J Vet Intern Med 2012;26:1350–1357

Congenital Hypothyroidism with Goiter in Tenterfield Terriers

S.E. Dodgson, R. Day, and J.C. Fyfe

Background: A cluster of cases of congenital hypothyroidism with goiter (CHG) in Tenterfield Terriers was identified and hypothesized to be dyshormonogenesis of genetic etiology with autosomal recessive inheritance.

Objectives: To describe the phenotype, thyroid histopathology, biochemistry, mode of inheritance, and causal mutation of CHG in Tenterfield Terriers.

Animals: Thyroid tissue from 1 CHG-affected Tenterfield Terriers, 2 affected Toy Fox Terriers, and 7 normal control dogs. Genomic DNA from blood or buccal brushings of 114 additional Tenterfield Terriers.

Methods: Biochemical and genetic segregation analysis of functional gene candidates in a Tenterfield Terrier kindred. Thyroid peroxidase (TPO) iodide oxidation activity was measured, and TPO protein and SDS-resistant thyroglobulin aggregation were assessed on western blots. *TPO* cDNA was amplified from thyroid RNA and sequenced. Exons and flanking splice sites were amplified from genomic DNA and sequenced. Variant *TPO* allele segregation was assessed by restriction enzyme digestion of PCR products.

Results: Thyroid from an affected pup had lesions consistent with dyshormonogenesis. TPO activity was absent, but normal sized immunocrossreactive TPO protein was present. Affected dog cDNA and genomic sequences revealed a homozygous *TPO* missense mutation in exon 9 (R593W) that was heterozygous in all obligate carriers and in 31% of other clinically normal Tenterfield Terriers.

Conclusions: The mutation underlying CHG in Tenterfield Terriers was identified, and a convenient carrier test made available for screening Tenterfield Terriers used for breeding.

Key words: Carrier test; DNA sequence; Enzyme assay; Thyroid peroxidase; Western blot.

Hypothyroidism in the newborn (CH) is a metabolic disruption characterized by a constellation of developmental abnormalities that includes dwarfism, delayed epiphyseal ossification, abnormal hair texture, unresponsiveness, and lethargy.[1] CH can be further subdivided into disorders characterized by enlarged thyroid glands (CHG) and those that are not. As opposed to CH caused by failed thyroid development, goiter indicates that thyroid tissue is present. In addition, goiter indicates that regulation via the hypothalamo-pituitary-thyroid axis is intact; however, the thyroid gland is unable to synthesize sufficient hormone, a so-called dyshormonogenesis, to inhibit pituitary release of thyrotropin (TSH). Unrelenting stimulation of thyroid follicular epithelial cells via TSH receptor-mediated signal transduction causes a characteristic histopathology, including a diffuse microfollicular pattern, follicular cell hyperplasia obliterating the colloid spaces, transition to cuboidal or columnar cell morphology, and scant, palely eosinophilic colloid.[1–3]

Hypothyroidism with goiter can be an acquired disorder caused by dietary iodine excess or deficiency[4] or from exposure to a variety of goitrogenic compounds that inhibit iodide uptake into the gland or inhibit the activity of thyroid peroxidase (TPO).[5] Commercial dog and cat food compounding has largely eliminated iodine deficiency in these species, but iodine excess is occasionally seen in animals fed improperly compounded food.[6,7]

Dyshormonogenesis of suspected genetic origin suggests a short list of functional candidate genes deduced from known thyroid gland physiology, all of which have been implicated in human forms of CHG.[1,8,9] Inherited causes of goiter include inability of the thyroid to concentrate iodide, defects of organification (ie, covalent bonding of iodine to thyroglobulin [TG] in the follicular colloid), defects in TG synthesis, and inability to recycle iodine by deiodinating unused iodothyronines. The organification process requires, the multiple activities of thyroid peroxidase (TPO), a heme-protein that uses

Abbreviations:

CHG	congenital hypothyroidism with goiter
PCR	polymerase chain reaction
SSR	simple sequence repeat
TG	thyroglobulin
TPO	thyroid peroxidase
TSH	thyrotropin

Standard single letter code for amino acid residues:
R, arginine; W, tryptophan; V, valine; I, isoleucine; G, glycine; D, aspartic acid; Y, tyrosine

From the Laboratory of Comparative Medical Genetics, College of Veterinary Medicine, Michigan State University, East Lansing, MI (Dodgson, Fyfe); and the Alpine Animal Doctors, Porepunkah, Victoria, Australia (Day).

Sites: *The local veterinarian (RD) contributed clinical assessment, sample collection, and contacts with participating breeders. Enzymology, histopathology, and molecular investigations were performed at the College of Veterinary Medicine, Michigan State University, East Lansing, MI.*

Meeting presentation: *These data were presented as a poster in preliminary form at the 5th International Conference: Advances in Canine and Feline Genomics and Inherited Diseases, Baltimore, MD, September 22–25, 2010.*

Corresponding author: J.C. Fyfe, DVM, PhD, 2209 Biomedical Physical Sciences, 567 Wilson Road, Michigan State University, East Lansing, MI 48824; e-mail: fyfe@cvm.msu.edu.

Submitted April 20, 2012; Revised August 15, 2012; Accepted September 5, 2012.

Copyright © 2012 by the American College of Veterinary Internal Medicine

10.1111/j.1939-1676.2012.01015.x

H_2O_2 to oxidize and bind iodide to tyrosyl residues in TG.[10] TPO also catalyzes the oxidative coupling of iodotyrosines to generate the iodothyronines, T4 and T3. Thus, CHG may be caused by a defect in any of a variety of genes whose products are important for the synthesis of thyroid hormones. Dyshormonogenesis causing CHG typically exhibits autosomal recessive inheritance, and signs become evident in the newborn or early childhood period.

