KIT gene mutation causes deafness and hypopigmentation in Bama miniature pigs

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Abstract

Background: Waardenburg syndrome (WS), a common type of syndromic hearing loss. A large group of patients affected by WS were found no mutations in the above gene panel, indicating that there are still potential genes responsible for WS yet to be detected. **Methods:** In our previous study, we established a *KIT* (OMIM# 164920) mutation (c.2418T>A, p.Asp806Glu) pig pedigree with an autosomal dominant inheritance model. This model presented congenital bilateral severe sensorineural hearing loss with hypopigmentation, exact the same as human WS. **Results:** Histological analysis of the *KIT* mutant cochlea showed nearly normal structures of the organ of Corti, stria vascularis (SV) and spiral neuron ganglions at E85. Scanning electron microscopy (SEM) exhibited the auditory hair cells began to degenerate at E100, and totally gone at P1. Transmission electron microscope (TEM) showed disorganization of SV and intermediate cells in the middle layer of SV had gone. The absence of endocochlear potentials also demonstrated the dysfunction of stria. **Conclusions:** *KIT* mutation (c.2418T>A, p.Asp806Glu) interrupted the development of melanocytes in cochlea, which led to the malformation and dysfunction of SV, resulting in degeneration of hair cells and finally hearing loss. Therefore, *KIT* was highly supposed to be a newly found gene associated with WS.

Keywords

KIT gene; Waardenburg syndrome; miniature pigs;

Introduction

More than one hundred genes have been identified to cause hereditary hearing loss to date(1). Waardenburg syndrome (WS) is one of the most common types of syndromic hearing loss, characterized by the association of sensorineural hearing loss and hypopigmentation of various organs, including skin and hair, with or without heterochromia iridis(2). WS accounts for 2–5% of congenital deafness with the prevalence of 1/42,000(2). WS is classified to 4 types (WS1, OMIM# 193500; WS2, OMIM# 193510; WS3, OMIM# 148820; WS4, OMIM# 277580) according to clinical presentation(3). It is an autosomal dominantly inherited disease due to disorders of neural crest cells. *PAX3* (OMIM# 606597), *MITF* (OMIM# 156845), *SNAI2* (OMIM# 602150), *SOX10* (OMIM# 602229), *EDNRB* (OMIM# 131244) and *EDN3* (OMIM# 131242) are the several reported genes related to WS and have been added to the screening panel in recent years(3–5). However, only a small portion of WS patients were found to be caused by the mutations in the above gene panel, the etiology of the relative large group of WS patients remains unknown, indicating that there are still potential genes responsible for WS yet to be detected(4,5).

It is believed that all of the WS-associated genes acted in the common pathways that regulating the developmental process of neural crest-derived melanocytes by influencing MITF functions(5). MITF is suggested to mutually interact with KITLG-KIT signaling in melanocyte development. Activation of KIT by binding of its ligand KITLG (OMIM #184745) on the cell surface, leads to the downstream activation of MITF. Furthermore, this signaling cascades are also involved in the development and migration of neural crest-derived melanocytes, including intermediate cells of SV(6–10). In rodents, loss-of-function mutations of *MITF* led to the absence of melanocytes which gave rise to hypopigmentation and hearing loss, similar to the phenotypes in cases with *KIT* and *KITLG* mutations(6,10–12). More

importantly, Zazo Seco (2015) and Ogawa (2017) reported that mutations at *KITLG* could cause asymmetric and unilateral hearing loss and WS2 in humans, adding a new candidate to WS screening panel(13,14).

In addition to WS, there are other syndromes manifesting hearing loss and hypopigmentation due to melanocyte defects, like Piebaldism (OMIM# 172800). It is an autosomal dominant disorder characterized by a congenital white forelock, scattered normal pigmented and hypopigmented patches on the skin, which is caused by loss-of-function mutations in *KIT*, encoding the receptor of KITLG(7,13,14). Sporadic cases of Piebaldism with *KIT* mutations presented with congenital hearing loss, indicating that mutations at *KIT* and *KITLG* might be part of the same disorder(17,18).

KIT gene is located on 4q11-12 in humans, chromosome 5 in the mice, and 8p12 in pigs(19,20). It encodes a 145-kD glycosylated transmembrane protein consists of an extracellular domain, a transmembrane region, and a tyrosine kinase domain. The extracellular domain contains 5 Ig-like domains, function in binding KITLG, and receptor dimerization. Activation of KIT by binding of KITLG causes autophosphorylation at tyrosine residues, then triggers the downstream signaling cascades(7,9,21,22).

