Single Amino Acid Substitution in Aquaporin 11 Causes Renal Failure

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ABSTRACT

A screen of recessive mutations generated by the chemical mutagen n-ethyl-n-nitrosourea (ENU) mapped a new mutant locus (5772SB) termed sudden juvenile death syndrome (sjds) to chromosome 7 in mice. These mutant mice, which exhibit severe proximal tubule injury and formation of giant vacuoles in the renal cortex, die from renal failure, a phenotype that resembles aquaporin 11 (Aqp11) knockout mice. In this report, the ENU-induced single-nucleotide variant (sjds mutation) is identified. To determine whether this variant, which causes an amino acid substitution (Cys227Ser) in the predicted E-loop region of aquaporin 11, is responsible for the sjds lethal renal phenotype, Aqp11/sjds compound heterozygous mice were generated from Aqp11/sjds and Aqp11/sjds/sjds intercrosses. The compound heterozygous Aqp11/sjds offspring exhibited a lethal renal phenotype (renal failure by 2 wk), similar to the Aqp11/sjds/sjds and Aqp11/sjds/sjds phenotypes. These results demonstrate that the identified mutation causes renal failure in Aqp11/sjds/sjds mutant mice, providing a model for better understanding of the structure and function of aquaporin 11 in renal physiology.


Phenotype-driven screen of recessive mutations generated by n-ethyl-n-nitrosourea (ENU), a powerful chemical mutagen, mapped a new 5772SB mutant locus termed sudden juvenile death syndrome (sjds) at chromosome 7 in mice. Deletion-mapping studies narrowed the sjds locus to a region in the vicinity of the Myo7a locus. Sjds homozygous mice consistently die before 20 d of age, whereas heterozygous sjds and wild-type siblings are phenotypically normal. The identified interval of mouse chromosome 7, to which sjds locus mapped, also contains the recently described aquaporin 11 (Aqp11) gene. Aquaporins belong to the family of integral membrane protein channels responsible for selective transport of water, glycerol, and other small, nonpolar solutes through cell membranes. The transmembrane segment of these channels is formed by six helices and two re-entrant loops (B and E) each containing a highly conserved Asn-Pro-Ala (NPA) signature motif at the center of aquaporin monomer. AQP11 was referred to a new subcellular aquaporin superfamily with poorly conserved NPA motifs. Human...
AQP11 protein sequence is 92% similar and 83% identical to mouse AQP11 protein sequence. AQP11 was reported to be expressed intracellularly in the proximal tubular cells in kidney, \( Aqp11^{−/−} \) knockout mice exhibited pronounced cyst formation in kidney cortex and severe vacuolization of proximal tubular epithelium and died as a result of advanced renal failure at the age of approximately 3 wk. Whereas the conventional models for transport of water and other solutes did not allow the study of AQP11 function and published results were controversial,\(^8\),\(^9\) recently, Yakata et al.\(^10\) were able to show water channel activity of recombinant mouse AQP11 reconstituted into liposomes.

We report the identification of a single nucleotide T-to-A mutation in \( Aqp11 \) cDNA resulting in substitution of highly conserved downstream Cys\(^{227} \) to Ser\(^{227} \) (BAC45005) in the predicted structurally important E-loop region of AQP11 protein in \( sjds \) mutant mice. The goal of these studies was to test whether the \( sjds \) phenotype arose from a point mutation in \( Aqp11 \) using genetic complementation analysis by breeding together \( Aqp11^{+/−} \) heterozygous mice and heterozygous mice that carried the recessive \( sjds \) mutation. The resulting 26% offspring had a compound heterozygous genotype \( Aqp11^{−/−}/sjds \) and showed characteristic lethal renal phenotype. This study directly showed that the identified T-to-A mutation is an inactivating mutation that causes functional disruption of AQP11 and results in severe proximal tubule injury leading to subsequent renal failure in \( sjds \) mice. We present the \( sjds \) mutant mice as an excellent tool to explore the AQP11 function of the new subfamily of aquaporins, because the identified Cys\(^{227} \) to Ser\(^{227} \) mutation may provide unique insight into structure and function of AQP11 and novel molecular mechanisms of renal failure.

