Comparison of the gene encoding the thyroid-stimulating hormone receptor between hyperthyroid and non-hyperthyroid domestic cats.

by

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Abstract

The prevalence of feline hyperthyroidism (FH) is increasing, yet the etiology of FH remains to be elucidated. The purpose of this study was to compare exons 1 to 5 of the thyroid-stimulating hormone receptor (TSHR) gene and the predicted encoded amino acids between hyperthyroid and unaffected cats.

Genomic DNA was isolated from whole blood of hyperthyroid and non-hyperthyroid cats. Exons 1 to 5 of the TSHR gene were amplified and sequenced. DNA and amino acid sequences were compared to identify differences between phenotypes.

We identified 4 polymorphisms; 1 SNP in Exon 1 (Al-10→Val), 2 SNP in Exon 4 (silent mutations) and 1 SNP in Exon 5 (Al-139→Gly). In Exon 4, some cats had an intronic 30 base pair insertion and one hyperthyroid cat had a 2 base pair deletion upstream of the coding sequence.

Finding a genetic basis for FH would have important implications for its treatment, screening and prevention.

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List of Abbreviations

FH	Feline Hyperthyroidism
TNG	Toxic Nodular Goiter
T4	Thyroxine
T3	Tri-iodothyronine
TSHR	Thyroid-stimulating hormone receptor
EDTA	Ethylenediaminetetraacetic acid
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
dNTP	Deoxynucleotide
UV	Ultraviolet
SNP	Single Nucleotide Polymorphism
bp	Base Pair

Introduction

Over the last four decades the prevalence of feline hyperthyroidism (FH) has increased dramatically to epidemic proportions[1-5], with the prevalence reaching up to 10% in certain geographical areas.[2,3,6,7] It is the most common endocrine disease in senior cats in the United States.[5,8]

Feline hyperthyroidism is analogous to toxic nodular goiter (TNG) in humans. Both FH and TNG are caused by hypersecretion of thyroid hormones by one or more hyperplastic or neoplastic nodules of the thyroid gland and independent of thyroid-stimulating hormone (TSH). The hypersecretion results in elevated circulating levels of thyroxine (T4) and tri-iodothyronine (T3) with subsequent multi-systemic clinical signs. The classic clinical signs of FH are weight loss, polyphagia, polyuria, polydipsia, agitation, vomiting, diarrhea and an unkempt hair coat. If untreated, affected cats can become severely debilitated with muscle wasting and cachexia. Potential consequences of FH include thyrotoxic heart disease, hypertension and insulin resistance. Left untreated, FH can be fatal. The prevalence increases with advancing age.[2,3,5]

Several treatment options exist for FH including administration of radioiodine, medical management with methimazole, surgical thyroidectomy and dietary therapy using an iodine-restricted prescription diet. The choice of therapy depends on many factors such as the cat's age, comorbidities, cost and the availability of treatment options. No treatment option is perfect, and each has disadvantages and potential complications.

Radioactive iodine is considered the treatment of choice[8-10], however, it is not always readily available and the average cost is $1,500-2,000. Surgical thyroidectomy requires anesthesia, and affected cats may not be good surgical candidates. In addition, cost is comparable to radioiodine and post-operative complications include hypothyroidism, hypoparathyroidism and laryngeal paralysis.[11] The rate of iatrogenic hypothyroidism is quite high; in cats with FH that underwent bilateral thyroidectomy, 67% of cats developed hypothyroidism post-operatively. Furthermore, iatrogenic hypothyroidism contributes to development of azotemia and reduced survival time in azotemic cats.[12] Medical therapy can be highly effective; however, therapy is lifelong, typically requiring twice-daily administration. With oral administration, side effects occur in up to 20% of cats, and life-threatening complications such as liver failure, thrombocytopenia and agranulocytosis are possible.[13] Iatrogenic hypothyroidism may be common.[14] Prevalence of large thyroid tumors, multifocal disease, and suspected malignancy increase over time with medical therapy.[5] With dietary intervention, efficacy is lower[15,16], and safety remains a concern, especially for non-hyperthyroid cats consuming the diet. Thus, better therapies or, even more optimally, decreased disease prevalence are important issues for feline geriatric health worldwide. In order to eradicate and/or treat a disease, it is best to address the underlying pathophysiology.

Despite its high prevalence, the cause of FH is unknown.[1-3,5-7,17-19] Cells from feline thyroid nodules function autonomously, resulting in unregulated cell growth and hormone secretion.[2,3,20-23] Nutritional deficiencies or excesses, environmental factors and genetics have all been proposed to result in thyroidal dysfunction and eventual hyperthyroidism.[1-7,17-19] Several epidemiological studies have attempted to identify

environmental or dietary risk factors that lead to FH, but a dominant one has yet to be identified.[1-3,5-7,17-19] Multiple mutations have been identified in humans and cats in the TSH receptor (TSHR) gene that may ultimately result in thyroid autonomy.[23]

The TSHR protein is a G protein-coupled receptor. The gene consists of 2292 base pairs and encodes a 763 amino acid protein.[22] The amino acid sequence of the feline TSHR is 90% identical to the human protein. The feline TSHR lacks glutamate 360, a residue conserved in all other species.[22] It contains seven transmembrane regions that have effector properties mediating G-protein activation, three intracellular loops, an intracellular carboxy-terminus and an extended extracellular domain involved in binding TSH. The gene has 10 exons. The extracellular domain is encoded by 9 exons, whilst the transmembrane and intracellular domains are encoded by exon 10.[24]

Binding of TSH to its receptor and subsequent G protein activation controls cyclic adenosine monophosphate (cAMP) concentrations in thyroidal cells. Elevation of intracellular cAMP concentrations, in turn, results in growth and differentiation of thyroidal follicular cells, as well as thyroid hormone secretion.[21,23-26] The TSHR is coupled to both inhibitory (Gi) and stimulatory (Gs) G proteins.[21,25,26]

