Molecular Genetic Characterization of Thyroid Dyshormonogenesis in a French Bulldog

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Background: A case of congenital hypothyroidism with goiter (CHG) in a juvenile French bulldog was identified and hypothesized to be caused by dyshormonogenesis of genetic etiology.

Objectives: To describe case management, unusual phenotypic aspects, and a CHG-causing mutation in a French bulldog.

Animals: Thyroid tissue and blood from a CHG-affected French bulldog and 4 normal control dogs and buccal brush samples of 125 French bulldogs were studied.

Methods: Standard clinical assessment and laboratory tests were applied. Thyroid peroxidase (TPO) iodide oxidation activity was measured *in vitro*, and TPO protein was assessed on Western blots. Thyroid peroxidase exons and flanking splice sites were amplified from genomic DNA and sequenced. Thyroid peroxidase cDNA was amplified from thyroid RNA and sequenced.

Results: At 9 months of age, the affected dog had signs of cretinism, but near-normal skeletal maturation. The enlarged thyroid glands exhibited noninflammatory fibrosis and aberrant follicular organization. Thyroid peroxidase activity and immunocrossreactive protein were undetectable. There was a T>C mutation of the intron 12 splice donor consensus that caused abnormally spliced mRNA, consistent with absent TPO function. The mutant allele was not observed in 125 clinically normal French bulldogs.

Conclusions: Presumptive CHG in a French bulldog with unusual clinical presentation is described. Genetic etiology was confirmed by identifying the underlying *TPO* mutation.

Key words: Goiter; Inborn error; Mutation; RNA splicing; Thyroid peroxidase.

Although hypothyroidism in dogs is most commonly an adult-onset disorder,[1] it sometimes occurs in the neonatal period,[2] and as in humans, early development of goiter signals hypothyroidism because of dyshormonogenesis.[3] Congenital hypothyroidism with goiter (CHG) causes developmental delay and a constellation of signs collectively known as cretinism, in addition to the metabolic abnormalities observed in adult patients. Affected pups typically exhibit delayed opening of eyes and ear canals, poor nursing, inactivity, unresponsiveness to environmental stimuli, hypomyelination

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Abbreviations:

CHG	congenital hypothyroidism with goiter
TPO	thyroid peroxidase
T4	thyroid hormone
TSH	thyroid stimulating hormone
DNA	deoxyribonucleic acid
cDNA	complementary DNA
RNA	ribonucleic acid
PCR	polymerase chain reaction
RT	reverse transcription
EDTA	ethylenediaminetetraacetic acid
SDS	sodium dodecyl sulfate
bp	base pair
kDa	kilodalton
DUOX2	dual oxidase 2

Standard single letter code for amino acids
A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; G, glycine; I, isoleucine; L, leucine; N, asparagine; P proline; Q, glutamine
Standard single letter code for nucleotides
A, adenosine; C, cytosine; G guanosine; T, thymine; R, either purine

of the central nervous system, disproportionate dwarfism caused by epiphyseal dysplasia, and macroglossia. Canine CHG most often is an inborn error of metabolism, but may be an acquired disorder caused by late fetal or neonatal iodine excess[4,5] or deficiency[6] or by exposure of the neonate or pregnant dam to a variety of drugs.[7,8]

Inherited CHG is an autosomal recessive disorder caused by breed-specific mutations of the thyroid peroxidase gene (*TPO*) in toy fox, rat, and Tenterfield terriers,[9–11] and in Spanish water dogs.[12] An unusual presentation of CHG in a juvenile French bulldog precipitated a candidate gene investigation of the molecular basis of its disorder. We report here, unusual

Fig 1. The patient of this report before diagnosis at approximately 9 months of age. In panel (**A**) chronic protrusion of the tongue is evident. Bilateral ventral cervical swellings that converge on the midline are evident in the close-up shown in panel (**B**).

phenotypic features of the case and a *TPO* splice site mutation that abrogates TPO expression and function.

