Late-Onset Manifestation of Antenatal Bartter Syndrome as a Result of Residual Function of the Mutated Renal Na⁺-K⁺-2Cl⁻ Co-Transporter

Carsten A. Pressler,* Jolanta Heinzinger,* Nikola Jeck,* Petra Waldegger,* Ulla Pechmann,* Stephan Reinalter,* Martin Konrad,+ Rolf Beetz,+ Hannsjörg W. Seyberth,* and Siegfried Waldegger*

*Department of Pediatrics, Philipps University of Marburg, Marburg, Germany; †Department of Pediatrics, Inselspital, Bern, Switzerland; and ‡Department of Pediatrics, Johannes-Gutenberg University of Mainz, Mainz, Germany

Genetic defects of the Na⁺-K⁺-2Cl⁻ (NKCC2) sodium potassium chloride co-transporter result in severe, prenatal-onset renal salt wasting accompanied by polyhydramnios, prematurity, and life-threatening hypovolemia of the neonate (antenatal Bartter syndrome or hyperprostaglandin E syndrome). Herein are described two brothers who presented with hyperuricemia, mild metabolic alkalosis, low serum potassium levels, and bilateral medullary nephrocalcinosis at the ages of 13 and 15 yr. Impaired function of sodium chloride reabsorption along the thick ascending limb of Henle’s loop was deduced from a reduced increase in diuresis and urinary chloride excretion upon application of furosemide. Molecular genetic analysis revealed that the brothers were compound heterozygotes for mutations in the gene coding for the NKCC2 co-transporter. Functional analysis of the mutated rat NKCC2 protein by tracer-flux assays after heterologous expression in Xenopus oocytes revealed significant residual transport activity of the NKCC2 p.F177Y mutant construct in contrast to no activity of the NKCC2-D918fs frameshift mutant construct. However, coexpression of the two mutants was not significantly different from that of NKCC2-F177Y alone or wild type. Membrane expression of NKCC2-F177Y as determined by luminometric surface quantification was not significantly different from wild-type protein, pointing to an intrinsic partial transport defect caused by the p.F177Y mutation. The partial function of NKCC2-F177Y, which is not negatively affected by NKCC2-D918fs, therefore explains a mild and late-onset phenotype and for the first time establishes a mild phenotype-associated SLC12A1 gene mutation.

at the ages of 13 and 15 yr. Molecular genetic analyses revealed that the brothers were compound heterozygotes for mutations in the SLC12A1 gene. Because one of the mutations only partially impaired NKCC2 function, variable degrees of NKCC2 dysfunction may be associated with variations of the clinical picture. This is the first report of patients with a mild and late-onset phenotype associated with SLC12A1 gene defects and indicates that partially impaired NKCC2 function should be considered even beyond the neonatal period in patients who present with symptoms of renal salt wasting.

Materials and Methods

In Vivo Assessment of TAL Function

Diuresis and urinary chloride excretion in response to oral furosemide application (2 mg/kg body wt) was determined essentially as described by Koeckerling et al. (4). In brief, urine volume and urinary chloride excretion during 3 h were determined at the same time on 2 consecutive days under standard diet and fluid intake. The first day served as the control period. On the second day, urine collection during 3 h was performed immediately after a single oral dose of furosemide. The difference in urine volume and chloride excretion between day 1 and day 2 (∆V, ∆Cl) was considered to be induced by furosemide administration. A possible bias by variable furosemide concentrations was excluded by normalization of ∆V and ∆Cl to urinary furosemide excretion.

SLC12A1 Sequence Analysis

Informed consent was obtained from the patients and their parents before blood samples were taken for genomic DNA isolation. All 26 exons together with adjacent intronic sequences of the SLC12A1 gene (1) were PCR amplified from genomic DNA according to standard protocols. PCR products with aberrant electrophoretic mobility determined by single-strand conformational polymorphism analysis were sequenced in both directions. All nucleotide variations observed were confirmed from independent PCR and DNA samples from 50 healthy, unrelated white individuals and served as normal controls.