The TSHR has basal constitutive activity, stimulating Gs independently of TSH and conferring intrinsic autonomy of thyroid follicular cell growth and function.[22,25,26] As hyperthyroidism develops, subpopulations of follicular cells replicate, eventually becoming fully autonomous from TSH stimulation. A theory of nodule generation has been developed.[3] First, environmental factors, such as nutritional goitrogens, cause diffuse thyroid hyperplasia. The increased thyroidal cell proliferation together with possible DNA damage due to action of hydrogen peroxide, a by-product of thyroid

hormone synthesis, increase the number of cells that mutate. Some spontaneous mutations confer constitutive activation of the TSHR, and, thus, autonomy of function. Genetic susceptibility to mutations also plays a role. To date, most mutations in the human TSHR in patients with TNG have been located in the transmembrane domain. Less commonly, mutations have been identified in the TSHR Gs protein subunit.[21,23,24,27]

Potentially up to 82% of autonomously functioning thyroid nodules in humans are due to TSHR mutations[28]; identified mutations are located mainly in exons 9 and 10 of the gene.[29] Analysis of the TSHR gene in hyperthyroid cats has been limited. Two studies failed to identify a functional genetic mutation in the TSHR gene.[20,21] Thyroid nodular DNA was used, as somatic mutations occurring within nodules may be more likely to cause FH than germline mutations. However, both studies had small sample sizes and examined only part of the TSHR gene. Only part of exon 10 was sequenced, and not the portion most often affected in humans. Only one study included exons 1 to 9, but only 10 cats were included.[21] In addition, DNA was extracted from the entire thyroid lobe rather than adenomatous thyroid nodules, possibly masking mutations present in the nodules themselves.[20,21]

A more recent study examining 135 nodules from 50 hyperthyroid cats identified ten missense mutations and one silent mutation in exon 10 in 35% of nodules.[23] Five of the 10 missense mutations are associated with human TNG. Thus, exon 10 is likely to be important in development of FH as it is in the similar human disease. A total of 9 somatic mutations were identified, with the most common being Met-452-Thr.[23] As up to 82% of autonomously functioning thyroid nodules in humans are related to TSHR mutations, more mutations may exist in feline cases than previously identified. At least

31 somatic and 17 germline mutations of the TSHR have been associated with TNG.[23,24] Some of the mutations identified in FH have been observed as germline mutations in human hyperthyroid disease.[23]

Examining the TSHR gene in domestic cats with FH and comparing the genetic sequence to cats without FH may highlight amino acid differences that play an important role in pathogenesis of FH. Additionally, examining the TSHR gene in a large number of hyperthyroid cats may also increase the likelihood of identifying novel mutations. As several germline mutations of the TSHR have been associated with TNG in people, examining whole blood may identify germline mutations in cats with FH.

Identifying areas of interest in the TSHR will facilitate and focus future studies on pathophysiology using larger numbers of cats. As small molecules that are TSHR antagonists are being developed and may be ideal for treating hyperthyroidism[30], knowledge of the section of the TSHR to be targeted for treatment of FH will be invaluable. Additionally, if a germline mutation is found in a high proportion of cats with FH, a screening test can be developed to determine which cats are at risk to develop FH.

Thus, the purpose of this study was to compare the sequences of exons 1 to 5 of the TSHR gene and predicted encoded amino acids between hyperthyroid cats and unaffected cats. Exons 1 to 5 were chosen as the initial part of a larger project to examine exons 1 to 10. Finding a genetic basis for FH would have important implications for the treatment, screening and prevention of the disease.

Material and Methods

Animals

EDTA-anticoagulated whole blood samples were obtained from 15 hyperthyroid cats and 5 non-hyperthyroid cats. Blood from the hyperthyroid cats was provided by Dr. Mark Peterson at the Animal Endocrine Clinic, New York. Blood from the unaffected cats was obtained from the Auburn University Clinical Pathology Laboratory. All blood samples were excess, remaining after required diagnostic procedures, and not collected solely for the purposes of the study.

The diagnosis of FH was established on the basis of consistent clinical signs (e.g. weight loss despite a normal to increased appetite, polyuria, polydipsia and a palpable thyroid nodule), thyroid hormone testing and scintigraphic evidence as previously described.[31] Non-hyperthyroid cats were all greater than 13 years of age, lacked a history of hyperthyroidism and had thyroid testing consisting of a total T4 (TT4) to rule out hyperthyroidism. Cats with equivocal TT4 concentrations had a free T4 measured using the modified equilibrium dialysis method to rule out hyperthyroidism..

Molecular Studies

DNA was isolated from whole blood (QuickGene Mini80 kit; AutoGen Inc., Holliston, MA, USA) within 1 month of collection; blood was stored in the refrigerator until processing. Desired segments were amplified using PCR with primers designed to

flank the gene encoding exons 1-5 of the TSHR. Primers for exons 1 through 5 (Table 1) were designed based on the sequence for the domestic cat TSHR (GenBank NM_001009288.1).

Table 1. Primer pairs used to amplify Exons 1 through 5 of the feline TSHR gene. BP: base pair, Tm: Annealing temperature.

Exon	Primer	Expected product BP	Tm
1	GCC TAG GGA AAC CGA ACA CTT (forward) GCA ATC CCA GGT TTG GGC AGT (reverse)	526	60
2	GGA TGT CAT AAG GAC CAG GAT (forward) GGC TGG CAC AGT TGC AAA CTT (reverse)	230	58
3	GGG AAG CTT TCA GGT ATC ACA (forward) CGA ACA CCC AGC TGT TAA CAG (reverse)	373	58
4	GGC TGA TTC CTG GCA GAG ACA (forward) GGC TCT GGT CCA CAG CAT CCT (reverse)	290	62
5	GGG AAC AAC ATC CCA TTC AAG (forward) CCA GTG TGT TTG TAA CAG CTG (reverse)	389	58

PCR reactions of 25 µL total volume were performed using the HotStar HiFidelity Polymerase Kit (Qiagen, Hilden, Germany). Each reaction contained 1 µL extracted DNA, 2µM forward/reverse primer (Eurofins MWG Operon Sequencing Lab, Louisville, KY, USA), PCR buffer (Qiagen, Hilden, Germany), dNTP's (Qiagen, Hilden,

Germany), and Taq polymerase (Qiagen, Hilden, Germany). The remaining volume was made up with molecular grade water.

