A new kind of naturally occurring mouse model for Usher Syndrome generated by crossing with CBA/J mice

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Abstract

Background: Our laboratory previously discovered a strain of Kunming mice, referred to as

KM$^{ush/ush}$ mice, that exhibits notable abnormalities in electroretinogram (ERG) readings and elevated thresholds for auditory brainstem responses (ABRs), which resemble the characteristics of Usher Syndrome (USH). In the current investigation, our objective was to perform crossbreeding between KM$^{ush/ush}$ mice and CBA/J mice with the aim of establishing novel recombinant inbred lines and subsequently analyzing their phenotypic and genotypic characteristics.

Methods: ERG, ABR testing, fundus morphology, histological examination of the retina and inner ear, qRT–PCR, western blotting, DNA sequence analysis and behavioral experiments were performed to assess the phenotype and genotype of the progeny lines.

Results: The results revealed no significant waveforms in the ERG and normal ABRs in the F1 hybrid. The F2 hybrid exhibited segregation of hearing loss phenotypes. The J1$^{ush/ush}$ mice had a retinitis pigmentosa (RP) phenotype with elevated ABR thresholds, while the J2$^{ush/ush}$ mice exhibited only the RP phenotype. Interestingly, J1$^{ush/ush}$ mice showed significantly elevated ABR thresholds from Day P28 compared with wild-type mice, but histological analyses showed no significant structural changes in the organ of corti or spiral ganglia, and qRT–PCR and DNA sequencing showed that the Adgrv1 gene and its expression were significantly altered in J1$^{ush/ush}$ mice. Further elevation of ABR hearing thresholds by P56 manifested only as a reduced density of spiral ganglion cells, which was significantly different from the previous pattern of cochlear alterations in CBA-2$^{ush/ush}$ mice.

Conclusions: We successfully introduced the hearing loss phenotype of inbred mice with Usher syndrome into CBA/J mice, which provides a good animal model for future studies on the important physiological role played by the Adgrv1 gene in the inner ear structure and for

therapeutic studies targeting Adgrv1-mutant Usher Syndrome.

Keywords: Usher Syndrome, Adgrv1, Electroretinogram, Auditory brainstem response, Inbred strain, Mutation

Background

Usher Syndrome (USH) is a group of autosomal recessive disorders characterized by retinitis pigmentosa (RP) and various degrees of hearing loss with or without vestibular abnormalities. Regarding RP, USH is genetically heterogeneous [1]. Additionally, USH is the most common cause of deaf-blindness worldwide, the combined prevalence of which exceeds 400,000 cases, seriously reducing patients' quality of life [2]. Patients are classified into three subtypes - USH1, USH2, and USH3 - according to the extent of hearing loss, vestibular function, visual field impairment, progression and age of onset [3]. The limitations of this classification are gradually becoming apparent. With the utilization of next-generation sequencing technology, the list of known pathogenic mutations in USH genes has expanded, twelve genes have been identified that correspond to the clinical subtype (https://hereditaryhearingloss.org), and the nonspecificity and variability of the clinical picture have further complicated the clinical diagnosis. The current treatment for USH is mainly symptom-based, using hearing aids or cochlear implants to ameliorate sensorineural hearing loss [4] and visual aids and retinal prostheses to delay vision loss caused by RP [5]. Nevertheless, the existing therapeutic effect is limited, and none of these methods are capable of resolving the pathophysiologic mechanisms underlying USH. Currently, to treat and cure

the inner ear and retina, various gene therapies are being developed [3], including utilizing CRISPR tools [6] and intravitreal injection of an antisense oligonucleotide as a mutation-specific treatment for USH2A. However, the outcomes of these studies have not yet been reported, and currently, there is no widely applicable, safe, and effective treatment method for patients with Usher Syndrome.

USH2 is the most common subtype of USH, accounting for more than half of all USH patients [7, 8]. Three causative genes have been associated with this subtype, namely, *Ush2a* (USH2A), *Adgrv1* (USH2C), and *Whrn* (USH2D). *Adgrv1* mutations are present in approximately 5-19% of clinical cases, second only to *Ush2a* [9]. *Adgrv1* is expressed in several tissues, including brain, lung, kidney, eye, and inner ear [10], and the expression of its protein product (very large G-protein-coupled receptor 1, Vlgr1) is sensitive to changes in the extracellular Ca^{2+} concentration in hair and photoreceptor cells. Defects in Vlgr1 may cause imbalances in the extramembrane Ca^{2+} concentrations of both of these cell types [11], but the exact mechanism remains unclear.

