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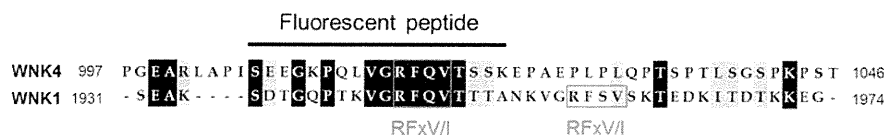
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SUPPLEMENTARY ONLINE DATA

Chemical library screening for WNK signalling inhibitors using fluorescence correlation spectroscopy

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WNK4-RFQV : TAMRA-SEEGK**Q**LVGR**FQ**VTSSK
 WNK1-RFQV : TAMRA-SDTG**Q**PTKVGR**FQ**VTTTA

Figure S1 Alignments of WNK4 and WNK1 sequences around RFxV/I motif and TAMRA-labelled WNK4 and WNK1 peptides used for FCS assay

Identical residues are highlighted in black and similar residues are in grey.

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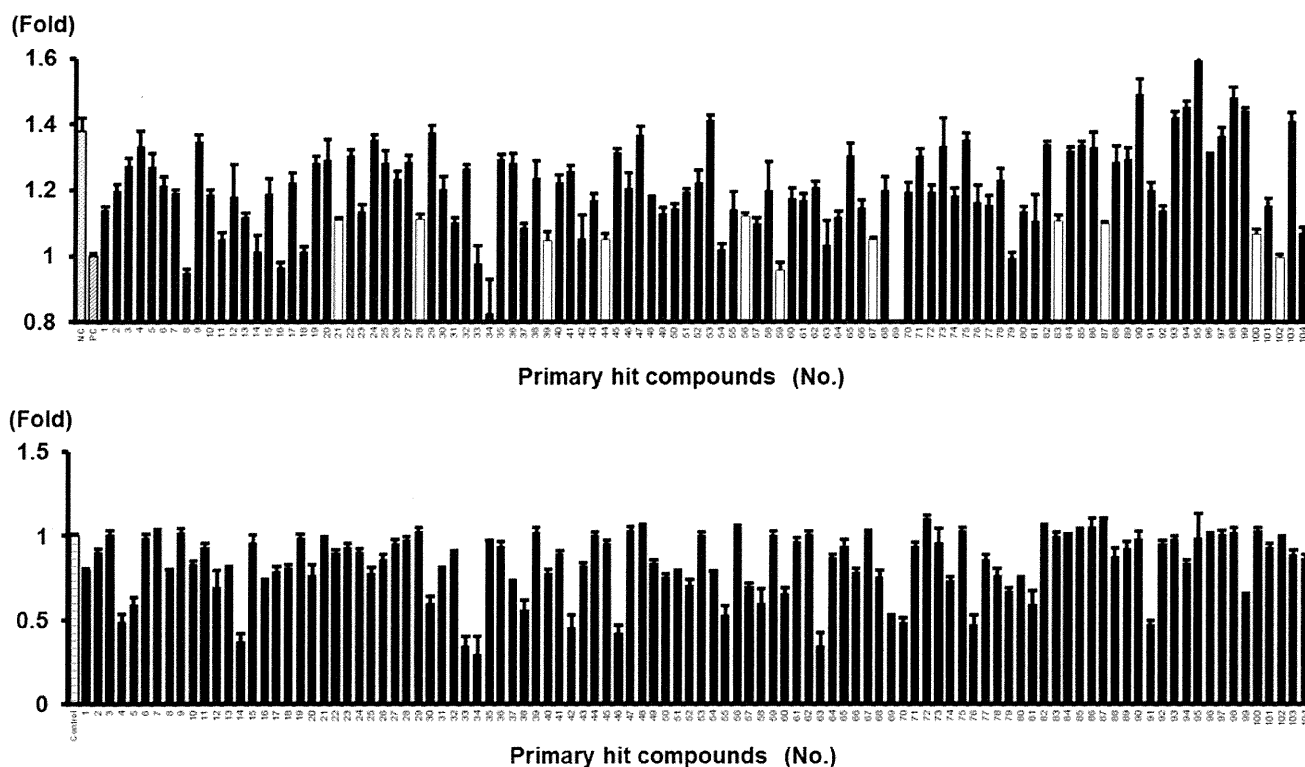


Figure S2 Confirmation of the primary hit compounds

The upper panel shows the secondary screening of the primary hit compounds. The assay was performed under the same conditions as the initial screening (the concentrations of screening compounds were 100 μM). The lower panel shows the effects of the primary hit compounds on the diffusion time of TAMRA-labelled WNK4 peptide without GST-SPAK-CCT. The compounds shown as white bars in upper panel were effective in the restoration of the diffusion time, which was not due to the direct effect on the WNK4 peptide (lower panel). NC, negative control; No., number; PC, positive control.

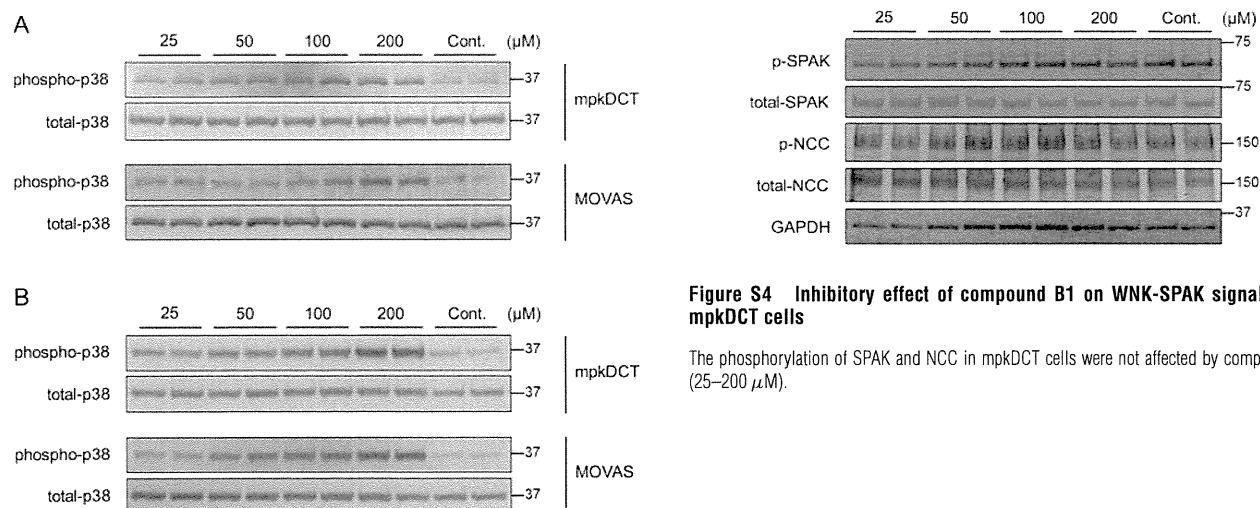
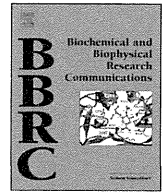


Figure S3 Effect of compounds A and B on p38 MAPK in mpkDCT and MOVAS cells

(A) Phospho-p38 MAPK and total p38 MAPK endogenously expressed in mpkDCT and MOVAS cells were not reduced by compound A (25–200 μM). (B) Results similar to (A) were obtained by the treatment with compound B in mpkDCT and MOVAS cells.

Figure S4 Inhibitory effect of compound B1 on WNK-SPAK signalling in mpkDCT cells

The phosphorylation of SPAK and NCC in mpkDCT cells were not affected by compound B1 (25–200 μM).



Decrease of WNK4 ubiquitination by disease-causing mutations of KLHL3 through different molecular mechanisms



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ABSTRACT

Recently, we demonstrated that WNK4 is a substrate for KLHL3–Cullin3 (CUL3) E3 ubiquitin ligase complexes and that impaired WNK4 ubiquitination is a common mechanism for pseudohypoaldosteronism type II (PHAII) caused by WNK4, KLHL3, and CUL3 mutations. Among the various KLHL3 mutations that cause PHAII, we demonstrated that the R528H mutation in the Kelch domain decreased the binding to WNK4, thereby causing less ubiquitination and increased intracellular levels of WNK4. However, the pathogenic mechanisms of PHAII caused by other KLHL3 mutants remain to be determined. In this study, we examined the pathogenic effects of three PHAII-causing mutations in different KLHL3 domains; the protein levels of these mutants significantly differed when they were transiently expressed in HEK293T cells. In particular, S410L expression was low even with increased plasmid expression. The cycloheximide chase assay revealed that an S410L mutation in the Kelch domain significantly decreased the intracellular stability. Mutations in E85A in the BTB domain and C164F in the BACK domain decreased the binding to CUL3, and S410L as well as R528H demonstrated less binding to WNK4. *In vitro* and *in vivo* assays revealed that these mutants decreased the ubiquitination and increased the intracellular levels of WNK4 compared with wild-type KLHL3. Therefore, the KLHL3 mutants causing PHAII investigated in this study exhibited less ability to ubiquitinate WNK4 because of KLHL3's low stability and/or decreased binding to CUL3 or WNK4.

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1. Introduction

Pseudohypoaldosteronism type II (PHAII) is a hereditary disease characterized by hypertension, hyperkalemia, and metabolic acidosis [1]. Mutations in the with-no-lysine kinase 1 (WNK1) and WNK4 genes are known to be responsible for PHAII [2]. Several *in vitro* and *in vivo* studies have been performed to clarify the molecular pathogenesis of PHAII [3,4]. We discovered a novel phosphorylation signal cascade in the kidney that consisted of WNK-oxidative stress-responsive kinase 1 (OSR1) and STE20/SPS1-related proline/alkaline-rich kinase (SPAK)–NaCl cotransporter (NCC) [5–8]. The constitutive activation of this cascade is the pathogenic molecular mechanism for PHAII caused by WNK4.

In 2012, two new genes KLHL3 and Cullin3 (CUL3) were reported as being responsible for PHAII [9,10]. KLHL3 is a member of the BTB–BACK–Kelch family of proteins, some of which were reported to function as substrate adapters in CUL3-based E3 ubiquitin ligase complexes [11–13]. We have previously reported that WNK4 is a target for ubiquitination by the KLHL3–CUL3 E3 ligase

complex [14] and that the PHAII-causing mutations of WNK4 and KLHL3 (i.e., D564A and R528H, respectively) impaired the binding of WNK4 and KLHL3, thereby reducing WNK4 ubiquitination and increasing the intracellular protein levels of WNK4. Although the role of WNK4 in NCC regulation is controversial, our recent study using WNK4 transgenic mice clearly indicated that increased WNK4 expression activated the WNK–OSR1/SPAK–NCC cascade and caused PHAII [14]. Taken together, these findings indicated that PHAII was caused by impaired ubiquitination of WNK4. Two recent reports have supported this conclusion [15,16]. Furthermore, it has been reported that the KLHL3–CUL3 E3 ligase complex ubiquitinates WNK1 *in vitro* [17], and very recently, we demonstrated that KLHL2, which possesses 84% amino acid identity with KLHL3 in the Kelch domain, also acts with CUL3 as an E3 ubiquitin ligase for WNK kinases [18]. Therefore, various combinations of KLHL2 and KLHL3 with the four types of WNK kinases (WNK1–4) may regulate the WNK signal cascades in different types of cells.

