A dog pedigree with familial medullary thyroid cancer

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Abstract. Multiple endocrine neoplasia (MEN) is defined as concurrent neoplasia or hyperplasia in more than one endocrine gland. MEN is well known in humans and has also been reported in small animals. We report on a dog family of a mixed breed with Alaskan malamute as a major influence, where three members developed thyroid carcinomas and another dog had clinical signs mimicking the other three but without a confirmed diagnosis. The age of onset of the tumour was between 96-109 months. Clinical, biochemical and immunohistochemical examinations revealed that the affected individuals typically demonstrated symptoms including calcitonin positive thyroid cancer, hypothyroidism and chronic dermatitis. In addition, elevated serum calcium and multi-nodular adrenocortical hyperplasia were demonstrated in a single member. The diagnosis observed is similar to the familial form of medullary thyroid carcinoma (FMTC) in human. This is the first report of FMTC in dog. Up to 95% of FMTC and MEN2 is known to be caused by activating mutations in the *RET* gene. The dog *Ret* gene was analysed as a candidate in this pedigree. The complete dog *Ret* genomic sequence was predicted *in silico*. The lack of demonstratable *Ret* mutation suggests the involvement of alternative predisposing mutation in this pedigree. The unique occurrence of familial MTC makes this potentially an important model in further defining the genetic basis of MTC.

Introduction

Multiple endocrine neoplasia (MEN) is defined as concurrent neoplasia or hyperplasia in more than one endocrine gland. This is well known in humans, but has also been reported in small animals (Blackwood L, 15th ECVIM-CA Congress, Glasgow, UK, 2005). Two main types of MEN are recognised in human. MEN 1 is an autosomal dominant syndrome characterised by parathyroid hyperplasia, pituitary adenomas and endocrine pancreatic tumours. MEN2 is an autosomal dominant syndrome with a high degree of penetrance and variable clinical expressions. It exists in three clinically distinct forms: MEN2A, MEN2B and familial medullary thyroid carcinoma (FMTC) (1). MEN2A is the commonest subtype of MEN2 with a higher penetrance accounting for more than 90% of MEN2. MEN2A patients develop medullary thyroid carcinoma (MTC), pheochromocytoma and primary hyper-parathyroidism. MEN2B, the most distinct and aggressive variant, occurs in ~5% of all cases of MEN2 (2). MEN2B shares the same clinical features as MEN2A except with an earlier onset age, absence of hyperparathyroidism and it has other developmental abnormalities such as decreased upper/lower body ratio, marfanoid habitus, skeletal deformations, mucosal neuromas, ganglioneuromatosis of the intestinal tract, and myelinated corneal nerves (3-5). The third form of MEN2 is FMTC, with MTC as the only clinical feature. It is worth noting that MTC is the common feature of all these three variants of MEN2 and also occurs with a high penetrance.

MTC arises from the parafollicular or C cells of the thyroid, which have a unique ability to secrete calcitonin (CT). CT serves as the major clinical marker for the presence of MTC (6). The vast majority of MEN2 patients exhibit germ-line mutations in the *RET* (rearranged during transfection) proto-oncogene. Up to date, exons 8, 10, 11, 13, 14-16 of *RET* are hotspots for mutations identified to be associated with MEN2 syndrome in human (2).

Sporadic MTC has been previously reported in animals such as dog, red fox, bulls, rats, horse and ferret (7-12). Thyroid tumours are uncommon in the dog and account for 1-4% of all dog tumours (13). The most common thyroid tumours in dogs are of follicular origin (12). MTC is reported to correspond to 36% of the dog thyroid carcinomas and calcitonin is present in 70-100% of cases (14,15).

For the first time we report and describe the occurrence of a familial thyroid carcinoma in dog. This led to the identification of dog *Ret* gene *in silico*, investigation and screening of this family for the occurrence of *Ret* mutations.

Materials and methods

Pedigree description. The pedigree consists of a mixed breed with Alaskan malamute as a major influence (Fig. 1, Table I). The father (II:1) first presented in November 1998 with symptoms of itching and loss of luster to the hair coat. A skin biopsy showed severe para- and hyperkeratosis and chronic

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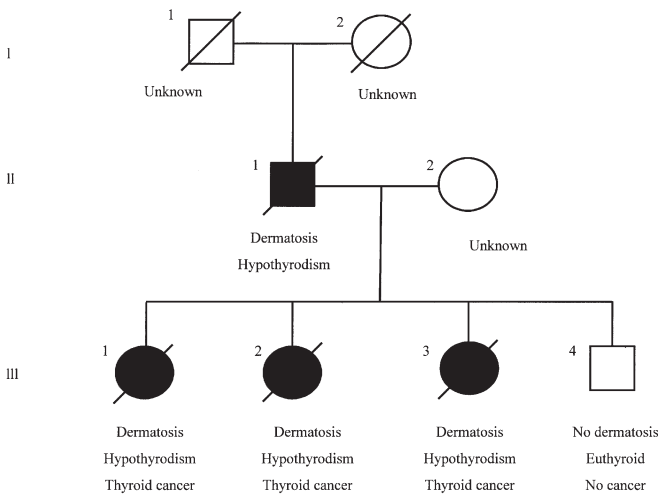