Case Report

A female French bulldog was adopted at 9 months of age and presented for evaluation (Fig 1). The early history was incomplete, but dullness and growth delay had been recognized from a few months of age. The dog weighed 6.5 kg, was small for age and breed, and was mentally dull and withdrawn. The dog had disproportionately short legs, a large protruding tongue, generalized seborrhea, and areas of alopecia. Neurologic examination identified mild ataxia with proprioceptive deficits (2/4) in all 4 limbs, and the dog was suspected to be deaf. The dog had multifocal demodecosis and isospora oocysts and giardia cysts were detected in feces. An approximately 3 × 6-cm mass was present on either side of the trachea near the thoracic inlet (Fig 1B), but submandibular and other lymph nodes were within normal limits. Radiographs showed hemivertebrae at T_1, each of T_{4-6}, T_{12}, and L_3; and a butterfly vertebra at T_8. Limb and axial epiphyses were normally mineralized and closed to a degree appropriate for age (Fig 2). Needle aspirates of the ventral cervical masses disclosed glandular epithelial tissue with no cytologic evidence of inflammation or neoplasia, but were otherwise inconclusive.

A CBC and serum biochemistry disclosed slight decreases of hemoglobin [13.3 g/dL; reference interval (RI), 13.4–20.7] and mean corpuscular hemoglobin concentration (31.7 g/dL; RI, 32.6–39.2), and mild eosinophilia (1,788/μL; RI, 700–1,490). Serum phosphorus concentration (6.8 mg/dL; RI, 2.5–6.1) and alanine aminotransferase (158 U/L; RI, 18–121), aspartate aminotransferase (68 U/L; RI, 16–55), and creatine kinase (259 U/L; RI, 10–200) activities each were slightly increased. Serum total (0.7 μg/dL; RI, 1–4) and free thyroid hormone (T4) concentrations (3.9 pmol/L; RI, 7.7-47.6) were low, and serum thyroid stimulating hormone (TSH) concentration was increased (0.70 ng/mL; RI, 0.05–0.42).
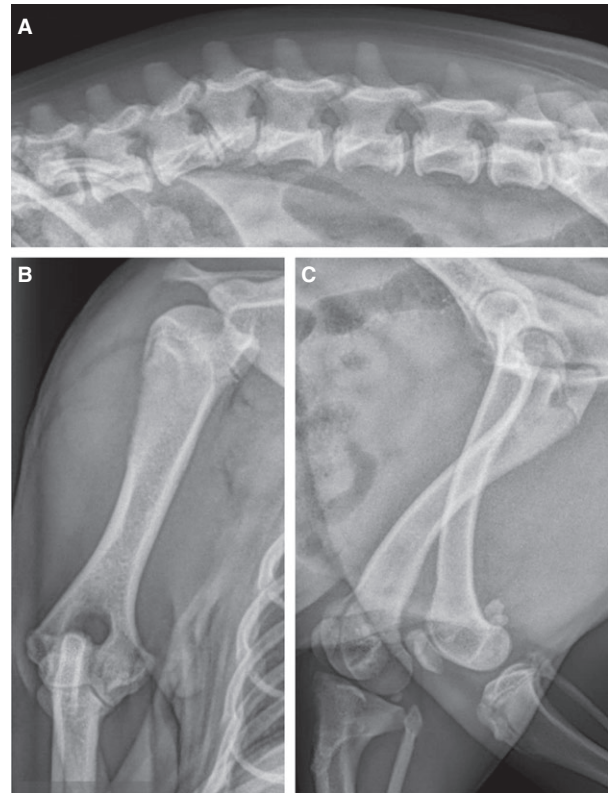

Fig 2. Radiographs of the affected dog at 9 months of age, before diagnosis and treatment for hypothyroidism. Panel (**A**) is a lateral view of lumbar vertebral column. A hemivertebra is evident at L3. Panels (**B**) and (**C**) are a VD view of the right forelimb and a lateral view of the pelvis and hind limbs, respectively.

Thyroid hormone replacement treatment was initiated with 0.1 mg of levothyroxine[a] PO q12h. The dog received weekly SC injections of doramectin[b] (6 mg/kg) until 2 consecutive skin scrapings were negative for demodex mites, and 2 SC injections of cefovecin sodium[c] (51 mg) 10 days apart. It was also treated with toltrazuril sulfone (50 mg/kg PO q24h for 3 days) and metronidazole (25 mg/kg PO q24h for 5 days) and bathed 2–3 times per week with shampoo[d] containing 1% ketoconazole and 2% chlorhexidine until seborrhea subsided.