**RESULTS**

**Impaired Renal Function In \( sjds \) Mutant Mice**

Albino homozygous mice carrying \( sjds \) locus (see the Concise Methods section) consistently died before 20 d of age, whereas wild-type (chinchilla coat) and heterozygous (light buff coat) siblings seemed normal. We evaluated renal function in mutant \( sjds \) mice (\( n = 15 \)) and compared with wild-type mice (\( n = 7 \)) at 18 to 20 d of age. Homozygous \( sjds \) mice exhibited increased blood urea nitrogen (BUN; 95.3 ± 47.6 mg/dl) 4.7-fold higher than in wild-type mice (20.3 ± 3.8 mg/dl; \( P < 0.005 \); Figure 1A). Plasma creatinine, determined by HPLC, was also increased to 0.719 ± 0.721 mg/dl in mutant mice (\( n = 15 \)) versus 0.112 ± 0.042 mg/dl (\( n = 9 \)) in control mice (\( P = 0.005 \)). Some of these 15 \( sjds \) mice had extremely high creatinine levels (2.221 ± 0.06 mg/dl; \( n = 4 \)), comparable to anephric mice.\(^11\) BUN and plasma creatinine values directly indicated renal failure as a cause of death in \( sjds \) mice.

**Renal Histopathology in \( sjds \) Mutant Mice**

Pathologic examination of \( sjds \) kidneys revealed that kidneys were pale (Figure 1B), and histomorphology showed characterisitics of tubular and cellular swelling, formation of giant vacuoles (“spider web”) in renal cortex, and disruption of brush border in proximal tubule epithelial cells. This was accompanied by tubular membrane alterations consistent with severe epithelial cell injury in kidneys from \( sjds \) mutant mice (Figure 1, C and D). Electron microscopy revealed drastically altered proximal tubule epithelial cells including destruction of brush border and disorganization of the mitochondrial matrix in \( sjds \) kidney (Figure 1F) compared with wild-type kidney (Figure 1E).

**Detection of Nephron Segmental Identity in \( sjds \) Mutant Mice**

To identify further the injured nephron segments in \( sjds \) mice, we performed immunostaining by proximal tubule marker aquaporin 1 (AQP1) antibodies.\(^12\) Control kidney (Figure 2A, arrow) exhibited intense AQP1 immunoreactivity of proximal tubule brush border. In \( sjds \) mutant mice, damaged tubules showed reduced AQP1 labeling (Figure 2B, *) with the most severely injured tubules showing absence of AQP1 staining (Figure 2B, **). Staining with lotus tetragonolobus lectin, an-
other proximal tubule marker,13 was identical to AQP1 immunostaining (data not shown).

Tubules expressing Tamm-Hoersfall protein (THP), a thick limb–specific marker,14 never exhibited tubular injury (Figure 2, C and D, arrowheads). Injured tubules were not positive for the distal convolute tubule marker thiazide-sensitive co-transporter (TSC)15 (data not shown). These observations identify the proximal tubule as the major site of injury in sjds mice.

Sequence Analysis of Aqp11 cDNA from sjds Mutant Mice

The sjds locus mapped to a 6- to 11-cM subregion of the Fah-Hbb interval of the mouse chromosome 7 in the vicinity of the Myo 7a locus.1 AQP11, a new member of major intrinsic protein family, have been also mapped to the same region of chromosome 7 (Figure 3A). Striking similarities in life span and renal phenotype that we observed in sjds mutants,16 and Aqp11−/− knockout mice9 prompted us to test whether sjds mice carried a mutation in Aqp11 gene. Sequencing of the Aqp11 full-length cDNA from sjds mutants (n = 7) and alignment analysis with the reported mouse Aqp11 cDNA (BAC45005) using Basic Local Alignment Search Tool (BLAST) revealed a T-to-A transversion that occurred at codon 718 in exon 2 of Aqp11 gene and resulted in mutation of Cys227 (TGC) to Ser227 (AGC). This mutation was present in all tested sjds mutant mice (Figure 3B). Using Vector NTI Bioinformatics software (Invitrogen, Carlsbad, CA) and Sosui analysis server (http://www.sosui.proteome.bio.tuat.ac.jp), the analysis of AQP11 hydrophobic transmembrane regions showed that Cys227 was predicted to reside within the AQP11 nonhelical part of the second re-entrant E-loop (Figure 3C). Cys227 is a highly conserved amino acid in most of the living organisms except for plant Small Basic Intrinsic Proteins.7 The sequencing results of the wild-type and mutant Aqp11 cDNA completely co-segregated with the characteristic kidney morphology in all wild-type and sjds mutant mice. No other mutations in sjds Aqp11 cDNA were detected. The mutation created an Alu1 site of the mutant Aqp11 gene allowing genotyping of sjds mice.

