Identification of a novel *c-KIT* mutation inducing piebaldism and hearing loss in pigs by ENU mutagenesis

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Abstract

As a rare autosomal dominant hypopigmentation disease, piebaldism is characterized by the presence of patchy albinism on the skin, and is mainly caused by the loss-of-function mutations in *c-KIT* gene. Congenital hearing loss is occasionally found as an expanded syndrome of piebaldism. However, the correlation between *c-KIT* mutations and piebaldism with hearing loss has not yet been described. Herein, we created a mutant strain of miniature pig through the N-ethyl-N-nitrosourea (ENU)-mediated mutagenesis, which showed severe piebaldism and congenital profound hearing loss. A genome-wide association study (GWAS) linked these phenotypes to the c.2430T>A transition mutation in exon 17 of *c-KIT*, resulting in an Asp810Glu substitution in the tyrosine kinase domain. The Asp810 was localized at the highly conserved DFG motif and the transition from Asp to Glu would deplete the kinase activity of c-KIT protein, finally inducing the degeneration of intermediate cells and hair cells in inner ear. The $c\text{-}KIT^{c.2430T>A/+}$ pig is the first large animal model with a *c-KIT* loss-of-function mutation, showing piebaldism with hereditary hearing loss. It will serve as an experimental model for exploring the function of c-KIT during biological processes and the candidate therapies for *c-KIT* mutations related diseases.

Keywords

piebaldism; c-*KIT* mutation; hearing loss; DFG motif; hair cells

Introduction

Piebaldism (MIM #172800) is a rare autosomal dominant disorder characterized by a congenital white forelock and leukoderma on the frontal scalp, forehead, ventral trunk, and extremities. This phenotype was induced by the abnormal migration of neural crest derived melanoblasts to the skin during embryogenesis or the local postnatal migration of skin melanoblasts and melanocytes(Alexeev & Yoon, 2006; Bondanza et al., 2007; Grichnik, 2006). Various "loss-of-function" mutations in *c-KIT* have been demonstrated in about 75% of the patients with piebaldism(Ezoe et al., 1995; Murakami et al., 2004; Murakami et al., 2005). Also, a satisfactory correlation has been described between the mutant genotype and clinical phenotypes: missense mutations in the extracellular ligand-binding domain and nonsense mutations preserved approximately 50% of the *c-KIT* function and induced mild piebaldism by haploinsufficiency; mutations in the tyrosine kinase domain preserved approximately 25% of the *c-KIT* function and induced severe piebaldism through a dominant-negative effect(Oiso, Fukai, Kawada, & Suzuki, 2013; Spritz, 1994; Ward, Moss, & Sanders, 1995).

Interestingly, piebaldism combined with congenital deafness was firstly reported in two Hopi Indian brothers in Arizona(Woolf, 1965). Until 1998, Spritz et al. firstly described the pathogenicity of heterozygous c-KIT (p.R796G) in piebaldism with congenital deafness(Spritz & Beighton, 1998). Recently, the homozygous deletion of exons 20 and 21 in*c-KIT* was reported to induce piebaldism and deafness in humans(Kilsby et al.,

2013), and the heterozygous mutation of A608D was found in one adult patient with non-hereditary unilateral deafness(Hamadah et al., 2019). Furthermore, several KIT mutations (KIT$^{W\text{-}V}$, KIT ^{Wads} , and KIT^{F856S}) have been identified in mice with hypopigmentation and hearing loss(Hoshino et al., 2000; J. CABLE, 1994; Ruan, Zhang, & Gao, 2005). Nevertheless, the low and irregular coincidence of piebaldism with hearing loss seemed to be an expanded and occasional manifestation of *c-KIT* mutations. Thus, piebaldism was speculated as an inherited condition in an intermediate rather than a dominant manner(Hamadah et al., 2019; Spritz & Beighton, 1998).