Sporadic and familial cases of CHG occur in dogs and cats.[2,3,11–16] Autosomal recessive CHG occurs in Toy Fox and Rat Terriers caused by the same *TPO* mutation, likely because they share recent ancestry.[3,15] The Tenterfield Terrier is a similar looking breed that split from Australian Miniature Fox Terriers in 1993 (http://www.ankc.org.au/Breed_Details.aspx?bid=212). In a recent review of CH in dogs and cats,[16] the authors mentioned CHG caused by an iodine organification defect diagnosed in 1996 among New Zealand Miniature Fox Terriers, but a disease-causing mutation was not determined. Litters of Tenterfield Terriers with puppies affected by CHG were brought to our attention in December, 2008. The studies described herein followed a candidate gene approach and identify the genetic basis of CHG in these dogs, allowing development of a breed-specific DNA-based carrier test.

Materials and Methods

Animals

CHG was suspected in 3 of 5 pups in a litter of Tenterfield Terrier puppies presented at 5 weeks of age. A sample of cells from 1 affected pup was obtained by buccal brushing before euthanasia of all 3 (sodium pentobarbital, 86 mg/kg, IV). Immediately upon euthanasia, thyroids of the brush-sampled affected pup were dissected. A portion was snap frozen in liquid nitrogen and stored thereafter in liquid nitrogen or at −80°C. The remaining thyroid tissue was fixed in neutral buffered formalin, embedded in paraffin, and 8-μm sections were stained with H&E. Subsequently, samples of whole blood in sodium EDTA or buccal brushings were collected from 114 related Tenterfield Terriers, including the clinically normal sire, dam, and male littermate of the presented affected pups. Owners of these dogs were contacted by the National Tenterfield Terrier Council (Australia), sample collection was granted exemption from Institutional Animal Care and Use Committee (IACUC) oversight, samples were collected with owner informed consent, and blood samples were collected by cooperating local veterinarians. Club-certified, 3–5 generation pedigrees were submitted along with each sample. Thyroids from control dogs were collected after euthanasia for unrelated reasons under protocols approved by the MSU IACUC.

TPO Enzymatic Assay

Snap-frozen thyroids of CHG-affected and clinically normal dogs stored at −80°C for variable periods up to 14 years were thawed in cold 150 mM NaCl, dissected free of connective tissue, and minced with a razor blade on an iced glass plate. The pieces were washed 3 times in 10 mL of cold 150 mM NaCl and homogenized using a motorized tissue disruptor[a] on ice in 5 volumes (wt/vol) of 50 mM Tris-HCl, pH 7.0, containing 0.4 mM KI. The homogenates were centrifuged for 20 minutes at 700 × *g* at 4°C, and the resulting supernatants were centrifuged for 1 hour at 27,000 × *g* at 4°C. The second supernatants were frozen for subsequent thyroglobulin analysis, and pellets were resuspended in 3 mL of 67 mM Na phosphate buffer, pH 7.0, containing 0.5 mM KI using 10 strokes of a motor driven Teflon pestle in a Potter–Elvehjem tissue grinder on ice. Aliquots were made 0.7% Na cholate and gently agitated overnight at 4°C. Assays of TPO iodide oxidation activity were carried out essentially as described[3,17] using spectrophotometric monitoring of $\Delta\ A^{353}$. Assays were performed on 2 tissue preparations per dog and in duplicate, using 20 and 40 μL of each preparation. A unit of activity was defined as $\Delta\ A^{353}/\text{min} = 1$, and specific activities were expressed as U/mg protein. Total protein in each preparation was determined using a Coomassie blue dye-binding assay.[b]

Western Blotting

Ten μg of thyroid proteins from the 27,000 × *g* pellets prepared for enzyme assays, above, were separated using electrophoresis on 7.5% SDS-polyacrylamide gels under reducing conditions and electroblotted to polyvinylidene difluoride (PVDF) membranes as previously described.[18] Membranes were blocked by incubation in 25 mM Tris-HCl, pH 9.0, containing 250 mM NaCl and 2% Tween-20, without protein. Blocked membranes were incubated sequentially with rabbit polyclonal anti-mouse TPO peptide (residues 31–150) antibody[c] diluted 1 : 200, goat polyclonal anti-rabbit IgG-horseradish peroxidase conjugate[d] diluted1 : 2000, and chemiluminescence detection reagents[e].

Thyroglobulin western blots were prepared from the 27,000 × *g* supernatant fractions saved during sample preparation for TPO analysis, above. Supernatants were thawed and centrifuged again at 100,000 × *g* for 1 hour at 4°C. Between 2 and 6 μg of protein from the resulting supernatants were electrophoresed on 5% SDS-polyacrylamide gels under nonreducing conditions and electroblotted as above. The membranes were blocked as above, incubated sequentially with a high titer anti-TG dog serum[f] diluted 1 : 2000, goat polyclonal anti-dog IgG-horse radish peroxidase conjugate[g] diluted 1:2000, and chemiluminescence detection reagents[e], as previously described.[3] Control blots for both proteins were produced by leaving the primary antibody incubation step out of the detection procedure or by substituting an irrelevant rabbit or dog antiserum.

