Novel KCNQ4 variants in different functional domains confer genotype- and mechanism-based therapeutics in patients with nonsyndromic hearing loss

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INTRODUCTION

The KCNQ4 (Kv7.4) gene, which encodes a voltage-gated potassium channel protein, can cause autosomal dominant nonsyndromic hearing loss (DFNA2) when mutated1–4, accounting for approximately 9% of all ADNSHL cases5. To date, over 30 variants have been shown to cause nonsyndromic sensorineural hearing loss (NSHL)5–8. KCNQ4 is highly expressed in the basolateral membrane of outer hair cells (OHCs) in the cochlea and is involved in the formation of M-type potassium currents that repolarize the cells, reduce cell excitability, and regulate many physiological responses1,9. KCNQ4 plays an essential role in the recycling of potassium ions (K⁺), allowing for sound transduction and maintenance of the resting membrane potential and osmotic equilibrium10. Correspondingly, kcnq4−/− mice primarily show degeneration of OHCs and progressive hearing loss, mirroring DFNA2 in humans11.

The KCNQ4 protein comprises six transmembrane domains (S1–S6), namely, four voltage-sensor domains (S1–S4) and a pore region (S5, pore loop, and S6); the N- and C-termini are intracellular6. As KCNQ4 channels form homo- and heteromeric assemblies of the four pore-forming subunits, variants in a single subunit may disrupt the channel function of the tetramer via dominant-negative inhibition12. The majority of KCNQ4 variants responsible for DFNA2 are clustered in the S5–S6 region, surrounding the ion permeating pore region (amino acids 271–292). Pore variants, particularly in the potassium ion selectivity...
filter with a signature GYG motif or core pore domain are closely associated with loss-of-function; disruption of ion permeation, independent of channel gating, and dominant-negative inhibitory effects have been suggested as the pathogenesis underlying DFNA2.

Voltage-gated ion channels are promising drug targets. The most popular strategies for modulating voltage-gated ion channel function with small molecules have been targeted at voltage-sensor domain (VSD) activation, pore domain (PD) opening, or permeation. To the best of our knowledge, only channel openers have been reported as potent molecules for restoring impaired KCNQ4 channel activity. However, channel openers primarily modify gating kinetics rather than ion conductance. Thus, KCNQ4 mutant channels containing pore variants tend to be less amenable to the pharmacological application of channel openers. Recently, VSD-PD coupling has emerged as a potential pharmacological target, regulating a current without significantly changing the critical properties of voltage dependence and time dependence. In addition, the phospholipid phosphatidylinositol 4,5-phosphate 5-kinase (PIP5K) has been reported to act as an obligate ligand that activates KCNQ channels by regulating the VSD-PD coupling state. More specifically, defective residues in potential PIP2-binding sites in KCNQ channels have been reported to affect gating kinetics and regulate the specificity of tetramer assembly, leading to various human phenotypes, such as long QT syndrome and epilepsy. Moreover, a structural target for PIP2 binding related to VSD-PD coupling has been revealed, as evidenced by cryo-EM-based KCNQ4 structures, including KCNQ4 and thus, PIP2 regulation may contribute to the development of DFNA2 therapeutics.

Here, we introduce two novel DFNA2-causing missense KCNQ4 variants located far distal to the essential pore-loop region, where loss-of-function variants occur. The p.Gly319Asp (p.G319D) variant involves the C-terminal part of the transmembrane S6 segment; p.Asp311Gln (p.R331Q) is in the proximal part of the cytoplasmic C-terminus, which has been considered a potential PIP2-binding domain. Considering that PIP2 binding guarantees KCNQ4 channel activity and regulates pharmacological sensitivity to KCNQ channel openers, these variants are potential targets for pharmacological therapy to restore lost function.

**MATERIALS AND METHODS**

**Subjects**
All procedures in this study were approved by the Institutional Review Boards of Seoul National University Hospital (IRB-H-2005-041-281) and Seoul National University Bundang Hospital (IRB-B-1007-105-402). Four families (SB228, SB155, SB356, and SB62) were enrolled. The affected individuals underwent comprehensive phenotypic evaluations, including medical history interviews, physical examinations, imaging, and audio-lingual assessments.

**Molecular genetic testing and diagnosis**
As previously described, exome sequencing was performed followed by bioinformatics analyses. Rare single-nucleotide variations, indels, or splice-site variations were chosen using a comprehensive filtering process. The pathogenic potential of novel variants was evaluated according to the American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) guidelines.