In pigs, *c-KIT* is highly related with the dominant white color phenotype by two mutations: the copy number variations of the entire gene (˜450 kbp) and a G to A substitution in the first nucleotide of intron 17(Giuffra et al., 2002; Johansson, Pielberg, Andersson, & Edfors-Lilja, 2005; Pielberg, Olsson, Syvanen, & Andersson, 2002). To date, six *c-KIT* alleles have been reported in pigs: i , the recessive allele in wild boar;I^{Be} , the dominant allele for the belt phenotype in Hampshire pigs; I^{P} , the half dominant allele for the patch phenotype in Pietrain; $I(I^{1}$, I^{2} , orI^{3}), the completely dominant allele for white color in Landrace and large white pigs. The polymorphisms in the c-*KIT* sequence could discriminate the European from the Asian groups, the two main clusters produced during the domestication history(Niu, Shi, Xie, Liu, & Zhong, 2018). On the other hand, pigs exhibit more similar audiological characteristics to humans than rodents, including the cochlear structure, the range of audible frequency, and sensitivity to sound(Chen et al., 2016; Guo et al., 2015; Hai, Guo, et al., 2017), making it more advantageous in the research and treatment of hearing loss.

Previously, we reported the first large-scale ENU-mediated mutagenesis program in pigs in the world using Chinese Bama miniature pig and identified 36 dominant and 91 recessive novel strains(Hai, Cao, et al., 2017). One of the dominant inherited strain exhibited a severe phenotype of piebaldism and congenital profound hearing loss. The genome-wide association study (GWAS) and linkage analysis identified a *de novo* mutation in*c-KIT* (*c.* 2430T>A), which induced the missense mutation of Asp810Glu in c-KIT protein. Then, we described theI^{Be} genotype of *c-KIT* in wild-type Bama pigs. In addition, the pathological results revealed the collapse of Reissner's membrane and the absence of hair cells in the mutant inner ear. Finally, we reanalyzed the mutations in *c-KIT* gene to provide new clues for understanding the etiology and development of piebaldism with hearing loss.

Results

Severe piebaldism and profound hearing loss in mutant pigs derived from ENU mutagenesis

During N-ethyl-N-nitrosourea (ENU) mutagenesis, we designed a three-generation breeding scheme to retrieve dominant and recessive mutations in the F1 offspring(Hai, Cao, et al., 2017). A dominant mutation was identified in a male pig (G1-016105) with pigmentation abnormalities. The G1-016105 was mated with a wild-type Bama sow, resulting in 63 offspring from 10 litters (Fig. 1a). Among them, 27 offspring had the "two-ends" black color, and 36 showed severe piebaldism similar to G1-016105, including 16 males and 20 females (Fig. 1b). The mutant piglets showed symmetrical pigmentation abnormalities with white hair and skin on the forehead, cheek, the entire ear, and the distal part of the tail, which is highly similar to the distribution of piebaldism in humans (Fig. 1c). Moreover, the iris color of mutant pigs was normal. The blood cell analysis of peripheral blood did not find any marked difference in the mutant pigs (Table. S1).

As the hypopigmentation in the skin is primarily associated with hearing loss in human Waardenburg syndrome (WS)(Saleem, 2019), we conducted an auditory brainstem response (ABR) test in the mutant and wild-type littermates at postnatal day 7. Mutant pigs did not produce any recognizable waveforms up to 100 dB sound pressure level (SPL) stimuli in the range of 1–32 kHz, whereas wild-type pigs produced ABR thresholds at 10–30 dB SPL (Fig. 1d, 1e). The ABR test on every offspring showed a linkage between piebaldism and hearing loss. The frequency of the two phenotypes was compared with expected Mendelian ratios using a χ^2 test ($\chi_c{}^2$=1.0159<$\chi_{0.05(1)}{}^2$), suggesting that the mutant phenotype is inherited in an autosomal dominant pattern (Table 1).

