.5 months; min-max: 1-139 months). The most frequent complaint was growth failure (11; 52.3%) at diagnosis. Other frequent symptoms were vomiting (4; 19%) and polyuria-polydipsia (4; 19%). Four patients were diagnosed incidentally, one of them was diagnosed before an inguinal hernia operation, and three of them were diagnosed while being investigated for fever. Hypercalciuria was present in 16 patients (76.1%) and 11 patients had nephrocalcinosis (52.3%) at the time of presentation (Supp. Table II). Demographic, biochemical characteristics, medication at the last visit, and initial and final eGFRs of patients are given in Supp. Table III.

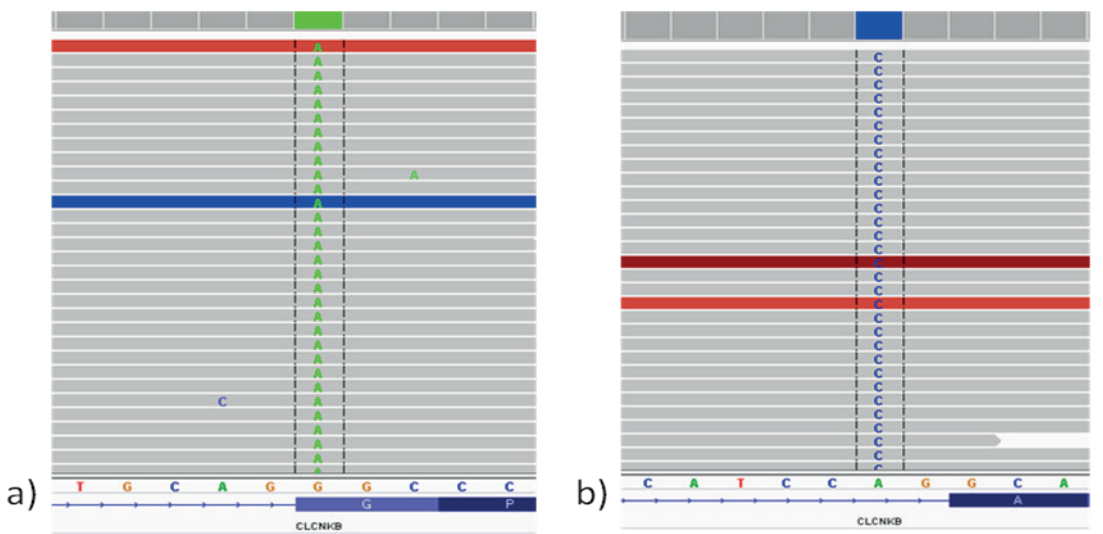

Fig. 1. The Integrative Genomic Viewer (IGV) sequence data of some novel mutations detected in *CLCNKB* gene (*NM_000085*) **a)** c.499G>A (p.Gly167Ser) **b)** c.1930-2A>C