Thermocycler conditions for amplification (GeneAMP PCR system 2400; Perkin Elmer, Waltham, MA, USA) were an initial denaturation at 95°C for 15 min, followed by 28 cycles of subsequent denaturing, annealing and extension phases. The annealing temperature was set depending on the guanine and cytosine nucleotide content of the primers (Table 1). There was a final extension phase at 72°C for 10 min. Each PCR run included a negative control where molecular grade water was used in place of DNA. PCR products were separated by electrophoresis in a 1.5% agarose gel with added nucleic acid dye (Gel Red; Biotium, Oakland, CA, USA). To judge product size, a DNA ladder (All-purpose HiLo™ DNA marker; Bionexus, Oakland, CA, USA) was included in each gel.

Target bands were excised from the gel and DNA extracted (QIAquick Gel Extraction Kit; Qiagen,Valencia, CA, USA). The concentration of the extracted DNA was measured via spectrophotometry (Nanodrop, Thermo Fisher Scientific, Waltham, MA, USA). Harvested products were submitted to a commercial laboratory for sequencing (Eurofins MWG Operon Sequencing Lab, Louisville, KY, USA).

The coding sequence of the amplified DNA and predicted amino acid sequences for hyperthyroid and non-hyperthyroid cats were compared to the published *felis catus* TSHR DNA sequence (NCBI reference number 018728.2). The predicted DNA and protein sequences were compared (DNASTAR MegAlign, Lasergene, Madison, WI). The amino acid sequences were compared to the expected amino acid sequence translated

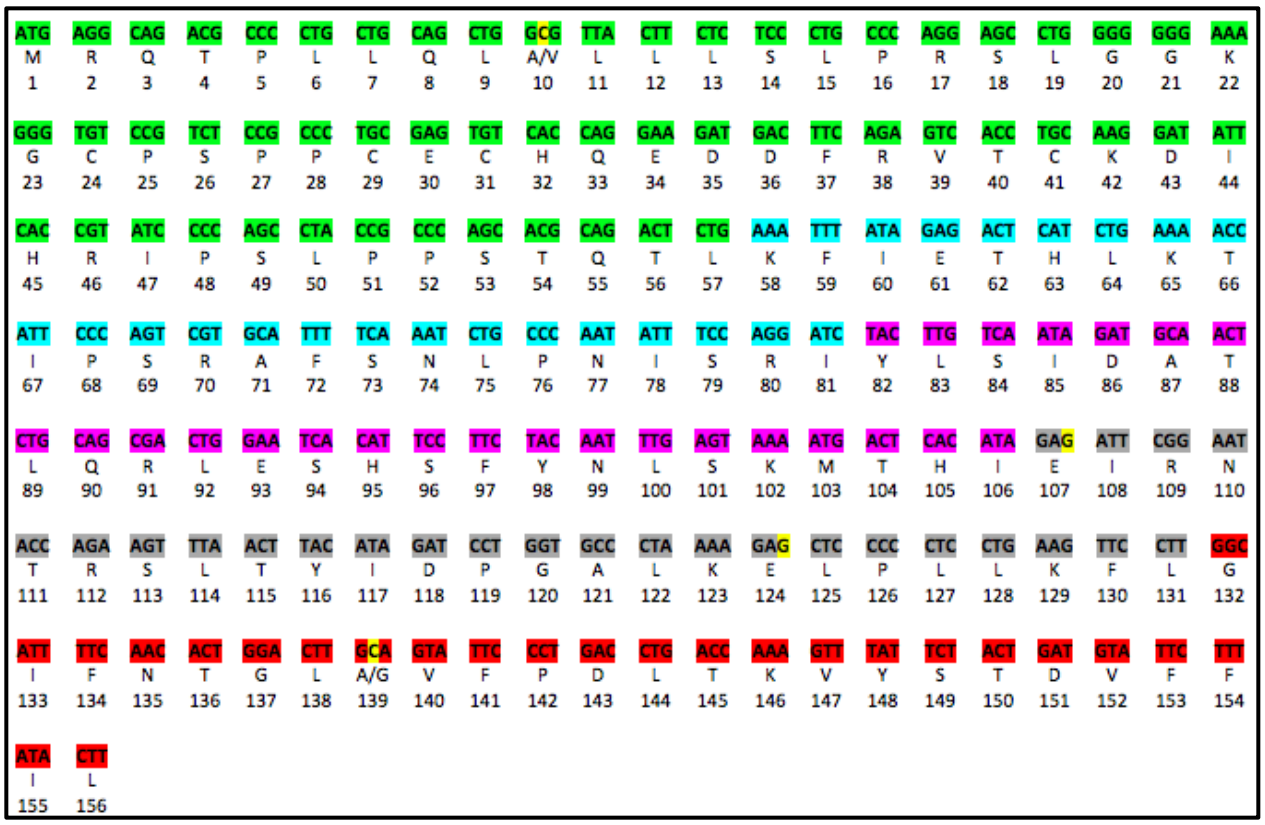
from the DNA sequence. When a mutation in the DNA was noted, the DNA extraction, PCR and sequencing were repeated to verify results.

Results

Whole blood samples were obtained from 15 hyperthyroid cats and 5 non-hyperthyroid cats. There were 8 neutered males and 7 spayed females in the hyperthyroid group. Breeds of hyperthyroid cats included 14 domestic short hair (DSH) cats and 1 Norwegian Forest cat. The mean age was 12 years (range 9 to 17 years). There were 3 neutered males and 2 spayed females in the non-hyperthyroid cat group. The mean age was 17 years (range 16 to 18 years old). Breeds included 4 DSH and 1 Norwegian Forest cat.