TPO Sequence Analysis

Genomic DNA was isolated from blood or buccal cells using standard methods.[19] A $(GAAA)_n$ repeat (dog chromosome 17: 3,609,770-3,609,945) 170 kb centromeric to *TPO* was amplified using polymerase chain reaction (PCR) primer sequences, 5′-GAAAATGAGTATCCGCGTTCAGA-3′ and 5′-CGGAGGCTCTGAGGACAGAA-3′. All *TPO* exons were amplified and sequenced using PCR primers (Table 1) designed from sequences flanking exon-intron boundaries in the canine genome reference sequence (CanFam 2; boxer) accessed via the UCSC Genome Bioinformatics Browser (http://genome.ucsc.edu/). PCR was performed essentially as previously described,[3] but with deoxyribonucleotide concentrations of 0.25 mM and 50 cycles at 94°C for 1 minute, various annealing temperatures (Table 1) for 2 minutes, and 72°C for 3 minutes. The full-length affected dog *TPO* cDNA sequence was determined by sequencing overlapping products amplified by reverse transcription (RT) coupled PCR using RNA isolated from snap frozen thyroid as template, primers from the canine reference

sequence (Table 2), and methods as previously described.[3] Amplification products were analyzed using electrophoresis on 2–4% agarose gels and submitted to a core facility[h] for sequencing using automated dideoxynucleotide chain termination methods. Sequences were assembled[i] and compared with the Boxer reference genome sequence and the Toy Fox Terrier cDNA sequence previously determined in this laboratory (new sequences incorporated into an updated GenBank® accession # AY094504.2).

Genotyping

The same exon 9 amplicon that was used for sequencing (Table 1) was amplified using PCR from genomic DNA of 115 sampled Tenterfield Terriers. Aliquots of the products were digested overnight at 37°C with the restriction endonuclease *Nci I*, and digestion fragments were separated on 4% agarose gels.

A modified exon 6 amplicon was amplified by PCR using the forward primer previously used for sequencing (Table 1) and the reverse primer, 5′-TCACTTCCTAGCTCTCCCATTC-3′. Products were amplified from genomic DNA of clinically normal Tenterfield Terriers that were heterozygous at the exon 9 sequence variation or unrelated control dogs in PCR reactions performed as described above for *TPO* exon sequencing. Products were digested with the restriction endonuclease *Bcc I* for 6 hours at 37°C, and digestion fragments were separated on 3% agarose gels.

Molecular Modeling

The canine TPO protein sequence was used as query in an online BLASTp search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify homologous proteins in various organisms. Amino acid conservation at sites of the observed *TPO* sequence variations was determined by alignment of sequences of the animal haem peroxidase superfamily (pfam 03098) from the Conserved Domains Database[20] (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml), as well as by SIFT[21] (http://sift.jcvi.org/www/SIFT_seq_submit2.html) and PolyPhen-2 analyses[22] (http://genetics.bwh.harvard.edu/pph2/), each performed online. Putative structural effects of *TPO* sequence variants were further assessed using the PyMol program[23] and 1.9 Å (0.19 nm) resolution X-ray crystallographic data of the homologous protein, human myeloperoxidase (PDB accession1DNW).

Results

Clinical Presentation and Family History

A litter of 5 Tenterfield Terrier puppies was examined at 5 weeks of age, including 2 males and 1 female that showed signs of cretinism. Growth failure of the affected pups had been evident to the owner from 2.5 to 3 weeks of age. The 3 affected pups were half the size of normal littermates with short legs and had not opened their eyes until 4 weeks of age (normal opening of palpebral fissues is 10–15 days of age). They had unusually fluffy coat texture for age without guard hairs, were lethargic in movements, and had bilaterally enlarged thyroid glands. Their corneas were slightly opaque, and they became unusually tense upon handling. Two of them could not nurse and required hand feeding. The bitch had been on a complete and balanced commercial diet throughout gestation and lactation, and there was no history of exogenous iodine exposure to the bitch or pups. The owner declined antemortem endocrine testing, but allowed buccal cell sampling and thyroid gland preservation of 1 affected pup after euthanasia. The affected puppies were euthanized, and a thyroid gland measuring ~3.6 × 2.5 × 2.5 cm was dissected from one. Microscopic examination of the thyroid tissue revealed diffuse follicular epithelial cell hyperplasia (Fig S1). Follicle spaces were largely obliterated by cuboidal to columnar epithelial cells piled up as blunt papillae and having multiple small cytoplasmic vacuoles. The remaining colloid spaces were small and had scant, poorly staining colloid.

Matings of related Tenterfield Terriers by other breeders had produced similarly affected offspring. Relationships between members of the litter presented to us and others reported by breeders are shown on the pedigree in Figure 1. Goiter had been confirmed at

Table 1. *TPO* primers for exon amplification from genomic DNA.

Exon	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Amplicon Size (bp)	PCR Anneal Temp. (°C)
1	AGTCTCCCAGGGCTTCAGAC	GGGTCGATGGTCACAGACCTAGTTGTT	293	58-54 TD[a]
2	TGGCTGTGGTAGAGGGTGAT	GAGCTCCTCCTTGGGAGACT	287	54
3	TAGCGGTTGCTTCTGAGTGA	TGAACGAGCTCCTGCAAAC	427	58-54 TD
4	CACCTGCTGCACAACAGTCA	AGGAGCCTGGGAGACAGGA	706	58-54 TD
5	CGTGTCACGTTTATTTCCACA	GGAGATGGGCTCTGAGCTT	496	54
6	CGTCCCACTAGCAGACACAA	CACACAGACGTGGACACCTT	629	58-54 TD
7	GTGAGGGCAGGACACAGTTC	GGCTCAGCTGCGTGTGTAAC	747	58-54 TD
8	AAGGTCCGAGCTGTGAGGAT	CATTCACGTTGTGCGGTTCT	780	58
9	GAAGAACCGCACAACGTGAA	AGCGAGAGGTGAGCACATGA	463	58
10	CCTGACTCTGGGCTGGACTA	GCTCCCTTTGTGTGATGACC	513	58-54 TD
11	CACCCTCGGCTCTTTCCTC	TCCACGTGCTACGACACAATC	551	60
12	CAGGCTCCACCTTCCATCTC	GTCTGAGGACCGCCGATTT	257	58
13	CCACGTGCTCTGACCTGTGT	GTCCTTGAGCCTGGGAGAGA	290	60
14	GTGCTCCGGAGAGCCTGTT	GGGAGAAAGCAACCTCGTGT	284	58
15	CTGAGCAGGCCATGTTGTGT	AGAGCAGGAAGGTGCGAAGT	439	58
16	GTACGCACAACCGCAAGAGA	GTCCTGACCGGGCCTCTAAC	555	58

