CLINICAL AND GENETIC SPECTRUM OF BARTTER SYNDROME TYPE 3

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ABSTRACT

Background: Bartter syndrome type 3 is a clinically heterogeneous hereditary salt-losing tubulopathy. It is caused by mutations of *CLCNKB*, which encodes the ClC-Kb chloride channel involved in NaCl reabsorption in the renal tubule. We report phenotype/genotype correlation and follow-up for a large cohort of patients.

Methods: Genetic analyses were performed by direct sequencing and multiplex ligation-dependent probe amplification; medical charts were analyzed retrospectively for 115 patients with *CLCNKB* mutations. Functional analyses were performed in *Xenopus laevis* oocytes for eight missense and two nonsense mutations.

Results: Sixty mutations, including 27 previously unreported mutations, were detected. The phenotype was ante/neonatal Bartter Syndrome (polyhydramnios or diagnosis in the first month of life) in 30% of cases, classic Bartter Syndrome (diagnosis during childhood, hypercalciuria and/or polyuria) in 45% and Gitelman-like syndrome (fortuitous discovery of hypokalemia with hypomagnesemia and/or hypocalciuria in childhood or adulthood) in 25%. Nine of the 10 mutations expressed *in vitro* decreased or abolished chloride conductance. Severe (large deletions, frameshift, nonsense, and essential splicing) and missense mutations resulting in poor residual conductance were associated with younger age at diagnosis. Electrolyte supplements and indomethacin were frequently used to induce catch-up growth with few adverse effects. After a median follow-up of 8 years (1-41) in 77 patients, chronic renal failure was detected in 19 patients (25%): One required hemodialysis and four underwent renal transplantation.

Conclusion: We report the first genotype/phenotype correlation for Bartter syndrome type 3: Complete loss-of-function mutations were associated with younger age at diagnosis. Chronic kidney disease was observed in all phenotypes.

INTRODUCTION

Bartter syndromes (BS) and Gitelman syndrome (GS) are autosomal recessive salt-losing tubulopathies caused by defective salt reabsorption. They are characterized by hypokalemia, metabolic alkalosis, and secondary aldosteronism, with normal or low blood pressure[1,2]. BS are classified by phenotype (antenatal, classic) or genotype (types 1 to 5). Antenatal BS (ABS) is the most severe form, characterized by polyhydramnios, premature birth, life-threatening episodes of neonatal salt and water loss, hypercalciuria, and early-onset nephrocalcinosis[3]. Classic BS (CBS) occurs in infancy or early childhood and is characterized by marked salt wasting and hypokalemia, leading to polyuria, polydipsia, volume contraction, muscle weakness, growth retardation and, sometimes, nephrocalcinosis[4]. BS types 1, 2, and 3 are caused by mutations of genes expressed in the thick ascending limb of Henle's loop encoding the luminal Na^+-K^+-$2Cl^-$ cotransporter (*SLC12A1;* OMIM #601678), the luminal K^+ channel ROMK (*KCNJ1;* OMIM #241200), and the basolateral chloride channel ClC-Kb (*CLCNKB;* OMIM #607364), respectively[5-7]. Loss-of-function mutations of *BSND*, encoding barttin, an essential beta subunit for chloride channels, cause BS type 4a with sensorineural deafness (OMIM #602522)[8]. Simultaneous mutations of *CLCNKB* and *CLCNKA* cause type 4b BS (OMIM #613090)[9]. Finally, severe gain-of-function mutations of the extracellular Ca^{2+}-sensing receptor gene can result in a Bartter-like syndrome (BS type 5, OMIM #601199)[10,11]. GS (OMIM #263800) is a milder disease frequently associating hypomagnesemia and hypocalciuria. GS is often asymptomatic or associated with mild symptoms such as muscle weakness, salt craving, paresthesia, and tetany. GS is related to loss-of-function mutations of the *SLC12A3* gene encoding the apically expressed thiazide-sensitive NaCl cotransporter (NCC) of the distal convoluted tubule (DCT)[12].

The first BS3 patients described had a clinical phenotype corresponding to CBS[7]. Considerable phenotypic variability has since been described: *CLCNKB* mutations can also

underlie the ABS, neonatal BS (NBS), and Gitelman-like (GLS) phenotypes[13-15]. This study aimed to shed light on the phenotypic heterogeneity of BS3 by investigating phenotype-genotype correlations in a very large French cohort and by evaluation of published results and original data for *in vitro* expression.

