Characterization of the Kyoto Circling (KCI) Rat Carrying a Spontaneous Nonsense Mutation in the Protocadherin 15 (*Pcdh15*) Gene

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Abstract: Protocadherin-15 (*Pcdh15*) plays important roles in the morphogenesis and cohesion of stereocilia bundles and in the maintenance of retinal photoreceptor cells. In humans, mutations in *PCDH15* cause Usher syndrome type 1F (USH1F) and non-syndromic deafness DFNB23. In mice, repertoires of *Pcdh15* mutant alleles have been described as Ames waltzer mutations. For further understanding of *Pcdh15* function in vivo and to develop better clinical treatment for the disabling symptoms of USH1F and DFNB23 patients, animal models suitable for clinical as well as pharmacological studies are required. Here we report the characterization of a *Pcdh15* mutant allele, Kyoto circling, (*Pcdh15*^{kci}) in the rat. Rats homozygous for *Pcdh15*^{kci} display circling and abnormal swimming behaviors along with the lack of an auditory-evoked brainstem response at the highest intensities of acoustic stimulation. Positional cloning analysis revealed a nonsense mutation (c. 2911C>T, p. Arg971X) in the *Pcdh15* gene, which is predicted to result in the truncation of the PCDH15 protein at the 9th domain of cytoplasmic cadherin domains. Histological study revealed severe defects in cochlear hair cell stereocilia, collapse of the organ of Corti, and marked reduction of ganglion cells in adult *Pcdh15*^{kci} mutants. Severe reduction of sensory hair cells was also found in the saccular macula. Since the rat is more advantageous for clinical and pharmacological studies than the mouse, the KCI rat strain may be a better disease model for *Pcdh15*-deficit USH1F and DFNB23.

Key words: deafness, disease model, protocadherin 15, rat, USH1F

Introduction

Genetic analyses of congenital deafness in mice and rats and hereditary neurosensory disorders in humans

largely serve to identify the genes responsible for hearing impairments [1, 16]. Genetic analysis of mouse Ames waltzer (*av*) mutation, which causes deafness and vestibular dysfunction associated with degeneration of

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the inner ear neuroepithelia, identified protocadherin 15 (*Pcdh15*) as the gene responsible for hearing impairment [5]. Several different alleles have been identified in the *av* locus and include *av^J*, *av^{2J}*, *av^{3J}*, *av^{6J}*, *av^{Tg}* (*av^{TgN2742Rpw}*), *av^{Jfb}*, and *av^{5J}* (*av^{nmf19}*) [4, 5, 8, 17, 18]. Extensive analyses of these *av* mutants show that mutation in *Pcdh15* affects hair bundle morphogenesis and polarity [8, 12, 17] and mechanotransduction [7]. A detailed study on the localization and function of PCDH15 in hair cells by Senften *et al.* strongly supports the role of *Pcdh15* in bundle morphogenesis and polarity [14]. More recently, Kazmierczak *et al.* have shown by immunohistochemical studies using rodent hair cells and biochemical experiments that PCDH15 interacts with cadherin 23 to form tip-link filaments that connect the stereocilia and are thought to gate the mechanoelectrical transduction channel [10].

In humans, missense mutations of the *PCDH15* gene cause non-syndromic deafness, DFNB23, recessive prelingual hearing loss with normal vestibular responses and electroretinogram [3]. Meanwhile, nonsense mutations of the *PCDH15* cause Usher syndrome type 1F (USH1F), a recessive disorder characterized by congenital profound hearing loss, vestibular problems, and delayed retinitis pigmentosa [2, 6]. The prevalence of USH1F in USH1 patients varies among the cohort, but it is a relatively common subtype of USH1. To treat the deafness of USH1F patients, cochlear implantation is widely used. Recently, an aminoglycoside-dependent therapeutic approach has been attempted *in vitro* as a novel and definitive treatment of USH1F [13]. Whatever therapeutic approaches for USH1F and DFNB23 are chosen, it is currently necessary to validate them in an animal model that mimics the mutant phenotype of human diseases. The laboratory rat (*Rattus norvegicus*) provides important mammalian models for various human diseases. Due to its suitable body size and great adaptability, the rat serves as an animal model especially in neurological, behavioral, surgical and pharmacological studies. An experimental system with the rat model for USH1F and DFNB23 would be advantageous, especially when the causative gene of the rat model is identified as a mutation of *Pcdh15*.

