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A Novel *AQP2* Sequence Variant Causing Aquaporin-2 Retention in the Cytoplasm and Autosomal Dominant Nephrogenic Diabetes Insipidus

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INTRODUCTION

Congenital nephrogenic diabetes insipidus (NDI) is a rare hereditary disease characterized by excessive diuresis and compensatory polydipsia. No causal treatment of hereditary NDI exists, leaving the patient with severe symptoms, impaired quality of life, and at risk of mental impairment due to recurrent dehydration episodes in childhood. In ~90% of cases, NDI is caused by sequence variants in the *AVPR2* gene. This gene is located on the X-chromosome and encodes the vasopressin V2 receptor[1,2] (V2R). In ~10% of the cases, NDI is caused by sequence variants in the gene for aquaporin-2 (*AQP2*), on chromosome 12.[3,4] Variants in these genes can lead to impaired arginine vasopressin (AVP)-mediated water reabsorption in the renal collecting duct.[1] Water homeostasis critically depends on the proper responsiveness of the V2R and regulated trafficking of aquaporin-2 water channels (AQP2)[5] (Supplementary Figure S1A). Autosomal dominant inheritance with a mutation in 1 allele of the *AQP2* gene is extremely rare.[6] To our knowledge, only 11 disease-causing sequence variants leading to autosomal dominant NDI have been described. The phenotype of autosomal dominant NDI caused by heterozygous *AQP2* sequence variants is generally less severe than the X-linked form caused by *AVPR2* sequence variants. The common denominator for dominant mutations is their localization in the gene segment encoding the carboxyl (C) terminus of AQP2. The mutant and wild-type AQP2 monomers form hetero-tetramers, which are retained in different cellular compartments (Supplementary Figure S1B). The analysis of *AQP2* mutants provides a unique opportunity to gain mechanistic insight into AVP-mediated water reabsorption, which is a prerequisite for devising new strategies for the treatment of diabetes insipidus and to alleviate symptoms. In the present study, we undertake the following: (i) describe a family with a novel heterozygous sequence variant in the *AQP2* gene, (ii) characterize the clinical phenotype, (iii) investigate the underlying mechanism (Supplementary Figure S1C), and (iv) explore the clinical outcome of treatment with supraphysiological doses of vasopressin.

RESULTS

Case Presentation: NDI

The patient is a male who was born as the sixth of the 7 siblings. His father, grandmother, and 3 of his siblings reported symptoms of polydipsia and polyuria. None of them were aware of any disease in the family. His mother experienced no symptoms. X-linked inheritance can be excluded based on the pedigree with likely father-to-son transmission of the disease, and similar polyuric-polydipsic symptoms in both sexes in 2