RESULTS

Population

We retrospectively analyzed results for 115 patients (56 male and 59 female patients) from 111 families with *CLCNKB* mutations evaluated at the Genetics Department of Georges Pompidou European Hospital over the last 15 years. A history of consanguinity was recorded for 22 families; the geographic origin is shown in Supplementary Tables 1 to 3.

Initial clinical presentation

Thirty-four patients from 32 families (29.5%) presented with A/NBS, 51 patients from 49 families (44.5%) presented with CBS, and 30 patients from 30 families (26%) presented with GLS.

Mutations and Large Rearrangements

Genetic status and mutation type were determined for each initial phenotype group (Table 1). The detailed genotypes of each patient are summarized in Supplementary Tables 1 to 3. The deletion of a single allele was excluded in patients with homozygous point mutations and no consanguinity, and molecular abnormalities of the other genes implicated in GS and BS were excluded in patients with only one heterozygous mutation. The breakpoints of large rearrangements were not characterized; in consequence, we cannot exclude the possibility that patients with homozygous deletions from non-consanguineous families harbored two different deletions. Testing was carried out for both parents in 22 families and only the mother in seven

families. In all cases, parents were heterozygous for the homozygous mutation detected in the proband or for one of the two mutations detected in compound heterozygous probands.

Sixty different mutations were detected: 55% missense, 13% frameshift, 12% nonsense, 10% large deletions, and 10% splice-site mutations (Figure 1). Twenty-seven of these mutations were previously unknown (Figure 2A and B, Supplementary Tables 1 to 3). Two of the three splice-site mutations disrupt the obligatory consensus donor or acceptor splice site and were considered pathogenic as likely to cause exon skipping and frameshift. The variant at position -6 in the acceptor site of exon 14 is a known rare variant (rs369329893, allele frequency in African populations of 0.02%) for which MaxEntScan predicts a 100% decrease in splice-site score and SpliceSiteFinder predicts activation of an intronic cryptic acceptor site. Unfortunately, no mRNA from this patient was available for analysis.

Two of the 13 previously unreported missense mutations were present in the same allele in patient BR050 (p.Arg395Trp and p.Ala469Pro), and p.Gly465Arg was detected in the same allele as the known mutation p.Pro124Leu in three patients (BR116-1, BR157-1, and GT657-1). Eight of the 13 missense variations affected conserved amino acids and were predicted by at least four out of five tools used for *in silico* analysis as potentially pathogenic. The remaining five missense changes (p.Ser218Asn, p.Ala254Val, p.Arg395Trp, P.Ile447Thr, and p.Ala469Pro) were classed as variations of unknown significance (VOUS) (Supplementary Table 4). Among these changes, only the p.Arg395Trp has been described in databases (rs34255952) with an allelic frequency of 2% in African Americans and has not been detected in European Americans. Of the 33 missense mutations detected in our population, 13 were previously shown to result in loss of function[16]. *In silico* predictions are presented in Supplementary Table 5 for missense mutations for which *in vitro* analysis was not performed.

Functional expression of ClC-Kb mutants in *Xenopus* oocytes

We investigated the effect of two new missense mutations predicted to be pathogenic (p.Gly345Ser, p.Ala510Thr), two new VOUS (p.Arg395Trp, p.Ala469Pro), four previously described missense mutations (p.Gly296Asp, p.Ser297Arg, p.Gly424Arg, and p.Gly433Glu) and two nonsense mutations (p.Trp391Ter and p.Arg595Ter) on chloride conductance in *Xenopus* oocytes; p.Gly424Arg and p.Gly433Glu are located in α-helix N of ClC-Kb, which is involved in the selectivity filter; p.Gly296Asp and p.Ser297Arg are located in the α-helix J, which interacts with barttin; p.Ala510Thr is found in the α-helix Q involved in the dimer interface, and Arg595Ter is present in the CBS1 domain involved in channel common gating and trafficking. The p.Gly345Ser and p.Ala469Pro mutations affect α-helices K and O, respectively, and the p.Trp391Ter and p.Arg395Trp mutants affect the L-M linker (Figure 2B). Nine of these 10 mutations significantly decreased or abolished normalized conductance (Figure 3). The p.Trp391Ter, p.Gly296Asp, p.Gly424Arg, p.Gly433Glu, p.Ala469Pro, and p.Arg595Ter mutations abolished conductance, whereas p.Ser297Arg, p.Gly345Ser and p.Arg395Trp decreased conductance to 61%, 57%, and 65% of wild-type values, respectively (significantly different from oocytes expressing WT ClC-Kb and non-injected oocytes). By contrast, p.Ala510Thr had no influence on channel conductance. Finally, the p.Arg395Trp/p.Ala469Pro double mutation decreased conductance to 37% of wild-type values.