Furthermore, several *KIT* mutations at tyrosine kinase domain including W(v), W(41), W(s), KIT(wads) have been identified in deaf mice and rats with decreased number of intermediate cells and malfunction of SV(23–28). Thus, *KIT* is highly suspected as a potential gene underlies WS.

Animal model provides a useful tool to study the pathogenic mechanisms of deafness.

Rodents are the most commonly used laboratory animals of otologic researches. However, huge differences in inner ear development, morphology and electrophysiology between human and mouse limit the application of mice as an ideal

deafness model. According to our previous studies, Bama miniature pig is an ideal mammalian model for otologic research(29–31). Bama miniature pigs, a natural Chinese miniature pig breed, are characterized by their genetic stability and white coat color with large black patches around the head and buttocks. In addition to the genome, the size, composition, morphology and the function of auditory system highly assembles that of human. Pigs have a larger cochlea than rodents and are ideal for evaluating the fine anatomic changes of cochlear and audiological studies(29–31). In our previous study, we performed random mutagenesis of the porcine genome by ENU treatment and successfully established a novel dominant mutant line with c.2418T>A missense mutation in exon 17 of the *KIT* gene, resulting in amino acids substitution (p.Asp806Glu), named $KIT^{D806E/+}$ Porcine model. The $KIT^{D806E/+}$ Porcine model manifested congenital hearing loss combined with hypopigmentation, the same phenotype as our previous reported Rongchang Pig WS models, which highly resembled the features of human WS(32,33).

In this study, we performed the auditory tests, morphological analysis from E85 to P1 and Endocochlear Potential (EP) recordings of $KIT^{D806E/+}$ Porcine model aiming at exploring the role of KIT functioning in auditory system. Our results firstly described the pathological changes of cochlea in the $KIT^{D806E/+}$ porcine model and help us better understanding the mechanism of *KIT* associated hearing loss in human beings.

Materials and Methods

Animals

Bama miniature pigs (Department of Laboratory Animal Science, College of Basic Medicine, Army Military Medical University, Chongqing, P.R. China) were maintained under a specific pathogen-free environment. The experimental procedures and animal welfare were performed in accordance with the care and use of laboratory animals and the related ethical regulations of Army Military Medical University.

Ethics approval

All animal experiments were performed according to the guidelines for the Care and Use of Laboratory Animals established by the Chongqing Association for Laboratory Animal Science and approved by the Animal Ethics Committee of Army Medical University, China.

Phenotype record, auditory brainstem response test and endocochlear potential recording

We recorded the phenotype of the F2 and F3 offspring, including the coat color, iris color and sex. Then, the auditory brainstem responses (ABRs) were evaluated as we previously described(30). All the animals were measured using an Tucker-Davis-Technology RZ6 system to evaluate the thresholds and confirm whether the mutants were deaf. The pigs were anesthetized (3% pentobarbital sodium, 1 ml/kg, i.m.) and placed in a sound-attenuating chamber. The reference electrode was fixed in the lobulus auriculae of both ears, the recording electrode was positioned subcutaneously in the cranial vertex, and the grounding electrode was placed in the nasal tip. Stimuli were presented 1,024 times from 90 to 30 dB SPL in steps of 10 dB SPL and then repeated in steps of 5 dB SPL when approaching the threshold. Details of endocochlear potential (EP) measurement are provided on our previous study(30).

Celloidin embedding- Hematoxylin–Eosin Stain, scanning electron microscopy and transmission electron microscopy

The celloidin embedding- hematoxylin–eosin (CE-HE) stained cochlea sections, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) samples were made as we previously described(30,31). The Bama miniature pigs were sacrificed in accordance with the care and use of laboratory animals, and the cochlea were dissected from the temporal bones and post-fixed in 4% paraformaldehyde or 2.5% glutaraldehyde at room temperature overnight. Cochlea were washed in 1% phosphate-buffered saline (PBS) and decalcified in 10% EDTA for approximately one month.

