Insights into phenotypic differences between humans and mice with p.T721M and other C-terminal variants of the SLC26A4 gene

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Recessive variants of the SLC26A4 gene are an important cause of hereditary hearing impairment. Several transgenic mice with different Slc26a4 variants have been generated. However, none have recapitulated the auditory phenotypes in humans. Of the SLC26A4 variants identified thus far, the p.T721M variant is of interest, as it appears to confer a more severe pathogenicity than most of the other missense variants, but milder pathogenicity than non-sense and frameshift variants. Using a genotype-driven approach, we established a knock-in mouse model homozygous for p.T721M. To verify the pathogenicity of p.T721M, we generated mice with compound heterozygous variants by intercrossing Slc26a4<sup>T721M</sup>/T721M mice with Slc26a4<sup>919-2A>G</sup>/919-2A>G mice, which segregated the c.919-2A>G variant with abolished Slc26a4 function. We then performed serial audiological assessments, vestibular evaluations, and inner ear morphological studies. Surprisingly, both Slc26a4<sup>T721M/T721M</sup> and Slc26a4<sup>919-2A>G/T721M</sup> showed normal audiovestibular functions and inner ear morphology, indicating that p.T721M is non-pathogenic in mice and a single p.T721M allele is sufficient to maintain normal inner ear physiology. The evidence together with previous reports on mouse models with Slc26a4 p.C565Y and p.H723R variants, support our speculation that the absence of audiovestibular phenotypes in these mouse models could be attributed to different protein structures at the C-terminus of human and mouse pendrin.

Recessive variants in the SLC26A4 (PDS, GeneID: 5172) gene are a common cause of hereditary hearing impairment (HHI). In certain populations, SLC26A4 variants can be identified in approximately 15% to 20% of patients with HHI. SLC26A4 encodes pendrin, a chloride/iodide/bicarbonate transporter expressed in the thyroid, inner ears, kidneys, lungs, liver, and heart. Recessive SLC26A4 variants lead to Pendred syndrome (PS; MIM #274,600) and non-syndromic DFNB4 (MIM # 600,791). DFNB4 is characterized by isolated sensorineural hearing impairment (SNHI), which is associated with a common inner ear malformation called enlarged vestibular aqueduct (EVA; MIM 603,545), whereas patients with PS have goiter in addition to EVA. To date, approximately...
SLC26A4 variants have been identified (https://databases.lovd.nl/shared/genes/SLC26A4). Clinically, patients with SLC26A4 variants, either with DFNB4 or PS, usually suffer from progressive or fluctuating SNHI. The pathogenesis of SNHI in patients with DFNB4 and PS has been partially elucidated in various mouse models. Several mouse models have been generated, including knock-out Slc26a4-/- mice, Slc26a4loop/loop mice with the p.S408F variant, Slc26a4919-2A>G mice with the c.919–2 A > G variant, Slc26a4H723R/H723R mice with the p.H723R variant, Slc26a4 L236P/L236P mice with the p.L236P variant, Slc26a4 C565Y/C565Y mice with the p.C565Y variant, conditional knock-out Tg[E]; Tg[R]; Slc26a4Δ/Δ mice, and humanized hH723R Tg mice with the p.H723R variant in the human SLC26A4 sequence. None of these models recapitulated SNHI phenotypes in humans. Knock-out Slc26a4-/-, Slc26a4loop/loop, and Slc26a4919-2A>G mice showed congenital profound SNHI that was too severe compared to their human counterparts. Slc26a4H723R/H723R and Slc26a4 C565Y/C565Y mice showed normal hearing without any hearing loss phenotypes. Although Tg[E]; Tg[R]; Slc26a4Δ/Δ mice demonstrated hearing loss of various severities, doxycycline was required to induce the phenotype. The auditory phenotypes of Slc26a4 L236P/L236P and hH723R Tg mice were milder than those of Slc26a4-/-, Slc26a4loop/loop, and Slc26a4919-2A>G mice, yet the absence of progressive hearing loss could not reflect the clinical symptoms in patients with SLC26A4 mutations.