Clinical Data at Diagnosis

Table 2 summarizes clinical and biochemical characteristics at birth and at diagnosis. As expected, gestational age (GA) at birth was significantly lower in the A/NBS group than in the CBS and GLS groups, but similar between the CBS and GLS groups. Age at diagnosis was significantly lower in the A/NBS group than in the other two groups and in the CBS group than in the GLS group. Polyhydramnios was found in 29 patients with A/NBS (85%), at mean GA of 28 weeks, and amniotic fluid had to be drained in four cases. Ten patients (5

A/NBS and 5 CBS) had birth weights below the 10th percentile, and four patients (2 A/NBS and 2 CBS) had birth heights below the 10th percentile for gestational age at birth.

Plasma sodium and chloride concentrations were significantly lower and plasma renin and magnesium concentrations were significantly higher in CBS and A/NBS patients than in GLS patients; plasma potassium and total CO_2 concentrations were similar in all groups. Strong hypochloremia is a known phenotypic hallmark of BS3[17-19]. We therefore compared the relationship between plasma sodium and chloride concentrations between patients with BS types 1 and 2 (*n*=21) and patients with BS3 (*n*=51). This curve was shifted downwards in BS3 patients, indicating that plasma chloride depletion could not be accounted for by hyponatremia (Figure 4). No difference was observed in parameters at diagnosis when confirmed homozygous patients are compared with compound heterozygous patients (data not shown).

Clinical and Biological Data during Follow-up

Clinical manifestations during follow-up and treatment were recorded for 77 patients (Table 3). Median follow-up was eight years and was similar in the three groups. The main treatments administered to these patients were NaCl and KCl supplementation and non-steroidal anti-inflammatory drugs (NSAIDs, mainly indomethacin). The main adverse effects were abdominal pain (*n*=5), weight gain (*n*=1), esophagitis (*n*=1), and diarrhea (*n*=1). One of the main criteria of a successful treatment is a normal growth; in this cohort, 63 out 77 patients (82%) had a height between -2 SD and +1 SD or a normal height as adults. Fourteen patients had height below -2SD: five A/NBS patients including one with GH deficiency (IGF1 = 38 ng/ml before GH initiation at 15 years of age), eight CBS patients including two with GH deficiency and two with chronic kidney disease (CKD), and one GLS patient with no identified cause of failure-to-thrive.

Abnormalities in psychomotor and neurological development included psychomotor retardation in four A/NBS and four CBS patients (Table 3). Five CBS patients required psychiatric follow-up (hyperactivity, anorexia, or eating disorders).

Irregular heart rate or ECG abnormalities were documented in six patients: one A/NBS patient had premature ventricular beats with prolonged QT interval, three CBS patients had a right bundle branch block or U wave, and one GLS patient presented torsade de pointe attacks. None of the patients in this cohort had high blood pressure.

Fourteen patients developed nephrolithiasis or nephrocalcinosis during follow-up. None of the patients required shock-wave lithotripsy. Other renal and urological abnormalities diagnosed in eight A/NBS and nine CBS patients are detailed in Table 3. Proteinuria data were available for 43 patients, nine of whom displayed glomerular proteinuria over 50 mg/dl.

Nineteen (10 female and 9 male patients) of the 77 patients (25%) presented CKD (Table 4). Ten patients presented stage 2 CKD: four A/NBS, four CBS, and two GLS patients. Two patients reached stage 3 CKD (1 A/NBS and 1 GLS). One CBS patient reached stage 4 CKD. Six patients reached stage 5 CKD (3 A/NBS and 3 CBS patients), at a mean age of 25 years (6 - 49). Renal biopsies were performed in five out of 19 patients with CKD. Four patients with stage 5 CKD had diffuse glomerular and tubulointerstitial lesions with enlarged glomeruli presenting focal lesions of segmental glomerulosclerosis/hyalinosis (FSGS). One patient with stage 2 CKD had minimal glomerular and tubular alterations (Table 4 and Supplementary Table 6). Patients with CKD were older than patients without CKD; they did not differ in terms of birth weight, AINS treatment, urologic or renal abnormalities, or hypokalemia severity (Table 6).