Eighteen days after beginning treatment, the dog was more alert and active, but remained ataxic and unresponsive to auditory stimuli. Brainstem auditory-evoked response testing indicated that hearing was intact. One month later a 4-hour postpill measurement of serum total T4 concentration was 1.2 μg/dL, in the low reference range, and levothyroxine administration was increased to 0.2 mg PO in the AM and 0.1 mg PO in the PM. Four weeks later the dog's serum total T4 concentration remained in the low reference range (1.6 μg/dL), and TSH remained increased (0.51 ng/mL). Levothyroxine administration was further increased to 0.3 mg PO q12h, and 4 weeks later serum total T4 concentration was 5.2 μg/dL and TSH concentration was <0.1 ng/mL. By that time, the dog had normal skin and hair coat, was much more active, and socialized well

with people and other dogs. At 8 months after initial presentation, the gait remained abnormal, and both hind limbs moved together when running, and the size of the thyroid masses decreased to approximately 3 × 3 cm. The dog still appeared to be deaf and continued to urinate and defecate in inappropriate places despite continued house training efforts by the owner.

The dog underwent routine ovariohysterectomy and incisional thyroid biopsy at 11 months of age. Portions of thyroid were placed in 10% neutral-buffered formalin and an RNA preservation solution[e] and frozen in liquid nitrogen for histopathology and molecular and biochemical analyses, respectively. Whole blood was collected in EDTA anticoagulant for DNA isolation. Light microscopic examination of thyroid sections identified areas of various size follicles (50–300 μm) with low cuboidal epithelium and lightly eosinophilic colloid devoid of re-absorption vacuoles (Fig 3). In places, the follicles were separated by widened, collagenous septae. In other areas, the follicular structure was completely disorganized, with abundant collagen deposition and many small follicles (15–30 μm) with low cuboidal epithelium and lightly staining colloid or abnormally large follicles (>400 μm) of irregular shape. In a few large follicles, cuboidal epithelial cells lined papillae that protruded into the colloidal spaces. Immunohistochemistry with macrophage and T-lymphocyte markers confirmed that the abundant fibrosis was not accompanied by infiltration of inflammatory cells.

Methods

Thyroid biopsy of the affected dog was performed with informed owner consent. Normal control dog thyroids were obtained from adult dogs euthanized for other reasons under protocols approved by the Michigan State University Institutional Animal Care and Use Committee. Thyroid peroxidase iodide oxidation activity was assessed in the affected and control dog thyroid tissues as previously described.[9,11] DNA was isolated from whole blood using a commercial kit and the manufacturer's protocol.[f] The PCR primers flanking each of the 16 TPO exons with consensus splice sites were published previously.[11] Additional primers for cDNA amplification in this study (Table 1) were designed using GenBank accession AY094504.2 and software available online.[g] PCR amplification from genomic DNA was performed as previously described,[11] and products were sequenced directly on both strands by routine Sanger cycle-sequencing methods using the PCR primers and dideoxynucleotide chain termination. Sequences were assembled[h] and compared to the CanFam 3.1 canine (boxer) reference sequence.

RNA was isolated from thyroid tissue using a commercial kit according to the manufacturer's protocol with on-column DNAase digestion.[i] Three μg of total RNA were reverse transcribed[j] (RT) using an exon 16 gene-specific primer (Table 1) in 50 μL reactions at 50°C. The resulting cDNA was purified by micro-adsorption chromatography[k] and amplified by PCR as described previously[12] using primers located in exons 10 and 14 (Table 1). The RT-PCR amplicons were examined by agarose gel electrophoresis and sequenced as above. French bulldog buccal brush samples and pedigree information were obtained from the Canine Health Information Center (CHIC) DNA repository. Genotyping was by PCR amplification of genomic DNA (Table 1) and Bsr I restriction endonuclease digestion of the products.

