

## BRIEF REPORT

## Salt Wasting and Deafness Resulting from Mutations in Two Chloride Channels

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**I**NHERITED KIDNEY DISORDERS ASSOCIATED WITH SALT WASTING, HYPOKALEMIA, and metabolic alkalosis (Bartter's syndrome) are clinically classified into several variants<sup>1</sup>: classic Bartter's syndrome,<sup>2</sup> Gitelman's variant of Bartter's syndrome,<sup>3</sup> and antenatal Bartter's syndrome (originally described as the hyperprostaglandin E syndrome).<sup>4</sup> The classic form and Gitelman's variant are characteristically accompanied by hypokalemia during early infancy or adolescence, whereas the antenatal form is marked by polyhydramnios in the mother, followed by severe volume depletion in the infant during the early neonatal period. Molecular genetic analyses of the different forms of Bartter's syndrome have revealed mutations in various genes encoding ion channels and transporters that mediate transepithelial salt reabsorption along distal nephron segments: the sodium–potassium–chloride cotransporter NKCC2<sup>5</sup> and the potassium channel ROMK<sup>6</sup> in antenatal Bartter's syndrome (genetically defined as Bartter's types I and II, respectively); the chloride channel ClC-Kb in classic Bartter's syndrome (Bartter's type III)<sup>7</sup>; and the sodium–chloride cotransporter (NCCT)<sup>8</sup> in Gitelman's variant.

In a newly identified phenotype of antenatal Bartter's syndrome, both severe renal salt wasting and sensorineural deafness are present; it is called antenatal Bartter's syndrome with sensorineural deafness (BSND, or Bartter's type IV).<sup>9</sup> In contrast to the other Bartter variants, the underlying genetic defect does not affect a bona fide ion-transport protein.<sup>10</sup> Rather, mutations in the *BSND* gene product, a protein called barttin, indirectly interfere with epithelial salt transport by impairing the barttin-dependent insertion in the plasma membrane of ClC-Kb and the closely related chloride channel ClC-Ka, both of which associate with barttin in the epithelial cells of the kidney and the inner ear<sup>11,12</sup> (Fig. 1).

In this report, we describe a child with renal salt wasting and deafness who had no mutation in the *BSND* gene. Given the known role of the barttin protein, we tested the hypothesis that the child's disease resulted from combined impairment of the chloride-channel functions of ClC-Ka and ClC-Kb and that the impairment was caused by a digenic defect in the closely adjacent genes encoding ClC-Ka (*CLCNKA*) and ClC-Kb (*CLCNKB*) on chromosome 1p36.

### CASE REPORT

The patient's clinical course and biochemical findings have been reported in detail elsewhere.<sup>13</sup> In brief, the patient was born to consanguineous parents (first cousins) at 28 weeks of gestation with a birth weight of 1250 g (40th percentile for gestational age). Severe maternal polyhydramnios had made repeated amniocenteses necessary during the last six weeks of gestation. Within 72 hours after birth, the child began to have polyuria and volume depletion associated with hypokalemia and metabolic alkalosis, necessitating supplementation with water, sodium, and potassium chloride. Ante-

natal Bartter's syndrome (hyperprostaglandin E syndrome) was diagnosed on the basis of excessive urinary prostaglandin E-M excretion, and treatment with indomethacin was initiated 10 days after birth. The indomethacin was subsequently discontinued because of an unsatisfactory response, and treatment with rofecoxib was initiated and the patient monitored. However, for fluid and electrolyte balance, he required supplementary potassium and sodium (up to 15 mmol per kilogram of body weight per day and 4 mmol per kilogram per day, respectively, added to his normal enteral formula). Electric-response audiometry of the brain stem revealed bilateral sensorineural deafness at the age of eight weeks, which prompted genetic analysis for a *BSND* gene defect. The parents provided written informed consent for the genetic analyses, and these investigations were approved by the ethics committee of the University of Marburg (Marburg, Germany). Written informed consent was also obtained from 51 control subjects of Middle Eastern Arabian descent, who provided DNA for analysis of the genes responsible for rare kidney diseases.

