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Whole genome sequencing identifies a homozygous nonsense mutation in the *JPH2* gene in Shih Tzu dogs with progressive retinal atrophy

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Summary

Progressive retinal atrophy (PRA), common autosomal recessive disorder affecting several dog breeds including Shih Tzu, is characterized by degeneration of photoreceptors leading to blindness. To identify PRA genetic variants, three affected and 15 unaffected Shih Tzu and 20 non-Shih Tzu were recruited. Dogs underwent ophthalmologic examination and electroretinography, revealing hallmark retina pathological changes and an abnormal electroretinography in all affected dogs but not in unaffected dogs. WGS was performed. Non-synonymous homozygous variants were searched in coding regions of genes involved in retinal diseases/development; the criterion was that variants should only be present in affected dogs and should be absent in both unaffected and 46 genomes of dogs (from an available evolutionary database). Only one out of the 109 identified variants is predicted to harbor a high-impact consequence, a nonsense c.452A>C (p.L151X) in the *JPH2* gene. The genotype of *JPH2* variant in all 38 dogs was determined with Sanger sequencing. All three affected dogs, but none of the 35 unaffected, were homozygous for the nonsense variant. *JPH2* has been previously found to be expressed in several excitable cells/tissues including retina photoreceptors. Hence, *JPH2* is a candidate gene for PRA in Shih Tzu.

Keywords dogs, eye diseases, junctophilin, retinal degeneration, retinal diseases, retinitis pigmentosa, retinopathy

Introduction

Progressive retinal atrophy (PRA) is a heterogeneous group of inherited retinal disorders affecting several dog breeds, a condition that is clinically and genetically similar to retinitis pigmentosa (RP) in their human counterparts (Miyadera *et al.* 2012; Bunel *et al.* 2019). Canine PRA is commonly classified according to age of onset (early vs. late) and type of breed. The prevalence of PRA varies with the type of dog breed as well as the breed's geographical origin. Over 100 breeds are known to be affected by different PRA types and numerous PRA forms have been described in the literature

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(Christmas 1992; Miyadera *et al.* 2012; Papaioannou & Dubielzig 2013; Bunel *et al.* 2019; Winkler *et al.* 2020).

Progressive retinal atrophy is characterized by a progressive night vision impairment to total blindness from initial degeneration of rod followed by cone photoreceptors. There is another form of photoreceptor cell degeneration led by cone cells with rods being affected later. Ophthalmologic examination reveals changes in the fundus such as tapetal hyperreflectivity, pigmentation changes, atrophy of the optic disk and retinal vascular attenuation in both forms. Thus, at the early stage of the disease course, electroretinography can be useful to distinguish PRA from cone-led diseases (Miyadera *et al.* 2012). The age of onset and its clinical course varies between breeds (Bunel *et al.* 2019). Progressive retinal atrophy can be inherited as autosomal-recessive or -dominant and x-linked traits, although the most common mode of inheritance is recessive owing to the genetic history of dog breeding – inbreeding and use of popular sires increases the probability of spreading a mutation and obtaining dogs homozygous for that mutation (Miyadera *et al.* 2012). Over 20 genes underlying canine PRA have been identified, such as *ADAM9*, *BEST1*, *C2orf71*, *CNGB3*, *CNGB1*, *NPHP4*, *RD3*, *STK28L*, *RPGR*, *CCDC66*, *COL9A2*, *COL9A3*, *NHEJ1*, *PDE6A*, *PDE6B*, *RHO*, *RPGRIP1*, *RPE65*, *SLC4A3*, *PRCD* and *SAG*, from which a wide range of molecular pathways have been unveiled (Miyadera *et al.* 2012; Ahonen *et al.* 2013; Goldstein *et al.* 2013; Cooper *et al.* 2014; Downs *et al.* 2014a; Winkler *et al.* 2020). Most of the genes have overlapped with human retinal disorder genes. Moreover, in humans, there are over 200 genes identified as causing retinal disorders. Progressive retinal atrophy and other ocular co-morbidities in Shih Tzu have been reported (Christmas 1992; Papaioannou & Dubielzig 2013; Krishnan *et al.* 2020); however, the causative gene for PRA has not been identified in this dog breed.