Identification of the c.2430T>A mutation in exon 17 of *c-KIT* in mutant pigs

To identify the potential causative gene in the G1-016105 mutant strain, linkage analysis and family-based genome-wide association study (GWAS) were performed in 16 mutants and 17 wild-type littermates (including G1-016105, three wild-type sows, and their offspring). Consequently, parametric analysis revealed a significant linkage at the 32 - 48 M region of chromosome 8 (P value $< 1\times10^{-5}$; LOD=7.8) (Fig. 2a, b). The annotation of the pig reference genome suggested the presence of about 141 genes in this candidate linkage disequilibrium interval. *c-KIT* (chr8: 43550231–43601377) occurs in this region (Fig. 2c) and is associated with the coat color in pigs; hence, it was selected for further analysis. All coding sequences of *c-KIT* genomic DNA were sequenced, and a point mutation (T>A) was identified at the 2430^{th} nucleotide (CDS2430 bp) in exon 17 that completely co-segregated with the mutant phenotype (Fig. 2d). This mutation (*c-KIT* c.2430 A>T) induced the substitution of the 810^{th} aspartic acid with glutamate acid in the second tyrosine kinase domain of the pig c-KIT protein (p.D810E) (Fig. 2d). Intriguingly, this mutation was not found in the SNP database (dbSNP, https://www.ncbi.nlm.nih.gov/snp/) or other pig breeds, indicating that it was novel and created by ENU mutagenesis. Further sequence alignment with BLAST (https://blast.ncbi.nlm.nih.gov/) and ClustalW (https://www.genome.jp/tools-bin/clustalw) revealed that this site Asp810 (Asp812 in mice) in the protein tyrosine kinase domain was conserved among all mammalian c-KIT proteins (Fig. 2e). Moreover, Asp810 belonged to the DFG motif (Asp810, Phe811, Gly812) and localized in the N-terminus of the activation loop.

Genotype of *c-KIT* in Chinese Bama miniature pigs

As previously described, the genotype of *c-KIT* in pigs is various and complex with changes in copy number and splice mutation (Fig. 3a). Firstly, we described the genotype of *c-KIT* in Bama pigs, which regulates the "two-ends" black phenotype (Fig. 3b). To determine the copy number of *c-KIT* , we designed primers and probes to amplify *c-KIT* and a single copy control sequence (*ESR*) using the droplet-digital PCR (ddPCR) in five wild-type pigs (Fig. 3c). After amplification, the number of positive and negative droplets was determined based on the fluorescence signal (Fig. 3d), and the *c-KIT* and *ESR* copy numbers in each sample were calculated by Poisson statistics based on the ratio of positive to total partitions (Fig. 3e). The comparative copy numbers of *c-KIT* and *ESR* in all five samples implied the presence of two *c-KIT* copies in the Bama pig diploid genome (Fig. 3f). Therefore, this genotype is the I^{Be} allele of *c-KIT* in pigs.

Also, we analyzed the whole *c-KIT* sequence in Bama pigs. Finally, a total of 26 polymorphisms (23 single nucleotide polymorphisms (SNPs) and three indels) were identified in the genomic DNA: 12 (nine SNPs and three indels) in introns and 14 in exons (Table. S3). Of the 14 SNPs located in exons, two were in the 3'-untranslated region (UTR) and 12 were synonymous (n=10) or non-synonymous mutations (n=2). The missense mutations in exon 3 (g.58390G>A, p.R173K; g.58455 G>A, p.V195M) have been previously reported in cDNA or genomic sequences of *c-KIT* (Fontanesi et al., 2010; Lai et al., 2007). The BLAST analysis of the sequence of the KIT protein (NP_001037990.1) predicted an amino acid substitution (p.D309G, exon 5) in the third Ig-like loop in the extracellular domain of Bama pigs.

Moreover, we detected two critical mutations in *c-KIT* : a splice mutation in exon 17 and a 4 bp indel in intron 18 (intron 18: g.29_32delAGTT). As described previously(Marklund et al., 1998), we obtained a 175-bp fragment with KIT 17 primers and a 160–164 bp fragment with Intron 18 primers. Sequence analysis showed high conservation of the GT dinucleotide in the 5' splice site of exon 17 (Fig. 3f) in Bama pigs, which confirmed the correlation between the *c-KIT* splice mutation and the dominant white phenotype. Moreover, the 4-bp deletion mutation in intron 18 was absent in Bama pigs.