Figure 2. Immunostaining of nephron segment markers in serial sections of kidney tissue from wild-type and the sjds mutant mice, using peroxidase-diaminobenzidine procedure. (A and B) The sections were counterstained with periodic acid-Schiff reagent. AQP1 as a marker of proximal tubules in wild-type (A, arrow) and sjds mutant (B, *) kidneys. (C and D) THP as a marker of thick ascending limb (arrow heads) in wild-type (C) and the sjds mutant (D) kidneys.
mutant mice. A 272-bp mutant allele PCR product was digested with Alu1 to 110- and 162-bp fragments, whereas the PCR product of wild-type allele remained unchanged as expected (Figure 4A). sjds genotype entirely co-segregated with renal lethal phenotype and was transmitted with a 25% frequency, consistent with recessive Mendelian segregation, in each generation from nine subsequent crosses (G9). We concluded that identified T-to-A mutation in Aqp11 is associated with sjds lethal phenotype in these mutants that we designated as Aqp11sjds/sjds mice.

Aqp11 Gene Expression in Aqp11sjds/sjds Mice

A representative reverse transcriptase–PCR (RT-PCR) analysis for mutant and wild-type Aqp11 gene expression showed that the level of mutant Aqp11 transcripts in spleen and kidneys of different mutant mice were reduced but detectable compared with the wild-type transcripts and normalized to mouse glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (Figure 4, C and D), which showed signal reduction and correspondence with the injured cortex region in kidney from 7-d-old mutant mouse compared with wild-type control when hybridized with the antisense riboprobe.

Figure 4. (A) Genotyping of the sjds mutation in Aqp11 gene. Digestion with Alu1 decreased the sjds allele to 162- and 110-bp bands in Aqp11+/sjds (+/sjds) heterozygous and Aqp11sjds/sjds (sjds/sjds) homozygous mice. (B) Representative RT-PCR analysis of Aqp11 gene expression (516-bp PCR product) in kidney from wild-type (+/+), and spleen and kidneys from different Aqp11sjds/sjds (sjds/sjds) mice. RT-PCR product of mouse G3PDH (983 bp) was generated from each sample as a loading control. (C and D) Representative photomicrographs of in situ hybridization in postnatal day 7 control (C) and mutant (D) mouse kidneys.

Generation and Genotyping of Aqp11−/sjds Compound Heterozygous Mice

To determine whether the mutation in Aqp11 is an inactivating mutation and is responsible for renal phenotype in Aqp11sjds/sjds mice, we generated Aqp11−/sjds compound heterozygous mice performing genetic complementation analysis. Aqp11−/sjds mice were intercrossed with heterozygous Aqp11+/− mice, yielding seven litters with 57 pups total (Figure 5A). We examined the phenotype and genotype of offspring that received both Aqp11sjds and Aqp11− alleles and of wild-type offspring as a control.

Sjds mutation and Aqp11− knockout allele express the recessive phenotype.9,16 We were expecting that if Aqp11 gene contains sjds mutation, then 25% of offspring would be compound heterozygous mice without any wild-type Aqp11 allele and would show the expression of mutant renal phenotype. If they are at different loci, then compound heterozygous progeny with wild-type alleles at both loci would exhibit wild-type phenotype.

PCR genotyping showed that 26.3% (15 of 57) of offspring of Aqp11+/sjds and Aqp11+/− parents carried both Aqp11null and Aqp11sjds alleles, consistent with recessive Mendelian segregation. Aqp11−/sjds mice, as expected, were heterozygous by both Aqp11sjds and Aqp11− knockout alleles. Aqp11−/− knockout mice were generated with the targeted allele that contained a deletion of Aqp11 gene region including exon 2, whereas an ENU-induced T-to-A mutation took place in Aqp11sjds/sjds

Figure 5. (A) Breeding scheme to generate compound heterozygous Aqp11−/sjds mice containing Aqp11− and Aqp11sjds alleles. (B through D) Representative genotyping of the Aqp11sjds and Aqp11− alleles in the Aqp11 gene in three different compound heterozygotes (Aqp11−/sjds) compared with wild-type mouse (+/+). (B) The sjds mutation creates an Alu1 site in the exon 2 of mutant Aqp11. Alu1 digestion of the Aqp11 272-bp PCR product generates 110- and 162-bp fragments in all Aqp11−/sjds mice. Aqp11− knockout allele is not recognized by sjds genotyping because targeted allele has a deletion of Aqp11 exon 2. (C) In Aqp11 knockout genotyping, the Aqp11− allele was recognized by wild-type 402-bp PCR product. (D) The Aqp11− knockout allele was identified by amplification of a 262-bp PCR product.
mice. Thus, for sjds genotyping, we used primers designed for exon 2, which would not recognize Aqp11 mutant allele and detect Aqp11 compound heterozygotes containing one null and one mutated Aqp11 alleles were identified by Aqp11 homozgyosity and detected by AluI digest products of expected size, 110 and 162 bp (Figure 5B).