For the histological analysis, the cochlea were dehydrated using graded ethanol and dipped in graded celloidin. The cochlea were sectioned (15 μm) using a freezing microtome (Leica CM1900) and stained with hematoxylin and eosin. The sections were visualized under a Leica DMI3000 microscope. For the SEM analysis, decalcified cochlea was post-fixed for 2 h in 1% osmium, dehydrated in graded ethanol (50% - 100%), and dried in a critical point dryer (HCP-2, Hitachi) using liquid CO_2. Fixed sections were then coated using a sputter coater and examined under a scanning electron microscope. For the TEM analysis, after dehydrated in graded ethanol, cochlear tissues were then embedded in Epon resin and sectioned on an Reichart Ultracut E ultramicrotome (Boeckeler Instruments, Tucson, NM, USA). Ultrathin sections were mounted on formvar-coated slot grids, stained with lead citrate and uranyl acetate and examined via transmission electron microscope.

Results

Hypopigmentation and deafness of $KIT^{D806E/+}$ pigs

The wild-type Bama miniature pigs covered white coat color with large black patches around the head and buttocks. While the mutant pigs showed pigmentation abnormalities, including systemic white hair with small dark spots on the head and buttocks but normal irises. For auditory tests, Click and 1-32KHz tone-pips auditory brainstem response (ABR) were conducted on the wild-type and mutant pigs. The wild-type pigs had an ABR waveforms similar to humans and its hearing thresholds were at 20 dB SPL in Click, and 20-60 dB SPL in 1-32KHz tone-pips. While the waveforms could not be induced in the mutant, even at 90 dB SPL, in all frequencies, indicating severe hearing loss. Moreover, the hypopigmentation feature was co-segregated with hearing loss.

Cochlear morphology in $KIT^{D806E/+}$ pigs

Firstly, we compared the CE-HE sections of cochlea from $KIT^{D806E/+}$ and $KIT^{+/+}$ pigs at P1 via light microscopy. Fig 2 A-D showed the cochlear structure of $KIT^{+/+}$ pigs, including the organ of Corti, Reissner's membrane (RM), basilar membrane (BM), spiral ganglion neurons (SGNs) and SV. Similar to humans, the cochlea consists of three scala, including the scala vestibule (SVE), the scala media (SM) and the scala tympani (ST). The organ of Corti was lying on BM, and tectorial membrane (TM) was contacting with inner hair cells (IHCs) and outer hair cells (OHCs). However, the stereocilia of hair cells could not be observed clearly in CE-HE sections. On the contrast, we could not observe any identical abnormalities in the cochleae of $KIT^{D806E/+}$ pigs, no thinner SVs nor underdeveloped hair cells (Fig 1 E-H).

Abnormal hair cell morphology in $KIT^{D806E/+}$ pigs

Aiming to find the potential causes of deafness, and to finer structures of hair cells, we further examined the morphology of hair cells by SEM. In order to inspect

the pathological changes of hair cells, we observed the hair cells at E85, E100 and P1 via SEM. The structures of porcine cochlea were similar to human beings as one row of inner hair cells (IHCs) located in the inner most row toward the central region of the cochlea and three rows of outer hair cells (OHCs) located toward the peripheral region. IHCs and OHCs were separated by the head plate of inner pillar cells. The hair bundles of IHCs are arranged in a line. While the hair bundles of OHCs are positioned in a V-shaped pattern. The structures of hair cells at E85 were well developed (Fig 3 A-C) and stayed the same at E100 (Fig 4 A-C) and P1 (Fig 5 A-C) in wild types. For the mutants, no significant abnormality was observed at E85 (Fig 3 D-F). While stereocilia bundles began to degenerated in the basal and middle turns at E100 (Fig 4 D-F). Then, pathological changes aggravated at P1 as stereocilia bundles were not present in the cuticular plates of all turns (Fig 5 D-F).

Disorganized structures and impaired function of stria vascularis in $KIT^{D806E/+}$ pigs

As the morphological changes of hair cells from E85 to E100, a potential mechanism should be responsible for degeneration of well-developed hair cells. KIT signaling pathway plays an essential role of the development and migration of neural crest-derived melanocytes, including the intermediate cells of SV. Dysfunction of SV can lead to sensorineural hearing loss. To investigate the pathological changes of SV in $KIT^{D806E/+}$ pigs, we examined the morphology of the SV by TEM and its function by recording EP.