D810E mutation depleted the kinase activity of c-KIT protein and induced the destruction of the inner ear structure

The DFG motif flipped between the "on" and "off" states in the activation process of the c-KIT protein(Mol et al., 2004; Mol et al., 2003). Asp810 was directed towards the positively charged guanidinium group of Arg815 in the autoinhibited conformation and coordinated the Mg^{2+} ion bridging of the α- and β-phosphates in the active conformation (Fig. 4a). Therefore, we deduced that the substitution of Asp810 with Glu810 elongated the side chain at this specific site and exerted a deleterious impact on the kinase activity of

the protein. This prediction was further confirmed by SIFT (https://sift.bii.a-star.edu.sg/) and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) (Supplemental Data). In addition, c-KIT has been reported to indirectly regulate the transcription activity of microphthalmia-associated transcription factor (MITF) through the MAP kinase machinery to affect the survival, proliferation and apoptosis of melanocyte derived intermediate cells(Hemesath, Price, Takemoto, Badalian, & Fisher, 1998; Ronnstrand, 2004). RT-PCR revealed the downregulation of MITF-regulated genes (*PAX3* , *TYR* , *DCT* , and *MC1R*) (Fig. 4b), implying the reduction and dysfunction of the mutant KIT protein activity. However, the D810E mutation did not affect the transcription of *KIT* , *MITF* , or *SOX10* .

To investigate the pathology of hearing loss, we dissected the cochleae from wild-type and mutant pigs for microcomputed tomography (micro-CT) scanning and three-dimensional reconstructions to observe the cochlear structure and integrity. The three-dimensional structure of the cochlea showed three semicircular canals perpendicular to each other and three and one-half turns of the cochlear capsule; any marked difference was not detected between wild-type and mutant pigs (Fig. 4c). The results of celloidin embedding and hematoxylin-eosin staining (CE-HE) showed that, in wild-type pigs, the organ of Corti, the Reissner's membrane (RM), spiral ganglion (SG), tectorial membrane (TM), and the stria vascularis (SV) were arrayed normally in the cochlear duct (Fig. 4d). However, an extensive collapse of Reissner's membrane onto the SV and the disordered organ of Corti were observed in $c\text{-}KIT^{c.2430T>A/+}$ pigs, which induced the profound hearing loss.

The cochlear hair cells degenerated completely in $c\text{-}KIT^{c.2430T>A/+}$ pigs

The morphology of hair cells in the apical, middle and basal turns was examined via scanning electron microscopy (SEM) (Fig. 5). Three rows of outer hair cells (OHCs) and one row of inner hair cells (IHCs) were regularly arranged along the cochleae in wild-type pigs (Fig. 5, upper pannel). The stereociliary bundles of OHCs were in the form of a "V" shape in the middle and basal turns, whereas the stereociliary bundles had a bundle arrangement in the apical turns. The typical morphology of a complete OHC is shown in Fig.5. Compared with the wild-type pigs, the OHCs and IHCs of the $c\text{-}KIT^{c.2430T>A/+}$ pigs appeared to be completely degenerated, and stereociliary bundles were not observed in the cuticular plates (Fig.5, lower pannel). In addition, the number of OHCs and supporting cells was severely reduced, leaving empty structures as shown in Fig.5.

Discussion

In this study, we identified the novel mutation of c-KIT (p. Asp810Glu) which has not been reported previously and induced severe piebaldism with profound hearing loss in pigs. The heterozygous $c\text{-}KIT^{c.2430T>A/+}$ pig is the first big mammalian animal with a loss-of-function mutation in the *c-KIT* gene, thereby providing an important experimental model for exploring the pathological effect of *c-KIT* mutation and the candidate therapies for *c-KIT* mutation related diseases.

As a type III receptor tyrosine kinase, the binding of SCF to the first three Ig-like domains causes dimerization of two c-KIT proteins and promotes the kinase domains to act in *trans* as a substrate and enzyme for each other(Lemmon, Pinchasi, Zhou, Lax, & Schlessinger, 1997; Liu, Chen, Focia, & He, 2007; Yuzawa et al., 2007). To date, a total of 35 missense mutations, 17 deletions, four insertions, three nonsense mutations, seven nucleotide splice-site mutations, and one pericentric chromosomal inversion in *c-KIT* have been described (Table S3). Of the 35 missense mutations, three are located in the extracellular domain, 19 in the first tyrosine kinase domain (TK1), and 13 in the TK2 domain (Fig. 6a). Moreover, only R796G induced congenital and profound hearing loss in offspring(Spritz & Beighton, 1998), while A608D induced unilateral deafness in only one patient(Hamadah et al., 2019).