Various animal or cell models have contributed to the discovery of the molecular mechanisms of USH and the preclinical evaluation of novel therapeutics [12]. Our laboratory prescreened a naturally occurring mouse model with RP and deafness under the Kunming (KM) background, named $KM^{ush/ush}$ [13]. To understand the genetic background of $KM^{ush/ush}$ mice and investigate the causes of its auditory and ocular impairments, we crossed $KM^{ush/ush}$ mice with CBA/Caj mice ("normal hearing" controls) [14] to segregate the ocular and auditory phenotypes and found that retinal degeneration in $KM^{ush/ush}$ mice was caused by a *Pde6b* mutation, while auditory impairment was caused by an *Adgrv1* mutation. The resulting

CBA-2$^{ush/ush}$ mice represent an animal model for a naturally inherited phenotype of hearing loss, with a mutation in the human Usher syndrome 2C gene, *Adgrv1*. This is the first report of a mouse strain with hearing loss being isolated from an RP ancestor, and this animal model can be used to study the pathological mechanisms of RP/USH. However, the cochleae of CBA-2$^{ush/ush}$ mice show obvious morphological changes at an early stage, which is not suitable for the study of therapeutic approaches to USH and related drug development. Furthermore, in the subsequent breeding process, CBA-2$^{ush/ush}$ and CBA-3$^{ush/ush}$ mice showed inbreeding-related declines, which greatly hindered the subsequent study of the pathological mechanisms of RP/USH. CBA/J and CBA/CaJ are almost interchangeable as "normal-hearing" controls for hearing and deafness research, but there are significant differences in the pattern of inner ear damage in sensorineural deafness [15]. Therefore, in this experiment, we utilized CBA/J mice, which are widely used in the study of hearing and cochlear function [16]. To introduce the pathogenic gene from KM$^{ush/ush}$ mice into the CBA/J background, we crossed KM$^{ush/ush}$ mice with CBA/J mice. After inbreeding the F1 hybrid, two phenotypic segregations of ERG without waveforms and elevated ABR thresholds appeared in the F2 hybrid. We used functional, morphological, molecular biology, and other technical methods to confirm that the Usher phenotype (with the KM background) was still present, and after identifying the phenotype and genotype of the corresponding offspring (in which traits were segregated after crossbreeding), we bred new mice with the CBA/J background. These mice are a novel animal model for studying the *Adgrv1* mutation in a CBA/J background and for audiology research in general.

Methods

Animal model generation

KM$^{ush/ush}$ mice were obtained from the SPF animal facility of the Aerospace Clinical Medicine Department of the Air Force Medical University (License No. #SYXK2012-004). The mice had a KM background and were maintained and expanded as a colony through 38 generations of sibling mating. This strain contains mutations in both *Pde6b* and *Adgrv1* [13]. CBA/J mice (#11004A) were purchased from Beijing Huafukang Biotechnology Co., Ltd. Male KM$^{ush/ush}$ mice were crossed with female CBA/J mice to obtain the F1 hybrid, and F1 hybrid mice were inbred to obtain F2 hybrid. Each generation of mice was subjected to electroretinogram (ERG) and auditory brainstem response (ABR) evaluations at 28 days old (P28). F2 hybrids segregated according to RP and hearing loss phenotypes were grouped with mice with the same ERG and ABR phenotypes and then inbred and retained for further passages (between littermates with the same phenotype as their respective mothers). They were designated J2$^{ush/ush}$ (reduced ERG amplitude, normal ABR threshold, below 50 dB) and J1$^{ush/ush}$ (reduced ERG amplitude, elevated ABR threshold, above 50 dB) mice. All animals were housed under the same conditions in the SPF animal facility and exposed to 35 lx of light. All animal experiments were conducted according to the Association for Research in Vision and Ophthalmology (ARVO) Statement on the Use of Animals in Ophthalmic and Vision Research and approved by the Animal Care and Use Committee of the Air Force Medical University. Tissues from at least 3 animals were used in each experiment for statistical validation.

133

134 ***Electroretinogram (ERG)***

135 ERGs were recorded at P28. The experimental animals were placed in a dark-adapted
136 chamber for >12 h before the experiment. Then, the animals were deeply anesthetized with an
137 intraperitoneal injection of 1% sodium pentobarbital and 10% Sumianxin II. Their pupils
138 were dilated using compound tropicamide eye drops, and corneal surface anesthesia was
139 performed using oxybuprocaine hydrochloride eye drops. The action electrode was placed on
140 the corneal surface, the reference electrode was inserted under the skin of the cheek, and the
141 grounding electrode was inserted under the skin of the tail. Dark-adapted 3.0 ERG responses
142 were recorded using a computerized system (MonPack 3; Metrovision, France) according to
143 ISCEV guidelines [17].

144

145 ***Auditory brainstem response (ABR)***

146 ABR recording was carried out at P14, P28, and P56 using an ABR workstation
147 (Otometrics, Taastrup, Denmark) in a sound attenuation chamber. Test electrodes were
148 placed subcutaneously (cranial apex for recording electrodes, postauricular mastoid for
149 reference electrodes, and caudal for grounding electrodes). Short tones (click mode) starting
150 at 95 dB sound pressure level (SPL) were used, and if an obvious and reproducible waveform
151 was detected, quieter tones (decreasing in increments of 5 dB) were played until no obvious
152 waveform was detected. The threshold value was taken as the hearing threshold of the mouse.
153 If no waveform was detected at 95 dB, the hearing threshold was recorded as 95 dB. The
154 ABR waveform for a short tone (click mode) stimulus usually consists of four or five

response peaks, denoted as I, II, III, IV, and V [18].

