Brief Communication

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Inheritance, Mode of Inheritance, and Candidate Genes for Primary Hyperparathyroidism in Keeshonden

Richard E. Goldstein, Daniel Z. Atwater, Dava M. Cazolli, Orly Goldstein, Claire M. Wade, and Kerstin Lindblad-Toh

Background: Primary hyperparathyroidism (PHPT) is caused by inappropriate secretion of parathyroid hormone (PTH) by autonomously functioning neoplastic or hyperplastic parathyroid "chief" cells. Keeshonden are thought to be over-represented in studies on canine PHPT, but no proof of heritability or mode of inheritance has been published. The canine disease clinically resembles human familial isolated hyperparathyroidism (FIHP).

Hypothesis: Primary hyperparathyroidism in Keeshonden is genetically transmitted and is caused by a mutation in 1 of 4 genes implicated in human FIHP: *MEN1*, *CASR*, *HRPT2*, or *RET*.

Animals: Pedigrees consisting of 1647 Keeshonden were created including 219 Keeshonden with known PHPT phenotypes (69 positive). DNA samples were obtained from 176 of the 219 Keeshonden (34 positive).

Methods: Heritability and mode of inheritance were determined by segregation analysis. Canine homologs to the human genes were identified. Exons and surrounding intron regions were sequenced and scanned for sense-altering polymorphisms or polymorphisms that segregated with the disease. Messenger RNA from a parathyroid tumor of an affected Keeshond was analyzed for polymorphisms and splice alterations.

Results: PHPT follows an autosomal dominant mode of inheritance in Keeshonden with possible age-dependent penetrance. No polymorphisms identified in the genes analyzed were associated with a change in predicted protein or in hypothesized splice sites.

Conclusions and Clinical Importance: PHPT is an autosomal dominant, genetically transmitted disease in Keeshonden. Once the mutation locus is identified, genetic testing should quickly decrease the incidence of PHPT in this breed. It is unlikely that mutations in *MEN1*, *CASR*, *HRPT2*, or *RET* cause PHPT in Keeshonden.

Key words: Canine genetics; Hypercalcemia; Parathyroid disease.

Primary hyperparathyroidism (PHPT) is a common cause of hypercalcemia in dogs.[1,2] It is caused by inappropriate secretion of parathyroid hormone (PTH) by autonomously functioning parathyroid "chief" cells. Parathyroid hormone secretion is normally regulated tightly by negative feedback in the presence of increased ionized calcium concentrations in serum. In PHPT, PTH secretion persists despite increased calcium concentrations, often resulting in marked hypercalcemia. Eighty to 85% of cases of PHPT in dogs result from a solitary parathyroid adenoma. Parathyroid hyperplasia affecting multiple glands occurs in most of the remaining cases, while malignant parathyroid carcinoma is considered to be very rare in dogs.[2] PHPT affects middle-aged to older dogs with a 11.2 year mean age of diagnosis,[3] rarely affecting dogs younger than 4 years of age.[2] Early clinical signs are mild and may include increased drinking and urination, calcium-containing urolithiasis, and gradual onset of weakness, lethargy, and weight loss. As the hypercalcemia persists and becomes more profound, damage to bones and kidneys may occur.[2]

The diagnosis of PHPT is based on the identification of normal or increased serum PTH concentrations with abnormally high total and ionized calcium concentrations in serum. In a recent review, PTH concentrations were increased in approximately 25% of PHPT cases, and within the reference range in 75%.[3] The enlarged parathyroid gland or glands can often be identified during cervical ultrasonography.[4] Treatment of PHPT involves surgical resection or ultrasound-guided ablation (chemical or heat) of the affected parathyroid glands. When performed properly, all treatment modalities are typically successful with a relatively low percentage of recurrence.[5]

Little is known about the genetics of this condition in dogs despite reported breed predispositions.[1,2] A familial form of neonatal hyperparathyroidism was reported in a single litter of German Shepherd puppies. An autosomal recessive mode of inheritance was suspected.[6] The Keeshond is the breed most likely to be affected by the more common adult onset PHPT. Keeshonden represented 44 of 168 (26%) of the dogs in a University of California Davis study[2] and approximately 40% of the dogs diagnosed at the Cornell University Hospital for Animals in the last 10 years. A review of cases of PHPT in dogs identified Keeshonden as the most likely breed to be affected by PHPT with 214 positive samples and an average American Kennel Club registration of 4,375 dogs, yielding an

From the Department of Clinical Sciences, Cornell University, Ithaca, NY (R.E. Goldstein, Atwater, Cazolli); and the James A. Baker Institute, Cornell University, Ithaca, NY (O. Goldstein); and the Broad Institute of Harvard and MIT, Boston, MA (Wade, Lindblad-Toh); and the Center for Human Genetic Research, Massachusetts General Hospital, Boston, MA (Wade). Dr Cazolli is presently affiliated with the Fifth Avenue Veterinary Specialists,

Reprint requests: Dr Richard E. Goldstein, Department of Clinical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853; e-mail: rg225@cornell.edu.

