Somatic mutations of the thyroid-stimulating hormone receptor gene in feline hyperthyroidism: parallels with human hyperthyroidism

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Abstract

Hyperthyroidism is the most common endocrinopathy in cats, and is both clinically and histopathologically very similar to human toxic nodular goitre (TNG). Molecular studies on human TNG have revealed the presence of mis-sense mutations in the thyroid-stimulating hormone receptor (TSHR) gene, most frequently in exon 10. Our hypothesis was that similar mutations exist in hyperthyroid cats. Genomic DNA was extracted from 134 hyperplastic/adenomatous nodules (from 50 hyperthyroid cats), and analysed for the presence of mutations in exon 10 of the TSHR gene. 11 different mutations were detected, one silent and 10 mis-sense, of which nine were somatic mutations. 28 of the 50 cats (67/134 nodules) had at least one mis-sense mutation. The mis-sense mutations were Met-452→Thr in 17 cats (35 nodules), Ser-504→Arg

(two different mutational forms) in two cats (two nodules), Val-508→Arg in one cat (three nodules), Arg-530→Gln in one cat (two nodules), Val-557→Leu in 13 cats (36 nodules), Thr-631→Ala or Thr-631→Phe (each mutation seen in one nodule of one cat), Asp-632→Tyr in six cats (10 nodules) and Asp-632→His in one cat (one nodule). Five of these mutations have been associated previously with human hyperthyroidism. Of the 41 cats for which more than one nodule was available, 14 had nodules with different mutations. The identification of a potential genetic basis for feline hyperthyroidism is novel, increases our understanding of the pathogenesis of this significant feline disease, and confirms its similarity to TNG.

Journal of Endocrinology (2005) **186**, 523–537

Introduction

Feline hyperthyroidism (FH) is a very common endocrine condition, resulting in debilitating disease in a significant percentage of middle-aged and older cats (Holzworth *et al.* 1980, Hoenig *et al.* 1982, Peterson *et al.* 1983, Thoday & Mooney 1992, Peterson *et al.* 1994). It is analogous, clinically and pathologically, to toxic nodular goitre (TNG) in elderly humans, although in cats there is no sex predisposition (Peterson & Becker *et al.* 1983, Peter *et al.* 1985, Capen 2002). In both species, hyperthyroidism is caused by thyroid-stimulating hormone (TSH)-independent overactivity of one or more benign hyperfunctioning adenomatous thyroid nodules (Peterson *et al.* 1994). This results in high circulating concentrations of thyroxine (T₄) and tri-iodothyronine (T₃) hormones (Thoday & Mooney 1992), which cause multisystemic clinical signs including weight loss, increased appetite, tachycardia and polyphagia (Peterson *et al.* 1983, Capen 2002). In both species, thyroid carcinoma is a rare cause of hyperthyroidism (Leav *et al.* 1976, Holzworth *et al.* 1980,

Hoenig *et al.* 1982, Capen 2002, Hegedus 2004, Pacini *et al.* 2004).

The aetiopathogenesis of FH and TNG is complex and multifactorial, and is not fully elucidated. However, numerous studies have identified genetic lesions within key components of the TSH receptor (TSHR) signalling pathway in human TNG (Tonacchera *et al.* 2000, Yen *et al.* 2000, Corvilain *et al.* 2001, Kopp 2001). Most mutations have been identified in the TSHR gene, with up to 82% of cases of human TNG having identifiable TSHR mutations (Parma *et al.* 1997). These mutations are generally within exon 10 of the TSHR gene, specifically within the transmembrane domain, and a 'hot spot' for gain-of-function mutations has been identified at amino acids 619–650 (Yen *et al.* 2000, Kopp 2001).

The feline and human TSHR are very similar at both genetic and functional levels (Nguyen *et al.* 2002). However, few studies have investigated the prevalence of TSHR mutations in cats (Pearce *et al.* 1997, Nguyen *et al.* 2002, Peeters *et al.* 2002), and only one study has detected an exon 10 TSHR transmembrane mis-sense

mutation, *in vitro*, in one thyroid cell line (Nguyen *et al.* 2002). However, some studies have only looked at part of the TSHR gene, excluding areas where mutations have been reported in the human condition (Pearce *et al.* 1997, Peeters *et al.* 2002), and only a small number of samples have been investigated (Pearce *et al.* 1997, Nguyen *et al.* 2002, Peeters *et al.* 2002). More importantly, investigators have not attempted to detect mutations in individual nodules (Pearce *et al.* 1997, Peeters *et al.* 2002). Hyperplastic nodules are surrounded by apparently normal paranodular thyroid tissue (Ferguson *et al.* 1990), and therefore DNA extracted from the whole thyroid lobe will represent both diseased and normal tissue, the latter causing dilution of the diseased tissue DNA, potentially masking any mutations. In addition, different mutations have been found in different nodules taken from individual human thyroid glands (Fuhrer *et al.* 1996, Holzapfel *et al.* 1997a, Duprez *et al.* 1997a, Parma *et al.* 1997, Tonacchera *et al.* 1998a, 2000, Fuhrer *et al.* 2003), and such mutations would also be masked by extraction of DNA from whole thyroid lobes.

In this study, we investigated the prevalence of mutations in the TSHR gene in individual nodules from cats with thyroid nodular adenomatous hyperplasia and/or thyroid adenomas.

Materials and methods

Sample recruitment

Formalin-fixed thyroid lobes were obtained after therapeutic thyroidectomy from cats with FH (confirmed by elevated resting total thyroxine (3,5,3¹,5¹-tetraiodothyronine) concentrations in plasma or serum, and compatible histopathological findings), from veterinary surgeons throughout the UK. Samples were sequentially assigned a T number upon arrival in the laboratory. Bilateral lobes were designated A and B. Individual nodules were identified by gross examination of each affected lobe, and numbered sequentially. Each thyroid lobe was cut in half so that all identified nodules were bisected. One half of the lobe was submitted for histopathological evaluation, to identify the type of lesion and its compatibility with FH (Capen 2002). The bisected nodules in the other half of each thyroid lobe were dissected individually and submitted for DNA extraction. Where available, concurrent blood samples were used for extraction of control DNA, and an additional 15 blood samples were obtained from the Clinical Pathology Service within the Faculty of Veterinary Science, University of Liverpool, from cats being treated for diseases other than FH.