Because the above concept is based on the analysis of a single mutant of KLHL3 and WNK4 that causes PHAII [14], we conducted the current study to examine the pathogenic effects of other mutants of KLHL3 and further establish the molecular pathogenesis of PHAII. KLHL3 is composed of the BTB, BACK, and Kelch domains. The R528H mutation, which we previously characterized, is located

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in the Kelch domain. Consistent with its role of substrate binding in the Kelch domains, R528H revealed less binding to WNK4, thereby causing less ubiquitination and increased intracellular protein levels of WNK4 [14]. Therefore, we selected other mutants from different domains in this study (Fig. 1), namely E85A (BTB domain) and C164F (BACK domain). Moreover, we focused on another mutant in the Kelch domain, S410L, because it may have a novel pathogenic effect that is not observed with R528H.

2. Materials and methods

2.1. Plasmids

Expression plasmids for 3×FLAG-tagged human WNK4 have been previously described [19]. The cDNA encoding Halo-tagged human wild-type (WT)-KLHL3 (Halo-WT-KLHL3) in the pFN21A vector was purchased from Promega. The disease-causing mutations (E85A, C164F, S410L, and R528H) were introduced using a QuickChange Site-directed Mutagenesis Kit (Stratagene). Human CUL3 cDNA was isolated using the reverse transcription-polymerase chain reaction (RT-PCR) using human prostate mRNA as a template and then cloned into the 3×FLAG-CMV10 vector (Sigma-Aldrich). The HA₄-tagged ubiquitin expression vector was kindly provided by T. Ohta (School of Medicine, St. Marianna University).

2.2. Cell culture and transfections

HEK293T cells were cultured at 37 °C in a humidified 5% CO₂ atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. HEK293T cells (3 × 10⁵ cells/6-cm dish or 1 × 10⁵ cells/3.5-cm dish) were transfected with the indicated amount of plasmid DNA using the Lipofectamine 2000 reagent (Invitrogen).

2.3. Immunoprecipitation

HEK293T cells transfected with the indicated amount of DNA were lysed in a buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 1 mM sodium orthovanadate, 50 mM sodium fluoride, and protease inhibitor cocktail] for 30 min at 4 °C. For proteasome inhibition, HEK293T cells were treated with 1 μM epoxomicin (Peptide Institute, Osaka, Japan) for 3 h before harvesting. After centrifugation at 12,000×g for 15 min, the protein concentration of the supernatants was measured, and equal amounts were used for immunoprecipitation with anti-FLAG M2 beads (Sigma-Aldrich) or anti-Halo beads (Promega) for 2 h at 4 °C. Thereafter, the immunoprecipitates were washed with the lysis buffer and eluted in SDS sample buffer after boiling for 5 min. To detect WNK4 ubiquitination in the denatured samples, the cells transfected with various plasmids were lysed in 2% SDS buffer [2% SDS, 150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 2 mM sodium orthovanadate, 50 mM sodium fluoride, and 1× protease

inhibitor cocktail] and then boiled for 10 min, followed by sonication. Before immunoprecipitation, the lysates were diluted at 1:10 in a dilution buffer [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, and 1% Triton X-100], incubated at 4 °C for 1 h with rotation, and centrifuged at 12,000×g for 15 min.

2.4. Antibodies

The blots were probed using the following primary antibodies: anti-FLAG (Sigma-Aldrich), anti-Halo (Promega), anti-HA (Merck Millipore), and anti-GAPDH (Cell Signaling Technology). Alkaline phosphatase-conjugated anti-IgG antibodies (Promega) were used as the secondary antibodies for immunoblotting, and the signals were detected by the Western blue Stabilized Substrate for Alkaline Phosphatase (Promega).

2.5. Cycloheximide chase assay

HEK293T cells (6 × 10⁵ cells in 6-well plates) were transfected using the Lipofectamine 2000 reagent with 1 μg of Halo-WT-KLHL3, or 4 μg of either Halo-S410L or R528H-KLHL3. The cells were then cultured for 24 h, followed by incubation with 100 μM cycloheximide. After incubation for the indicated time periods, the cells were washed with PBS and suspended in 50-μl lysis buffer. Thereafter, the cleared lysate (10 μl) was subjected to immunoblot analyses with anti-Halo (Promega).

2.6. Fluorescence correlation spectroscopy (FCS)

Fluorescent TAMRA-labeled WNK4 peptides covering the PHAII mutation sites were prepared (Hokkaido System Science Co., Ltd., Hokkaido, Japan). The sequences of these peptides have been previously reported [14]. Human full-length KLHL3 (wild-type and mutants) was cloned into pGEX6P-1 vectors. The recombinant GST-fusion KLHL3 protein expressed in the BL21 *Escherichia coli* cells was purified using glutathione Sepharose beads. The TAMRA-labeled WNK4 peptides were incubated at room temperature for 30 min with different concentrations of GST-KLHL3s (0–2 μM) in 1× PBS containing 0.05% Tween 20 (reaction buffer). Further, FCS was performed to measure single-molecule fluorescence using the FluoroPoint-light analytical system (Olympus, Tokyo, Japan) [14,18,20]. The assays were performed in 384-well plates, with all the experiments including 10s of data acquisition. The measurements were repeated five times per sample.

2.7. In vitro ubiquitination assay

cDNA encoding human WNK4 (490–626) with a C-terminal His-tag was amplified by PCR and cloned into a pGEX6p-1 vector. Recombinant GST-fusion WNK4 protein expressed in BL21 *E. coli* cells was purified using glutathione Sepharose beads. KLHL3-CUL3 complexes were immunoprecipitated from the lysates of HEK293T cells transiently expressing Halo-KLHL3. We have previously reported that CUL3 overexpression is not necessary for the *in vitro* ubiquitination assay, because HEK293T cells contain abundant endogenous CUL3 [18]. Thereafter, the complexes were incubated in a 20-μl reaction buffer [50 mM Tris-HCl (pH 7.4), 2.5 mM MgCl₂, 0.5 mM DTT, 2 mM ATP] for 2 h at 30 °C with purified GST-WNK4-His (1 μg), 100 ng recombinant human E1 (Boston Biochem.), 500 ng recombinant human Ubch5a/UBE2D1 (Boston Biochem.), and 2.5 μg recombinant HA-tagged human ubiquitin (Boston Biochem.). The reaction was terminated by addition of SDS-PAGE sample buffer, followed by boiling for 5 min. The reaction mixtures were then subjected to immunoblot analyses with anti-Ub (Cell Signaling Technology) or His (Abcam) antibodies.

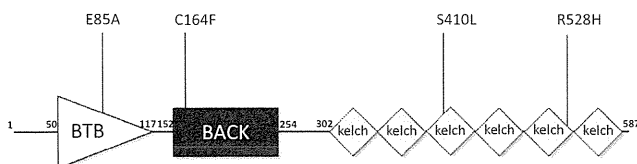


Fig. 1. Structure of human KLHL3 and PHAII mutations analyzed in this study. KLHL3 is composed of BTB, BACK, and Kelch repeat domains. We selected four PHAII-causing KLHL3 mutants for the purpose of evaluation in this study.

2.8. Statistical analysis

Comparisons between the two groups were performed using the unpaired *t*-tests. Analysis of variance with the Tukey's post hoc test was used to evaluate the statistical significance of the comparisons between multiple groups. *P* values <0.05 were considered statistically significant. Data are presented as the mean ± S.E.M.

3. Results and discussion

3.1. Low expression levels of S410L and R528H in HEK293T cells

To analyze each mutant causing PHAII (Fig. 1), we transiently expressed the Halo-tagged KLHL3 (wild-type and mutant) in HEK293T cells. We observed that the expression levels of S410L and R528H appeared to be lower compared with those in the wild-type (WT) and other mutants (Fig. 2A). To confirm this finding, we compared the expression levels of the wild-type and the abovementioned two mutants using different amounts of expression plasmids. As presented in Fig. 2B, the expression levels of these mutants, particularly S410L, was considerably lower compared with that in the wild-type. Because all the KLHL3 expression vectors were the same, we suspected that the missense mutants may have degraded more rapidly in the cells, probably by an endoplasmic reticulum quality control system.

3.2. Significantly decreased stability of S410L

To evaluate the mechanism responsible for the low expression levels of S410L and R528H, we measured the stability of these mutants using the cycloheximide chase assay. As presented in Fig. 2C, S410L was degraded more rapidly than WT-KLHL3. The half-life of S410L was about 6 h, whereas the protein levels of WT-KLHL3 were stable for at least 12 h after cycloheximide treatment.

Contrary to our expectations, the stability of R528H was not decreased compared with that of WT-KLHL3. These results suggest that the pathogenic effects of the S410L mutation may be attributable to the decreased intracellular expression levels resulting from decreased stability. It is possible that the low R528H expression may be attributable to its low translation efficiency, although this remains to be established.

3.3. Impaired binding of the mutant KLHL3 to WNK4 or CUL3

Using immunoprecipitation, we previously demonstrated that the R528H mutation in the Kelch domain decreased its binding to the acidic domain of WNK4 [14]. The current study involved direct measurements of the binding ability of various KLHL3 mutants, including R528H, to WNK4. These measurements were performed using our recently developed FCS method [14,18]. We measured the diffusion time of the TAMRA-labeled WNK4 peptide using FCS in the presence of different concentrations of GST-fusion proteins of WT and the mutant KLHL3; the binding of KLHL3 to this peptide was assessed by the increased diffusion time of the fluorescent peptide [14,18]. In agreement with our previous study [14], we demonstrated that WT-KLHL3 increased the diffusion time of the TAMRA-labeled peptide (Fig. 3). However, S410L and R528H had little effect on the diffusion time, similar to that observed for GST alone. This indicated that both S410L and R528H decreased the ability to bind to WNK4. Therefore, S410L may have two pathogenic roles in the cells, namely increased degradation and decreased binding to WNK4. These changes may be related to the more severe phenotype of patients with this mutation. For example, Louis-Dit-Picard et al. [10] reported a 29-year-old male patient with a heterozygous S410L mutation, who revealed a blood pressure of 165/98 mm Hg and serum potassium levels of 6.7 mmol/L even under hydrochlorothiazide treatment.