**Plasmid construction, cell culture, and transfection**
The WT KCNQ4 cDNA was cloned into the SgfI and KgoCl sites of pEGFP (enhanced green fluorescent protein)-N1. The monkey embryonic kidney cell line COS-7 (Korea Culture Line Bank, Korea) was maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. Before transient transfection, the cells were plated at a density of 70–80% confluency in a lab Tek II chamber. The cells were transfected with a plasmid expressing wild-type or mutant KCNQ4 using Lipofectamine 3000 (Invitrogen, Seoul, Korea) and incubated at 37 °C for 24 hours. The cells were then stained with concanavalin A.

**Chemicals and solutions for electrophysiology**
The external bath solution for whole-cell voltage-clamp recording consisted of 147 mM NaCl, 5 mM KCl, 1.5 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, and 10 mM D-glucose, adjusted to pH 7.4 with N-methyl-D-glucamine (NMDG). The internal patch pipette solution contained 130 mM KCl, 10 mM NaCl, 10 mM EGTA, 10 mM HEPES, 3 mM Mg-ATP, and 0.5 mM CaCl2, adjusted to pH 7.0 with HCl. The calculated free Ca2+ concentration was ~10−10 M. Linopirdine dihydrochloride (Tocris Bioscience, Bristol, UK), retigabine (Glentham Life Science, Corsham, UK), ML213 (Tocris Bioscience, Bristol, UK), and zinc pyrithione (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in DMSO and prepared as 1000x stock (5-30 mM) solutions. Each stock solution was diluted into the external bath solution before use.
Evaluation of cellular trafficking of wild-type and mutant KCNQ4

After incubation, transfected cells were fixed in 4% paraformaldehyde for 15 min, followed by washing with PBS, which was repeated three times. The cells were incubated with primary antibodies (ANTI-FLAG Sigma-Aldrich Corp., St. Louis, MO, USA) at 24 °C for 160 min, washed three times with chilled (4 °C) PBS, and incubated with secondary antibodies (Fab2/goat antimouse IgG (H + L), Invitrogen, Seoul, Korea) at room temperature for 90 min. The samples were mounted with VECTASHIELD mounting medium (Vector Laboratories, CA, USA), and images were taken using a confocal microscope (Carl Zeiss, LSM710).

Cell surface biotinylation assay

A total of 5.0 × 10⁴ COS-7 cells (in a T75 flask) were transfected with KCNQ4 WT and mutant plasmids (WT, p.R331Q and p.A271_D272del) using Lipofectamine Plus reagent (Life Technologies, Inc.) and incubated at 5% KCNQ4 proteins on the cell surface) were isolated using a PIERCE™ cell surface protein isolation kit (Thermo Scientific) and analyzed with a Myc-tag (Abcam)-based ELISA kit (Thermo Scientific).

Statistical analysis

Data were plotted with Origin software (version 6.1, OriginLab), and the results are shown as the mean ± standard error of the mean (SEM), with n denoting the sample number. Statistical significance was determined by paired or unpaired Student’s t-test or ANOVA, and differences were considered significant at P < 0.05.

RESULTS

Clinical phenotypes of four KCNQ4 variants

Notably, diverse audiological configurations were observed in four DFNA2 families with segregating KCNQ4 variants. Audiological configurations were classified into three types: high-frequency (Families SB228 and SB155), mid-frequency (SB356), and low-frequency (SB62) hearing loss (Fig. 1a). SNHL in families SB228, SB356, and SB62 was autosomal dominant; that in SB155 was sporadic (Fig. 1a). The age of onset for SNHL and the age of ascertainment for proband SB228-442 were in the early 20s and at 33 years, respectively. The affected individuals in the SB228 family, including the proband’s sibling and father, developed high-frequency hearing loss in their late 20s and at the time of the study were using hearing aids due to progressive hearing deterioration. In the SB155 family, proband SB155-272 initially exhibited minimally progressive symmetrical high-frequency hearing loss with a downslipping configuration at the age of 11 years (Fig. 1a). Contrary to family SB228, both parents in SB155 had normal hearing thresholds across all frequencies. In the SB356 family, proband SB356-697 (33 years of age at ascertainment) manifested moderate-to-severe hearing loss, displaying predominantly mid-frequency hearing loss (symmetric notch at 1 kHz up to 60 dB HL in both ears), namely, cookie-bite configuration (Fig. 1a). However, there was no change in hearing thresholds over a 1-year follow-up period in the affected individual. The hearing status of the proband’s father was allegedly poor but had not been documented. In the SB62 family, proband SB62-110 (58 years of age at ascertainment) manifested symmetrical low- and mid-frequency hearing loss of approximately 40 dB HL (Fig. 1a). The pedigree indicated an autosomal dominant inheritance of hearing loss. During the 10-year follow-up period, SB62-110 experienced a definite progressive deterioration of bilateral hearing across all frequencies, as well as decreased speech discrimination scores. The proband’s audiogram eventually showed hearing thresholds at all frequencies of 65 dB HL in both ears. The proband’s mother had also experienced hearing loss in both ears at the age of 60 years; however, a corresponding hearing test had not been performed. No cochleovestibular malformations were noted by radiological evaluation in the affected individuals.