DNA extraction

Prior to extraction, individual tissue nodules were washed in two changes of 70% ethanol for 30 min each, to remove

formalin from the tissue. Genomic DNA was extracted separately from each dissected nodule and blood sample, according to the manufacturer's instructions (DNeasy Tissue Extraction Kit; Qiagen, Venlo, the Netherlands), with the exception of proteinase K digestion, where the samples were digested for 2 h at 60 °C followed by an overnight incubation of 42 °C, both incubations with constant agitation. Both tissue and blood DNA samples were eluted in molecular-grade water (VWR International, Poole, Dorset, UK) in 100 and 400 µl volumes respectively. Samples were extracted in batches and each batch included a DNA-negative control-extraction sample, where no tissue or blood was present. The quality of the extracted DNA was assessed by agarose gel electrophoresis.

Primers

Feline-specific oligonucleotide primers were designed within exon 10 of the feline TSHR gene to yield a 936 bp PCR product covering codons 386–698 encompassing the transmembrane domain (MWG Biotech, Ebersberg, Germany). These primers were designed based on the available published genomic data (cat (GenBank accession no. AF218264); human (NM_000369); dog (X17146); pig (NM_214297); cow (NM_174206); sheep (Y13434); rat (NM_012888); mouse (NM_011648); African green monkey (AY1683990); Rhesus monkey (AY169400)) (Fig. 1). Primers were: FeTSHRF, 5'-ACTACACTGTG TGTGGAGGCAA-3', and FeTSHRR, 5'-TGCCAAA CTTGCTGAGCAGGATA-3'. To ensure the feline specificity of these TSHR primers, they were tested on human DNA obtained from blood, and under the same conditions as below, no amplification of the TSHR gene resulted (data not presented).

PCR and sequence analysis

PCR reactions of 50 µl total volume were performed using the Qiagen Hot Start Kit (Qiagen). Each reaction contained 1 µl extracted DNA, 5 µl 10 × PCR buffer, 0.8 mM dNTPs (Abgene, Epsom, Surrey, UK), 200 nM forward/reverse primer (MWG Biotech) and 1.25 units *Taq* polymerase, and the remaining volume was made up with molecular-grade water. Thermal cycle conditions for TSHR amplification were an initial denaturation of 95 °C for 15 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, with a final elongation step of 72 °C for 10 min. Each PCR included a known positive control, a water negative control and the corresponding DNA-extraction-batch negative control. Amplified products were purified using the Qiagen Purification Kit (Qiagen) according to the manufacturer's instructions, eluted in 30 µl molecular-grade water and sequenced using the PCR primers (Dundee Sequencing Service, University of Dundee, Dundee, Scotland, UK and Lark

(A) Consensus	HYYVFEEQE	DEIIGFGQEL	KNPQEETLQA	FDSHY	DYTVG	G.NE	DMVCTP
Cat	-	...l.....				g.....	
Human					i.....	ds.....	
Dog		...l.....				g.....	
Cow		q.				gs.....	
Sheep	d..			..n.....		gs.e.....	
Pig						gs.....	
Mouse		..vv.....		..e.....		d.....	
Rat						d.....	
AF Green Mon						d.....	
Rhesus Monk						d.....	

Consensus	KSDEFNP	CD	IMGYKFLRIV	VWVFSLLALL	GNVSVLLILL	TSHYKLTVPR
Cat					i.....	
Human					iv.....	n..
Dog					iv.....	
Cow					v.....	
Sheep					v.....	
Pig			..r.....		v.....	
Mouse			..r.....		i.....	
Rat				..pm.....	..fv.....	
AF Green Mon						n..
Rhesus Monk						n..

S1

Consensus	FLMCNLA	FAD	FCMGMYLLLI	ASVDLYTHSE	YYNHAI	DWQT	GPGCNTAGFF
Cat							a.....
Human							
Dog							
Cow			...l.....	q.			
Sheep			...l.....	q.			
Pig				q.			
Mouse			...v.....				
Rat			...v.....	t.			
AF Green Mon							
Rhesus Monk							

S2

Consensus	TVFAS	ELSVY	TLTVITLERW	YAITFAMRLD	RKIRLRHAYA	IMVGGWCCF
Cat					..m.....	
Human					c.	
Dog						
Cow				h.....	...w...v	..l.....
Sheep				h.	...w...v	..l.....
Pig						..a.....
Mouse					t	..a...s.
Rat					t	..a...s.
AF Green Mon					c.	
Rhesus Monk					c.	

S3ab

S4

S5

(B)	Consensus	LLALLPLVGI	SSYAKVSICL	PMDTETPLAL	AYIILVLLLN	IVAFVIVCSC
	Cat					...i....
	Human				..vf..t..	c.
	Dog					...i....
	Cow					.i..i...a.
	Sheep					.i..i...a.
	Pig					...t....
	Mouse	m..		...d....	...v....	v...v....
	Rat	m..		...d....	...a....	v...v....
	AF Green Mon				..vf..t..	c.
	Rhesus Monk				..vf..t..	c.
		S6				
	Consensus	YVKIYITVRN	PQYNPGDKDT	KIAKRMAVLI	FTDFMCMAPI	SFYALSALMN
	Cat		...t....			
	Human	h.....			...i....	il.
	Dog					
	Cow		.h.....	r.....		
	Sheep		.h.....	r.....		
	Pig					
	Mouse		...r....			
	Rat		...r....			
	Af Green Mon					il.
	Rhesus Monk				...i....	il.
				S7ab/S8ab		
	Consensus	KPLITVTNSK	ILLVLFYPLN	SCANPFLYAI	FTKAFQRDV	F ILLSKFGI CK
	Cat				...t....	
	Human	s...				
	Dog					
	Cow					m.....
	Sheep					m.....
	Pig					...f...
	Mouse					
	Rat	g v.				...l...
	AF Green Mon	s...				
	Rhesus Monk	s...				