Fundus photography and fluorescein fundus angiography (FFA)

Mice were anesthetized with intraperitoneal injections of 1% sodium pentobarbital and 10% Sumianxin II, and the pupils were dilated with 0.5% tropicamide-norepinephrine ophthalmic drops. The cornea was covered with sodium hyaluronate gel. The lens of the fundus imaging system (OPTO-RIS; OptoProbe, Canada) was aligned with the animal's pupil, and photographs were taken when the image was clear. One percent fluorescein sodium was injected intraperitoneally, and mice were photographed after 2 min, the injected fluorescein sodium was completely discharged 24 h later. Images were assessed to determine whether the structure of the optic disc was normal and whether there was exudation and/or hemorrhage in the fundus.

Measurement of retinal outer nuclear layer (ONL) thickness

The mice were euthanized at P28, and their eyeballs were rapidly removed. Appropriate amounts of eyeball fixative were administered into the eyeballs using a syringe, and the eyeballs were also immersed in eyeball fixative and fixed at 4 ℃ for 48 h. The completely fixed eyeballs were dehydrated, paraffin-embedded, and sectioned. HE staining was performed according to the manufacturer’s instructions. After staining, the sections were sealed with neutral gum. Retinal section images were taken using a digital imaging system (DP71; Olympus, Japan) to observe the ONL thickness in a randomly selected field of view on both sides of the optic nerve.

177

178 ***Microanalysis of cochlear cross-sections***

179 After the mice were sacrificed at P14, P28, or P56, their inner ears were dissected. The
180 cochlea was perforated apically to allow adequate penetration of the fixative (4%
181 paraformaldehyde). After fixation at 4 °C for 24 h, the cochlea was decalcified with 10%
182 EDTA at 4 °C for 1 week. Specimens were dehydrated and embedded in paraffin before
183 being sectioned, placed on slides, and stained with HE. Cochlear section images were taken
184 using a digital imaging system (DP71; Olympus, Japan) to identify the basal profile of
185 Rosenthal's canal and to determine its total area at low magnification. The magnification was
186 then increased by 16- to 25-fold, and the spiral ganglion cells were counted. The spiral
187 ganglion cell density was calculated by dividing the number of spiral ganglion cells by the
188 measured area.

189

190 ***Western blotting***

191 Retinal tissues were collected and homogenized. After centrifugation, extracts
192 containing equal amounts of protein (10 μg) were aliquoted, electrophoresed, transferred to a
193 PVDF membrane, and probed with primary antibodies against PDE6B (#NBP2-58654;
194 Novus Biologicals USA; at a 1:1000 dilution) and GAPDH (#10494-1-AP; Proteintech,
195 Rosemont, IL, USA; at a 1:5000 dilution) at 4 °C overnight. The membrane was incubated
196 with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (#EK020;
197 Zhuangzhi, Xi'an, China; 1:8000 dilution) at room temperature for 2 h. The protein bands
198 were detected by an enhanced chemiluminescence system (Thermo Fisher Scientific, USA).

The intensity of the protein bands was determined using ImageJ software (National Institutes of Health, USA). GAPDH was used as a loading control.

qRT–PCR

Cochlear RNA was extracted using a TSINGKE TSP413 RNAprep FastPure kit (Beijing Tsingke Biotechnology Co., Ltd.). Target genes were reverse transcribed and amplified using TSINGKE TSK322S SynScript™ III cDNA Synthesis Mix according to the manufacturer's instructions, and real-time PCR (RT–PCR) was carried out using TSINGKE TSK322S SynScript™ III cDNA Synthesis Mix (SYBR Green I) and TSE201 2×TSINGKE® Master qPCR Mix (SYBR Green I). Primers for subsequent RT-qPCR were designed using the Beacon Designer 7 (Table 1). All reactions were performed in triplicate. β-Actin was used as an endogenous control. Gene expression levels were normalized to the expression levels of β-actin.

Exon trapping and sequence analysis

Genomic DNA was extracted from the tail tissues of mice. Exon sequencing of the targeted genes was performed by Tsingke Biotechnology Co., Ltd. Briefly, DNA quality was assessed using 1% agarose gel electrophoresis and PicoGreen dsDNA assays (Invitrogen, USA). All of the coding exons of the target genes were assessed to detect single nucleotide variants and insertions/deletions. The design of primers for Sanger sequencing was accomplished through the utilization of Beacon Designer 7. (Table 1).

Behavioral experiment

The sensory behavior of the mice was measured using an open-field apparatus (50 × 50 × 50 cm, O'Hara and Co. Ltd., Japan). Each mouse was placed at the center of the open-field apparatus. The center area was defined as a square 10 cm away from the wall. A video imaging system (EthoVisionXT; Noldus Information Technology, The Netherlands) was used to record the distance each mouse walked and the time it stayed in the central area for 5 min [19]. Additionally, the elevated plus maze (40 cm length, 10 cm width, 50 cm height; O'Hara and Co., Ltd., Japan) was used. The closed arms were enclosed by a 20 cm high black wall. Each mouse was placed in the central area of the maze, facing one of the open arms. The time spent in the open arms was measured for 5 min with the EthoVisionXT video-imaging system [20].