To further explore the mechanism under *c-KIT* mutations inducing hearing loss, we labeled and reanalyzed the missense mutations in the crystal structure of the active c-KIT protein (Fig. 6b). Two types of active sites of type III receptor tyrosine kinase are essential for the normal kinase activity of KIT: ATP binding sites and polypeptide substrate binding sites. A total of 4/19 sites (L595P, G601R, A621D, and A621T) in TK1 are ATP binding sites, while 2/13 (D792Y and P832L) in TK2 are polypeptide substrate binding sites (Fig.

6b). Only the R796G mutation is located in the core of the active pocket, and Arg796 functions as both types of the active sites. As for the residue Asp810, it is directed towards the positively charged guanidinium group of Arg815 in the c-KIT autoinhibited conformation and is rotated to ligate the Mg^{2+} ion and nucleotide phosphates during the activation process(Mol et al., 2004; Mol et al., 2003). The carboxylate side chain of Asp810 maintains approximately the same position in both enzyme conformations. Herein, in the mutated D810E, glutamic acid is similar to aspartic acid in polarity and acidity but with additional carbon. Therefore, there would be no space in the active pocket to accommodate the elongated side chain of Glu810. Thus, we deduced that the core location and function of Arg796 and Asp810 make them indispensable for the c-KIT function, and the induced mutation expanded the hearing loss with piebaldism.

Previous studies have reported approximately 163 *c-KIT* mutations in mice, although only $KIT^{W\text{-}V}$ and KIT^{Wads} homozygotes exhibit a recessive hearing loss trait(Cable, Jackson, & Steel, 1995; J. CABLE, 1994; Ruan et al., 2005). However, the phenotypes induced by the D810E mutation in Bama pig were transmitted in an autosomal dominant pattern, and the homozygotes faced embryonic lethality. This phenomenon indicates that the*c-KIT* dosage may exert a critical effect on pigs than mice and that the genetic, physiological, and structural similarities between pigs and humans make the pigs superior to mice for studying inherited deafness. Reportedly, the homozygous mutation (F809L) of Phe811 in mice induces hair pigmentation, macrocytic anemia, hepatic steatosis, and postnatal lethality (Magnol et al., 2007); however, no hearing phenotype was described. A missense mutation (G812V) of Gly812 has been identified in a patient with severe piebaldism, showing white forelocks and extensive leukoderma of the forehead without hearing loss (Kuster, 1987; Spritz, Holmes, Itin, & Kuster, 1993). These different phenotypes derived from the three sites of the DFG motif may be related to the function and characteristic of specific and substituted amino acids, respectively. The specific association among *c-KIT* mutation, the residual activity of the KIT protein, and hearing loss still need further study.

Materials and Methods

Editorial Policies and Ethical Considerations

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.

Animals

Bama pigs were maintained at the Center of Experimental Animal in the Army Medical University and allowed *ad libitum* access to a commercial pig diet (nutrient levels according to the United States National Research Council) and clean water throughout the experimental period. The generation of mutant pigs has been described previously(Hai, Cao, et al., 2017). Briefly, the G0 pigs (10-month-old, male) were injected three times intravenously with ENU at a dose of 85 mg/kg at 1-week intervals to generate random genome-wide mutations in germ cells. After a recovery period of one month, the treated G0 boars were mated with wild-type sows to produce G1 progeny. G1-016105 used in this study was one of the dominant progenies found in G1 and was crossed with ten wild-type females for inheritance testing.

Auditory electrophysiology

Auditory brainstem response (ABR) was used to evaluate the hearing function of wild-type and mutant pigs, as described by Guo et al(Guo et al., 2015). Briefly, the ABRs were evoked with clicks and tone pips at 1, 2, 4, 8, 16, 24, and 32 kHz. The response was amplified, filtered, and averaged using the intelligent hearing system (TDT, USA). And the ABR threshold was determined by visual inspection.