The discovery of the junctophilin (JPH) family of proteins in 2000 was consistent with a consistent body of literature linking these proteins to key roles in all excitable cells with implications for cellular physiology and pathophysiology (Landstrom *et al.* 2014; Jiang *et al.* 2019). This JPH family is responsible for maintaining subcellular architecture and regulating critical calcium-handling proteins in a variety of neuronal networks and muscle tissues. Impaired *JPH1–JPH4* genes have been implicated in behavioral and learning deficits, motor control, Huntington disease-like pathology, skeletal muscle myopathy, cardiomyopathy, heart failure and arrhythmia (Landstrom *et al.* 2014; Jiang *et al.* 2019). The phenotypic analysis of knockout rodents lacking JPH subtypes has demonstrated their key contribution to physiological functions in such muscles and neurons. *Junctophilin-2* (*JPH2*) gene has been found in the retina as well as in other tissues/organs in dogs (Robinson-Rechavi *et al.* 2020). However, it has not been reported to be associated with RP and its physiological role in canines is not known.

Herein, WGS was performed aiming to identify a possible causal mutation in Shih Tzu dogs affected by PRA.

Materials and methods

Study sample

All study protocols were performed with approval from the Faculty of Veterinary Science, Mahidol University's Institutional Animal Care and Use Committee (certificate number 2015-04-10) and followed all principles of animal welfare. Three PRA-affected Shih Tzu dogs, 15 unaffected Shih Tzu dogs and 20 other unaffected dogs of other breeds, including five Beagles, five French Bulldogs, four Mixed Breed dogs, one English Bulldog, one Bulldog, one Rottweiler, one Siberian Husky, one Pekingese and one Pug, were recruited through Prasu-Arthorn Animal Hospital, Faculty of Veterinary Science, Mahidol University. The inclusion criteria for the Shih Tzu were that all participating dogs must be privately owned pets and older than 7 years of age. After recruitment, all dogs were examined by a veterinary board-certified ophthalmologist for eye screening to confirm PRA-related pathologies and assigned to PRA-affected and -unaffected groups. Electroretinography was carried out in all affected and unaffected Shih Tzu dogs. In addition, echocardiography was performed in two affected dogs as one of the three affected dogs died before the echocardiography appointment. This cardiological examination was essential to rule out cardiac disease as the *JPH2* gene has been reported to cause cardiac pathologies in humans and rodent models (Landstrom *et al.* 2014). Dogs presenting with clinical signs consistent with PRA, such as vascular attenuation, tapetal hyperreflectivity, atrophy of the optic nerve, retina pigmentary changes, blindness and abnormal electroretinography recordings were categorized as PRA affected.

DNA was extracted and isolated from whole blood using a Gentra Puregene Blood Kit (Qiagen) and a DNeasy blood and tissue kit (Qiagen) using EDTA as an anti-clotting agent. DNA concentrations were measured using an ND-1000 NanoDrop spectrophotometer (Thermo Scientific).

Whole genome sequencing

WGS was performed in two affected and one unaffected Shih Tzu using the Illumina paired-ends Hiseq 4000 platform from Macrogen Inc. The total outputs of two PRA-affected and one unaffected dogs were 90, 88 and 75 Gb respectively. Raw data reads were mapped to CANFAM 3.1 reference genome using the Burrows–Wheeler Aligner (Li & Durbin 2009). The coverages were 34.88×, 33.75× and 29.6× depth respectively. SAMTOOLS was used to remove

duplicated reads and call variants as compared with the reference genome resulting in variant call format (VCF) files.

As the autosomal recessive pattern is the most common mode of inheritance for PRA, the homozygous state was assumed in both affected dogs, and that was different from the unaffected dog and the reference dog genome (which can be heterozygous or homozygous wt). In other words, both affected dogs had variants called '1/1', whereas the rest of the dogs had variants called either '0/0' or '0/1' in the VCF file. Thus, we searched for variants that satisfied this assumption. After that we carried out variant annotation for the remaining variants using Ensembl VARIANT EFFECT PREDICTOR (McLaren *et al*. 2016).

Initially, the candidate gene screening approach in known human RP genes and canine PRA genes (Table S1) was performed to screen for pathogenic or likely-to-be pathogenic variants. A non-synonymous variant in *NPHP4* gene was identified and further Sanger sequenced. Subsequently, the publicly available VCF files of 46 dogs (11 indigenous dogs from southern East Asia, 12 indigenous dogs from northern East Asia, four dogs from Africa (Nigeria) and 19 dog breeds from the Old World and The Americas such as Tibetian Mastiff, Sloughi, Samoyed and Chihuahua that evolutionarily were domesticated) were downloaded from Dog 10K genomes project (Wang *et al*. 2016; Wang *et al*. 2019). Then, we merged the VCF files of the two affected and one unaffected dogs with the VCF file from Dog 10K genomes project using BCFTOOLS 1.7 (Narasimhan *et al*. 2016). We retained only the intersecting portion between our VCF file and Dog 10K genomes project VCF file, then we searched for variants that satisfied the autosomal recessive mode of inheritance criteria mentioned above for all genes. There was only one position in *JPH2* gene that had stop gain consequence and high impact prediction. The variant in *JPH2* gene was then Sanger sequenced. A summary of our WGS analysis pipeline is shown in Fig. S1.