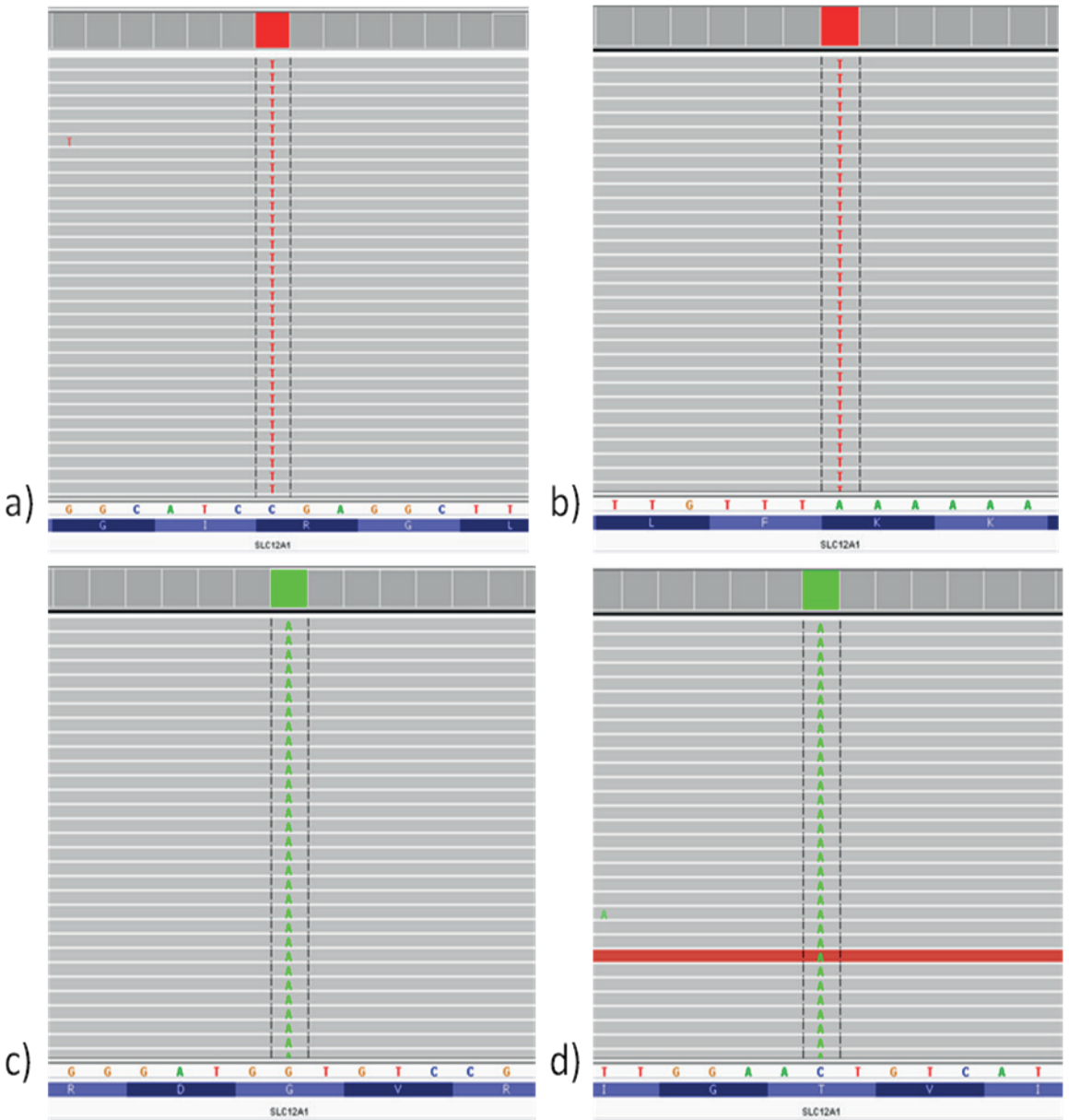

Fig. 2. The Integrative Genomic Viewer (IGV) sequence data of some novel mutations detected in *SLC12A1* gene (NM_000338) **a)** c.2572C>T (p.Arg858*) **b)** c.2584A>T (p.Lys862*) **c)** c.2276G>A (p.Gly759Asp) **d)** c.1034C>A (p.Thr345Asn)

The median age at the last visit was 48 months (IQR: 25-149 months; min-max: 14-245 months). The median follow-up period was 39 months (IQR: 17-105.5 months; min-max: 3-236). Medical treatment at the last follow-up visit included potassium supplementation (18; 85.7%), sodium supplementation (4; 19%), indomethacin (12; 57.1%) and spironolactone (10; 47.6%). The median eGFR was 79 ml/min/1.73m^2 (IQR: 56-113.5 months; min-max: 31-172 ml/min/1.73m^2) at presentation and 101 ml/min/1.73m^2 (IQR: 77-117 months; min-max: 8-192 ml/min/1.73m^2) at the last clinic visit.