Primers designed for Aqp11 knockout genotyping recognize Aqp11 mutant allele as wild-type allele and detect heterozygous Aqp11 knockout allele in Aqp11 compound mice. Aqp11 heterozygosity was detected with 402-bp PCR product identifying wild-type Aqp11 allele (Figure 5C) and 262-bp PCR product identifying knockout allele in each of Aqp11 compound heterozygous mice (Figure 5D).

Lethal Renal Phenotype in Aqp11 Compound Heterozygotes

Life Span.
Mice died between the ages of 10 to 20 d in the majority of Aqp11 compound heterozygotes (n = 15), which was comparable with life span of both Aqp11 compound mice (n = 15) and Aqp11 mice (n = 15; between 14 and 23 d). Only five Aqp11 compound mice survived up to 20 d of age. Thus, a different mouse line with genetically modified Aqp11 gene showed similarity in the life span regardless of the mouse genetic background of Aqp11 compound (complex BALBc/C57BL6, and noninbred background mix) and Aqp11 (CD1/C57BL6) parents.

Renal Histology.
As indicated in Figure 6, there is a striking resemblance in the morphology and histology of kidneys from Aqp11 compound, Aqp11, and Aqp11 mice at the age of 20 d (Figure 6, A through C). Macroscopic observations revealed rough pale kidneys in all genetically modified Aqp11 allele carriers compared with wild-type littermates. Histologic examination of Aqp11 kidneys (Figure 6F) showed enlarged swollen tubules with transitional overblown epithelia cells with damaged brush border and characteristic “spider web” formation in the cortex, similar to Aqp11 compound and Aqp11 kidneys (Figure 6, D and E). Renal phenotype in Aqp11 compound heterozygous mice was evident and did not seem to be influenced by different backgrounds in parental lines.

Renal Function.
Aqp11 compound mice exhibited severe renal dysfunction with a significant four-fold increase of BUN level of 98.1 ± 34.4 mg/dl (n = 5) at 20 d of age compared with control wild-type littermates (23.4 ± 3.1 mg/dl; n = 7; P < 0.005; Figure 6G). Importantly, BUN levels in compound heterozygous Aqp11 compound mice were comparable with BUN levels in Aqp11 compound mutants (95.3 ± 47.6 [n = 15] versus control 20.3 ± 3.8 mg/dl [n = 7]; P < 0.005) and in Aqp11 knockout mice (93.6 ± 37.1 [n = 4] versus control 21.2 ± 5.1 mg/dl [n = 5]; P < 0.005; Figure 6G).

Identification of Injured Proximal Tubules in Aqp11 Compound Heterozygotes
Swollen and injured tubules in Aqp11 compound mice were identified as proximal tubules on the basis of immunostaining of serial kidney tissue sections with nephron tubule markers. In Aqp11 compound mice, positive immunolabeling with AQPI, a prox-
imal tubule marker, was observed in injured tubules in renal cortex (Figure 6H). Positive immunostaining for lotus tetragonolobus lectin, another proximal tubule marker, confirmed these observations (data not shown). The same tubules were negative for THP immunostaining in tissue serial sections of the same kidney (Figure 6I) as well as for TSC, the distal convoluted tubule marker (data not shown).

**DISCUSSION**

ENU transfers its ethyl group to oxygen or nitrogen in DNA, resulting in base-pair substitution predominantly of A/T to T/A transversion and A/T to G/C transition.\(^17,18\) We report the identification of a new, ENU-induced single-nucleotide T-to-A recessive mutation resulting in Cys\(^{227} \) to Ser\(^{227} \) amino acid substitution in AQP11, a new major intrinsic protein family member, as a cause of severe proximal tubule injury followed by renal failure in mice. In this study, we demonstrated that by the age of 20 d, sjds mutant mice exhibit severely injured tubules in deep cortex identified as a proximal tubules, with formation of giant vacuoles and disrupted brush borders in tubular epithelium accompanied by striking increase of BUN and plasma creatinine as a clear indicator of renal failure.