Figure 1. Pedigree of the dog family with thyroid carcinoma. Square denotes male and circle represents female. Affected individuals are highlighted black, whereas unaffected individuals remain white. A slash represents a deceased individual.

superficial pustular dermatitis, with no signs of sebaceous adenitis or pemphigus (analysed at REST associates, Cambridge, UK). Treatment with low dose prednisolon, anti-parasitic drugs and cefalexin resulted in partial remission when the prednisolon dose was kept at 0.5 mg/kg SID. After discovering a severe hypothyroidism, levothyroxine (0.03 mg/kg SID) was added to the treatment regime resulting in an almost complete remission. In July 2003 the dermatological symptoms returned. The dog also exhibited subtle, abnormal signs of reduced activity, diminished appetite, tremor and muscle atrophy on the skull. Biochemical analysis showed anaemia and elevated ALP (assumingly due to steroid treatment). The dog was treated with clindamycin. In August 2003 the condition was dramatically worsened and the dog was treated for anaemia and acute hypovolemic shock. Despite supportive care the condition was not stabilised and the dog was euthanised. The owner declined autopsy.

Four months later the first out of four dogs (III:1) from the same litter was admitted to the Small Animal University Hospital, SLU with similar clinical symptoms as the father (II:1). The dog had chronic dermatitis, a 10x5 cm mass located in the subcutis adjacent to the cervical trachea and was in a semiconscious condition. Explorative surgery revealed a tumour mass that emanated from the thyroid and infiltrated the cervical musculature and regional lymph nodes. Since the condition was not improved and haemolytic anaemia was suspected, the dog was euthanised.

A few months later, the same owner entered the clinic with two littermates (III:2 and III:3) who had developed similar symptoms as their sister. Thyroid ultrasound revealed highly vascularised masses in both dogs, which were biopsied and histopathologically diagnosed as thyroid cancer. The owner declined thyroidectomy. They also had prominent hypothyroidism. They were treated with oral levothyroxine (0.03 mg/kg SID). However after two months, the treatment was discontinued since the dogs became hyperactive that could not be controlled by dose reduction. In April 2004 the second sister (III:2) was euthanised as the disease had progressed. The youngest sister (III:3) was also euthanised in June 2005. The fourth littermate (III:4), a male, did not show any symptoms of the disease at clinical, biochemical or radiological examinations.

All dogs were thoroughly investigated with clinical status, biochemistry, imaging analyses and histopathology as detailed in Table I. Regular autopsy was performed in III:1 and III:2, and in III:3 an extended autopsy was carried out.

Blood and tissue samples. The following clinical samples were collected for the study: peripheral blood samples from III:1-4; paraffin-embedded tumour samples from primary tumours of III:1-3, lymph node metastasis of III:2; thyroid tumour biopsy of III:3; a fresh frozen tumour sample from III:2 and III:3; paraffin-embedded autopsy samples of III:1 including kidney, lung, liver and normal lymph node; and paraffin-embedded autopsy samples of III:3 from pituitary and adrenal. A normal thyroid from a healthy dog was analysed as control. All samples were obtained with informed consent and all treatments and sampling of dogs conformed to the decision of the Swedish Animal Ethics Committee (No. C 23/4).

Histopathological examination and immunohistochemistry. Paraffin-embedded sections of 4 μm thickness were prepared from the primary thyroid tumour of (III:1, III:2 and III:3), dewaxed, rehydrated and stained with haematoxylin and eosin as routine stain. Additional slides were pre-treated with citrate buffer at pH 6.0 in a microwave oven for 20 min. After rinsing, the endogenous peroxidase activity was blocked by treatment with 0.5% hydrogen peroxide for 30 min. The sections were then rinsed, incubated with blocking serum (1% bovine serum albumin) for 20 min, and incubated with the primary antibody overnight at 4˚C. They were treated with the secondary antibody diluted 1:100 (anti-mouse IgG or anti-rabbit IgG with <1% cross reactivity to dog IgG) for 30 min, followed by rinsing and incubation with avidin-biotin-peroxidase complex (Vectastain Elite, Vector, UK) for 30 min. The peroxidase reaction was developed using 3,3-diaminobenzidine for 6 min. After counterstaining with Mayer's haematoxylin, the slides were dehydrated and mounted. Paraffin sections from thyroid collected from a normal dog were analysed in parallel as references. Staining for chromogranin A (1:150, NCL-Chrom, Novocastra, UK and 1:1500 and 1:3000, Yanaihara Institute, Shizuoka, Japan), chromogranin B (1:100, PH191, Binding Site, UK), synaptophysin (1:20, RM9111-S, Lab Vision, CA, USA) and RET (1:20, BAF482, R&D Systems, MN, USA and 1:20, sc-167, Santa Cruz, CA, USA) were excluded because of negative results in the normal dog control indicating lack of cross reactivity to the dog epitopes. For calcitonin (1:1000, A576, DakoCytomation, Denmark), NSE (pre-diluted, Ventana, AZ, USA), PTH (1:3000, OP4, Novocastra), S-100 protein (1:2000, Z311, Dako), thyroglobulin (1:12000, A251, Dako) the expected staining results were obtained in normal dog thyroid.