The SV under TEM revealed ultrastructural differences of between the $KIT^{+/+}$ and $KIT^{D806E/+}$ cochlea. For the wild-types, the SV consisted of three layers: the marginal cell (MC) layer, the intermediate cell (IC) layer and the basal cell (BC) layer. MC contacted the scala media. And BC attached the spiral ligament. The MCs connected with each other via tight junctions and formed a barrier to block the endolymph. Elongated processes of MCs interacted with the ICs and capillaries (Fig 6 A-C). In contrast, ICs were visible reduced in mutants. And three layers of SV was

disorganized. (Fig 6 D-F). Then, EP was used to test the function of SV. As we expected, EP of the middle turn of wild-types was about 60mV (Fig 7 A). While it was reduced to nearly 0 in $KIT^{D806E/+}$ pigs, which exhibited the dysfunction of SV (Fig 7 B).

HGVS variant nomenclature

The Human Genome Variation Society recommendations were used to standardize the nomenclature of *KIT* mutant variants. RefSeq sequence NM_001044525.1 was used for the coding region transcript. And NP_001037990.1 was used for the amino acid sequence resulting from translation of this transcript.

Discussion

In this study, we described the phenotype and the morphological changes from embryonic to postnatal stages found in the cochlea of Bama miniature pigs induced by KIT mutation. The CE-HE sections of KIT mutant cochlea was roughly normal, no disrupted hair cells nor thinner SVs was found. Then OHCs and IHCs began to degenerate since E100, and stereocilia bundles were totally disappeared at P1 by SEM. Further studies found reduced ICs and malformation of SV. Then, we examined the function of SV by EP recording. And EP at middle turn of $KIT^{D806E/+}$ cochlea was close to 0 comparing to 60 mV of wild-types. Our results demonstrated that the hearing loss in this $KIT^{D806E/+}$ porcine model was primarily caused by the malformation and dysfunction of the SVs and subsequent progressive degeneration of sensory epithelium of the developing inner ears. Our findings on this $KIT^{D806E/+}$ miniature pigs are similar to our previous reports about MITF-M mutant Rongchang porcine model(32,33).

It is known that hearing loss in WS is caused by disorders of melanocytes in the middle layer of the SV called intermediate cells, due to abnormal proliferation, survival, migration, or differentiation of neural crest cells (4,5,34). Various studies have proofed that the histopathological changes of WS were primarily reduced number of intermediate cells in the SV both in patients and animal models, then subsequently degeneration of organ of Corti as its secondary alterations(4,34). In the $KIT^{D806E/+}$ porcine cochlea, the reduction but not absence of intermediate cells in the SV was revealed and decreased EP was recorded. As the progressive apoptosis of hair bundles began at E100, the Reissner's membrane and cochlear duct did not shown obvious changes after birth. Therefore, the pathological changes of $KIT^{D806E/+}$ pigs are not so severe as MITF-M mutant pigs(32,33).

Most current animal disease models, especially for inherited deafness, were established using mice. However, the biological differences between mice and humans limit the use of these models in further studies. Bama miniature pigs could be used as a useful model to study inherited deafness as the similarity of the genetic and physiological characteristics and their inner ear structural with humans(29–31). In mice and rats, mutations at KIT, also known as W locus, resulted in depigmentation of the skin and hair as well as deafness. Many mutant strains including W(s), W(v), KIT(wads) mice and W(s) rats have been demonstrated to have interrupted process of development and migration of neural crest- derived melanocytes, resulting in decreased number of intermediate cells and malformation of SV, then failing to generate a large enough EP and subsequent hearing loss(23–28). These pathological changes are in accordance with $KIT^{D806E/+}$ pigs, indicating they might be part of the same disorder. However, unlike other KIT mutant rodents that cochlea-saccular retrogressions were mainly observed at postnatal period, our porcine models and humans shared a similar process that cochlear disorders began at embryonic stages(17,18,23–28). Likewise, studies on *KIT* mutation cats and canines showed similar phenotypes, but the pathological changes had not been revealed(35–37).