Parallel to the transgenic mouse models, studies on cell lines have provided crucial insights into the pathogenicity of SLC26A4 variants. It has been reported that different SLC26A4 variants may result in different degrees of protein misexpression and/or dysfunction. Among the SLC26A4 variants, whose pathogenicity has been investigated in cell lines, the p.T721M variant is especially interesting. The expression of pendrin in cell lines with p.T721M could not be rescued after salicylate treatment, but that in cell lines with p.H723R could, indicating that p.T721M might confer a stronger pathogenicity than p.H723R. Based on these results, we hypothesized that mice with the Slc26a4 p.T721M variant might exhibit auditory phenotypes milder than those of c.919-2A>G mice but more severe than those of p.H723R mice. In this study, we generated a knock-in mouse model with the p.T721M variant, as well as compound heterozygous (Slc26a4919-2A>G/T721M) mice in which we tried to manipulate the severity of phenotypes by abolishing the other functional Slc26a4 allele. We then characterized the audiovestibular phenotypes and inner ear pathology in these mouse models (Fig. 1).

Results

Auditory phenotypes. Wild-type mice (Slc26a4+/+), heterozygous mice (Slc26a4+/T721M), and homozygous mice (Slc26a4919-2A>G/T721M) (n = 10 each) were subjected to audiological evaluations at 12, 28, and 44 weeks (Fig. 2). Both Slc26a4+/T721M and Slc26a4919-2A>G/T721M mice had normal hearing up to 44 weeks, indicating that the p.T721M allele did not cause deafness in mice.
Figure 2. Auditory phenotypes. (A) The ABR waveforms in clicks did not differ significantly among heterozygous Slc26a4<sup>−/−</sup>/T721M, homozygous Slc26a4<sup>−/−</sup>/T721M/T721M, compound heterozygous Slc26a4<sup>919-2A>G/T721M</sup> and wild-type Slc26a4<sup>+/+</sup> mice. The ABR waveforms of Slc26a4<sup>919-2A>G/T721M</sup> mice with profound hearing loss were also determined for comparison. (B) Heterozygous, homozygous, and compound heterozygous mice showed normal hearing thresholds across different frequencies, similar to those in wild-type Slc26a4<sup>+/+</sup> mice. There were no significant differences among these four groups of mice at 12, 28, and 44 weeks.
To confirm the pathogenicity of the p.T721M allele in mice, we invalidated the Slc26a4 allele by intercrossing Slc26a4<sup>T721M</sup> mice with Slc26a4<sup>T721M</sup> mice to generate compound heterozygous mice (i.e., Slc26a4<sup>919-2A>G/T721M</sup>). Similar to heterozygous mice with the c.919-2A > G variant (i.e., Slc26a4<sup>T721M</sup> mice), Slc26a4<sup>919-2A>G/T721M</sup> mice (n = 10) had normal hearing up to 44 weeks. Furthermore, physical collisions such as falling from rotarods did not induce hearing loss in Slc26a4<sup>919-2A>G/T721M</sup> and Slc26a4<sup>919-2A>G/T721M</sup> mice (data not shown). These findings indicate that the p.T721M allele is not pathogenic and a single allele with p.T721M is sufficient to maintain the auditory function in mice with the p.T721M variant.

**Inner ear morphology and pendrin expression.** Cochlear morphology was investigated in homozygous (Slc26a4<sup>T721M/T721M</sup>) and compound heterozygous mice (Slc26a4<sup>919-2A>G/T721M</sup>). Cochlear morphologies of wild-type mice and profoundly deaf Slc26a4<sup>919-2A>G</sup> mice were also obtained for comparison. The endolymphatic sac was severely enlarged only in Slc26a4<sup>919-2A>G</sup> mice, and remained normal in size in Slc26a4<sup>T721M/T721M</sup> and Slc26a4<sup>919-2A>G/T721M</sup> mice (Fig. 3A). Similarly, abnormal morphological findings in Slc26a4<sup>919-2A>G/T721M</sup> mice, including dilatation of the scala media, atrophy of the stria vascularis, and degeneration of the cochlear hair cells, were not observed in Slc26a4<sup>T721M/T721M</sup> and Slc26a4<sup>919-2A>G/T721M</sup> mice (Fig. 3B–D). Quantitative analyses of the endolymphatic space revealed that the cross-sectional area of the scala media in Slc26a4<sup>919-2A>G</sup> mice was significantly larger than those in wild-type, Slc26a4<sup>T721M/T721M</sup> and Slc26a4<sup>919-2A>G/T721M</sup> mice (271.6 ± 2.2, 59.6 ± 2.0, 56.5 ± 3.1; 58.9 ± 2.2 *1000 μm², respectively, n = 3 each) (Fig. 3E).