GWAS and candidate gene screening

Genomic DNA samples from 33 Bama pigs of the G1-016105 strain (including G1-016105, three wild-type sows, and their 29 littermates) were genotyped using porcine SNP60 BeadChips (Illumina Inc., USA). A family-based genome-wide association analysis (GWAS) detected the SNP loci exhibiting a significant cor-

relation with piebaldism using PLINK software. Parametric linkage analysis was performed using Merlin software based on an autosomal-dominant model, and the LOD score was calculated as the evidence for linkage. The whole coding sequence of the candidate gene *c-KIT* was amplified and sequenced to identify the causative mutation. All primers are listed in Table S2.

Droplet-digital PCR (ddPCR)

The ddPCR workflow was executed as reported previously(Hindson et al., 2011; Pinheiro et al., 2012). Briefly, 20 μL reaction mixtures (supermix, primers, and template) were loaded and partitioned into a maximum of 20,000 droplets in the generator cartridge (Bio-Rad, USA). Then, these droplets were transferred to a 96-well PCR plate and placed on a thermal cycler (Bio-Rad) for amplification. The reaction was carried out at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 1 min, and an extension at 98 °C for 10 min. The reaction was held at 4 °C. Subsequently, the droplets were passed through the QX200 reader (Bio-Rad) and were assigned as positive or negative based on the fluorescence signal, which is used to determine the presence of target DNA. Data were analyzed using QuantaSoft version 1.3.2.0 software (Bio-Rad). Primers and probes are shown in Fig. 3.

Whole length analysis of the *c-KIT* gene

The coding region of *c-KIT* was sequenced, as reported by Fontanesi et al(Fontanesi et al., 2010). The sequence was aligned against the pig genomic sequence AC141857 (clone RP44-473N18) containing 21 *c-KIT* exons using BLASTN analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The splice mutation site in exon 17 was amplified with primer KIT17, designed by Marklund et al(Marklund et al., 1998). The 4-bp indel in intron 18 was amplified using primer Intron 18, and the 164/168 bp product was sequenced. The thermal cycling profile was 95 °C for 2 min, followed by 35 cycles of 95 °C for 10 s, 58 °C for 10 s, and 72 °C for 10 s, with a 5 min extension at 72 °C and holding at 4 °C. PCR products were purified by 2% gel electrophoresis and sequenced.

Real-time PCR (RT-PCR)

Total RNA was extracted from cochleae using TRIzol reagent (Invitrogen, USA). Random hexamers were used for synthesizing the first cDNA strand synthesis with 1 μg of total RNA (Molecular Biology). Then, the expression of an array of genes (*MITF* , *SOX10* , *PAX3* ,*TYR* , *DCT* , and *MC1R*) involved in the MITF signaling pathway was evaluated using EvaGreen Supermix on a CFX96 apparatus (Bio-Rad). The thermal cycling profile was 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 58 °C for 10 s, and extension at 95 °C for 5 s, 65 °C to 95 °C, and 0.5 °C for 5 s. The PCR primers are listed in Table S1. The expression of each target gene in WT and mutant littermates was standardized against that of *actin* mRNA using the $2^{-\Delta\Delta^{\circ}\tau}$ method.

Structural analysis

The structural data of autoinhibited and active c-KIT were downloaded from the Protein Data Bank: 1PKG (activated) and 1T45 (autoinhibited). The various mutation sites found in humans, mice, and pigs were reanalyzed using PyMOL software. The D810E mutation site was labeled and exported as a PNG image.

Micro-CT scanning

In order to assess the 3D structure and integrity of the inner ear, cochleae samples from wild-type and heterozygous pigs at postnatal day 7 were dissected and fixed with 4% paraformaldehyde and scanned by a micro-CT system (Quantum FX, Perkin Elmer). The parameters were as following: 90 kV, 160 μA, 24 mm of field of view, and 4.5 min of duration. The structure of the inner ear was analyzed and reconstructed using Amira 2.0 software.

Celloidin embedding and hematoxylin-eosin staining (CE-HE)

After fixation with 4% paraformaldehyde, the wild-type and mutant cochleae were washed in 1% phosphate-buffered saline (PBS) and decalcified in 10% EDTA for approximately 1 month. Following dehydration with

graded ethanol and embedding with celloidin, the cochleae were sectioned into 15 μm slices using a frozen microtome CM1900 (Leica, Germany) and stained with hematoxylin-eosin (HE). Finally, the sections were visualized under a Leica DMI3000 microscope (Leica, Germany).