#### METHODS

##### PREPARATION AND ANALYSIS OF DNA

Genomic DNA was prepared from blood leukocytes with a commercially available kit (Amersham). The polymerase chain reaction (PCR) was used to generate products spanning exons and flanking intronic sequences of the *BSND* gene and the *CLCNKA* gene, which were then analyzed by direct sequencing. The resulting *BSND* and *CLCNKA* sequences were compared with published sequences (GenBank accession numbers NM\_057176 and NM\_004070, respectively). The presence of a newly generated *Bsi*YI site by the G240C mutation in exon 3 of the *CLCNKA* gene was ruled out by restriction analyses of PCR products derived from 102 control chromosomes from the 51 healthy control subjects.

##### EXPRESSION IN *XENOPUS LAEVIS* OOCYTES AND VOLTAGE-CLAMP ANALYSIS

Single oocytes were obtained from *Xenopus laevis* frogs by transabdominal incision and resection of ovarian lobes, followed by collagenase treatment for two hours, as previously described.<sup>12</sup> Defolliculated xenopus oocytes were injected with complementary RNA (cRNA) transcribed in vitro (mMessage mMachine kit, Ambion), including 10 ng of *ClC-Ka* constructs and 5 ng of *barttin* cRNA, and were

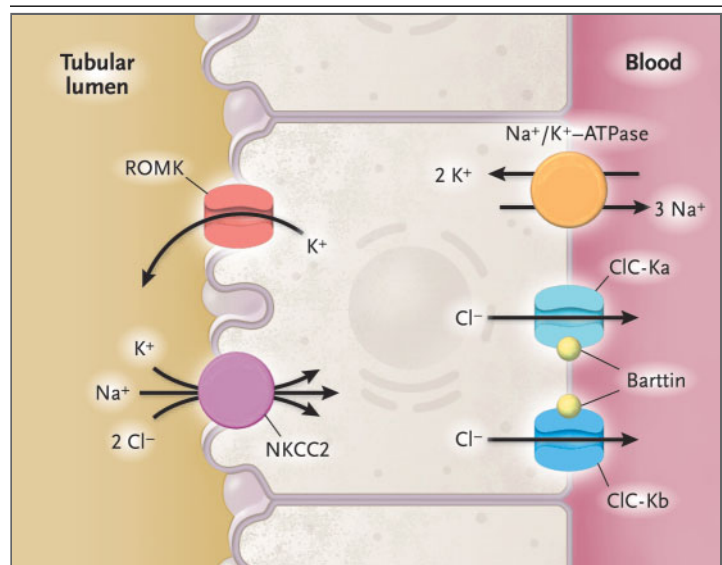
stored at 16°C in frog Ringer's solution. Two-electrode voltage-clamp measurements were performed (GeneClamp 500 amplifier, Axon Instruments) at room temperature two to five days after injection.

##### STATISTICAL ANALYSIS

Statistical analysis was performed on at least seven oocytes derived from one preparation. Experiments were repeated in five batches of oocytes derived from different frogs. To rule out the possibility that differences in cRNA quality accounted for differences in the levels of current, experiments were repeated with two independent preparations of cRNA. The error bars in diagrams representing the results were calculated from the standard error. Student's t-test was used to assess statistical significance, which was assumed at a P value of less than 0.05.