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odds ratio of 50.7 for PHPT in Keeshonden. Other breeds that had over 100 positive samples were Golden Retrievers (odds ratio of 1.6) and Dachshunds (odds ratio of 2.0).[1]

Familial hyperparathyroidism is commonly diagnosed in humans.[7] It encompasses 5 clinically defined disorders including multiple endocrine neoplasia types 1 and 2A (MEN1 and MEN2A), hyperparathyroidism-jaw tumor syndrome (HPT-JT), familial hypocalciuric hypercalcemia (FHH), and familial isolated hyperparathyroidism (FIHP).[8] Among these, MEN1, MEN2A, HPT-JT, and FHH are characterized by inactivating mutations in the *MEN1*, *RET*, *HPRT2*, and *CASR* genes, respectively. These diseases are all associated with hyperparathyroidism of varying penetrance caused by parathyroid neoplasia, hyperplasia, or both, in addition to other endocrinopathies.[7–10] Human FIHP is defined as familial PHPT alone, in the absence of additional endocrinopathies.[11] The clinical presentation of canine PHPT often closely matches human FIHP. The diagnoses of the exact syndrome and the causative gene in humans can be complicated. In some cases, patients with family history of MEN1, HPT-JT, and FHH syndromes present with PHPT alone and are therefore diagnosed as suffering from FIHP.[8] The causative genes of most cases of FIHP are unknown, but when mutations are recognized they are also most frequently in the same *MEN1*, *CASR*, and *HRPT2* genes.[8–10] The fact that the causative mutations are yet undiscovered in most cases of human FIHP reflects the difficulty in detecting germline mutations and also the likelihood that mutations in other, unrecognized genes may be involved.[12]

We hypothesized that PHPT is genetically transmitted in Keeshonden and is caused by an exonic mutation in the canine orthologs of the *MEN1*, *CASR*, or *HRPT2* genes, or a mutation in the canine ortholog of the *RET* gene region that is commonly associated with human HPT.

Materials and Methods

Phenotypic Determination

The phenotypic status of all dogs included in the study was established based on the following criteria by which dogs were considered positive for PHPT if they had all of the following:

a. Abnormally high serum total calcium concentration (measured by the dog's veterinarian or at Cornell University Hospital for Animals, Ithaca, NY).
b. Abnormally high serum ionized calcium concentration assayed at a veterinary endocrine reference laboratory.[a]
c. PTH concentrations that were above or within the reference range when assayed by a veterinary endocrine reference laboratory.[a]

In over 80% of the cases the diagnosis was also confirmed by histopathologic analysis of excised parathyroid glands.

Dogs were considered negative for PHPT based on any of the following:

a. Serum total calcium concentrations (corrected for albumin concentrations) within the reference range (measured by the dog's veterinarian or at Cornell University Hospital for Animals).
b. Serum ionized calcium concentrations within the reference range assayed at a veterinary endocrine reference laboratory.[a]

Because of the late onset nature of this disease, only negative dogs over the age of 12 were considered free of the disease and included in the study population.

Keeshond DNA Collection and Pedigree Analysis

A Keeshond DNA bank was established by means of ethylenediamine tetraacetic acid (EDTA) whole-blood provided by dog owners and veterinarians across the United States. DNA was purified with QIAmp DNA purification kits.[b] Information supplied by owners and veterinarians was used to compile a database documenting the PHPT status, age, date of diagnosis, and 5-generation pedigree of each dog.

Pedigrees were compiled and analyzed by commercially available software.[c] A segregation analysis to determine the mode of inheritance was performed from the pedigree by the program "Geneprob."[13] This program uses a Gibbs sampling method to allocate likelihood values to different genotypes for each individual, given prior assumptions about the inheritance model provided by the user. Three inheritance models were tested, completely dominant, incompletely dominant, or recessive. Because the occurrence of the disease did not appear to be restricted by sex, only autosomal models were tested. The normal allele probabilities were estimated at 0.82, 0.83, and 0.42 for complete dominance, incomplete dominance, and recessive, based on the number of affected and unaffected individuals and assuming Hardy-Weinberg equilibrium. Hardy-Weinberg equilibrium assumes random mating, which typically is not the case in breeds of dogs with small populations. Therefore its use in this study was limited to provision of estimates of allele frequencies only of non–closely related Keeshonden for modeling purposes.