Assessment of growth

At the time of presentation, the mean height SDS, weight SDS and BMI SDS were -2.32±1.39; -3.54±2.10 and -3.44±3.02, respectively. At the last visit the mean height SDS, weight SDS and BMI SDS were -2.00±2.03; -1.91±2.27 and -0.64±1.79, respectively. The improvements in the last weight SDS and BMI SDS according to the first presentations of the patients were found to be statistically significant (p=0.003; p<0.001, respectively), but there was no statistically significant difference in height SDS. Similarly, when the aBS and cBS groups were evaluated separately, there was an improvement in weight and BMI SDS in both groups at the initial and final visits, but no difference was found in height SDS (Table II). At the time of presentation, the percentage of patients with height SDS <-2 and BMI SDS <-2 were 57.1% (12/21) and 61.9% (13/21), respectively. At the last visit the percentage of patients with height SDS <-2 and BMI SDS <-2 were 47.6% (10/21) and 23.8% (5/21), respectively. The percentage of patients with a final BMI SDS <-2 according to the first presentation was found to be significantly higher (p=0.008). There was no difference in the percentage of those with height SDS <-2. On the other hand, according to the changes in height SDS over time, ten patients (47.6%) remained stable, eight patients improved (38%) and only three patients deteriorated (14.2%).

Comparison of the initial and final findings of patients with classical BS and antenatal BS

The presenting and follow-up findings of 10 patients with cBS and 11 patients with aBS were compared (Table II). There was no difference between the two groups in terms of age of diagnosis and follow-up time. Prematurity was significantly higher in the patients with aBS, compared to the patients with cBS (p=0.017). As a presenting complaint, growth failure was more frequent in the cBS group (7/10; 70%) than aBS group (4/11; 36.3%); but polyuria-polydipsia was more frequent in the aBS group (1/10; 10% vs. 3/11; 27.2%). The mean plasma potassium and chloride concentrations were significantly lower in the patients with cBS at the time of diagnosis (p=0.009 and p=0.020, respectively). Hypercalciuria was non-significantly lower in patients with cBS than patients with aBS at the presentation (6/10; 60% vs. 10/11; 91%) and the last visit (1/10; 10% vs. 6/11; 57.5%) Nephrocalcinosis was present in only one patient with cBS, this patient had a truncating mutation, on the other hand, all the ten hypercalciuric patients with aBS had nephrocalcinosis at the time of diagnosis and the last visit. We had only one patient in the aBS group who was normocalciuric and did not have nephrocalcinosis. (Table II, Suppl. Table II). The mean height SDS at the time of presentation was significantly worse in patients with aBS than in patients with cBS (p=0.03). Similarly, at the last visit the mean height SDS of patients with aBS was lower than patient's with cBS with a border p-value (p = 0.051). The percentage of patients with height SDS <-2 at the presentation and the last visit were higher in aBS group than cBS (p=0.03 and p=0.08, respectively). The mean weight SDS at the time of presentation was worse in patients with aBS than in patients with cBS, very close to statistical significance (p=0.06). Other initial and final anthropometric values (weight SDS at the last visit, BMI SDS and percentage of patients with BMI SDS <-2) of both groups were not statistically different (Table II). Although the difference was statistically not significant, eGFR at presentation and the last visit was worse in the antenatal group than in the classical group (Table II). In our study, the only patient who had chronic kidney disease (CKD) stage V at the last visit and underwent hemodialysis was in the aBS group.

Comparison of the initial and final findings of patients with classical BS

The median age was 11.5 months (IQR: 6.2-18 months; min-max: 1-76 months) and 49 months (IQR: 25-77.5 months; min -max:18-196 months) at diagnosis and the last visit, respectively.

With the effect of treatment, the mean plasma sodium, potassium and chloride concentrations were significantly higher at the last visit (p=0.008, p=0.00, p=0.12, respectively). The mean BMI SDS at presentation was significantly higher than the mean BMI SDs at the last follow-up (p=0.032) (Table II).