Thyroid proteins were isolated from snap frozen tissue and subjected to SDS-polyacrylamide gel electrophoresis as previously described.[11] Western blots were prepared on polyvinylidene difluoride membranes and developed by sequential incubation with rabbit polyclonal anti-TPO peptide (mouse residues 31–150)[l] diluted 1 : 1,000 and an antirabbit IgG-horse radish peroxidase conjugate[m] diluted 1 : 5,000. Cross-reactive proteins were identified by the incubation of membranes with chemiluminescence detection
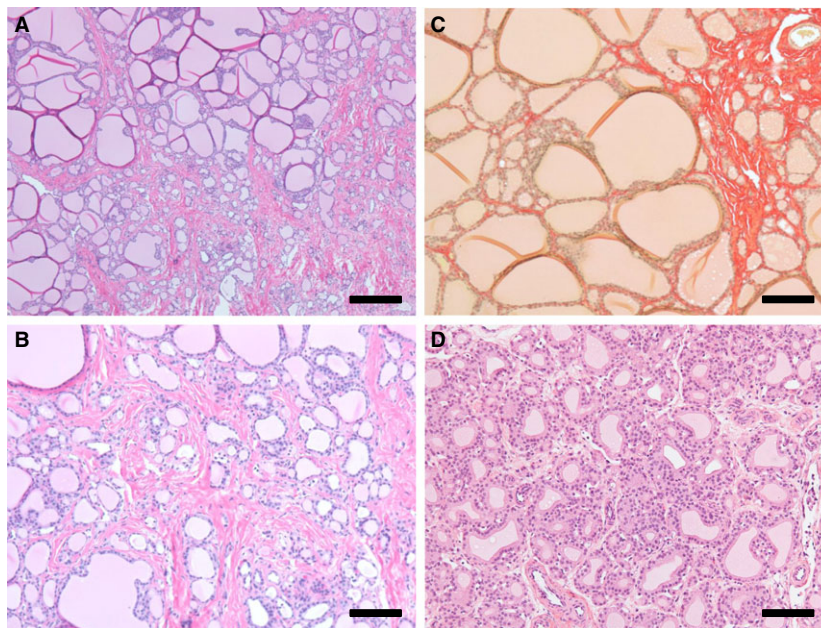

Fig 3. Thyroid gland histology. Panel (**A**) is a photomicrograph of thyroid tissue of the hypothyroid French bulldog biopsied at 10.5 months of age (H&E stain; bar = 300 μm). Panels (**B**) (H&E) and (**C**) (picrosirus red) are the same tissue at higher magnification (bars = 120 μm). Panel (**D**) is a section of goitrous thyroid from a 13-month-old Spanish water dog (H&E; bar = 120 μm) affected by a previously described *TPO* null mutation.[12] Tissue of each dog was biopsied while on oral thyroid replacement treatment.

Table 1. RT and PCR primers new in this study.

Location	Reaction	Sequence 5′>3′	T_m (°C)
TPO cDNA primers			
Exon 16	Gene-specific RT primer	CCACGTGGCTCCTACTGGATGT	61
Exon 10	PCR forward	TCATCGGGAGGCAGATGAAG	63.6
Exon 14	PCR reverse	ACGCGATGGAGACCAAGGA	64.1
Genotyping primers			
Exon 12	PCR forward	TCCCCGACAGCCTGGACAAT	67.3
Intron 12	PCR reverse	CGTCTGAGGACCGCCGATTT	66.9

reagents.[n] Positive and negative control samples were included on each blot, and specificity was determined from duplicate blots incubated with irrelevant primary antibody.

Results

Thyroid peroxidase iodide oxidation activity was undetectable in the membrane fraction of thyroid tissue homogenates of the affected dog, and activity was 2.74 ± 0.45 U/mg protein (mean $\pm$ SD) in 3 normal control dog samples. Additionally, the expected approximately 105–110 kDa TPO protein[13] of normal dogs was undetectable in the affected dog thyroid tissue sample on Western blots (Fig 4E).

Upon amplification and sequencing the *TPO* exons and flanking splice sites, we found no variant of the TPO protein-coding sequences in the affected dog, but there was a homozygous T>C transition in the +2 position of the intron 12 splice donor site (CFA17:801,598; *TPO* c.2242 + 2T>C; Fig 4A). To investigate the potential effect of this putative mutation, we amplified a portion of *TPO* cDNA from thyroid tissue of the affected and a normal dog by RT-PCR using a gene-specific RT primer in exon 16 and a PCR primer pair annealing in exons 10 and 14. The main cDNA product amplified from the affected dog was 423 bp, 171 bp smaller than the 594 bp predicted by the reference sequence as well as what was amplified from normal dog thyroid cDNA (Fig 4C). In addition, a second, but, less robust amplification product of 545 bp consistently was present in the affected dog reactions.