FASTQ files of WGS of PRA affected dogs and VCFs of control dogs were deposited at the Genome Sequence Archive under accession number CRA001375 and are publicly available at http://bigd.big.ac.cn/gsa (Wang *et al*. 2017; National Genomics Data Center Members & Partners 2020).

Sanger sequencing to determine genotype of the candidate variants

The candidate variants in the *NPHP4* and *JPH2* genes were Sanger sequenced in all three affected dogs. Seven unaffected Shih Tzu dogs and 35 unaffected dogs (15 Shih Tzu and 20 non-Shih Tzu breeds) were Sanger sequenced for *NPHP4* and *JPH2* variants respectively. Primers specific to the *NPH4* and *JPH2* genes were designed by Primer3 version 0.4.0 (Untergasser *et al*. 2012). The primer set for *NPH4* (forward, 5′-GCTGAGAGCAGGAGCAACATA-3′; reverse, 5′-GAAAGCAGGGGTAGAGAGATGA-3′) generated a 453 bp PCR product size. The primer set for *JPH2* gene (forward, 5′-CAGAAGCTCAGAGGGGTTACA-3′; reverse, 5′-TGGGATTTTTCTGGTTCTCTT-3′) generate a 233 bp PCR product size. The PCR mixture contained 2 µl of template DNA, 2.5 µl of 10× Expand High Fidelity buffer with 15 mM $MgCl_2$, 1 µl of 1 mM dNTPs, 1.3 units of Expand High Fidelity Enzyme mix (Roche Life Science) and 0.4 µM of each forward and reverse oligonucleotide primer for each gene. Sterile nuclease-free water was added to increase the PCR mixture volume to 25 µl. PCR for the *NPH4* gene was performed under the following conditions: 95°C for 5 min for initial denaturing, followed by 30 cycles of 15 s at 94°C, 30 s at 56°C, and 45 s at 72°C, and terminated with a cycle at 72°C for 7 min. As for the *JPH2* gene, PCR was run under these conditions: 2 min at 94°C for initial denaturing phase, followed by 30 cycles of 15 s at 94°C, 30 s at 48°C, and 45 s at 72°C, and was terminated at 72°C for 7 min.

Results

Three dogs were clinically classified as affected with PRA and 35 dogs were deemed normal or unaffected by the condition. The onset of PRA signs in the three affected Shih Tzu was reported by owners at 5, 7 and 9 years of age. Fundus examination of the affected dogs revealed retina vascular attenuation, tapetal hyperreflectivity, atrophy of the optic nerve head and retina pigmentary changes. Electroretinography further confirmed the PRA diagnosis.

Screening for variants segregated among affected dogs in known human RP and canine PRA genes (Table S1) revealed a non-synonymous substitution variant c.136G>A (p.Val46Met) in *NPHP4* gene located in chromosome 5 concordant with the autosomal recessive trait of PRA. Subsequently, Sanger sequencing of this variant in the other affected dog and in six unaffected dogs revealed that the affected and five unaffected dogs were homozygous for the missense variant, and one unaffected dog was homozygous for the wt.

When the analysis was performed with publicly available VCF files of 46 domesticated dogs, which are a part of an evolutionary *Canis lupus familiaris* study database across the Old World continents and The Americas, East Asia and Africa (Wang *et al*. 2016; Wang *et al*. 2019), 7600 homozygous variants were present only in the genome of the two affected dogs. Out of these, 109 variants were non-synonymous with 108 being missense. All 108 missense variants were predicted to be moderate (at most) in their impact on the protein's functions. Only one variant, a nonsense c.452A>C (p.L151X) in the *JPH2* gene (Fig. 1), was predicted to have a high impact. This nonsense variant would lead to a premature stop codon and shorten the translated polypeptide sequence from 674 to 150 amino acid residues.