Comparison of the initial and final findings of patients with antenatal BS

The median age was 5 months (IQR: 3-20 months; min-max: 2-139 months) and 47 months (IQR: 23-189 months; min-max: 14-245 months) at diagnosis and the last visit respectively. The mean plasma potassium concentrations were

Table II. Comparison of the findings of patients at presentation and at last visit according to clinical groups: classical Bartter syndrome (cBS) and antenatal Bartter syndrome (aBS)

Parameters	At presentation		p	At last visit		p	p*	p**
	Classical BS (n=10)	Antenatal BS (n=11)		Classical BS (n=10)	Antenatal BS (n=11)			
Female/male	5/5	7/11	0.520				-	-
Age at presentation, months	17.3±21.4	25.4±44.5	0.387				-	-
Age at the last visit, months				61.7±52.9	103.9±93	0.756	-	-
Follow-up, months				44.4±35.9	78.4±72.6	0.189	-	-
Prematurity, n (%)	3 (30.0)	10 (90.9)	**0.017**				-	-
Polyhydramnios, n (%)	6 (60.0)	9 (81.8)						
Growth failure, n (%)	7 (70.0)	4 (36.3)	-			-	-	-
Polyuria-Polydipsia, n (%)	1 (10.0)	3 (27.2)	-	1 (10.0)	3 (27.2)	-	-	-
Vomiting, n (%)	3 (30.0)	0 (0)	-	3 (30.0)	0 (0)	-	-	-
Incidental, n (%)	1 (10.0)	3 (27.2)	-	2 (20.0)	3 (27.2)	-	-	-
Hearing loss, n (%)	0 (0)	1 (9.1)	-	0 (0)	1 (9.1)	-	-	-
Blood pH	7.53±0.8	7.48±0.09	0.219	7.48±0.06	7.45±0.06	0.263	0.086	0.379
Blood HCO^3 (mmol/L)	36.6±9.5	30.6±5.7	0.104	33.4±5.4	33.6±9.7	0.970	0.267	0.371
Plasma sodium (mEq/L)	133.6±5.1	138.6±6.8	0.072	138.7±2.8	140.2±5.9	0.482	**0.008**	0.540
Plasma potassium (mEq/L)	2.5±0.4	3.1±0.5	**0.009**	3.4±0.5	3.6±0.6	0.490	**0.000**	**0.011**
Plasma chloride (mEq/L)	84.4±10.03	93.2±7.73	**0.020**	93.8±3.76	95.3±10.66	0.656	**0.012**	0.283
Plasma calcium (mg/dl)	10.6±0.81	10.8±0.81	0.654	10.6±0.53	10.1±0.62	0.052	0.798	**0.027**
Plasma magnesium (mg/dl)	2.2 ± 0.5	2.4 ± 0.4	0.352	1.9±0.3	2.2±0.5	0.173	0.059	0.319
Hypercalciuria, n (%)	6 (60.0)	10 (90.9)	0.121	1 (10.0)	6 (54.5)	0.063	0.063	0.25
Nephrocalcinosis, n (%)	1 (10.0)	10 (90.9)	**0.001**	1 (10.0)	10 (90.9)	**0.001**	1	1
eGFR (ml/min./1.73m^2)	102.3± 40	77.4±37.6	0.159	119.9±40.7	83.2±34.7	0.173	0.203	0.624
Height SDS	-1.64±1.16	-2.9±1.3	**0.03**	-1.2±1.4	-2.8±2.3	0.051	0.354	0.803
Height SDS<-2, n (%)	3 (30.0)	9 (81.8)	**0.03**	2 (20.0)	6 (54.5)	0.08	1	0.5
Weight SDS	-2.6±1.6	-4.4±2.2	0.06	-1.5±1.5	-2.3±2.8	0.973	0.056	**0.026**
BMI SDs	-2.5±1.9	-4.3±3.6	0.314	-1.1±1.5	-0.3±1.9	0.291	**0.032**	**0.003**
BMI SDs <-2, n (%)	7 (70.0)	7 (63.6)	0.659	3 (30.0)	2 (18.1)	0.635	0.125	0.125
CKD stage, n (%)								
Stage II	3 (30.0)	3 (27.2)	-	2 (20.0)	3 (27.2)	-	-	-
Stage III	1 (10.0)	5 (45.5)	-	0 (0)	2 (18.1)	-	-	-
Stage V	0 (0)	0 (0)	-	0 (0)	1 (9.1)	-	-	-