Sequencing of the 423 bp product identified loss of the 171 bp of exon 12 exactly, with no other inserted or deleted sequence (Fig 4B). The deletion of exon 12 maintained the translation reading frame, but predicted loss of 57 amino acid residues. Sequence of the 545 bp product demonstrated that the 5′ 122 bp of exon 12 were included between exons 11 and 13, but the 3′ 49 bp of exon 12 were deleted. This longer sequence predicted a shift of the translation reading frame at the site of exon 12 truncation and altered the deduced amino acid sequence thereafter until a premature stop 44 codons later, at the last codon of exon 13.

The T>C transition in the intron 12 splice donor site created a new *Bsr I* restriction endonuclease recognition site allowing design of a convenient genotyping assay. A 216 bp portion of genomic DNA flanking the mutation site was amplified by PCR and subjected to *Bsr I* digestion. When present, the mutant allele was cut into fragments of 156 and 60 bp. The digestion assay was validated for heterozygous allele detection by mixing normal and affected dog DNA 1 : 1 before PCR amplification. The mutant allele was not observed in any of 125 clinically healthy French bulldog samples tested.

Discussion

Congenital hypothyroidism with goiter has been observed in toy fox terriers,[9] Tenterfield terriers,[11] and Spanish water dogs[12] as autosomal recessive disorders, each segregating a breed-specific null mutation of the *TPO* gene. The toy fox terrier *TPO* mutation also was introduced into rat terriers, apparently by cross-breeding, and produced at least 1 rat terrier family affected by CHG.[10] Thyroid peroxidase gene mutations also are the most common cause of inherited CHG in humans.[14,15] Thyroid peroxidase is a multi-functional enzyme required for thyroid hormone synthesis.[13] Lack of TPO activity causes failure of iodide incorporation into thyroglobulin, the so-called organification defect demonstrated in radioiodine uptake and perchlorate discharge studies.[9,16] The consequent failure of thyroid hormone synthesis, or dyshormonogenesis, leads to unrelenting TSH stimulation and diffuse thyroid follicular epithelial cell hyperplasia recognized as goiter.

In each of the previously described breeds, the inherited disorder caused thyroid hormone deficiency in the newborn period and life-threatening developmental delays that were apparent in the first few weeks of life. Survivors required intensive nursing care and assisted feeding until diagnosis and institution of PO thyroid hormone replacement treatment. In the present case, a juvenile French bulldog exhibited signs of hypothyroidism and enlarged thyroid glands. Thyroid gland tissue lacked detectable TPO activity and immunoreactive protein *in vitro*, and an intron 12 splice donor site mutation produced *TPO* mRNA lacking all or part of exon 12. Despite an incomplete history and no pedigree information, the genetic etiology of TPO deficiency in this dog indicates that the dysfunction was present from birth.

