Defects in KCNJ16 cause a novel tubulopathy with hypokalemia, salt wasting, disturbed acid-base homeostasis, and sensorineural deafness

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Significance Statement

We report a novel disease phenotype comprising a tubulopathy with severe hypokalemia, renal salt wasting, disturbed acid-base homeostasis and sensorineural deafness associated with variants in *KCNJ16* ($K_{ir}5.1$). In the kidney, the inwardly rectifying potassium channel subunit KCNJ16 forms functional heteromers with KCNJ10 in the distal nephron and with KCNJ15 in the proximal tubule. Functional studies of mutant KCNJ16 in *Xenopus* oocytes demonstrate a disturbed function of channel complexes with both KCNJ10 and KCNJ15. Individuals with KCNJ16 variants may present with metabolic acidosis or alkalosis reflecting a differential effect on proximal tubular bicarbonate reabsorption as well as distal tubular salt and potassium conservation. These findings together establish a multifaceted role of KCNJ16 in tubular transport processes, potassium and pH sensing.

ABSTRACT

Background

The transepithelial transport of electrolytes, solutes and water in the kidney is a well-orchestrated process involving numerous membrane transport systems. Basolateral potassium channels in tubular cells not only mediate potassium recycling for proper Na^+,K^+-ATPase function, but are also involved in potassium and pH sensing. Genetic defects in KCNJ10 cause EAST/SeSAME syndrome characterized by renal salt wasting with hypokalemic alkalosis associated with epilepsy, ataxia, and sensorineural deafness.

Methods

A candidate gene approach and whole-exome sequencing were used to determine the underlying genetic defect in eight patients with a novel disease phenotype comprising a hypokalemic tubulopathy with renal salt wasting, disturbed acid-base homeostasis and sensorineural deafness. Electrophysiological studies and surface expression experiments were performed to investigate the functional consequences of newly identified variants.

Results

We identified mutations in the *KCNJ16* gene encoding KCNJ16, which along with KCNJ15 and KCNJ10 constitutes the major basolateral potassium channel of the proximal and distal tubule, respectively. Co-expression of mutant KCNJ16 together with KCNJ15 or KCNJ10 in *Xenopus* oocytes resulted in significantly reduced currents.

Conclusions

Bi-allelic variants in KCNJ16 were identified in patients with a novel disease phenotype comprising a variable proximal and distal tubulopathy associated with deafness. Variants affect the function of heteromeric potassium channels resulting in disturbances in proximal tubular bicarbonate handling as well as distal tubular salt reabsorption.

INTRODUCTION

In the distal convoluted tubule (DCT) of the kidney, the essential role of basolateral potassium recycling for transepithelial salt reabsorption was highlighted by the discovery of loss-of-function variants in *KCNJ10* (K_{ir}4.1) in children with EAST/SeSAME syndrome[1;2]. Affected individuals display a phenotype resembling Gitelman syndrome with salt wasting, hypokalemic metabolic alkalosis, hypomagnesemia, and hypocalciuria[3]. In addition, they exhibit sensorineural deafness, ataxia and intellectual disability attributed to a disturbed function of KCNJ10 in the stria vascularis of the inner ear and in the central nervous system (CNS). Mice deficient for Kcnj10 largely replicate the human EAST/SeSAME phenotype[1].

Though KCNJ10 was shown to form functional homomeric channels *in vitro*[4], studies in kidney epithelia demonstrated a predominant role of heteromeric channels of KCNJ10 and KCNJ16 (K_{ir}5.1) *in vivo* with differences in gating and single channel conductance, and especially sensitivity to changes in intracellular pH[5;6]. These differing physiological properties also allowed to identify KCNJ10/KCNJ16 heteromers as the predominant inwardly rectifying potassium channel in the basolateral membranes of cells lining the DCT and cortical collecting duct (CCD)[7]. Thus, while KCNJ16 is unable to constitute functional homomeric channels, a critical role in the control of basolateral potassium fluxes and acid-base metabolism in kidney via heteromerization with KCNJ10 was suggested[6;8;9]. By sensing plasma potassium and intracellular pH with consequent adjustment of the activity of the apical NCC, basolateral KCNJ10/KCNJ16 channels are thought to modify sodium delivery to downstream tubular segments to modulate potassium and proton secretion[10].

The hypokalemic metabolic alkalosis in EAST syndrome is analogous to Gitelman syndrome and thought to result from reduced activity of the sodium chloride cotransporter (NCC). This causes salt wasting and an activation of the renin-angiotensin-aldosterone system (RAAS),

which, in turn, facilitates potassium and proton secretion[11;12]. Interestingly, while mice with a targeted disruption of *Kcnj16* exhibit hypokalemia, they show hyperchloremic metabolic acidosis instead of the alkalosis seen after inactivation of *Kcnj10*[13].

KCNJ16 has also been shown to interact with KCNJ15 ($K_{ir}4.2$)[5], which is expressed on the basolateral membrane of proximal tubular cells. Notably, mice deleted for Kcnj15 display metabolic acidosis with a reduced threshold for bicarbonate and impaired ammoniagenesis[14]. This raises the possibility that the acidosis seen in *KcnJ16*$^{-/-}$ mice may reflect a dysfunction of KCNJ15/16 heteromers. Thus, evidence from these animal models suggests a role for Kcnj16 both in proximal tubule, associated with acidosis, as well as in distal tubule, but there associated with metabolic alkalosis.

Together, these findings raise interesting questions about the role of KCNJ16 in acid-base homeostasis. However, investigating this role in human physiology has so far not been possible, as no subjects with bi-allelic loss-of-function variants have been reported. Here, we investigate seven individuals from six families exhibiting a tubulopathy with hypokalemia, salt wasting, disturbed acid-base homeostasis, and sensorineural deafness.