Figure 1 Consensus sequence of the transmembrane domain of exon 10 of the TSHR gene showing the position of the 11 mutations/polymorphisms, S1–S8b (10 mis-sense, one silent) identified in thyroid adenomas and adenomatous hyperplastic nodules from hyperthyroid cats. Open boxes, primer-binding sites; dots show that a given amino acid is the same as the consensus sequence; a dash in the first line of the cat sequence indicates that Glu-360 in the human TSH receptor gene is not present in the equivalent published feline sequence. Therefore, beyond this point, the analogous human codon number is one greater than that of the feline TSHR. GenBank accession numbers: cat (AF218264); human (NM 000369); dog (X17146); pig (NM 214297); cow (NM 174206); sheep (Y13434); rat (NM 012888); mouse (NM 011648); African green monkey (AY1683990); Rhesus monkey (AY169400).

Technologies Sequencing Service, Lark Technologies, Takeley, Essex, UK). Forward and reverse sequences were aligned (MatchTool Navigator; Applied Biosystems), to produce a consensus sequence for each sample. Mutations were defined based on a comparison of this consensus sequence with the published feline TSHR gene sequence (AF218264) using programmes from the Wisconsin package (Genetics Computer Group; Devereaux *et al.* 1984). All codons were numbered according to the published feline sequence. The feline sequence has a deletion equivalent to codon 360 (glutamic acid) in the human TSHR. Therefore, beyond this point, the analogous human codon number is one greater than that of the feline TSHR.

Results

Sample data

Thyroid lobes were received from a total of 128 cats. Of these, 74 were excluded because the extracted DNA was of poor quality, due to inadequate or prolonged formalin fixation, and four samples were excluded because they were not diseased thyroid tissue (two lymph nodes, one blood clot and one normal thyroid lobe). From the remaining 50 cats, a total of 134 nodules and 19 blood samples were included. Tissue from one thyroid lobe was included from 48 cats, and from both lobes for the remaining two. Three of the 50 cats had no

distinguishable nodules in the submitted thyroid tissue, so DNA was extracted from the whole lobe.

The 50 cats comprised 44 domestic short-hair (88%), three domestic long-hair, one Siamese, one British Blue and 1 unknown breed. 25 (50%) were male and 25 (50%) female. The mean age for the cats was 13 years (range 7–17·5 years). Histopathology identified thyroid adenomas in 49 cases (98%), and in eight (16%) of these nodular adenomatous hyperplasia was also observed. A single adenoma was diagnosed in 32 cases (64%), but adenomas were often lobulated, so that more nodules were identified grossly than adenomas were identified histologically. In lobes with more than one histologically confirmed adenoma, up to four individual tumours were identified. In one case, only nodular adenomatous hyperplasia was detected. Clinical details and histopathological diagnoses for the 50 cats in this study are summarized in Table 1.

Genetic analysis of the TSHR gene

Direct sequencing of the transmembrane domain of exon 10 of the TSHR gene produced 855 bp of double-stranded consensus sequence, spanning codons 399–684. A consensus was identified only where both forward and reverse sequences agreed. When the consensus sequences were compared with the published sequence, a total of 168 polymorphisms were identified, affecting eight codons. 166 (99%) were seen in both forward and reverse sequences. The remaining two were only seen in one sequence direction, and not the other. This was consistent over several repeats. These two mutations have been included in the results (see mutations S3b and S7a below).

In order to determine the reproducibility of the sequencing, DNA from six blood samples and 42 nodules from 20 cats were selected randomly, re-amplified and sequenced. In all cases, the same sequence, including heterozygous polymorphisms, was identified in these repeat consensus sequences as was detected in the first PCR/sequencing reaction (data not shown).

When the consensus sequences were compared with the published sequence, a total of 10 mis-sense mutations and one silent mutation were observed (Figs 2 and 3). Of the 134 nodules analysed, 66 had the same amino acid sequence as the published sequence, 47 had one mis-sense mutation, 19 had two mis-sense mutations and two had three mis-sense mutations. The frequencies with which the mutations were identified are summarized in Table 2.

Of the 41 cats for which more than one nodule was available, 14 had nodules with different mutations (Table 3). In contrast, in the remaining 27, all nodules from the same cats had the same sequence. 16 of these had either the published sequence or S1 polymorphism (see Figs 2 and 3 for details of mutations), four the S6 mutation either alone or with the S1 mutation, and the other seven had one or more of the other mis-sense mutations.

Somatic mutations

Nine somatic mutations were identified at six codon locations (Figs 2 and 3): Met-452→Thr (S2), Ser-504→Arg (two mutational forms, S3a and S3b), Val-508→Arg (S4), Arg-530→Gln (S5), Thr-631→Ala (S7a), Thr-631→Phe (S7b), Asp-632→Tyr (S8a) and Asp-632→His (S8b). All were heterozygous.

35 of 134 nodules (26%) in 17 of the 50 cats (34%) had a mutation in the second transmembrane domain, resulting in Met-452→Thr (S2) (Figs 2 and 3). Seven of these cats harboured this S2 mutation in all nodules (17 nodules in total). This was the most common mutation.