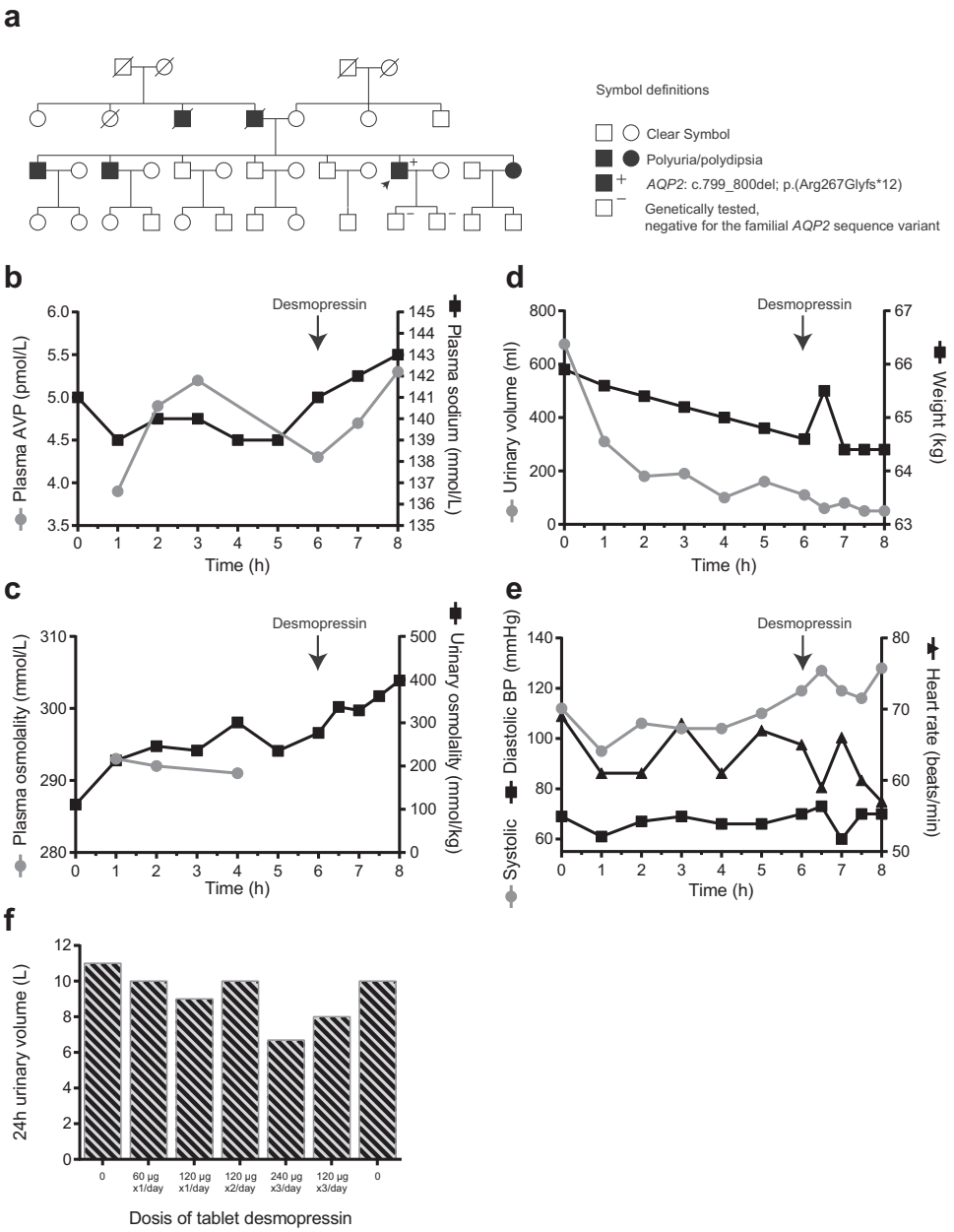

Figure 1. (a) Pedigree of the family showing autosomal dominant inherited congenital nephrogenic diabetes insipidus. (b–e) Diagrams show the effects of water deprivation and desmopressin (dDAVP) administration in the patient expressing the sequence variant AQP2-R267G. The patient was water-deprived for 8 hours and the indicated parameters were determined before and after initiation of dDAVP treatment. dDAVP was administered from sixth hour. (f) During prolonged treatment (18 months) with incremental doses of dDAVP, 24-hour urinary output was reduced. Termination of dDAVP resulted in a gradual increase in 24-hour urinary volume.

generations indicate autosomal dominant inheritance (Figure 1a). As a child, the patient suffered from enuresis until the age of 10 years and reported a continuous need to drink and urinate several times at night since then. At age of 23 years, the patient was admitted to hospital with similar symptoms and a water deprivation test was carried out. The conclusion was psychogenic polydipsia, and the patient was recommended to reduce water intake. At age of 28 years, the patient was hospitalized for 1 week for reevaluation of the continuous symptoms. Gradually, his water intake was reduced under constant monitoring of serum electrolytes and physical well-being. At termination, the patient was discharged from the hospital with water intake at 2.5 L/d and normal values of electrolytes, creatinine, and body weight. The only symptom the patient experienced was headache. The diagnosis of psychogenic polydipsia was maintained. The patient experienced continuous symptoms and could not diminish water intake without side effects. The patient, 36-year-old when examined in the present case story, was admitted to the outpatient clinic of the Department