We observed that the behavior of E85A (BTB domain mutant) and C164F (BACK domain mutant) was similar to that of

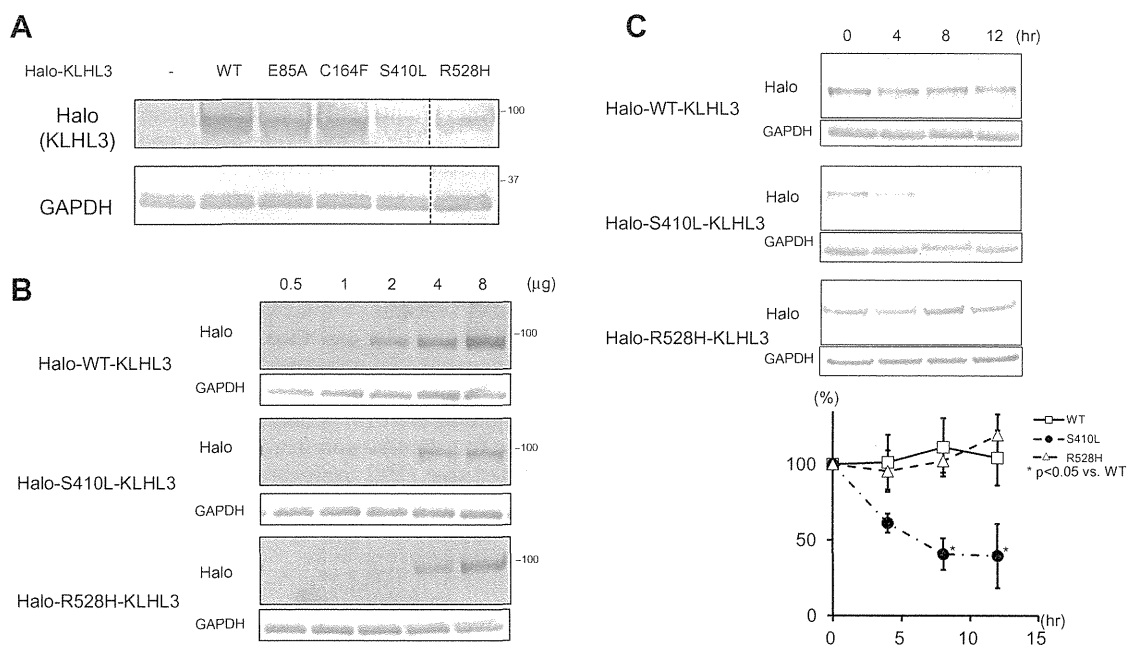


Fig. 2. Transient expression and stability assay of Halo-tagged KLHL3 mutants in the HEK293T cells. (A) We transfected HEK293T cells (1×10^5 cells/3.5-cm dish) with a Halo-KLHL3-pFN21A vector (using 2 μ g plasmid). The expression levels of S410L and R528H appeared to be lower compared with those for the other genes. (B) Wild-type, S410L, and R528H KLHL3 were expressed in HEK293T cells (1×10^5 cells/3.5-cm dish) by different amounts of the expression plasmids. The expression of S410L and R528H was lower compared with that of the wild-type KLHL3. (C) Cycloheximide chase assay of Halo-WT-KLHL3, Halo-S410L-KLHL3, and Halo-R528H-KLHL3. Halo-S410L-KLHL3 was degraded more rapidly compared with Halo-WT-KLHL3 and Halo-R528H-KLHL3. The half-life of Halo-S410L-KLHL3 was approximately 6 h.

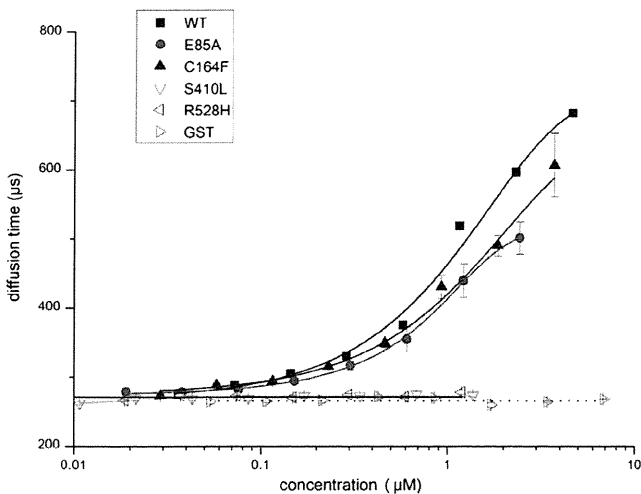


Fig. 3. FCS assay of the binding of KLHL3 and WNK4. The diffusion time of the TAMRA-labeled WNK4 peptide containing the acidic motif (a binding domain to KLHL3) was measured using FCS in the presence of different concentrations of GST fusion proteins of the wild-type and the KLHL3 mutant. The WNK4 peptide bound to the wild-type, E85A, and C164F GST-KLHL3. On the other hand, GST alone and S410L and R528H did not affect the diffusion time, indicating that these mutants could not bind to WNK4.

WT-KLHL3 in the FCS assay, indicating that these mutants maintained the ability to bind to WNK4. This result was not unexpected because the BTB and BACK domains of KLHL proteins are both thought to be involved in binding, not to the substrates (WNK kinases) but to CUL3 [17,21,22]. As presented in Supplementary Fig. 1, we confirmed the decreased binding of these mutants to CUL3 using a coimmunoprecipitation experiment.

3.4. Decreased ability of KLHL3 mutants causing PHAII to ubiquitinate WNK4 and to decrease the intracellular WNK4 protein levels

We previously reported that the expression of WT-KLHL3 significantly decreased the coexpressed protein levels of WNK4, but this effect of KLHL3 was impaired in a PHAII-causing R528H mutant [14]. Wu et al. recently reported a similar effect in other mutants associated with PHAII, including R528H and C164F, in *Xenopus oocytes* [16]. As presented in Fig. 4A, coexpression of PHAII-causing mutants revealed less ability to decrease the intracellular WNK4 protein levels compared with that of WT-KLHL3. Therefore, we performed *in vitro* and *in vivo* assays to evaluate WNK4 ubiquitination using the KLHL3 mutants E85A, C164F, and S410L. We have previously demonstrated that the GST-WNK4-His protein (residue from 490 to 626 containing the binding domain to KLHL3) was directly ubiquitinated by the KLHL3-CUL3 complex *in vitro* and that the R528H mutation caused a significant decrease in WNK4 ubiquitination [14]. Further, as presented in Fig. 4B, the ability of the mutant KLHL3-CUL3 complexes (E85A, C164F, and S410L) to ubiquitinate WNK4 was lesser compared with that of the wild-type KLHL3. In addition, we performed an *in vivo* ubiquitination assay to confirm this finding. Following the coexpression of FLAG-tagged WNK4, Halo-tagged KLHL3, and hemagglutinin (HA)-tagged ubiquitin in HEK293T cells, the cells were treated with 1 μ M epoxomicin, and WNK4 immunoprecipitated with the FLAG antibody. In order to exclude the ubiquitination signals from other proteins that coimmunoprecipitated with WNK4, immunoprecipitation was performed under denaturing conditions. The ubiquitination signals were observed as a smear band of over approximately 200 kDa, which is the apparent molecular size of

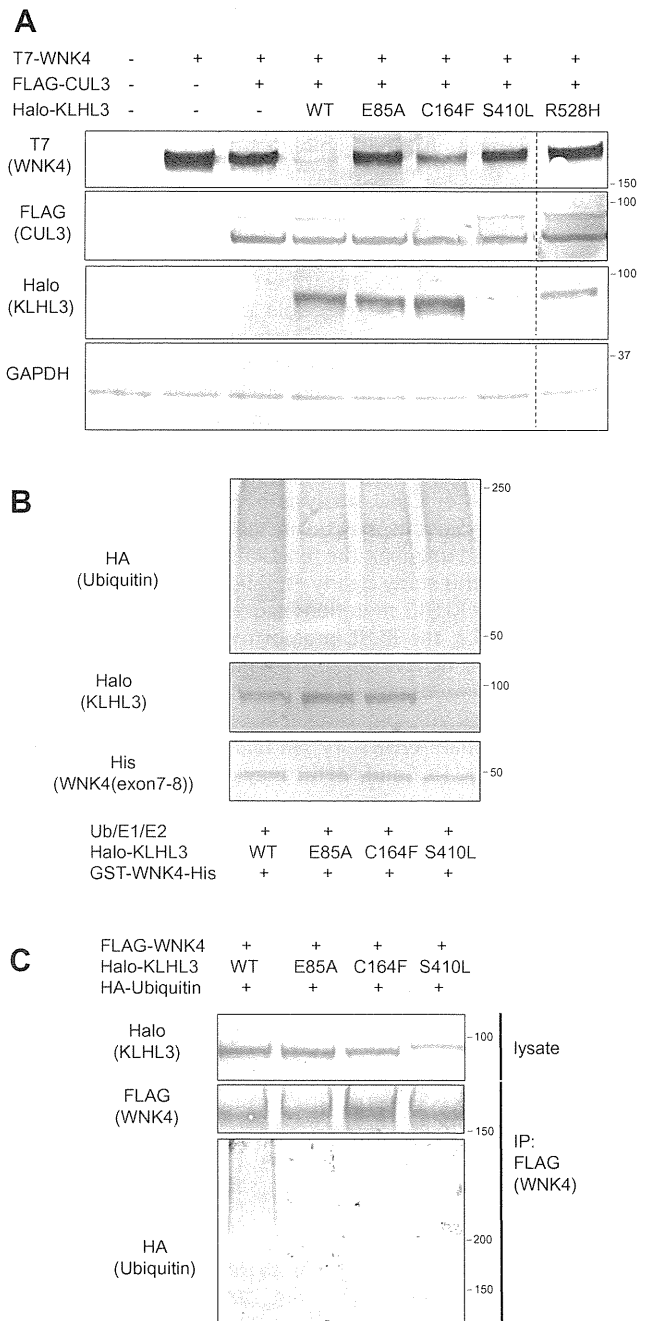


Fig. 4. Effect of the wild-type and mutant KLHL3 on the protein levels and ubiquitination of WNK4. (A) T7-tagged WNK4 and FLAG-tagged CUL3 were coexpressed with Halo-tagged KLHL3. Coexpression of the wild-type KLHL3 caused a significant decrease in the abundance of WNK4, but the KLHL3 mutants were less able to reduce WNK4. (B) *In vitro* ubiquitination assay of WNK4. As demonstrated in our previous report [14, 18], the wild-type KLHL3 complex ubiquitinated the GST-WNK4-His protein. However, the KLHL3 mutant failed to ubiquitinate WNK4 as efficiently as wild-type KLHL3. (C) *In vivo* ubiquitination assay of WNK4. Wild-type KLHL3 coexpression increased the WNK4 ubiquitination. However, the KLHL3 mutants were less able to ubiquitinate WNK4.