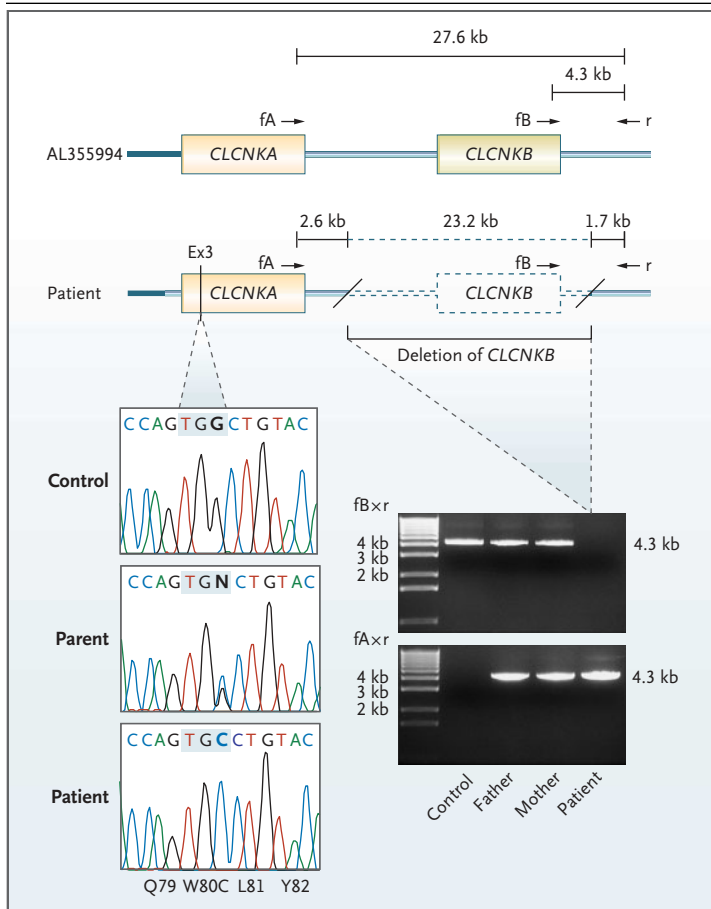
#### RESULTS

PCR amplification of exonic and adjacent intronic *BSND* sequences<sup>10</sup> and direct sequencing of the PCR products gave rise to normal (wild-type) sequences



**Figure 1. Transport Proteins Involved in Transepithelial Salt Reabsorption along the Distal Nephron.**

Apical (luminal) uptake of sodium chloride in the thick ascending limb cells is mediated by the sodium-potassium-chloride cotransporter (NKCC2). Potassium recirculates into the lumen by way of apical potassium channels (ROMK), whereas sodium is extruded basolaterally by sodium-potassium ATPases. Basolateral release of chloride occurs by way of ClC-K-type chloride-channel proteins (ClC-Ka and ClC-Kb), which require the beta subunit barttin to form functional ion channels.



**Figure 2. Genetic Analysis of the *CLCNKA* and *CLCNKB* Gene Defects.**

The schematic illustration of *CLCNKA* and *CLCNKB* shown in the top portion of the figure was deduced from the wild-type genomic sequence (AL355994). The relative positions of the two forward primers fA and fB, directed against corresponding sequences within intron 18 of *CLCNKA* and *CLCNKB*, respectively, and of the reverse primer (r) are indicated by arrows. Amplification by the polymerase chain reaction (PCR) with primers fB and r generated a 4.3-kb amplicon, which was detected in a control subject and in the patient's heterozygous parents. In the case of the patient, who had a homozygous *CLCNKB* deletion, no such amplicon was generated (bottom right-hand portion of the figure). PCR amplification with primers fA and r resulted in a 4.3-kb fusion fragment in the case of homozygosity or heterozygosity for the *CLCNKB* deletion but failed to generate an amplicon from control DNA. (A theoretically expected 27.6-kb fragment was too large to be amplified under the conditions used.) Genomic sequence analysis of exon 3 of *CLCNKA* in a control subject, the father, and the affected patient are shown in the bottom left-hand portion of the figure.

for the complete barttin coding region and the flanking intron sequences of the exon-intron boundaries. Moreover, homozygosity, as would be expected for an autosomal recessive inheritance in a consanguineous family, was ruled out by linkage analysis for the genomic loci of the gene encoding

barttin as well as the genes encoding NKCC2 and ROMK.