The last eGFR follow-up data for 30 patients with BS1, 34 patients with BS2, and 11 patients with BS4a were compared with eGFR for the 77 patients with BS3. These groups had similar age distribution, and eGFR decreased with age (Supplementary Figure 1). In BS1 and BS4

patients, eGFR decrease was more severe and there were higher proportions of patients with CKD 3 to 5 than in BS2 and BS3 (Supplementary Figures 1 and 2).

Genotype/Phenotype Correlation

Large deletions were more frequent in patients with earlier onset, and severe phenotypes and missense mutations were more common in the GLS phenotype (Table 1). Similar results were obtained if other potentially severe mutations (frameshift, nonsense, and essential splicing) were considered with large deletions: Severe mutated alleles were more frequent in A/NBS and CBS (74 and 66% respectively) patients than in GLS patients (42%). Further, missense mutations were more frequent in patients with less severe phenotypes: 58% in GLS patients, versus 34% and 26% in CBS and A/NBS patients, respectively.

We classified mutations into two groups: complete loss-of-function (CL) and partial loss-of-function (PL) groups (Table 5). We included p.Trp610Ter, the only C-terminus-truncating mutation expressed *in vitro* and yielding a residual current[20]. Each mutated allele was classified separately, independently of the initial phenotype, and only patients for whom both alleles could be classified were analyzed (*n*=85) (Supplementary Tables 1 to 3). ClC-Kb functions as homodimer. The residual activity of the CL/PL genotypes may therefore correspond to homodimers of PL mutants, with similar consequences to PL/PL genotype. We therefore analyzed CL/PL genotypes together with PL/PL genotypes. With this classification, 56 patients had a CL/CL genotype, and 29 patients had a CL/PL or PL/PL genotype. CL/CL genotypes were associated with a significantly younger age at diagnosis than CL/PL and PL/PL genotypes. No difference was observed for the other biological parameters analyzed (Table 5).

DISCUSSION

Bartter syndromes are phenotypically and genotypically heterogeneous. Phenotype/genotype correlations highlighting the link between particular traits and genetic types (*i.e.* transitory

hyperkalemia in BS2, severe hypochloremia in BS3, and hearing loss in BS4) have been identified in previous studies[17,19]. BS3 is particularly heterogeneous in terms of clinical presentation, accounting for the diverse initial diagnoses attributed (A/NBS, CBS, or GLS). We investigated the basis of this variability in a cohort of 115 patients harboring *CLCNKB* mutations, and studied the phenotype/genotype correlation based on clinical presentation and follow-up as well as on *in vitro* functional studies of missense mutants.

More than 54 mutations of this gene have been reported in free access HGMD (www.hgmd.cf.ac.uk) and scientific publications[4,7,17, 19, 21-25]. They include a high frequency of large rearrangements favored by the close location of the homologous *CLCNKA*. We detected 60 different mutations, 27 of which had not been previously reported (13 missense, 5 frameshift, 3 nonsense, 3 splice-site mutations, and 3 large deletions). Thirteen of these mutations (frameshift, nonsense, splice-site mutations, and large deletions) were predicted to result in the production of unstable mRNAs or truncated or absent proteins. Eight of the 13 previously unknown missense mutations were predicted to be pathogenic *in silico* (Supplementary Table 4). Three out of other five, classified as VOUS, were expressed in *X. laevi*s oocytes (p.Arg395Trp, p.Ala469Pro, and p.Gly345Ser) as were two previously described mutations (p.Gly424Arg and p.Gly433Glu) detected as the only heterozygous mutation in two patients. All these mutations significantly decreased chloride conductance. The p.Ala510Thr, predicted *in silico* as pathogenic had a chloride conductance similar to that of the wild-type channel. The molecular abnormality of patient heterozygous for this variant thus remains unidentified.