Two different mutations were located at codon 504 (S3a/b), both of which resulted in Ser-504→Arg. Each of these mutations was seen in one nodule from one cat, and S3b was only strongly visible in one sequencing direction. Mutation S4 (Val-508→Arg) involved two altered nucleotides and was found in all three nodules taken from one cat only. Mutation S5 (Arg-530→Gln), in the region of the second intracellular loop, was found in only two out of 134 nodules, both from the same cat. Two different mutations (S7a and S7b; Thr-631→Ala and Thr-631→Phe) were located in the sixth transmembrane domain at codon 631, and each was found in only one nodule from a single cat, and S7a was only strongly visible in one sequencing direction. The final mis-sense mutation detected was also found in two different forms (S8a/S8b) in codon 632. An aspartic acid residue was replaced with either a tyrosine (S8a) or a histidine residue (S8b). 10 out of 134 nodules (7·5%) in six out of 50 cats (12%) harboured the Asp-632→Tyr (S8a) mutation, and two cats had this mutation in all their nodules (four nodules in total). Only one nodule from one cat showed the S8b substitution.

Matched blood samples were available for seven cats whose thyroids harboured the S2 mutation, one cat whose thyroid harboured the S3b mutation, one with the S4 mutation and one with the S8a mutation. No matched blood samples were available for S3a, S5, S7a, S7b or S8b. None of these mutations (S2, S3a, S3b, S4, S5, S7a, S7b, S8a and S8b) were present in the blood samples from the hyperthyroid cats, nor in the 15 blood samples from cats not being treated for hyperthyroidism.

Silent mutations/natural polymorphisms

99 of the 134 hyperplastic nodules (74%) from 37 of the 50 cats (74%) harboured a silent substitution (S1) compared with published sequence (GAT/GAC, both aspartic acid) at codon 402, in the extracellular domain. This was heterozygous in 58 out of 99 nodules from 22 cats, and homozygous in 41 out of 99 nodules in 15 cats. All nodules from the 37 cats with this apparent silent mutation harboured the same sequence (Fig. 2). In addition, 15 of 19 control blood samples showed the

Table 1 Clinical details of 50 hyperthyroid cats included in this study. Numbers of adenomas identified histopathologically are given in parentheses. Gross nodule numbers identified exceed the numbers of adenomas due to the lobulated nature of adenomas and, where present, nodular hyperplasia. The cases for which total T₄ levels were unavailable included five cases where the cat had palpable goitre and compatible clinical signs, but the client had refused T₄ measurement on financial grounds (T18, T33, T39, T77, T109); one case which had had recurrence of clinical signs after previous unilateral thyroidectomy where the client had refused repeat T₄ measurement (T85); and one case which had transferred from another veterinary practice already on medical management with NCZ (T27).

Thyroid sample number	Blood sample	Thyroid lobe involvement	Total number of nodules identified	Breed	Sex	Age	Pre-treatment total T ₄ levels (nM)	Current thyroid medication	Histopathological diagnosis
T2	No	Unilateral	5	DSH	M	12	169	TMZ	Lobulated micro and macrofollicular adenomas (4)
T5	No	Unilateral	4	DLH	MN	10	296	NCZ	Lobulated microfollicular adenoma
T8	No	Unilateral	NDN	DSH	FN	14	135	CZ	Microfollicular adenoma
T10	No	Unilateral	3	DSH	MN	17	65	TMZ	Multinodular adenomatous hyperplasia
T12	No	Unilateral	2	DSH	F	12	90.1	UNK	Lobulated macrofollicular adenoma
T15	Yes	Unilateral	2	UNK	F	17.5	218	UNK	Lobulated microfollicular adenoma
T16	Yes	Unilateral	3	DSH	FN	15	156	NCZ	Lobulated microfollicular adenoma
T18	Yes	Unilateral	2	DSH	FN	10+	UNK	None	Microfollicular adenoma, multinodular adenomatous hyperplasia
T19	No	Unilateral	3	DSH	M	14.5	192	TMZ	Lobulated microfollicular adenoma
T20	Yes	Unilateral	2	DSH	F	10+	175	UNK	Lobulated microfollicular adenomas (2)
T21	No	Unilateral	NDN	DSH	M	12	81	TMZ	Microfollicular adenoma
T22	Yes	Unilateral	3	DSH	FN	12.5	148	NCZ	Lobular microfollicular adenoma
T23	No	Unilateral	3	DSH	MN	13	194	TMZ	Nodular adenomatous hyperplasia, microfollicular adenoma
T25	Yes	Unilateral	1	DSH	MN	12	120	TMZ	Microfollicular adenoma
T27	No	Unilateral	2	DSH	MN	10	UNK	NCZ	Microfollicular adenoma
T33	Yes	Unilateral	2	DSH	F	10	UNK	NCZ	Lobulated microfollicular adenoma/nodular adenomatous hyperplasia
T35	Yes	Unilateral	3	DSH	F	UNK	139	NCZ	Lobulated microfollicular adenoma/nodular adenomatous hyperplasia
T37	Yes	Unilateral	3	DSH	MN	7	147	NCZ	Lobulated microfollicular adenoma
T39	No	Unilateral	1	DSH	M	13	UNK	TMZ	Microfollicular adenoma
T41	Yes	Bilateral	3A 1B	DSH	MN	14	205	NCZ	Lobulated microfollicular adenoma in both lobes
T44	No	Bilateral	3A 1B	DSH	F	13	240	UNK	A: Lobulated microfollicular adenomas (2), nodular adenomatous hyperplasia B: Lobulated microfollicular adenoma, nodular adenomatous hyperplasia
T45	No	Unilateral	1	DSH	FN	13	98	None	Microfollicular adenoma
T46	No	Unilateral	2	DSH	M	UNK	74.5	None	Microfollicular adenoma
T47	No	Unilateral	5	DSH	FN	12	90	UNK	Microfollicular adenomas (3), nodular adenomatous hyperplasia
T48	Yes	Unilateral	2	DSH	MN	11.5	44.4	None	Microfollicular adenoma, papillary adenoma
T52	No	Unilateral	NDN	DSH	MN	13	224	NCZ	Macrofollicular adenoma
T57	No	Unilateral	3	DSH	FN	17	142	TMZ	Lobulated microfollicular adenomas (2)
T58	No	Unilateral	1	DSH	F	12	145	None	Lobulated microfollicular adenoma
T60	No	Unilateral	2	DSH	FN	13	177	TMZ	Lobulated microfollicular adenomas (2)
T61	No	Unilateral	2	DSH	MN	14	UNK	UNK	Lobulated microfollicular follicular adenoma, nodular adenomatous hyperplasia
T62	Yes	Unilateral	2	Siamese	MN	14	84.8	NCZ	Microfollicular adenomas (2)

