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Differential diagnosis of classical Bartter syndrome and Gitelman syndrome: Do we need genetic analysis?

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ABSTRACT

Objective: Classical Bartter syndrome (cBS) and Gitelman syndrome (GS) are genotypically distinct, but there is a phenotypic overlap among these two diseases, which can complicate the accurate diagnosis without genetic analysis. This study aimed to evaluate the correlation between clinical and genetic diagnoses among patients who have genetically defined cBS and GS.

Patients and Methods: The study included 18 patients with homozygous/compound heterozygous *CLCNKB (NM_000085)* (n:10/18) and *SLC12A3 (NM_000339)* (n:8/18) mutations. Biochemical, clinical and radiological data were collected at presentation and at the last visit.

Results: In cBS group age at diagnosis, median plasma potassium and chloride concentrations were significantly lower and median plasma HCO3 and blood pH values were significantly higher. Patients with GS had significantly lower median plasma magnesium concentrations and urinary calcium/creatinine ratio. One child with GS had normocalciuria, two children with cBS had hypocalciuria and hypomagnesemia. Low estimated glomerular filtration rate (eGFR) (ml/dk/1.73m2) and growth failure were more evident in cBS group. In patients with cBS, nine different CLCNKB gene mutations were detected, five of them were novel. 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. In patients with GS, six different *SLC12A3* gene mutations were found.

Conclusions: It may not always be possible to clinically distinguish cBS from GS. We suggest to perform a genotypic classification if genetic analysis is possible.

Keywords: Bartter syndrome, Gitelman syndrome, Kidney tubuler disease, Hypokalemic metabolic alkalosis

1. INTRODUCTION

Bartter (BS) and Gitelman syndromes (GS) are autosomal recessive inherited salt-losing tubulopathies, characterized by hypokalemic hypochloremic metabolic alkalosis and normal blood pressure in the context of elevated renin and aldosterone levels [1]. Clinically, these tubulopathies are often categorized into 3 major subgroups: antenatal BS (aBS), classical BS (cBS) and GS. BS type-3 also known as cBS is caused by loss of function mutations in *CLCNKB* gene. *CLCNKB* gene encodes a chloride channel protein CIC-Kb, expressed in the thick ascending limp (TAL) of Henle's loop, the distal convoluted tubule (DCT), the connecting tubule and the collecting duct [1-3]. CIC-Kb has a role in the reabsorption of sodium chloride in the TAL and the loss of function mutations in *CLCNKB* gene cause electrolyte

abnormalities called 'loop phenotype'. The use of loop diuretics results in the same electrolyte abnormalities [2,3]. GS was initially thought to be a subtype of BS but advances in molecular genetics showed that it was a separate entity. GS is caused by loss of function mutations in the gene *SLC12A3*, encoding thiazide-senstitive NaCl cotransporter called NCCT of the DCT [3]. The NCCT is responsible for the sodium reabsorption in the DCT and like with loop dysfunction, impaired sodium reabsorption in the DCT causes characteristic electrolyte abnormalities, called 'DCT' phenotype [3].

Classical BS and GS share some metabolic abnormalities such as hypochloremic hypokalemic metabolic alkalosis. Clinical and metabolic findings like hypercalciuria, normomagnesemia,

early onset at younger age or severity of the disease are used to distinguish cBS from GS. In most cases, the correct diagnosis can be made without genetic analysis. However, it may not always allow distinction between them. Because hypercalciuria is not present always in all cases of cBS and some patients with cBS have hypomagnesemia, which is a typical feature of GS. Also, neither hypocalciuria nor hypomagnesemia are consistent findings in GS [4,5]. This study aimed to clarify clinical and laboratory findings among patients with genetically defined cBS and GS and evaluate the correlation between clinical diagnoses and genetic diagnoses.

2. PATIENTS and METHODS

Patients and biochemical analysis

The study included eighteen patients with homozygous or compound heterozygous *CLCNKB* (n:10/18) and *SLC12A3* (n:8/18) mutations presenting to the pediatric nephrology division of a university hospital. Biochemical, clinical and radiological data were collected at presentation and at the last clinic follow-up visit. Hypomagnesemia was defined as a serum magnesium concentration less than 1.7 mg/dl, hypokalaemia was defined as a serum concentration less than 3.5 mEq/L, hypochloremia was defined as a serum chloride concentration less than 98 mg/dl. Normocalciuria was defined with the normal ranges for the patient's age. Estimated glomerular filtration rate (eGFR) was calculated using the modified Schwartz formula, with a k-value of 0.413. Body height percentiles of patients were recorded at the presentation and at the last follow-up visit. Growth failure was defined as a height percentile less than 3. Kidney ultrasound imaging was performed at presentation and in the follow-up of all children.