The PCR products included non-coding regions on either end of the exon; for ease, the PCR products are named by the exon contained, e.g. the PCR product that contains Exon 1 along with non-coding regions on either end will be called "Exon 1". The position of a SNP in an exon is numbered according to Figure 1.

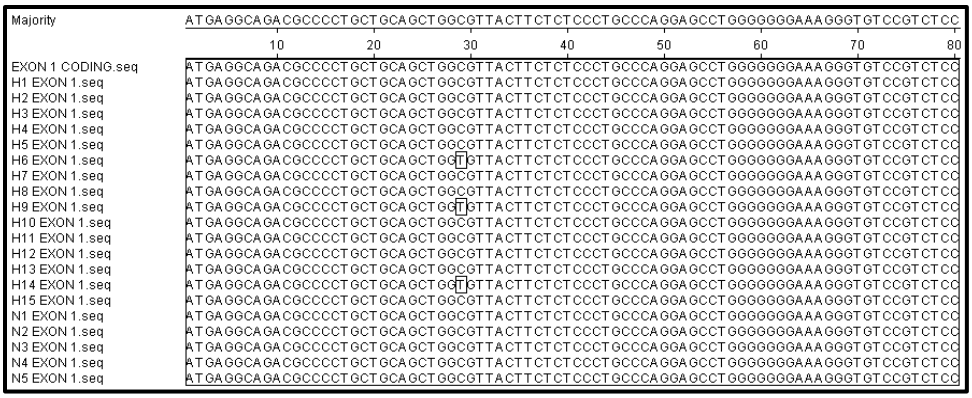
Figure 1. Codons of the TSHR gene for exons 1 to 5. Single nucleotide polymorphisms are highlighted in yellow. The letters underneath the codon represent the encoded amino acid. Where an SNP resulted in an amino acid change, both wild type and mutated amino acids are shown. Exons are divided by color. Green= Exon 1, Blue= Exon 2, Purple= Exon 3, Grey= Exon 4, Red= Exon 5.



ATG	AGG	CAG	ACG	CCC	CTG	CTG	CAG	CTG	GCG	TTA	CTT	CTC	TCC	CTG	CCC	AGG	AGC	CTG	GGG	GGG	AAA
M	R	Q	T	P	L	L	Q	L	A/V	L	L	L	S	L	P	R	S	L	G	G	K
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
GGG	TGT	CCG	TCT	CCG	CCC	TGC	GAG	TGT	CAC	CAG	GAA	GAT	GAC	TTC	AGA	GTC	ACC	TGC	AAG	GAT	ATT
G	C	P	S	P	P	C	E	C	H	Q	E	D	D	F	R	V	T	C	K	D	I
23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44
CAC	CGT	ATC	CCC	AGC	CTA	CCG	CCC	AGC	ACG	CAG	ACT	CTG	AAA	TTT	ATA	GAG	ACT	CAT	CTG	AAA	ACC
H	R	I	P	S	L	P	P	S	T	Q	T	L	K	F	I	E	T	H	L	K	T
45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66
ATT	CCC	AGT	CGT	GCA	TTT	TCA	AAT	CTG	CCC	AAT	ATT	TCC	AGG	ATC	TAC	TTG	TCA	ATA	GAT	GCA	ACT
I	P	S	R	A	F	S	N	L	P	N	I	S	R	I	Y	L	S	I	D	A	T
67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88
CTG	CAG	CGA	CTG	GAA	TCA	CAT	TCC	TTC	TAC	AAT	TTG	AGT	AAA	ATG	ACT	CAC	ATA	GAG	ATT	CGG	AAT
L	Q	R	L	E	S	H	S	F	Y	N	L	S	K	M	T	H	I	E	I	R	N
89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110
ACC	AGA	AGT	TTA	ACT	TAC	ATA	GAT	CCT	GGT	GCC	CTA	AAA	GAG	CTC	CCC	CTC	CTG	AAG	TTC	CTT	GGC
T	R	S	L	T	Y	I	D	P	G	A	L	K	E	L	P	L	L	K	F	L	G
111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132
ATT	TTC	AAC	ACT	GGA	CTT	GCA	GTA	TTC	CCT	GAC	CTG	ACC	AAA	GTT	TAT	TCT	ACT	GAT	GTA	TTC	TTT
I	F	N	T	G	L	A/G	V	F	P	D	L	T	K	V	Y	S	T	D	V	F	F
133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154
ATA	CTT																				
I	L																				
155	156																				

The predicted size of Exon 1 and the contained coding sequence were 526 base pairs (bp) and 170 bp, respectively. Comparison of the coding sequence for Exon 1 revealed a single nucleotide polymorphism (SNP) from C to T at position 10 in 3 hyperthyroid cats (Figure 2). All 3 cats were heterozygous for the mutation (Figure 3). The SNP resulted in a missense mutation, with an amino acid change from alanine to valine at amino acid position 10 (Al-10→Val) (Figure 4).

Figure 2. DNA alignment of Exon 1 for 15 hyperthyroid and 5 euthyroid domestic cats. The top line (Exon 1 CODING) shows the exon from the published sequence (NCBI reference number 018728.2). H1 to H15 are sequences from the hyperthyroid cats and N1 to N5 sequences from the non-hyperthyroid cats. The boxes indicate location of a SNP from C to T at position 10**.**



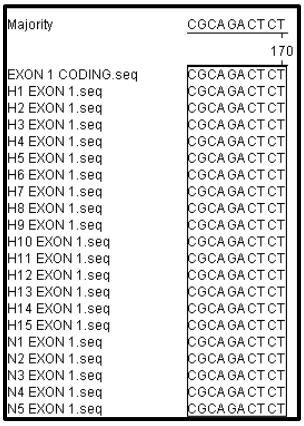


Figure 3. Example sequence data for homozygosity or heterozygosity for SNP from C to T in Exon 1. The arrow indicates the affected base. A) Homozygous for C (wild type); B) Heterozygous for C (wild type) and T (mutation).