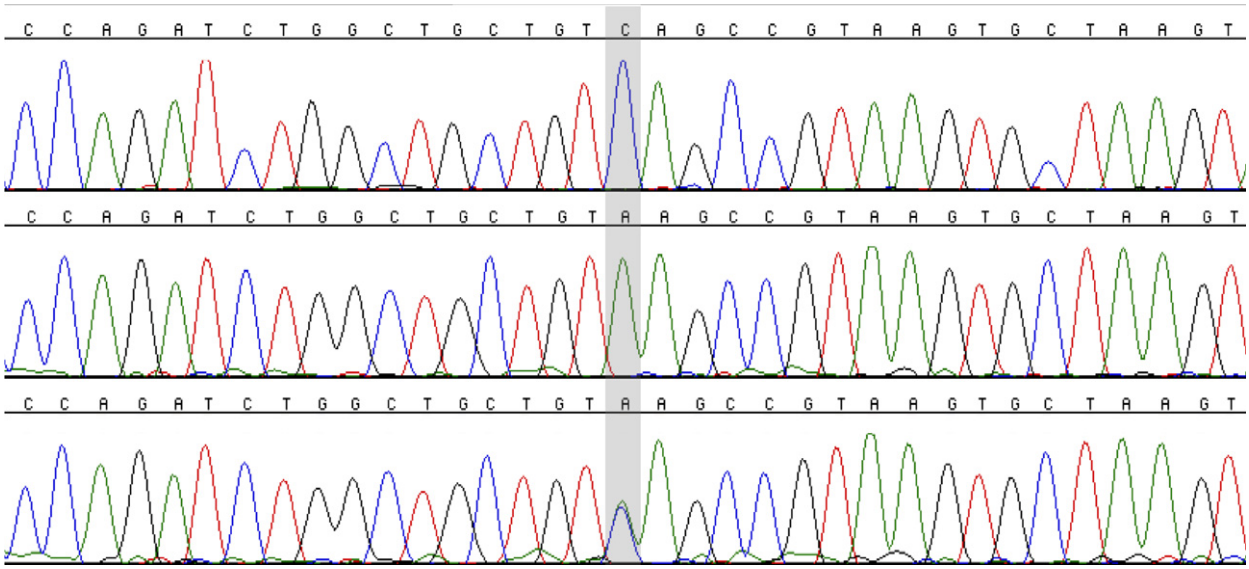

Figure 1 Sanger sequencing in the *JPH2* region. Chromatograms of the c.452A>C (p.L151X) mutation in the *JPH2* gene (gray shadow region) in a Shih Tzu affected by PRA showing a homozygous CC genotype (top panel), an unaffected dog showing a homozygous AA genotype (middle panel) and another unaffected dog showing a heterozygous AC genotype (lower panel).

Subsequently, Sanger sequencing showed that all three affected dogs were homozygous for the nonsense, whereas 11 and four unaffected Shih Tzu dogs were heterozygous for the nonsense and homozygous for the wt respectively. To determine whether c.452A>C is present in other breeds, we screened 20 dogs from non-Shih Tzu breeds, which revealed its heterozygous presence in three French bulldog, one Pug, one English bulldog and one Beagle. The remaining 14 dogs were homozygous for the wt. This suggests that the homozygous nonsense mutation in *JPH2* may underlie PRA in Shih Tzu. A comprehensive examination of all cardiac signs and symptoms including echocardiography revealed no abnormal findings in PRA-affected dogs.

Discussion

To our knowledge, this is the first genetic study searching for and identifying genetic variants as potential etiological causes for common eye degenerative diseases causing PRA in Shih Tzu dogs. The WGS identified a homozygous nonsense variant in *JPH2* gene c.452A>C (p.L151X) in all three studied Shih Tzu. In the 15 unaffected Shih Tzu, 46 primitive dogs (from a publicly available evolutionary database) and 20 additional dogs of non-Shih Tzu breed, although the heterozygous state was present, none were homozygous for the nonsense. Progressive retinal atrophy is a heterogenous group of retinal disorders and the mutant allele frequency ranges between 0.0052 and 0.396 depending on the gene mutations, breed and dog's geographical location (Kukekova *et al.* 2009; Downs *et al.* 2011; Downs *et al.* 2013; Downs & Mellersh 2014; Downs *et al.* 2014b; Palanova *et al.* 2014; Koll *et al.* 2017; Karlskov-Mortensen *et al.* 2018; Andrade *et al.* 2019). Moreover, the mutation in one gene can be found in many different breeds. Thus, further investigation in a larger Shih Tzu and other breeds population would be required to investigate the variant allele frequency and determine whether the variant in *JPH2* is a common variant or a pathogenic one.