Rats showing abnormal behaviors characterized by constant circling movements were found in the F₃ gen-

eration of Crl:CD(SD) rats purchased from Charles River Laboratory Japan (Kanagawa, Japan) in 2003. Preliminary genetic analysis showed that these abnormal traits were inherited in an autosomal recessive manner. Although inbreeding has not been fully completed (F18), we called the rats Kyoto Circling (KCI) and named the causative gene *kci*.

In this report, we describe the identification of the *kci* as a nonsense mutation of the *Pcdh15* gene, and the histopathological characteristics of the KCI rat.

Materials and Methods

Animals

KCI rats were provided by the National Bio Resource Project for the Rat in Japan and kept in our animal facility for all experiments in this study. BN/SsNSlc rats were purchased from Japan SLC, Inc. (Shizuoka, Japan). KCI rats were bred by a brother-sister mating of *kci/+* heterozygous females with *kci/kci* homozygous males. Animal care and experimental procedures were approved by the Animal Research Committee, Kyoto University and were conducted according to the Regulation on Animal Experimentation at Kyoto University.

Auditory brainstem response measurement

Auditory brainstem response (ABR) measurements were performed in three individuals each for *kci/+* heterozygous and *kci/kci* homozygous rats at 9 weeks of age. The following experiments were performed using animals anesthetized with ketamine (80 mg/kg, i.p.) and xylazine (8 mg/kg, i.p.). Stainless steel needle electrodes were inserted subcutaneously into the vertex (indifferent), one side (active), and the other side (ground) of the retroauricular region. The ABR was obtained by averaging 1,000 evoked responses to click stimuli at intensities of 43, 52, 63, 72, 81, and 90 dB peak equivalent sound pressure levels (peSPL) with 50-ms intervals generated by an acoustic stimulator (MEB-5504, Nihon Koden, Tokyo, Japan). Clicks were delivered through an inner ear type earphone facing the meatus acusticus externus. ABR thresholds were determined for each stimulus frequency by identifying the lowest intensity producing a reproducible ABR pattern on the computer screen (at least two consistent peaks).

Table 1. Primers used for amplifying rat *Pcdh15* cDNA

Primer name	Forward (5'>3')	Reverse (5'>3')	Position*
<i>cPcdh15.1</i>	ATGTCCCCACAGTTT	CGTTGCCAGTCAACATGAGT	412–1066
<i>cPcdh15.2</i>	CCAGAAGATCCGACATCCAA	CTGCAGTCAGCTGGATGACA	1009–2027
<i>cPcdh15.3</i>	GTTTACACGGACATGAGTCC	GAACACGGGAGCGTTATCAT	1978–2577
<i>cPcdh15.4</i>	GCCACTGTGAACATAGTGGT	GGAAACTGCACATCATCCAC	2527–3344
<i>cPcdh15.5</i>	GTTTATGCTGAAGACGCAG	GCTATAGTCTTCTAGGGAG	3268–4338
<i>cPcdh15.6</i>	GTTGTAGAGTCCATTGGTGC	CCACACCCTCTGGATCTTTT	4279–5145
<i>cPcdh15.7</i>	GTTAAGAGTCAGTCCCTGAG	TTACAAGGACGTT	5095–6234

*Nucleotide positions of 5' and 3' ends of PCR products for rat *Pcdh15* cDNA (XM_001080000).