mRNA in situ hybridisation for calcitonin. mRNA *in situ* hybridisation was performed on 4 μm paraffin-embedded sections according to the protocol supplied by GeneDetect, Limited. The sections were hybridised with 200 ng/ml of anti-

Table I. Clinical finding of the dog family.

Parameter	II:1	III:1	III:2	III:3	III:4
Diagnosis	Affected	Affected	Affected	Affected	Unaffected
Sex (M/F)[a]	M	F	F	F	M
Age of disease onset	96 months	107 months	109 months	109 months	NA
Clinical status					
Fatigue	Yes	Yes	Yes	Yes	No
Chronic dermatitis	Yes	Yes	Yes	Yes	No
Weight loss	Yes	Yes	Yes	Yes	No
Muscle atrophy	Yes	Yes	Yes	Yes	No
Swollen neck	Yes	Yes	Yes	Yes	No
Response to levothyroxine	Yes	Yes	Yes	Yes	NA
Multifocal liver fibrosis	NA	Yes	Yes	Yes	No
Hepatic necrosis	NA	Yes	Yes	Yes	No
Hematology and biochemistry					
Anemia	Yes	Yes	Yes	No	No
Elevated ALP	Yes	Yes	Yes	Yes	No
Hypothyroidism	Yes	Yes	Yes	Yes	No
Positive TgAb	Yes	Yes	Yes	Yes	No
Hypercalcemia	No	No	No	Yes	No
Imaging performed					
Neck ultrasound	NI	Yes	Yes (extended)	Yes	Yes
Trunk X-ray	NI	Yes	Yes	Yes	Yes
Histopathology					
Autopsy performed	No	Yes	Yes	Yes (extended)	NA
Skin biopsy performed	Yes	Yes	Yes	Yes	NA
Tumour size	NI	10x5 cm	6x4 cm	10x4 cm	NA
Thyroid carcinoma	NI	Yes	Yes	Yes	No
Regional metastases	NI	Yes	Yes	Yes	NA
Immunohistochemistry					
NSE	NA	Yes	Yes	Yes	NI
PTH	NA	No	No	No	NI
S100	NA	No	No	No	NI
Calcitonin	NA	Yes	Yes	Yes	NI
Thyroglobulin	NA	Yes	Yes	No	NI

[a]M, male; F, female; NA, not applicable; NI, not investigated; ALP, alkaline phosphatase; TgAb, thyroglobulin autoantibody.

sense biotin-labelled (3'-end labelling) 48-mer oligonucleotide specific for dog calcitonin (5'-TCTCTGGCATCGGCTTCG GGGCTGAAACACCTGGCAAGAAAAGGGACA-3'). All sections were hybridised without probe and with antisense non-labelled calcitonin probe as negative controls. The expected staining result was successfully performed on the normal dog thyroid, but not the tumour samples.

In silico analysis of dog Ret gene. To obtain full-length cDNA and genomic structure of the dog *Ret* gene, the human *RET* sequence (GenBank accession no. AJ243297) was used to perform a BLAST search in the HTGS database of the Dog Genome (http://www.ncbi.nlm.nih.gov/genome/seq/CfaBlast.html). The *Canis familaris* BAC clone, RP81-410M10 (working draft Sequence, 7 ordered pieces, GenBank accession no. AC123973.2) has the highest alignment match with human *RET*. The sequence of this BAC clone was aligned with the partial dog *Ret* sequence (AF364316) for confirmation using BLAST (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi). Genomic dog *Ret* sequence, intron-exon borders and the coding region were determined by the GenScan prediction program (http://genes.mit.edu/GENSCAN.html). From the coding region, dog *Ret* amino acid sequence was deduced using ORF (Open Reading Frame) Finder (http://www.ncbi.

Table II. Primers used for PCR amplification and sequencing of the dog *Ret* gene.