The affected dog appeared to be deaf, and normal BAER test results suggested a central nervous system cause. This presentation is distinct from the syndromic sensorineural deafness observed in human patients with congenital hypothyroidism caused by mutation of *SLC26A4*, an anion exchanger expressed in thyroid epithelium and the inner ear. However, hearing loss in humans who are hypothyroid of various causes is common and may be conductive, sensorineural, central, or mixed.[17]
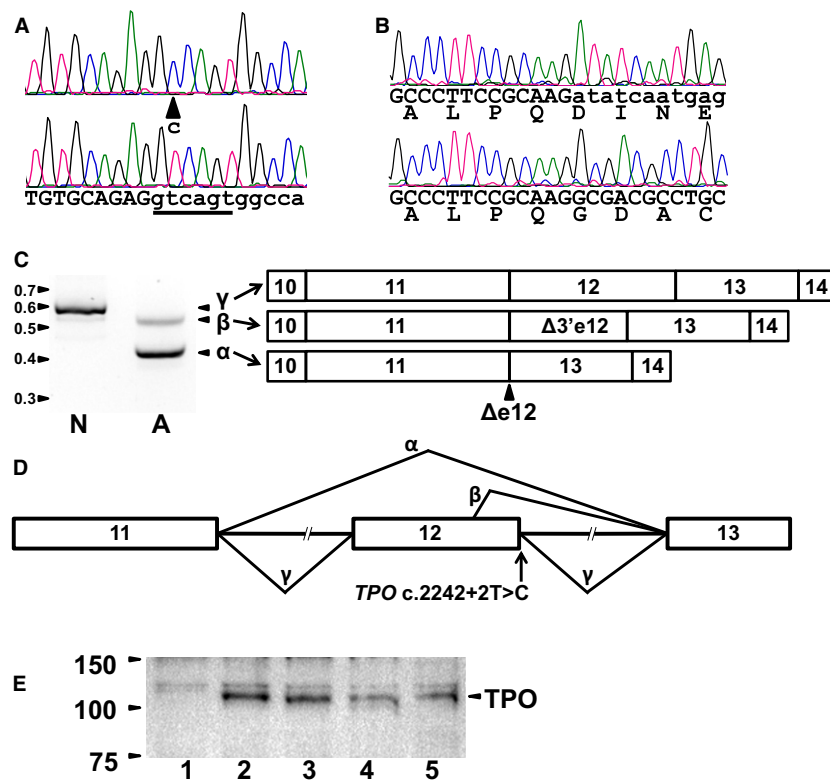
Fig 4. An intron 12 splice donor mutation alters *TPO* gene expression. Panel (**A**) shows genomic sequence of the affected (above) and a normal dog (below) at the boundary of exon 12 and intron 12. Exonic sequence is in capitals, intronic sequence is in small letters, and the normal splice donor consensus sequence is underlined. The transition of T>C in the affected dog sequence is indicated. Panel (**B**) shows cDNA sequence of the affected dog (above) and a normal dog (below) at the 3′ boundary of exon 11. In the normal dog, the sequence after exon 11 is the expected 5′ end of exon 12, whereas in the affected dog, the sequence after exon 11 is the 5′ end of exon 13 (small letters). The predicted amino acid sequence of each is shown in single letter code below the DNA sequences. Panel (**C**) shows a 3% agarose gel separating thyroid *TPO* RT-PCR amplicons of a normal dog (N) and the affected dog (A) produced in reactions using PCR primers in *TPO* exons 10 and 14. The normal dog product was ~590 bp (γ), whereas the products from the affected dog were ~540 bp (β) and ~420 bp (α), as estimated from the gel. Migration of 0.1 kbp molecular weight markers are indicated to the left. To the right of the gel are schematics deduced by sequencing the 3 RT-PCR products. Exons are numbered according to the normal sequence (γ). Δ3′e12 indicates the presence in product β of exon 12 sequence truncated after the 5′ 122 bp, leaving out the 3′ 49 bp. Panel (**D**) is a cartoon of genomic DNA illustrating the normal splicing pattern (below) of primary RNA transcripts producing the normal RT-PCR product, γ, and the 2 altered splicing patterns (above) that produced the abnormal RT-PCR products, α and β, of the affected dog. Exons are large open boxes, and introns are horizontal lines between the boxes. Exons 10 and 14 are truncated at the sites of PCR primer annealing, but the lengths of exons 11 (209 bp), 12 (171 bp), and 13 (132 bp) are proportional to the nucleotide sequence. The introns are shortened, as indicated by the breaks; intron 11 is actually 714 bp, and intron 12 is 2,671 bp. Position of the intron 12 splice donor site mutation is indicated. Panel (**E**) is a Western blot of thyroid membrane fraction proteins separated by SDS-PAGE on a 7.5% gel. Ten μg of total protein were loaded in each lane. Migration of molecular weight markers (kDa) are indicated on the left. The TPO immuno cross-reactive band is indicated on the right. Shown are results of the CHG-affected dog of this report (lane 1), and 4 normal dogs (lanes 2–5).