Identification of novel KCNQ4 variants

We identified four potential causative variants of SNHL: three are novel (c.811_816del:p.A271_D272del (SB155), c.G956 > A:p.G319D (SB356), and c.G992 > A:p.R331Q of KCNQ4 (SB62)) (Fig. 1b). The other is a previously reported in-frame deletion (c.806_808del:p.S269del) found in SB228.21,25 Specifically, none of the biological parents in SB155 were phenotypically affected, nor did they carry p.A271_D272del, as evidenced by haplotype phasing from trio ES data26,27, thus indicating that the p.A271_D272del arose de novo as a causative variant in the SB155 family (Fig. 1b). As confirmed by haplotype phasing from trio ES data26,27, the biological parents in SB155 were not phenotypically affected or carriers of the variant. The novel in-frame deletion variant, p.A271_D272del is located in the pore-loop domain of KCNQ4 (Fig. S1), where the majority of known KCNQ4 variants are linked to the hearing loss cluster. This deletion of six bases (GCCGAC) results in the loss of alanine and aspartic acid. This deletion is not listed in Korean Reference Genome Database (KRGDB, 1722 individuals); global minor allele frequency, including in Exome Aggregation Consortium (ExAC) and Genome Aggregation Database (gnomAD) (http://gnomad.broadinstitute.org/), was not found. These residues are highly conserved among KCNQ4 orthologs (http://genome.ucsc.edu/) (Fig. 1c), coupled with a high Genomic Evolutionary Rate Profiling (GERP +++) score of 5.08. This variant is consistently predicted as “disease-causing” through in silico analyses, including combined annotation-dependent depletion (CADD) (https://cadd.gs.washington.edu/). Confirmation of de novo occurrence of p.A271_D272del indicates “moderate evidence” for PS2 in relation to hearing loss. Moreover, PM1 can be applied because the variant p.A271_D272del is located in the KCNQ4 pore-forming region, as evidenced by a well-studied functional domain without a benign variant. Therefore, the variant p.A271_D272del may be classified as “likely pathogenic” based on the ACMG/AMP guidelines used to classify variants, including PS2, moderate, PM1, PM2, and PS3 supporting (Table 1).

Another novel missense variant, c.G956 > A:p.G319D (SB356 family) in the distal end of the transmembrane S6 segment (Fig. S1) is highly conserved among KCNQ4 orthologs (Fig. 1c), as supported by a high GERP ++ score of 5.27. The variant was not found in public databases. This variant is consistently predicted as “disease-causing” by in silico analyses, including CADD and rare exome variant ensemble learner (REVEL) (https://sites.google.com/site/revelgenomics/). Accordingly, p.G319D can be classified as a “variant of uncertain significance (VUS)” based on ACMG/AMP guidelines (Table 1).

The last novel missense variant, p.R331Q (SB62 family), is the first reported variant involving the proximal cytoplasmic C-terminus (Fig. S1). This residue is highly conserved among KCNQ4 orthologs (Fig. 1c), as reflected by a high GERP ++ score of 5.44. This variant has an extremely low MAF, satisfying PM2 criteria. CADD and REVEL show higher scores of 33 and 0.768, respectively, indicating “disease-causing” pathogenicity. Additionally, the mutant protein exhibited almost completely abrogated voltage-activated potassium currents compared with the WT protein (Fig. 2), supporting PS3. PS3 can also be applied due to a higher REVEL score of 0.8. Collectively, the p. R331Q variant was classified as “VUS” (Table 1).