Statistical analyses

All experimental data were analyzed using GraphPad Prism 9, and the experimental results are expressed as the mean±SEM. T test was used to compare data between groups, and statistical significance was defined as $P<0.05$.

Table 1

Primers used in the present study.

Primer name	Sequences (5'-3')	Destination
Adgrv1(Mus)-F	GAACTGCCCATCAGTGGGAT	RT-qPCR
Adgrv1(Mus)-R	TGCATTCTGATTTCCTGCTAGA	

β-Actin(Mus)-F	AACAGTCCGCCTAGAAGCAC	RT-qPCR
β-Actin(Mus)-R	CGTTGACATCCGTAAAGACC	
Adgrv1-31-F	ACATCTGTTCATGTAGCAGAAAATC	Sanger sequencing
Adgrv1-31-R	GCCACTACATCAATATCAGAAGTAC	
Pde6b-7-F	CTATCTAAACTGCGAACGGTATTCA	Sanger sequencing
Pde6b-7-R	TGTCAGAGACTCCATAAGGACTTCA	

Results

Stable inheritance of USH phenotypes in KM$^{ush/ush}$ mice with well-maintained pathogenic genotypes

Phenotypic and genotypic analysis of KM$^{ush/ush}$ mice at P28 revealed dark-adapted 3.0 ERG responses with a marked disappearance of waveforms (Fig. 1a). Fundus imaging results suggested severe retinal degeneration with attenuation of the retinal vessels (Fig. 1b). The ONL in the retina of KM$^{ush/ush}$ mice completely disappeared (as seen in retinal sections; Fig. 1c). Western blotting showed that the relative protein expression of Pde6b in the retinal tissues of KM and KM$^{ush/ush}$ mice was 1.12±0.19 and 0.56±0.07, respectively ($P < 0.05$) (Fig. 1d). Most of the ABRs of the KM$^{ush/ush}$ mice did not exhibit response waveforms at 85 dB stimulation at P28, whereas the normal control KM mice had ABR thresholds below 40 dB; the difference in hearing thresholds was significant ($P < 0.0001$) (Fig. 1e). Cochlear histological sections showed that KM$^{ush/ush}$ mice at P56 did not exhibit significant alterations in their inner hair cells, but their outer hair cells were significantly atrophied at the base of the cochlea. The spiral ganglion cell densities in KM and KM$^{ush/ush}$ mice were significantly

different (40.25 ± 2.28 and 33.25 ± 2.86, respectively; $P < 0.05$) (Fig. 1f). qPCR results showed that Adgrv1 mRNA expression levels in the cochlear tissues of KM and $KM^{ush/ush}$ mice were 1.32±0.04 and 0.20±0.01, respectively. Adgrv1 mRNA levels were significantly lower in the cochlear tissues of $KM^{ush/ush}$ mice than in those of KM mice (Fig. 1g). Sequencing results identified a point mutation from C to A at position 49 of exon 7 of the *Pde6b* gene in $KM^{ush/ush}$ mice, resulting in a nonsense mutation at codon 347 from tyrosine (TAC) to a termination codon (TAA). Exon sequence analyses revealed that $KM^{ush/ush}$ mice possessed a deletion mutation of a single base at the 52nd nucleotide of exon 31 of the *Adgrv1* gene. This deletion caused a frameshift mutation that resulted in the formation of a termination codon (TAA) after codon 2250 (Fig. 1h).

Phenotyping of CBA/J mice

Compared to C57BL/6 mice, no obvious waveforms from the dark-adapted 3.0 ERG recording could be identified in $KM^{ush/ush}$ mice (Fig. 2a). Fundus photography and FFA examination found that CBA/J mice showed obvious signs of retinal degeneration compared to normal C57BL/6 mice, including pale optic papillae and atrophy and sclerosis of the retinal vasculature (Fig. 2b). HE staining of paraffin sections of retinal tissues showed the loss of normal structures in the ONL of the retina in CBA/J mice (Fig. 2c). The ocular phenotype of CBA/J mice was associated with the mutated $Pde6b^{rd1}$ gen[21]. Regarding their cochlear phenotype, the mean hearing threshold of CBA/J mice (n=6) was 25.83±3.74 dB, and all thresholds were less than 50 dB as obtained by ABR audiometry.