In this large BS3 cohort, we confirmed the phenotypic variability, consisting of about 30% A/NBS, 45% CBS, and 25% GLS (Table 2). In order to determine if the type of mutation influences the phenotype, we first correlated them with initial clinical presentation. Large deletions and severe mutations were associated with all clinical presentations, but were more

frequent in A/NBS and CBS. Next, eighty-five patients with two mutated alleles were analyzed for phenotype-genotype correlations, taking into account the type of mutation and *in vitro* expression results, regardless of initial clinical presentation. Patients with complete loss-of-function (CL/CL) were significantly younger at diagnosis than patients harboring one or two alleles with a partial loss-of-function (CL/PL or PL/PL), suggesting that the type of mutation may influences the clinical presentation of BS3.

Surprisingly, the milder GLS phenotype did occur in patients harboring severe mutations or deletions, suggesting that the phenotype severity is not only driven by *CLCNKB* allelic variability. Phenotypic heterogeneity of BS3 has been attributed to distribution of the ClC-Kb channel along the nephron and to possible compensatory function of the ClC-Ka channel. ClC-Kb channel is expressed in the thick ascending limb (TAL), distal convoluted tubule (DCT), and collecting duct, where it transfers chloride (Cl^-) ions to the basolateral side[26]. Impaired ClC-Kb function in the TAL results in lower levels of Cl^- exit, NaCl reabsorption through the Na-K-2Cl cotransporter, and divalent cation reabsorption, accounting for the Bartter phenotype. Defective basolateral Cl^- exit in the DCT decreases NaCl reabsorption via the Na-Cl cotransporter (NCC), accounting for the GLS phenotype in other patients. Two recent studies in which the mouse *Clcnk2* gene (corresponding to *CLCNKB* in humans) was disrupted confirmed that ClC-K2 is the principal chloride channel in all three nephron segments and that TAL impairment is not compensated by ClC-K1 (corresponding to the human ClC-Ka channel, which is also expressed in the TAL)[27,28]. Nevertheless, it cannot be excluded that allelic variants of genes encoding KCl cotransporters or other chloride channels may also compensate for renal sodium loss accounting for phenotypic variability[27, 29].

Next generation sequencing (NGS) approaches allow parallel analysis of several genes, which is particularly useful in diseases with genetic heterogeneity, such as ABS, or in diseases with phenotypic variability such as the BS type 3. A genetic confirmation is important for the

follow-up as well as to improve our knowledge of the natural history of these syndromes. In consequence we recommend the use of NGS panels to diagnosis confirmation. NGS could also be useful to determine whether additional genes are involved in the observed clinical variability. *In vitro* studies of additional mutant proteins can contribute in the future to improve our understanding of the phenotype/genotype correlation and of the precise pathogenic mechanism of mutants. These studies have the potential interest to define targeted therapeutic approaches such as channel openers or pharmacological chaperones[30,31] .

Despite missing data for some phenotypic criteria due to the retrospective nature of this study, several patterns emerged from our analysis. First, growth retardation was common but frequently improved with treatment. Fourteen patients presented with persistent growth retardation; two of these patients had CKD, a common cause of growth retardation due to a combination of abnormalities of the growth hormone axis, vitamin D deficiency, hyperparathyroidism, inadequate nutrition, and drug toxicity and three patients presented with growth hormone (GH) deficiency[32]. BS and potassium deficiency have already been reported to be associated with GH deficiency[33-35]. One previous study showed that GH and IGF1 did not stimulate longitudinal growth unless hypokalemia was corrected[36]. In two patients with hypokalemia (median=2.5 mmol/L), growth improved after GH supplementation but remained below -2SD.

Second, hypochloremia is a hallmark of BS3: An analysis of the data available at diagnosis showed that hypochloremia was more severe in A/NBS and CBS patients than in GLS patients. ClC-Kb is expressed not only in the diluting segment but also in the intercalated cells of the collecting duct (CD). Defects in this segment may impair chloride exit and transepithelial chloride reabsorption through the pendrin Cl/HCO_3 exchanger, potentially accounting for the stronger chloride depletion in BS3 patients than in BS1 or BS2 patients[19]. We found a downward shift of the relationship between plasma chloride and sodium

concentrations consistent with a defect in adaptation to chloride depletion in BS3 as compared to BS1 and BS2 patients. These results are consistent with the phenotype of mice with *Clcnk2* disruption[27] and suggest that sodium and potassium supplementation should be provided as chloride salts in BS3 patients.