This *JPH2* genetic nonsense mutation is expected to lead to a shorter polypeptide sequence. The *JPH2* gene is moderately expressed in the retina in dogs (Bastian *et al.* 2020; Robinson-Rechavi *et al.* 2020), supporting its mutant role in retinal diseases. Moreover, key genes such as *JPH2* are expressed at higher levels in the human neurosensory retina (macula and at the periphery) as compared with choroid and sclera regions (Li *et al.* 2014). Other genes such as *BDKRB2*, *CYP4B1*, *DES*, *HBB*, and *SAA1* can also be highly expressed; however these genes have not been reported either in the literature or in public databases for retinal diseases (Daiger *et al.* 1998).

Junctophilins connects endo/sarcoplasmic reticulum to plasma membrane in excitable cells to mediate the communication between the cell surface and intracellular channels. The junctophilin protein domain includes eight amino-terminal membrane occupation and recognition nexus (MORN) motifs (I and II), the joining region, the alpha-helical region, the divergent region and the C-terminal transmembrane motif (Nishi *et al.* 2000; Landstrom *et al.* 2014; Jiang *et al.* 2019). All *JPH* subtypes (1–4) are highly conserved across various species and different isoforms, particularly MORN motifs (Garbino *et al.* 2009). In human, there are five exons in the *JPH2* gene (Landstrom *et al.* 2014) and two isoforms produced by alternative splicing have also been reported (The UniProt Consortium

2020). The variant c.452A>C (p.L151X) in the *JPH2* gene was found to be in the exon 2 of human *JPH2* where it could be part of MORN I, which is localized at the plasma membrane (Nishi *et al*. 2000).

Interestingly, the phenotypical analysis of transgenic mice lacking *JPH* subtypes has demonstrated their key contribution to physiological functions in neurons as well as smooth/cardiac/skeletal muscles. Junctophilin 1 plays a key role in maintaining skeletal muscle excitation–contraction coupling by mediating Ca^{2+} release from sarcoplasmic reticulum, whereas junctophilin 3 and 4 maintain efficient Ca^{2+} signaling cross-talk between intracellular signaling and the plasma membrane in the neurons (Landstrom *et al*. 2014; Jiang *et al*. 2019). Junctophilin 2 has a functional interaction with potassium ion channels in native cardiomyocytes (Fan *et al*. 2018) and *JPH2* variants may have a role in human cardiomyopathies (Matsushita *et al*. 2007; Landstrom *et al*. 2007; Vanninen *et al*. 2018; Sabater-Molina *et al*. 2016) and atrial fibrillation derived from dysfunctional ryanodine receptor Ca^{2+} ion channels (Beavers *et al*. 2013). Despite the evidence of cardiac pathologies found in human studies of subjects displaying *JPH2* mutations, the clinical examination of PRA-affected dogs did not reveal any cardiac abnormalities. The *JPH2* gene has also been found in canine retina on the public Bgee database (Robinson-Rechavi *et al*. 2020), confirming our reported findings. Although *JPH2* gene is expressed in both retina and heart, this discovered variant may lead to a clinical sign in only the retina because genes that can compensate for the defective *JPH2* are expressed adequately in the heart but not in the retina.

These findings may require further examination of retinal tissue biopsies from deceased PRA-affected Shih Tzu to confirm the mutated protein sequences generated by the variant c.452A>C (p.L151X). Transgenic mice and conditional knock-out or knock-in models for the *JPH2* gene are necessary to understand the full spectrum of functional abnormalities in the anatomical structures of the retina and compare them with the potentially impaired ion channels in cardiac tissue (Landstrom *et al*. 2007; Matsushita *et al*. 2007; Beavers *et al*. 2013; Landstrom *et al*. 2014; Fan *et al*. 2018). Furthermore, silencing the *JPH2* gene in retinal photoreceptor cells (rods or cones) *in vitro* could unveil the potential role of the *JPH2* gene in PRA-affected dogs.

In conclusion, a homozygous nonsense mutation c.452A>C (p.L151X) in the *JPH2* gene is potentially associated with PRA in Shih Tzu dogs.

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Conflicts of interest

The authors declare no conflicts of interest.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 A summary of WGS analysis pipeline.

Table S1 A list of human retinitis pigmentosa and canine progressive retinal atrophy genes.