Dog *Ret* target	Correspond. human *RET*	Forward primer		Reverse primer		Product size (bp)
		Name	Sequence 5'-3'	Name	Sequence 5'-3'	
Ex 5	Ex 2	cfaRETex2F	CTGGGAGGTCTGCTCTGG	cfaRETex2R	GAGCCTCAGTGTGGGTGTG	403
Ex 6	Ex 3	cfaRETex3F	CTGGACTTTCCACCAGCC	cfaRETex3R	GCCATACCTGGCATACAGC	428
Ex 7	Ex 4	cfaRETex4F	TAGCTCACCCTGATTCCTGG	cfaRETex4R	ATAAATGGTGGACGGAGTGG	385
Ex 9	Ex 5	cfaRETex5F	AAGTCTCAGGCTCCAGGGTC	cfaRETex5R	CTGTTGGAAGAGTGGACTGC	330
Ex 10	Ex 6	cfaRETex6F	CAGTGGCATGGAGCAGTG	cfaRETex6R	GTTAAACAGAGTGCCCTCCC	349
Ex 11	Ex 7	cfaRETex7F	TTCAGGCTGCCCAGAAAC	cfaRETex7R	CCCGTAGTTTTACCACACCC	399
Ex 12	Ex 8	cfaRETex8F	ATGCTGGCTGGACACTGG	cfaRETex8R	ACTCAGCCACCATTACCCC	247
Ex 13	Ex 9	cfaRETex9F	CCTTCTGGCTCTGGCAGTC	cfaRETex9R	CATTGGCTGGCTACCCCTAC	248
Ex 14	Ex 10	cfaRETex10F	GGGTGGTCCTGGTTTGTC	cfaRETex10R	GGGTTTCACCTGCTTTCC	282
Ex 15	Ex 11	cfaRETex11F	GCCACAGCAGCCTTCCAG	cfaRETex11R	CCCACACCTCCCAGCTTC	429
Ex 16	Ex 12	cfaRETex12F	ACCCTCACCAACTTTCTCCC	cfaRETex12R	CTTTAGAGGGCCCTAGGCTG	304
Ex 17	Ex 13	cfaRETex13F	CCAGGGATGGTCACAAGAAG	cfaRETex13R	AAGCAGCCCAGAACCCTTAG	239
Ex 18-19	Ex 14-15	cfaRETex14/15F	TTGGTAGGATACAGAGCCTGC	cfaRETex14/15R	CTACCCACTGCAGGAACCC	605
Ex 20	Ex 16	cfaRETex16F	CCCAGGGTGTTGTCAGAGTG	cfaRETex16R	GGTCCCTGCCATTTGCTG	195
Ex 21	Ex 17	cfaRETex17F	ACATATCGTCTGCTGAGGGG	cfaRETex17R	GGAGCAGGGAGGGAATGAG	278
Ex 22	Ex 18	cfaRETex18F	TGTGATTGGGCAATCCTTG	cfaRETex18R	GAACTGAGGTGAGGCTGGAG	236
Ex 23	Ex 19	cfaRETex19F	AAGGGGCGAGTCCAGTTG	cfaRETex19R	GAGAGCTTTGGTTTCATTCAGA	320
Ex 24	Ex 20	cfaRETex20F	TTTGCCAAGGCCTTACTGTC	cfaRET3utrR	GCAGACTTTCCATTCTCGGC	298

Correspond., corresponding.

nlm.nih.gov/gorf/gorf.html). The predicted dog *Ret* amino acid was compared to its human counterpart using ClustalW (http://www.ebi.ac.uk/clustalw) sequence alignment program.

Mutational screening of Ret

Genomic DNA extraction. Ten milliliters of blood from individual lll:2, lll:3, lll:4, normal control and an unrelated dog were used for genomic DNA extraction using Wizard® Genomic Purification Kit (Promega). Genomic DNA was also extracted from tumour tissue of individual lll:2 using Qiagen DNA Mini Kit.

DNA amplification. Primers used in amplification of exons 5-7 and 9-24 corresponding to exons 2-20 of its human counterpart are described in Table II. Briefly, the PCR reaction was performed in GeneAmp® PCR System 9700 (PE, Applied Biosystems, Foster City, CA) employing 50 ng genomic DNA in a 50-μl volume containing 1 U AmpliTaq Gold Polymerase (Applied Biosystems), 200 μM dNTP, 2.5 μM of $MgCl_2$ and 25 pmol of each specific primer (Proligo, France). Cycling conditions to generate PCR products included an initial phase of 10 min at 96˚C, followed by 35 cycles of 30 sec at 96˚C, 45 sec at 65˚C and 1 min at 72˚C and a final extension step of 10 min at 72˚C.

SSCP and sequencing analysis. The presence or absence of variation of each amplicon was assessed by single strand conformation polymorphism (SSCP) analysis using GeneGel SSCP Starter Kit (Amersham Biosciences). The nucleotide sequences of amplified products that show an abnormal electrophoretic mobility on SSCP analysis and those amplicons that were reported as mutation hot-spots in human MEN2 were directly sequenced using an automated system employing BigDye® Terminator v3.1 (Applied Biosystems) and analysed on an ABI3700 sequencer (PE, Applied Biosystems).