Molecular Genetics studies

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). For all patients sequencing of *CLCNKB (NM_000085)* and *SLC12A3* genes *(NM_000339)* were performed using two different methods: Sanger sequencing and Next-Generation Sequencing (NGS). All samples were first analyzed by NGS and after detection of sequence changes, confirmation of them were performed using Sanger sequencing with targeted sequencing for those already detected mutations/variations. The Nephropathies Solution (NES) kit by Sophia Genetics (Switzerland) was used for NGS (Illumina Nextseq 500) and single nucleotide polymorphisms (SNPs) and copy number variations (CNVs) were analyzed through Sophia-DDM-v4 platform [ ]. For both of these genes, specific primers were designed for all exons and exon-intron junctions and Sanger sequencing was performed via ABI PRISM 3130xl Genetic Analyzer (AppliedBiosystems, Foster City, CA, USA). SNPs were analyzed with Chromas software version 2.4.1. 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 Human Gene 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.

Statistical analysis

All data were analyzed using the Statistical Packages for the Social Sciences (SPSS Inc., Chicago, IL, USA) 21.0 package. A One Sample Kolmogorov-Smirnov test was used to determine the normality of data. Results were expressed as mean with standard deviation (mean ± SD) in case of normal distribution and median (range) in case of non-normal distribution. Differences between categorical data were evaluated using t-test and Mann-Whitney non-parametric U test in normal distribution and non-normal distribution, respectively. A p-value of less than 0.05 was considered statistically significant.

3. RESULTS

Biochemical features and clinical features at presentation and at the last follow-up visit are summarized in Table I. Ten patients presented with cBS and eight patients with GS. Five patients (50%) with cBS and five patients (62.5%) with GS were male. Patients with cBS had a significantly lower median age at diagnosis than the patients with GS (11.5 vs. 128 months, respectively). The median follow-up time was 33 months in patients with cBS and 47 months in patients with GS.

Table I. *Clinical, demographic and biochemical characteristics of the patients*

	Classical Bartter Syndrome (n=10)	Gitelman Syndrome (n=8)	p
Age, months	11,5 (1-76)	128 (44-209)	**<0.001**
Female/male, n (%)	5/5 (50%)	3/5 (60%)	0.664
Follow-up, months	33 (3-120)	47 (19-174)	0,200
Prematurity, n (%)	3 (30%)	0 (0%)	0,090
Blood pH	7.57 (7.40-7.70)	7.44 (7.40-7.59)	**0.003**
Blood HCO3 (mEq/L)	41.1 (21.8-51.5)	30.6 (27.4-32.4)	**0.010**
Plasma potassium (mEq/L)	2.2 (1.9-3.1)	2.8 (2.2-3.7)	**0.002**
Plasma chloride (mg/L)	85 (67-96)	95 (88-101)	**<0.001**
Plasma magnessium (mg/dl)	2.15 (1.6-2.7)	1.4 (1.30-1.50)	**0.001**
Urinary calcium/creatinine ratio (mg/mg)	0.6 (0.03-1.9)	0,01 (0.01-0.67)	**<0.001**
eGFR (ml/dk/1.73 m²) (at presentation)	103 (49-172)	137 (99-172)	**0.043**
eGFR (ml/dk/1.73 m²) (at the last visit)	103 (76-192)	112 (89-271)	0.410
Height <3.persentile (at presentation) , n(%)	5 (50%)	2 (25%)	0.280
Height <3.persentile (at the last visit), n(%)	3 (30%)	1 (12.5%)	0.370

Values are represented as median (min – max), eGFR, estimated glomerular filtration rate

Median plasma potassium and chloride concentrations were significantly lower in cBS group (2.2 vs. 2.8 mEq/L; 85 vs. 95 mg/L, respectively). Median plasma HCO3 concentrations and blood pH values were significantly higher in cBS group (41.1 vs. 30.6 mEq/L; 7.57 vs. 7.44 respectively). Patients with GS had significantly lower median plasma magnesium concentrations (1.40 vs.2.15 mg/dl respectively) and urinary calcium/creatinine ratio (0,01 vs. 0,6 respectively). One child with GS had normocalciuria and two children with cBS had both hypocalciuria and hypomagnesemia at presentation. Median eGFR (ml/dk/1.73m²) at presentation were significantly lower in cBS patients than patients with GS (103 vs. 137 ml/dk/1.73m² respectively). At the last follow-up, patients with cBS had still lower eGFR (103 vs. 112 ml/dk/1.73m² respectively). Growth failure was the most frequent complaint of patients with cBS and it was more evident in patients with cBS (50% at presentation;

30% at last follow-up visit). Ultrasonography performed at presentation and in the follow-up showed nephrocalcinosis in only one child with cBS.