WNK4. As presented in Fig. 4C, WNK4 ubiquitination by these KLHL3 mutants was lower compared with that by WT-KLHL3.

Following our publication that impaired WNK4 ubiquitination was a common molecular mechanism in PHAII and was caused by mutations in WNK4 (D564A), KLHL3 (R528H), and CUL3 (skipping of exon 9), Shibata et al. analyzed the same R528H mutant

and reported similar results [15]. In addition to R528H, Wu et al. examined other PHAII-causing mutants (A77E, C164F, Q309R, and L387P) in *Xenopus* oocytes and confirmed that they were less effective in decreasing the WNK4 protein levels compared with WT–KLHL3 [16]. However, with the exception of R528H, the effect of these mutations on WNK4 ubiquitination has not been investigated. A more thorough investigation of these disease-causing mutants of the KLHL3 gene is therefore necessary to establish the pathogenesis of PHAII. Evidence to date indicates these mutations are distributed in different functional domains of the KLHL3 protein and that KLHL proteins other than E3 ligase (e.g., actin-binding protein) may have a pathogenic role in PHAII [10]. The current study clearly demonstrated that each mutation associated with PHAII led to the common consequence of decreased WNK4 ubiquitination, with this effect being mediated through different molecular mechanisms. The mutations in the BTB and BACK domains affected the interaction with CUL3, whereas those in the Kelch domains affected the binding to WNK4. In addition to the impaired binding to WNK4 or CUL3, analysis of S410L in this study suggested that some mutants may be unstable within the cells, which would be expected to impair the functions of KLHL3. Therefore, the data obtained in this study clearly establishes that impaired WNK4 ubiquitination by KLHL3 mutations causes PHAII. Recently, we reported that KLHL2, the KLHL protein member that is closest to KLHL3, could also behave as an E3-ubiquitin ligase with CUL3 [18]. In addition, we observed that KLHL2 had the ability to bind to all WNK kinases, although Ohta et al. reported that KLHL3 could bind and ubiquitinate WNK1 [17]. In this regard, the regulation of WNK kinases by KLHL2 and KLHL3 may occur in various types of cells in the body and may be involved not only in the pathogenesis of PHAII but also in other pathophysiological conditions.

In summary, we clarified the pathogenic mechanisms of PHAII-causing mutations in different domains in KLHL3. Although the molecular mechanisms were different, impaired WNK4 ubiquitination is a common consequence of these mutations.

Acknowledgments

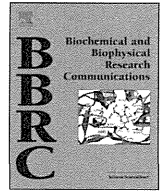
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbrc.2013.08.035>.

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Treatment with 17-allylamino-17-demethoxygeldanamycin ameliorated symptoms of Bartter syndrome type IV caused by mutated *Bsnd* in mice



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ABSTRACT

Mutations of *BSND*, which encodes barttin, cause Bartter syndrome type IV. This disease is characterized by salt and fluid loss, hypokalemia, metabolic alkalosis, and sensorineural hearing impairment. Barttin is the β -subunit of the CIC-K chloride channel, which recruits it to the plasma membranes, and the CIC-K/barttin complex contributes to transepithelial chloride transport in the kidney and inner ear. The retention of mutant forms of barttin in the endoplasmic reticulum (ER) is etiologically linked to Bartter syndrome type IV. Here, we report that treatment with 17-allylamino-17-demethoxygeldanamycin (17-AAG), an Hsp90 inhibitor, enhanced the plasma membrane expression of mutant barttins (R8L and G47R) in Madin–Darby canine kidney cells. Administration of 17-AAG to *Bsnd*^{R8L/R8L} knock-in mice elevated the plasma membrane expression of R8L in the kidney and inner ear, thereby mitigating hypokalemia, metabolic alkalosis, and hearing loss. These results suggest that drugs that rescue ER-retained mutant barttin may be useful for treating patients with Bartter syndrome type IV.

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1. Introduction

Mutations of *BSND* that encode barttin cause Bartter syndrome type IV [1]. This disease is characterized by salt and fluid loss, hypokalemia, metabolic alkalosis, and sensorineural hearing impairment. Patients with Bartter syndrome are typically treated with potassium-sparing diuretics such as spironolactone or amiloride, angiotensin inhibitors, and potassium supplementation and frequently treated with nonsteroidal anti-inflammatory drugs to suppress the elevation of renal prostaglandin E2. However, these treatments only partially ameliorate Bartter syndrome symptoms. Sensorineural hearing impairment accompanies renal symptoms; however, no treatment is available for this impairment.

Barttin is the β -subunit of CIC-K chloride channels that are expressed in the distal renal tubules and inner ear [2]. Barttin is coexpressed with CIC-K1 in apical and basolateral membranes of the thin ascending limb of Henle's loop [3,4] and with CIC-K2 in the basolateral plasma membranes of nephron segments from the thick ascending limb of Henle's loop to intercalated cells in collecting ducts [5–7]. Constitutive barttin knockout mice die a few days after birth because of severe dehydration [8]. Moreover, barttin hypomorphic mice suffer from severe polyuria,

hypokalemia, and metabolic alkalosis [9]. These findings indicate that CIC-K/barttin plays a crucial role in transepithelial chloride transport in the kidney.

Barttin colocalizes with CIC-K1 and CIC-K2 in the basolateral membrane of marginal cells of the stria vascularis and vestibular dark cells [2]. Mechanical vibration is enhanced and converted to electrical signals through outer and inner hair cells in the organ of Corti. To allow a depolarizing influx of potassium ions through apical mechanosensitive cation channels of hair cells, the endolymph that fills the cavity of the scala media is high in K^+ and has a positive potential [10,11]. These conditions are generated by the stria vascularis, which comprises a multilayered epithelium [11,12]. Inner ear-specific barttin knockout mice show a significant decrease of endocochlear potential (EP) and are congenitally deaf [8], indicating that barttin/CIC-K is required to generate EP.

Patients with Bartter syndrome type IV harbor different *BSND* mutations [1,13–20]. The R8L missense mutant barttin does not activate the CIC-K chloride channel currents when introduced into *Xenopus laevis* oocytes, and R8L barttin and CIC-K are not expressed on the plasma membrane [2,21]. We showed that the R8L barttin stably expressed in Madin–Darby canine kidney (MDCK) cells was trapped in the endoplasmic reticulum (ER) and did not reach the plasma membrane [22]. Recently, we confirmed this phenotype in the kidneys of *Bsnd*^{R8L/R8L} knock-in mice (R8L knock-in mice) [9].

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Certain pathogenic proteins are misfolded and are retained in the ER, including the cystic fibrosis transmembrane conductance regulator (CFTR) [23–26], aquaporin 2 (AQP2) [27,28], V2 vasopressin receptor [29,30], podocin [31], and Tamm–Horsfall Protein (THP) [32]. Attempts have been made to rescue these ER-trapped mutant proteins. For example, the Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG) increases cell-surface expression of AQP2-T125 M and partially corrects nephrogenic diabetes insipidus phenotype in AQP2^{T126M} mice [28]. Moreover, 17-AAG increases the stability of mutant pendrin by inhibiting its ubiquitin-mediated degradation [33].

In the present study, we investigated the effect of 17-AAG treatment on mislocalized R8L barttin in MDCK cells and in R8L knock-in mice. We found that 17-AAG increased the expression of R8L barttin on the plasma membrane and partially ameliorated the phenotypes of Bartter syndrome type IV, including hearing loss.

2. Material and methods

2.1. Cell culture and analysis of cell-surface expression of barttin

We used MDCK cells stably expressing wild-type (WT) and R8L mutant barttin, which are described in our previous study [22]. G47R mutant barttin stably expressing MDCK cells were generated with site-directed mutagenesis and G418 selection as previously described [22]. Cell-surface proteins were harvested using a

published side-specific biotinylation technique [34]. MDCK cells were incubated in the wells of 6-well TransWell filters (Corning) for at least 3 days to form confluent monolayers and were then incubated with 17-AAG (LC Laboratories), curcumin (Sigma-Aldrich), trimethylamine oxide (TMAO) (Sigma-Aldrich), or DMSO (0.15%) as control for 2 h before biotinylation. Cells were biotinylated using EZ-Link Sulfo-NHS-SS-biotin (Thermo Scientific) for 30 min. After quenching the remaining biotin with 50 mM NH₄Cl, cells were lysed with lysis buffer (150 mM NaCl, 20 mM Tris-HCl, 5 mM EDTA, and 1% Trion-X-100). Biotinylated proteins were bound to NeutrAvidin resin (Thermo Scientific), eluted using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) buffer containing 50 mM dithiothreitol at 60 °C for 20 min and then subjected to immunoblotting. Blots were incubated with an anti-barttin antibody diluted to 1:200 with 5% skim milk in Tris-buffered saline with tween (TBST) [9]. Immunofluorescence (IF) detection of barttin in MDCK cells grown on filters was performed as follows. The cells were fixed with 3% paraformaldehyde (PFA) for 15 min, permeabilized with 0.1% Triton X-100 in PBS for 10 min, blocked in 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 30 min, and then incubated with the anti-barttin antibody (1:200 dilution with 1% BSA in PBS) for 2 h at room temperature. Alexa-Fluor[®]-labeled secondary antibodies (Life Technologies) were used to detect the barttin-primary antibody complexes. IF images were observed using an LSM510 Meta fluorescence microscope (Carl Zeiss).