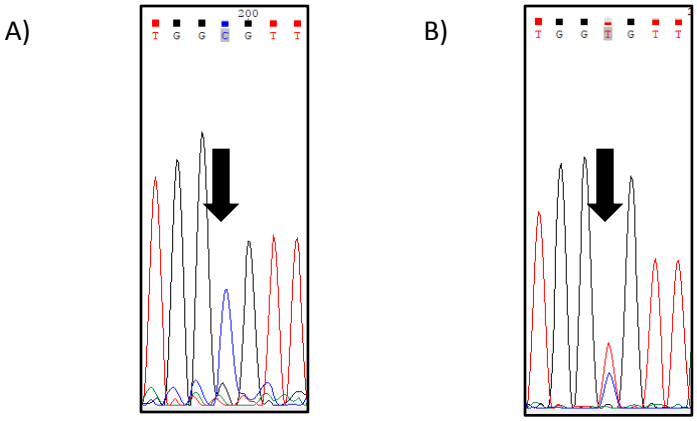


Figure 4. Protein alignment of Exon 1. The boxes highlight the missense mutation in the Exon 1 amino acid sequence. The mutation creates an amino acid change from wild type alanine to valine (Al-10→Val).

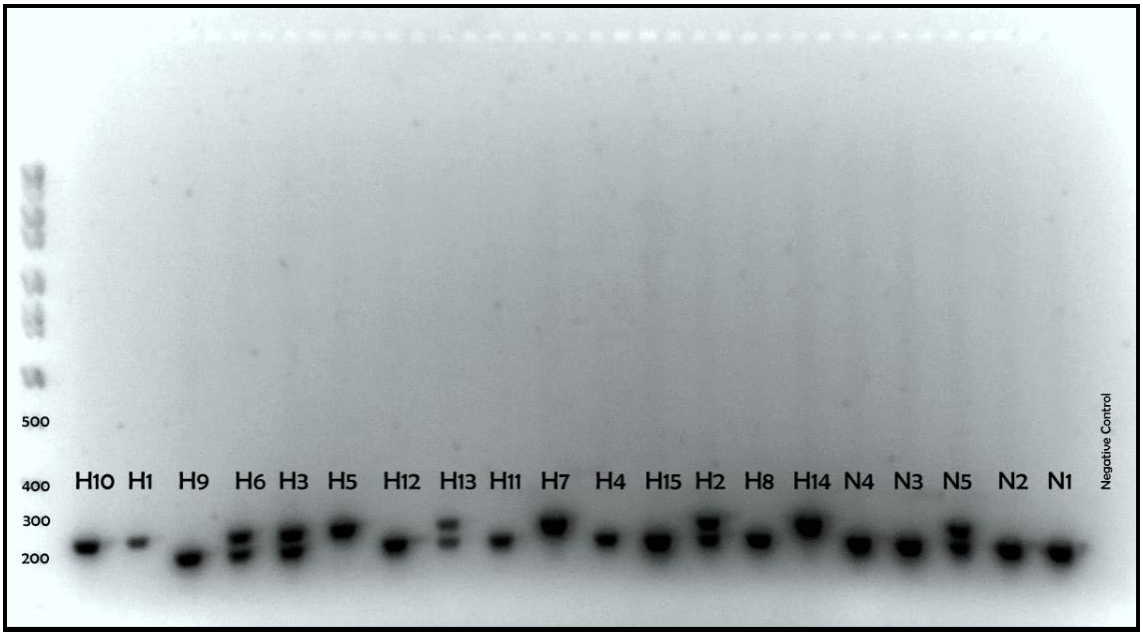
Majority	MRQTPLLQLALLLSLPRSLGGKGCPSPPCECHQEDDFRVTCKDIHRIPSLPPSTQT
	10 20 30 40 50
EXON 1 PRO.pro	MRQTPLLQLALLLSLPRSLGGKGCPSPPCECHQEDDFRVTCKDIHRIPSLPPSTQT
H1 EXON 1 PRO.pro	MRQTPLLQLALLLSLPRSLGGKGCPSPPCECHQEDDFRVTCKDIHRIPSLPPSTQT
H2 EXON 1 PRO.pro	MRQTPLLQLALLLSLPRSLGGKGCPSPPCECHQEDDFRVTCKDIHRIPSLPPSTQT
H3 EXON 1 PRO.pro	MRQTPLLQLALLLSLPRSLGGKGCPSPPCECHQEDDFRVTCKDIHRIPSLPPSTQT
H4 EXON 1 PRO.pro	MRQTPLLQLALLLSLPRSLGGKGCPSPPCECHQEDDFRVTCKDIHRIPSLPPSTQT
H5 EXON 1 PRO.pro	MRQTPLLQLALLLSLPRSLGGKGCPSPPCECHQEDDFRVTCKDIHRIPSLPPSTQT
H6 EXON 1 PRO.pro	MRQTPLLQL**V**LLLSLPRSLGGKGCPSPPCECHQEDDFRVTCKDIHRIPSLPPSTQT
H7 EXON 1 PRO.pro	MRQTPLLQLALLLSLPRSLGGKGCPSPPCECHQEDDFRVTCKDIHRIPSLPPSTQT
H8 EXON 1 PRO.pro	MRQTPLLQLALLLSLPRSLGGKGCPSPPCECHQEDDFRVTCKDIHRIPSLPPSTQT
H9 EXON 1 PRO.pro	MRQTPLLQL**V**LLLSLPRSLGGKGCPSPPCECHQEDDFRVTCKDIHRIPSLPPSTQT
H10 EXON 1 PRO.pro	MRQTPLLQLALLLSLPRSLGGKGCPSPPCECHQEDDFRVTCKDIHRIPSLPPSTQT
H11 EXON 1 PRO.pro	MRQTPLLQLALLLSLPRSLGGKGCPSPPCECHQEDDFRVTCKDIHRIPSLPPSTQT
H12 EXON 1 PRO.pro	MRQTPLLQLALLLSLPRSLGGKGCPSPPCECHQEDDFRVTCKDIHRIPSLPPSTQT
H13 EXON 1 PRO.pro	MRQTPLLQLALLLSLPRSLGGKGCPSPPCECHQEDDFRVTCKDIHRIPSLPPSTQT
H14 EXON 1 PRO.pro	MRQTPLLQL**V**LLLSLPRSLGGKGCPSPPCECHQEDDFRVTCKDIHRIPSLPPSTQT
H15 EXON 1 PRO.pro	MRQTPLLQLALLLSLPRSLGGKGCPSPPCECHQEDDFRVTCKDIHRIPSLPPSTQT
N1 EXON 1 PRO.pro	MRQTPLLQLALLLSLPRSLGGKGCPSPPCECHQEDDFRVTCKDIHRIPSLPPSTQT
N2 EXON 1 PRO.pro	MRQTPLLQLALLLSLPRSLGGKGCPSPPCECHQEDDFRVTCKDIHRIPSLPPSTQT
N3 EXON 1 PRO.pro	MRQTPLLQLALLLSLPRSLGGKGCPSPPCECHQEDDFRVTCKDIHRIPSLPPSTQT
N4 EXON 1 PRO.pro	MRQTPLLQLALLLSLPRSLGGKGCPSPPCECHQEDDFRVTCKDIHRIPSLPPSTQT
N5 EXON 1 PRO.pro	MRQTPLLQLALLLSLPRSLGGKGCPSPPCECHQEDDFRVTCKDIHRIPSLPPSTQT