BS: Bartter syndrome, CKD: chronic kidney disease, eGFR: estimated glomerular filtration rate, BMI: body mass index, SDS: standard deviation score.
Stage II: e GFR 60-90 ml/min./1.73m^2; Stage III: eGFR 30-59 ml/min./1.73m^2; Stage V: eGFR <15 ml/min./1.73m^2
P *: comparison of findings at the time of presentation and at the last visit in cBS group.
P** : comparison of findings at the time of presentation and at the last visit in aBS group.

significantly higher at the last visit (p=0.011) and plasma calcium concentrations were significantly lower at the last visit (p=0.027). The mean weight SDS and BMI SDS were significantly higher at the last visit (p=0.026 and p=0.003, respectively) (Table II).

Comparison of the findings of patients with missense and truncating mutation

The median age of patients with a missense mutation was 7 months (IQR: 4-10.5 months; min-max: 3-15 months) at diagnosis. The median age of patients with a truncating mutation was 14 months (IQR: 3.5-62.25 months; min-max:1-139 months). The difference was statistically significant (p=0.034). BMI SDS were significantly higher at the last visit both in patients with missense mutation and truncating mutation (p=0.006 and 0.006, respectively). BMI SDS at the last visit were also significantly higher in patients with truncating mutation than missense mutation (p=0.021) (Suppl. Table IV).

Discussion

The present study describes the clinical features and genotypes of 21 children with BS from a single center and it also evaluates the clinical, radiological and biochemical data of patients with type I-II-IV BS known as aBS and patients with type III BS known as cBS.

The most commonly affected gene was *CLCNKB* and this result was compatible with previous studies.[3] c.867-2delA mutation was not reported in population studies. The mutation taster predicted this variant as pathogenic, but according to HSF, it had no significant impact on splicing signals. c.499-2insG and c.1930-2A>C mutations which were not reported in population studies, were predicted as pathogenic according to mutation taster and HSF. The c.499G>A (p.Gly167Ser) mutation was not reported in gnomAD, 1000G and exAC databases and was predicted as pathogenic according to in silico tools. The c.1930-2A>G mutation at the same position was previously reported.[14] The c.66G>A (p.Trp22*) variant was considered pathogenic because it caused premature protein termination and was not reported in public databases.-

Previous studies from different countries had shown that whole gene deletion was the most common mutation in *CLCNKB* gene, this mutation was detected in only one patient in our cohort.[3,15-17] This finding indicates that Turkish patients may have different ancestry. Although the c.1830G>A (p.Trp610*) mutation in the *CLCNKB* gene was one of the common mutations in Japanese and Korean patients[3,18], the absence of this mutation in all of our patients supports this hypothesis. In addition, the fact that we could not detect any hot spot mutations in our cohort also showed the genetic diversity in Turkish patients.

Seven different *SLC12A1* gene (NM_000338) mutations, five of which were novel, were detected in eight patients. The allele frequency of the novel c.2572C>T (p.Arg858*) and c.2584A>T (p.Lys862*) mutations were 0.00000797 and 0.00000398 in gnomAD, respectively. Both variants were extremely rare and were considered pathogenic because they had a truncated effect on the protein. c.1034C>A (p.Thr34Asn) mutation's allele frequency was 0.00003185 in GnomAD. Provean, SIFT, Polyphen and Mutation taster in silico tools predicted this mutation as pathogenic. The c.2276G>A (p.Gly759Asp) mutation was not reported in GnomAD, ExAC and 1000G databases and was predicted as pathogenic via in silico tools. Both novel missense mutations altered the conserved amino acid residues. c.2485+5G>A mutation's allele frequency was 0.000004013 in GnomAD and predicted as pathogenic according to Mutation taster and HSF in silico tools.