Table 1 Continued

Thyroid sample number	Blood sample	Thyroid lobe involvement	Total number of nodules identified	Breed	Sex	Age	Pre-treatment total T ₄ levels (nM)	Current thyroid medication	Histopathological diagnosis
T64	Yes	Unilateral	4	DSH	FN	12	>300	NCZ	Microfollicular adenomas (3)
T66	No	Unilateral	2	DSH	MN	12	202	TMZ	Lobulated microfollicular adenoma
T68	Yes	Unilateral	3	DSH	M	16	68.1	TMZ	Lobulated microfollicular adenoma (2)
T70	Yes	Unilateral	1	DSH	FN	13.5	82	NCZ	Macrofollicular adenoma
T71	Yes	Unilateral	2	DLH	F	12.5	209	NCZ	Lobulated microfollicular adenoma
T74	No	Unilateral	3	DSH	MN	9.5	245	TMZ	Microfollicular adenomas (3)
T77	Yes	Unilateral	2	DSH	FN	15.5	UNK	None	Lobulated microfollicular adenoma
T81	No	Unilateral	4	DSH	FN	17	140	TMZ+NCZ	Lobulated microfollicular adenoma
T85	No	Unilateral	4	DLH	FN	16	UNK	UNK	Lobulated microfollicular adenomas (4)
T86	No	Unilateral	2	DSH	FN	11	250	NCZ	Microfollicular adenomas (2)
T87	Yes	Unilateral	2	DSH	MN	17	136	TMZ	Micro- and macrofollicular adenomas (2)
T90	Yes	Unilateral	7	DSH	FN	13.5	111	NCZ	Lobulated microfollicular adenoma
T91	No	Unilateral	2	British blue	M	13	98.7	NCZ	Microfollicular adenomas (2)
T92	No	Unilateral	2	DSH	M	16	135	NCZ	Microfollicular adenomas (2)
T94	No	Unilateral	4	DSH	M	13	151	NCZ	Microfollicular, partly cystic adenoma, nodular adenomatous hyperplasia
T107	No	Unilateral	4	DSH	FN	8	262	TMZ	Lobulated microfollicular adenoma
T109	No	Unilateral	7	DSH	M	15	UNK	UNK	Lobulated microfollicular adenoma
T112	No	Unilateral	2	DSH	MN	14	148	NCZ	Microfollicular adenoma, cystic adenoma (2)
T119	No	Unilateral	1	DSH	FN	9.5	97	None	Microfollicular adenoma

NCZ, neomecrazole; TMZ, thiamazole; CZ, carbimazole; NDN, no distinct nodules; DSH, domestic short hair; DLH, domestic long hair; MN, male neutered; FN, female neutered; UNK, unknown; A, lobe A; B, lobe B; T₄, 3,5,3',5'-tetraiodothyronine. T₄ reference interval, 19–60 nM.

Figure 2 TSHR sequences showing 11 different mutations/polymorphisms detected in feline thyroid adenomas/adenomatous hyperplastic nodules, compared with the corresponding 'wild-type' sequence. Eight mis-sense mutations and one silent mutation were detected in both the forward and reverse sequences. Mutations S3b and S7a were strongly visible in only one sequencing direction. Natural polymorphisms S1 and S6 were also detected as homozygous mutations in some samples. Code for nucleotide anomalies: Y, C/T; K, T/G; S, G/C; R, G/A; W, A/T; M, A/C; *, double mutation detected in one codon. Codon numbers used refer to feline sequence, with the equivalent human codon locus (HCL) in brackets. This figure appears in colour at <http://joe.endocrinology-journals.org/content/vol1186/issue3/>.

Figure 3 Schematic representation of the transmembrane domain of exon 10 of the feline TSHR showing the localization of mutations/polymorphisms found in this study (S1–S8b): affected codons are shown in black with white lettering. Dark-grey shading represents the site of primer binding. This illustration is derived from the equivalent human TSHR structure (Kopp 2001): the amino acid sequence presented is that of the feline TSHR. For all samples, 855 bp of double-stranded DNA sequence were obtained spanning amino acids 399–684.

heterozygous/homozygous silent mutation S1, and all of these cats also had the same mutation in all thyroid nodules (41 out of 41). A similar polymorphism was detected in 12 of the 15 blood samples from control cats. This suggests that this is a normally polymorphic site in the feline TSHR, which is unlikely to be of functional significance.

A mutation in codon 557 of the fourth transmembrane domain (S6; Val-557→Leu) was observed in 36 out of 134 nodules (27%), in 13 out of 50 cats (26%). However, this mutation was seen in all nodules taken from these 13 affected cats. In 32 nodules, from 12 cats, this mis-sense mutation was heterozygous. The remaining four nodules, three from one lobe and one from the contralateral lobe in the same cat, had a homozygous mutation. Interestingly, five of the 19 blood samples also harboured Val-557→Leu (four heterozygous, one homozygous) and all nodules from the accompanying thyroid lobes from these five cats had this sequence in all nodules (17 out of 17). Blood samples

were not available for eight cats where this mutation was detected in thyroid nodules, but all 19 nodules from these cats had the same mutation. In addition, two of the 15 blood samples from non-hyperthyroid cats revealed the same mutation. These findings suggest that this variation from the published sequence represents natural polymorphism.