Scanning electron microscopy (SEM)

The morphology of the stereocilia of cochlear hair cells was examined using SEM, as described previously(Guo et al., 2015). Briefly, cochlea samples from wild-type and heterozygous pigs at postnatal day 7 were fixed with 2.5% glutaraldehyde, decalcified in 10% EDTA, post-fixed in 1% OsO_4, dehydrated in graded ethanol (50–100%), and dried in a critical point dryer (HCP-2, Hitachi) using liquid CO_2. Then, fixed sections were coated using a sputter coater and examined under a Hitachi S-3700N scanning electron microscope (SEM, Japan) from the basal, middle, and apical areas of each cochlea.

Abbreviations

ENU, N-ethyl-N-nitrosourea; WS, Waardenburg syndrome; ABR, auditory brainstem response; SPL, sound pressure level; GWAS, genome-wide association study; ddPCR, droplet-digital PCR; CE–HE, celloidin embedding and hematoxylin-eosin staining; SEM, scanning electron microscopy.

Web resources

BLAST,*https://blast.ncbi.nlm.nih.gov/*

ClustalW,*https://www.genome.jp/tools-bin/clustalw*

SIFT,*https://sift.bii.a-star.edu.sg/*

Polyphen-2,*http://genetics.bwh.harvard.edu/pph2/*

HGMD,*http://www.hgmd.cf.ac.uk/ac/index.php*

dbSNP,*https://www.ncbi.nlm.nih.gov/snp/*

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Authors' contributions Conceived and designed the experiments: HW, SMY, YW, and WWG. Performed the experiments: TTL, LL, YZ, CX, CHL, and LHL. Data analysis: TTL, LL, HTS, and FX. Manuscript preparation: TTL, LL, YW, and WWG.

Availability of data and material the data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of interest the authors have declared that no conflict of interest exists.

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Table 1. Chi-square test of G2 birth ratio of normal to mutant.

Phenotype	Observed number (O)	Expected number (E)	O-E	x_c^2
Normal	27	31.5	-4.5	0.5079
Mutant	36	31.5	4.5	0.5079
Sum.	63	63	0	1.0159

$$x_c^2 = \sum \frac{(|O-E|-0.5)^2}{E} = 1.0159 < x_{0.05(1)}^2$$

Chi-square test for the phenotype of piebaldism with 63 individuals. Normal: pigs with "two-ends" black phenotype; Mutant: pigs with hypopigmentation in head and hip; Sum: total of the number of pigs. The value of $x_{0.05(1)}^2$ was 3.84.

Figure Legends

Figure 1. Severe piebaldism and profound hearing loss in mutant pigs. (**a**) The pedigree of the G1-016105 mutant strain, including the ENU treated G0 male pig, the G1 male founder pig (G1-016105), and 63 G2 pigs produced by the wild-type sow. The black boxes and circles represented the pigs with pigmentation abnormalities. (**b**) Statistical data of sex distribution and pigmentation pattern in the progenies of G1-016105 mating with ten wild-type females. (**c**) The hair color of wild-type Bama pigs was "two-ends" black in head and hip, while the mutant pigs showed hypopigmentation in the head, ears, and hip. The retinal iris of the mutant pigs showed normal pigmentation and color. Scale bar: 5 cm. (**d**) The results of ABR tests of wild-type and mutant pigs. The threshold of wild-type pigs was about 10–20 dB, while the mutant pigs showed profound bilateral deafness. (**e**) Tone burst results of wild-type and mutant pigs. Wild-type pigs responded to sound stimuli from 1–32 kHz, while the mutant cochleae remained irresponsive at all frequencies.