Mutation Screening of Candidate Genes

Canine orthologs to human genes *MEN1* (NM 130799), *CASR* (NM 000388), *HRPT-2* (NM 024529), and *RET* (NM 001014432) were identified by the canine BLAT search at the University of California Santa Cruz Genome Bioinformatics website.[d] An open reading frame was hypothesized for each candidate gene by investigating canine sequences for probable start, stop, and splice sites. Hypothetical splice sites were chosen that conferred the most similarity to the human homologous protein sequence. Polymerase chain reaction (PCR) primers flanking each hypothesized exon were designed by PrimerQuest software.[e]

DNA from 4–12 PHPT-positive and 2–8 PHPT-negative dogs was amplified with standard PCR protocols on an Eppendorf Mastercycler Gradient thermocycler.[f] The samples selected for exon scanning included DNA from a PHPT-positive male dog, a PHPT-negative female, and 1 of 3 PHPT-positive progeny of those 2 dogs as well as DNA from at least 1 other PHPT-positive and 2 PHPT-negative dogs. The DNA from the blood of a PHPT-positive dog, from which a tumor tissue sample was available, was also sequenced with each primer set. Amplified PCR product was purified with QIAquick PCR Purification kits[f] and sequenced with the same primers at the Cornell Biotechnology Resource Center with the Applied Biosystems Automated 3730 DNA Analyzer.[g] All exon sequences and flanking intron sequences were scanned for polymorphisms that might affect RNA processing and translation or that segregated with PHPT in the sampled dogs. In the case of the *RET* gene, only exons 10 and 11 were sequenced to scan for the well-characterized mutations that cause HPT in humans. Exons 9, 12, and 20 were

Table 1. Mutation screening of candidate genes for primary hyperparathyroidism in Keeshonden.

Gene	GenBank Accession Number	Human Disease	Nucleotide Similarity Between Canine and Human Genes (%)	Amino Acid Similarity Between Canine and Human Proteins (%)	Single Nucleotide Polymorphisms Identified		
					All	Exonic	AA or splice change
MEN1	DQ366289	MEN-1	90.8	98.5	9	3	0
CASR	DQ366290	FHH, NSHPT	89.8	94.2	14	7	0
HRPT2	DQ366291	HPT-JT	94.9	100.0	15	0	0
RET	DQ366292	MEN-2A	85.8	88.1	7	3	0

AA, amino acid; MEN, multiple endocrine neoplasia; FHH, familial hypocalciuric hypercalcemia; NSHPT, neonatal severe primary hyperparathyroidism; and HPT-JT, hyperparathyroidism-jaw tumor syndrome.

also sequenced to aid in the identification of reverse transcription primers.

RNA Analysis

A parathyroid neoplasm from a PHPT-positive Keeshond was stored in RNAlater[h] and subsequently purified with an RNeasy RNA purification kit.[b] The purified mRNA was amplified by reverse transcriptase (RT)-PCR with MEN1-specific (5′-TCA GAG GCC CTT GCG CTG GCG-3′), CASR-specific (5′-TTA CGA ATG CAG TAT GTT TTC CGT-3′), HRPT2-specific (5′-TCA GAA TCT CAA GTG GGA TTT ATG-3′), and RET-specific (5′-TTA ACT ATC AAA TGC GTC CAT TAA-3′) primers with the Invitrogen Thermoscript RT-PCR System.[i] The cDNA was amplified and sequenced by protocols described in previous Mutation Screening of Candidate Genes section. The RNA from healthy parathyroid tissue of a PHPT-negative mixed breed dog was also analyzed. For the *RET* gene, a 586 base pair (bp) portion containing the well-characterized HPT-causing mutations within exons 10 and 11 was reverse transcribed and sequenced.

Results

Keeshond Family Collection and Segregation Analysis

Whole-blood from 176 Keeshonden was received between June 2004 and November 2005 from owners and veterinarians. Of those dogs, 34 were PHPT positive and 142 were PHPT negative at the time of sample submission. Five generations' ancestry data were used to construct a pedigree of 1647 related dogs. The PHPT status of an additional 43 dogs within the pedigree was determined, 35 of which were PHPT positive.

The mean age of PHPT diagnosis was 9.8 ± 1.9 SD years and ranged from 6.2 to 13.7 years. Among 69 PHPT-positive dogs, 39 (57%) were male and 30 (44%) were female. Among 140 PHPT-negative dogs, 63 (45%) were male, and 77 (55%) were female.