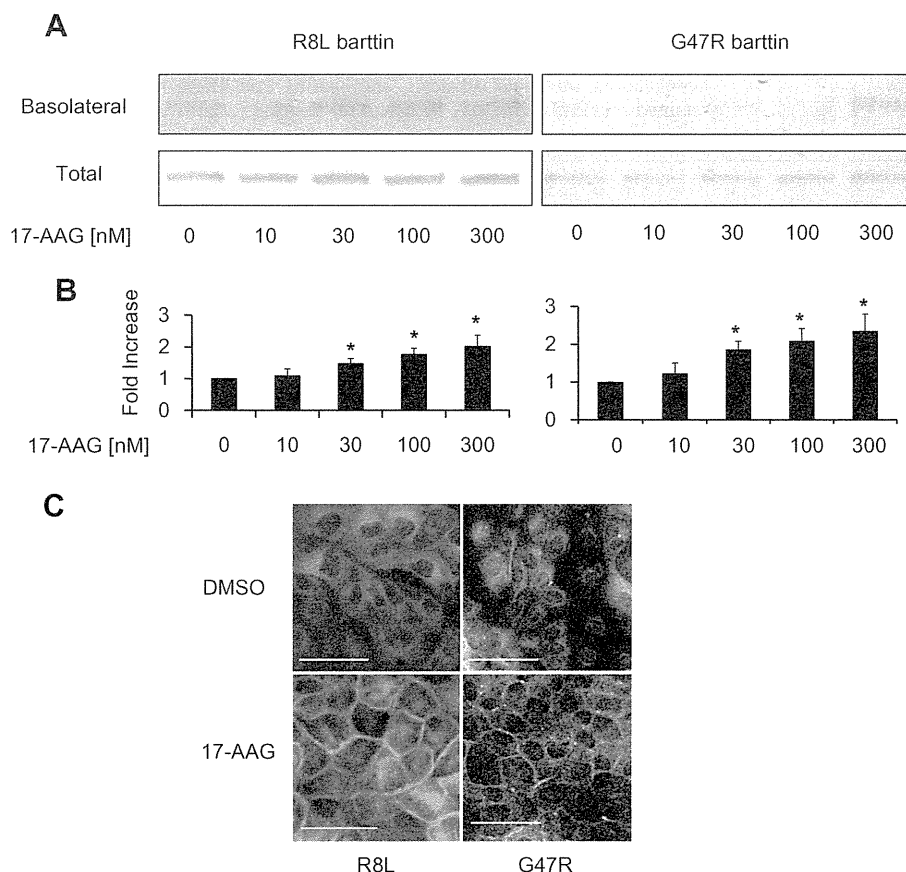


Fig. 1. Effect of 17-allylamino-17-demethoxygeldanamycin (17-AAG) on cell-surface expression of R8L and G47R mutant barttins. R8L and G47R were stably expressed in Madin–Darby canine kidney (MDCK) cells. The cells were incubated with 17-AAG 2 h before biotin labeling and IF analysis. (A) Expression of R8L and G47R in basolateral plasma membranes increased after treatment with >30 nM 17-AAG. (B) Densitometric analyses of the data shown in Fig. 1A. $N = 4$ (R8L), $N = 3$ (G47R), * $p < 0.05$. (C) Immunofluorescence analysis of R8L and G47R showing increased expression on the plasma membrane after treatment with 17-AAG (300 nM). At least three independent experiments yielded similar results. Scale bars = 50 μ m.

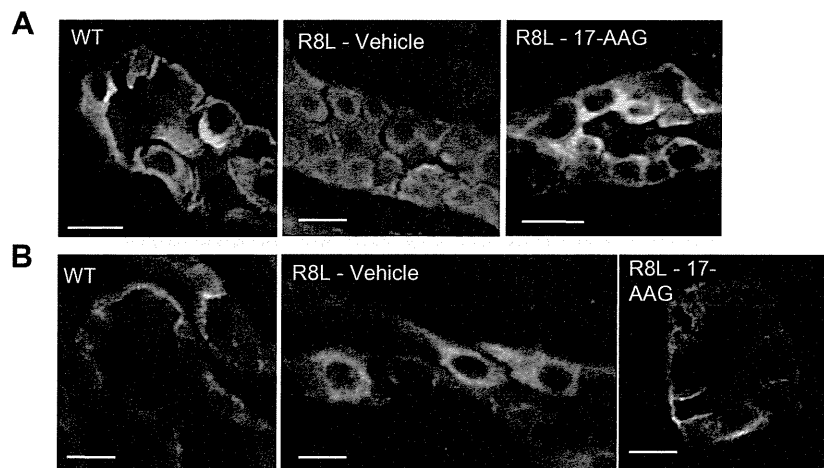


Fig. 2. Representative images of R8L barttin and CIC-K in the kidney of an R8L knock-in mice after 17-allylamino-17-demethoxygeldanamycin (17-AAG) treatment. 17-AAG (25 mg/kg) or dimethyl sulfoxide (DMSO; vehicle control) was intraperitoneally administered. (A) 17-AAG restored the plasma membrane localization of R8L barttin (red) in connecting tubules. Apical green staining identifies AQP2. (B) Localization of CIC-K in intercalated cells in the cortical connecting duct was corrected by 17-AAG treatment in R8L knock-in mice. WT: wild-type mice, R8L: R8L knock-in mice. Scale bars = 10 μm . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Analysis of blood samples obtained from R8L knock-in mice before and after 17-allylamino-17-demethoxygeldanamycin (17-AAG) treatment.

	DMSO + Low NaCl diet (N = 10)		17-AAG + Low NaCl diet (N = 9)	
	Before treatment (regular)	After treatment (low NaCl)	Before treatment (regular)	After treatment (low NaCl)
Na (mEq/L)	147 \pm 0	143 \pm 1	146 \pm 1	142 \pm 1
K (mEq/L)	4.5 \pm 0.2	3.9 \pm 0.2	4.7 \pm 0.1	4.5 \pm 0.1
Cl (mEq/L)	111 \pm 1	106 \pm 1	111 \pm 1	108 \pm 1
pH	7.294 \pm 0.015	7.345 \pm 0.016	7.285 \pm 0.012	7.321 \pm 0.018
HCO ₃ ⁻ (mEq/L)	22.8 \pm 0.4	25.6 \pm 0.6	22.6 \pm 0.4	24.1 \pm 0.8

The knock-in mice were injected with 17-allylamino-17-demethoxygeldanamycin (17-AAG) (25 mg/kg) or dimethyl sulfoxide (DMSO; vehicle control) daily for 7 days. The mice were fed a low salt diet during treatment. Plasma HCO₃⁻ levels and pH of DMSO-treated mice fed a low salt diet increased. However, the increase of plasma HCO₃⁻ and pH in 17-AAG-treated mice was not significant. DMSO-treated mice fed a low salt diet were hypokalemic; however, 17-AAG-treated mice were not.

^{*} $p < 0.05$ vs. Pre.

[†] $p < 0.05$ vs. DMSO. Na, sodium; K, potassium; Cl, chloride; HCO₃⁻, bicarbonate.

2.2. Immunofluorescent analysis of mouse tissues

The Animal Care and Use Committee of Tokyo Medical and Dental University approved the experiments conducted using animals. Mice were intraperitoneally administered 17-AAG (25 mg/kg) 2 h before tissue preparation. 17-AAG was dissolved in 50% DMSO, and the same volume of 50% DMSO was used as a vehicle control. Tissues were prepared as described previously [35]. Mice were deeply anesthetized with ether, and tissues were harvested and fixed with 4% PFA in PBS by perfusion through the left ventricle. The kidneys were removed and placed in the fixative. To prepare inner ear specimens, the temporal bones were removed and placed in the fixative. Small openings were made at the round and oval windows and at the apex of the cochlea, and the perilymphatic space was then gently perfused with fixative. After 1 h, the right temporal bones were decalcified by immersion in 5% ethylenediaminetetraacetic acid with stirring at 4 °C for approximately 7 days. Kidney and right inner-ear specimens were soaked for several hours in 20% sucrose in PBS, embedded in Tissue-Tek OCT compound (Sakura Finetech Co., Ltd.), and snap frozen. For whole-mount staining of the stria vascularis, fixed left-inner ears were microdissected, and the isolated stria vascularis was soaked in fixative. The whole-mount samples were treated with 0.5% Triton X-100 in PBS for 30 min. Frozen sections and whole-mount samples were blocked using 1% BSA in PBS and incubated with primary antibodies as follows: anti-barttin (diluted 1:200 with 0.1%

BSA in PBS) [9] and anti-CIC-K (diluted 1:100) [36]. The secondary antibodies were conjugated to Alexa-Fluor[®] (diluted 1:200). Samples were mounted using VECTASHIELD[®] with DAPI (Vector Laboratories).

2.3. Analysis of blood

Blood was collected from the submandibular vein after administering light anesthesia with ether. Blood data was determined using an i-STAT[®] analyzer (Fuso, Osaka, Japan). Baseline blood data were acquired from mice fed a normal diet (0.4% NaCl). The mice were then fed a low salt diet (0.01% NaCl) that induces hypokalemia and metabolic alkalosis in R8L barttin knock-in mice. We administered intraperitoneal injections of 25 mg/kg 17-AAG once daily for 7 days. Blood samples were collected after the treatment period. All nutrients were obtained from the Oriental Yeast Co., Ltd., (Tokyo, Japan).

2.4. Auditory brainstem response (ABR)

ABR recording was performed as previously described [35]. Mice were anesthetized using pentobarbital before placing stainless steel needle electrodes dorsolaterally to the ears. Waveforms of 512 stimuli (9 Hz) were averaged, and the visual detection threshold was determined using varying sound pressure levels. Baseline hearing thresholds were recorded first. After 2 h of

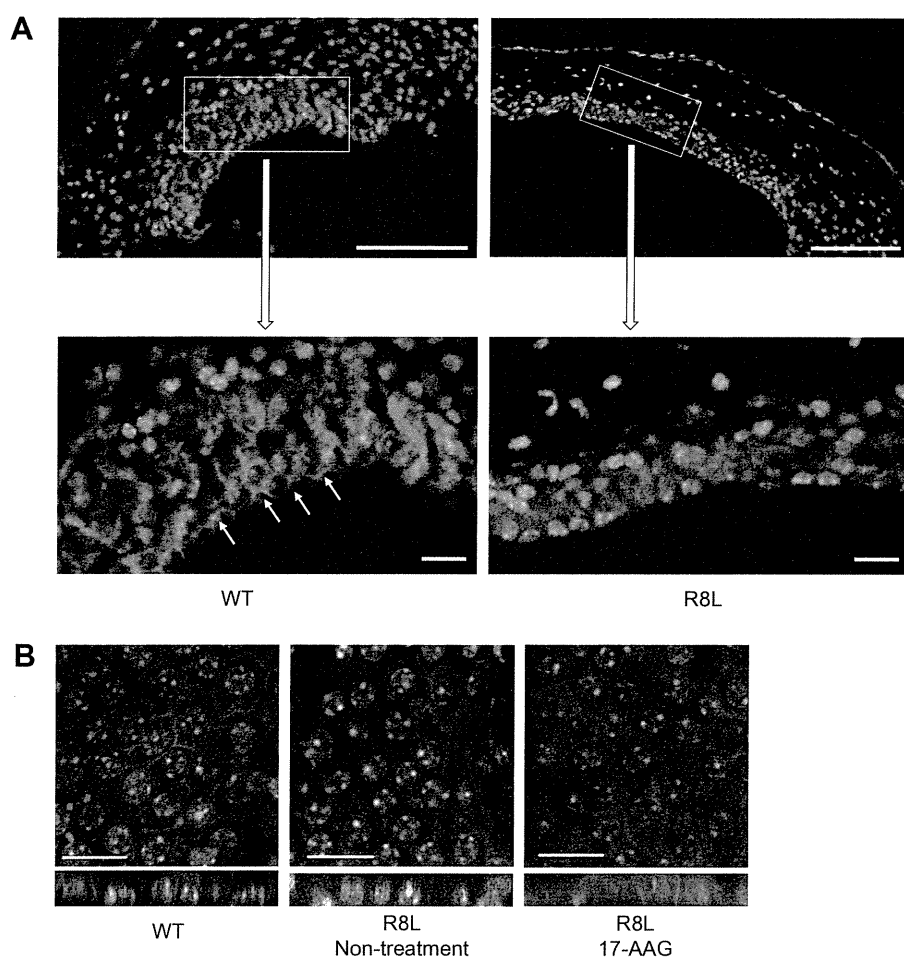


Fig. 3. Representative images of barttin in the stria vascularis. (A) Representative images of barttin in frozen sections of the stria vascularis. Wild-type (WT) barttin appeared to be present on the lateral walls of marginal cells (arrows), whereas R8L expression on the plasma membrane was not clear. Blue indicates nuclear (DAPI) staining. Scale bars = 100 μm in upper panels, 20 μm in lower panels. (B) Representative images of barttin (red) in whole mounts of the stria vascularis. Blue indicates nuclear (DAPI) staining. Scale bars = 20 μm . Lower panels show z-stacks of upper panels. Wild-type (WT) barttin was observed on the lateral walls of marginal cells (left panel), whereas R8L expression on the plasma membrane was not clear (middle panel). 17-AAG treatment partially restored the plasma membrane expression of R8L (right panel). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2

Hearing thresholds (dB) of wild-type (WT) and R8L knock-in mice assessed using auditory brainstem response (ABR).