Results

Clinical presentations. Four affected and one clinically unaffected members of the pedigree were studied. The chronic hyperplastic dermatosis presented as sparse hair coat with alopecia (bilateral, symmetric and non-pruritic), mainly affecting the flank, neck, posterior thighs and tail (rat-like tail) (illustrated for lll:3 in Fig. 2A). From the histopathological examination of the skin biopsy, the hair coat was found to have hyperpigmentation, hypertrichosis, hyperkeratosis, acanthosis and distortion with large sebaceous and dilated sweat glands. The thickened and puffy skin due to oedema produced exaggerated skin folds on forehead and neck. This caused anxiety with discomfort, hyperactivity and change in behaviour. Slow hair growth with few hair follicles in the anagen phase, slow wound healing and easy bruising were also observed. In all the affected dogs, the chronic dermatosis included alopecia, hyperpigmentation, pyodermia and itching.

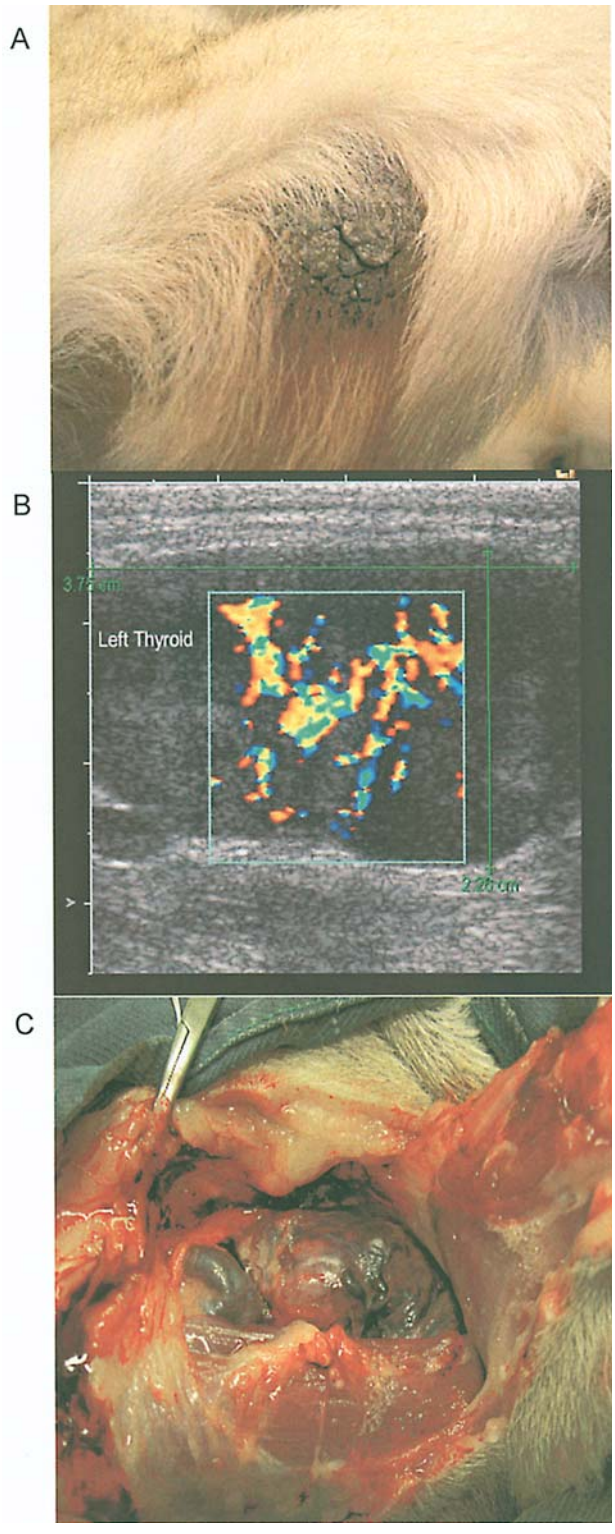

Figure 2. (A), Presentation of skin dermatosis in dog lll:3. The hair coat was sparse in a bilateral and symmetrical pattern with alopecia. (B), Color doppler ultrasound imaging of dog lll:3 showing the intensive vascularisation of the entire thyroid cancer. (C), Thyroid tumour *in situ* of dog lll:3. Note the large, aberrant vessels around the tumour.

Symptoms of fatigue, weight loss, muscle atrophy, hypothyroidism and neck oedema were also observed in individuals ll:1, lll:1, lll:2 and lll:3. Biochemically, these dogs were found to have severe anaemia, hypothyroidism with low free and total serum T4, elevated TSH and positive thyroglobulin autoantibody (TgAb). High serum calcium was noted in one

Table III. Genomic structure of the dog *Ret* gene corresponding to the human cDNA (AJ243297).