aTD = touchdown, where annealing temperature decreases 1°C/cycle until the lower temp. is reached.

Table 2. *TPO* primers for cDNA amplification from thyroid RNA.

cDNA fragment	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Amplicon Size (bp)	PCR Annealing Temp. (C)
−122 × 215	AGGGCTTCAGACGCAAGCTG	TCCTCACGGTGTGCTGGATG	337	58-54 TD[a]
39 × 761	GAGAGCACTCGCCGTCCTG	CGACGTCGTGGTCGATGTACTG	723	58-54 TD
504 × 1311	CGCCTGCAACAACAGAGACCAC	CCAGTGAGCGTTGAGAGCCTTG	808	58-54 TD
974 × 1818	AGATGAACGGGCTGACGTCCTT	GAACTCCCTCCACGCGTTGTAA	845	58-54 TD
1700 × 2440	AGGAGCTGACCGAGAGGCTGTT	GGCTCGCGTCTTCACACTCATT	741	58-54 TD
2178 × 2928	CTTCGAGCCGTGCGAGAACATT	GGGTCGGGGTTGTTTCCAGA	751	58-54 TD

aTD = touchdown, where annealing temperature decreases 1°C/cycle until the lower temp. is reached.

necropsy in one of the reported pups (solid diamond with asterisk in lower right of Figure 1). Noteworthy features of the family history were that affected pups of both sexes had been produced in matings in different kennels of clinically normal parents (obligate carriers of the disease trait under a hypothesis of autosomal recessive inheritance), all of which were descended from at least 1 common ancestor.

Candidate Gene Exclusion

Alleles of simple sequence repeat (SSR) markers near candidate genes were assessed for segregation in linkage with disease locus alleles. An SSR 170 kb centromeric to *TPO* (dog chromosome 17:3,609,770–3,609,945) exhibited 3 distinct alleles in a subset of the pedigree (Fig 2). The marker alleles segregated perfectly with disease alleles deduced on the assumption of autosomal recessive inheritance. The affected pup was the only dog among 53 Tenterfield Terriers studied that was homozygous for the largest allele, whereas all clinically normal, but obligate carriers were heterozygous, having only 1 copy of the largest allele. Alleles of SSRs near other candidate genes did not segregate with the deduced disease alleles (data not shown).

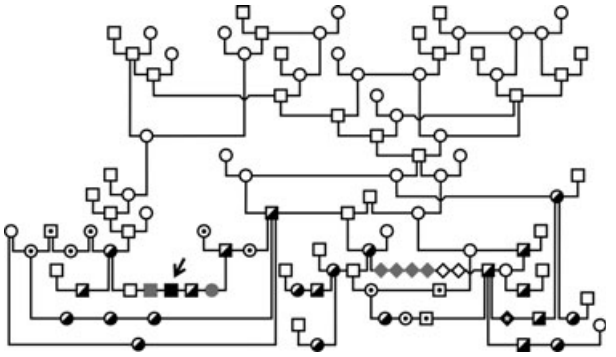


Fig 1. Congenital hypothyroidism with goiter (CHG) segregating in a Tenterfield Terrier family. Squares indicate males, circles indicate females, and diamonds indicate dogs of unknown sex. Solid-filled symbols represent affected pups with necropsy confirmation of goiter, and gray-shaded symbols represent pups suspected to be affected based on observed or reported clinical signs but without laboratory confirmation. Half-filled symbols represent CHG carriers identified by genotyping for the *TPO* mutation as described herein (Fig 4). Unfilled symbols containing a single dot indicate clinically normal dogs that were determined to be homozygous normal by genotyping. Unfilled symbols with no inclusion indicate clinically normal dogs for which a sample was not available for analysis. The arrow indicates the single affected pup for which DNA and thyroid samples were available for study.

Biochemical Analysis

We performed a kinetic iodide oxidation assay of thyroid tissue of the affected Tenterfield Terrier and several control dogs, including age-similar pups and dogs whose tissues had been snap frozen and stored for similar or longer periods. We found no consistent difference in TPO activity between young and older dogs or between tissues stored for varying periods. In 7 control dog thyroids, TPO activity was 1.55 ± 0.37 U/mg protein (mean ± SD), similar to previously reported normal values from this laboratory.[3] However, TPO activity was undetectable in the affected Tenterfield Terrier thyroid and in newly repeated assays of the previously reported affected Toy Fox Terriers.[3]

Western blots of TG isolated from thyroids of the affected Tenterfield Terrier, one of the previously reported[3] affected Toy Fox Terriers, and 3 unrelated normal dogs revealed immunoreactive TG of ~330 kDa in all dogs (Fig 3A). In normal controls, these proteins migrated as 2 closely spaced bands, but as a single band in the affected dogs. Furthermore, SDS-resistant, high molecular weight TG dimers and tetramers were observed only in tissue of the 3 normal dogs. Western blots of TPO protein isolated from affected and normal dog thyroids (Fig 3B) revealed a ~110 kDa immunoreactive TPO protein band in all normal dog thyroids that was missing from tissue of the previously reported[3] affected Toy Fox Terriers. However, the CHG-affected Tenterfield Terrier also exhibited a normal sized immunoreactive TPO band.