Frequency		3-Week-old mice	5-Week-old mice	10-Week-old mice
8000 Hz	WT	16.0 \pm 2.0	19.9 \pm 1.7	21.8 \pm 1.4
	R8L	36.0 \pm 2.8	54.9 \pm 2.9 [†]	70.4 \pm 3.3 ^{†§}
20,000 Hz	WT	6.7 \pm 2.3	14.1 \pm 2.8	13.4 \pm 0.0
	R8L	42.6 \pm 5.5	50.9 \pm 2.0	63.4 \pm 2.9 ^{†§}

The numbers of mice are shown in Fig. 4A.

^{*} $p < 0.05$ vs. WT.

[†] $p < 0.05$, 3-week-old mice.

[§] $p < 0.05$, 5-week-old mice.

recovery from anesthesia, 17-AAG (25 mg/kg) or vehicle (equal volume of 50% DMSO) was intraperitoneally injected and hearing thresholds were determined again.

2.5. Statistical methods

All values are expressed as the mean \pm standard error of the mean (SE). Statistical analyses of the effects of treatment were performed using a paired *t*-test. Other statistical analyses were performed using an unpaired *t*-test. *p*-Values < 0.05 were considered statistically significant.

3. Results

3.1. Cell-surface expression of mutant barttins by MDCK cells is increased by treatment with 17-AAG

Analysis of MDCK cells stably expressing human R8L and G47R barttins showed that barttin localization was primarily intracellular (Fig. 1). We next tested whether agents that rescue ER-trapped mutant membrane proteins (17-AAG, curcumin, and TMAO) [23,25,26,28,33] were effective in relocalizing the mutant barttins. IF and site-specific biotin labeling revealed that these agents effectively increased the basolateral plasma membrane expression of the mutant proteins (Fig. 1 and Fig. S1). Moreover, the basolateral expression of WT barttin did not increase in response to 17-AAG (Fig. S2). We also assessed the effects of 17-AAG on barttin mutants G10S, I12T, Q32X; however, G10S and I12T mutant barttin were expressed on the plasma membrane before the treatment [17,22,37], and the low level of Q32X barttin expression did not permit the acquisition of meaningful data.

3.2. Partial rescue of renal phenotypes of R8L knock-in mice

We previously showed that R8L and CIC-K expression in the basolateral membrane is significantly decreased in the renal

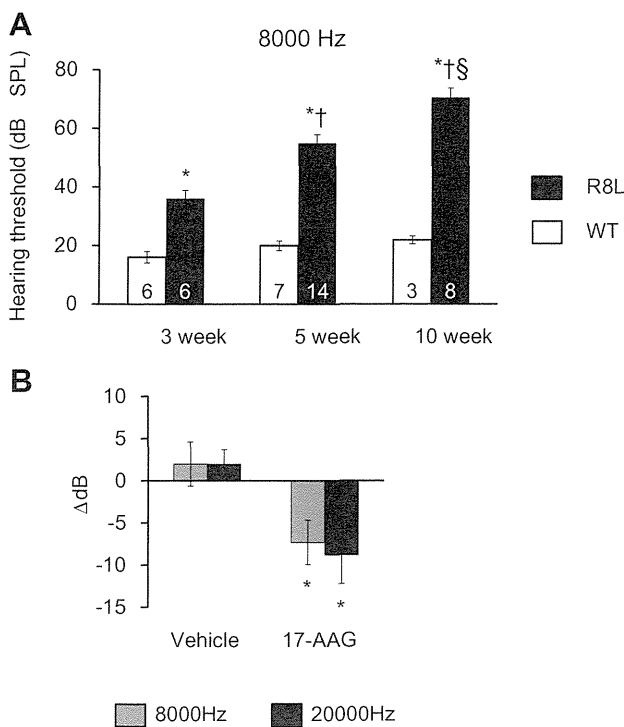


Fig. 4. Hearing impairment in R8L knock-in mice and its amelioration with 17-AAG treatment (A) Hearing thresholds at 8000 Hz in wild-type (WT) and R8L knock-in mice measured using auditory brainstem response (ABR). Numbers of mice are shown on the columns. * $p < 0.05$ vs. WT, † $p < 0.05$ vs. 3-week-old mice, § $p < 0.05$ vs. 5-week-old mice. (B) Variation of hearing thresholds before and after treatment. 17-allylamino-17-demethoxygeldanamycin (17-AAG): $N = 13$; vehicle: $N = 10$. * $p < 0.05$ vs. vehicle.

tubules of R8L knock-in mice [9]. IF analysis of the R8L mutants shows that 17-AAG restored the basolateral localization of barttin in the connecting tubules (Fig. 2) and corrected the intracellular localization of ClC-K in the intercalated cells of cortical collecting ducts (Fig. 2).

The R8L knock-in mice suffered from metabolic alkalosis and hypokalemia when fed a low-salt diet [9]. We confirmed our previous findings in the vehicle-treated R8L knock-in mice (Table 1). However, administration of 17-AAG for one week corrected the low salt-induced metabolic alkalosis and ameliorated hypokalemia (Table 1). In wild-type mice, administration of 17-AAG did not significantly affect these parameters (Table S1).

3.3. Partial rescue of sensorineural hearing impairment of R8L knock-in mice

IF analysis revealed that R8L was present in the stria vascularis (Fig. 3A). In higher magnification (lower panels), wild-type barttin appeared to be localized on the lateral walls of marginal cells (arrows), whereas R8L expression on the plasma membrane was not clear. However, it was difficult to assess from this view whether localization of R8L to the plasma membrane was impaired because marginal and intermediate cells are extensively interdigitated. Therefore, we analyzed whole-mount samples (Fig. 3B) and confirmed the finding shown in Fig. 3A. Furthermore, we could observe that 17-AAG treatment partially restored the plasma membrane expression of R8L (Fig. 3B, right panel).

We evaluated hearing impairment using ABR. R8L knock-in mice lost hearing at 8 and 20 kHz compared with control WT littermates at 3-, 6-, and 10-weeks of age (Table 2, Fig. 4A). Although 17-AAG treatment did not affect the hearing thresholds of WT mice (Table S2), it slightly, but significantly, improved the hearing

thresholds of R8L knock-in mice (Fig. 4B). DMSO treatment did not affect hearing thresholds.

4. Discussion

We previously described the renal phenotypes of R8L barttin knock-in mice [9]. Bartter-like phenotypes (loss of salt from the kidney, hypokalemia, and metabolic alkalosis) were only observed when these mice were fed a low salt diet. In contrast to the renal phenotypes, we found here that the hearing of R8L knock-in mice was significantly impaired under normal conditions. Because ABR analysis generated semi-quantitative data indicating impaired function of ClC-K/R8L barttin *in vivo*, we used this parameter to assess the reversal of symptoms by administering 17-AAG to R8L knock-in mice. We observed significant improvement in the hearing threshold as well as the increased expression of barttin at the plasma membrane. Metabolic alkalosis and hypokalemia were also ameliorated by 17-AAG treatment, which increased the plasma membrane expression of R8L barttin in renal tubules. 17-AAG, a semisynthetic chemical analog of the natural product geldanamycin, inhibits Hsp90 function [38]. Hsp90 participates in a diverse range of cellular processes, including chaperoning of newly synthesized proteins, stress responses, signal transduction, and transcriptional regulation [39]. We chose 17-AAG for the present study because 17-AAG is effective at low concentrations *in vitro* and has been used in other mouse models of human diseases [28] as well as for treating patients with cancer [40,41]. We previously demonstrated that decreased transepithelial chloride transport and plasma membrane localization of barttin significantly correlate in the thin limb of Henle's loop of R8L knock-in mice, providing compelling evidence that impaired plasma membrane localization of R8L barttin accounts for pathogenesis. We demonstrate that 17-AAG was effective for rerouting barttin to the plasma membrane of MDCK cells expressing R8L and G47R barttin. Therefore, 17-AAG may be useful for treating patients with Bartter syndrome type IV who harbor other *BSND* missense mutations.

The mechanism underlying the ability of 17-AAG to rescue mutant barttins is unknown. ER-retained mutant barttins may be misfolded and are restored to their native conformations by 17-AAG. This assumption is supported by findings that TMAO and curcumin were also effective in increasing barttin expression at the plasma membrane (Fig. S1). However, it was difficult to confirm this hypothesis from these experiments because the maturation of barttin (an unglycosylated protein) cannot be monitored by its glycosylation. Findings related to those reported here demonstrate that the treatment of human cell lines with 17-AAG facilitates the folding of pendrin through heat shock transcription factor 1-dependent induction of molecular chaperones [33], suggesting that the same mechanism mediates the effects of 17-AAG on R8L and G47R barttin. Restoration from the misfolding by 17-AAG might not only correct the mislocalization of R8L but also decrease ER-associated degradation of R8L, both of which might be involved in the increased plasma membrane expression of R8L by 17-AAG.

In conclusion, we demonstrated that the hearing of patients with Bartter syndrome type IV may be restored by treatment with drugs such as 17-AAG. Further research is required to discover more effective drugs that correct the aberrant intracellular localization of misfolded membrane proteins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbrc.2013.10.129>.