Figure 2. Localization of the ENU-induced mutation site in exon 17 of the *c-KIT* gene. (**a**) (**b**) GWAS analysis results showed a highly significant linkage between piebaldism and the 32–48 cM region on chromosome 8. $P < 1\times10^{-5}$. LOD=7.8. (**c**) 141 genes were annotated in the candidate linkage disequilibrium interval, including the candidate gene *c-KIT* , indicated as red. (**d**) Sanger sequencing identified a site mutation (c.2430T>A) in exon 17 of the *c-KIT* gene, which altered aspartic acid (D) into glutamic acid (E) on the 810^{th} site of the c-KIT protein. The p.D810E mutation was localized in the second tyrosine kinase domain. And the sequence of mutation site showed the transition (GAT > GAA). (**e**) Alignment of the second kinase domain of c-KIT proteins showed that the Asp810 residue is highly conserved across different

species and located in the DFG (Asp810, Phe811, and Gly812) motif at the N-terminus of the activation loop (A-loop).

Figure 3. The genotype of the *c-KIT* gene in Bama miniature pigs. (**a**) Six different *c-KIT* alleles (i , i^{Be} , i^{P} , I^{1} , I^{2} , I^{3}) exist in pigs. (**b**) The picture of a wild type Bama pig with "two-ends" black hair color, which is similar to the *belt* phenotype in Hampshire. Scale bar: 5 cm. (**c**) The primers and probes used in ddPCR for *c-KIT*and *ESR* . (**d**) One example of the ddPCR results of*c-KIT* and *ESR* in the same DNA sample. The Y-axis shows the amplitude of fluorescence in every droplet, and the X-axis shows the number of droplets detected. The value of the purple line showed the threshold to decide positive or negative. (**e**) The statistical data of the copy numbers of *c-KIT* and *ESR* in all five DNA samples. X: DNA sample; Y: 1000 copies/uL. (**f**) The copy number of *c-KIT* in the Bama pig genome was decided by the ratio of *c-KIT* to *ESR*copy number. The data implied the presence of a single copy of*c-KIT* on one chromosome. (**g**) The products of target fragment amplification with KIT17 primers and sequence analysis of the splice site mutation of exon 17.

Figure 4. D810E mutation disrupts the c-KIT-MITF signaling pathway. (**a**) The crystal structure of the activated c-KIT protein. The DFG motif is localized in the core of the c-KIT protein active pocket and was manifested in the right side. ATP: blue; Mg^{2+}: red; PTR568: orange. The DFG motif and the other important residues are shown. (**b**) qPCR results of MITF-related genes (*SOX10, PAX3, DCT, TYR,* and *MC1R*). (**c**) The micro-CT scanning and 3-Dimensional reconstruction results of cochleae were shown at upper panel. ASC, anterior semicircular canal; PSC, posterior semicircular canal; LSC, lateral semicircular canal; OW, oval window; RW, round window. The results of celloidin embedding and hematoxylin-eosin staining (CE-HE) of wild-type and mutant cochleae were shown at lower panel. RM, the Reissner's membrane; SG, spiral ganglion; SV, the stria vascularis.

Figure 5.Morphological changes of hair cells under scanning electron microscopy. The bundles of three rows of outer hair cells (OCHs) and one row of inner hair cells (IHCs) are regularly arranged in the apical, middle, and basal turn of the cochlea from a wild-type animal. Scale bar: 10 μm. High-resolution images show the bundles of OHCs from the apical and middle turn. Scale bar: 2 μm. An entire OHC can be observed in the basal turn. Scale bar: 10 μm. OHCs and IHCs of$c\text{-}KIT^{c.2430A>T/+}$ animals appear to be completely degenerated in the apical, middle, and the basal turn. Scale bar: 10 μm. Stereociliary bundles are not present in the cuticular plates of the apical, middle, and basal turn. Scale bar:2 μm.

Figure 6. Representation of the structure of KIT, illustrating the observed missense mutations in patients with piebaldism.(**a**) The complete list of KIT missense mutants retrieved from the literature for piebaldism. ECD, extracellular domain; TMD, transmembrane domain; JMD, juxtamembrane domain; KID, kinase insert domain; TK, tyrosine kinase domain. The red star represents the position 810, where the Asp is converted to Glu point mutation found in this article. (**b**) The overall structure of activated c-KIT kinase labeled with the missense mutations located in the tyrosine kinase domain. The yellow labels Indicate that the sites belong to TK1, while the orange indicates TK2. The purple labels show the ATP binding pocket of KIT kinase. ATP: green; Mg^{2+}: red; PTR568 from another KIT proteins: blue. Asp810 and Arg796 are indicated.