METHODS

Patients and Genetic Screening

Details on the patient cohort and genetic screening procedures are provided in the Supplemental Material. Ethical approval for this study was obtained from the University of Münster, Germany. Informed consent was obtained from all individuals (whenever appropriate) and their families. Genomic DNA of affected individuals and available family members was extracted from peripheral venous blood by standard methods. The entire coding sequence of *KCNJ16* was screened by direct sequencing of both strands (primer sequences available upon request). Next generation sequencing techniques (tubulopathy panel or whole exome sequencing) were applied in patients from families C, D, E, and G.

Molecular Biology

Full-length genes for KCNJ10 (NM_002241), KCNJ16 (NM_001270422) and KCNJ15 (NM_001276435) used for studies in *Xenopus laevis* oocytes were cloned between the 5' and 3' UTR of the Xenopus β-globin gene in pSGEM or pTLN vectors to increase expression efficiency[15]. For surface luminescence measurements, an external hemagglutinin (HA) epitope tag was introduced via QuikChange site-directed mutagenesis (Strategene, Germany) into the extracellular loop of KCNJ10 and KCNJ15 at positions 98 and 97 respectively. Both ends of the epitope were flanked by PGG residues to enhance accessibility and flexibility of the extracellular HA-tag, creating a sequence, which reads as GDLLE(98)PGGYPYDVPDYAGGPL(99)DPPA and GDLEP(97)PGGYPYDVPDYAGGPG(98)EPIS, respectively.

Complementary RNA (cRNA) was transcribed in vitro from linearized plasmids containing the cDNA of interest using T7 or SP6 kit (Ambion, Huntingdon, UK). cRNA was purified by

LiCl/ethanol precipitation or RNA clean and concentrator-5 kit (Zymo Research, Freiburg, Germany). Yield and concentration were quantified spectrophotometrically and the quality of RNA was confirmed by agarose gel electrophoresis.

Electrophysiological Measurements

Defolliculated oocytes from *Xenopus laevis* obtained from Ecocyte Bioscience (Dortmund, Germany) or collagenase type II digested oocytes obtained from frogs bred at Portsmouth University, UK were treated as described previously[16] and were injected with 50 nl nuclease free water containing cRNA (1-2 ng per oocyte of *KCNJ10* or *KCNJ15*, respectively, for single injections and 0.5-1ng, respectively, of each subunit for co-injections with KCNJ16), and then stored at 16°C in ND96 solution containing (in mM): 96 NaCl, 2 KCl, 1 MgCl2, 1.8 CaCl2 and 5 HEPES (pH 7.4), supplemented with 100 µg/ml gentamycin and 2.5 mM sodium pyruvate. Two to three days following injection, two-electrode voltage-clamp measurements were performed at room temperature with a Gene Clamp 500 amplifier (Axon Instruments, Union City, USA) or a TurboTec 01C (npi, Tamm, Germany). All measurements were done in bath solution ND96 containing (in mM): 96 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$ and 5 HEPES (pH 7.4) with or without added KCl to increase total K^+ to 20 mM. Currents were elicited by 200 ms pulses applied in 20 mV increments to potentials ranging from -120 mV to +60 mV from a holding potential of -80 mV or -40 mV, accordingly. Statistical analyses (ANOVA) were performed on oocytes derived from one preparation. The error bars in the diagrams indicate the standard error of the mean (SEM). Experiments were repeated in at least 3 different batches of oocytes derived from different frogs.

Surface Luminescence Assay

Surface expression of HA-tagged KCNJ10 or KCNJ16 in *Xenopus laevis* oocytes was analyzed two days after injection with the cRNA (10 ng/oocyte of HA-tagged KCNJ alone or together with the wildtype or mutant subunit), as described previously[16]. The primary and secondary antibodies used in this assay are 1 mg/ml rat monoclonal anti-HA antibody (clone 3F10, Roche Pharmaceuticals, Basel, Switzerland) and 2 mg/ml peroxidase-conjugated affinity-purified F(ab)2 fragment goat anti-rat immunoglobulin G antibody (Jackson ImmunoResearch, West Grove, PA, USA), respectively. For each construct, surface expression of at least 8 oocytes was analyzed in one experiment, and at least two experiments were carried out. The luminescence produced by water injected oocytes was used as a reference signal (negative control). Protein immunoblotting for oocyte experiments was performed as described previously [16], in order to verify equal expression of all HA-tagged fusion proteins in *Xenopus* oocytes. Primary and secondary antibodies used for blotting are rat monoclonal anti-HA antibody (1:500) (clone 3F10, Roche Pharmaceuticals, Basel, Switzerland) or rabbit anti-HA antibody (1:1000) (ab9110, Abcam, Berlin, Germany) and peroxidase-conjugated affinity-purified F(ab)2 fragment goat anti-rat immunoglobulin G antibody (1:10,000) (Jackson ImmunoResearch, West Grove, PA, USA) or StarBright™ Blue 520 Goat Anti-Rabbit IgG (1:5000) (12005870, Biorad, Feldkirchen, Germany), respectively.

Microdissection of renal tubules, RNA isolation, and quantitative RT-PCR

Nephron segments were obtained from the kidneys of adult (8-10 week) male C57BL6J mice and digested with type-2 collagenase, as previously described [17]. Glomeruli and tubules were isolated manually according to the morphological differences. Total RNA was extracted with RNAqueous® kit (Invitrogen, Carlsbad, CA). One µg of RNA was used to perform the reverse

transcriptase reaction with iScript™ cDNA Synthesis Kit (BioRad). Changes in mRNA levels of the target genes were determined by relative RT-qPCR with a CFX96™ Real-Time PCR Detection System (BioRad) using iQ™ SYBR Green Supermix (Bio-Rad)(for detailed methods see Supplemental Material). The relative changes in targeted genes over Gapdh mRNA were calculated using the $2^{-\Delta\Delta Ct}$ formula.