Intriguingly, however, there are several differences in our case from previous descriptions of dogs exhibiting CHG caused by known *TPO* mutations. The dog survived, albeit somewhat cretinized, without diagnosis or specific treatment until several months of age. In the present case, the affected dog's thyroid tissue was fibrotic and did not exhibit the diffuse epithelial hyperplasia that is a hallmark of unrelenting TSH stimulation in dyshormonogenesis. Moreover, the goiters shrank somewhat after prolonged thyroid hormone replacement treatment. Additionally, near-normal epiphyseal ossification and closure were observed at the time of presentation and diagnosis. This finding is in stark contrast to previous cases of dogs presenting with apparent

congenital hypothyroidism at ages of 5 months to 4 years with delayed closure of long bone and vertebral body physes and lacking epiphyseal ossification.[16,18–21] However, in those late-diagnosed dogs, epiphyseal ossification proceeded to maturity after hormone replacement treatment began. Although by definition *TPO* mutations are congenital, the hypothyroidism they cause varies in severity, and in the present case the extent of hypothyroidism that was present for the several months after birth is unknown. Given the incomplete history in the present case, the dog may have been treated with more or less adequate thyroid hormone replacement for some time earlier in its life. Also, despite the lack of detectable TPO activity and

protein *in vitro*, the dog could have had some residual TPO activity that allowed hormone synthesis sufficient to support epiphyseal ossification.

Nearly 80% of >115 000 human disease-causing mutations cataloged to date are found in protein-coding sequences and comprise missense and nonsense mutations, insertions or deletions, or small indels.[14] Approximately 9% are splice-site mutations. Splicing is a regulated modification of the primary RNA transcript during gene expression in eukaryotic cells to remove introns (non-protein-coding sequences) that are interspersed between exons, the coding sequences in genomic DNA. The nuclear splicing machinery is a group of small nuclear ribonucleic protein complexes that recognize specific consensus sequences at the exon/intron boundaries. The splice donor consensus sequence at the 5′ end of an intron is AG/GTRAGT, where the slash indicates the exon/intron boundary and R indicates either G or A. The underlined GT in positions +1 and +2 of the intron are nearly 100% conserved, whereas the consensus at other positions is less stringent. Thus, a T>C transition at the +2 position of intron 12 in the affected dog predicted inactivation of that splice donor, necessitating the splicing machinery to remove the intron by joining exon 13 with the next available splice donor sequence. Approximately 1% of authentic human introns have AG/GCAAGT splice donor sequences, but they often are highly regulated in a tissue-specific fashion that involves both *cis*- and *trans*- acting factors.[22] They also occur occasionally in dogs, cats, and other mammals as normal sequence supporting constitutive splicing (e.g., *CUBN* intron 32). Regardless, the RT-PCR experiment reported verified that the 5′GT consensus is required in *TPO* intron 12 for normal splicing. Further evidence that the T>C transition is a disease-causing mutation was that we failed to observe the mutant allele in any of 125 healthy French bulldogs, estimated to represent approximately 165 independent chromosomes.

In the current case, most often the alternative site is the splice donor at the 5′ end of intron 11, thus removing both introns 11 and 12 as well as exon 12 in a single event (Fig 4D). In a minority of transcripts, the 5′ end of exon 13 is joined to a cryptic splice donor in exon 12 (CAG/GTGGCC, the slash indicating the 3′ end of the truncation), thereby removing intron 12, but also 49 bp of exon 12. A faint approximately 545 bp RT-PCR product of the normal dog visible in panel C of Fig 4 suggests that the exon 12 cryptic splice site rarely may be used in normal thyroid tissue, although the frameshifted product is likely subject to nonsense-mediated mRNA decay[23].

Thyroid peroxidase is an N-glycanated, type I apical membrane protein that in concert with DUOX 2 mediates 3 sequential, H_2O_2-requiring steps of thyroid hormone biosynthesis, iodide oxidation, iodination of tyrosine residues, and coupling of iodotyrosines in thyroglobulin.[13] These functions are mediated by the catalytic domain encoded by *TPO* exons 5–11. Exon 12 of *TPO* encodes a small protein domain called a complement control protein domain[o] of unknown function in

TPO immediately C-terminal of the catalytic domain. Although loss of exon 12 in the predominant form of the affected dog *TPO* cDNA (designated α in Fig 4C) does not interrupt the translation reading frame, it is likely that such an internal deletion inhibits protein folding and stability or subsequent translocation to its functional position on the apical membrane.

Despite a consistent pattern of CHG phenotypic characteristics in dogs caused by *TPO* mutations that has emerged in the last 12 years, our report indicates that unusual thyroid histology or skeletal maturation does not preclude a genetic etiology of the disorder and risk in subsequent generations.

Footnotes

Acknowledgments

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