As for the condition in human beings, *KIT* is associated with piebaldism, an autosomal dominant disorder of neural crest- derived melanocyte development and migration resulting in congenital white forelock and hypopigmented patches of skin from which melanocytes are absent(15,16,38,39). Besides, in occasional cases, the piebaldism has been found to be associated with congenital hearing loss, the same features of WS(17,18,40). Hulten et al. (1987) described a Pakistani case of presumed homozygous piebaldism. The proband manifested the congenital absence of pigmentation in the eyes and skin and hair associated with deafness, hypotonia and delayed developmental milestones. His parents, both affected with piebaldism. However the disease locus had not been mentioned(40). Kilsby et al. (2013) described

another Pakistani case of presumed homozygous piebaldism manifesting albinism and deafness caused by homozygous deletion of exons 20 and 21 of *KIT*, resulting in loss of the final 77 amino acids of the full 976 amino acid protein. Both of his parents are heterozygotes for this mutation(18). Spritz et al. (1998) reported a heterozygous mutation of the KIT gene (p.Arg796Gly) in a South African girl of Xhosa stock, presenting congenital piebaldism and profound hearing loss(17). Hamadah et al. (2019) reported a family with expanded syndrome of piebaldism, while p.Ala608Asp induced unilateral deafness in only one patient. Those KIT mutant cases manifested hypopigmentation associated with congenital deafness, overlapping the features of WS, and were analogous to our $KIT^{D806E/+}$ porcine model (align to p.Asp810Glu in human). All of these mutations located at tyrosine kinase domain of KIT (Fig 8).

KIT plays a role partly overlap to MITF in cell survival, proliferation, differentiation and migration of neural crest cells, which are the precursors of melanocytes, hematopoietic stem cells, germ cells, mast cells and the interstitial cells of Cajal(9,21,41–44). In humans, gain-of-function mutations in the *KIT* gene have been associated with many neoplastic diseases. While Loss-of-function KIT mutations are associated with piebaldism, due to abnormal development of neural crest- derived melanocytes(11,23,24). It is not unexpected piebaldism occasionally associated with deafness, since intermediate cells of SV are neural crest- derived. The similar mechanism was confirmed in *MITF* associated WS of both human and animal models(6,8–11,33,46).

Previous reports indicated that there are ion channels in the SV to facilitate K^+ recycling in the cochlea, and a high level of K^+ in the endolymph is fundamental to sensory hair cell mechano-transduction. K^+ circulation of the cochlea consists of following processes: K^+ stream from the endolymph into the perilymph via the sensory hair cells, K^+ uptake from the perilymph to the fibrocytes of the spiral

ligament, K^+ funneling into the basal and intermediate cells of the SV via gap junctions, K^+ outflowing from the intermediate cells to the intrastrial fluid, and K^+ secretion into the endolymph by the marginal cells of the SV. We speculate that the $KIT^{D806E/+}$ mutation disrupts the balance of K^+ homeostasis because of the lack of intermediate cells in the SV. As a result, EP was nearly completely absent because of the lack of K^+ in the scala media which is full of endolymph. Moreover, hair cell transduction was inhibited and further impacted the depolarization of hair cells and release of neurotransmitters, which ultimately resulted in deafness(47,48). Furthermore, the absence of intermediate cells of SV could lead to K^+ accumulated in intrastrial fluid, resulting in disorders of strial capillaries. This would cause an increase of endothelial permeability, and subsequently result in series changes of ischemia and hypoxia of inner ear(27). Then as we found, the well-developed hair cells began to degenerate since E100, which can be observed by SEM. However, the molecular mechanisms by which *KIT* mutations causing hereditary deafness remain unknown. Reports have shown that bind to KITLG, the activation of KIT leads to the downstream phosphorylation of MITF, which occurs specifically via the Ras-Erk signaling pathway, the PI3 kinase survival pathway and miRNAs(8,24,48). Other studies have shown that MITF is necessary for KIT expression in melanoblasts(7,23). It is reported nearly every WS-associated genes shared the same pathways by influencing MITF expression and function(5,6,10,12). We speculate that the $KIT^{D806E/+}$ mutation in Bama miniature pigs may affect MITF expression, which leads to pigmentation defects and deafness. Additional analyses of the *MITF* gene are underway to better understand its role in the $KIT^{D806E/+}$ deafness model. Furthermore, *KITLG* was a newly identified gene associated with WS(13,14). As a key role of KITLG/KIT-MITF signaling in neural crest- derived melanocyte development, *KIT* could be a candidate for WS(6,9,21,45).

In conclusion, the $KIT^{D806E/+}$ pigs showed autosomal dominant congenital hearing loss and hypopigmentation. Its mechanism is the malformation and dysfunction of SV leading to secondary hair cell degenerations, due to abnormalities

of neural crest-derived melanocytes. The deafness phenotype of $KIT^{D806E/+}$ pigs is the first discovered in pigs, and this mutation is phenotypically distinct from other *KIT* alleles. The $KIT^{D806E/+}$ pig establishes a powerful model for studying the mechanism of KIT in hearing loss and developing therapies to combat hearing loss. Furthermore, *KIT* could be a potential gene associated with WS.