Genetic mapping

(BN/SsNSlc × KCI)F₁ rats were backcrossed to KCI to obtain N₂ rats. Homozygous *kci/kci* animals were identified on the basis of the appearance of circling behavior and inability to swim at 3–4 weeks of age. A total of 259 N₂ progeny were produced in this study. Genomic DNA was prepared from tail biopsy using the automatic DNA purification system (PI-200, Kurabo, Japan). For the initial mapping of *kci*, we employed pooled-SSLP analysis [15]. DNA from 14 randomly selected rats of each genotype at the *kci* locus was standardized to 20 ng/μl and equal amounts of individual DNA were pooled with respect to each genotype. The *kci/kci* and *kci/+* DNA pools were genotyped for 61 microsatellite markers distributed among all autosomal chromosomes. The KCI rats used in the genetic study were homozygous for all of these markers. For the fine mapping of *kci*, all N₂ animals were genotyped.

RNA extraction and RT-PCR

Total RNA was extracted from the brain of 7-week-old animals with ISOGEN (Nippon Gene, Japan) according to the manufacturer's instructions and was stored in RNA Storage Solution (Ambion). Five micrograms of total RNA was used for first-strand cDNA synthesis with Superscript II reverse transcriptase (Invitrogen), and a 1-μl aliquot of 50 μl of reaction mixture was used as a template for PCR. Rat *Pcdh15* cDNAs were amplified with 7 sets of primers (Table 1). These PCR products overlapped each other and spanned the entire coding sequence of *Pcdh15*.

Sequencing

PCR products were treated with ExoSAP-IT (Amer-

sham Biosciences) to digest single-strand DNAs and excess primers. Cycle sequencing was performed with the BigDye Terminator Ready Reaction Mix v3.1 according to the manufacturer's instructions (Applied Biosystems). PCR samples were purified with CENTRI-SEP spin columns and then loaded into an ABI PRISM 3100 genetic analyzer (Applied Biosystems).

Histopathology

We examined the inner ears of 4 *kci/kci* homozygous mutant rats and 4 control (*kci/+* heterozygous) rats at 16 weeks of age. Perfusion fixation through the left ventricle was conducted with Karnovsky solution (5% glutaraldehyde and 4% paraformaldehyde) under anesthesia. For light microscopy, the removed cochlea were fixed in 10% neutral-buffered formalin for 24 h and decalcified in ethylene diamine tetraacetic acid (EDTA). The specimens were then dehydrated in graded ethanol, embedded in paraffin and stained with hematoxylin and eosin (HE) or embedded in epoxy resin and stained with toluidine blue.

For scanning electron microscopy (SEM), the removed cochleae were immersed in 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 to 48 h. After dehydration and critical point drying under a dissecting microscope, the vestibule and membrane tectoria ductus cochlearis were removed. The blocks of tissues were covered with ionized gold and visualized under a scanning electron microscope (JSM-5200, JEOL, Tokyo, Japan). The surface view of the organ of Corti was analyzed.

For retina histology, eyes were removed from 4 *kci/kci* homozygous mutant rats and 2 control rats at 16 weeks of age after anesthetic overdose followed by cervical

dislocation. Eyes were fixed overnight in Davidson's fixation solution, embedded in paraffin, and stained with HE.

Results

Mutant phenotype

Mutant offspring are identifiable at approximately 15 days of age by manifestation of twisting the neck toward the back when lifted by the tail. After weaning, mutant rats fail to show a startle response and display head tossing and bidirectional circling behavior. Circling behavior is observed as early as 14 days of age and persists throughout life. When the KCI rats were placed into a deep tank filled with warm water (35°C), they immediately began rotating along their long axis and sank. While underwater, the rats still rotated along their body length. The rats seldom resurfaced before they were rescued. These findings suggest that KCI rats might have lost their balance and have defects in the inner ear, which senses linear and angular acceleration.