Effects of novel variants on KCNQ4 channel function

To test the effects of KCNQ4 variants on voltage-gated channel activity, we recorded whole-cell currents in HEK293T cells transiently transfected with plasmids expressing the WT and mutant proteins via patch-clamp recording. Whole-cell currents for HEK293T cells transfected with the empty pRK5 and GFP vectors were used as negative control; cells transfected with p.S269del KCNQ4, which is known to cause DFNA2, were used as a null-function control. The linopirdine-sensitive potassium currents of the WT protein exhibited a typical KCNQ4 channel current, with voltage-dependent slow activation and outward rectification (Fig. 2a, b).
Cells expressing the WT protein exhibited voltage-dependent potassium currents with a peak current density of 121.8 ± 25.4 pA/pF at +40 mV ($n = 12$). In contrast, the outward potassium currents generated by mutant channels (p.A271_D272del, p.R331Q, and p.G319D) were barely detectable, similar to the null-function and negative controls (Fig. 2b). Thus, our newly identified KCNQ4 variants all showed complete loss of function in a homologous expression setting.

We further investigated whether the lost channel activity of these mutant proteins, especially in a homologous setting was restored by known KCNQ channel openers, such as retigabine (Ret, 10 μM), zinc pyrithione (ZnPy, 10 μM), or a combination of them (Ret/ZnPy). The combined application of channel openers (Ret/ZnPy) potentiated the KCNQ4-mediated potassium currents of the WT protein by more than twofold (Fig. 2c, d). However, homotetrameric channels containing the novel KCNQ4 variants (p.A271_D272del, p.R331Q, and p.G319D) displayed almost no potassium current, even with channel openers, and the currents were comparable to those in the null-function and negative controls.

**Dominant-negative effects of novel KCNQ4 variants**

To better understand the pathogenesis of our novel KCNQ4 variants in a heteromeric system, we measured potassium currents in HEK293T cells coexpressing WT and each variant at various molar ratios (WT/variant ratios ranging from 4:0 to 0:4). The resulting KCNQ4-mediated potassium currents appeared to inversely correlate with the concentration of mutant cDNA (Fig. 2c).
Table 1. KCNQ4 variants in the current study and pathogenicity prediction analysis.

| HGVS | Zygosity | Inheritance | Acronyms | Criteria | Graded | Ethnicity | MAF Global | ACMG/AMP 2018 guidelines |
|------|----------|-------------|----------|----------|--------|-----------|-----------|--------------------------|
|      |          |             |          |          |        |           |           |                          |
|      |          |             |          |          |        |           |           |                          |
|      |          |             |          |          |        |           |           |                          |
|      |          |             |          |          |        |           |           |                          |
|      |          |             |          |          |        |           |           |                          |
|      |          |             |          |          |        |           |           |                          |
|      |          |             |          |          |        |           |           |                          |
|      |          |             |          |          |        |           |           |                          |
|      |          |             |          |          |        |           |           |                          |

SB228-442 Chr1:41285116-c.806_808del p.Ser269del
Het Dominant 19.1 NA 5.08 Absent Absent Absent PM1, PM2, PM4
Het De novo* 22.7 NA 5.08 Absent Absent Absent PS2_moderate, PS3_supporting
SB155-271 Chr1:41285121-c.811_816del p.Ala271_D272del
Het Dominant 29.7 0.938 5.27 Absent Absent Absent PM2, PP3, VUS
SB356-697 Chr1:41285847 c.956 G > A p.Gly319Asp (p.G319D)
SB62-110 Chr1:41285883 c.992 G > A p.Arg331Gln

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- **Table 1.** KCNQ4 variants in the current study and pathogenicity prediction analysis.
- **HGVS:** Human Genome Variation Society (https://www.hgvs.org/).
- **Sequence Variant Nomenclature** (http://varnomen.hgvs.org/).
- **CADD:** Combined Annotation Dependent Depletion (https://cadd.gs.washington.edu/).
- **REVEL:** Rare Exome Variant Ensemble Learner (https://sites.google.com/site/revelgenomics/).
- **KRGDB:** Korean Reference Genome Database (http://coda.nih.go.kr/coda/KRGDB/index.jsp).