Among our patients, a known gross deletion (exon 2-4 deletion) in the *BSND* gene (NM_057176) was found in one patient. Mutations in the *BSND* gene cause type IV BS. Our patient presented with severe clinical symptoms. Hearing loss is the most important

characteristic of this type of BS and hearing loss was one of the presenting symptoms of our patient. Remarkable biochemical and clinical features of our patient were extremely low levels of serum chloride and the lowest initial eGFR with 31 ml/min./1.73 m^2. *BSND* mutations are one of the factors that have been reported to influence renal survival.[3] Bircan et al.[19] reported the same mutation in a 2-month-old boy. There were some similarities between the case presented in this report and our patient. Both patients needed a large amount of fluid and electrolytes, which could only be provided through a nasogastric tube and gastrostomy. Our patient was the only patient in the antenatal group who did not have hypercalciuria or nephrocalcinosis. In the patient reported by Bircan et al.[19], transient hypercalciuria was present. But Spanish, Turkish, Italian and Portuguese reports have described different mutations with hypercalciuria or normocalciuria.[20-23] Case presentations and series do not agree on hypercalciuria. In fact, inhibition of NaCl reabsorption in cBS causes hypercalciuria, whereas decreased NaCl reabsorption in the DCT is associated with hypocalciuria as in Gitelman syndrome.[22] The absence of hypercalciuria in BS type IV unlike other antenatal types can be explained by the fact that these two opposite effects are present in BS type IV because of the extent of the functional loss of barttin in the kidney tubules.[22]

In BS type II with *KCNJ1* mutations, transient hyperkalemia and also metabolic acidosis can be seen. In this study, *KCNJ1* (NM_000220) mutation (c.365T>A, p.Val122Glu) was detected in two patients (P12, P13) who were related. They were neither hyperkalemic nor acidotic, however, the bicarbonate values of these patients were not very high and even close to normal (36.6 mEq/L and 27.2 mEq/L, respectively) and the potassium values were also not low enough to need replacement and were even normal (3.9 mEq/L and 3.6 mEq/L, respectively).

An earlier age of diagnosis is mostly typical for aBS[9] and, in our cohort, the age at diagnosis was lower in the aBS group with 5 months compared to the cBS group with 11.5 months; but it was not statistically significant. This finding was consistent with other studies[3,15,24] but note that, at 84 months and 139 months, the two eldest children of our cohort were in the aBS group. A novel homozygous splice-site mutation (c.2485+5G>A) in *SLC12A1* gene was detected in these two patients who were cousins. It is not yet known whether this new mutation will affect the age at diagnosis in patients with type I BS.

Recently, it has been shown that patients with BS may be complicated by a nephrogenic diabetes insipidus (NDI) like phenotype.[25] Although most of our patients had normal serum sodium levels, we had two patients, who had remarkable hypernatremia (P15, P16). These two patients were diagnosed with BS type I with no identifiable mutations in AVPR2 or AQP2. Also, the serum sodium levels of those patients were not as high as they might be in patients with NDI. Secondary NDI seen in BS patients has been reported to be specific for BS type I and II, but it is unlikely to be a mutation specific complication.[25] Serum sodium levels were not statistically different in patients with cBS and aBS. But note that, hyponatremia was detected and treated only in the cBS group and in one patient with BS type IV in our cohort. Our patients with BS type I and II were normonatremic or hypernatremic. It is well known that hypochloremia and hypokalemia are more severe in patients with cBS.[9] In our study, plasma potassium and chloride concentrations were significantly lower in cBS group, as expected.

Growth retardation seen in BS is known to be the result of volume depletion.[26] In our cohort, the most common presenting complaint in the aBS group was polyuria-polydipsia and growth retardation was more common in the aBS at the last visit (Suppl. Table II). All findings in our study show that while there was an improvement in weight and BMI SDS in our patients at follow-up, there was no significant improvement in height SDS. Growth hormone (GH) deficiency has been reported in children

with BS.[27] Hochberg et al.[28] showed GH and insulin-like growth factor-1 did not stimulate longitudinal growth unless hypokalemia was corrected. In our study, GH was used in only one patient (P14), who was diagnosed with BS type I. She had one of the lowest initial eGFR among all patients and needed hemodialysis during follow-up.

The mechanism of CKD development is multifactorial. Nephrocalcinosis, chronic hypokalemia, long-term treatment with NSAIDs, damaging effect of elevated aldosterone levels on podocytes and BSND mutations are some of the possibilities for CKD development in patients with BS.[24] Prematurity is also thought to be a cause of CKD development.[24] It is assumed that patients with aBS are often born prematurely.[29-31] Similarly, prematurity was seen more commonly in our patients with aBS than cBS (90% vs 30%) (Table II). The fact in our study that eGFR values were lower in the antenatal group, also supports the relationship between prematurity and CKD.