	Sequence of Ret Splice junctions	Dog *Ret* Size (bp)	Human *RET* Size (bp)	Correspond. to human *RET* exon/ intron no.
Ex 1	GCAAGgtaaaacagc	[a]143+UTR	253	NC
Int 1	ccctgccccagCCCTC	2898	23127	NC
Ex 2	GTGCTgtgcgtggct	122		NC
Int 2	gtgtcttcagCTGCC	449		NC
Ex 3	AGGAGgtcagccctg	98		NC
Int 3	tccttcccagTCCAG	556		NC
Ex 4	AGCAGgtggggccaa	25		NC
Int 4	ctttctgcagCTCCA	1948		NC
Ex 5	CCGCAgtaagggagc	267	264	Ex 2
Int 5	ctctgtctacagATGGC	798	1619	Int 2
Ex 6	AGAAGgtgagtgctg	288	288	Ex 3
Int 6	tggtgtgtagGTGAG	2158	2322	Int 3
Ex 7	AGGAGgtgcctgtct	245	242	Ex 4
Int 7	ttctcctaagGGACC	879	1182	Int 4
Ex 8	CACAGgtcagtctgt	168		NC
Int 8	ttgtctgcagGGCAC	140		NC
Ex 9	CTACAgtaagagggg	196	196	Ex 5
Int 9	ccacccacagGGCTG	1296	2459	Int 5
Ex 10	CCCAGgtgagcctgc	200	200	Ex 6
Int 10	ccatgcctagATTGG	2060	1976	Int 6
Ex 11	GACATgtgagtgcca	259	259	Ex 7
Int 11	ccacctgcagATGTG	711	633	Int 7
Ex 12	CAAAGgtaggcccca	126	126	Ex 8
Int 12	tgtattatagGGATC	544	628	Int 8
Ex 13	CCTCCgtaagcaacc	111	111	Ex 9
Int 13	ctgtcaccagGAGGT	540	592	Int 9
Ex 14	CCAGGgtgagtgggt	123	120	Ex 10
Int 14	ctgcccacagACCCA	930	804	Int 10
Ex 15	TCCCGgtgaggctct	257	257	Ex 11
Int 15	ccccacatagGAGGA	3064	1847	Int 11
Ex 16	GAAAGgtacctgtcc	148	148	Ex 12
Int 16	tgcatttcagAGAAT	1485	1641	Int 12
Ex 17	GGATGgtaaggccgg	108	108	Ex 13
Int 17	ctgcccccagGGCCA	994	1050	Int 13
Ex 18	TGAAGgtgtgtgcca	215	215	Ex 14
Int 18	ttccctgcagCTTGT	87	335	Int 14
Ex 19	GCAAGgtaccaggtc	123	123	Ex 15
Int 19	ccctttatagGGTCG	1981	1742	Int 15
Ex 20	GATGTgtaagtgtgg	71	71	Ex 16
Int 20	ctccccttagGTGGT	1277	1654	Int 16
Ex 21	GAGATgtgagcaggc	138	138	Ex 17
Int 21	ttcccgccagGTATG	1110	1074	Int 17
Ex 22	ACAGAgtgagtacctgg	100	100	Ex 18
Int 22	tctcttccagGACTA	1510	1592	Int 18
Ex 23	TCTAGcaacgttgtg	180	148+UTR	Ex 19
Int 23	tcatttttaggGCATG	1811	1389	Int 19
Ex 24	GTTAAcatttctttg	[a]158+UTR	1390	Ex 20

Ex, exon; Int, intron. NC, exon/intron sequences not corresponding to the human exon/intron sequences. Uppercase and lowercase letters, exonic and intronic sequences, respectively. [a]5'-end of exon 1 and 3'-end of exon 24 have not yet been determined. Correspond., corresponding.