Third, nineteen patients presented CKD, and seven of these patients also had proteinuria (Table 4). Five patients underwent renal biopsy, which revealed diffuse glomerular and tubulointerstitial lesions with enlarged glomeruli in four cases, suggesting compensatory hypertrophy to nephron reduction (Table 4 and Supplementary Table 6). Six patients diagnosed before the age of 8 years displayed progression to ESDR at a median age of 24 years, associated in four cases with FSGS. Proteinuria, a low glomerular filtration rate and FSGS have been reported in BS and GS patients[22,37-39]. It has been suggested that FSGS is a secondary lesion due to adaptation to salt loss, resulting in chronic stimulation of the renin-angiotensin system[37,39,40]. In the present study FSGS occurred in late stage of CKD suggesting a large contribution of nephron reduction. We failed to identify other risk factors of CKD progression including birth weight, age at diagnosis, long-term NSAID treatment, persistent hypokalemia, and other renal abnormalities (Table 6). CKD has been described in other types of BS[19,22]. In our BS cohort, CKD was observed in all BS types but the proportion of patients with preserved renal function (i.e., eGFR > 90 ml/min/1.73 m^2) was higher in patients with BS types 2 and 3 and the proportion of patients with moderate to severe kidney disease (i.e., eGFR < 60 ml/min/1.73 m^2) in patients with BS types 1 and 4 suggesting that the laterBS subtypes 1 and 4 are associated with more severe renal prognosis. The mechanism of CKD development is probably multifactorial, and its elucidation will require prospective studies. Case reports are rare for BS patients undergoing renal transplantation. The post-transplantation period was uneventful in our four patients, with the complete disappearance of BS and no recurrence of FSGS, as previously described[41-43].

In conclusion, BS3 syndrome, which is caused by *CLCNKB* mutations, is highly variable phenotypically. We show, for the first time, that there is a correlation between severe mutations and a significantly younger age at diagnosis, suggesting that milder defects of ClC-Kb function may account for some of this variability. We also confirmed the severe chloride depletion previously observed in BS3 patients and report that 25% of cases suffer from CKD. Long-term prospective follow-up of this cohort will identify other severity parameters involved in this genotype/phenotype correlation and will allow us to evaluate whether early diagnosis and treatment have an influence on the evolution to CKD.

CONCISE METHODS

Patients

The study included 115 patients (from 111 families) with *CLCNKB* mutations referred to the Genetics Department of Georges Pompidou European Hospital (Paris, France) from January 2001 to December 2014 for genetic analysis after the diagnosis of BS or GS. The study was approved by the "Comité de Protection des Personnes, Paris-Île de France XI (Ref. 09069)" and informed consent for genetic studies was obtained from each proband or from their parents for minors. Genetic investigations were performed after the clinical and biological diagnosis of salt-losing tubulopathy. Patients with a history of polyhydramnios or clinical manifestations in the first month of life were considered to have A/NBS. Patients diagnosed during childhood, with hypercalciuria and/or polyuria, were considered to have CBS, and children, adolescents, or adults for whom hypokalemia and hypomagnesemia and/or hypocalciuria were discovered fortuitously were considered to have GLS. Genetic investigations were extended to both parents in 22 families and to the mother only in another seven families. Twenty-three patients from this cohort have been described before[19,23,25] (Supplementary Tables 1 to 3).

Detection of point mutations

DNA was extracted with a salt-based method or with blood DNA midi kits (Qiagen, Venlo, the Netherlands). *CLCNKB* exons and flanking intron sequences were amplified by PCR, sequenced with BigDye Terminator v3.1 cycle sequencing kits and run on an ABI Prism 3730XL DNA Analyzer (Perkin Elmer Applied Biosystems®, Foster City, CA, USA), as previously described (16). DNA mutations were identified with Sequencher software, by comparison with the reference sequence for *CLCNKB*: NM_000085.4. Each mutation was confirmed by sequencing a second independent PCR product.

Detection of large rearrangements

Large rearrangements were detected by quantitative multiplex PCR of short fluorescent fragments (QMPSF) before June 2010 and by multiplex ligation-dependent probe amplification (MLPA) thereafter. We adapted the QMPSF method for the detection of large deletions of *CLCNKB*[44]. The procedure is described in detail in the Supplementary Materials and the primers used, covering all exons, are listed in Supplementary Table 7. For MLPA, we used the SALSA® MLPA® P266-B1 CLCNKB Kit (MRC Holland, Amsterdam, the Netherlands). The P136 Kit contains 29 probes: probes for 14 of the 20 exons of *CLCNKB* (exons 4, 7, 9, 12, 16 and 20 are not represented), four probes for upstream genes (*PRM2*, *CASP9* and the homologous *CLCNKA* gene), and 11 reference probes. The procedure is described in detail in the Supplementary Materials.