Aquaporins play critical roles in the ability of kidney to form both concentrated and dilute urine.\(^15,19,20\) It has been shown that significant electrolyte abnormalities are associated with altered expression of AQP2 and that aquaporins may mediate intracellular movement of water and other small, nonpolar solutes.\(^21–23\)

The sjds locus had been mapped to chromosome 7 at the time when Aqp11 was unknown and precise identification of sjds mutation was not feasible.\(^1\) Ishibashi and co-workers\(^7,24\) identified new members of AQP family, AQPX1 and AQPX2 (subsequently designated AQP11 and AQP12, respectively), which were referred to as a new aquaporin superfamily with poorly conserved NPA motifs that distinguished them from other AQP. Both AQP11 and AQP12 showed a low homology with other aquaporins but demonstrated 32% identity with each other.\(^8\) Although AQP11 is widely expressed in multiple organs, its critical role in kidney function was shown in Aqp11 null mice.\(^8,9\) In these mice, Morishita et al.\(^9\) demonstrated proximal tubule origination of cyst formation in renal cortex and an endosomal acidification defect in cultured proximal tubule cells. In addition, the authors demonstrated an intracellular endoplasmic reticulum localization of AQP11 in proximal tubule epithelium. Gorelick et al.\(^8\) showed confirmed AQP11 expression in Xenopus oocytes injected with rat Aqp11 and myc-tagged human Aqp11 but detected no transport of water, glycerol, urea, or ions. In contrast, the most recent stop-flow studies\(^10\) showed positive water conduction by recombinant AQP11 protein expressed in Sf9 insect cells and reconstituted into liposomes.

Although aquaporins play important roles in cell transporting systems, of all aquaporins, disruptions of only Aqp2 and Aqp11 genes were shown to be lethal.\(^9,25\) Previously, we reported a perinatal lethal phenotype in sjds mutant mice generated during ENU-induced mutagenesis.\(^16\) For the first time, we report the identification of this recessive point mutation resulting in Cys\(^{227} \) to Ser\(^{227} \) substitution in AQP11. We demonstrated that the proximal tubule injury characterizing Aqp11\(^{sjds/sjds} \) mutant line was of comparable severity to Aqp11 null strain (Table 1).\(^9\) Some variability in the life span and the kidney size in mice with a genetically modified Aqp11 gene might be a result of different background of Aqp11\(^{sjds/sjds} \) and Aqp11\(^{−/−} \) mice or of a different effect of mutant AQP11 protein in Aqp11\(^{sjds/sjds} \) mice versus its complete absence in Aqp11\(^{−/−} \) knockout mice. Before approximately day P5, the kidneys appear normal in Aqp11\(^{sjds/sjds} \) and Aqp11\(^{−/−} \) knockout mice. The first lesions appear in proximal tubules between 1 and 2 wk of age in both mouse lines. A previous study\(^8\) demonstrated no changes in mitochondria in the kidney section from a 7-d-old Aqp11\(^{−/−} \) knockout mouse. Controversially, we have observed the formation of giant vacuoles along with

**Table 1.** Phenotypic similarity between 20-d-old Aqp11\(^{sjds/sjds} \) and Aqp11\(^{−/−} \) knockout mice^a^

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Aqp11(^{sjds/sjds} ) (n = 15)</th>
<th>Aqp11(^{−/−} ) (n = 4)</th>
<th>Aqp11(^{+/+} ) (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Life span</td>
<td>14 to 23 d</td>
<td>15 to 60 d(^b)</td>
<td>&gt;1 yr</td>
</tr>
<tr>
<td>Body weight at weaning date (g)</td>
<td>7.3 ± 1.5(^a)</td>
<td>6.7 ± 2.5(^c)</td>
<td>10.7 ± 0.6</td>
</tr>
<tr>
<td>Kidney appearance</td>
<td>Pale</td>
<td>Dark</td>
<td></td>
</tr>
<tr>
<td>Origin of vacuoles and dilation</td>
<td>Proximal tubules</td>
<td>Proximal tubules(^a)</td>
<td>−</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>8.7 ± 0.7(^b)</td>
<td>8.7 ± 0.9(^b)</td>
<td>10.5 ± 0.5</td>
</tr>
<tr>
<td>Cause of death</td>
<td>Kidney failure</td>
<td>−</td>
<td>Normal renal morphology</td>
</tr>
<tr>
<td>Renal histomorphology</td>
<td>PT dilation, giant vacuoles in PT epithelia, lumen obliteration by tubular epithelia with occupation of whole cytoplasm by vacuoles (&quot;spider webs&quot;)</td>
<td>Normal renal morphology</td>
<td></td>
</tr>
<tr>
<td>Ultrastructural analysis of PT epithelial cells</td>
<td>Cytoplasm vacuolization, nuclear swelling, visible mitochondrial injury</td>
<td>Normal epithelial ultra structure</td>
<td></td>
</tr>
<tr>
<td>Urine osmolality (mOsm)</td>
<td>200 to 600</td>
<td>200 to 700</td>
<td>995 to 3130</td>
</tr>
</tbody>
</table>

^a^Data are as means ± SD. Hb, hemoglobin; PT, proximal tubule.