Segregation analysis was carried out on 189 phenotyped individuals (63 affected and 126 normal) from the pedigree. The probabilities of each model being true were 100% for incompletely dominant, 96% for completely dominant, and 74% for recessive. The incomplete dominant mode of inheritance was accepted as the best fit. Pedigree analysis suggested that all affected individuals were heterozygous for the PHPT trait. If this is the case, it indicates that the homozygous state is likely to be lethal. This analysis confirms heritability and supports a dominant mode of inheritance with a high, age-dependent penetrance.

Mutation Screening of Candidate Gene

DNA purified from 4 to 12 (the number of dogs varied from gene to gene) PHPT-positive (median age 11 years, range 7–14.5 years) and from 2 to 8 PHPT-negative dogs (median age 13.4 years, range 12–15.7 years) was used for exon scanning. A total of 45 polymorphisms were identified in the Keeshond DNA samples relative to the published canine genome sequence.[d] Of the polymorphisms, 3/9 *MEN1* polymorphisms, 7/14 *CASR* polymorphisms, 0/15 *HRPT2* polymorphisms, and 3/7 *RET* polymorphisms were within putative exons. None of the intronic polymorphisms occurred in hypothesized splice sites, and all exonic polymorphisms appeared silent. None of the polymorphisms appeared to segregate convincingly with PHPT.

RNA Analysis

The *MEN1* RNA sequence from the parathyroid tumor and healthy parathyroid gland perfectly matched the predicted sequence, closely resembling human *MEN1* splice variant 2. The germline sequence of the subject from which we obtained the parathyroid tumor was homozygous at all *MEN1* markers identified. The *CASR* RNA sequences from both the parathyroid tumor and the healthy parathyroid tissue showed the expected splicing pattern and did not differ in predicted protein sequence. Both showed heterozygous expression at several markers on exon 6, and neither contained novel polymorphisms. The *HRPT2* RNA sequences from both the tumor and healthy tissue perfectly matched one another and also matched the predicted sequence. Heterozygosity of *HRPT2* expression in the parathyroid could not be evaluated, as we did not identify any polymorphisms within the *HRPT2* exons in this study. The germline sequence of the PHPT-positive subject from which we extracted tumor RNA was heterozygous at 14/15 *HRPT2* intron markers. The *RET* sequence from both the PHPT-positive and PHPT-negative tissue samples were identical. Canine cDNA nucleotide data for each gene were deposited

in the GenBank database (accession numbers in Table 1).

Discussion

This study of PHPT in Keeshonden establishes the heritability and likely mode of inheritance of PHPT in any dog breed or other domestic animal. The dominant mode of transmission and thus a lack of a large number of silent carriers as occurs in recessive disorders should allow rapid elimination of this disease from the Keeshond breed when genetic testing becomes available. The Keeshond is the first non-human species shown to have a heritable form of PHPT, as do humans. Thus, the importance of this finding goes beyond its obvious contribution to canine medicine. The similarities between the genetics of this disease in the Keeshond and in the human are many. In both Keeshonden and humans the condition appears to be caused by a single gene mutation, transmitted via simple Mendelian genetics with a high degree of penetrance. Even more interesting is the fact that in most human cases and in the Keeshond, the mode of inheritance appears to be autosomal dominant, making a tumor suppressor or regulatory gene defect likely. We hypothesized that PHPT in Keeshonden is caused by a mutation in the canine homolog of one of the genes commonly indicated in the clinically similar human condition known as FIHP. But after direct sequencing of each of these genes in a sample of PHPT-positive and PHPT-negative Keeshonden, we did not discover any mutations that appeared to cause an interruption in transcription or translational changes. The RNA sequence recovered from the parathyroid tumor of a PHPT-positive Keeshond was functionally identical to that from the healthy parathyroid, and differed from it and the predicted sequences only by silent point mutations that are known to occur both in PHPT-positive and PHPT-negative Keeshonden. Therefore, we conclude that it is unlikely that the genetic cause of PHPT in Keeshonden is a mutation in the *MEN1*, *CASR*, *HRPT2*, or *RET* genes. Mutations in a nontranscribed region of these genes such as a promoter region have not been ruled out in this study as a cause of PHPT. Such mutations, though, are highly unlikely to be the cause of PHPT in Keeshonden because of the complete lack of an association between polymorphisms within and around the evaluated genes and the disease. Such polymorphisms should segregate with the diseased allele because of linkage disequilibrium.[14] It is likely that a mutation in a gene not previously associated with HPT is the cause of this disease in Keeshonden and possibly in human families with FIHP, as only a minority of human families suffering from FIHP have been diagnosed with a mutation in a known gene.[15] The gene responsible for the disease in Keeshonden could also be responsible for at least a portion of those many human cases. Although most canine genetic disorders are breed specific, the same mutation could also be responsible for the disease in additional canine breeds that are at risk for the disease such as Golden Retrievers and Dachshunds. A genome-wide linkage or association study should shed insight into the genetic cause of PHPT in Keeshonden, in additional canine breeds, and possibly in humans.