Bioinformatic analysis of mutations

The software used to interpret variants is described in the Supplementary Methods.

Functional expression in *Xenopus laevis*

Voltage clamp experiments were performed as previously described in *Xenopus laevis* oocytes (25, 27). We injected 10 ng ClC-Kb cRNA and 5 ng barttin cRNA into defolliculated oocytes, which were then incubated in modified Barth's solution at 16°C. Two-electrode voltage-clamp experiments were performed at room temperature with TURBO TEC-10CX (npi electronic GmbH, Tamm, Germany) and PClamp 8 software (Axon Instruments, Union City, CA, USA), two to three days after injection. Conductance at +60 mV ($G_{+60\ mV}$) was calculated by dividing the current at +60 mV by the difference in current between +60 mV and the reversal potential.

Statistics

Clinical data were analyzed with GraphPad Prism Software (La Jolla, CA, USA) and SPSS software, release 20.0.0 (SPSS, Chicago, IL, USA). Kruskal-Wallis tests were used to compare the three groups (A/NBS, CBS, and GS). In cases of statistical significance, Mann-Whitney *U* tests were used to compare the groups in pairs. Dichotomous variable were compared using the chi-squared test or Fisher's exact test as appropriate. Variables for which more than 50% of the data were missing in one group were excluded from the analysis. Clinical manifestations were analyzed by descriptive methods, using the number of subjects for each group. Data for *in vitro X. laevis* studies were analyzed by ANOVA and Holm-Sidak tests, with Sigma Stat software.

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Figure Legends

Figure 1: Mutations of *CLCNKB* detected in Bartter type 3, and classified by type (*n*=60).

Figure 2: Locations of novel *CLCNKB* mutations and of mutations expressed *in vitro.* A. *CLCNKB* gene structure, showing the newly discovered large deletions and splicing mutations. B. Schematic topological model of the ClC-Kb protein: the lower part of the model corresponds to the intracellular region, and the upper part is extracellular. Each rectangle represents one of the 18 α-helices and the two cystathionine-β-synthase (CBS) domains. The α-helices involved in the selectivity filter, those interacting with Barttin, and those located at the dimer interface are shown in blue, green, and pink, respectively. Previously unknown missense (●) and nonsense (✸) mutations are shown in red; previously described mutations are shown in blue (●); mutations expressed *in vitro* are underlined.

Figure 3: Functional studies of selected ClC-Kb mutants (n=10). Conductance at +60 mV for non-injected oocytes (NI) and for oocytes into which mutant ClC-Kb cRNA was injected, normalized with respect to the mean value for wild-type (WT) ClC-Kb and expressed as the mean ± SEM. The mutants were exposed to a solution at pH 7.4 containing 10 mM Ca^{2+} (panel A) or to a solution at pH 9.0 containing 20 mM Ca^{2+} (panel B). As ClC-Kb current increases at high external Ca^{2+} concentration or high pH, these solutions were chosen to obtain a submaximal current. $, $P < 0.05$ for the difference between NI or mutant ClC-Kb and WT; *, $P < 0.05$ for the difference between WT or mutant ClC-Kb and NI. Number of measurements for panel A: NI (*n*=63), WT (*n*=109), p.Trp391Ter (*n*=12), p.Arg395Trp (*n*=16), p.Arg395Trp-p.Ala469Pro (*n*=20), p.Gly424Arg (*n*=16), p.Ala469Pro (*n*= 10). For panel B: NI (*n*=9), WT (*n*=16), p.Gly122Val (*n*=6), p.Gly296Asp (*n*=5), p.Ser297arg (*n*= 7), p.Gly345Ser (*n*=8), p.Gly433Glu (*n*=8), p.Ala510Thr (*n*=8) and p.Arg595Ter (*n*=3).

Figure 4: Correlation between plasma sodium and plasma chloride concentrations in patients

with BS type 1 and 2 (black symbol, grey area) and in patients with BS type 3 (white symbols, orange area).