^b^P < 0.005, ^c^P < 0.05 versus control group.
severe mitochondrial injury in proximal tubular cells in both Aqp11sjds/sjds and Aqp11 null kidneys from mice at ages older than 15 d. These findings suggest that cellular swelling precedes the injury of the mitochondria and is a primary event in proximal tubular cell damage followed by induction of mitochondrial injury in both Aqp11sjds/sjds and Aqp11−/− knockout lines. Phenotypic similarity in these mice led us to hypothesize that a newly identified sjds mutation is responsible for lethal phenotype in Aqp11sjds/sjds mutant mice.

We used a genetic complementation analysis to show that the identified Cys227 to Ser227 mutation is an inactivating mutation and a sufficient cause of severe proximal tubule injury and renal failure. We generated Aqp11−/sjds compound heterozygotes from Aqp11sjds+/− and Aqp11+/− intercrosses, in the frequency consistent with Mendelian recessive segregation. Renal phenotype evaluation in Aqp11−/sjds mice showed an impressive resemblance to Aqp11sjds/sjds and Aqp11−/− phenotypes. Aqp11−/sjds mice exhibited proximal tubule injury, vacuole origin, cyst formation, and renal failure at perinatal age similar to Aqp11 null and Aqp11sjds/sjds mice. These studies clearly demonstrated that identified single amino acid substitution of Cys227 to Ser227 in AQP11 impaired its functional activity and resulted in kidney failure in affected mice, confirming a vital role of AQP11 in renal physiology. Our studies prominently demonstrated that Aqp11 gene disruption results in kidney injury and lethal phenotype in mice regardless of the mouse genetic background.

Our observations are in accordance with previous studies of the aquaporin family demonstrating that the mutation of only a single functionally important residue might disrupt protein function. For example, the substitution of Arg187 for Cys187 in AQP2 leads to a severe form of nephrogenic diabetes insipidus, whereas inherited cataracts is the result of Glu142 substitution for Gly142 in AQP0, a major intrinsic protein not expressed in the kidney. Importantly, Cys227 is a highly conserved amino acid residue residing within predicted AQP11 nonhelical segment of the second re-entrant E-loop (Figure 3C). In the high-resolution atomic model of AQP11, E-loop dips into transmembrane pore in AQP11 monomer. Near the center of the molecule, the overlap of E-loop with B-loop, which folds back into transmembrane pore from the opposite site, forms the aqueous pathway in each AQP11 monomer. Thus, it is conceivable that Cys227 is positioned in the pore of the AQP11 monomer, being essential for the functioning of AQP11.

AQP11 NPA motif, along with the surrounding amino acid residues including Cys227, might determine the pore selectivity of transport channel. The effect of experimental amino acid substitution in E-loop region on oligomerization of major intrinsic protein was analyzed previously. The transport specificity of aquaglyceroporins was linked to their oligomeric state and showed the involvement of structurally and functionally important E-loop region of aquaporin molecule. These studies showed that glycerol permeation was associated with monomers, whereas water permeation was associated with tetramers. A substitution of two amino acids in loop E in AQPcic (widely known stable tetramer) by corresponding amino acids of GlPF induced a switch from water to glycerol channel and from tetrameric to monomeric state of protein. Our preliminary studies suggest that deoligomerization might take place in Aqp11 mutant. Thus, it is not excluded that expressed mutant AQP11 in Aqp11sjds/sjds mice have impaired substrate specificity as a result of substitution Cys227 to Ser227 resulting in the accumulation of atypical transported substrate in concentration that could be toxic for cell and formation of vacuoles in proximal tubule epithelium. This cytotoxicity might be reflected by mitochondrial injury detected in sjds proximal epithelium. Alternatively, the mutation of Cys227 to Ser227 might lead to the misfolding of AQP11 and loss of function. The studies to discriminate these possibilities are in progress.

In summary, AQP11 is a new and unique member of aquaporin family whose function is critically vital for mammalian renal physiology and survival. We report for the first time the identification of the new recessive mutation of highly conserved Cys227, a critical feature of AQP11 oligomer, leading to severe proximal tubule injury and complete abruption of renal function in mice. Integrated assessment of AQP11 function in Aqp11sjds/sjds mutant mice might result in a breakthrough for solving of the AQP11 role in kidney physiology.

**CONCISE METHODS**

**Animals**

All experimental procedures were in compliance with the Vanderbilt University Guide for Care and Use of Laboratory Animals. Mice were housed in a pathogen-free veterinary facility accredited by the American Association for the Accreditation of Laboratory Animal Care.