Molecular analyses, revealed mutations that are shown in Tables II and III. In ten patients with cBS, nine different *CLCNKB(NM_000085)* gene mutations were detected (Table II). Five of them were novel mutations. 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. In eight patients with GS, six different *SLC12A3 (NM_000339)* gene mutations were detected (Table III). Only one patient had compound heterozygous mutation and the majority of mutations caused a frameshift c.237_238dupCC (p.Arg80Profs*35) mutation.

Table II. Nine variants with five novel variants (showed in bold) in classical Bartter syndrome-related gene

Patients	Gene	Status	Mutation	Position	Type of mutation	Reference
B1	*CLCNKB*	Homozygous	c.371C>T (p.Prol24Leu)	Exon 5	Missense	Simon et al.,1997 [6]
B2	*CLCNKB*	Homozygous	c.371C>T (p.Prol24Leu)	Exon 5	Missense	Simon et al., 1997 [6]
B3	*CLCNKB*	Homozygous	**c.867-2delA**	Intron 8	Splice-site	This study
B4	*CLCNKB*	Homozygous	Exon 2-20 deletion	Exon 2-20	Gross deletion	Simon et al.,1997[6]
B5	*CLCNKB*	Homozygous	**c.499-2insG**	Intron 4	Splice-site	This study
B6	*CLCNKB*	Homozygous	c.910C>T (p.Arg304*)	Exon 10	Nonsense	Messa et al., 2020[7]
B7	*CLCNKB*	Homozygous	c.910C>T (p.Arg304*)	Exon 10	Nonsense	Messa et al., 2020[7]
B8	*CLCNKB*	Homozygous	**c.1930-2A>C**	Intron 18	Splice-site	This study
B9	*CLCNKB*	Homozygous	**c.499G>A (p.Gly167Ser)**	Exon 5	Missense	This study
B10	*CLCNKB*	Compound heterozygous	**c.66G>A (p.Trp22*)** c.865G>C (p.Gly289Arg)	Exon 2 Exon 9	Nonsense Missense	This study and Sahbani et al.,2020[8]

Table III. Six variants in Gitelman syndrome-related genes

Patients	Gene	Status	Mutation	Location	Type of mutation	Reference
G1	*SLC12A3*	Homozygous	c.1180+1G>T	IVS 9	Splice-site	Coto et al., 2004[9]
G2	*SLC12A3*	Compound heterozygous	c.237_238dupCC (p.Arg80Profs*35) c.514T>C (p.Trp172Arg)	Exon 1 Exon 4	Frameshift Missense	Mastroianni et al., 1996[10] Syren et al., 2002[11]
G3	*SLC12A3*	Homozygous	c.625C>T (p.Arg209Trp)	Exon 5	Missense	Simon et al., 1996[3]
G4	*SLC12A3*	Homozygous	c.1175C>T (p.Thr392Ile)	Exon 9	Missense	Colussi et al., 2007[12]
G5	*SLC12A3*	Homozygous	c.237_238dupCC (p.Arg80Profs*35)	Exon 4	Frameshift	Mastroianni et al., 1996[10]
G6	*SLC12A3*	Homozygous	c.237_238dupCC (p.Arg80Profs*35)	Exon 4	Frameshift	Mastroianni et al., 1996[10]
G7	*SLC12A3*	Homozygous	c.237_238dupCC (p.Arg80Profs*35)	Exon 4	Frameshift	Mastroianni et al., 1996[10]
G8	*SLC12A3*	Homozygous	c.1964G>A (p.Arg655His)	Exon 16	Missense	Simon et al., 1996[3]

4. DISCUSSION

The present study describes the initial clinical and biochemical characteristics and the genetic findings of the patients with cBS and GS. According to our findings, age at diagnosis, the urinary calcium/creatinine ratio, plasma chlorid, magnesium, bicarbonate levels may be useful markers for differentiating cBS and GS. Although, cBS and GS are genotypically distinct, there is considerable overlap in clinical presentation. cBS may sometimes mimic GS and some patients with cBS appear to have a phenotypic switch [13]. cBS is generally considered to be a disorder of TAL, whereas GS is a disorder of DCT. Initially, some cBS patients present with loop (or BS) phenotype but later they revert to a DCT (or Gitelman like) phenotype [14]. The expression of *CLCNKB* also in DCT may explain the phenotypic similarity of cBS and GS.

It is known that cBS often presents clinically before the age of two [15]. In this study, the age at diagnosis differed significantly between two diseases. Only one patient with cBS was diagnosed after the age of two years, whereas the youngest patient in GS group was 44 months old at the time of diagnosis.