In situ hybridization

Fluorescent multiplex in situ hybridization (RNAscope) assays (Advanced Cell Diagnostics, Hayward, CA) were used to visualize single RNA molecules per cell in 10 µm cryo-sections of wild-type mouse kidney fixed with 10% neutral buffered formalin. Kidney sections were incubated with probes for *Kcnj16*, as well as tubular marker genes *Aqp1* (proximal tubule), *Umod* (thick ascending limb), and *Avpr2* (collecting duct), respectively (for details see Supplemental Material). Images were obtained with a confocal microscope SP8 (Leica Microsystems, Wetzlar, Germany).

Statistical tests

Data are reported as means ± SEM. Statistical significance was determined using Student's t-test or ANOVA, as appropriate. In the figures, statistically significant differences to control values are marked by asterisk (* $p<0.0001$); n.s indicates non-significant differences ($p>0.05$). Membrane potential measurements in *Xenopus* oocytes expressing KCNJ15 alone or with wildtype KCNJ16 were performed in a batch separate from the batch used for both voltage and current measurements featuring KCNJ15 and KCNJ16 WT or mutants, and a two-sided t-test with Welch correction for unequal variance was used instead of ANOVA used for the other comparisons.

RESULTS

Patient Characteristics

We initially studied an infant (A-II-7) who presented at five days old with polyuria and weight loss. Laboratory analyses revealed profound hypokalemia and RAAS activation indicative of renal salt wasting. In addition, sensorineural hearing impairment was diagnosed by brainstem-evoked response audiometry (Table 1). Under the suspicion of Bartter syndrome, the known causative genes were analyzed but no causative variants were identified. Due to the diagnosis of deafness, also mutations in KCNJ10 were excluded. Interestingly, the child, who had been alkalotic initially, developed metabolic acidosis during follow-up. Of note, there were neither signs of ataxia nor epilepsy.

The index case in family B (B-II-1) had presented at 14 months old during an episode of gastroenteritis with severe hypokalemia and metabolic acidosis (Table 1). Unexpectedly, these findings persisted after fluid and electrolyte replacement and the child commenced long-term potassium and bicarbonate supplements. At the age of 8 years a moderate hearing impairment was noticed (Figure S2). The younger brother (B-II-2) was evaluated at 18 months old and was also found to exhibit metabolic acidosis and hearing impairment. An acid loading test demonstrated a lack of ammonia excretion despite an intact ability to acidify the urine. Individual C-II-1 was diagnosed at 5 years old during an acute febrile illness with severe hypokalemia (1.2 mmol/L) and metabolic acidosis (Table 1). Under supplementation with potassium and bicarbonate, her acid-base status normalized while plasma potassium levels remained in the low normal range. Additional findings in this girl comprised RAAS activation as well as an intact ability to acidify the urine as observed in patient B-II-2. Individual D-II-2 was diagnosed at 4 years old with hypokalemia, mild normochloremic metabolic acidosis, and hyperreninemia. She had suffered from chronic constipation since her second year of life

which improved under potassium and salt supplementation. Sensorineural hearing loss was diagnosed at 4.5 years of age and she received hearing aids at the age of 5 years.

In individual E-II-1, hypokalemia was detected at 26 years of age during a work-up for fatigue, abdominal pain, and fainting episodes with palpitations and loss of consciousness. Additional laboratory findings included RAAS activation and a borderline metabolic acidosis. As in individual B-II-2, an acid loading test demonstrated a defect in ammonia excretion despite an acidic urine pH. All affected individuals from families B to E exhibited high frequency hearing impairment consistent with sensorineural deafness (Supplemental Material).

Individual F-II-1 presented at 16 years of age with fatigue, muscle pain and weakness. Laboratory findings showed severe hypokalemia (1.5 mmol/L), normal bicarbonate (22 mmol/L) and rhabdomyolysis (creatine kinase 9027 IU/L). She had presented with salt craving and chronic constipation in her past medical history and was diagnosed with bilateral sensorineural hearing loss at the age of 5 years (threshold 60 dB at 4000 Hertz) and received hearing aids at the age of 7 years. A tendency towards metabolic acidosis was observed during follow-up.

Individual G-II-2, a woman of Arab descent, presented at 22 years of age with dyspnea and hypokalemia (2.5 mmol/L). Her medical history included sensorineural deafness diagnosed at the age of 14 years. A nephrological work-up revealed a Gitelman-like phenotype with renal salt wasting, an activated RAAS, hypokalemia, metabolic alkalosis, hypomagnesemia (lowest S-Mg 0.52 mmol/L), and hypocalciuria.

In summary, the tubulopathy in these patients included severe hypokalemia together with polyuria, salt craving, and RAAS activation suggestive of salt wasting. Regarding acid-base homeostasis, findings were variable with either metabolic alkalosis or acidosis. Notably, one patient “switched” from metabolic alkalosis in infancy to acidosis later in childhood.

Hypomagnesemia and hypocalciuria, typical findings in Gitelman syndrome, were present in single individuals (especially A-II-7 and G-II-2), while urinary concentrating ability was largely intact (D-II-2, G-II-2). There was no indication of nephrocalcinosis. Besides metabolic acidosis, we did not observe additional specific signs of proximal tubular dysfunction such as phosphate wasting, glucosuria, aminoaciduria, or tubular proteinuria (data not shown). Uniformly, sensorineural hearing impairment was diagnosed in childhood or adolescence (Supplemental Material). In contrast, there were no signs of ataxia or epilepsy.