Auditory brainstem response

In addition to this balance disorder, the KCI rats showed no response to sounds such as knocking and clapping. To test the auditory organ function, we measured ABR in KCI homozygous (*kci/kci*) rats and their normal littermates (*kci/+*). In *kci/+* heterozygotes, ABRs composed of I, II, III, IV, and V peaks were observed at all of the intensities examined (Fig. 1A), but no *kci/kci* homozygotes exhibited ABR up to the maximum level of acoustic stimulation (Fig. 1B), indicating that the KCI rats were completely deaf.

Genetic analysis

Pooled-SSLP analysis showed a linkage relationship between *D20Rat4* and the *kci* locus. A distinct reduction of the BN allelic fragment of the *D20Rat4* was seen in the *kci/kci* pool relative to both the F_1 hybrid and the *kci/+* pools. A genetic linkage study of 259 (BN/SsNSlc $\times$ KCI) F_1 $\times$ KCI backcross progeny using 3 additional markers on Chr 20 narrowed down *kci* to a 2.3-cM interval between *Rab36* and *D20Rat75* (Fig. 2A). Within this interval, three genes, *Rab36* (member RAS oncogene family), *Gnaz* (guanine nucleotide binding protein, alpha

z subunit), and *Pcdh15* (protocadherin 15), and three predicted transcripts (RGD1561987, LOC502417 and RGD1563351) have been mapped, and these genes were considered as candidates for *kci* (Fig. 2B). *Pcdh15* was considered to be the strongest candidate among them, because mutations of this gene are responsible for deafness in humans and mice.

Although the expression level and size of the *Pcdh15* transcript are not altered in KCI rats, sequencing analyses of the entire coding region revealed the substitution of a cytosine to a thymidine residue at nucleotide position 2911 from the start of the coding region (c. 2911 C>T) (Fig. 2C), which was verified by PCR-RFLP analysis (Fig. 2D). This substitution introduces a stop codon at the 971st amino acid of the PCDH15 protein of the KCI rat (p. Arg971X). In the presence of the premature stop codon, the PCDH15 protein expressed in the *kci* allele would be truncated after the ninth extracellular cadherin domain (Fig. 2E). The *kci* nonsense mutation was completely associated with mutant phenotypes in 259 backcross progeny and not shared among 62 Crj:CD(SD) rats (data not shown). These findings suggest that the nonsense mutation of *Pcdh15* is responsible for the *kci* mutant phenotype.

Histopathological analysis

As illustrated in Fig. 3, stereocilia of both inner and outer hair cells of affected mutants were severely disorganized compared to those of control animals in which stereocilia were of normal configuration. The normal "V"-shaped arrangement of stereocilia was completely disrupted in all three rows of outer hair cells (Fig. 3). Stereocilia were misoriented and thickly fused. Inner hair cell stereocilia also showed a disorderly arrangement compared to controls (Fig. 3).

In the cochlea of *kci/kci* rats, severe to total loss of inner and outer hair cells was found (Fig. 4). The organ of Corti was collapsed into a poorly differentiated mass of cells in which the normal arrangement of fluid spaces was not noticeable. The number of cochlear nerve fibers in the osseous spiral lamina was dramatically reduced (Figs. 4A and 4B). Despite the severe degeneration of the organ of Corti in affected animals, the configuration of the cochlear duct remained normal. Reissner's membrane was in its normal position and no abnormalities of

Fig. 1. Representative ABR waveforms of *kci/+* heterozygous (A) and *kci/kci* homozygous (B) rats at 9 weeks of age. Five major peaks were detected for *kci/+* heterozygous rats at the various intensities tested (43–90 dB). No peaks were obtained for *kci/kci* homozygous rats.

the stria vascularis were observed by light microscopy. The number of spiral ganglion cells was also reduced (Figs. 4C and 4D).

In the saccula macula of *kci/kci* rats, the number of sensory hair cells was severely reduced compared to control rats, although that of supporting cells seemed to be normal. The remaining hair cells appeared to be degenerated and the otolithic membrane was very severely damaged (Figs. 4E and 4F).