Cloning of the NKCC2 Gene from Rat Kidney

Freshly prepared rat kidney tissue was homogenized in cold Trizol reagent (Invitrogen, Carlsbad, CA; www.invitrogen.com), and total RNA was prepared according to the manufacturer’s recommendations. After mRNA purification with oligo-dT–coupled beads (Oligotex mRNA purification system; Qiagen, Valencia, CA; www.qiagen.com), reverse transcription to cDNA was performed with SuperScript reverse transcriptase (Invitrogen). We used a proofreading polymerase (pfu-Polymerase; Stratagene, La Jolla, CA; www.stratagene.com) to amplify the complete open reading frame of the NKCC2 cDNA (NCBI GenBank accession no. XM.579419) with the primers (in 5′-3′ direction) TGG AAG ATG TCA GTG GCC ACC CCT TCC (5′-end) and TC CCT CGC CTC TTA AGA GTA AAA TGT C (3′-end; the start ATG and the reverse complementary stop TTA are indicated in bold). The resulting PCR product was cloned into pCRII-Topo (Invitrogen) and sequenced in both directions. To optimize translation efficacy in Xenopus oocytes, we then transferred the verified cDNA sequence to pOGII, which contains the 5′- and 3′-untranslated regions of the Xenopus β-globin gene. Site-directed mutagenesis was performed according to the Quick-Change protocol (QuickChange Site Directed Mutagenesis Kit; Stratagene).

Expression in Xenopus laevis Oocytes and Tracer Flux Measurements

Ten or 20 ng of in vitro transcribed rat cRNA (mMessage mMachine kit; Ambion, Austin, TX; www.ambion.com) for the NKCC2 constructs were injected in defolliculated Xenopus oocytes, which were kept at 16°C in ND96 storage solution that contained 96.0 mmol/L NaCl, 2.0 mmol/L KCl, 1.8 mmol/L CaCl2, 1.0 mmol/L MgCl2, 5.0 mmol/L HEPES (pH 7.4), 2.5 mmol/L sodium pyruvate, 0.5 mmol/L theophylline, and 20 μg/ml gentamicin. Four days after injection, 36Cl uptake was determined in chloride-free ND96 uptake solution (which contained [in mmol/L] 96.0 Na-aspartate, 80.0 D-Mannitol, 2.0 KOH, 1.8 Ca-acetate, 1.0 Mg-acetate, and 5.0 HEPES [pH 7.4]) supplemented with 5.0 mmol/L Na36Cl. After equilibration for 15 min in 36Cl-free uptake solution, the tracer was added and cells were kept in uptake solution at room temperature for 60 min, then cooled on ice and washed five times in ice-cold chloride-free ND96 (without tracer). After mechanical lysis, intracellular radioactivity was determined by scintillation counting for each oocyte. Mean and SEM of the determined counts per minute (cpm) are shown for at least 15 oocytes per data point from the same preparation. Experiments were repeated in at least three different batches of oocytes that were derived from different frogs. The error bars in the diagrams were calculated from the SEM. Statistical significance was analyzed with t test (unpaired test with unequal variance) and was assumed at a P ≤ 0.05.

Quantification of NKCC2 Surface Expression

For quantification of wild-type and mutated NKCC2 surface expression, a hemagglutinin (HA) epitope was inserted into the extracellular loop between the transmembrane domains VII and VIII (as predicted by hydropathy analysis). As determined by tracer-flux experiments, the extracellular HA epitope did not interfere with 36Cl uptake activity (data not shown). Surface quantification of NKCC2-HA constructs after expression in Xenopus oocytes was performed as described previously (5). In brief, viable NKCC2-HA–expressing oocytes were labeled with rat anti-HA mAb (3F10, 1 μg/ml). After rigorous washing in cold ND96 solution, bound antibodies on individual oocytes were detected with horseradish peroxidase–coupled goat anti-rat antibodies (Fab fragments, 1:500 dilution; Jackson ImmunoResearch, West Grove, PA) in a peroxidase-catalyzed luminescence reaction using SuperSignal ELISA Femto Maximum Sensitivity chemiluminescent substrate (Pierce, Rockford, IL). The luminescence signal as determined by a luminometer (Berthold Lumat LB9507; Berthold Technologies, Bad Wildbad, Germany) is given in relative light units. Oocytes that expressed wild-type NKCC2 without HA epitope served as control. The experiments were repeated in three different batches of oocytes that were derived from different frogs. For each data point, approximately 20 oocytes that were derived from one single preparation were analyzed individually, and the relative light units are given as mean ± SEM.