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- *Note that the biological parents of SB155 were not phenotypically affected or carriers of the variant, as confirmed by haplotype phasing from trio exome sequencing data.*

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**WT-p.G319D concatemers exhibit hyperactive channel activity with negative shifts in the voltage dependence of activation**

The potassium currents of the heteromeric p.G319D channels were larger than those of homomeric WT channels when the cells expressed both the WT and p.G319D mutant proteins (3:1 and 2:2 ratios; Fig. S2b). Notably and unexpectedly, a significant difference in the mean value of linopirdine-sensitive currents was found for the WT-p.G319D concatemer (174.9 ± 51.5 pA/pF at +20 mV, P < 0.05) compared with that of WT-WT concatemers (95.2 ± 10.4 pA/pF; Fig. 2f). In another set of experiments, the total potassium current of the WT-p.G319D concatemer (198.7 ± 15.0 pA/pF at +40 mV) was approximately twice the amplitude of the WT-WT concatemer (110.2 ± 7.8 pA/pF) (Fig. 3a). Additionally, the WT-p. G319D concatemer showed a marked shift in the current-voltage (I-V) relationship (Fig. 2f and Fig. 3b) and the voltage dependence of activation toward negative potentials. The calculated half-activation voltage (V_{0.5}) of the WT-p.G319D concatemer was −38.2 ± 0.6 mV (V_{0.5} of the WT-WT concatemer = −21.1 ± 1.0 mV).
Based on these results, we concluded that p.G319D did not exert a dominant-negative inhibitory effect on WT channels, unlike the other novel variants, but that it is the first hypermorphic DFNA2 variant with a gain of function when heterotetramerized with WT KCNQ4 subunits. In addition, cell surface expression analyses showed that both mutant KCNQ4 (p.G319D and WT-p. G319D concatemer) and WT proteins localized to the plasma membrane (Fig. S4).
Rescue effects of the PIP5 kinase and KCNQ activators on the impaired potassium currents of novel KCNQ4 variants

Rescue effects of the PIP5 kinase and KCNQ activators on current density (pA/pF at +40 mV) and half-activation voltages (V_{0.5}, act, mV) measured from the KCNQ4 channels were shown in Table S1 and Table S2, respectively. Increasing the PIP2 concentration by expressing PIP5K enhanced WT and WT-WT channel currents by more than twofold (Fig. 3b, d), with a negative shift in V_{0.5} values by approximately –10 mV (Fig. 3c, e). With PIP5K expression, KCNQ channel openers (Ret 10 µM, ZnPy 10 µM, and ML213 3 µM) further enhanced channel currents in an additive fashion, but no clear potentiating effect was observed after combination
Fig. 3 Rescue effects of the PIP5 kinase on KCNQ4 mutant channels. a The effects of phosphatidylinositol 4-phosphate 5-kinase (PIP5K) expression were compared between homomeric mutant channels (p.R331Q and p.G319D) and heteromeric tandem concatemer channels assembled from WT-p.R331Q and WT-p.G319D. Total K⁺ currents were measured in the absence (−PIP5K) or the presence of PIP5K expression (+PIP5K) in HEK293T cells. b Comparison of I-V curves between homomeric and tandem concatemer channels assembled from WT-WT, WT-p.R331Q, and WT-p.G319D. c Steady-state activation curves of the homomeric and tandem concatemer channels are compared (n = 10–12). d, e Comparison of K⁺ current densities measured at −40 mV and half-activation voltages (V0.5). WT-p.R331Q and WT-p.G319D concatamer mutant channels, but not homomeric mutant channels, were activated by PIP5K d, with a concomitant shifting of activation curve to negative voltage ranges e. Two pore-region mutant channels (p.S269del and p.A271_D272del) were unresponsive to PIP5K expression. The horizontal dotted lines in the graphs indicate the values obtained from homomeric WT channels (WT). The names of the groups are indicated in insets on the graph. Mean ± SEM (n = 10–12); **P < 0.01, ***P < 0.005; NS, not significant.

**DISCUSSION**

In this study, we explored the application of genotype/pathophysiology-based customized pharmacology to functionally rescue impaired KCNQ4-mediated potassium currents related to novel DFNA2 variants. Our two novel missense mutant channels (p.R331Q and p.G319D) responded to PIP5K- and KCNQ-regulating drug treatment, but only when the variants were assembled with WT KCNQ4 subunits. Specifically, application of PIP5K, channel openers, or a combination of both, if not to the same degree, restored the ion conductance of the p.R331Q variant in the forced heteromeric state compared with the lack of response to any of these treatments in the homomeric state. Furthermore, the development of a customized pharmacological approach for DFNA2 variants in this study is especially fueled by the identification of the first hypermorphic DFNA2 variant, p.G319D, and this variant may be amenable to KCNQ channel inhibitors and PIP2 suppression to regulate excessive potassium outflow. Thus, our results suggest the potential for tailored therapeutics for KCNQ4 variants and contribute to future clinical practices with respect to DFNA2.