In the retina, no anatomical defects were noted in any *kci/kci* homozygous or heterozygous rats (Fig. 5). Retinas from all animals included all normal retinal layers, and no abnormalities were noted in the cellular structure as examined at the light microscopic level.

Discussion

The behavior of rats homozygous for the *kci* mutation is very similar to those described previously in mouse *Pcdh15*-mutant alleles [4, 5, 8, 17, 18]. The mutation in the *kci* allele is a nonsense mutation (c. 2911 C>T, p. Arg971X) and is predicted to result in truncated PCDH15 protein at the 9th domain of extracellular cadherin domains. This substitution was completely correlated with behavioral abnormalities in backcross progeny and was not shared by the outbred colony from which founders of KCI were discovered. Based on genotype-phenotype correlation and a significantly similar phenotype with *Pcdh15*-mutant Ames waltzer mice, we concluded that *Pcdh15* was the gene responsible for the mutant phenotype of the KCI rat. The *kci* is designated *Pcdh15^{kci}*.

Fig. 2. Identification of the rat *kci* mutation. (A) Genetic linkage map around the *kci* locus (left). Distribution of haplotypes observed among 10 progeny carrying a recombinant chromosome between *D20Rat59* and *D20Rat75*. Black boxes, homozygote for the *kci* allele. White boxes, heterozygote for the *kci* and BN alleles. (B) The *kci* locus was physically localized to the 2.6-Mb region defined with *Rab36* and *D20Rat75*. Within the *kci* locus, three genes (white boxes) and three predicted transcripts (gray boxes) have been mapped. (C) Sequence analysis of *Pcdh15* cDNA from wild-type and *kci/kci* rats. In the *kci/kci* rat, a nucleotide conversion C to T (red) occurred at the position of nucleotide 2911 of the rat *Pcdh15* cDNA. The *kci* mutation generates a premature termination at codon 971 of the putative PCDH15 protein. Due to the *kci* mutation, a *Kpn*I site (GGTACC) is lost and a *Dde*I site (CTNAG) is generated. (D) Molecular diagnosis for the *kci* mutation. PCR products amplified with a pair of primers, rPcdh15kci-F (5'-GGGTTGCCAGCAAGTCGG-3') and rPcdh15kci-R (5'-CTTAAAAATTGT-TGTAGGCTC-3'), were subjected to restriction digestion with *Kpn*I (upper) or *Dde*I (lower). A 141-bp PCR product from the wild-type allele was digested with *Kpn*I to 114-bp and 27-bp fragments, but not with *Dde*I, while a 141-bp PCR product from the *kci* allele was digested with *Dde*I to 114-bp and 27-bp fragments, but not with *Kpn*I. Note that the 27-bp fragment was too small to be seen. The CD(SD) rat was used as a control (+/+). (E) Schematic representation of PCDH15 indicating cadherin repeats (C1-C11), transmembrane domain (TM), and cytoplasmic domain. In the KCI rat, the protein is prematurely truncated and lacks the last two cadherin domains, transmembrane and cytoplasmic domains.

Fig. 3. Scanning electron micrograph of the organ of Corti from control (*kci/+*) (A, C) and homozygous (*kci/kci*) (B, D) rats at 16 weeks of age. The single row of inner hair cells is indicated by an arrow and the three rows of outer hair cells (A) are labeled 1, 2, and 3. Stereocilia in the control show a normal configuration. In the *kci/kci* rat, stereocilia of both inner and outer hair cells are severely disorganized. Most outer hair cells lose their stereocilia and the remaining stereocilia are shortened, fused, and disorientated. Bar=10 μ m (A, B). Bar=5 μ m (C, D)