Genetic Analyses

Genetic analyses revealed *KCNJ16* variants in all affected individuals of families A to G following an autosomal recessive mode of inheritance (Figure 1A, supplemental Table S1). In individual A-II-7, we identified a homozygous missense variant (c.409C>T, p.R137C) in the pore-forming region of KCNJ16[18] (Figure 1B). In individuals from families B, E, and F, the *KCNJ16* gene was screened by conventional Sanger sequencing. Genomic DNA of individual C-II-1 and D-II-2 had been initially screened using a tubulopathy panel[3] and subsequently by whole exome sequencing (Supplemental Material). *KCNJ16* variants in families B to F comprised a premature stop codon (c.526C>G, p.R176*) as well as three additional missense variants (c.395T>G, p.I132R, c.404G>C, p.G135A, and c.749C>T, p.P250L)(Figure 1B). The analysis of parents' DNA in families B, C, and E confirmed that the variants were present in compound-heterozygous state (Table 1). In individual G-II-1, after an initial tubulopathy panel was negative [19], diagnostic whole exome sequencing identified a homozygous missense variant in *KCNJ16* in a run of homozygosity (c.296C>T, p.T64I). *In silico* analyses (Polyphen2; genetics.bwh.harvard.edu/pph2, SIFT; provean. jcvi.org) predicted all variants to be deleterious, affecting highly conserved amino acid residues. Like the p.R137C variant, p.I132R

and p.G135A are located near the selectivity filter in the pore-forming domain[18], whereas p.R176* and p.P250L are located in the intracellular C-terminus (Figure 1B)[20]. All variants are listed in the Genome Aggregation Database with allele frequencies <0.001 in the European population (gnomAD; www.gnomad.broadinstitute.org).

Segmental Distribution of KCNJ16 Along the Nephron

The quantitative expression analysis of *Kcnj16*, *Kcnj10* and *Kcnj15* mRNAs revealed that *Kcnj16* is abundantly expressed in both the proximal and distal segments of mouse kidney, whereas *Kcnj10* was mostly detected in distal segments and *Kcnj15* predominantly in proximal tubule (Figure 2A). The localization of *Kcnj16* transcripts along the proximal tubules, thick ascending limbs and collecting ducts was confirmed by multiplex *in situ* hybridization (RNAscope) in mouse kidney, against validated segmental markers (Fig 2B/C).

Functional Studies

For functional analyses, mutations were introduced into full-length cDNA encoding human KCNJ16, and co-expressed with KCNJ10 and KCNJ15 in *Xenopus* oocytes. Currents were measured by two-electrode voltage-clamp as described[16; 21]. As previously reported, expression of KCNJ10 yielded the typical inwardly-rectifying current-voltage relationship[21], while injection of KCNJ16 alone did not produce any measurable currents (Figure 3A). Next, co-injection of KCNJ10 with wildtype KCNJ16 resulted in a more pronounced inward-rectification and an increase in KCNJ10-evoked currents that was reduced with mutant KCNJ16 (Figure 3A+3B). For comparison of results between various batches and different experimental settings, results were normalized to currents evoked by co-expression of KCNJ10 and wildtype KCNJ16 (Figure 3C). Co-injection with p.I132R and p.R137C, located near the selectivity filter

of the channel pore, resulted in a further reduction of current amplitudes compared to the expression of KCNJ10 alone, suggesting an inhibitory effect on KCNJ10 function. The formation of functional KCNJ10 homomers may explain the preservation of small residual currents. Interestingly, a similar effect as for the two mentioned pore mutants was also observed for the p.T64I mutant observed in patient G-II-2 with the Gitelman syndrome-like phenotype and metabolic alkalosis.

In order to recapitulate the compound-heterozygous genotype in affected individuals from families B to F, we co-expressed combinations of KCNJ16 variants together with KCNJ10 (Figure 3B). Here, KCNJ16 missense variants located near the ion channel pore together with C-terminal variants uniformly resulted in a significant reduction of current amplitudes to levels of KCNJ10 expression alone.

To further characterize the functional consequences of KCNJ16 mutants, we also analyzed the membrane expression of wildtype and mutant proteins in *Xenopus* oocytes. HA-tagged KCNJ10 co-expressed along with KCNJ16 (WT and p.R137C) yielded currents identical to those observed for the untagged constructs. A comparable decrease in currents was observed when HA-tagged channels (KCNJ10 and KCNJ15) were co-expressed with mutant KCNJ16-p.R137C (data not shown). In accordance with the electrophysiological data, co-expression of wildtype KCNJ16 led to a significant increase in HA-tagged KCNJ10 surface expression (Figure 3D/E). In contrast, co-expression of C-terminal mutants p.R176* and p.P250L resulted in a reduction of KCNJ10 surface expression to levels of KCNJ10 when expressed alone. An additional inhibition of KCNJ10 surface expression was observed upon co-expression with KCNJ16 mutants p.T64I, p.I132R, and p.R137C.

To investigate the effects of KCNJ16 mutants on heteromeric channel function in more detail, we also performed single-channel recordings after co-expression of wildtype and mutant

KCNJ16 with KCNJ10 in HEK293 cells (for details see Supplemental Material). Here, co-expression of wildtype KCNJ16 yielded a significant increase in current amplitudes compared to the expression of KCNJ10 alone. A similar increase was observed after co-expression of KCNJ16-p.P250L suggesting the presence of mutant heteromeric channels at the cell surface. In contrast, co-expression of KCNJ16-p.I132R and -p.R137C resulted in smaller current amplitudes most likely corresponding to KCNJ10 homomers. The open probabilities of the investigated mutants remained largely unchanged in comparison to KCNJ10 alone and KCNJ10-KCNj16 wildtype.