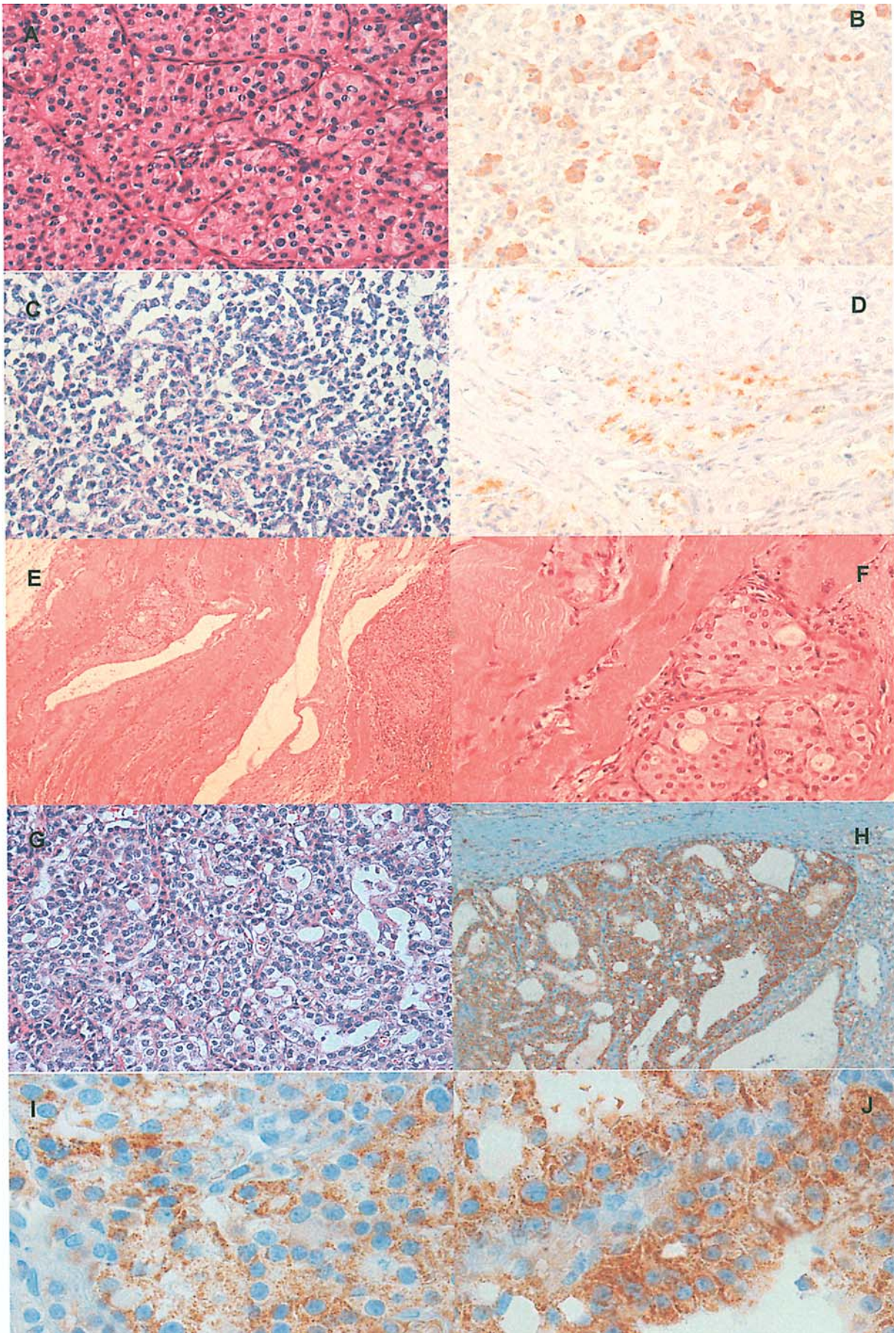

Figure 3. (A), H&E staining of the thyroid from lll:1 (x180). (B), Immunohistochemical (IHC) staining for calcitonin of the thyroid cancer from lll:1. Cells with positive expression stained brown (x180). (C), H&E staining of the thyroid cancer from lll:2 (x180). (D), IHC expression of calcitonin in the thyroid cancer from lll:2 (x180). (E and F), H&E staining of the thyroid cancer infiltrating the adjacent muscle from lll:2 (x45 and x180). (G), H&E staining of the thyroid cancer from lll:3 (x180). (H, I and J), IHC expression of calcitonin in the thyroid tumour of lll:3 (x90, x180 and x440).