Hypochloremia, hypokalemia and metabolic alkolosis are hallmarks of cBS. In our study, serum chloride and potassium levels were significantly lower and bicarbonate levels were higher in patients with cBS than GS as expected.

Hypercalciuria and normomagnesemia are used to distinguish cBS from GS. Hypercalciuria and nephrocalcinosis which are very important risk factors for chronic kidney disease progression, are less commonly seen in patients with cBS than aBS and not an expected finding in GS. Impaired salt reabsorption in the TAL leads to an impaired paracellular cation uptake, mostly manifesting as hypercalciuria as with *CLCNKB* mutations [1]. In contrast to loop dysfunction, impaired salt reabsorption in the DCT paracellular calcium reabsorption in the TAL is unaffected and patients typically have hypocalciuria due to a compensatory increase in salt and calcium reabsorption in the proximal tubule as with *SLC12A3* mutations [1]. Hypomagnesemia is present in 20-52% of patients with BS and this is caused by magnesium wasting [16]. Among our patients, hypercalciuria was found in six of ten patients with cBS. However, some patients with cBS may have hypomagnesemia and hypocalciuria [14,17-19]. Zelikovic et al., had reported that four patients in the 12 family members with *CLCKNB* mutations had concominant hypomagnesemia and hypocalciuria [20]. In our study, two children with cBS (B4, B7) had both hypocalciuria and hypomagnesemia at presentation. Patient B4 had a gross deletion (Exon 2-20 deletion) and patient B7 had a homozygous nonsense mutation (p.Arg304*) in exon 10 of *CLCNKB* gene. But we did not observe hypomagnesemia and hypocalciuria in another patient (B6) with the same mutation as patient B7. Recently, Ring et al. and Kamel et al., also reported that patients with GS may have normocalciuria [4,5].

In the present study, patients with GS had a significantly lower median plasma magnesium concentrations and urinary calcium/creatinine ratio. But similar to the above mentioned studies, one child with GS (G5) had normocalciuria. Patient G5 had a

homozygous frameshift mutation (p.Arg80Profs*35) in exon 4 of *SLC12A3* gene. However, our other patient (G6) with the same mutation had hypocalciuria. It is still unclear why the same genetic defect causes different clinical outcomes but Zelikovic et al., hypothesized that a modifying genetic effect on any one of channels participating in Cl⁻ transport in the TAL and the DCT may influence the disease phenotype [20]. Genetic analysis is pivotal for an accurate diagnosis in such patients.

Impaired GFR is observed more commonly in patents with BS than GS. It has been reported that 25% of patients with cBS suffer from CKD [15,21-24]. In our study, four patients with cBS had impaired GFR at admission, whereas only two patients had CKD stage-II at the end of follow-up. However, all patients with GS had an eGFR greater than 90 ml/dk/1.73 m² at admission and at the end of follow-up period. The mechanism of CKD development is probably multifactorial. Nephrocalcinosis, chronic hypokalaemia, prematurity, long-term treatment with NSAIDs and damaging effect of elevated aldosterone levels on podocytes are some of the possibilities for CKD development in patients with BS and GS.

One recent study covering 30 patients with cBS detected no genotype-phenotype association [25], whereas Seys et al., reported an association between complete loss of function (CL/CL) mutations of *CLCNKB* mutations and severe phenotypes [21]. Although, we did not demonstrate by functional analysis, there was only one large deletion (Exon 2-20 deletion, patient B4) in our cohort that could be predicted to cause complete loss of function. This patient had the lowest eGFR at presentation. Although, previous studies from different countries [18,21,26] reported that whole gene deletion was the most common mutation in *CLCNKB* gene, in our cohort only one patient had this mutation. When our patients were evaluated in terms of genotype-phenotype correlation, no significant correlation was found even among patients harboring the same mutation.

The major limitations of our study were the low number of our patients and the relatively short period of follow up. Long term follow up of a larger number of patients could allow us to identify a significant difference in growth between these two study groups. Nonethless, the strength of our study is that the diagnosis of our patients were confirmed by very detailed genetic analyses. Prospective studies with larger number of patients, longer follow-up time and genetic confirmation will allow better demonstration of the genotype-phenotype correlation.

Conclusions

According to our findings, age at diagnosis, plasma chlorid, magnesium, bicarbonate levels and the urinary calcium/creatinine ratio may be useful markers for differentiating cBS and GS. But, in some cases, cBS cannot be distinguished clinically from GS, especially in patients with overlapping features. For this reason we suggest to perform a gene-based classification especially in countries where genetic analysis is possible.

study protocol was described to all patients and their parents and written informed consent was obtained from all individual participants older than 18 years of age and from the parents of all children included in the study.

Conflict of Interest: The authors have no potential conflicts to disclose.

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