279 ***Pedigree analysis of hybrids obtained by crossing KM$^{ush/ush}$ mice with CBA/J mice***

280 The mean ABR threshold of the F1 hybrid mice (obtained by crossing KM$^{ush/ush}$ mice
281 with CBA/J mice) was 24.44±5.50 dB, and the dark-adapted 3.0 ERG response waveform
282 disappeared. The F2 hybrids (obtained from inbreeding of the F1 hybrid) were classified
283 according to their ABR characteristics. The ERG waveform was absent in the ABR of the
284 J2$^{ush/ush}$ mice. In addition to the absence of the ERG waveform, the ABR of the J1$^{ush/ush}$ mice
285 also showed hearing thresholds over 50 dB. J1$^{ush/ush}$ and J2$^{ush/ush}$ mice did not appear to
286 undergo inbreeding decline, as previously observed in CBA-1$^{ush/ush}$ and CBA-3$^{ush/ush}$ mice
287 [22]. The female CBA/J mice were backcrossed with F4 J1$^{ush/ush}$ hybrids segregated
288 according to RP and hearing loss phenotypes, and the F2 hybrid mice obtained from this
289 inbreeding demonstrated phenotypic segregation of the RP phenotype and deafness (Fig. 3).
290
291 ***Ocular phenotypes of J1$^{ush/ush}$ and J2$^{ush/ush}$ mice***

292 At P28, dark-adapted 3.0 ERG waveforms were significantly absent in J1$^{ush/ush}$ and
293 J2$^{ush/ush}$ mice, in contrast to C57BL/6 mice with normal visual system development (Fig. 4a).
294 The results of both fundus photography and FFA suggested severe retinal degeneration with
295 attenuation of the retinal vessels (Fig. 4b). The ONL in the retina of J1$^{ush/ush}$ and J2$^{ush/ush}$ mice
296 was completely lost, as seen in the retinal sections (Fig. 4c).
297
298 ***Auditory phenotypes of J1$^{ush/ush}$ and J2$^{ush/ush}$ mice***

299 At P14, the ABR thresholds of J1$^{ush/ush}$ and J2$^{ush/ush}$ mice were 71.67±4.71 and
300 70.00±6.45, respectively. This difference was not statistically significant (P>0.05), which

may be because their auditory systems were not yet fully developed. The histopathological tissue sections showed immature ganglion cells. The ABR thresholds of $J1^{ush/ush}$ and $J2^{ush/ush}$ mice at P28 were 67.50 ± 4.79 and 20.83 ± 3.44, respectively. The ABR hearing thresholds were higher in $J1^{ush/ush}$ mice than in $J2^{ush/ush}$ mice of the same age ($p < 0.0001$). The histopathological sections showed that $J1^{ush/ush}$ mice showed no significant changes in their inner hair cells but had significant atrophy of the outer hair cells. The ganglion cell densities at P28 in $J1^{ush/ush}$ and $J2^{ush/ush}$ mice were 42.50 ± 1.12 and 44.25 ± 1.30, respectively, which were not significantly different. At P56, the ABR thresholds of $J1^{ush/ush}$ and $J2^{ush/ush}$ mice were 81.67 ± 4.71 and 19.17 ± 3.44, respectively. The ABR threshold of $J1^{ush/ush}$ mice was further elevated and significantly higher than that of $J2^{ush/ush}$ mice of the same age ($p < 0.0001$). At P56, the ganglion cell densities of $J1^{ush/ush}$ and $J2^{ush/ush}$ mice were 33.25 ± 3.49 and 43.50 ± 3.64, respectively, which were significantly different ($P<0.05$) (Figs. 5 and 6).

Genotyping of $J1^{ush/ush}$ and $J2^{ush/ush}$ mice

Western blotting results showed that Pde6b protein expression was significantly reduced in the retinal tissues of $J1^{ush/ush}$ and $J2^{ush/ush}$ mice. Sequencing of the exons of the Pde6b gene in the tail tissues showed that both $J1^{ush/ush}$ and $J2^{ush/ush}$ mice possessed a point mutation (from C to A) at position 49 of exon 7 of the Pde6b gene. This generated a stop codon (TAA) at codon 347 instead of a tyrosine (TAC), resulting in a nonsense mutation that led to a significant decrease in Pde6b protein expression in the retinal tissues of $J1^{ush/ush}$ and $J2^{ush/ush}$ mice (Fig. 7a and c). qPCR results showed that the relative Adgrv1 mRNA expression levels in the cochlear tissues of $J1^{ush/ush}$ mice and $J2^{ush/ush}$ mice were 0.21±0.01 and 1.34±0.04,

respectively (P < 0.0001). Sequencing of Adgrv1 exons in tail tissues showed that J1$^{ush/ush}$ mice had a single-base deletion at nucleotide 52 of exon 31 of the Adgrv1 gene, which caused a frameshift mutation and led to the formation of a termination codon (TAA) after codon 2250, resulting in a significant decrease in Adgrv1 mRNA levels (Fig. 7b and c).

Sensory behavioral studies of J1$^{ush/ush}$ mice

Sensory behavioral studies of KM and J1$^{ush/ush}$ mice at P56. In the elevated plus maze (EPM), wild-type KM mice (with normal visual and auditory function) and J1$^{ush/ush}$ mice (n=6) explored the open arm for 6.21±3.65 and 41.11±15.53 seconds, respectively. J1$^{ush/ush}$ mice explored the open arm region for significantly longer periods (P<0.001). In the open field test, the wild-type KM mice and J1$^{ush/ush}$ mice (n=6) explored the central region for 4.57±1.52 and 14.73±8.59 seconds, respectively. J1$^{ush/ush}$ mice explored the central region for significantly longer periods than wild-type KM mice (P<0.05; Fig. 8).