of Nephrology with symptoms of polydipsia and polyuria and a suspected diagnosis of NDI. He was a nonsmoker and did not take any regular medication. At presentation, the patient was physically normal, body weight was 65.9 kg (BMI 22.8). Ultrasonographic examination revealed that both kidneys were normal. The patient had 24-hour urinary volumes of 10 L/d to 13 L/d and an equivalent large water intake. Baseline plasma parameters (Supplementary Table S1) were all within normal ranges including plasma-sodium at 141 mmol/l (Figure 1b). Baseline urinary osmolality was 111 mmol/kg (Figure 1c) with corresponding urinary sodium <20 mmol/l (not depicted), indicating a urinary concentration defect. The proband completed the water deprivation test without reaching stop-criteria. Upon water deprivation, plasma-sodium remained within reference value, although a gradual increase from 139 mmol/l at 1 hour after water deprivation to 143 mmol/l at the end of the test was observed (Figure 1b). Despite a high and increasing plasma-AVP concentration in response to water deprivation (3.9–5.3 pmol/l) (Figure 1b) and a concomitant increase in plasma-sodium, a continued urinary output between 100 ml/h to 360 ml/h was registered (Figure 1d). Urinary osmolality increased from 111 mmol/kg to 398 mmol/kg (Figure 1c). The weight loss matched the diuresis (65.9 kg to 64.4 kg at termination, Figure 1d). Blood pressure increased slightly from 95/61 mmHg 1 hour after water deprivation to 128/70mmHg at the end with a stable heart rate (Figure 1e). Prolonged treatment with desmopressin (dDAVP) was tested by incremental doses and 24-hour urine sampling (Figure 1f). There was a gradual response where 24-hour urinary output was reduced from 11 L/d to 6.8 L/d on the maximal tested dosage of dDAVP at 240 μg x 3 per day. The proband complained about headache, a known side effect of dDAVP, and he terminated the medication which resulted in a parallel and gradual increase in 24-hour urinary volume (Figure 1f).

Analysis of the *AQP2* Gene

Sequencing of the *AQP2* gene (NM_000486) was performed on genomic DNA extracted from peripheral lymphocytes from the proband. A novel heterozygous *AQP2* frameshift, c.799_800del, was detected, leading to substitution of Arginine 267 for Glycine in the C terminus of the AQP2 protein and a new amino acid sequence beyond Gly267. The frameshift extends the C terminus by 13 amino acids: p.(Arg267Glyfs*12). Analysis of the *AQP2* gene sequence of both sons revealed no mutation, consistent with the observation that none of them had baseline polyuria.

Functional Analysis of AQP2-R267G on AQP2 Glycosylation and Cellular Trafficking

To determine the effects of the sequence variant on the *AQP2* protein, Western blotting was carried out to analyze the expression level and glycosylation. For this, wild-type or mutant AQP2 was transiently expressed in Madin-Darby canine kidney cells and human embryonic kidney 293 cells (Figure 2a and 2b; Supplementary Figures S3 and S4). Wild-type AQP2 is expressed as nonglycosylated, complex glycosylated and high mannose forms. Mutant AQP2 showed a significant increase in the complex-glycosylation form compared to wild-type AQP2 in human embryonic kidney 293 cells. AQP2 was detected with both an antibody directed against the C terminus (E-2) or the N terminus (N-20) of AQP2. The mutation did not affect the recognition of the C-terminal epitope by the antibody. Glycosylated AQP2 was not detectable in our Madin-Darby canine kidney cells (not shown) although a weak glycosylation in Madin-Darby canine kidney cells has previously been reported.[7] Nevertheless, in SDS-PAGE the elongated mutant form migrated at a higher molecular weight than the wild-type, as was the case in human embryonic kidney 293 cell lysates.