The predicted size of Exon 2 was 230 bp. The coding sequence was predicted to be 72 bp. No mutation was found in any of the 20 cats in the coding sequence of Exon 2 (data not shown).

The predicted size of Exon 3 was 373 bp. The coding sequence was predicted to be 75 bp. No mutation was found in any of the 20 cats in the coding sequence of Exon 3 (data not shown).

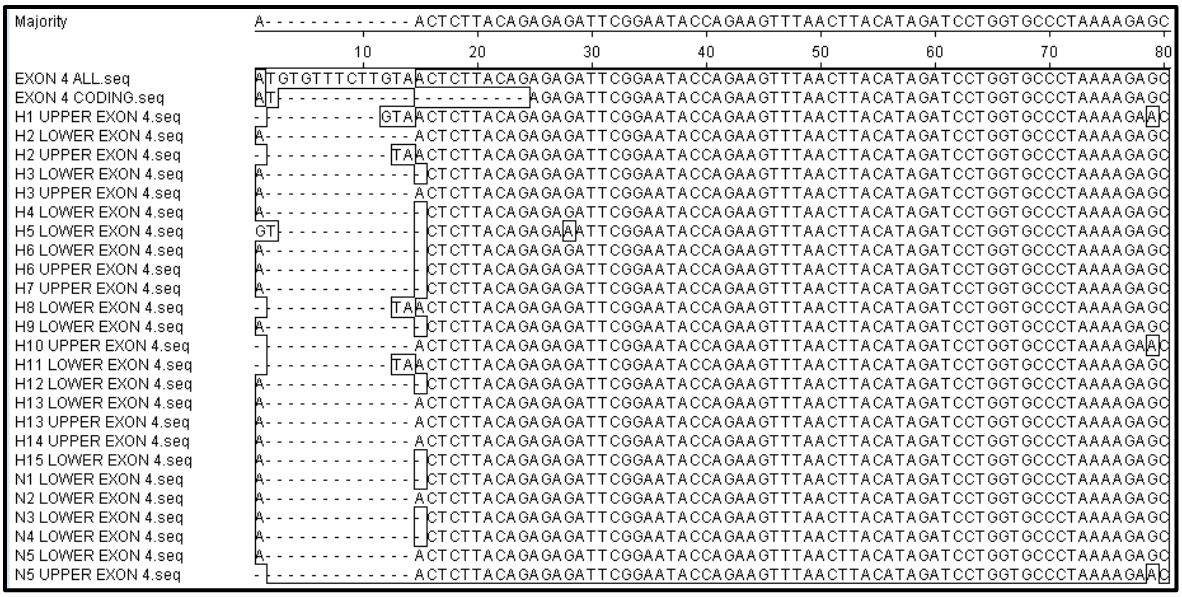
Bands of two different sizes were seen in the gel containing the PCR products of Exon 4 (Figure 5). The lower band was the predicted size of 290 bp, whilst the upper band was approximately 320 bp. The coding sequence of Exon 4 was predicted to be 75 bp. Four hyperthyroid cats and 1 non-hyperthyroid cat had both upper and lower bands. The upper band was seen in 5 hyperthyroid cats. The lower band was seen in 6 hyperthyroid cats and 4 non-hyperthyroid cats. In total, 9 hyperthyroid cats and only 1 non-hyperthyroid cat had the upper band, and only hyperthyroid cats (n=5) were homozygous for the change (i.e. only had the upper band).

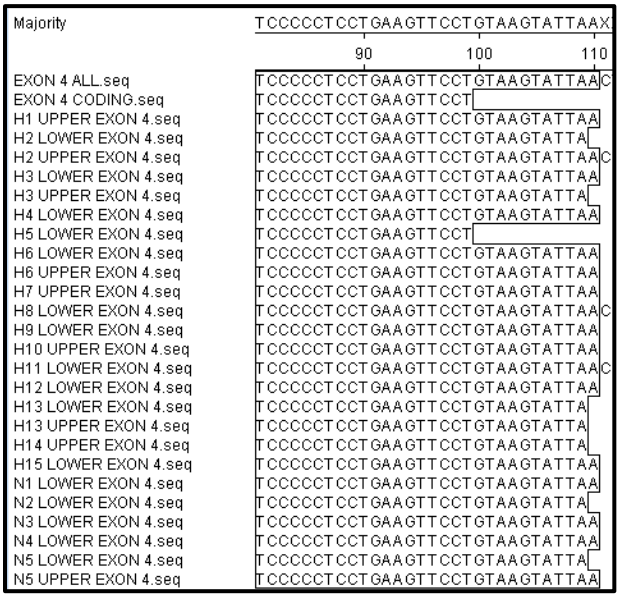
Figure 5. Picture of the gel containing the PCR products of Exon 4. The lower band was the predicted sequence size of 290 bp.