We then examined the expression of pendrin in the cochlea of Slc26a4<sup>T721M/T721M</sup> and Slc26a4<sup>919-2A>G/T721M</sup> mice by immunolocalization (Fig. 3F). In both strains, pendrin was normally distributed in the spiral prominence and root cells, indicating that the expression of pendrin was normal in the p.T721M mice. In addition, the contour of the stria vascularis (indicated by white dashed lines in the figure) showed atrophic changes in Slc26a4<sup>919-2A>G</sup> mice, but not in Slc26a4<sup>T721M/T721M</sup> and Slc26a4<sup>919-2A>G/T721M</sup> mice.

**Vestibular function evaluation.** Vestibular morphology was also investigated in homozygous mice (Slc26a4<sup>T721M/T721M</sup>) and compound heterozygous mice (Slc26a4<sup>919-2A>G/T721M</sup>) (Fig. 4A). Both strains of mice showed normal vestibular morphologies. In contrast, giant otoliths were observed in Slc26a4<sup>919-2A>G</sup> mice. Fluorescence confocal microscopy demonstrated that vestibular hair cells in Slc26a4<sup>T721M/T721M</sup> and Slc26a4<sup>919-2A>G/T721M</sup> mice did not degenerate (Fig. 4B).

Fifteen mice of each group including Slc26a4<sup>T721M</sup>, Slc26a4<sup>T721M/T721M</sup>, and Slc26a4<sup>919-2A>G/T721M</sup> mice, were subjected to vestibular evaluations. Similar to the normal auditory phenotypes, neither heterozygous mice (i.e., Slc26a4<sup>T721M</sup> and Slc26a4<sup>919-2A>G/T721M</sup>) nor homozygous mice (i.e., Slc26a4<sup>T721M/T721M</sup>) showed vestibular deficits such as head tilting and circling behavior, and both groups performed normally on the rotarod (Fig. 4C) and in swimming tests (Fig. 4D). These findings indicate that a single p.T721M allele is sufficient to maintain normal vestibular function in mice.

**Discussion**

The knock-in mouse generated in this study, the Slc26a4<sup>T721M/T721M</sup> with the Slc26a4 p.T721M variant, demonstrated normal audiovestibular phenotypes and inner ear morphologies, similar to wild-type mice. To investigate whether the p.T721M variant could contribute to SNHI in mice through the haplo-insufficiency mode, we further generated mice with compound heterozygous variants (Slc26a4<sup>919-2A>G/T721M</sup>) by intercrossing Slc26a4<sup>T721M/T721M</sup> mice with Slc26a4<sup>919-2A>G</sup> mice, which segregated the c.919-2A > G variant with abolished function. Compound heterozygous mice for p.T721M and c.919-2A > G (i.e., Slc26a4<sup>919-2A>G/T721M</sup>) mice also had normal audiovestibular phenotypes, indicating that a single p.T721M allele was sufficient to maintain normal inner ear physiology in the mice.