Discussion

In this study, we have identified somatic mutations in the transmembrane region of exon 10 of the TSHR gene in thyroid adenomas and nodules of adenomatous hyperplasia from cats diagnosed with FH. To date, this is the largest number of samples recruited from hyperthyroid cats and analysed for TSHR genetic aberrations, and the first study specifically examining transformed/hypertrophic thyroid nodules.

Table 2 Summary of the frequency of somatic mutations detected in 134 nodules from 50 hyperthyroid cats, in blood samples from 19 of these hyperthyroid cats and in control blood samples from 15 cats not being treated for hyperthyroidism. NDN (no distinct nodules) in Table 1 counted as one nodule

	S1*	S2	S3a	S3b	S4	S5	S6*	S7a	S7b	S8a	S8b
	Asp-402→Asp (HCL-403)	Met-452→Thr (HCL-453)	Ser-504→Arg (HCL-505)	Ser-504→Arg (HCL-505)	Val-508→Arg (HCL-509)	Arg-530→Gin (HCL-531)	Val-557→Leu (HCL-558)	Thr-631→Ala (HCL-632)	Thr-631→Phe (HCL-632)	Asp-632→Tyr (HCL-633)	Asp-632→His (HCL-633)
No. of Nodules	99/134 (74%)	35/134 (26%)	1/134 (0.7%)	1/134 (0.7%)	3/134 (2.2%)	2/134 (1.5%)	36/134 (27%)	1/134 (0.7%)	1/134 (0.7%)	10/134 (7.5%)	1/134 (0.7%)
No. of Cats	37/50 (74%)	17/50 (34%)	1/50 (2%)	1/50 (2%)	1/50 (2%)	1/50 (2%)	13/50† (26%)	1/50 (2%)	1/50 (2%)	6/50 (12%)	1/50 (2%)
Blood samples from HC	15/19 (79%)	0/19 (0%)	0/19 (0%)	0/19 (0%)	0/19 (0%)	0/19 (0%)	5/19† (26%)	0/19 (0%)	0/19 (0%)	0/19 (0%)	0/19 (0%)
Blood samples from NHC	12/15 (80%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	2/15 (13%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	0/15 (0%)

HC, hyperthyroid cats; NHC, non-hyperthyroid cats; HCL, Human codon locus equivalent codon number. *Both homozygous/heterozygous mutations included in calculations. †Of the 13 cats with this S6 mutation, only five had an accompanying blood sample, all of which showed the same mutation.

Table 3 Mutations (shown in bold) identified in 14 hyperthyroid cats from which individual thyroid nodules contained different polymorphisms

Cat	Nodule	S1 mutation Asp-402→Asp (HCL-403)	S2 mutation Met-452→Thr (HCL-453)	S3a/b mutation Ser-504→Arg (HCL-505)	S4 mutation Val-508→Arg (HCL-509)	S5 mutation Arg-530→Gln (HCL-531)	S6 mutation Val-557→Leu (HCL-558)	S7a/b mutation Thr-631→Ala Thr-631→Phe (HCL-632)	S8a/b mutation Asp-632→Tyr Asp-632→His (HCL-633)
T2	1, 4 and 5 2 3	GAT GAT GAT	ATG/ACG ATG ATG	AGT AGT AGT	GTG GTG GTG	CGG CGG CGG	GTG GTG GTG	ACT ACT ACT	GAC GAC/TAC GAC
T35	1 and 2 3	GAT/GAC GAT/GAC	ATG ATG/ACG	AGT AGT	GTG GTG	CGG CGG	GTG/TTG GTG/TTG	ACT ACT	GAC GAC
T44	A1, A2, A3 B1	GAT GAT	ATG/ACG ATG	AGT AGT	GTG GTG	CGG CGG	TTG TTG	ACT ACT	GAC GAC
T48	1 2	GAT GAT	ATG ATG/ACG	AGT AGT	GTG GTG	CGG CGG	GTG GTG	ACT ACT	GAC GAC
T57	1 2 and 3	GAT/GAC GAT/GAC	ATG ATG/ACG	AGT AGT	GTG GTG	CGG CGG	GTG GTG	ACT/GCT* ACT	GAC GAC
T62	1 2	GAT/GAC GAT/GAC	ATG/ACG ATG	AGT AGT	GTG GTG	CGG CGG	GTG GTG	ACT ACT	GAC GAC
T64	1 2 and 3 4	GAT/GAC GAT/GAC GAT/GAC	ATG ATG/ACG ATG	AGT AGT AGT/CGT*	GTG GTG GTG	CGG CGG CGG	GTG GTG GTG	ACT ACT ACT	GAC GAC GAC
T74	1 2 3	GAC GAC GAC	ATG/ACG ATG/ACG ATG	AGT AGT AGT	GTG GTG GTG	CGG CGG CGG	GTG GTG GTG	ACT ACT ACT	GAC GAC GAC
T81	1 and 3 2 and 4	GAC GAC	ATG ATG	AGT AGT	GTG GTG	CGG CGG	GTG GTG	ACT ACT	GAC GAC/TAC
T85	1 and 3 2 and 4	GAT/GAC GAT/GAC	ATG ATG/ACG	AGT AGT	GTG GTG	CGG CGG	GTG GTG	ACT ACT	GAC/TAC GAC
T86	1 2	GAT GAT	ATG ATG	AGT AGT	GTG GTG	CGG CGG	GTG/TTG GTG/TTG	ACT ACT/TTT	GAC/TAC GAC
T87	1 2	GAT/GAC GAT/GAC	ATG ATG/ACG	AGT AGT	GTG GTG	CGG CGG	GTG GTG	ACT ACT	GAC GAC
T91	1 2	GAT GAT	ATG ATG	AGT AGT/AGG	GTG GTG	CGG CGG	GTG GTG	ACT ACT	GAC GAC
T94	1,2,3 and 4 5	GAT GAT	ATG ATG	AGT AGT	GTG GTG	CGG CGG	GTG GTG	ACT ACT	GAC GAC/CAC

HCL, human codon locus equivalent codon number. A, lobe A; B, lobe B. *, Mutation only detected in one sequencing direction.