Next, we investigated the functional consequences of KCNJ16 mutants by co-expressing KCNJ15 and KCNJ16 in *Xenopus* oocytes (Figure 4). Here, we specifically co-expressed combinations of KCNJ16 variants identified in compound-heterozygous state in addition to homozygous KCNJ16 variants p.R137C and p.T64I. First, co-injection of KCNJ15 with wildtype KCNJ16 resulted in a significant increase in KCNJ15-evoked currents. The co-expression of patient-specific combinations not only reduced this increase in KCNJ15-evoked currents seen after co-expression of wildtype KCNJ16, but largely abrogated KCNJ15/KCNJ16-mediated currents (Figure 4A). Small residual currents and a preserved inward rectification were only observed for KCNJ16-p.T64I and for co-expression of p.I132R/p.P250L (Figure 4B). Currents normalized to co-expression of KCNJ15 and wildtype KCNJ16 are shown in Figure 4C. In line with the observation for KCNJ10 and KCNJ16, co-expression of wildtype KCNJ16 resulted in a significant increase in current and hyperpolarization of KCNJ15-expressing oocytes (Figure 4C/D). In agreement with current-voltage relationships, the analyses of membrane potentials demonstrated significantly depolarized membrane potentials for all variants and combined variant expressions (Figure 4D).

Finally, we investigated the membrane expression of KCNJ15/KCNJ16 heteromers (Figure 4E). In accordance with the increased current and more hyperpolarized membrane voltage, co-expression of wildtype KCNJ16 also led to a significant increase in HA-tagged KCNJ15 surface expression. In contrast, co-expression of mutant KCNJ16 resulted in a reduction of KCNJ15 surface trafficking to levels observed for KCNJ15 when expressed alone. This effect was similar for all KCNJ16 variants.

DISCUSSION

Inwardly rectifying potassium channels are found in almost every cell type of the human body playing key roles in controlling membrane potential and cellular excitability[20]. In the kidney tubule, they are an integral component of polarized epithelia enabling transport processes across apical and basolateral membranes[22;23]. An example of a defect of apical potassium recycling in the thick ascending limb (TAL) of Henle's loop is Bartter syndrome type 2 due to bi-allelic mutations in *KCNJ1*[24]. The discovery of recessive mutations in KCNJ10 in individuals with EAST/SeSAME syndrome established the essential role of basolateral potassium recycling for salt reabsorption in the DCT[1]. Epilepsy, ataxia, intellectual disability, and sensorineural deafness observed in EAST/SeSAME syndrome indicated important functions for KCNJ10 also in the CNS and the inner ear.

We here describe a novel disease phenotype combining a tubulopathy and sensorineural deafness caused by bi-allelic loss-of-function variants in *KCNJ16*. Of note, affected individuals do not exhibit epilepsy or ataxia as observed in EAST/SeSAME syndrome. Similar to KCNJ10, expression analyses demonstrated KCNJ16, among additional organs, in the kidney and the inner ear[25]. In the kidney, KCNJ16 shows a broad expression pattern in epithelial cells of

proximal tubule (PT; proximal convoluted and straight tubule, PT segments S1-S3), loop of Henle (thin segment and TAL), DCT, and CCD (principal cells) (Figure 2)[7;22;26;27]. These expression analyses confirm high-throughput quantitative transcriptomic and proteomic profiling data from microdissected rat kidney tubule segments[28;29]. In the inner ear, KCNJ16 expression was detected in fibrocytes in the spiral ligament of the lateral cochlear wall[25], while KCNJ10 is expressed in strial intermediate cells[30;31]. KCNJ16 is also abundantly expressed in the CNS[32;33], but in a region-specific fashion[32]. Due to the expression of KCNJ16 in several brainstem nuclei, its distinct pH sensitivity, and a blunted response to intracellular acidification in *Kcnj16*$^{-/-}$ mice, a role in neuronal pH/CO_2 chemosensitivity has been proposed[34]. We observed no obvious clinical phenotype pointing to disturbed breathing control in our cohort.

Interestingly, Kcnj16$^{-/-}$ mice recapitulated the hypokalemia shown in *Kcnj10*$^{-/-}$ mice, but differed most notably by exhibiting metabolic acidosis[13]. Electrophysiological recordings of DCT basolateral membranes indicated an increased potassium conductance mediated by remaining Kcnj10 homomers with decreased pH sensitivity. In contrast to Kcnj10 deficiency with an abrogated salt reabsorption in the DCT, these mice showed an exaggerated response to hydrochlorothiazide. Such an upregulation of NCC-mediated salt reabsorption in the DCT has been associated with a phenotype of arterial hypertension and hyperkalemia as seen in human pseudohypoaldosteronism type 2 (PHAII). Instead, *Kcnj16*$^{-/-}$ mice showed hypokalemia and normal blood pressure, as well as increased water intake. Accordingly, the authors suggested a “non-distal” origin of hypokalemia and metabolic acidosis in *Kcnj16*$^{-/-}$ mice[13].

Indeed, the variable phenotype with respect to acid-base homeostasis, comprising both metabolic acidosis as well as alkalosis, represents one of the most fascinating clinical findings in this group of individuals with KCNJ16 defects. Importantly, when an acid loading test was

performed, patients failed to increase urinary ammonia excretion, while the ability to acidify the urine remained intact. These findings also argued against a pure distal renal tubular acidosis caused by KCNJ16 defects. As indicated above, KCNJ16 is expressed in proximal tubule where its expression overlaps with that of KCNJ15 (Figure 2)[28;29]. Indeed, functional studies had already demonstrated that KCNJ16 is able to build functional heteromers with KCNJ15[5]. These heteromers significantly differ from KCNJ15 homomers concerning open probability as well as single channel conductance[5].

Our results now also indicate a disturbed function of KCNJ15/KCNJ16 heteromers caused by mutant KCNJ16. This is consistent with recent data from *Kcnj15*$^{-/-}$ mice that exhibit hyperchloremic metabolic acidosis, a reduced threshold for bicarbonate reabsorption and a defect in urinary ammonia excretion reminiscent of the findings in humans with KCNJ16 defects presented here[14]. The finding of a defective ammonia production in our patients despite intact urinary acidification is indeed consistent with a proximal tubular disturbance. In the PCT, membrane depolarization, as observed in our heterologous expression system upon expression of mutant KCNJ15/KCNJ16 heteromers, could impair basolateral bicarbonate exit via the Na-HCO_3-cotransporter NBCe1 (SLC4A4)[35]. This in turn would lead to intracellular alkalinization potentially inhibiting ammoniagenesis[36]. These findings together support the assumption that the variable phenotype with respect to acid-base homeostasis reflects different effects of mutant KCNJ16 on heteromers with KCNJ15 (acidosis) or KCNJ10 (alkalosis). It is unclear at this point if this represents a specific genotype-phenotype effect, or whether other as yet unidentified factors play a role.