Previous studies have demonstrated that even a single-nucleotide change close to the pore region may drastically impede ion permeation, rendering channels nonfunctional, independent of channel gating. Similarly, the two KCNQ4 pore-region variants identified in our study produced no detectable potassium currents and were completely nonrescuable by the application of PIP5K, channel openers, or both. In contrast, channel openers were previously found to partially restore the activity of heterogeneous KCNQ4 channels containing pore variant subunits; however, this mitigation of dominant-negative inhibitory effects may have relied exclusively on the potentiation of residual homomeric WT channel function. Indeed, expression of homogenous forced heteromeric channels (i.e., tandem concatemers) was insensitive to pharmacological treatments, which resembles the previously reported unresponsiveness of WT:p.W276S or WT:p.G285S to a combination of channel openers. Collectively, the lack of effect of pharmacological treatment on channels containing the pore-region variants identified in our studies may be attributed to either the lack of residual homomeric WT channels in the concatemer status or the inability of residual homomeric WT channels to respond to channel openers, even under PIP5K-enhanced conditions, or both. Thus, further exploration of novel therapeutics to potentiate currents through totally abolished KCNQ4 channels due to pore-region DFNA2 variants is warranted, though such efforts with a focus on the enhancement of the specificity of KCNQ4 openers have already been initiated.

Channels containing a novel p.R331Q variant showed impaired potassium currents not only in homotetrameric states but also in heterotetrameric states, indicating its inhibitory mechanism on the pathogenesis of DFNA2. Of note, variants affecting basic and charged amino acids of the proximal C-terminus in most KCNQ
Fig. 4 Rescue effects of KCNQ openers on KCNQ4 mutant channels assembled from tandem concatemers. a Total K⁺ currents were measured in the absence (-PIP5K) or presence (+PIP5K) of PIP5K expression in HEK293T cells, and responses to KCNQ openers were recorded. Representative current traces recorded before (Ctrl) and after 10 μM ZnPy treatment are presented for each concatemer channel. b Rescue effects of KCNQ openers (10 μM Ret, 10 μM ZnPy, and 3 μM ML213) on KCNQ4 mutant channels assembled from homomeric settings or heteromeric tandem concatemers were compared in the absence or presence of PIP5K expression. Current densities measured at +40 mV were compared as bar graphs. c Half-activation voltages ($V_{0.5}$) were calculated from activation curves, and the values are plotted as bars. Horizontal dotted lines in the graphs indicate the current density level or $V_{0.5}$ values of the control homomeric WT channels (WT, Ctrl) measured in the absence of PIP5K and KCNQ openers. The names of the groups are indicated in insets on the graph. Mean ± SEM. n = 10–12.
channels are associated with various human diseases\(^\text{13}\). Moreover, variants in the proximal C-terminus of KCNQ channels have been shown to significantly attenuate affinity for PIP\(_2\)\(^\text{29}\), leading to the decoupling of the voltage-sensing domain (VSD) with the pore domain (PD) and resulting in the failure of pore opening. Indeed, the residues around Arg331 in the KCNQ4 channel are well conserved and are predicted to be a target region for PIP\(_2\) binding\(^\text{30,31}\). Expectedly, the interaction between PIP\(_2\) and the Arg331 residue of KCNQ4 was supported by Protein Data Bank (Fig. S5). Furthermore, KCNQ1 double variants (Lys358Ala/

Fig. 5 Downregulation of enhanced channel activity of WT-p.G319D tandem concatemers by a KCNQ inhibitor or screening of PIP\(_2\). Linopirdine (3–10 \(\mu\)M) inhibition of WT-p.G319D concatemer current is presented with I-V curves a and activation curves b. In the absence c, d or presence e, f of PIP5K expression, intracellularly applied poly-L-lysine (PLL, 10 or 30 \(\mu\)g/ml) attenuated the WT-p.G319D concatemer current c, e, with a concomitant shift of activation curves toward positive potentials d, f. The reducing effects of linopirdine and PLL on the WT-p. G319D channel current (\(n=10\)) were compared with the effects on the WT-WT current (\(n=6\)). Mean ± SEM.
Arg360Ala), located at the equivalent position of the proximal C-terminal residue (Arg331) of KCNQ4, show no ion conductance due to disrupted PIP2 binding affinity, leading to long QT syndrome. Based on these results, the null activity of KCNQ4 channels containing p.R331Q is likely due to an altered electrostatic interaction with PIP2 and subsequent abolishment of the ion conductance via impaired coupling with the PD and/or VSD.