Results

Patient 1 is the first of two sons of healthy nonconsanguineous parents. He was born 3 wk past term after a normal pregnancy and showed normal thriving. The clinical and laboratory data are summarized in Table 1. A routine check at the age of 15 yr in preparation for a tonsillectomy revealed hyperuricemia (0.76 mmol/L; normal range 0.18 to 0.42 mmol/L), hypokalemia (2.5 mmol/L plasma potassium), and metabolic alkalosis (venous blood pH of 7.52) with a bicarbonate concentration of 30.7 mmol/L (normal range 21 to 28 mmol/L). Plasma sodium, calcium, and magnesium concentrations were
normal, as was plasma creatinine. A more elaborate workup that was initiated by these findings showed elevated serum renin (50.5 pg/ml) and aldosterone levels (865 pg/ml). Urinalysis after 24-h urine collection revealed mild polyuria (2850 ml/24 h or approximately 20 to 80 ml/kg per 24 h) with a urine osmolality of 248 mOsmol/kg. Fractional sodium excretion was between 1050 and 4000 ml (approximately 20 to 80 ml/kg per 24 h). Urinary excretion of PGE₂ was normal, as was that of PGE-M. Sonography of the kidneys showed bilateral medullary nephrocalcinosis. Oral potassium chloride (0.6 mmol/kg body wt) and allopurinol (7.5 mg/kg body wt) were started.

In both siblings, the constellation of hypokalemia, (compensated) metabolic alkalosis, mild polyuria, increased fractional sodium excretion, and hypercalciuria with nephrocalcinosis pointed to a sodium chloride transport defect along the TAL of Henle’s loop. To evaluate the function of the TAL in vivo, we applied the NKCC inhibitor furosemide to both patients and the effects on diuresis and urinary chloride excretion were determined. In case of impaired TAL sodium chloride reabsorption, addition of the loop diuretic furosemide should exert no or only minor effects on diuresis and renal chloride excretion. When compared with healthy control subjects and patients with typical clinical features of aBS/HPS (4), the stimulatory effect of furosemide on diuresis and urinary chloride excretion for both siblings was between the means of both groups (Figure 1). A partially impaired capacity for sodium chloride reabsorption along the TAL therefore could explain the electrolyte disturbances of both patients.

The NKCC2 isoform of the sodium-potassium-chloride co-transporters is the pacemaker of TAL sodium chloride reabsorption. We therefore analyzed the sequence of the NKCC2 encoding gene SLC12A1 in both patients. As shown in Figure 2, mutations were detected in both SLC12A1 alleles of both patients: A missense mutation (c.530 T > A) resulting in an phenylalanine to tyrosine exchange at amino acid position 177 (p.F177Y) was inherited from the father, and a frameshift mutation (c.2751dupT) that results in a frameshift with premature stop codon at amino acid position 918, deleting a major part of the intracellular C-terminus (p.D918fs), was inherited from the mother. As expected, the parents were found to be heterozygous for only one mutation. These mutations were neither

<p>| Table 1. Clinical and laboratory data of both patients (at the initial evaluation) and their parentsa |
|-----------------------------------------------|---------------|----------------|---------------|---------------|</p>
<table>
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<th>Patient 1</th>
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<th>Father</th>
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<td>Urinary PGE-M excretion (ng/h per 1.73 m²)</td>
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aPGE₂, prostaglandin E₂; PGE-M, prostaglandin E₉₅. 
described before nor observed in 100 control chromosomes from healthy individuals (data not shown).

Genetic defects of the NKCC2 transporter were the first to be identified in aBS/HPS (1). Up to now, NKCC2 defects were unambiguously associated with prenatal manifestation and severe, life-threatening volume depletion in the early neonatal period (6). So far analyzed, complete loss of human NKCC2 transport activity was described for the SLC12A1 mutations associated with the severe phenotype (3). The relatively mild phenotype observed in these cases therefore might be explained by some residual activity of the affected NKCC2 proteins. To clarify this point, we performed \(^{36}\)Cl-uptake experiments after heterologous expression of wild-type rat NKCC2 and mutated proteins in Xenopus oocytes. The rat ortholog of NKCC2, which shows 93\% overall sequence identity with the human isoform, was used for these experiments because it gave more robust uptake signals. As shown in Figures 3 and 4, top, expression of wild-type rat NKCC2 (NKCC2-WT) resulted in an approximately three-fold increase of \(^{36}\)Cl uptake when compared with noninjected oocytes. As expected, expression of a mutated NKCC2 protein that was identified in a patient with a prenatal onset aBS/HPS (p.L196P) did not induce chloride.