The Slc26A4 p.T721M variant has been documented sporadically in hearing-impaired families worldwide, including two Mediterranean families, two Iranian families, two Japanese family, three Chinese families, and one Taiwanese family. Although this variant is widely distributed across different populations, its prevalence is relatively low compared to other Slc26A4 variants, such as c.919-2A > G or p.H723R. According
to the American College of Medical Genetics and Genomics (ACMG) guidelines in the Varsome platform, SLC26A4 p.T721M is classified as “pathogenic” by fulfilling the criteria of PM1, PM2, PP2, PP3, and PP5. It is located in the hotspot region of the SLC26A4 gene, where the majority of pathogenic variants occur (PM1). Its allele frequency is <0.0001 across various ethnic groups in gnomAD (PM2). The majority of non-VUS missense variants in SLC26A4 have been reported as “pathogenic” in UniProt (PP2). In well-established databases (e.g., ClinVar, DVG, and UniProt) and in several prediction algorithms (e.g., SIFT, Polyphen2, LRT, FATHMM, Mutation Taster, etc.), p.T721M was categorized as “pathogenic” (PP3 and PP5). Clinically, both homozygosity for p.T721M and compound heterozygosity for p.T721M with another SLC26A4 mutation have been linked to non-syndromic DFNB4 or PS, as characterized by EVA, progressive or fluctuating severe-to-profound SNHI.
and/or goiter. Specifically, the hearing features have been detailed in two compound heterozygotes, one with symmetric SNHI (right/left: 103.75/110 dBHL) and the other with asymmetric SNHI (right/left: 112.5/68.75 dBHL). These typical clinical manifestations in p.T721M homozygotes and compound heterozygotes suggest that p.T721M is a pathogenic SLC26A4 variant in humans.

In previous cell line studies, a number of SLC26A4 variants, including p.P123S, p.M147V, p.L236P, p.S657N, p.T721M, and p.H723R, have been demonstrated to confer pathogenicity by affecting the trafficking process, rather than the expression level of pendrin. However, the affected trafficking process can be rescued by certain treatments. Low-temperature incubation has been reported to rescue the trafficking of pendrin with p.H723R, which originally accumulated in the endoplasmic reticulum, but not the trafficking of pendrin with p.L236P, which originally accumulated in centrosomal regions. Similarly, salicylate treatment could rescue the trafficking of pendrin with p.P123S, p.M147V, p.S657N, and p.H723R, and restore the function of pendrin as an anion exchanger, but not that of pendrin with p.T721M. These lines of evidence also support the view that the SLC26A4 p.T721M variant is pathogenic and implies that p.T721M is more pathogenic compared to other missense SLC26A4 variants, such as p.H723R.

To our surprise, the pathogenicity of p.T721M as predicted by the ACMG guidelines and evidenced by the cell line studies was not observed in our mouse model with the Slc26a4 p.T721M variant. These findings are consistent with our previous studies in mouse models with Slc26a4 p.H723R and p.C565Y variants. In the cochlea of mice, pendrin is expressed at the spiral prominence and outer sulcus cells, which is almost the same as its expression in the cochlea of primates. Therefore, the position of pendrin expression does not appear to be a major factor contributing to the phenotypic discrepancy between species.

Alternatively, the inter-species phenotypic discrepancy may be attributed to the structural differences between mouse and human pendrin.

To date, five mouse models with missense Slc26a4 variants, including p.L236P, p.S408F, p.C565Y, p.T721M, and p.H723R, have been documented in the literature (Fig. 5A). Abnormal audiovestibular phenotypes were observed in mice with p.L236P and p.S408F, but not in mice with p.C565Y, p.T721M, and p.H723R. Notably, p.L236P and p.S408F are located in the transmembrane domains of pendrin. In contrast, p.C565Y, p.T721M, and p.H723R are located in the C-terminus of pendrin comprised of amino acid residues 508–780. From an evolutionary perspective, the amino acid sequence of the pendrin C-terminus is less conserved, sharing only 86% identity between mice and humans. In contrast, the amino acid sequence of the transmembrane domains shared 92% identity between the two species.
in anion-binding transmembrane domains (TMs), including TM1, 3, 8, and 10. The findings indicate that deafness-associated (i.e., mice with p.S408F).

Methods

Ethics statement. All animal experiments were carried out in accordance with animal welfare guidelines and approved by the Institutional Animal Care and Use Committee of the National Taiwan University College of Medicine (approval no. 20160337). Also, all animal experiments followed the guidelines of ARRIVE 2.0.