Notably, a Gitelman-like phenotype is also observed in Dahl salt sensitive rats with *Kcnj16* knockout, contrasting the mouse phenotype and highlighting the spectrum of acid-base abnormalities associated with KCNJ16 dysfunction, as seen in our patients[37].

A similar phenotypic variability with predominant affection of different tubular segments has also been observed in other hereditary tubular disorders, i.e. Bartter syndrome type III, due to mutations in the basolateral chloride channel ClC-Kb: affected individuals may present early in life with profound salt wasting consistent with a defect in the TAL, whereas others display a milder DCT phenotype indistinguishable from Gitelman syndrome[38]. Indeed, some individuals may present initially with abnormalities typical of Bartter syndrome and subsequently "convert" to a Gitelman-like phenotype[39]. This is reminiscent of the "phenotypic conversion" observed in individual A-II-7, who presented initially with metabolic alkalosis and subsequently exhibited acidosis.

The most prominent feature of disturbed KCNJ16 function observed in all patients as well as knockout animals is profound hypokalemia due to renal potassium wasting[13;37]. Furthermore, they exhibit, to a varying degree, signs of renal salt wasting and activation of the RAAS. In distal tubulopathies, including Gitelman and EAST syndrome, sodium losses and hypovolemia typically activate the RAAS, promoting increased sodium reabsorption in the CCD at the expense of potassium and hydrogen, resulting in hypokalemic metabolic alkalosis. The salt wasting and RAAS activation seen in individuals with KCNJ16 variants argue for a similar mechanism in the development of hypokalemia. Indeed, our functional studies demonstrated decreased heteromeric currents as well as a reduced surface expression of KCNJ10 upon co-expression with mutant KCNJ16. These observations together argue for a decrease in basolateral potassium recycling in the DCT caused by KCNJ16 variants, contrasting with the increased conductance that has been observed in *Kcnj16*$^{-/-}$ mice[13].

The contribution of the assumed proximal tubular dysfunction in the development of hypokalemia in our cohort remains less clear. Usually in pure proximal tubular disorders, i.e.

renal Fanconi syndrome or proximal renal tubular acidosis, the concurrent hypokalemia is attributed to a disturbed proximal tubular ammoniagenesis and bicarbonate reabsorption. These lead to a disturbed NCC phosphorylation and a stimulation of the RAAS, which in turn increases electrogenic sodium reabsorption via the epithelial sodium channel and potassium secretion [40;41]. Moreover, a disturbed function of KCNJ10-KCNJ16 heteromers in principal cells of the collecting duct may contribute to the pathophysiology of renal potassium losses[42]. Whether or not these effects account for an aggravation of hypokalemia in our cohort remains to be determined.

The KCNJ16 variants identified here comprise missense as well as nonsense variants that were, in part, present in compound-heterozygous state. Electrophysiological recordings imply potential differences in their inhibitory effect in interacting channel subunits KCNJ10 and KCNJ15. In particular, two out of the three variants located in the ion channel pore, p.I132R and p.R137C, exhibited strong effects on both interacting ion channel subunits, KCNJ10 and KCNJ15. This might potentially explain the severe phenotype with early manifestation in infancy, profound renal salt wasting, and failure to thrive observed in individual A-II-7, with the p.R137C mutation in homozygous state. Affected individuals from families B to E, who are each compound heterozygous for one pore mutation and a C-terminal variant, primarily exhibited metabolic acidosis together with hypokalemia from infancy to young adulthood. Of these, individual E-II-2 carrying the p.P250L mutation, that showed significant residual activity *in vitro*, appeared to display the mildest phenotype with late manifestation in adulthood, borderline metabolic acidosis, and moderate hypokalemia. Also, in individual F-II-1, the changes observed in acid-base homeostasis were rather mild, especially in face of the profound changes in plasma potassium levels. Individual G-II-2 stands out in our cohort, as she

presented with metabolic alkalosis and a renal phenotype highly reminiscent of EAST/SeSAME syndrome. Interestingly, functional studies of mutant KCNJ16-p.T64I identified in this individual demonstrated a strong inhibition of KCNJ10 while the effect on KCNJ15/KCNJ16 heteromers was milder.

Another uniform phenotypic feature of all patients, irrespective of the observed disturbance in acid-base homeostasis, was sensorineural hearing impairment diagnosed in childhood or adolescence. Audiograms of individuals with KCNJ16 defects demonstrated a moderate hearing loss especially at higher frequencies very similar to the findings in individuals with EAST syndrome (Supplemental Material, Figures S1-S4)[1]. As the expression pattern of KCNJ16 in the inner ear differs from that of KCNJ10 (see above), the pathophysiology of KCNJ16 defects might involve a disturbed interaction with additional ion channel subunits also in the inner ear.

In summary, we describe a new clinical entity comprising a hypokalemic tubulopathy and sensorineural deafness associated with bi-allelic mutations in *KCNJ16* encoding inwardly rectifying potassium channel subunit KCNJ16. Functional studies demonstrate an impaired function of KCNJ16 heteromers with KCNJ10 as well as with KCNJ15. These effects on two interacting ion channel subunits presumably add up to a complex phenotype with defective proximal tubular bicarbonate reabsorption and with distal tubular salt wasting. Genetic screening for mutations in KCNJ16 should therefore be included in the diagnostic work-up for patients presenting with hypokalemia, suspected tubular disease, and hearing impairment, irrespective of the prevailing disturbance in acid-base homeostasis.