Immunofluorescence microscopy was used to analyze the effect of the R267G substitution on the localization of AQP2 (Figure 2c). For semiquantitative analysis, the AQP2 fluorescence signals at the plasma membrane and in the perinuclear region of the cells were determined and the ratios of plasma membrane to perinuclear AQP2 fluorescence intensities were calculated. Ratios greater than 1 indicated a predominant localization at the plasma membrane, ratios less than 1 indicated a predominant intracellular localization. Under resting conditions, both wild-type and the mutant AQP2 were detected in intracellular domains in Madin-Darby canine kidney cells. The stimulation of cAMP synthesis with the activator of adenylyl cyclases, forskolin, induced a redistribution of wild-type AQP2 from intracellular domains to the plasma membrane.[8,9] A fraction of the mutant AQP2, as the wild-type, was found at the plasma membrane in untreated cells. Forskolin did not affect the localization of the mutant AQP2. Further, colocalization studies revealed an accumulation of the mutant AQP2 in the Endoplasmic Eeticulam - Golgi intermediate compartment (ERGIC), (Figure 2c and 2d).

DISCUSSION

In the present study: (i) present a family with a novel heterozygous frameshift mutation in the *AQP2* gene that extended the C terminus by 13 amino acids, (ii)

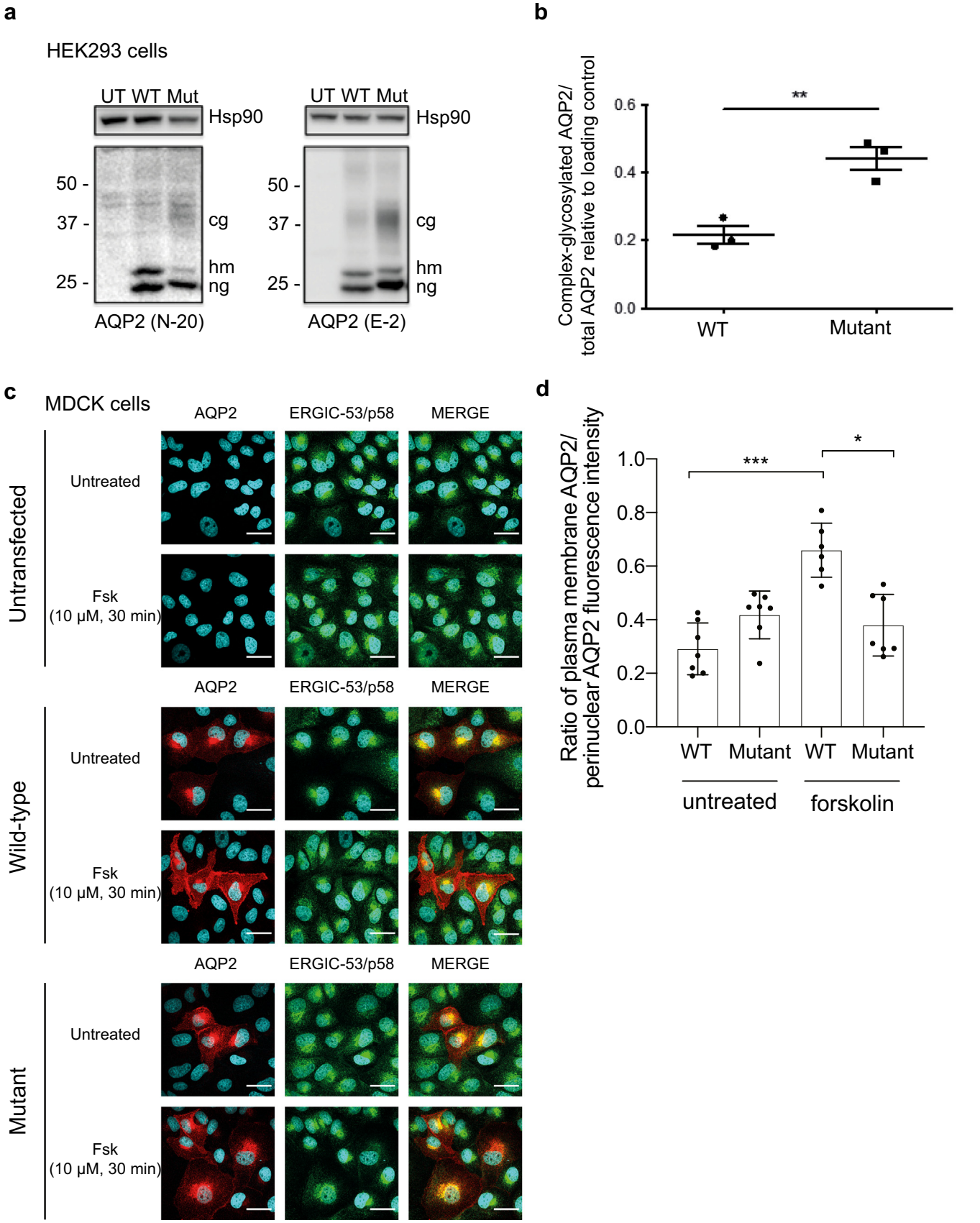

Figure 2. (a) The complex-glycosylation of mutant AQP2-R267G2 compared to wild-type AQP2 is increased in HEK293 cells. Cells were transfected to transiently express wild-type AQP2 (WT) or mutant AQP2 (Mut) or were left untransfected (UT). Cell lysates were prepared and complex-glycosylated, high mannose (hm), and nonglycosylated (ng) forms of AQP2 and Hsp90 (loading control) were detected by Western blotting using specific antibodies directed against the N terminus (N-20) or the C terminus (E-2) of AQP2. Antibody E-2 can still (continued)

characterize the clinical phenotype as autosomal dominant NDI with polydipsia-polyuria and impaired urinary concentration ability in response to water deprivation and dDAVP treatment, (iii) show an improvement of the urinary concentrating ability in response to incremental doses of dDAVP, and (iv) reveal abnormal AQP2 glycosylation and cellular retention of mutant AQP2 between Endoplasmic Reticulum and Golgi in the ERGIC (Figure 2c).