TPO Gene Sequence Analysis

The *TPO* cDNA of the affected pup and all 16 exons, including flanking splice sites from genomic DNA of the affected pup and his dam, were sequenced. There was no indication of abnormal splicing. Two protein coding sequence variations were detected both in genomic and cDNA for which the affected dog was

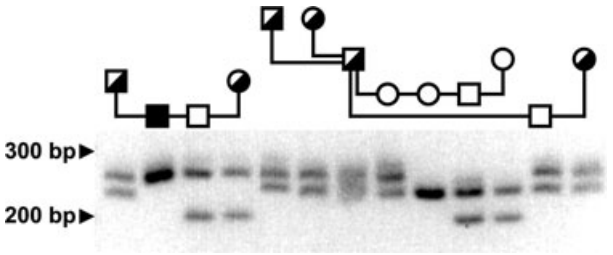


Fig 2. Failure to exclude *TPO* by simple sequence repeat (SSR) genotyping. An SSR at position 3,609,770-3,609,945 Mb of dog chromosome 17 in the canine genome was identified 170 kb centromeric of *TPO*. The SSR was amplified using PCR from genomic DNA and electrophoresed on a 3% agarose gel. PCR across the SSR generated variable length products defining 3 alleles at this locus in the subset of the pedigree shown. All unaffected dogs in this figure are heterozygous or homozygous for an allele different from those of the affected dog. The affected male is homozygous for the largest allele (filled symbol; lane 2), and all obligate carriers (half-filled symbols), ascertained by having produced an affected offspring, have 1 copy of the largest allele. This SSR was genotyped in 50 additional dogs of the kindred, and no clinically normal dog was homozygous for the largest allele.

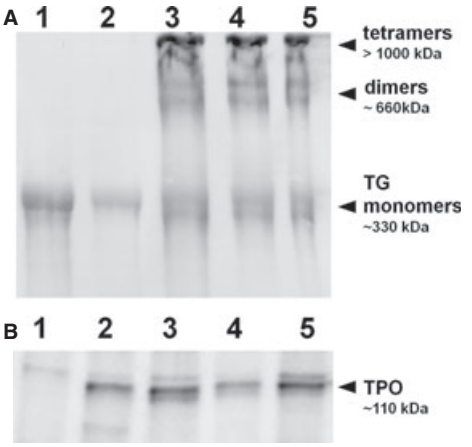


Fig 3. Western blots of thyroglobulin and TPO. Panel A shows a western blot of thyroglobulin in the 100,000 × *g* soluble fraction extracted from frozen thyroids of a CHG-affected Toy Fox Terrier (lane 1), the affected Tenterfield Terrier of this report (lane 2), and 3 unrelated normal dogs (lanes 3–5). Panel B shows a western blot of TPO in the 27,000 × *g* pellet extracted from a CHG-affected Toy Fox Terrier (lane 1), the affected Tenterfield Terrier of this report (lane 2), and 3 unrelated normal dogs (lanes 3–5).

homozygous for an allele different from those of either reference sequence, and for which the dam was heterozygous. We found a G → A transition in exon 6 (c. G694A) that predicted a valine (V)-to-isoleucine (I) substitution at amino acid residue 232 (numbering of dog TPO amino acids is offset by 12 from other species due to a canid-specific 5′ extension of the coding sequence) (p.V232I) and a C → T transition in exon 9 (c.C1777T) that predicted an arginine (R)-to-tryptophan (W) substitution at amino acid residue 593 (p.R593W).

Two approaches were used to determine which of these sequence variations might be pathogenic: segregation analysis in the CHG pedigree and evaluation of functional sequence conservation. Segregation of the exon 9 *TPO* sequence variation was determined by genotyping all 115 members of the Tenterfield Terrier pedigree. The C → T transition destroys an *Nci I* restriction enzyme cut site, facilitating design of a simple genotyping assay. PCR amplification of exon 9 produced a 463 bp amplicon from genomic DNA. The normal allele was distinguished by the presence of 209 and 31 bp *Nci I* digestion fragments that, in contrast, remained as an uncut 240 bp fragment when amplified from the mutant allele (Fig 4). Genotyping demonstrated that all 6 parents of affected pups were heterozygous, and that the affected dog, but none of 114 clinically normal Tenterfield Terriers, was homozygous for the T allele. Among the latter, 35 (31%) were identified as carriers.

Segregation of the exon 6 *TPO* sequence variation was determined similarly by genotyping a subset of clinically normal Tenterfield Terriers and unrelated dogs of other breeds. Exon 6 was amplified using PCR and the 540 bp amplicon was digested with *Bcc I*. The exon 6 G → A transition created a new *Bcc I* cut site so the G allele was characterized by a 298 bp fragment and the A allele by 142 and 156 bp fragments. The assay demonstrated that at least 4 clinically normal Tenterfield Terriers, all heterozygous for the exon 9 mutation, a Spanish Water Dog control, and a terrier-like mongrel control had the same homozygous exon 6 genotype (A/A) at this site as the affected dog (data not shown).