Notably, the present study demonstrated that forced heteromerization of p.R331Q with the WT channel, which mimics the heterozygous condition in vivo, induced recovery of impaired channel activity through PIP5K expression but that tandem concatemers of the WT and pore-region variant were nonrescueable even at the same high PIP5K expression. This suggests that disrupted PIP2 binding sites due to p.R331Q could be somehow related to the pathophysiology of the loss of function of mutated KCNQ4 channels containing p.R331Q. Our hypothesis is partly corroborated by previous work by Soldovieri et al., who reported that PIP5K enables a homomeric KCNQ2 mutant channel containing a variant of Arg325 residue that is equivalent position to the Arg331 of KCNQ4 to potentiate potassium currents. Regardless, it remains elusive why the current is not restored by cotransfection of PIP5K with homomeric p.R331Q KCNQ4 channel plasmids. Unlike for p.R331Q, the more drastic dominant-negative effect of pore variants on the WT subunit precluded the efficacy of PIP2 and KCNQ openers because the variants result in the collapse of ion permeation sites.

It should also be noted that ZnPy has been reported to rescue KCNQ channel activity in the absence of PIP2, even though retigabine acts as a primary modifier of gating kinetics by stabilizing the conformation of pore and voltage sensors. In addition, potentiation of WT-p.R331Q concatemer channel activity by a combination of ZnPy and PIP5K reached a similar level to that of the ZnPy and PIP5K-applied homomeric WT channel (Fig. 4b), which was not observed with the combination of retigabine with PIP5K. In accordance, stronger potentiation of KCNQ4-mediated potassium currents in OHCs was previously demonstrated when retigabine was combined with ZnPy. Therefore, retigabine and ZnPy may not only complement each other but also exert synergistic effects when combined with outstripped WT channel activity in terms of potentiating defective KCNQ4-mediated potassium currents. Our results are clinically encouraging because channel openers significantly restored the channel activity of forced homogenous heteromeric channels (WT-p.R331Q) that mimic the heterozygous condition in vivo, particularly when a high concentration of PIP2 was maintained (Fig. 6a).

Another novel missense variant, p.G319D, which is located in the C-terminal part of the transmembrane S6 segment, produced a nonconducting KCNQ4 channel in a homologous setting, suggesting that it acts as a pathogenic variant. The Gly348 residue in KCNQ1, corresponding to Gly319 in KCNQ4, is critical to channel gating, as alteration of the residue produced a nonconducting KCNQ1 channel. A GSG motif containing a Gly348 residue and a highly conserved glycine residue is located in the C-terminal part of S6. The GSG motif of KCNQ1 specifically interacts with the S4–S5 linker and the S5 segment, playing a pivotal role in the opening and closing of the channel gate. Experimental substitution of Gly348 with an alanine residue (G348A) shifts the voltage dependence of KCNQ1 channel activation by 25 mV toward a negative potential in homomeric settings, whereas G348W fails to generate any potassium current. These observations are somewhat consistent with our observations, whereby WT-p.G319D shifted voltage-dependent activation by approximately 20 mV to a negative potential compared with homomeric WT channels (V0.5: −38.2 ± 0.5 mV vs. −18.5 ± 1.0 mV). Our findings, together with the study in KCNQ1, suggest that the C-terminal part of S6 with the GSG motif plays a pivotal role in regulating KCNQ4 channel gating and kinetics. Interestingly, the homomeric mutant channel containing p.G319D was completely unresponsive to channel openers;

**Fig. 6** Schematic illustration of different pathophysiological mechanisms of KCNQ4 variants on DFNA2. a Loss-of-function caused by KCNQ4 p.R331Q and its restoration following phospholipid phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) activator (i.e., PIP5K) and channel openers. b Gain of function caused by KCNQ4 p.G319D and its restoration following interference of PI(4,5)P2 activation by intracellular application of a polycation poly-L-lysine (PI(4,5)P2 inhibitor) and channel inhibitor.
though the current from a homomeric channel containing p.G319D was substantially restored by the combined application of both channel openers. These two variants are located in similar topologies while providing enigmatic disparity. The difference in sensitivity between the two variants to channel openers may depend on the amino acid substituted and the size of the channel current involved in gating.