Footnotes

[a] Animal Health Diagnostic Laboratory, College of Veterinary Medicine, Michigan State University, East Lansing, MI
[b] QIAmp DNA purification kit and RNeasy RNA purification kit, QIAgen Inc, Valencia, CA
[c] Lineage pedigree analysis software (J. P. Pollack and K. Egan, http://www.ansci.cornell.edu/lineage/index.html)
[d] University of California Santa Cruz Genome Bioinformatics website, http://www.genome.ucsc.edu/
[e] PrimerQuest software (S. Rozen and H. J. Skaletsky, http://scitools.idtdna.com/Primerquest/)
[f] Eppendorf Mastercycler Gradient thermocycler, Eppendorf, Hamburg, Germany
[g] Applied Biosystems Automated 3730 DNA Analyzer, Applied Biosystems, Foster, CA
[h] RNAlater, Ambion Inc, Austin, TX
[i] Invitrogen Thermoscript RT-PCR system, Invitrogen Corporation, Carlsbad, CA

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References

1. Refsal KR, Provencher-Bolliger AL, Graham PA, et al. Update on the diagnosis and treatment of disorders of calcium regulation. Vet Clin North Am Small Anim Pract 2001;31:1043–1062.

2. Feldman EC. Primary Hyperparathyroidism (PHPTH) in dogs. In: Feldman EC, Nelson RW, eds. Canine and Feline Endocrinology and Reproduction, 3rd ed. St Louis, MO: WB Saunders; 2004:678–711.

3. Feldman EC, Hoar B, Pollard R, et al. Pretreatment clinical and laboratory findings in dogs with primary hyperparathyroidism: 210 cases (1987–2004). J Am Vet Med Assoc 2005;227:756–761.

4. Wisner ER, Nyland TG. Ultrasonography of the thyroid and parathyroid glands. Vet Clin North Am Small Anim Pract 1998;28:973–991.

5. Long CD, Goldstein RE, Hornof WJ, et al. Percutaneous ultrasound-guided chemical parathyroid ablation for treatment of primary hyperparathyroidism in dogs. J Am Vet Med Assoc 1999;215:217–221.

6. Thompson KG, Jones LP, Smylie WA, et al. Primary hyperparathyroidism in German Shepherd dogs: A disorder of probable genetic origin. Vet Pathol 1984;21:370–376.

7. Brandi ML, Falchetti A. Genetics of hyperparathyroidism. Urol Int 2004;1(Suppl):11–16.

8. Warner J, Epstein M, Sweet A, et al. Genetic testing in familial isolated hyperparathyroidism: Unexpected results and their implications. J Med Genet 2004;41:155–160.

9. Carling T. Molecular pathology of parathyroid tumors. Trends Endocrinol Metab 2001;12:53–57.

10. Cetani F, Pardi E, Borsari S, et al. Genetic analyses of the HRPT2 gene in primary hyperparathyroidism: Germline and somatic mutations in familial and sporadic parathyroid tumors. J Clin Endocrinol Metabo 2004;89:5583–5591.

11. Villablanca A, Wassif WS, Smith T, et al. Involvement of the MEN1 gene locus in familial isolated hyperparathyroidism. Eur J Endocrinol 2002;147:313–322.

12. Simonds WF, Robbins CM, Agarwal SK, et al. Familial isolated hyperparathyroidism is rarely caused by germline mutation in HRPT2, the gene for the Hyperparathyroidism-Jaw Tumor Syndrome. J Clin Endocrinol Metab 2004;89:96–102.

13. Kerr RJ, Kinghorn BP. An efficient algorithm for segregation analysis in large populations. J Anim Breed Genet 1996;113:457–469.

14. Ostrander EA, Wayne RK. The canine genome. Genome Res 2005;15(Suppl 12):1706–1716.

15. Klein RD, Salih S, Bessoni J, Bale AE. Clinical testing for multiple endocrine neoplasia type 1 in a DNA diagnostic laboratory. Genet Med 2005;7:131–138.