Molecular Modeling

Molecular assessment of the 2 observed sequence variations by comparison to the high resolution, X-ray crystallographic structure of human myeloperoxidase suggested that V232I is a change in an unconstrained residue at the periphery of the protein, whereas R593W is a change buried by van der Waals forces and hydrogen bonds in the catalytically essential heme-binding domain of TPO. Arginine (R) occupies the homologous position in 85 of 86 members of the animal haem peroxidase superfamily (pfam 03098). Modeling indicated that R593 forms 2 hydrogen bonds with aspartate 642, which in turn hydrogen bonds with tyrosine 635 (Fig S2). Consequently, both of these latter residues are as highly conserved as R593. Computerized SIFT (Sorting Intolerant From Tolerant) analysis predicted that R593W would not be tolerated, likely abrogating function of the enzyme (score of 0.00 for any substitution). R593 is in the middle of a 6 amino acid sequence where any substitution with any of the19 other amino acids is predicted to be functionally damaging. Nearly identical results were obtained using PolyPhen-2 (Polymorphism Phenotyping v2). In contrast, valine (V) at position 232 is not well conserved among species, often being substituted by another hydrophobic residue and occasionally by a

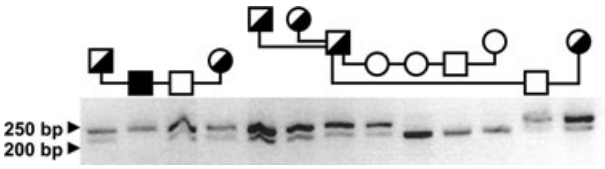


Fig 4. Mutation detection in genomic DNA segregates with CHG in the Tenterfield Terrier pedigree. PCR-amplified exon 9 products were digested with *Nci I*, and fragments were separated on a 4% agarose gel. The filled symbol indicates the affected dog (lane 2), half-filled symbols denote obligate carriers, and open symbols denote other clinically normal dogs. The larger band (240 bp) that is present in the affected pup and all obligate carriers is cut by *Nci I* into 209 and 31 bp fragments when amplified from the normal allele, but the 31 bp fragment is not shown. The 240 bp fragment is not cut by *Nci I* when amplified from the mutant allele. This test was applied to all 115 dogs of the kindred, and was used to identify the carriers and genetically normal dogs designated on the pedigree in Figure 1.

polar or charged residue. The SIFT score of 0.65 indicated that V232I would likely be tolerated without functional consequence.

Discussion

Clinical diagnosis of hypothyroidism typically hinges on determination of plasma thyroid hormone and TSH concentrations to assess thyroid and pituitary functions, respectively. Further clinical classification of thyroid dysfunction can be obtained from thyroid histology and studies of radioiodine uptake and perchlorate-induced discharge.[2,3] In the case reported herein, superficial clinical observation of the affected pups demonstrated cretinism, and thyroid histology demonstrated a dyshormonogenic type of goiter. A noteworthy family history of similarly affected pups produced in matings of related dogs in different households made CHG of environmental causes highly unlikely. Goiter itself indicates both an intact hypothalamo-pituitary-thyroid axis and unremitting TSH influence that, in the absence of environmental causes, is likely caused by a genetic defect of thyroid hormone synthesis. Because Tenterfield Terriers are so similar in form to Toy Fox and Rat Terriers, we first performed the breed-specific mutation test developed for those breeds,[3,15] but the affected Tenterfield Terrier was homozygous normal at the Toy Fox/Rat Terrier mutation site.

The owner declined any clinical testing except preservation of an enlarged thyroid gland of 1 affected pup concurrent with euthanasia. Despite disallowing a more complete clinical assessment of the disorder, the owner and other concerned Tenterfield Terrier breeders facilitated a genetic investigation by donating samples for DNA isolation from many related dogs. Therefore, even though tissue and DNA samples from only 1 affected dog were available, it was possible to determine the disease-causing mutation via a functional candidate gene approach. Most functional candidates were excluded by assessing segregation of gene-flanking SSR marker alleles, thereby focusing our attention on *TPO*.

TPO iodide oxidation activity was undetectable in the affected dog thyroid gland. TPO activity is also required for thyroglobulin (TG) maturation into high molecular weight dimers and tetramers that resist in vitro dissolution in SDS.[24–26] The affected dog TG resolved solely into monomers during SDS-polyacrylamide electrophoresis. Despite lacking both of these TPO activities, the affected dog thyroid had TPO immunocrossreactive protein of normal size, thus suggesting that a putative mutation had inactivated the enzyme, but had not substantially affected protein translation or stability.

Investigation of mRNA and DNA sequences of the *TPO* gene revealed 2 missense sequence changes that were homozygous in the affected dog. The V232I change did not segregate with deduced disease alleles in the Tenterfield Terrier family, is not a conserved residue, and the change was predicted to be benign. Most telling, however, was that the homozygous substitution with isoleucine at this position in healthy dogs demonstrates that this change had little or no functional consequence and can be considered a normal polymorphism. In contrast, the R593W change segregated perfectly, is a highly conserved residue, and was predicted with high probability to damage function of the enzyme. These results demonstrate, therefore, that the observed exon 9 sequence variation, but not that in exon 6, is the disease-causing mutation in Tenterfield Terriers. These studies also demonstrate that there are at least 3 *TPO* alleles segregating in the Tenterfield Terrier population, as was first suggested by the segregation of 3 alleles of an SSR near *TPO* (Fig 2). One allele encodes V232 and R593, another encodes I232 and R593, and the third is the disease allele, encoding I232 and W593. Presumably, the R593W mutation occurred on the normal functioning I232 allele at some time in a previous generation.