Importantly, we observed that the forced homogenous heterotetrameric WT-p.G319D concatemer produced comparable—or even increased—potassium currents compared with the WT-WT concatemer. To the best of our knowledge, this is the first hypermorphic KCNQ4 variant reported. Our data indicate that the dominant-negative inhibitory effect does not underlie the pathogenesis of DFNA2 related to p.G319D. Considering that activation of residual homomeric WT channels is key in determining the pharmacological responsiveness of the tetrameric KCNQ4 variant channel, the WT-p.G319D concatemer, which is presumably free from a dominant-negative effect, is expected to retain more channel activity than other heteromeric mutant channels, showing dominant-negative inhibition. A previous study demonstrated different gating mechanisms in the KCNQ1 channel between the two variants p.G348W and p.G348A. The p.G348A variant affects the gating properties of the KCNQ1 channel in such a way that it shifts the voltage dependence of activation toward a negative potential, which is associated with the open state. Similarly, the p.G319D variant may contribute to the stabilization of gating kinetics in a sustained open state if assembled as a heterotetrameric channel combined with the WT protein, as in the heterogeneous condition. It is easily conceivable that defective plasma membrane trafficking would not be the main pathogenic mechanism of the loss-of-function caused by p.G319D, as based on the robustly enhanced channel activity observed with heterogeneous KCNQ4 channels and immunofluorescence experiments of the mutant KCNQ4 protein (p.G319D) (see Fig. S4). Instead, a sustained open state of the KCNQ4 channel predicted from p.G319D-mediated hyperactivation of gating may lead to oversecretion of $K^+$ through the KCNQ4 channel from OHCs, potentially leading to $K^+$ depletion in these cells. Furthermore, loss of intracellular $K^+$ in OHCs may elicit chronic hyperpolarization, possibly affecting Ca$^{2+}$ influx through voltage-gated Ca$^{2+}$ channels or Ca$^{2+}$-modulated proteins (calmodulin) and causing their subsequent degeneration due to chronic cellular stress. Given that $K^+$ is the major charge carrier for sensory transduction in the inner ear, chronic $K^+$ depletion may hinder its proper recycling and trigger neurotransmission for the process of hearing. This, in turn, suggests that the hypermorphic p.G319D variant may cause hearing loss. Although $K^+$ depletion in OHCs due to KCNQ4 overactivation may be a somewhat new finding in the literature, hypokalemia per se might be a manifestation of various diseases, making hypermorphic KCNQ4 variants clinically important.

From this perspective, therapeutic agents that ameliorate KCNQ4 channel activity while maintaining sufficient current levels may allow for rescue of the DFNA2 phenotype due to the hypermorphic KCNQ4 variant (Fig. 6b). We, for the first time, demonstrated that a KCNQ inhibitor and PIP2 interference could potentially stabilize the enhanced potassium currents of the WT-p.G319D concatemer in transfected cells to a similar level as the WT-WT concatemer, suggesting a potential therapeutic strategy for yet-to-be-identified hypermorphic KCNQ4 variants. We believe that the development of knock-in mice expressing this hypermorphic KCNQ4 variant would provide an in vivo tool to address in-depth questions and establish therapies for hypermorphic KCNQ4 variants.

In this study, we observed diverse audiogram configurations not limited to orthodox high-frequency specific hearing loss. In particular, nonpore-region KCNQ4 variants were associated with nontypical high-frequency hearing loss, though the small sample number precludes any correlation between domain and phenotype. A possible correlation between auditory phenotypes, such as the onset of disease and affected frequencies, and genotypes (i.e., missense vs. deletion) has been proposed. Nevertheless, additional studies are necessary to draw any meaningful conclusions. It appears that differential spatiotemporal expression of KCNQ4 in spiral ganglion cells and inner hair cells may account for highly complex auditory phenotypes. Alternatively, the pathogenic effect of nonpore-region variants may vary, depending on which KCNQ4 isoforms are mainly affected by the variant, as each isoform may show a different tonotopic distribution. Complex alternative splicing of human KCNQ4 isoforms may further diversify the functional consequences of KCNQ4 variants in terms of auditory phenotypes.

DATA AVAILABILITY
The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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