Given these negative results, we sought another explanation for the child's disease and hypothesized that ClC-Ka and ClC-Kb chloride-channel function was directly impaired by mutations affecting the genes encoding these channels (*CLCNKA* and *CLCNKB*). Large-scale genomic rearrangements of the *CLCNKB* gene resulting in complete *CLCNKB* deletions have frequently been shown to underlie the classic Bartter's phenotype<sup>7,14</sup> raising the possibility that a contiguous-gene syndrome by deletion of the closely adjacent *CLCNKB* and *CLCNKA* genes could account for the phenotype of our patient, which resembled that of antenatal Bartter's syndrome with sensorineural deafness. Using a previously described genetic approach,<sup>7</sup> we detected a homozygous *CLCNKB* deletion. However, characterization of the extension of this deletion by a genome-walking technique toward the *CLCNKA* gene revealed that the 5' deletion breakpoint was located between the genes in this patient. Therefore, the *CLCNKA* coding region, together with its 3' untranslated region, was not affected by the genomic rearrangement.

We subsequently identified the 3' deletion breakpoint, which allowed us to amplify a fusion fragment specific for this deletion. As shown in Figure 2, simultaneous amplification of the deletion-specific fusion fragment and a normal (control) fragment specific for *CLCNKB* showed that our patient was homozygous for this deletion. As expected, both of his parents were positive for the deletion and the *CLCNKB*-specific fragment, whereas an unrelated person was homozygous for the *CLCNKB* fragment. Thus, the possibility of a contiguous-gene syndrome appears to be unlikely, even if a more complex rearrangement cannot be completely ruled out. The deletion-specific fusion fragment obtained from our patient was similar in size to those seen in patients with classic Bartter's syndrome, who have homozygous *CLCNKB* deletions (data not shown). A gross difference in the extent of the deletion thus probably does not explain the increased severity of the phenotype in our patient.

We therefore analyzed *CLCNKA* for missense mutations by an approach similar to that used in our routine *CLCNKB* screening.<sup>14</sup> This strategy allowed specific analysis of *CLCNKA* without causing undesired amplification of highly homologous *CLCNKB* sequences. It revealed a homozygous substitution of C for G within exon 3 of the *CLCNKA*

**Figure 3. Effect of the ClC-Ka W80C Mutation on ClC-Ka Chloride Channel Activity.**

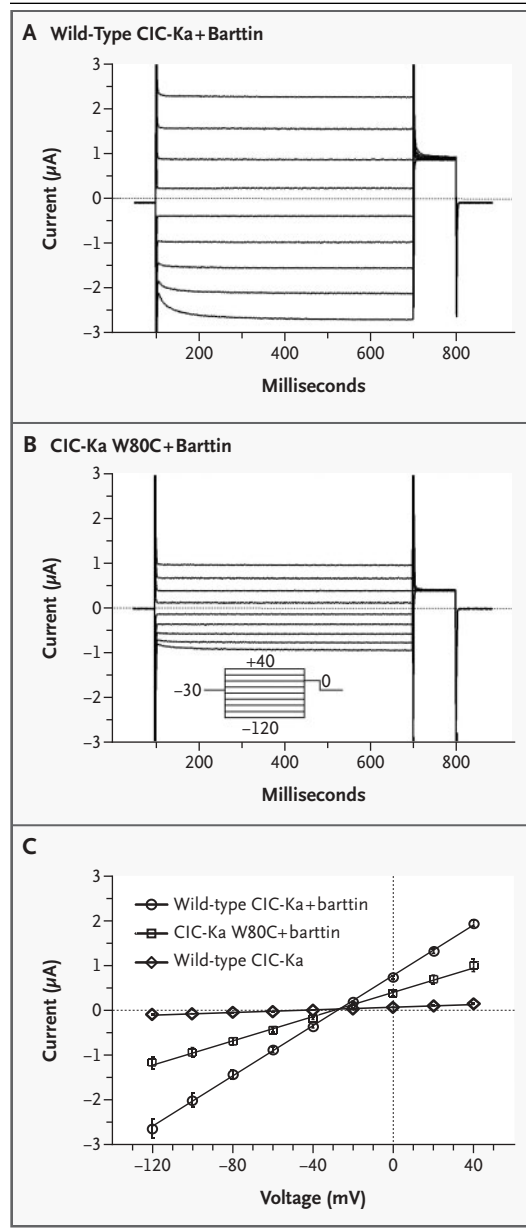
Currents induced by wild-type ClC-Ka (Panel A) and ClC-Ka W80C (Panel B) in the presence of barttin are depicted. As shown in Panel B, ion currents were activated by rectangular voltage steps from  $-120$  to  $+40$  mV, starting from a holding voltage of  $-30$  mV. Panel C shows a statistical analysis of the current–voltage relation derived from more than seven oocytes expressing the indicated construct and obtained from a single preparation. To demonstrate reproducibility, the mean reduction of outward current amplitude at  $+40$  mV was determined for each of five different batches of oocytes (with seven or more oocytes per construct for each batch). The mean ( $\pm$ SEM) of these five values (expressed as a percentage of the wild-type current) was  $48\pm 3$  percent for ClC-Ka W80C. Wild-type ClC-Ka expression in the absence of barttin gave rise to currents not significantly different from those of noninjected oocytes.