Patients with autosomal dominant NDI exhibit polyuric-polydipsic symptoms and lack of compensation by the wild-type AQP2 allele in the heterozygous state. Compared to NDI caused by recessive or compound heterozygous mutations, the symptoms are usually milder. This corresponds with the ability of the proband to partially increase the urinary concentration ability both in response to water deprivation (111 mmol/kg to 277 mmol/kg) and dDAVP (337 mmol/kg to 398 mmol/kg). Nevertheless, normal levels of urinary-osmolality were not reached. In patients with V2R mutations or autosomal recessive AQP2 mutations, urinary-osmolality remains below 150 mmol/kg and in patients with central diabetes insipidus, urinary-osmolality increases to more than 600 mmol/kg during dDAVP tests.[S1] This and the corresponding weight loss of 1.5 kg during the test illustrated an impaired urine concentration mechanism in line with the few known cases of autosomal dominant NDI.[S1-S7]

The late diagnosis of NDI in the patient of this study was likely due to the residual ability to concentrate urine and the lack of appropriate genetic analysis, emphasizing the importance of performing a genetic examination.

In NDI caused by V2R mutations or recessive AQP2 mutations, patients do not respond to dDAVP administration. In autosomal dominant NDI, a low level of AQP2 functionality remains likely due to the presence of a wild-type AQP2 allele. Transcription of the *AQP2* gene is dependent on and directed by cAMP that is elevated in response to V2R stimulation, either with endogenous AVP or dDAVP. Therefore, treatment of the patient bearing the R267G mutation with dDAVP most likely stimulated synthesis of 50% of the cellular AQP2, which accounts for the responsiveness to the supraphysiological doses of dDAVP and the ability to form functional homotetramers that reach the plasma membrane. Pharmacological treatment with incremental doses of dDAVP was terminated by the patient due to side effects. To our knowledge, prolonged treatment with dDAVP was reported in a previous heterozygous case.[S7] In that case, the dosages were 40 μg×3 per day which improved symptoms for 4 to 5 hours with subsequent improvement of sleep and well-being. In the present case, quantitative consecutive measurement of reduced 24-hour urine volume implies a partial response and indicates a therapeutic opportunity.