The ENU-induced mutant sjds mice were previously generated at Oak Ridge National Laboratory. Briefly, +/TyrG1 female offspring of ENU-mutagenized BALB/cRl (Tyr/TyrG1) G0 males were mated to noninbred males [Tyr−/−Del(Tyr)26DV1] heterozygous for the chin-chilla allele of Tyr opposite a 6- to 11-cM chromosome 7 Tyr deletion. Albino offspring [Tyr−/−Del(Tyr)26DV1] from G1 male #5772SB uniformly died between 13 and 20 d after birth, suggesting that a new recessive, ENU-induced mutation “m” (here termed sjds), closely linked to Tyr and responsible for this lethal phenotype, was present in the 5772SB line. Subsequently, the sjds mutation was propagated by intercrossing heterozygous siblings (Tyr−/− +/Tyr sjds) siblings.

Mice carrying the Aqp11tm1Lex knockout allele (abbreviated Aqp11−) were originally obtained from K. Ishibashi (Chiba East Na-
tional Hospital, Chiba, Japan) and bred on CD1 background at University of California, San Francisco, in Dr. Verkman’s laboratory. These mice were transferred to Vanderbilt University Medical Center after rederivation on the C57BL6 background in specific pathogen-free conditions at Charles River Laboratories (Wilmington, MA).

**Direct Sequencing of Aqp11 Gene**
Genomic DNA was isolated using DNA Purification System (Gentra Systems, Minneapolis, MN). Tails were dissected and stored at −80°C or directly lysed in lysis solution containing 20 mg/ml of proteinase K at 55°C overnight. Genomic DNA extraction was followed by ribonuclease A treatment and ethanol precipitation according to the manufacturer’s protocol.

PCR amplification was carried out by thermal cycling using PuReTaq ReadyToGo PCR beads (Amersham Bioscience, Amersham, UK) according to the manufacturer’s protocol in 25 μl of reaction mix containing 25 to 50 ng of genomic DNA and 10 pmol of each oligonucleotide set. The PCR conditions were denaturation at 95°C for 3 min, 95°C for 30 s, 58 to 60°C for 30 s, and 72°C for 30 s for 35 cycles with a final extension for 10 min at 72°C.

Oligonucleotides for PCR amplification and direct sequencing of PCR products were designed according to the sequence of murine Aqp11 gene (BAC45005). Amplification of DNA fragment containing the 879-bp exon 1 was performed in two different PCR reactions using two sets of oligonucleotides P1-1 (5′-GGGCTCTACAGCGTGT-3′ and 5′-ATCATCGGCAGGGGTGTGTA-3′) and P1-2 (5′-AGCTCACCCCTGGCTGTGCT-3′ and 5′-AGAACCTGTGTCTAGGGTCTC-3′), designed to amplify two overlapping DNA fragments of 580 and 520 bp in size, respectively. DNA fragments corresponding to exons 2 and 3 were amplified with P2 (5′-AGTCTTTGCCTTTTGTGTAACGG-3′) and 5′-GGGACTAGGCACACATAGGTCT-3′) and P3 (5′-CGAACAGCGATGGTCCTAGA-3′ and 5′-CATACAGTTTACTGACTTGGG-3′) primer sets.

PCR products containing full sequences of all three exons and adjoining intronic and splice sites regions were subjected to direct sequencing with corresponding primers and BigDye Terminator system 3.1 (Applied Biosystems, Foster City, CA).

**Aqp11 Protein Topology**
Transmembrane region analysis of AQP11 protein was performed using Vector NTI Bioinformatics software (Invitrogen) and Sosui analysis system (http://www.sosui.proteme.bio.tuat.ac.jp).

**Genotyping of Aqp11*<sup>jds</sup>* and Aqp11 Knockout Alleles**
To generate Aqp11<sup>−/−</sup> compound heterozygous mice, heterozygous Aqp11<sup>+/−</sup> mice were bred with heterozygous Aqp11<sup>+/−</sup> mice. Genomic DNA was isolated using DNA Purification System (Gentra Systems) as was described already.

**Aqp11<sup>jds</sup> Allele Genotyping.**
PCR amplification was carried out in 25 μl with 25 to 50 ng of genomic DNA and 10 pmol of each of Aqp11 exon 2 primers (5′-GACAGCTGGTTTCTCTTGGTAACGG-3′ and 5′-GGGACTAGGCACACATAGGTCT-3′). The PCR conditions were, after denaturation at 95°C for 3 min, 95°C for 30 s, 58 to 60°C for 30 s, and 72°C for 30 s for 35 cycles with a final extension for 10 min at 72°C. The PCR product (272 bp) was then digested by AluI and electrophoretically separated on a 2.0% agarose gel. The expected size of the digest was 162 and 110 (sjds mutant allele) and 272 bp (wild-type allele).