gene, resulting in the substitution of cysteine (encoded by TGC) for tryptophan (encoded by the triplet codon TGG) at amino acid position 80 of the translated ClC-Ka protein (hereafter referred to as ClC-Ka W80C) (Fig. 2).

To test whether the tryptophan-to-cysteine mutation in the outer region of the first transmembrane domain affected the function of the ion channel, we compared whole-cell ion currents of oocytes expressing barttin together with wild-type or mutated ClC-Ka chloride-channel proteins. As determined by two-electrode voltage-clamp analysis, co-expression of wild-type ClC-Ka and barttin gave rise to instantaneous, nearly voltage-independent currents with a linear current-voltage relation and a reversal potential at about  $-30$  mV. Currents with identical biophysical properties but with severely reduced amplitudes (approximately half the amplitude of normal currents) were observed after coexpression of ClC-Ka W80C and barttin (Fig. 3).

## DISCUSSION

In this report, we describe a digenic disorder involving impairment in the function of two chloride channels and resulting in a phenotype that combines severe renal salt wasting and deafness. This phenotype is very similar to that caused by a monogenic disorder affecting a single protein (barttin) that normally accounts for the proper function of the same chloride channels. In view of the suspected extreme rarity of such a digenic constellation, we sought and considered alternative explanations for



the observed combination of antenatal Bartter's syndrome and deafness. Isolated impairment of the function of the ClC-Kb channel is sufficient to compromise renal tubular salt reabsorption severely, as in classic Bartter's syndrome.<sup>7</sup> Moreover, in addition to parental consanguinity, prematurity is itself a risk factor for deafness, which affects up to 0.5 percent of preterm infants with birth weights of 1500 g or less.<sup>15</sup> This observation raises the possibility that renal salt wasting due to a ClC-Kb defect resulting from the deletion of the *CLCNKB* gene, in combination with deafness associated with consanguinity

or prematurity, could resemble the phenotype resulting from a *BSND* mutation.

Irrespective of deafness, however, a critical evaluation of our patient's clinical and biochemical data provides several lines of evidence that argue against the presence of an isolated *ClC-Kb* defect. First, none in a series of patients with genetically defined, hypokalemic salt-wasting tubular disorders, including some with complete *CLCNKB* deletions, had antenatal manifestations involving both polyhydramnios and birth before 32 weeks of gestation.<sup>16</sup> Polyhydramnios and preterm delivery, however, are typical characteristics of patients with *BSND* gene mutations,<sup>9,17</sup> and indeed they were characteristics of our patient. Second, urinary concentrating ability is typically not drastically impaired in patients with *CLCNKB* mutations.<sup>16</sup> In contrast, patients with *BSND* mutations have been shown to have a major defect in urinary concentrating ability<sup>17</sup> like that in our patient, whose maximal urine osmolality only reached 390 mOsmol per kilogram three days after the discontinuation of indomethacin (data not shown). Third, our patient's urinary excretion of prostaglandin E-M was inordinately high,<sup>13</sup> as is typical of patients with *BSND* gene mutations,<sup>17</sup> in whom the urinary excretion of this prostaglandin metabolite by far exceeds that in patients with isolated *CLCNKB* gene defects.<sup>16</sup>