A total of 11 mutations were detected in exon 10 of the TSHR gene (one silent, 10 mis-sense). Five of the 10 mis-sense mutations have previously been identified in human hyperthyroidism (Kosugi *et al.* 1994, Porcellini *et al.* 1994, Van Sande *et al.* 1995, De Roux *et al.* 1996, Russo *et al.* 1996, 1997, Spambalg *et al.* 1996, Tonacchera *et al.* 1996, 2000, Duprez *et al.* 1997a, Parma *et al.* 1997, Lavard *et al.* 1999, Mircescu *et al.* 2000, Trulzsch *et al.* 2001, Vanvooren *et al.* 2002, Fuhrer *et al.* 2003, Georgopoulos *et al.* 2003).

The most common somatic mutation detected was S2 (Met-452→Thr), identified in 34% of cats. This is equivalent to the human Met-453→Thr mutation, which has been observed as both a germline and somatic (usually heterozygous) mutation in sporadic human hyperthyroidism, and in hyperplastic nodules and thyroid carcinoma (De Roux *et al.* 1996, Duprez *et al.* 1997a, Parma *et al.* 1997, Lavard *et al.* 1999, Mircescu *et al.* 2000, Trulzsch *et al.* 2001, Vanvooren *et al.* 2002, Georgopoulos *et al.* 2003). This mutation has not previously been reported in cats.

The mutation Ser-505→Arg has been identified as a heterozygous germline mutation in familial human hyperthyroidism (Van Sande *et al.* 1995, Tonacchera *et al.* 1996). This is equivalent to S3a/b (Ser-504→Arg), which has never been reported in feline studies. In the current study, cats with this mutation became hyperthyroid in middle age, which would suggest acquired rather than congenital disease. Unfortunately, there were no concurrent blood samples available for the cats bearing this anomaly; however, this mutation was not detected in the blood of non-hyperthyroid cats. Further work is required to determine the true nature of this mutation. Another mutation, Ser-505→Asn, has been detected as a sporadic heterozygous germline mutation in four previous human studies (Schwab *et al.* 1996, Holzapfel *et al.* 1997b, Fuhrer *et al.* 1999, Wonerow *et al.* 2000), and also reported as a somatic heterozygous mutation in human hyperthyroidism (Trulzsch *et al.* 2001).

Mutations S4 and S5 have not been reported previously in either human or feline hyperthyroidism. The number of cats/nodules with these mutations was very small. The S4 (Val-508→Leu) mutation was detected in all three nodules taken from one cat, which had a matching blood sample lacking this mutation, so may represent a somatic mutation. In human hyperthyroidism, Val-509→Ala has been reported due to a heterozygous germline mutation (Duprez *et al.* 1994, Van Sande *et al.* 1995). Unfortunately, no blood sample was submitted from the single cat with the S5 (Arg-530→Gln) mutation. Neither the S4 nor the S5 mutation was detected in non-hyperthyroid cat blood. The significance of these mutations is unclear, and functional studies are required.

The S6 (Val-557→Leu) mutation/polymorphism has not been reported in human hyperthyroidism. It has, however, been identified in one of three cell lines estab-

lished from hyperthyroid cats (Nguyen *et al.* 2002); these authors also concluded this mutation probably represents a simple polymorphism, since it has been shown to have no apparent effect on function.

Two different heterozygous mutations were located at codon 631, Thr-631→Ala (S7a) and Thr-631→Phe (S7b). The equivalent mutation Thr-632→Ala has been reported in human hyperthyroidism as a heterozygous somatic mutation in thyroid carcinomas (Spambalg *et al.* 1996) and hyperthyroid nodules (Tonacchera *et al.* 2000, Trulzsch *et al.* 2001, Vanvooren *et al.* 2002). Germline mutations are not reported. Neither mutation has been reported previously in FH. In our study, each mutation was only identified in one nodule from one cat, neither of which had an accompanying blood sample. Neither mutation was present in non-hyperthyroid blood samples. The S7b mutation, Thr-631→Phe, has not been reported previously in either species.

The somatic heterozygous mutation Thr-632→Iso is common in human hyperthyroid nodules/hyperfunctioning adenomas (Kosugi *et al.* 1994, Paschke *et al.* 1994, Porcellini *et al.* 1994, Russo *et al.* 1996, Duprez *et al.* 1997a, Fuhrer *et al.* 1997, Holzapfel *et al.* 1997a, Parma *et al.* 1997, Tonacchera *et al.* 1998a, 1998b, 1999, 2000, Trulzsch *et al.* 2001), and has also been reported in thyroid carcinoma (Spambalg *et al.* 1996). This mutation also occurs as a sporadic heterozygous germline mutation (Kopp *et al.* 1997a, Biebermann *et al.* 2000). A further somatic mutation, Thr-632→Pro, has also been reported in autonomous thyroid nodules (Syrenicz *et al.* 1999). Thus the analogous feline mutations S7a and S7b may also be functionally significant, and the need for further investigation is indicated.