Discussion

This study is based on a KM mouse model that developed spontaneous, hereditary, deaf-RP in our laboratory. KM$^{ush/ush}$ mice exhibit an USH phenotype, identified by various evaluations, including electroretinograms, auditory brainstem response experiments, histopathological experiments, etc. KM$^{ush/ush}$ mice were verified to possess spontaneous, hereditary, double-gene (*Pde6b^{rd1}*, *Adgrv1*) mutations by molecular biology experiments and tail gene sequencing. The CBA/J strain is the only CBA substrain that carries the *Pde6b^{rd1}* mutation, which causes blindness by weaning age. These mice have been used to study

immunology and inflammation, metabolism, hearing and cochlear function, infectious disease, and fetal development [21, 23, 24]. The KM$^{ush/ush}$ gene was introduced into CBA/J mice, and after inbreeding of F1 hybrid, the ocular phenotype of F2 hybrid of mice showed absent ERG waveforms and degeneration of the fundal retinal pigmented epithelium, which was attributed to the fact that both the KM$^{ush/ush}$ and CBA/J mice carried the *Pde6b*^{rd1} gene mutation; it was further verified that the KM$^{ush/ush}$ mice were pure mutants of the *Pde6b*^{rd1} mice.

The auditory phenotypes in F2 hybrid of mice were segregated, and the cochlear phenotypes of J1$^{ush/ush}$ and CBA-2$^{ush/ush}$ mice appeared to be distinctly different according to ABRs and histopathological sections at various time intervals after birth [22]. While CBA-2$^{ush/ush}$ mice showed significant degeneration of their cochlea, Corti's apparatus, and spiral ganglion cells at P56, we observed changes in the auditory phenotypes of J1$^{ush/ush}$ mice as early as P14 and P28. We also found that J1$^{ush/ush}$ mice started to show an increased ABR hearing threshold at P28, with atrophied outer hair cells. In addition, decreased Adgrv1 mRNA expression was observed. At P56, the ABR hearing threshold of J1$^{ush/ush}$ mice had increased further, and decreased ganglion cell density was observed alongside atrophied outer hair cells. There are three key cellular components of the inner ear: hair cells, neurons, and strial cells. Different environmental and genetic risk factors may affect these components, leading to degenerative changes via various mechanisms. Hearing loss can be attributed to the accelerated loss of hair cells (sensory presbycusis), loss of neurons (neural presbycusis), loss of strial cells with attendant endocochlear potential (EP) decline (strial presbycusis), or some combination of these [25]. In previous studies, CBA/J and CBA/CaJ mice have been used as

"normal hearing" controls [26, 27]. Many studies refer to CBA/J and CBA/CaJ mice collectively as "CBA mice", but the CBA/J and CBA/CaJ strains diverged 80 years ago and exhibit >2000 polymorphisms [28]. Thus, they demonstrate very different degenerative changes in the cochlea. CBA/CaJ mice mainly exhibit hearing loss due to spiral ganglion cell degeneration and decreased EP due to reduced strial cells, while hearing loss in CBA/J mice is mainly attributed to hair cell loss [15]. It was found that early increases in the hearing threshold in CBA/J mice were not accompanied by significant hair cell degeneration and that the stria vascularis and EP also remained relatively intact from 3 to 25 months of age regardless of whether the animals had normal or elevated ABR thresholds [29]. Since this phenomenon may be related to the etiology of hearing loss in CBA/J and CBA/CaJ mice, the increase in the hearing thresholds of $J1^{ush/ush}$ mice may be attributed solely to cochlear ribbon synaptic damage at P28. It is possible that the cochlear ganglion cells are affected later, contributing to the further increased hearing thresholds at P56. Furthermore, there is little progression in hearing loss among USH2C patients before the age of 40 [30], which illustrates that $J1^{ush/ush}$ mice may present clinical phenotypes more similar to those of USH2C.

Adgrv1, previously known as Vlgr1, neuroepithelium-notable (neurepin) or G protein-coupled receptor 98 (Gpr98), is the largest G protein-coupled receptor (GPCR) and plays important roles in the sensory and central nervous systems [31]. Mutations in the *Adgrv1* gene in humans are associated with USH type II, which is characterized by combined congenital deafness and blindness. The *Adgrv1* gene plays an important role in hair cell development in the cochlea and is a key component of the ankle-link complex (ALC) [10], but the physiological role of Adgrv1-mediated signaling pathways in ALC formation and the

regulation of ALC dynamics has not been fully elucidated. Novel representative animal models are essential for investigating the mechanisms of Adgrv1 action. Recently, researchers established Adgrv1 Y6236fsX1 mutant mice as an animal model of the deafness-associated Y6244fsX1 mutation and demonstrated that the Y6236fsX1 mutation disrupts the interaction between Adgrv1 and other ALC components, resulting in stereocilia disorganization and mechanoelectrical transduction (MET) deficit[32].