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Hereditary nephrogenic diabetes insipidus in Japanese patients: analysis of 78 families and report of 22 new mutations in AVPR2 and AQP2

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Abstract

Background Familial form of nephrogenic diabetes insipidus (NDI) is a rare hereditary disease caused by arginine vasopressin type 2 receptor (AVPR2) or water channel aquaporin 2 (AQP2) gene mutations. It is speculated that 90 % of NDI families carry disease-causing mutations in AVPR2 and 10 % carry the mutations in AQP2; however, these percentages have not been supported by actual data. It is also unknown whether these percentages vary in different ethnic groups.

Methods Gene mutation analyses were performed for 78 Japanese NDI families. All exons and intron–exon boundaries of the AVPR2 and AQP2 genes were directly sequenced.

Results A total of 62 families (79 %) carried disease-causing mutations in AVPR2, while nine families (12 %) carried mutations in AQP2. We identified 22 novel putatively disease-causing mutations (19 in AVPR2 and 3 in AQP2). Regarding AVPR2, 52 disease-causing mutations were identified. Among them, missense mutations were most common (54 %), followed by deletion mutations. In the 64 women who had monoallelic disease-causing AVPR2 mutations, 16 (25 %) had NDI symptoms, including 4 complete NDI subjects. Regarding AQP2, 9 disease-causing mutations were identified in nine families. Two missense mutations and one deletion mutation showed a recessive inheritance, while one missense mutation and five small deletion mutations in the C-terminus of AQP2 showed a dominant inheritance.

Conclusions Most Japanese NDI families carry disease-causing mutations in AVPR2 and 12 % carry mutations in AQP2. A total of 22 novel putatively disease-causing mutations were identified. The relatively high occurrence of symptomatic carriers of AVPR2 mutations needs attention.

Keywords Aquaporin 2 · Urine concentration · Vasopressin · Vasopressin receptor · Kidney collecting duct

Introduction

Nephrogenic diabetes insipidus (NDI) is a human kidney disease in which the urine-concentrating ability of the kidney cannot respond to the antidiuretic hormone, arginine vasopressin, resulting in the massive excretion of diluted urine. Therefore, NDI patients manifest polyuria and polydipsia. The hereditary (congenital) form of NDI is relatively rare, and is known to be caused by mutations in two genes, the arginine vasopressin type 2 receptor (AVPR2) and the water channel aquaporin 2 (AQP2) [1–4]. These two genes encode two membrane proteins that are oppositely located at the basolateral and apical membranes of the collecting duct principal cells, respectively, and constitute the fundamental components of urine concentrating machinery [5, 6].

The AVPR2 gene is located on X chromosome (Xq28), and thus, NDI caused by AVPR2 gene mutations is transmitted in an X-linked recessive mode (OMIM 304800); males with one mutated gene are symptomatic, whereas heterozygous females are usually asymptomatic. The AQP2 gene is located on chromosome 12 (12q13.12), and NDI caused by AQP2 mutations shows both autosomal

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recessive and dominant inheritance (OMIM 125800, 107777) [7, 8]. Several review papers have claimed that about 90 % of NDI patients carry AVPR2 mutations and about 10 % carry AQP2 mutations; however, actual data in support of this estimate have not been shown [1, 3]. It is also unknown whether the genetic causes of NDI vary among different ethnic groups.

After the cloning of human AQP2 [9] and the first report of an NDI patient with mutated AQP2 [10], we have performed gene mutation analyses of Japanese NDI patients. At the end of July 2012, the total number of analyzed NDI families was 78, a significant number which may provide some insights into the genetic causes of hereditary NDI.

Materials and methods

All NDI families included in this study were referred to our department or visited our outpatient clinic for analysis of gene mutations. Diagnosis of NDI was made in individual facilities based on family histories, clinical symptoms (polyuria and polydipsia, fever due to dehydration), laboratory data (urine and serum osmolalities, serum sodium, serum vasopressin), and responses to water deprivation and/or vasopressin administration. Subjects who presented a milder form of NDI (partial NDI), such as having weaker responses to water deprivation and/or vasopressin administration, were included in this study. Written informed consent for gene mutation analysis was obtained in individual facility. Mutation analyses were performed in our laboratory for most families. Some earlier cases were analyzed in Daniel Bichet's laboratory in Montreal and reported previously [11]. Also, several cases have been reported separately before [12–16]. The AVPR2 and AQP2 genes are relatively small and all exons and intron–exon boundaries were sequenced with usual sequencing methods [12, 17, 18]. Usually, mutation analysis of AVPR2 was performed first. If no causative mutations were found, then AQP2 was analyzed.

Results and discussion

Causative genes in Japanese NDI families

A total of 78 families were referred to us and gene mutation analyses were performed for the AVPR2 and AQP2 genes (Table 1). Gene mutations that presumably cause NDI were identified in the AVPR2 gene in 62 families (79 %), and in the AQP2 gene in nine families (12 %). In the remaining seven families, no mutations were detected in either the AVPR2 or AQP2 genes (Table 1). Of these 78 families, 62 families were newly examined and reported in

Table 1 Causative genes in Japanese Nephrogenic diabetes insipidus (NDI) families

Causative genes	Number of families
<i>AVPR2</i>	62 (79 %)
New in this report	49
Previously reported	13
<i>AQP2</i>	9 (12 %)
New in this study	6
Previously reported	3
Not found	7 (9 %)
Total	78

this paper. A total of 22 novel putatively disease-causing mutations that have not been previously reported or included in the public database (HGMD: <http://www.hgmd.cf.ac.uk/ac/index.php>) were identified in this study (19 in AVPR2 and 3 in AQP2).

If the seven families with no mutations are excluded, AVPR2 accounts for 87 % of gene defect-identified cases, while AQP2 accounts for 13 %. These data provide clear evidence for the general assumption that 90 % of cases are caused by AVPR2 and 10 % are caused by AQP2 mutations [1, 3]. These data also indicate that the genetic mechanisms for congenital NDI are the same in the Japanese population.

More than 220 disease-causing mutations have been reported for AVPR2 [19], and 50 disease-causing mutations have been reported for AQP2 [7, 20]. Our present report of 22 new putatively disease-causing mutations significantly increases the numbers of known NDI-causing mutations by about 10 %. When new mutations are found, it must be determined if they are disease causative or not. This determination is usually done by examining associations between the genotype and phenotype within the family (a comparison of subjects harboring the mutation and expression of clinical symptoms) and by examining functions of the mutated gene product in heterologous expression systems. In the present study, the functions of the mutant proteins were not examined, which is a limitation of the present study.

Disease-causing AVPR2 mutations in 62 NDI families

A total of 52 putative disease-causing AVPR2 mutations were identified in 62 families (several mutations were shared by different independent families). Table 2 summarizes the types of AVPR2 mutations. Gene variants/polymorphisms that have been reported not to cause NDI [19] were excluded in this summary. Missense mutations were most common, accounting for half of the mutations, followed by deletion mutations, insertion mutations, and

nonsense mutations. Splicing mutations were the least common. This relative frequency of disease-causing AVPR2 mutations is consistent with the results of a worldwide summary of AVPR2 mutations, as shown in Table 2 [19], again confirming that the genetic mechanisms causing NDI are the same in different ethnic groups [19].

Of these AVPR2 mutations, 19 mutations were novel, and the other mutations were previously reported or recurrences of the previously reported mutations. Details of the novel AVPR2 mutations are summarized in Table 3. In brief, in a family carrying the missense mutation D85E, an index subject was a female patient manifesting complete NDI, and her father also manifested NDI. The index subject was heterozygous for this mutation. The codon Asp85 seems functionally important, because another missense mutation on this residue, D85N, was reportedly causative in six families [19]. L90P was observed in two unrelated families. In one family, the index case was a mother of a boy with NDI; they manifested partial and complete NDI, respectively, and the mother was a heterozygous carrier of the mutation. In another family, a boy showed complete NDI, and his mother was a heterozygous carrier of the mutation with no NDI symptoms. K116N mutation was found in a boy with complete NDI, and his mother was not a carrier of the mutation, implying that the mutation occurred de novo. M123R mutation was observed in two unrelated families in which the index patients were boys with complete NDI. DNA samples of other members of the families were not available, and a mother in one family had polyuria and polydipsia. M123R has not been previously reported, but another mutation on this residue, M123K, has been reported [11]. L131P were found in two young brothers with complete NDI, and their mother was a heterozygous carrier of the mutation with no NDI symptoms. Mutation on Leu131 has not been reported, but missense mutations on encompassing residues, I130L, I130F and A132D have been shown to be causative [19], indicating that this region is functionally important. W164R was found in a boy with NDI, and his mother was a heterozygous carrier of the mutation. On Trp164, another mutation, W164S, has been reported [17], and mutations on Ala165 and Ser167 were also shown to be causative [19]. Q225R was found in a boy with complete NDI, and his mother was a heterozygous carrier without symptoms, while his healthy brother was not affected. L316R was found in a boy with complete NDI, and his mother was a heterozygous carrier. Leu316 has not been the target of missense mutations, while encompassing residues Ser315 and Asn317 located in the 7th transmembrane domain of AVPR2 protein are the target of disease-causing mutations, S315R and N317K [19]. S329G was found in a boy with complete NDI. His mother and grandmother were asymptomatic heterozygous carriers of the mutation, and his uncle had the same

Table 2 Types of AVPR2 mutations in Japanese Nephrogenic diabetes insipidus (NDI) patients and comparison with a global summary

Types of mutations	Number of mutations identified in Japanese patients ^a	Relative frequency in a global summary ^b (%)
Missense	28 (54 %)	56
Nonsense	4 (8 %)	13
Deletion	13 (25 %)	29
Insertion	5 (10 %)	4
Splicing	2 (4 %)	1

^a A total 52 mutations were identified in this study

^b Relative frequency reported by Spanakis et al. [19]

Table 3 New putative disease-causing AVPR2 mutation

	Nucleotide change	Amino acid change
Missense	c.255C>A	D85E
	c.269T>C	L90P
	c.348G>C	K116N
	c.368T>G	M123R
	c.392T>C	L131P
	c.490T>C	W164R
	c.674A>G	Q225R
	c.947T>G	L316R
	c.985A>G	S329G
	c.985_986AG>CC	S329P
Nonsense	c.624G>A	W208X
Deletion	c.91_92 del AC	FS/190X
	c.521delA	FS/211X
	c.1055_1068delGTCCCCAAGATGAG 5'UTR-AVPR2_DEL 4,586 5'UTR-AVPR2_DEL 32,787	FS/376X Large del of AVPR2 Large del of AVPR2
Insertion	c.369_370insT	FS/191X
	c.498_499insTC	FS/212X
	c.738_739insG	FS/257X

mutation with complete NDI symptoms. S329P was found in a boy with complete NDI, and his mother was an asymptomatic heterozygous carrier. Another mutation on Ser329, S329R, has been reported [21].