Although many *TPO* mutations have been identified as causing CH in humans[1,9,27,j] and dogs,[3,15] the Tenterfield Terrier mutation is novel, occurring at a residue not previously implicated in the disease. Disease-causing missense mutations often disrupt protein structure, either by destabilizing the entire protein, leading to rapid degradation, or by altering some essential local structure and minimally altering protein stability. The highly conserved canine TPO residue R593 is in the ancient and highly conserved heme-binding domain found in many peroxidases and other oxidative enzymes throughout the animal kingdom. The protein sequence homology between TPO and myeloperoxidase (MPO) is so high in this region (74%) that MPO can efficiently iodinate TG and perform the iodotyrosyl coupling reaction in vitro.[28] Therefore, and because the TPO crystal structure has not been solved to date, we used a high-resolution MPO crystal structure to predict the structural consequences of an R593W mutation.

Structural modeling indicates that R593 forms a series of hydrogen bonds with aspartate 642 and tyrosine

635 (Fig S2). The three together are likely essential for maintaining the local protein structure and substitution with tryptophan (W) at position 593 would disrupt it. A significant aspect of the proper structure is that it positions the side-chain of R591, only 2 residues from R593, to directly coordinate the heme moiety. Binding of heme in this site is imperative for the enzyme's oxidative functions.[29,30] Thus, while the affected Tenterfield Terrier thyroid expresses immunocrossreactive TPO, binding of the prosthetic heme moiety or its enzymatic function of shuttling electrons is likely impaired. A human CHG missense mutation close to this one (p.G590R; G599 in dog) also disrupts TPO function.[27]

As is typical of enzymopathies in general and all cases of TPO deficiency in humans, Tenterfield Terrier CHG is an autosomal recessive disorder. *TPO* is an autosomal gene, and only dogs homozygous for the mutant allele express the disease. The mutation site genotyping assay reported herein is a convenient and reliable carrier test that is available to Tenterfield Terrier breeders. Among the 114 clinically normal dogs tested to date, 35 (31%) were identified as carriers, but bias of sample submission toward dogs related to affected puppies inflates the apparent carrier frequency over the actual in Tenterfield Terriers. Many of the dogs at the top of the pedigree in Figure 1 were registered as Miniature Fox Terriers before the split creating the Tenterfield Terrier breed in South Australia. It is likely, therefore, that CHG caused by TPO R593W mutation segregates among Miniature Fox Terriers as well. It is our hope, however, that breeders will test their stock for the disease allele and prevent further propagation of a debilitating and lethal disorder. A reliable genetic test, such as this is particularly important in breeds that have a small gene-pool so that breeders can exert selection against the disease mutation without decreasing the effective breeding population and leading to other problems. The mutation test could also be used diagnostically if one suspects CH in this breed before clinical detection of goiter. While treatment of the disorder using thyroid hormone replacement therapy is theoretically possible, it must be instituted early and aggressively to avoid permanent skeletal and/or CNS abnormalities or continued goiter enlargement that may eventually obstruct the main airway. Prevention by carrier detection is the better option.

Footnotes

[a] Polytron, Brinkman Instruments, Inc, Westbury, NY
[b] Bio-Rad Laboratories, Inc, Hercules, CA
[c] cat. # sc-134487, Santa Cruz Biotechnology, Inc, Santa Cruz, CA
[d] cat. # 141506, Kirkegaard & Perry Laboratories, Inc, Gaithersburg, MD
[e] Western Lightning ECL plus, PerkinElmer, Inc, Waltham, MA
[f] Endocrinology Laboratory, MSU Diagnostic Center for Population and Animal Health, East Lansing, MI
[g] cat. # 141906, Kirkegaard & Perry Laboratories, Inc, Gaithersburg, MD
[h] University of Michigan DNA Sequencing Core Laboratory, Ann Arbor, MI
[i] Lasergene SeqMan program by DNASTAR, Madison, WI
[j] Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php)

Acknowledgments

The authors thank the concerned breeders of Tenterfield Terriers for DNA samples and pedigree information, William Wedemeyer for protein structure insights, and Kaillathe Padmanabhan for online only supporting information Figure 2.

Grant support: The MSU Laboratory of Comparative Medical Genetics contributed laboratory supplies and costs. SED was supported by a Peabody Award from the Department of Microbiology & Molecular Genetics, Michigan State University.

Conflict of Interest: Authors disclose no conflict of interest.

References

1. Refetoff S, Dumont J, Vassart G. Thyroid disorders. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. The Metabolic & Molecular Bases of Inherited Disease, 8th ed. New York: McGraw-Hill; 2001:4029–4064.

2. Chastain CB, McNeel SV, Graham CL, et al. Congenital hypothyroidism in a dog due to an iodide organification defect. Am J Vet Res 1983;44:1257–1265.

3. Fyfe JC, Kampschmidt K, Dang V, et al. Congenital hypothyroidism with goiter in Toy Fox Terriers. J Vet Intern Med 2003;17:50–57.

4. Thompson T. Iodine-deficiency goitre in a bitch. N Z Vet J 1979;27:113.

5. Seelig DM, Whittemore JC, Lappin MR, et al. Goitrous hypothyroidism associated with treatment with trimethoprim-sulfamethoxazole in a young dog. J Am Vet Med Assoc 2008;232:1181–1185.

6. Castillo VA, Lalia JC, Junco M, et al. Changes in thyroid function in puppies fed a high iodine commercial diet. Vet J 2001;161:80–84.