Figure 1. Effect of furosemide on diuresis and urinary chloride excretion. Diuresis (top) and urinary chloride excretion (bottom) were determined before and after application of furosemide, and the difference was normalized to urine furosemide excretion as described by Koeckerling et al. (4). The mean values (± SEM) determined for healthy control subjects (n = 13) and patients with antenatal Bartter syndrome/hyperprostaglandin E syndrome (aBS/HPS; n = 8) were taken from Koeckerling et al. (4).

Figure 2. Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) (NKCC2) mutations and their topology. The p.F177Y and the p.D918fs mutations were identified in both patients. Both mutations affect highly conserved amino acid residues (middle: amino acid alignment of human [Homo sapiens (Hs)], rat [Rattus norvegicus (Rn)], spiny dogfish [Squalus acanthias (Sa)], and fruit fly [Drosophila melanogaster (Dm)] NKCC2 sequences). The p.L196P mutation that was detected in a patient with aBS/HPS served as a nonfunctional negative control. The position of the hemagglutinin (HA) epitope inserted for NKCC2 surface quantification is indicated between transmembrane domains VII and VIII close to two predicted glycosylation sites (protein model according to reference [15]).
uptake significantly different from control oocytes. The same was true for the construct with the p.D918fs mutation detected in one SLC12A1 allele of the two brothers. In contrast, expression of the p.F177Y mutation that was detected in the other allele showed partial chloride uptake, which amounted to 50% or more of the wild-type NKCC2 uptake. Therefore, the p.F177Y mutation in humans may result in a chloride-transporting protein with approximately half of the activity of the wild-type protein.

Functional NKCC2 transporters recently were shown to consist of two NKCC2 subunits (7). In view of the heterozygosity for both mutations, as observed in our patients, this dimeric architecture might implicate a negative impact of one mutated transporter on the function of the other. To rule out such a dominant negative effect of the p.D918fs mutation on the function of the other, to rule out such a dominant negative effect of the p.D918fs mutation on the function of the other, we performed coexpression experiments with co-injection of both mutants as well as co-injection of each of the mutants plus the wild type. As shown in Figure 4, neither of the constructs with p.F177Y or p.D918fs impaired transport activity of NKCC2 wild type. Moreover, the activity of the construct with p.F177Y remained unchanged in the presence of the p.D918fs mutant. These in vitro findings argue against a dominant negative effect of the p.D918fs mutation. To support this notion in vivo, we determined the osmolality of a morning urine sample of the patients’ mother, who is heterozygous for the p.D918fs mutation. In line with our experimental data, the measured urine osmolality of 930 mOsm/kg pointed to a normal renal concentrating capability and argues against an inhibitory effect of the p.D918fs mutation on NKCC2 wild-type function.

Impaired NKCC2 transport activity in case of the p.F177Y mutation might result from (1) decreased protein stability, (2) decreased expression in the plasma membrane via either impaired insertion in or increased clearance from the membrane, or (3) disturbed function of an otherwise normally processed protein. To reveal the underlying mechanism compromising transport activity of the construct with the p.F177Y mutation, we compared surface expression of wild-type and mutated proteins expressed in Xenopus oocytes. To this end, we inserted an HA epitope into an extracellular loop of the NKCC2 proteins (NKCC2-HA; the transport activity as determined by

Figure 3. Functional analysis of the NKCC2 mutations. Xenopus oocytes were injected with 10 ng of rat cRNA for the indicated constructs and $^{36}$Cl uptake subsequently was measured. Non-injected oocytes and oocytes that expressed the p.L196P mutation that was identified in a patient with aBS/HPS served as controls. In contrast to the p.D918fs frameshift mutation, the p.F177Y missense mutation induced a $^{36}$Cl uptake that was significantly different from noninjected oocytes. The columns represent the mean ± SEM of at least 15 oocytes per injected cRNA. A similar level of expression of wild-type and mutated NKCC2 proteins is demonstrated by Western blot analysis of HA-tagged constructs with a monoclonal anti-HA antibody. Note that the wild-type protein without HA epitope (WT) is not recognized by the antibody and that the p.D918fs construct is shifted to a lower molecular weight because of the deletion of a part of the C-terminal sequence.