Vestibular areflexia, which may present with gross motor delays in some USH patients, leads to decreased health-related quality of life, including poorer physical and psychological health, as well as higher rates of unemployment [33]. To better understand the somatosensory function of USH patients, we conducted J1$^{ush/ush}$ mouse behavioral experiments. To evaluate sensory function in rodents, methods such as the open field, dark avoidance, jumping platform, Y maze, eight-armed maze, and elevated cross experiments have been used [34, 35]. In the elevated cross maze, mice are curious about the open-arm area but prefer to stay in the closed-arm area due to the darkness, and the conflict between exploration and avoidance results in anxiety. The level of anxiety is evaluated by comparing the retention time of the mice in the open-arm and closed-arm areas [36]. The open-field experiment is mainly used to observe behavior in novel environments and is often used to study neurological function [34]. In this experiment, we found that J1$^{ush/ush}$ mice spent a significantly longer time exploring the open arm of the elevated cross maze and the central area of the open field when compared with KM mice with normal vision and hearing, which may be attributed to an impaired ability to perceive dangerous environments due to their dual sensory deficits. We simulated the behavioral manifestations of human visual and auditory disorders using an animal model with

hereditary visual-auditory deficits.

There are currently no effective treatments for USH, and many therapeutic strategies are in the preclinical or clinical I/IIa research phases, including gene replacement, gene editing, protein inhibition, and antisense oligonucleotide-based approaches. Many therapeutic studies for USH have been performed using patient-derived cells (usually fibroblasts) or mutant mice. Among these, gene replacement has been effective in several USH mouse models; for example, adeno-associated viral vectors were administered to *Myo7a*, *Whrn131*, and *Clrn1134* knockout mice, which successfully restored the expression of their respective defective genes. Double-overlapping AAV vectors have also been explored as a potentially safer large gene replacement vector and have achieved promising results [37, 38]. Lentiviral vectors, which are more capable of delivery but carry the risk of insertional mutagenesis, have been successful in delivering functional Myo7a to the retina of USH1B mice [39]. In the USH1C, USH1G, USH2D, and USH3 mouse models, injection of AAV vectors into the inner ear through the round window membrane or posterior semicircular canals significantly improved inner ear hair cell function [40]. Another therapeutic approach is gene editing, which includes the use of nucleases to eliminate mutations; homologous recombination with DNA templates containing wild-type sequences to correct DNA errors; and correction of point mutations, insertions/deletions, and splice point mutations. In recent years, the CRISPR/Cas9 system has gained popularity for gene editing due to its efficiency and ease of use. This technology has been successfully used for mutation repair in USH2A patient fibroblasts as well as patient-derived induced pluripotent stem cells, including cells containing pure heterozygous USH2A (Glu767Serfs*21) mutations or compound

heterozygous (Glu767Serfs*21/Cys759Phe) mutations [41]. The newly bred J1$^{ush/ush}$ mice used in this study contain spontaneous, hereditary *Adgrv1* mutations and do not show significant premature Corti's apparatus or spiral ganglion cell degeneration, which may be useful for investigating early therapeutic interventions for the treatment of *Adgrv1*-mutant USH.

Conclusions

In summary, we confirmed that the USH phenotype was present in laboratory KM background mice and bred new mice with a CBA/J background. It is necessary to validate the causality of USH genes after identifying genes associated with USH-like phenotypes, and for the future of USH treatment, we need to better define genotype-phenotype relationships and the nature of the disease, particularly for rare mutations. These crossbred mice may be useful for audiological research and represent a novel animal model of *Adgrv1* mutations, which can be used to study the physiological action of these variants.

List of abbreviations

ERG electroretinogram

ABR auditory brainstem response

USH Usher syndrome

qRT–PCR real-time quantitative polymerase chain reaction

Vlgr1 very large G-protein-coupled receptor 1

RP retinitis pigmentosa

ARVO Association for Research in Vision and Ophthalmology

FFA fluorescein fundus angiography

HE hematoxylin-eosin staining

ONL outer nuclear layer

INL inner nuclear layer

GCL ganglion cell layer

OC organ of Corti

SG spiral ganglion

n.s. not significant

EP endocochlear potential

Gpr98 G protein-coupled receptor 98

GPCR G protein-coupled receptor

ALC ankle-link complex

MET mechanoelectrical transduction

Declarations

Ethics approval and consent to participate

All animal experiments conducted in this study adhered to the ARRIVE guidelines, as well as the U.K. Animals (Scientific Procedures) Act, 1986. The animal study protocol received approval from the Animal Care Committee of the UIC and JBVAMC, and was conducted in accordance with the relevant guidelines outlined in the EU Directive 2010/63/EU for animal

477 experiments.

478 ***Consent for publication***

479 Not applicable

480 ***Availability of data and materials***

481 The original contributions presented in the study are included in the article material, further

482 inquiries can be directed to the corresponding authors.