Sequencing of the upper band revealed a 30 bp insertion in the intron of Exon 4 approximately 50 bp upstream of the coding sequence (data not shown). Two distinct SNP were also noted in the coding sequence of Exon 4. One polymorphism resulted in a change from G to A at position 107 in 1 hyperthyroid cat. The second polymorphism resulted in a nucleotide change from G to A at position 124 in 2 hyperthyroid cats and 1 non-hyperthyroid cat (Figure 6). All affected cats were heterozygous for the mutation. Both mutations were silent with respect to amino acid sequence (data not shown). In one of the hyperthyroid cats, a 2 bp deletion was detected in the first 10 bases upstream of the exon (data not shown).

Figure 6. DNA alignment of Exon 4 for 15 hyperthyroid and 5 euthyroid domestic cats. The top line (Exon 4 ALL) includes the exon and part of the intron, whilst the second line (Exon 4 CODING) shows the exon from the published sequence (NCBI reference number 018728.2). H1 to H15 are sequences from the hyperthyroid cats and N1 to N5 sequences from non-hyperthyroid cats. The boxes indicate the location of a SNP from G to A at position 107 and 124 of Exon 4**.**





The predicted size of Exon 5 was 389 bp. The coding sequence was predicted to be 75 bp. A SNP was found at position 139 in 5 hyperthyroid cats that resulted in a nucleotide change from wild type C to G (Figure 7); all cats were homozygous for the mutation. An additional 5 cats were heterozygous for C and G (4 hyperthyroid and 1 non-hyperthyroid cat) (Figure 8). The nucleotide change from C to G resulted in a

missense mutation, with an amino acid change from alanine to glycine at position 139 (Al-139→Gly) (Figure 9).

Figure 7. DNA alignment of Exon 5 for 15 hyperthyroid and 5 euthyroid domestic cats. The top line (Exon 5 CODING) shows the exon from the published sequence (NCBI reference number 018728.2). H1 to H15 are sequences from the hyperthyroid cats and N1 to N5 sequences from non-hyperthyroid cats. The boxes indicate the location of a SNP from C to G at position 139 in Exon 5.

Figure 8. Homozygosity or heterozygosity for SNP from C to G (mutation) in Exon 5. The arrow indicates the heterozygous location. A) Homozygous for G (mutation), B) Homozygous for C (wild type), C) Heterozygous for C

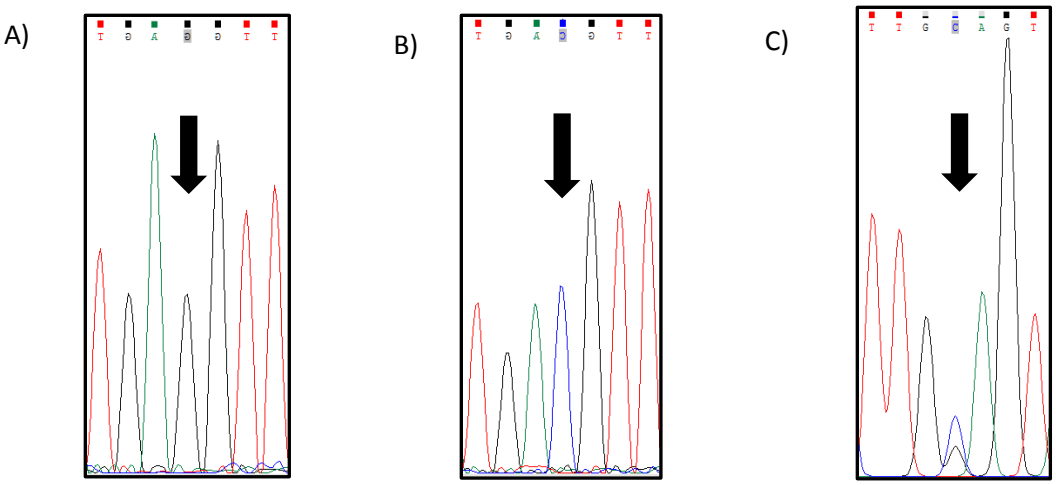


Figure 9. Protein alignment of Exon 5. The boxes highlight the missense mutation in the Exon 5 amino acid sequence. There was an amino acid change from wild type alanine to glycine (Al-139→Gly).

Majority	LGI FNTGLAVF PDLTKVYSTDVFFI
	10 20
EXON 5 PRO.pro	LGI FNTGLAVF PDLTKVYSTDVFFI
H1 EXON 5 PRO.pro	LGI FNTGLGVF PDLTKVYSTDVFFI
H2 EXON 5 PRO.pro	LGI FNTGLAVF PDLTKVYSTDVFFI
H3 EXON 5 PRO.pro	LGI FNTGLAVF PDLTKVYSTDVFFI
H4 EXON 5 PRO.pro	LGI FNTGLAVF PDLTKVYSTDVFFI
H5 EXON 5 PRO.pro	LGI FNTGLGVF PDLTKVYSTDVFFI
H6 EXON 5 PRO.pro	LGI FNTGLAVF PDLTKVYSTDVFFI
H7 EXON 5 PRO.pro	LGI FNTGLGVF PDLTKVYSTDVFFI
H8 EXON 5 PRO.pro	LGI FNTGLAVF PDLTKVYSTDVFFI
H9 EXON 5 PRO.pro	LGI FNTGLAVF PDLTKVYSTDVFFI
H10 EXON 5 PRO.pro	LGI FNTGLGVF PDLTKVYSTDVFFI
H11 EXON 5 PRO.pro	LGI FNTGLAVF PDLTKVYSTDVFFI
H12 EXON 5 PRO.pro	LGI FNTGLAVF PDLTKVYSTDVFFI
H13 EXON 5 PRO.pro	LGI FNTGLAVF PDLTKVYSTDVFFI
H14 EXON 5 PRO.pro	LGI FNTGLGVF PDLTKVYSTDVFFI
H15 EXON 5 PRO.pro	LGI FNTGLAVF PDLTKVYSTDVFFI
N1 EXON 5 PRO.pro	LGI FNTGLAVF PDLTKVYSTDVFFI
N2 EXON 5 PRO.pro	LGI FNTGLAVF PDLTKVYSTDVFFI
N3 EXON 5 PRO.pro	LGI FNTGLAVF PDLTKVYSTDVFFI
N4 EXON 5 PRO.pro	LGI FNTGLAVF PDLTKVYSTDVFFI
N5 EXON 5 PRO.pro	LGI FNTGLAVF PDLTKVYSTDVFFI