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Competing financial interests

The authors declare no competing financial interests.

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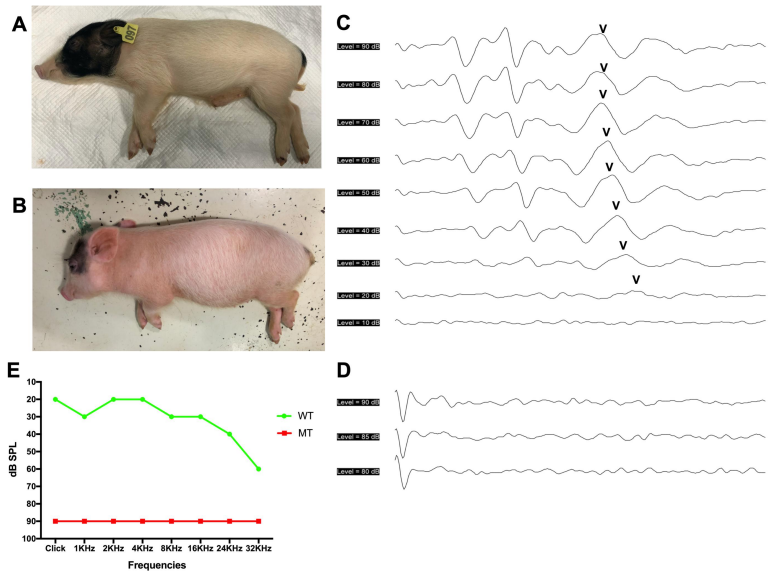

Figure 1. Hypopigmentation and deafness in KIT$^{D806E/+}$ pigs. **(A)** The hair color of wild-type Bama pigs was white coat coloured- belly with large black patches around the head and buttocks. **(B)** The mutant pigs showed systemic white hair with small dark spots on the head and buttocks. **(C)** The wild-type pigs had a hearing threshold of 20 dB SPL in Click. **(D)** Waveforms could not be induced in the mutant at 90 dB SPL. **(E)** Tone-pip ABR of wild-type and mutant pigs. The mutants remained irresponsive at 1–32 KHz frequencies.

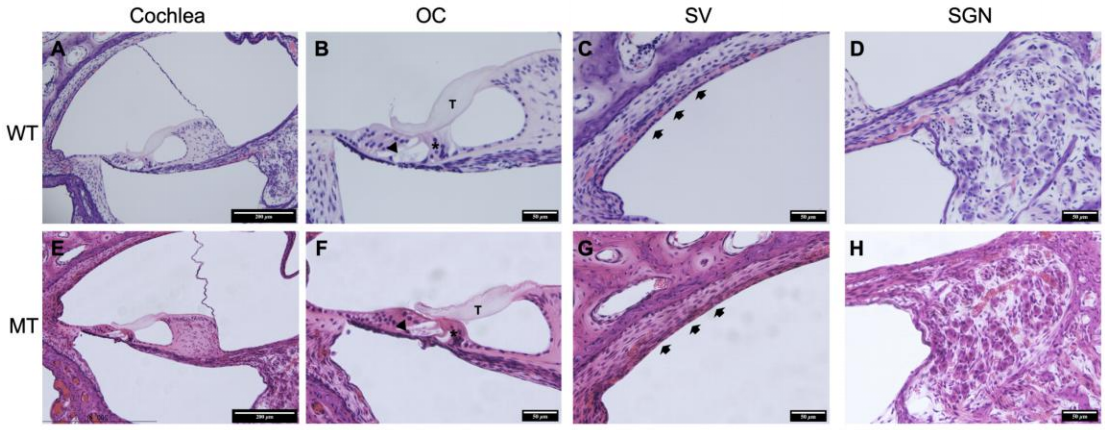

Figure 2. Generally normal structures of KIT$^{D806E/+}$ cochlea. **(A-D)** Cochlea of wild type pigs showed structures similar to humans. **(E-H)** Cochlea of mutants showed generally normal structures of tectorial membrane (T), inner hair cells (*), outer hair cells (arrow), SV (arrow head) and SGNs.