Figure 4. Transport activity (top) and surface expression (bottom) of NKCC2 wild-type and mutated proteins. Wild-type and mutated NKCC2 rat cRNA (20 μg for each construct) were injected alone or in combination as indicated. NKCC2 constructs with an extracellular HA epitope were used for luminometric surface quantification after heterologous expression in Xenopus oocytes. Oocytes that expressed NKCC2 without HA epitope (NKCC2-WT) served as a negative control. In contrast to the construct with p.D918fs (p.D918fs-HA) and p.L196P (p.L196P-HA identified in a patient with aBS/HPS) the construct with p.F177Y (p.F177Y-HA), which displayed residual transport activity, showed a surface expression that was not significantly different from the wild-type protein (NKCC2-HA). Coexpression of p.D918fs does not negatively affect transport activity or surface expression of NKCC2 wild type or p.F177Y. The columns represent the mean ± SEM of at least 15 oocytes per injected cRNA. The P values are given as numbers.
uptake measurements was not affected by this protein modification; data not shown). Quantification of HA expression on viable oocytes via an enzyme-linked luminometric assay then allowed determination of NKCC2 surface expression. As shown in Figure 4, a strong HA-dependent luminescence signal appeared after expression of wild-type NKCC2-HA. No surface expression was detected for the loss-of-function mutation p.L196P detected in a patient with aBS/HPS. Similarly, a severely reduced cell membrane expression was observed for the construct with the p.D918fs (p.D918fs-HA). In contrast, a luminescence signal similar to that of the wild-type protein appeared after expression of the p.F177Y mutation (p.F177Y-HA). The p.D918fs mutation therefore seemed to affect cell membrane insertion of NKCC2, whereas the p.F177Y mutation impaired directly the transport function of the NKCC2 protein without affecting protein stability or membrane insertion. Moreover, in co-injection experiments, neither p.F177Y nor p.D918fs affected the surface expression of the HA-tagged wild-type protein (NKCC2-HA + p.F177Y and NKCC2-HA + p.D918fs, respectively), and co-injection of both mutants (p.F177Y-HA + p.D918fs) showed no effect of the p.D918fs mutant on surface expression of p.F177Y-HA.

Discussion

In this report, we describe an unusually mild phenotype that resulted from a particular combination of mutations of the SLC12A1 gene coding for the NKCC2 sodium potassium chloride co-transporter. The clinical picture with mild polyuria and borderline hypercalciuria clearly contrasts with the prenatal-onset disease with polyhydramnios, prematurity, and severe neonatal volume depletion hitherto known to be associated with SLC12A1 gene mutations (aBS/HPS). Consistent with the in vitro finding of residual furosemide-sensitive chloride transport along the TAL, our functional results in vitro suggest significant residual transport activity in humans of the p.F177Y mutated NKCC2 protein, which may well account for the clearly attenuated phenotype of both patients. Their laboratory findings perfectly mirror the side effects of NKCC inhibition by chronic furosemide application (8): Decreased urine-concentrating ability, increased fractional sodium excretion, and hypercalciuria. Salt wasting and volume depletion in addition lead to secondary hyperaldosteronism (resulting in hypokalemia and metabolic alkalosis) and increase proximal tubular urate reabsorption entailing hyperuricemia.

According to our functional data, only approximately half of the normal NKCC2 activity would be expected along the TAL of the described patients. Although this extent of functional NKCC2 impairment plausibly reconciles with the mild clinical picture and the reduced renal tubular sensitivity for furosemide, a one-to-one translation from our heterologous expression data to in vivo conditions might not necessarily be warranted. Additional factors in TAL cells, which might not be relevant in the setting of heterologous overexpression of cRNA in Xenopus oocytes, might influence the activity of the mutated proteins. Despite these limitations of a heterologous expression system using rat cRNA, our data suggest that the investigated mutations affect different aspects of NKCC2 protein function.