AUTHOR CONTRIBUTIONS

K.P.S., E.J.H., R.W. and M.K. designed the study; E.J.H., V.G., N.G., C.R., B.K., S.T., P.H. are treating physicians; K.I., A.R., A.L.F., V.R., V.A., S.M., T.S.B., A.S.B., D.L., H.D., J.L., J.H.F.D., O.P., A.Z. carried out experiments; K.P.S., A.R., E.J.H., A.S., R.K., P.H., D.B., R.V.P., J.L., O.D., R.W., A.Z., M.K. analyzed the data; K.P.S., A.R., A.Z. made the figures; K.P.S., M.K. drafted the manuscript, K.P.S., A.R., E.J.H., C.R., S.W., A.S., R .K., P.H., D.B., R.V.P., O.D., R.W., A.Z., M.K. revised the paper; all authors approved the final version of the manuscript.

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DISCLOSURES

All authors have nothing to disclose.

SUPPLEMENTAL MATERIAL

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Table 1: Clinical characteristics and genetic findings of the patient cohort

	A-II-7	B-II-1	B-II-2	C-II-1	D-II-2	E-II-1	F-II-1	G-II-2
Sex	female	female	male	male	female	female	female	female
Consanguinity	yes	no	no	no	no	no	no	yes
Age at diagnosis	5 d	14 m	18 m	5 y	4y	26 y	16y	22 y
BLOOD (at presentation)								
Na^+ (mmol/L)(135-145)	141	141	140	124	139	137	138	140
K^+ (mmol/L)(3.5-5.5)	1.8	1.8	3.0	1.2	2,7	2.5	1.5	2.8
Cl^- (mmol/L)(100-109)	97	106	112	92	96	106	102	-
total Ca (mmol/L)(2.2-2.6)	2.4	2.57	-	2.25	2.50	2.17	2.02	2.36
Mg (mmol/L)(0.7-1.1)	0.64	0.74	-	0.99	0.84	0.82	0.75	0.76
Creatinine (µmol/L)(20-100)	47	71	27	33	34	39	59	58
Bicarbonate (mmol/L)(22-26)	27	18	17	17	21	22	20	32
URINE (at presentation)								
Urinary pH	7.0	7.1	5.3	5.3	5.5	5.6	4.4	7.0
FE-Na (%)(<1)	0.8	1.2	0.3	0.6	0.9	0.4	3.0	0.3
FE-K (%)(<15)	24	43	14	32	19	19	56	58
FE-Cl (%)	3.3	-	-	1.2	-	1.0	3.0	-
Ca/Crea (mmol/mmol)	0.01	2.70	-	0.54	0.27	0.40	0.16	0.01
FE-Mg (%)	6.0	-	-	1.4	-	1.3	2.0	3.5
CLINICAL FINDINGS								
Acidosis	yes	yes	yes	yes	yes	(yes)	(yes)	no
Nephrocalcinosis	no	no	no	no	no	no	no	no
Renal salt wasting	yes	yes	no	no	yes	yes	yes	yes
Hyperreninism	yes	yes	-	yes	yes	yes	yes	yes
Hyperaldosteronism	yes	yes	-	yes	no	(yes)	no	yes
Seizures	no	no	no	no	no	no	no	no
Ataxia	no	no	no	no	no	no	no	no
Sensorineural deafness	yes	yes	yes	yes	yes	yes	yes	yes
Acid loading test			intact urinary acidification iimpaired ammonia excretion	intact urinary acidification ammonia excretion not determined		intact urinary acidification impaired ammonia excretion		
TREATMENT								
Potassium (mmol/kg/day)	5	8	2	9	2	4	3	1
Bicarbonate (mmol/kg/day)	0.6	4.5	3.8	-	1	no	no	no
Additional medication	indomethacin magnesium	spironolactone	no	no	salt	no	magnesium	magnesium
KCNJ16 MUTATIONS								
Zygosity	homo	comp-het	comp-het	comp-het	comp-het	comp-het	comp-het	homo
Mutation type (location)	missense (pore)	missense (pore) + nonsense	missense (pore) + nonsense	missense (pore) + nonsense	missense (pore) + nonsense	missense (pore) + missense	missense (pore) + nonsense	missense
Nucleotide level	c.409c>t	c.409C>T + c.526C>T	c.409C>T + c.526C>T	c.395T>G + c.526C>T	c.395T>G + c.526C>T	c.395T>G + c.749C>T	c.404G>C + c.526C>T	c.191C>T
Protein level	p.R137C	p.R137C + p.R176*	p.R137C + p.R176*	p.I132R + p.R176*	p.I132R + p.R176*	p.I132R + p.P250L	p.G135A + p.R176*	p.T64I

FIGURE LEGENDS

Figure 1: Family pedigrees and localization of identified variants in KCNJ16/K_{ir}5.1 with Multiple Sequence Alignment **(A)** Pedigrees of seven families with eight affected individuals and homozygous or compound heterozygous variants in *KCNJ16* encoding the inwardly rectifying potassium channel subunit KCNJ16/K_{ir}5.1. Parental consanguinity is indicated by double bars in families A and G. Compound heterozygosity for KCNJ16 variants was analyzed by segregation analysis in the parents as indicated. **(B)** Localization of variants p.T64I, p.I132R, p.G135A, p.R137C, p.R176*, and p.P250L in the KCNJ16/K_{ir}5.1 protein. Missense variants p.I132R, p.G135A and p.R137C are located in the pore forming domain near the selectivity filter of the ion channel. Whereas p.T64I is located in the N-terminus near the first transmembrane domain, p.R176* as well as p.P250L are located in the C-terminus. **(C)** All affected amino acid residues are highly conserved between species as well as between interacting K_{ir} channel homologs KCNJ16, KCNJ10, and KCNJ15.