Taken together, these observations strongly support the notion that in our patient's case, an additional defect aggravated the consequences of a pure *CLCNKB* gene deletion. After the presence of *BSND* mutations had been ruled out, a mutation in the *CLCNKA* gene became the most obvious candidate. Indeed, the *ClC-Ka* W80C mutation identified in our patient severely impaired the function of his *ClC-Ka* chloride channels.

Both *ClC-Ka* and *ClC-Kb* belong to the *ClC* family of chloride channels. Though nearly identical at the protein level, *ClC-Ka* and *ClC-Kb* differ in their distribution pattern along the nephron. *ClC-Ka* predominates in the thin ascending limb of Henle's loop, whereas the expression of *ClC-Kb* is confined to the more distal segments of the nephron, extending from the thick ascending limb to the cortical collecting duct.<sup>18,19</sup> Along these nephron segments, both chloride channels are invariably associated with their beta subunit barttin, which is required for proper insertion in the plasma membrane of the functional channel complex.<sup>11,12</sup> Careful examination of the renal phenotype of patients with

*CLCNKB* defects suggests that basolaterally expressed *ClC-Kb* channels have an indispensable role in transcellular chloride reabsorption, primarily along the distal convoluted tubule and to a lesser extent along the thick ascending limb. This point can be deduced from the well-preserved urinary concentrating ability and absence of hypercalciuria in these patients<sup>16</sup>; in contrast, prominent hyposthenuria or isosthenuria and hypercalciuria, which eventually leads to nephrocalcinosis, are hallmarks of dysfunction of the thick ascending limb resulting from mutations in the cotransporter *NKCC2*<sup>5</sup> or the potassium channel *ROMK*.<sup>6</sup>

In contrast to *ClC-Kb*, no disease-associated defects have been reported to date in the *ClC-Ka* channel in humans. However, it has been shown that a mild diabetes insipidus develops in knockout mice deficient in *ClC-Ka*, suggesting the functional importance of *ClC-Ka* chloride channels.<sup>20</sup> This phenotype is attributed to an impairment of passive chloride reabsorption along the thin limb of Henle's loop, which in turn impairs water absorption in contiguous collecting ducts by reducing inner medullary tonicity.<sup>21</sup>

The patient we describe, who had a combined *ClC-Ka* and *ClC-Kb* defect, did not have phenotypic features that would be mirrored by a simple superposition of the two monogenic phenotypes. Deafness and fetal polyuria causing polyhydramnios and severe prematurity are extremely unusual in cases of either diabetes insipidus or classic Bartter's syndrome. As suggested for the mechanism underlying the mutant *BSND* phenotype,<sup>11,12</sup> these discrepancies might be explained by a mutual compensation of *ClC-K*-type chloride-channel function in cells that coexpress *ClC-Ka* and *ClC-Kb*, as is the case in the inner ear and along the distal nephron.<sup>10,11</sup> Only combined impairment of the two types of ion channels would then severely disturb the cell functions dependent on them.

In conclusion, we have shown that combined impairment of *ClC-Ka* and *ClC-Kb* results in a phenotype that mimics antenatal Bartter's syndrome with deafness through defects in barttin, the beta subunit common to the *ClC-K*-type chloride channels. This observation convincingly suggests that the *ClC-K*-type chloride channels are regulated by barttin. In addition, this case reveals the mutually compensating role of *ClC-K*-type chloride channels along the nephron and in the inner ear, while providing strong evidence of genetic heterogeneity

## in patients who have both severe renal salt wasting and deafness.

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