Figure 3. SEM image of basilar membrane at E85. **(A-C)** Normal arranged of inner hair cells (IHC) and three rows of outer hair cells (OHC1, 2, 3) in wild-types. **(D-F)** No significant abnormalities found in mutants.

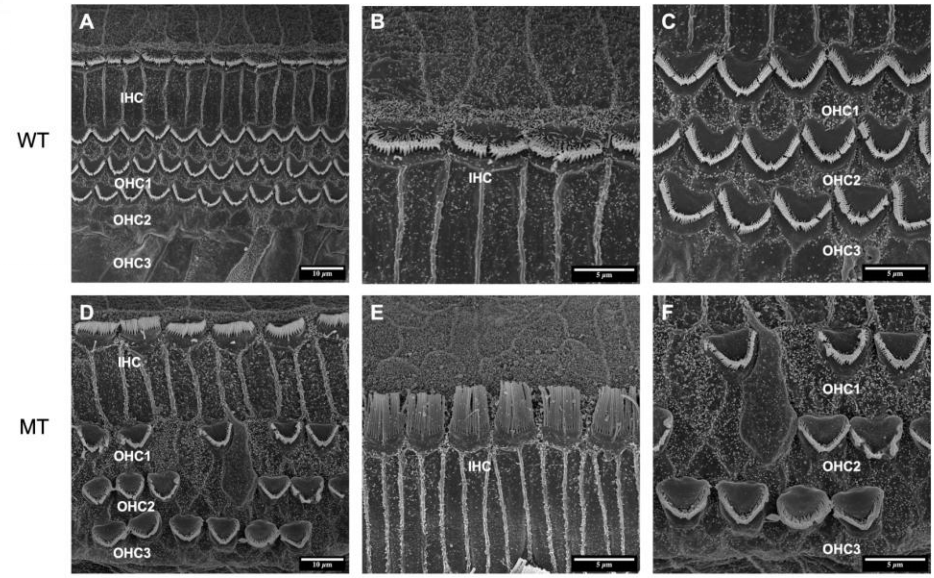

Figure 4. SEM image of basilar membrane at E100. **(A-C)** Normal arranged of inner hair cells (IHC) and three rows of outer hair cells (OHC1, 2, 3) in wild-types as E85. **(D-F)** Missing or fused stereocilias of OHC1-3 in mutants.

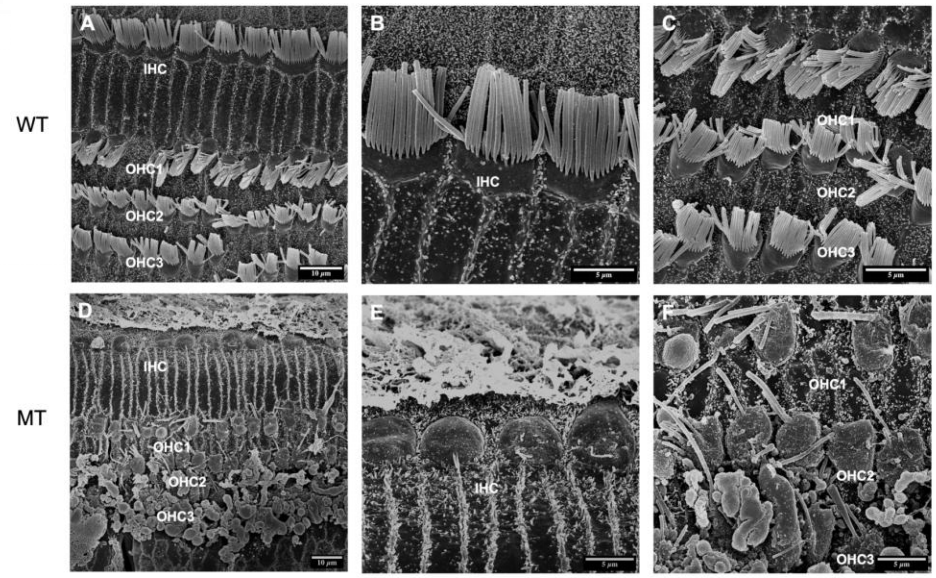

Figure 5. SEM image of basilar membrane at P1. **(A-C)** Normal arranged of inner hair cells (IHC) and three rows of outer hair cells (OHC1, 2, 3) in wild-types as E85 and E100. **(D-F)** Progressively degenerated OHCs and IHC.