Figure 2: Expression analysis of *Kcnj16*, *Kcnj10*, and *Kcnj15* by SYBR green–quantitative PCR on micro-dissected nephron segments (A) and by in situ hybridization in mouse kidney (B/C). (A) Segment–related genes (Glom=*Nphs2*; PCT=*Scl5a2*; PST=*Slc38a3*; TAL=*Slc12a1*; DCT=*Slc12a3* and CD=*Aqp2*) were used to validate the purity of each fraction. Quantification of targeted gene was done in comparison with *Gapdh* that was used as housekeeping gene. The quantitative RT-PCR was performed on 5 pools of ~70 isolated renal tubules, obtained from 5 kidneys / 3 mice. *Kcnj16* shows a broad expression pattern along the entire nephron (blue bars) whereas *Kcnj15* expression is primarily detected in the proximal tubule (orange) and *Kcnj10* is predominantly expressed in distal segments (yellow). (B/C) Fluorescent multiplex in situ hybridization (RNAscope) on 10µm cryo-sections from wild-type mouse

kidney. (B) RNAscope for *Kcnj16* (grey), *Umod* (red), and *Aqp1* (green) and (C) RNAscope for *Kcnj16* (grey), *Umod* (red), and *Avpr2* (green). Nuclei are counterstained with DAPI. White bar indicates 25µm. *Kcnj16* mRNA molecules are detected co-expressed with *Aqp1* (proximal tubule, PT), *Umod* (thick ascending limb, TAL), and *Avpr2* (collecting duct, CD).

Figure 3: Mutant KCNJ16 decreases both KCNJ10 (J10) + KCNJ16 (J16) currents and surface expression. **A-C** Variants in KCNJ16 were either expressed alone with KCNJ10 or in combinations as found in patients. Xenopus oocytes injected with 1 ng of KCNJ10 and 1 ng total cRNA encoding KCNJ16 mutants or WT were investigated by two-electrode voltage clamp after 48 hours in 2mM bath [K^+]. Currents were elicited by 200 ms pulses applied in 20 mV increments to potentials ranging from -120 mV to +60 mV from a holding potential of -80 mV. I-V curves for **(A)** J10+J16 (WT and mutants) and **(B)** J10+J16 (WT and compound heterozygotes) exhibit typical inward rectification and significant amplification of KCNJ10-evoked currents, upon co-expression with KCNJ16. **(C)** Normalized currents at -120 mV showing KCNJ16 variants individually and in combination as observed in patients. All variant combinations showed significant (ANOVA) decreases in currents compared to J10+J16 WT. Controls included are KCNJ16 and water injected oocytes. The error bars represent SEM for at least 10 oocytes from at least three batches (* $p<0.0001$ vs. KCNJ10+16). **(D)** Normalized surface expression of HA-tagged KCNJ10 alone or together with KCNJ16 (WT and mutants) showed a significant (ANOVA) decrease in the surface expression of KCNJ10(HA) upon co-expression with KCNJ16 mutants over their WT counterpart. The error bars represent SEM for at least 8 oocytes from at least two batches (* $p<0.0001$ Vs J10+16). **(E)** Corresponding Western blots of HA-tagged protein in total oocyte lysates. Water-injected oocytes were taken as control to determine antibody specificity.

Figure 4: Mutant KCNJ16 decreases both KCNJ15 (J15)+KCNJ16 (J16) currents and surface expression. A-D Variants in KCNJ16 were either expressed alone with KCNJ15 or in combinations as found in patients. *Xenopus laevis* oocytes injected with 1 ng of KCNJ15 and 1 ng total cRNA encoding KCNJ16 mutants or WT were investigated by two-electrode voltage clamp after 48 hours in 20 mM bath [K^+]. (A) Currents in response to voltage clamping from -120 to +80 mV in comparison to KCNJ15+KCNJ16 WT, with 20 mM extracellular K^+. Currents from KCNJ15+mutant KCNJ16 and water-injected oocytes were significantly different (ANOVA) from KCNJ15+KCNJ16 WT at all voltages except -40 mV. (B) The expanded current scale of the same data shows that p.T64I and p.I132R/P250L still exhibit typical inward rectification, and all variants show current reversal at around -40 mV, close to the predicted -41 mV, assuming an intracellular potassium concentration of 100 mM. This suggests at least minimal residual function for all mutations but KCNJ16-p.R137C. (C) The normalized currents, at -120 mV, are consistent with some residual function for KCNJ16-p.T64I and -p.I132R/P250L. (D) In agreement with this observation, membrane potentials showed a trend towards hyperpolarization for these two combinations. All variant combinations were significantly different (ANOVA) from KCNJ15+16WT. Note that due to the fast increase in expression over time, oocytes expressing KCNJ15 alone were analyzed for current in a different batch, and therefore, normalized to KCNJ15+KCNJ16 WT of that batch. The unpaired t-test in that batch showed a significant difference. The co-expression of KCNJ16 WT increased KCNJ15 currents by a factor of 5. The membrane voltages in for all oocytes injected with KCNJ15 alone and part of the KCNJ15/16WT and water-injected oocytes were also from a different batch and gave significant differences within their batches (* $p<0.01$). (E) Normalized surface expression of HA-tagged KCNJ15 alone or together with KCNJ16 (WT and mutants) showed a significant

(ANOVA) decrease in the surface expression of KCNJ15(HA) upon co-expression with KCNJ16 mutants over their WT counterpart when co-expressed with KCNJ10. The error bars represent SEM for at least 8 oocytes from at least two batches (* $p<0.0001$ vs KCNJ15+16). (F) Corresponding Western blots of HA-tagged protein in total oocyte lysates. Water-injected oocytes were taken as control to determine antibody specificity.