The *Pcdh15*^{*kci*} allele is a functional null, because the mutation introduces a stop codon, and it is included in the repertoire of rodent *Pcdh15* mutant alleles. Mature KCI rats show constant circling behavior and histological defects in both cochlear and vestibular hair cells, which are comparable with those observed in mouse *Pcdh15*-null alleles such as *av*^{*Tg*}, *av*^{*5J*}, or *av*^{*Jfb*} [4, 8, 17]. Behavioral and histological findings of KCI rats indicate that PCDH15 is also indispensable in stereocilia bundle morphogenesis in rats. In addition to analyses of different alleles of *av*, further extensive analyses of KCI rats will allow us to understand the function of *Pcdh15* in inner hair cell development and the cause of inner ear disorders in USH1F and DFNB23 patients.

As an animal model for USH1F and DFNB23, KCI rats have great advantages over the *av* null-mutant mice. Since the rat has suitable body size for artificial manipulation, the KCI rat could serve as a better disease model in the development of novel clinical treatments for USH1F and DFNB23. In the rat, ample data on physiology and pharmacology have been accumulated. Thus, the KCI rat could also serve as a better disease

model in the development of new drugs for USH1F and DFNB23.

Patients with USH1F suffer from progressive retinitis pigmentosa, in addition to profound congenital hearing loss and vestibular deficits [11]. Although cochlear implantation can recover auditory perception, there are no clinical treatments for recovery of visual perception, thus, animal models for retinitis pigmentosa in USH1F patients have been greatly anticipated. Although PCDH15 protein is known to be expressed in the rodent retina [9], we could not detect any evidence of retinal degeneration or disorganization in the KCI mutant rat. To identify functional abnormalities of the retina in the KCI rat, further analyses, such as electroretinograms and electron microscopic observations, will be necessary. In *av* mice, it has been reported that two nonsense *av* mutations, *Pcdh15*^{*av5J*} and *Pcdh15*^{*avJfb*}, show significantly attenuated but stable electroretinograms in the absence of histopathology of the retina [9].

In summary, we established the KCI rat strain and identified the causative gene of the KCI mutant phenotype as the Arg971X mutation of the *Pcdh15* gene. The

Fig. 4. Histology of cochlear (A, B), spiral ganglion (C, D), and saccular macula (E, F) in control (A, C, E) and *kci/kci* rats (B, D, F) at 16 weeks of age. (A) The organ of Corti from a control animal with normal inner hair cells (IHC) and outer hair cells (OHC) and intraepithelial fluid spaces. The osseous spiral lamina is filled with myelinated nerve fibers (N). (B) The collapsed organ of Corti and the degeneration of inner and outer hair cells in the *kci/kci*. There is also a dramatic reduction in the number of myelinated nerve fibers (arrow) in the osseous spiral lamina. (C) Cross sections of the spiral ganglion from a control cochlear. (D) The spiral ganglion (arrows) from the *kci/kci* rat showing reduced numbers of ganglion cells in an affected animal at 16 weeks of age. (E) The saccular macula from a control animal with normal sensory hair (H) and supporting (S) cells. (F) Cross section of the saccular macula demonstrating a marked decrease in the number of hair cells (H) in an affected animal. The otolithic membrane (OM) was also severely damaged. The supporting cells (S) appear normal. The specimens were embedded in epoxy resin and stained with toluidine blue (A, B) or embedded in paraffin and stained with hematoxylin and eosin (C–F). Bar=100 μ m (A, B). Bar=50 μ m (C–F).

Fig. 5. Retinal histology in *kci/kci* rats. Vertical retinal cross sections from a heterozygous (A) and a homozygous (B) KCI mutant rat at 16 weeks of age. Each layer is labeled: ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), and the layer of rods and cones (LRC). No anatomic defects were noted in the homozygous rat. Bar=50 μ m.

KCI rat showed abnormal morphologies or organizations of hair cells in the inner ear. Considering the importance of rats in the development of clinical treatments and diagnoses, and new drugs, the *Pcdh15*-deficit KCI rat has potential to serve as a good animal model for UDH1F and DFNB23.

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