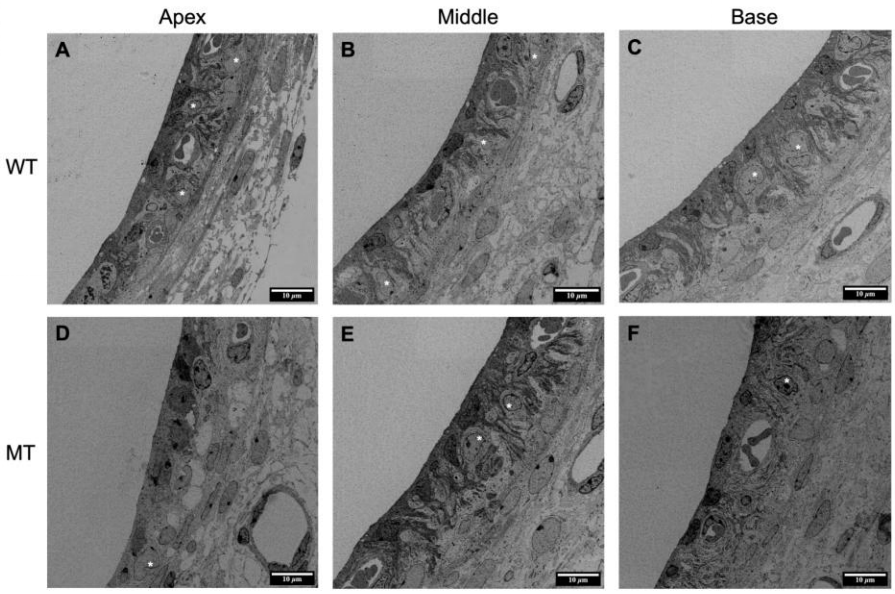

Figure 6. TEM image showed disorganization of SV in $KIT^{D806E/+}$ cochlea. **(A-C)** Normal structures of apical, middle and basal turn of wild-types, consisting of marginal cells (MC), intermediate cells (IC) and basal cells (BC). **(D-F)** Disorganized SVs and reduced ICs in mutants. * refers to intermediate cells.

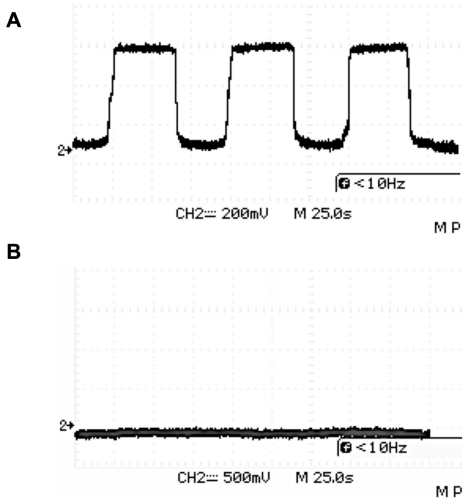

Figure 7. EPs in KIT$^{D806E/+}$ pigs were significantly lower than in wild-types. **(A)** Approximately 60mV of EPs in middle turn of wild-types. **(B)** EPs of mutants close to 0 in the same turn.

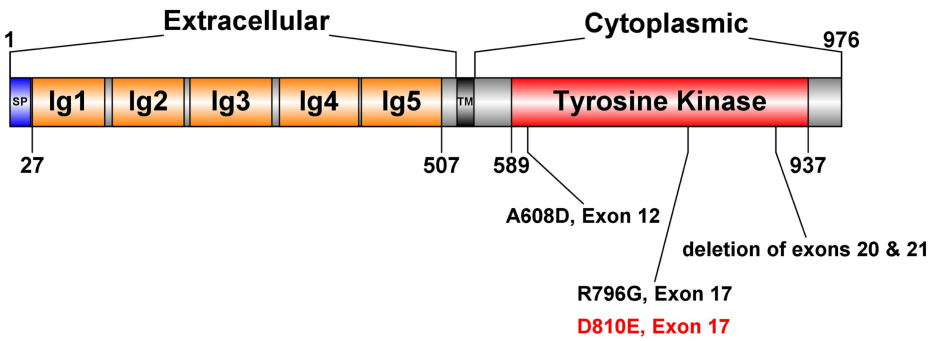

Figure 8. Representation of the structure of KIT, illustrating the observed missense mutations in patients with hearing loss. SP, signal peptide; TM, transmembrane domain. D810E in human aligns to D806E in pigs.