483 ***Competing interests***

484 The authors declare that the research was conducted in the absence of any commercial or

485 financial relationships that could be construed as a potential conflict of interest.

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492 ***Authors' contributions***

493 SY and ZZ designed the conceptual framework of the study. LS designed the experiments

494 and wrote the manuscript. LS, JY, ZL, WD, YW and CT performed the experiments. WX

495 analyzed the data. All authors contributed to the article and approved the submitted version.

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607 **Figure captions**
608 **Fig. 1.** Ocular and ear phenotypes and genotypes of $KM^{ush/ush}$ mice. a. Electroretinogram

(ERG) of KM$^{ush/ush}$ mice. b. Typical fundus images of KM and KM$^{ush/ush}$ mice (n = 6) at P28. c. Retinal sections with HE staining from KM and KM$^{ush/ush}$ mice (n = 4) at P28. ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. d. Representative western blotting bands of Pde6b in the retinas of KM and KM$^{ush/ush}$ mice at P28 (n = 3). * $p < 0.05$. e. Typical ABR waveforms at P28 and quantitative analysis of ABR thresholds in KM and KM$^{ush/ush}$ mice (n = 6). Roman numerals I-V indicate the 5 ABR waveform peaks in response to short tone stimuli. **** $p < 0.0001$. f. HE staining of typical cochlear sections at P56 in KM and KM$^{ush/ush}$ mice (n = 4). Red asterisks: inner hair cells; blue asterisks: outer hair cells; red arrowheads: spiral ganglion cell nuclei; OC: organ of Corti; SG: spiral ganglion; * $p < 0.05$. g. Cochlear Adgrv1 mRNA levels in KM and KM$^{ush/ush}$ mice at P28 (n = 3). ****$P<0.0001$. h. Exon sequencing of Pde6b in KM and KM$^{ush/ush}$ mice at P21 (n = 3) and exon sequencing of the Adgrv1 gene in KM$^{ush/ush}$ mice (n = 3). WT: NCBI sequence of a normal control mouse; arrow: mutation site.

Fig. 2. Ocular phenotype of CBA/J mice. a. Electroretinogram (ERG) of CBA/J mice. n = 6 eyes per group. b. Fundus FFA images of C57BL/6 and CBA/J mice at P28 (n = 6). c. HE-stained retinal sections of C57BL/6 and CBA/J mice (n = 6). ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer.

Fig. 3. Pedigree obtained from crossing KM$^{ush/ush}$ mice with CBA/J mice. ERG+: mice without significant ERG waveforms; ABR+: mice with elevated ABR thresholds; ※: progeny segregated according to RP and hearing loss phenotypes.

Fig. 4. Ocular phenotypes of J1$^{ush/ush}$ and J2$^{ush/ush}$ mice. a. Typical electroretinograms (ERGs) of C57BL/6, J1$^{ush/ush}$, and J2$^{ush/ush}$ mice at P28. n = 6 eyes per group. b. Typical fundus and

FFA images of J1$^{ush/ush}$ and J2$^{ush/ush}$ mice (n = 6) at P28. c. HE-stained retinal sections from J1$^{ush/ush}$ and J2$^{ush/ush}$ mice at P28 (n = 6). ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer.

Fig. 5. Quantitative analysis of ABR thresholds of J1$^{ush/ush}$ and J2$^{ush/ush}$ mice at various time intervals (n = 6). Roman numerals I-V indicate the 5 peaks in the ABR waveform in response to short tone stimuli. **** $p < 0.0001$; n.s.: not significant.

Fig. 6. Quantitative analysis of ganglion cell density of J1$^{ush/ush}$ and J2$^{ush/ush}$ mice at various time intervals (n = 4). Red asterisks: inner hair cell structure; blue asterisks: outer hair cells; red arrowheads: spiral ganglion cell nuclei; OC: organ of Corti; SG: spiral ganglion; * $p < 0.05$; n.s.: not significant.

Fig. 7. Genotypes of J1$^{ush/ush}$ and J2$^{ush/ush}$ mice. a. Representative western blotting bands of retinal Pde6b in KM, J1$^{ush/ush}$, and J2$^{ush/ush}$ mice at P28 (n = 3). b. Cochlear Adgrv1 mRNA levels in J1$^{ush/ush}$ and J2$^{ush/ush}$ mice at P28 (n = 3). ****P<0.0001. c. Sequencing of exons of the Pde6b and Adgrv1 genes in the tail tissues of J1$^{ush/ush}$ and J2$^{ush/ush}$ mice (n = 3). WT: Exon sequences of the controls in the NCBI; arrows: mutation sites.

Fig. 8. Sensory behavioral studies of J1$^{ush/ush}$ mice. a. Elevated plus maze (EPM) of KM and J1$^{ush/ush}$ mice at P56 (n=6). b. The open field test of KM and J1$^{ush/ush}$ mice at P56 (n=6). ***P<0.001; *P<0.05.

A new kind of naturally occurring mouse model for Usher Syndrome generated by crossing with CBA/J mice

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