**Aqp11 Knockout Allele Genotyping.**
Aqp11 knockout allele had a deletion in exon 2 and was identified as described previously.9 Briefly, primers 5′-GTCCTGCC-3′ and 5′-GCCCTCATTTTCAACTTTG-3′ identified wild-type Aqp11 allele by amplification of 402-bp PCR product, and the knockout Aqp11<sup>−/−</sup> allele was identified by 5′-CAGAATTTCCTTGTCCTGCC-3′ and 5′-GAGCGCCGATCGCTTCTATC-3′ primers, which amplified a 262-bp PCR product during 35 cycles of denaturing at 96°C for 17 s, annealing at 60°C (for Aqp11<sup>−/−</sup> knockout allele) or at 56°C (for Aqp11 wild-type allele) for 30 s, and extension at 72°C for 45 s.

**Evaluation of Renal Function**
Renal function studies included the measurement of BUN at time of killing. BUN was measured by an iSTAT analyzer (Heska Corp., Waukesha, WI) in 75 μl of whole blood. Plasma creatinine was measured as described previously using HPLC system (Perkin-Elmer, Waltham, MA).

**Light and Electron Microscopy**
Kidneys were fixed in 4% paraformaldehyde solution, dissected, and in paraffin, and 4-μm cross-sections were cut and stained with periodic acid-Schiff. Portions of cortex were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), processed, and embedded in Spurr resin. Thin sections were examined using a FEI/Phillips (Eindhoven, The Netherlands) CM12 transmission electron microscope.

**Immunohistochemistry**
Immunohistochemistry was performed according to a standard immunohistochemical technique.35 The following primary rabbit segment-specific antibodies were applied to the tissue sections and incubated overnight at 4°C: For proximal tubule marker, AQP1 (Abcam, Cambridge, MA);12 for thick ascending limb marker, Tamm-Horsfall protein 1:3000 (BTI, Stroughton, MA);14 for distal convoluted tubule marker, thiazide-sensitive co-transporter (anti-rTSC1; gift from Dr. Steve Gerbert).15 For proximal tubule staining, we also used biotinylated Lotus tetragonolobus lectin at 1:400 dilution for 30 min (Vector Laboratories, Burlingame, CA).13 The slides were incubated with secondary goat anti-rabbit IgG horseradish peroxidase–conjugated antibodies or horseradish peroxidase–conjugated streptavidin (Vectastain ABC Kit) for 30 min at 22°C followed by washing and incubation with 3,3-diaminobenzidine solution. The slides were counterstained with hematoxylin.

**RT-PCR Analysis of Aqp11 Gene Expression**
Total RNA was isolated from snap-frozen kidney tissue sections using TRIzol Reagent (Invitrogen) after RNA precipitation36 and RNA purification using RNeasy Midi Kit (Qiagen, Valencia, CA). Total RNA was reverse-transcribed by SuperScript II reverse transcriptase in the
presence of 40 U/μl RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen) for 60 min at 37°C. Sense 5’-CCACCTGAATCTGACAGTGATCTAC-3’ and antisense 5’-CACCTACAGGAAGGACACGTCACATATG-3’ primers from mouse Aqp11 (NCBI accession BAC45005) amplified a 516-bp RT-PCR product during 30 cycles of denaturing at 95°C for 2 min, 95°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 60 s. RT-PCR product of mouse Aqp11 and G3PDH, and manufacturer’s G3PDH-positive control were separated on a 2% agarose gel.

In Situ Hybridization

The sense and antisense Aqp11 cDNA were inserted into pCMVSPORT6 vector (Invitrogen) containing T7 promoter. For generation of the riboprobes for in situ hybridization, the plasmids were linearized and RNA was transcribed from the flanking T7 promoter in the presence of [α-35S]UTP RNA. Aqp11 mRNA was localized in 7-μm paraffin-embedded sections of kidney tissues. Before hybridization, sections were deparaffinized, refixed in 4% paraformaldehyde, and treated with triethanolamine plus acetic anhydride (0.25% vol/vol). Sections were dehydrated in 100% ethanol. Antisense or sense RNA probes were hybridized to the sections at 50 to 55°C for approximately 18 h. The sections were washed as described, and then they were treated with RNase (10 μg/ml) followed by washing in TEN (10 mM TRIS, 5 mM EDTA, and 500 mM NaCl) at 37°C. Slides were dehydrated with graded ethanols containing 300 mM ammonium acetate. For detection of the hybridized probe, slides were dipped in photo emulsion (Ilford K5) and exposed for 7 d at 4°C. After development in Kodak D19, photomicrographs were taken using a Zeiss Axioskop.

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DISCLOSURES

None.

REFERENCES