Discussion

Analysis of Exon 1 to 5 of the TSHR gene in hyperthyroid and non-hyperthyroid domestic cats revealed 4 SNP compared to the wild type sequence. Two SNP resulted in an amino acid change (Exon 1 and 5) whilst 2 SNP (in Exon 4) were silent mutations.

In Exon 1, a base change from C to T at codon 10 would result in an amino acid change from alanine to valine (Al-10→Val). Two SNP were detected in Exon 4, both being substitution for G by A (codons 107 and 124) and silent. Exon 4 had a 30 base pair insertion in the intron of the gene in some cats, and one hyperthyroid cat had a 2 bp deletion in the first 10 bases of the coding sequence. In Exon 5, an SNP resulted in a base change from C to G at codon 139, resulting in an amino acid change from alanine to glycine (Al-139→Gly).

The missense mutation in Exon 1 (Al-10→Val) was detected in only 3 hyperthyroid cats. Both amino acids belong to the same group distinguished by a hydrophobic side chain. Thus, this amino acid change is less likely to change the protein in a substantial manner, and the mutation is unlikely to be significant. The finding of the mutation in 3 hyperthyroid cats but none of the non-hyperthyroid cats is interesting, however, the sample size is small. To our knowledge, this mutation has not been reported previously in cats or people.

In Exon 4, a change from G to A at codon 107 was seen in 1 hyperthyroid cat. A base change from G to A at codon 124 was seen in 2 hyperthyroid cats and 1 non-

hyperthyroid cat. Both were silent mutations, not changing the amino acid. To our knowledge, this SNP has not been previously reported in people or cats.

In addition, a 30-bp insertion was found in the intron approximately 50 bp upstream of Exon 4 in some cats, causing the presence of a larger band on the gel. Interestingly, the larger band was seen predominately in hyperthyroid cats and only one heterozygous non-hyperthyroid cat, and thus could be significant. For example, an insertion in the intron could alter thyroidal axis function by altering gene expression. Due to location of the upstream primer, the sequence data for this particular part of Exon 4 was not clean. Further studies are required to accurately sequence the insert using primers that would target the intron between exon 3 and 4.

Interestingly in Exon 4, one hyperthyroid cat (H5) had a 2 base pair deletion 10 bp upstream of the coding sequence. This change could have implications for gene transcription. To determine if gene transcription would be altered, protein and mRNA expression could be evaluated by reverse transcription PCR.

The missense mutation (Al-139→Gly) in Exon 5 was seen only in 5 hyperthyroid cats. Alanine is an amino acid with a hydrophobic side chain. Glycine is structurally quite different, containing hydrogen as a side chain (as opposed to carbon); thus, it is considered more flexible than other amino acids. Glycine is also polar in nature, while alanine is hydrophobic. As Exon 1 to 9 are involved in the extracellular domain, this change could result in a functionally affected protein by altering the ligand binding site.

This specific mutation has not been noted in people, but, it has been found in 5 hyperfunctional feline nodules.[21] However, the amino acid change in the previous study[21] was reported as glycine to alanine, with glycine being the wildtype. When

comparing the sequence to the predicted sequence in GenBank (NCBI reference number 018728.2), the predicted amino acid in this position is alanine. Nonetheless, the authors hypothesized that this mutation was not functionally significant as sequence analysis of the corresponding regions on chromosomal DNA revealed the same mutation, thus, they predicted that it was not involved in tumorgenesis.[21] However, if the mutation is a germline mutation, it could be hypothesized that this mutation may make cats susceptible to hyperthyroidism. Additional studies with larger numbers of cats would be required to validate this finding.

As the current study does not include functional analyses, we can only suggest that a mutation may have functional significance. Further studies are indicated to determine the functional significance of these mutations in domestic cats.

Some limitations exist to this study. Previous studies examining mutations in the feline TSHR in cats have examined DNA from adenomatous/hyperplastic nodules. Somatic mutations may be more likely to cause hyperthyroidism. We did not use DNA extracted from nodules, but DNA extracted from whole blood. However, germline mutations have been related to hyperthyroidism in people[32-35], and analogous mutations have been detected in cats with FH.[23] If the mutations found are determined to be significant, a screening test for the development of hyperthyroidism in cats may be developed. Second, any of the non-hyperthyroid cats in the study could become hyperthyroid later in life; hyperthyroidism is a disease of geriatric cats with a mean age of 13 years. As a result, non-hyperthyroid cats were used only if they were older than 13 years old in order to minimize inclusion of cats that develop FH. Finally, the sample size

is small. However, potentially significant mutations have been identified that can be evaluated in further studies.

Conclusion

In summary, the cause of FH is unknown, and the disease can have extremely detrimental consequences if left untreated. Thus, the evaluation of the TSHR is crucial to better understanding FH and potentially to preventing it.

This study identified 4 polymorphisms in Exon 1, 4 and 5 of the TSHR. Additionally, a 30 base pair insertion in the intron of Exon 4 was found in some cats. The majority of cats with this intronic insertion were hyperthyroid. A 2 base pair deletion was also found at the start of the coding sequence of Exon 4 in one hyperthyroid cat.

There have been very few mutation identified in the extracellular portion of the TSHR gene in cats and humans. This study has highlighted several novel changes in the extracellular portion of the feline TSHR gene. However, further studies are required to determine the functional significance of these mutations.

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