The p.D918fs mutation clearly interferes with membrane trafficking of the NKCC2 protein, and a patient who is homozygous for this mutation is likely to present with severe aBS/HPS, whereas the p.F177Y mutation directly impairs transport function of NKCC2, whose abundance in the cell membrane seems not to be affected. One might speculate that homozygosity for this mutation would result in an even milder phenotype as observed for the described patients. Unfortunately, comprehensive structure-function data (i.e., crystallographic data) are not yet on hand for this family of transport proteins. The available data mainly are inferred indirectly from domain-swapping approaches between shark and mammalian NKCC and point to the importance of the second, fourth, and seventh predicted α-helical domains for ion transport (9). Therefore, we cannot predict the role of the affected amino acid residue at the beginning of the first α-helical domain in the transport cycle of the NKCC2 protein. With respect to the results from our coexpression experiments and the normal urine-concentrating capability of the mother, who is heterozygous for the p.D918fs mutation, we can exclude, however, a dominant negative effect of this mutation on NKCC2 wild-type or p.F177Y activity.

In line with the mild clinical presentation, urinary excretion of PGE₂, which is increased heavily in the prenatal-onset disease (2), was close to normal in our patients. Renal PGE₂ overproduction in aBS/HPS is thought to derive from impaired entry of chloride into the macula densa cells of the distal tubule, which in turn increases expression of cyclooxygenase 2, a key enzyme of the prostanoïd pathway (2,10,11). In contrast to epithelial cells of the TAL, which express the isoforms A and F of NKCC2, apical sodium chloride entry in macula densa cells is mediated by the isoform B. These isoforms result from alternative splicing of an exon encoding a 96-bp region of NKCC2, giving rise to three different protein sequences in the second transmembrane domain. Because the p.F177Y mutation affects the beginning of the first transmembrane domain, it is expected to occur in any of the described NKCC2 splice variants. Residual transport activity of the macula densa isoform B hence might explain normal urinary PGE₂ excretion as observed in our patients and thereby in addition would support the concept of residual NKCC2 transport activity.

Despite this strong evidence for residual NKCC2 function, the renal concentrating capability of our patients, as deduced from their urine osmolalities, surprisingly was not substantially higher than that of patients with untreated aBS/HPS (2). An impairment of the renal concentrating capability by the severe medullary nephrocalcinosis in addition to the partial NKCC2 defect might explain this discrepancy. Moreover, we cannot completely rule out the possibility of another mutation within unsequenced regions of the SLC12A1 gene, which might interfere with NKCC2 expression or function in vivo.

Although exceptional for NKCC2 mutations, mild and late-onset clinical manifestation of Bartter syndrome has been described for mutations that affect the CIC-Kb chloride channel (6,12,13). Distinct differences in disease severities hitherto hampered a direct comparison of severe renal salt wasting as observed in aBS/HPS caused by NKCC2 malfunction with the milder Bartter syndrome as a
result of impaired CIC-Kb function. Whereas plasma electrolyte abnormalities of patients who have CLCNKB gene defects resemble those of the here described mild NKCC2 defect, the former normally show milder impairment in urine-concentrating capability and normal urinary calcium excretion so that nephrocalcinosis usually does not develop (6). How could this discrepancy be explained? CIC-Kb together with the β-subunit barttin forms a basolateral chloride exit pathway not only in the TAL but also along the distal convoluted tubule (DCT), where luminal uptake of sodium chloride is mediated via the thiazide-sensitive sodium chloride co-transporter NCCT (2,14). In contrast to a NKCC2 defect, which affects sodium chloride reabsorption exclusively along the TAL, a CIC-kb defect thus would impair salt reabsorption along the TAL and the DCT. In contrast to the TAL, disturbed sodium chloride reabsorption along the DCT results in hypocaliuria. In case of a combined impairment of TAL and DCT, this calcium-saving effect of disturbed DCT sodium chloride reabsorption could counteract the reduced calcium reabsorption along the TAL, eventually resulting in normo- or even hypocaliuria (12). As learned from our two patients, the phenotypic differences observed in NKCC2 and CIC-Kb malfunction therefore may reflect not only differences in disease severity but also the different nephron segments affected by the mutations.

Conclusion

We have shown that a particular combination of NKCC2 mutations is associated with variations in disease severity. An incomplete loss-of-function effect of the described p.F177Y mutation might explain the mild phenotype. Reduced NKCC2 function therefore might be considered in patients who present with hypokalemia, metabolic alkalosis, hyperuricemia, and nephrocalcinosis even beyond the neonatal period.

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References


Access to UpToDate on-line is available for additional clinical information at http://www.jasn.org/