A nonsense mutation, W208X, was observed in a boy with complete NDI, and his asymptomatic mother and sister were heterozygous carriers of the mutation. To date, all reported nonsense mutations have been shown causative [19].

Five novel deletion mutations were found, and all these mutations cause either large losses of the gene, including the 5' untranslated region (two families), or frame shifts that result in premature truncation (two families) or

elongation (one family) of the coded proteins (Table 3). In a family with a 32,787 nucleotides deletion (the exact deletion size was determined in Daniel Bichet's lab in Montreal), two affected brothers showed complete NDI. Their mother and sister were asymptomatic heterozygous carriers of the mutation. In another family having a large deletion (4,586 nucleotides), a boy was affected with complete NDI and his mother was a heterozygous carrier. A 1-nucleotide deletion was observed in a complete NDI boy, and his mother was a heterozygous carrier of the mutation. In two families having 2-nucleotide or 14-nucleotide deletion mutations, the index patients were females who were diagnosed as having complete NDI after stimulation tests.

Three novel small (1–2 nucleotides) frame-shift insertion mutations were found in three families in which the index patients were males with complete NDI. All of these mutations are expected to introduce a premature stop codon, and the mutations were conserved within the families (Table 3).

Frequency of symptomatic carriers of AVPR2 mutations

Carriers of disease-causing mutations of AVPR2 (females having heterozygous mutations) sometimes manifest NDI symptoms [22, 23]; however, it is unknown how often this event occurs. In our present study, in 52 NDI families with AVPR2 mutations, at least one female member (usually a mother of an affected boy) were genetically analyzed and found to have the disease-causing allele. In a total of 64 such female subjects, 16 (25 %) had symptoms of polyuria and polydipsia, while 43 (67 %) were asymptomatic. Among the 16 symptomatic female subjects, 4 were diagnosed as having complete NDI, and 3 were the probands in each family. The types of mutations identified in these symptomatic carriers were: missense mutations (8), deletion mutations (6), nonsense mutation (1), and insertion mutation (1), indicating that this event occurs in any type of mutation.

The mechanism for the appearance of NDI symptoms in female carriers is explained by an extremely skewed inactivation of the normal allele of the X chromosome [24]; the frequency of this event was estimated to be very rare [9]. However, a recent study by Sato et al. [25] showed that a moderately skewed inactivation of the normal allele is enough to cause NDI symptoms. This result implies that symptomatic female carriers occur more often than previously thought. Our data are consistent with this speculation, and show that one fourth of carriers of AVPR2 disease-causing mutations present NDI symptoms. Thus, female patients with NDI symptoms require a careful examination, and gene mutation analysis for AVPR2 should be considered if other causes are unlikely.

AQP2 mutations causing NDI

Nine AQP2 mutations were identified in 9 NDI families (Table 4). The results from 3 of these families were previously reported [12]. These three families had monoallelic frame-shift deletion mutations (1–10 nucleotides) in the C-terminus of AQP2 (different mutations in each family), and showed an autosomal dominant inheritance with a slightly milder form of NDI [12]. The remaining six families were newly analyzed in the present study, and 6 different NDI-causing mutations were found (Table 4). These mutations consisted of 3 missense mutations and 3 deletion mutations (1–2 nucleotides deletions); 3 of them were novel mutations, and other three were recurrences of previously known mutations. Two missense mutations and one deletion mutation showed a recessive inheritance mode, while one missense mutation and two small deletion mutations manifested a dominant inheritance mode.

The family trees and results of mutation analysis of newly analyzed families are summarized in Fig. 1. In family 1, two missense mutations (A19V and G29S) were compound heterozygous in a male NDI patient and manifested by vasopressin-unresponsive polyuria (8–10 L/day). The patient's parents were asymptomatic. The father carried a novel A19V mutation, while the mother had a G29S mutation, which was previously reported to be causative [26]. In family 2, the G29S mutation (the same one found in family 1) was homozygous in the proband,

Table 4 Disease-causing *AQP2* mutations

Nucleotide change	Amino acid change	Inheritance mode	References
Newly analyzed			
Missense mutations			
c.56C>T	A19V	Recessive	New
c.85G>A	G29S	Recessive	Sahakitrungruang et al. [26]
c.761G>A	R254Q	Dominant	Savelkoul et al. [28]
Deletion mutations			
c.127_128delCA	FS/105X	Recessive	Tajima et al. [27]
c.750delIG	FS/334X	Dominant	New
c.775delIC	FS/334X	Dominant	New
Previously analyzed			
Deletion mutations			
c.721delG	FS/334X	Dominant	Kuwahara et al. [12]
c.763–772del	FS/331X	Dominant	Kuwahara et al. [12]
c.812–818del	FS/332X	Dominant	Kuwahara et al. [12]

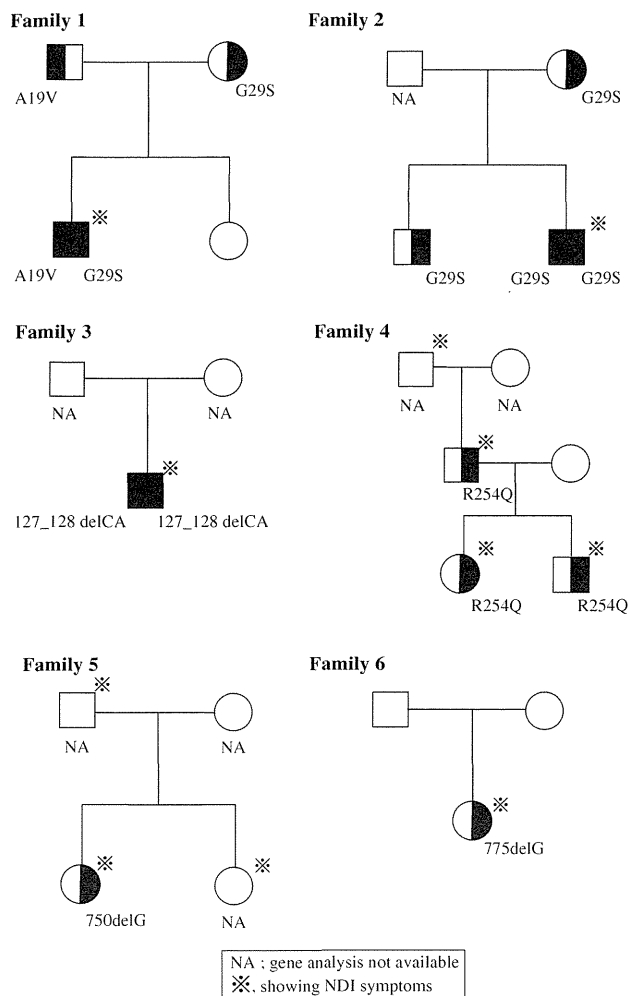


Fig. 1 AQP2 mutations newly found in Japanese NDI families. Six different AQP2 mutations were found in six Japanese NDI families. NA gene analysis not available. *Showing NDI symptoms

and his healthy mother and brother were heterozygous for the mutation. The patient exhibited polyuria (urine volume was 10–15 L/day), and the urine osmolality did not respond to vasopressin (maximum urine osmolality was about 100 mOsm/L). The appearance of NDI symptoms only when the mutations are compound heterozygous or homozygous strongly indicates that these two missense mutations are disease causative.

In family 3, a homozygous 2-nucleotide deletion mutation (c.127_128delCA) was found in a neonatal boy who exhibited polyuria and dehydration. His urine osmolality did not respond to vasopressin (< 150 mOsm/L). The resultant frame shift predicts new amino acids starting at codon 43, with a premature stop at codon 105. The same mutation was found in an unrelated Japanese family and has been reported by others [27].

In family 4, a monoallelic R254Q mutation was found in two siblings and their father. The father and paternal

relatives had NDI symptoms, but have not been clinically examined. The siblings (a 1-year-old boy and a 3-year-old girl) showed similar clinical characteristics of polyuria and polydipsia starting 4–6 months after birth, and slight responsiveness of urine osmolality to vasopressin (maximum urine osmolality was about 500 mOsm/L after vasopressin administration). Consistent with these observation, this mutation (R254Q) was recently reported as an NDI causative mutation with dominant inheritance [28]. Another missense mutation on this residue, R254L, was also reported to cause a similar NDI phenotype [29]. In the heterologous expression studies, the R254Q-AQP2 protein was impaired in its AVP-elicited phosphorylation of Ser256, which is a key step for AQP2 trafficking to the apical membrane [28]. The dominant inheritance can be explained by hetero-oligomerization of wild-type/mutant AQP2 proteins and dominant-negative effect of mutant protein on wild-type protein [7].

In a female patient of family 5, a novel heterozygous 1-nucleotide deletion mutation (750delG) was found. The patient’s sister and father were symptomatic. Her urine osmolality did not respond to vasopressin. This mutation causes a frame shift, with a new amino acids sequence starting from Val251 and ending at codon 334 in the C-terminal of AQP2. In Family 6, a 2-year-old girl was found to have a novel heterozygous 1-nucleotide deletion mutation (775delC) that causes frame shift with a new C-terminus starting at Leu259. The parents did not show NDI symptoms and did not carry the mutation, which indicated that the mutation occurred de novo. The girl showed polyuria and polydipsia and NDI was diagnosed by water deprivation and vasopressin administration tests. These identified two deletion mutations cause frame shifts from Val251 and Leu259 and a new C-terminal tail ending at codon 334 (Table 4). We previously reported three small deletion mutations in the C-terminus that cause similar frame shifts and show dominant inheritance [12] (Table 4). These frame-shift mutations share the loss of the last tail of the AQP2 protein, the site where PDZ proteins and ubiquitines interact, and the presence of extended C-terminal tails that contain missorting signals. As a result of these effects, these mutant AQP2 proteins making tetramers with wild-type proteins are incorrectly translocated to the basolateral membrane instead of the apical membrane [20, 30, 31]. This missorting is confirmed in knockin mice harboring a human C-terminal deletion mutation (c.763–772del) [32]. It is interesting that these deletion mutations are observed more often than missense mutations in Japanese patients, which is different from the frequencies in a total global summary [3, 20].

We could not detect mutations in the two genes in seven families (9 %, Table 1). It is said that causative gene mutations cannot be found in approximately 5 % of all

congenital NDI patients [4]. Possibilities such as the presence of mutations in the promoter regions of the AVPR2 or AQP2 genes are a likely explanation [4]. Our mutational analysis does not usually cover the promoter regions; thus, this possibility remains to be examined. To date, no genes other than AVPR2 and AQP2 have been attributed to NDI. However, it is possible that mutations in the genes encoding signaling cascade molecules connecting these two key membrane proteins cause NDI. Progress in gene mutational analysis methods such as whole-exome sequencing will address this possibility.

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Conflict of interest None.

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