The remaining two mutations, Asp-632→Tyr (S8a) and Asp-632→His (S8b), have both been previously reported at human codon locus 633 in human hyperfunctioning adenomas/nodules and thyroid carcinomas as somatic, heterozygous mutations (Kosugi *et al.* 1994, Porcellini *et al.* 1994, Van Sande *et al.* 1995, Russo *et al.* 1996, 1997, Parma *et al.* 1997, Trulzsch *et al.* 2001, Fuhrer *et al.* 2003) but neither have been reported in FH. Only one cat with the S8a or S8b mutation had a concurrent blood sample: DNA from this blood sample did not harbour either mutation, and neither mutation was detected in blood from non-hyperthyroid cats. Other identified somatic heterozygous mutations in human hyperthyroid nodules/hyperfunctioning adenomas at this codon location include Asp-633→Glu (Kosugi *et al.* 1994, Porcellini *et al.* 1994, Van Sande *et al.* 1995, Fuhrer *et al.* 1997, Parma *et al.* 1997, Tonacchera *et al.* 1998b, 1999, 2000, Trulzsch *et al.* 2001) and Asp-633→Ala (Parma *et al.* 1997).

Many more TSHR mutations have been detected in human hyperthyroidism, with at least 31 somatic and 17 germline mutations reported previously (Corvilain *et al.* 2001). All the mis-sense mutations detected in this study

were found at codons that were completely conserved in the TSHR of other species (Fig. 1). In addition, of the mutations found in our study that have been reported previously, S2, S3a and S8a have been shown to enhance the constitutive activity of the TSH receptor (Kosugi *et al.* 1994, De Roux *et al.* 1996, Tonacchera *et al.* 1996, Porcellini *et al.* 1997). The activating effects of mutations analogous to S7a and S8b have not yet been determined (Russo *et al.* 1996, 1997, Spambalg *et al.* 1996, Parma *et al.* 1997, Tonacchera *et al.* 2000, Trulzsch *et al.* 2001, Vanvooren *et al.* 2002). However, in humans, other mutations in the S7a and S8b codons have been shown to be activating (Kosugi *et al.* 1994, Paschke *et al.* 1994, Porcellini *et al.* 1994, 1995, Van Sande *et al.* 1995).

Not all nodules taken from an individual animal or thyroid lobe showed the same mutations, with different mutations in different adenomas and hyperplastic nodules. A similar scenario has been found in human hyperthyroidism (Fuhrer *et al.* 1996, 2003, Duprez *et al.* 1997a, Holzapfel *et al.* 1997a, Parma *et al.* 1997, Tonacchera *et al.* 1998a, 2000). Both our study and previous human studies indicate the importance of nodule dissection from hyperplastic thyroid tissue when analysing for genetic mutations.

To our knowledge, there have only been three previously published molecular genetic studies of the TSHR gene in FH (Pearce *et al.* 1997, Nguyen *et al.* 2002, Peeters *et al.* 2002). Pearce *et al.* (1997) did not identify any TSHR mutations in seven hyperthyroid cats, between codons 480 and 640 of exon 10. This region excludes areas where many mutations have been reported in humans, and the codons affected by S1 and S2. Peeters *et al.* (2002) investigated mutations mainly in the extracellular region of the TSHR gene spanning exons 1–9, with only a small proportion of exon 10 being studied, including less than half of the transmembrane domain. They also identified the silent mutation/polymorphism S1. In addition, they identified a mutation in exon 5, Gly-139→Ala, but this was not associated with disease. In both studies, DNA was extracted from the whole thyroid lobe, so that normal DNA from paranodular thyroid tissue may have masked any mutations present (Ferguson *et al.* 1990), and this technique also may reduce the chances of detecting multiple mutations in the same thyroid lobe. Finally, Nguyen *et al.* (2002) reported the S6 mutation (Val-557→Leu) in the exon 10 transmembrane domain in one of three thyroid cell lines obtained from autonomous nodules. This mutation probably represents a simple polymorphism since it has been shown to have no apparent effect on function. Our findings support this hypothesis, as this mis-sense mutation has been found in blood samples from both hyperthyroid and non-hyperthyroid cats in our study, with all tissue from the same cat always showing the same mutation.

As the current study does not include functional analyses, we can only suggest that the mutations are a probable cause of nodular proliferation and autonomous

function. In addition, 22 cats had no detected mis-sense mutations in any nodules, and an additional four cats harboured only the S6 polymorphism thought not to be associated with the disease (Nguyen *et al.* 2002). Activating mutations may occur in exons 1–9 in these cats; however, there have been very few mutations detected in the extracellular region of the human TSHR gene (Duprez *et al.* 1997b, Kopp *et al.* 1997b, Parma *et al.* 1997, Gruters *et al.* 1998, Biebermann *et al.* 2000). Mutations may also occur in other genes involved in the signalling transduction pathway of the TSHR, and mutations have previously been found in a Gs α subunit (a protein coupled to the TSHR) gene, in both human and feline hyperthyroidism (Lyons *et al.* 1990, O'Sullivan *et al.* 1991, Du Villard *et al.* 1995, Russo *et al.* 1995, Parma *et al.* 1997, Murakami *et al.* 1999, Tonacchera *et al.* 1999, Trulzsch *et al.* 2001, Peeters *et al.* 2002, Vanvooren *et al.* 2002, Georgopoulos *et al.* 2003).

In summary, we have identified nine somatic mutations in exon 10 of the TSHR gene, affecting a total of four domains in the transmembrane region. Only one of these has previously been reported *in vitro* in cell cultures from hyperthyroid cats. Five of the somatic mutations have previously been identified in human hyperthyroidism. This study represents the first report of somatic mis-sense mutations in FH, and further emphasises the complexity of the disease and its similarity to human TNG.

Acknowledgements

We would like to thank all the veterinary surgeons who kindly sent us thyroid lobes and to the owners of the cats for consenting to donate their cats' thyroids for the study, without which this investigation would not have been possible. We would also like to thank Dundee and Lark sequencing services for their technical support and Karen Coyne for help with the sequence analysis.

Funding

This work was supported by the Petplan Charitable Trust and the University of Liverpool, Faculty of Veterinary Science. The authors declare that there is no conflict of interest that would prejudice the impartiality of this work.

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Received 5 April 2005

Accepted 20 June 2005