7. Castillo VA, Pisarev MA, Lalia JC, et al. Commercial diet induced hypothyroidism due to high iodine. A histological and radiological analysis. Vet Q 2001;23:218–223.

8. Park SM, Chatterjee VKK. Genetics of congenital hypothyroidism. J Med Genet 2005;42:379–389.

9. Vulsma T, De Vijlder JJM. Genetic defects causing hypothyroidism. In: Braverman LE, Utiger RD, eds. Werner & Ingbar's The Thyroid: A Fundamental and Clinical Text, 9th ed. Philadelphia, PA: Lippincott, Williams & Wilkins; 2005:714–730.

10. Ruf J, Carayon P. Structural and functional aspects of thyroid peroxidase. Arch Biochem Biophys 2006;445:269–277.

11. Arnold U, Opitz M, Grossier I. Goitrous hypothyroidism and dwarfism in a kitten. J Am Anim Hosp Assoc 1984;20:753–758.

12. Jones BR, Gruffydd-Jones TJ, Sparkes AH, Lucke VM. Preliminary studies on congenital hypothyroidism in a family of Abyssinian cats. Vet Rec 1992;131:145–148.

13. Mazrier H, French A, Ellinwood NM, et al. Goitrous congenital hypothyroidism caused by thyroid peroxidase deficiency in a family of domestic shorthair cats. J Vet Intern Med 2003;17:395–396.

14. Brouwers J. GoÎtre et hérédité chez le chien. Ann de Méd Vét [Liège, FR] 1950;94:173–174.

15. Pettigrew R, Fyfe JC, Gregory BL, et al. CNS hypomyelination in Rat Terrier dogs with congenital goiter and a mutation in the thyroid peroxidase gene. Vet Pathol 2007;44:50–56.

16. Bojanic K, Acke E, Jones BR. Congenital hypothyroidism of dogs and cats: A review. NZ Vet J 2011;59:115–122.

17. Nakashima T, Taurog A. Improved assay procedures for thyroid peroxidase; application to normal and adenomatous human thyroid tissue. Clin Chim Acta 1978;83:129–140.

18. Xu D, Fyfe JC. Cubilin expression and posttranslational modification in the canine gastrointestinal tract. Am J Physiol Gastrintest Liver Physiol 2000;279:G748–G756.

19. Kawasaki ES. Sample preparation from blood, cells, and other fluids. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. PCR Protocols: Guide to Methods and Applications. San Diego, CA: Academic Press; 1990:146–152.

20. Marchler-Bauer A, Anderson JB, Chitsaz F, et al. CDD: Specific functional annotation with the Conserved Domain Database. Nucleic Acids Res 2009;37(D):205–210.

21. Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nat Protoc 2009;4:1073–1081.

22. Adzhubei IA, Schmidt S, Peshkin L, et al. A method and server for predicting damaging missense mutations. Nat Methods 2010;7:248–249.

23. DeLano WL. The PyMOL Molecular Graphics System (2002) DeLano Scientific, Palo Alto, CA (http://www.pymol.org)

24. Seed RW, Goldberg IH. Iodination in relation to thyroglobulin maturation and subunit aggregation. Science 1965;149:1380–1382.

25. Frati L, Bilstad J, Edelhoch H, et al. Biosynthesis of the 27 S thyroid iodoprotein. Arch Biochem Biophys 1974;162:126–134.

26. Arvan P, Di Jeso B. Thyroglobulin structure, function, and biosynthesis. In: Braverman LE, Utiger RD, eds. Werner & Ingbar's The Thyroid: A Fundamental and Clinical Text, 9th ed. Philadelphia, PA: Lippincott, Williams & Wilkins; 2005:77–95.

27. Ris-Stalpers C, Bikker H. Genetics and phenomics of hypothyroidism and goiter due to TPO mutations. Mol Cell Endocrinol 2010;322:38–43.

28. Taurog A, Dorris ML. Myeloperoxidase-catalyzed iodination and coupling. Arch Biochem Biophys 1992;296:239–246.

29. Furtmuller PG, Zederbauer M, Jantschko W, et al. Active site structure and catalytic mechanisms of human peroxidases. Arch Biochem Biophys 2006;445:199–213.

30. Fayadat L, Niccoli-Sire P, Lanet J, Franc J-L. Role of heme in intracellular trafficking of thyroperoxidase and involvement of H_2O_2 generated at the apical surface of thyroid cells in autocatalytic covalent heme binding. J Biol Chem 1999;274:10533–10538.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Photomicrograph of thyroid tissue from a 5-week-old Tenterfield Terrier exhibiting CHG. There is diffuse follicular epithelial cell hyperplasia. Follicle spaces are largely obliterated by cuboidal to columnar epithelial cells piled up as blunt papillae and exhibiting multiple small cytoplasmic vacuoles. The remaining colloid spaces are small and have scant, poorly staining colloid. The bar indicates 200 μm.

Figure S2. Local structure modeling demonstrating the predicted effect of the Tenterfield Terrier mutation in TPO. The structure of human myeloperoxidase, a TPO homologue, determined from high-resolution x-ray crystallographic data is illustrated herein as a discontinuous ribbon diagram using the PyMOL Molecular Graphics System. A star denotes the residue homologous to R593 which is replaced by tryptophan in CHG-affected dogs. The hydrogen bonds formed between the residues homologous to R593, aspartate 642, and tyrosine 637 in canine TPO are shown as dashed lines. Disruption of this hydrogen bonding sequence likely interferes with the maintenance of local protein structure necessary for arginine 591 to coordinate the heme group (in red), a key interaction required for TPO catalytic activity.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.