The *AQP2* sequence variant in the proband caused a frameshift with elongation of the C-terminal tail. This resulted in the retention of mutant AQP2 in the compartment between Endoplasmic Reticulum and Golgi, the ERGIC. This finding is reminiscent of a study by Kuwahara *et al.*[S8] (overview in Supplementary Figure S2) where the lack of residues 262 to 271 caused retention in the Endoplasmatic Reticulum. Moreover, all hitherto discovered sequence variants in *AQP2* that cause autosomal dominant NDI affect the C terminus of AQP2 (Supplementary Figure S2). Although such mutants often are functional water channels, they are misrouted to subcellular compartments other than the plasma membranes (Supplementary Figure S1B). *In vitro* investigations have revealed 3 different mechanisms as follows: (i) heterotetramerization with wild-type AQP2 and miss-sorting to the Golgi region which prevents functional tetramers to reach the plasma membrane,[S1] (ii) frameshift mutations with extended C termini and functional loss of the tail of AQP2 where PDZ proteins and ubiquitin interact, likely introducing aberrant sorting signals to the basolateral plasma membrane[S2-S9]or to late endosomes or lysosomes,[S3,S5,S10] and (iii) mutations in S254 that prevent or attenuate phosphorylation of the residue which is normally phosphorylated by Protein kinase A upon stimulation of the V2R.[S4,S6,S7,S11]

Figure 2. (continued) detect the mutant form with C-terminal elongation, and it was used for further experiments and for quantifying the glycosylation. (b) Signals emerging from the cg form of AQP2 in HEK293 cell lysates were semiquantitatively analyzed by densitometry and normalized to total AQP2 and the loading control. Statistically significant differences are indicated (mean ± SEM; $^{**}P < 0.01$). (c) The mutant AQP2-R267G is retained in the ERGIC, the ER-Golgi-intermediate compartment in MDCK cells. MDCK cells were transfected to transiently express wild-type AQP2 or mutant AQP2 or were left untransfected. Cells were left untreated or treated with forskolin (+Fsk). AQP2 (red) and ERGIC-53/p58 (green) were detected by immunofluorescence microscopy using specific primary and fluorophore-coupled secondary antibodies. Nuclei were stained with DAPI (blue). Shown are representative images from 3 independent experiments. Scale bar 30 μm. (d) Plasma membrane and perinuclear immunofluorescence AQP2 signal intensities were determined and the ratios of plasma membrane to perinuclear fluorescence signal intensities were calculated. Ratios > 1 indicate a predominant localization at the plasma membrane. Statistical analysis was carried out using one-way ANOVA and Kruskal-Wallis test multiple-comparison shown are means ± SD of 3 independent experiments with a total of 7 cells per condition. Statistically significant differences are indicated, $^{*}P < 0.1$, $^{***}P < 0.001$. cg, complex-glycosylated; HEK, human embryonic kidney 293 cells; hm, high mannose; MDCK, Madin-Darby canine kidney cells; mut, mutant; ng, nonglycosylated; UT, untransfected; WT, wild type.

In conclusion, we identified a novel autosomal dominant NDI-causing sequence variant in the *AQP2* gene and, with dDAVP, provide a therapeutic option to ameliorate symptoms. The late diagnosis of NDI in the proband emphasize the importance of genetic diagnostics in patients presenting with even mild nephrogenic water balance disorders without somatic explanations. The limited urinary concentration ability can be explained by the retention of R267G mutant AQP2 in the intermediate compartment between ER and Golgi (ERGIC).

DISCLOSURE

All the authors declared no competing interests.

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AUTHOR CONTRIBUTIONS

GH and EBD supervised dDAVP test, JMH performed genetic analysis. SB and TP conducted cloning and cell-based experiments. CB was consulting nephrologist. GH, PS, EK and BLJ drafted manuscript. All authors approved the final manuscript.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Figure S1. Scheme illustrating arginine vasopressin (AVP)-mediated water reabsorption in renal collecting duct principal cells

Figure S2. A schematic presentation of the AQP2 protein

Figure S3. Vector Map of AQP2 wild-type

Figure S4. Vector Map of AQP2 mutant

Table S1. Baseline parameters

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Supplemental material can be found on the Kidney International Reports website.

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