Construction of Slc26a4<sup>T721M/T721M</sup> knock-in mice. Transgenic mice were generated by the Transgenic Mouse Models Core (TMMC, Taiwan) using the CRISPR technology-associated RNA-guided endonuclease Cas9 to mutate the Slc26a4 gene and generate the Slc26a4<sup>T721M</sup> mouse line. Specific guiding RNAs (sgRNAs) were developed to target exon 15 of the Slc26a4 gene in C57BL/6 mice. The sgRNA and CRISPR/Cas9 RNA were delivered into the mouse zygote to generate founders. The two male founder mice that were obtained from each, harbored the p.T721M (c.2162C>T) variant in the Slc26a4 gene. After germline transmission of the targeted variant allele, we produced the congenic Slc26a4<sup>T721M/T721M</sup> mouse line used in this study by repeated backcrossing into the C57BL/6 inbred strain for 6–10 generations. Mice homozygous for the variant (Slc26a4<sup>T721M/T721M</sup>) were obtained by intercrossing heterozygous mice (i.e., Slc26a4<sup>T721M</sup>) (Fig. 1). Reverse transcription-PCR (RT-PCR) of mRNA of inner ear extract and direct sequencing confirmed a pure non-chimeric genetic background without unintentional wild-type Slc26a4 expression in Slc26a4<sup>T721M/T721M</sup> mice.

Auditory evaluations. Mice were anesthetized and placed in a head-holder within an acoustically and electrically insulated and grounded test room. We used an evoked potential detection system (Smart EP 3.90; Intelligent Hearing Systems, Miami, FL, USA) to measure auditory brainstem response (ABR) thresholds in mice. Click sounds, as well as 8, 16, and 32 kHz tone bursts at various intensities were generated to evoke ABRs. Response signals were detected using subcutaneous needle electrodes. The active electrodes were inserted into the vertex and the ipsilateral retroauricular region with a ground electrode on the back of each mouse. The ABRs were recorded from postnatal 12–44 weeks, to trace changes in auditory function.

Whole-mount studies of mouse inner ear specimens were performed as previously described. For light microscopy studies, tissues were stained with hematoxylin and eosin (H&E). The morphology of each sample was examined using a Leica optical microscope. For light microscopy studies, tissues were stained with hematoxylin and eosin (H&E). The morphology of each sample was examined using a Leica optical microscope.

Expression of pendrin. Tissue sections were prepared from the inner ears of wild-type, Slc26a4<sup>G919-2A</sup>, and Slc26a4<sup>T721M/T721M</sup> mice. The sections were mounted on silane-coated glass slides, deparaffinized in xylene, and rehydrated in ethanol. Tissues were stained with a 1:1000 dilution of rabbit anti-pendrin antibody kindly provided by Dr. Jinsei Jung, Yonsei University College of Medicine, Seoul, Republic of Korea. This was
followed by exposure to DAPI (1:5000) and Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) secondary antibody (1:200; Thermo Fisher Scientific). After incubation, the slides were washed with PBS and mounted with ProLong Antifade kit at 25 °C. Images were obtained using the aforementioned LSM 880 laser scanning confocal microscope.

Vestibular function evaluation. Mice were subjected to a series of tests at 8 weeks, including the swimming and rotarod tests. For the swimming test, the swimming performance of the mice was scored from 0 to 3, with 0 representing normal swimming and 3 representing underwater tumbling. For the rotarod tests, the mice were placed on the rotating rod for a maximum of 180 s. The speed of the rods was accelerated from 5 rpm to a maximum speed of 20 rpm in one min. The length of time each mouse remained on the rotating rod was recorded.

Statistical analyses. Data are presented as the mean ± standard deviation. Statistical analyses were conducted using an unpaired Student’s t-test with Bonferroni correction for continuous variables. Statistical significance was set at p < 0.05. All analyses were performed using SPSS software (version 15.0; SPSS Inc., Chicago, IL, USA).

Data availability

The authors confirm that the data supporting the findings of this study are available within the article.

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Author contributions
C.J.Hsu., T.C.L., C.C.W conceived and designed the study. I.S.Y. and S.W.L. generated the transgenic mouse. C.J.Hu., Y.C.L., Y.H.C. and Y.S.L. performed histological experiments and animal behavior analysis. C.Y.T. provided clinical data analysis. Y.H.C., Y.C.L., and C.J.Hu. performed the explants experiments analyzed the data. C.J.Hu., Y.C.L., Y.E.C., and C.C.W. drafted the manuscript. T.H.Y., C.C.W., C.J.Hsu., T.C.L., P.H.L., and Y.F.C. supervised the whole study and provided critical revision. All authors reviewed the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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