One of the reasons for CKD is nephrocalcinosis and it is less frequently seen in the cBS group than in the aBS group. Hypercalciuria and nephrocalcinosis are more common in BS Type I and II due to the defective cotransporter NKCC2 as in BS type I and the defective ROMK activity as in BS type II.[32] Similarly, we detected hypercalciuria and nephrocalcinosis in almost all (10/11; 90.9%) patients with aBS in our study. Also, consistent with previous studies, in our cohort, nephrocalcinosis was detected in only one patient with cBS who had hypercalciuria. But note that, this patient had the highest level of calciuria among patients with cBS and one of the highest levels in the entire cohort. It has been reported that impaired salt reabsorption in TAL can lead to an impaired paracellular cation uptake, mostly manifesting as hypercalciuria as in *CLCNKB* mutations.[33]

Although few studies[15,17] have shown that there is a genotype-phenotype correlation in patients with cBS, many studies[34,35] have not found a significant genotype-phenotype correlation. One recent study[34] covering 30 patients with cBS detected no genotype-phenotype association, whereas Seys et al.[17] reported an association between complete loss of function (CL/CL) mutations of *CLCNKB* and severe phenotypes. Although we did not demonstrate by functional analysis, there was only one large deletion (Exon 2-20 deletion, P4) in our cohort that could be predicted to cause complete loss of function. This patient had the lowest eGFR at presentation and one of the lowest eGFR at the last visit among patients with *CLCNKB* mutation.

Similar phenotypic characteristics were not found even among patients harboring the same mutation. Additionally, intrafamilial variability in clinical manifestations was also present. This poor genotype-phenotype correlation may be associated with modifier genes, environmental factors, or epigenetic mechanisms. However, when aBS and cBS patients were compared, significant phenotypic differences were found between them, (even between types of antenatal groups) as mentioned above. In addition, we have not found any significant difference between patients with truncating mutation and missense mutation except in terms of presenting age and final BMI SDs (Suppl. Table IV). In addition, the only patient with nephrocalcinosis and cBS also had a truncating mutation. This makes us think that the localization of the mutation and the protein it affects are more related to the phenotype than whether the mutation is truncating or not.

The most important limiting factor of our study is the relatively low number of patients. Nonetheless, the strength of our study is that the diagnosis of our patients was confirmed by a very detailed genetic analysis. And it revealed the long-term outcome with respect to growth and CKD and phenotype-genotype relationship of our patients. We were also not able to investigate the *MAGE-D2* mutation because the kit (NES) we used did not contain the MAGE-D2 gene so this gene could not be

analyzed. However, further studies are planned to design specific primers analyzing this gene in panel negative patients. In addition, it would have been much better if we could have looked at the difference in findings of patients with truncating and non-truncating mutations in the aBS and cBS groups, and even in patients with a single type of mutation. Unfortunately, our patient numbers were not sufficient for these comparisons. For all these reasons, to explain the genotype-phenotype correlation with all its clarity, there is a need for prospective studies with longer follow-up periods, in which all genes that may cause BS are studied and more patients can participate.

In conclusion, we present the clinical features, molecular diagnosis and the prognosis of 21 children with BS. We found ten novel mutations and this investigation further expanded the mutation spectrum of BS and provided valuable data for genetic counseling. Although CLCNKB gene mutations were the most common type of mutation seen in our patients, NGS panels are pivotal when searching for disease-causing genes, especially in populations having genetic diversity like Turkish people.

Ethical approval

The study was approved by the Institutional Ethics Committee of Marmara University Hospital (Protocol number: 09.2021.156).

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: SG, IG, CA; data collection: SG, CA, EDB, MS, SP, ONT, PA; analysis and interpretation of results: SG, NC; draft manuscript preparation: SG, IG, NC, CA. All authors reviewed the results and approved the final version of the manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

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