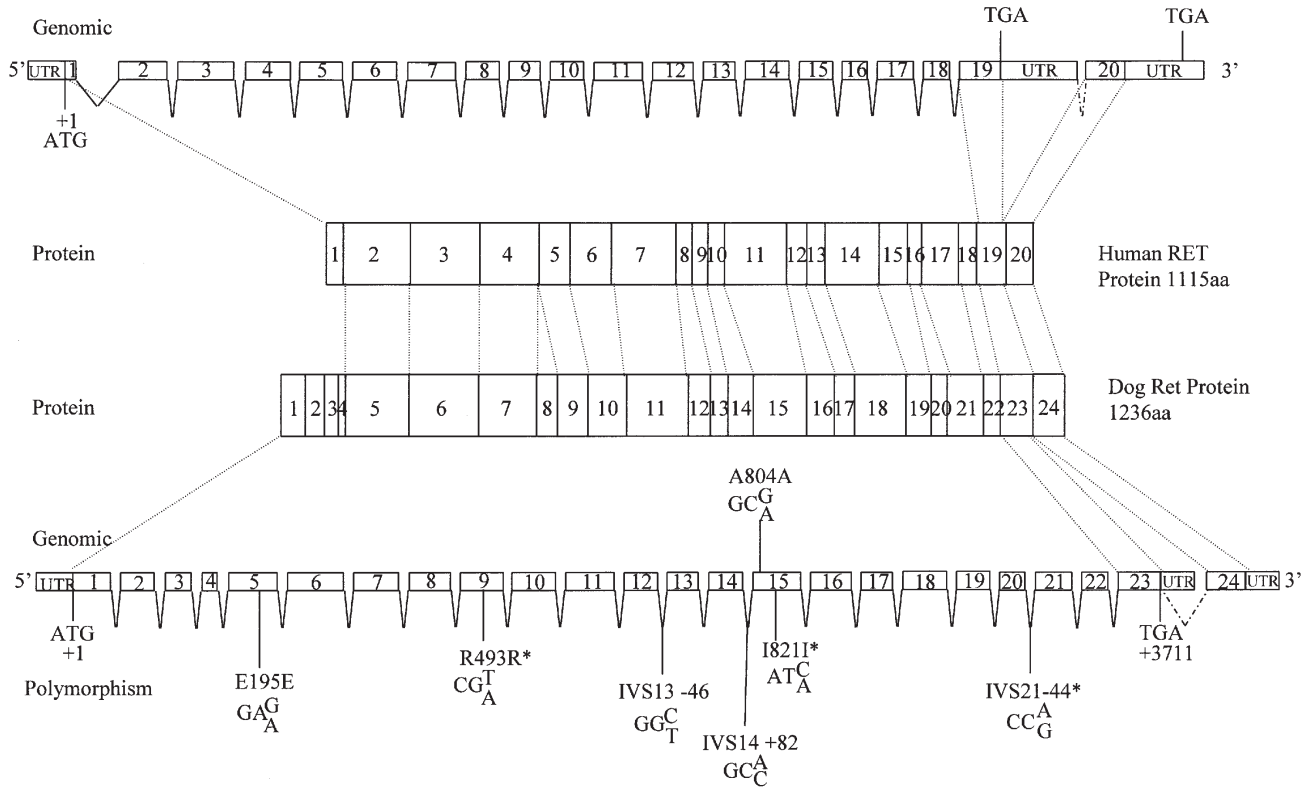

Figure 4. Genomic organisation of dog and human *RET*. Sequence variants detected in the dog *Ret* and their respective encoding proteins are shown at the bottom. Variants found in the pedigree are denoted as asterisk.

of the affected dogs only (lll:3). Neck ultrasound typically showed bilateral highly vascularised tumour masses (Fig. 2B). No other tumour was found at trunk imaging.

Autopsy was performed in lll:1, lll:2 and lll:3. This confirmed that the tumour emanated from the thyroid (Fig. 2C). lll:1 and lll:2 consisted of a differentiated, thyroid carcinoma with metastases to the cervical lymph nodes. A prominent oedema was present in the pharynx and subcutis in the head-neck region. Finally the dogs had multi-focal liver fibrosis and hepatic autolytic changes. An extended autopsy was also performed for lll:3 that showed similar findings as in lll:1. The thyroid carcinoma exhibited infiltrative growth into surrounding tissues and vessels, and in addition metastases were found in the thymus. Microscopically, the tumour was composed of a mixture of acinar and compact growing epithelial cells. No pheochromocytoma or pituitary adenoma could be found. However, unilateral multifocal adrenocortical hyperplasia was observed.

Histopathology and immunohistochemical findings. All three tumours examined (lll:1-3) showed similar histopathology and immunostaining results (Fig. 3; Table I). At histopathological examination, the tumours exhibited generally compact cell growth pattern or follicular pattern with intramuscular infiltration. The medium sized tumour cells had light to eosinophilic cytoplasm with round or slightly elongated nuclei. At immunohistochemistry all tumours were clearly positive for NSE and negative for PTH and S-100 protein. Various proportions of the tumour cells were positive for calcitonin. To confirm the accuracy of the calcitonin antibody, sections of normal dog thyroid were analysed with a small portion of C-cells expressing calcitonin. The mRNA *in situ* demonstrated calcitonin expression in C-cells from normal thyroid (data not shown). Immunohistochemistry with the calcitonin antibody on dog thyroid control sections produced a similar pattern of C-cells staining. The tumours also showed positive expression of thyroglobulin in a subset of tumour cells by immunohistochemistry except for lll:3, which was negative. Based on these findings, the tumours were classified as highly malignant, differentiated thyroid carcinoma, possibly of medullary or mixed follicular-medullary subtype. As differential diagnoses, thyroid paraganglioma, parathyroid carcinoma and follicular thyroid carcinoma were considered.

Dog Ret gene bioinformatics. A full-length cDNA and genomic structure of the dog *Ret* gene (GenBank submission BK005621) was determined by using the computational bioinformatics based on the human *RET* sequence (AJ243297). Analysis by GenScan (MIT, USA) on the sequence derived from dog BAC clone RP81-410M10 (GenBank accession no. AC123973.2) predicted 23 exons in the dog *Ret* gene, giving a 3711-bp of open reading frame (ORF). A separate putative 24th exon of the dog *Ret* was predicted and matched with the human *RET* exon 20 by aligning with its human counterpart. The predicted structure and exon-intron boundaries of dog *Ret* coincides with that of human *RET* as predicted by alignment of *RET* cDNA against the human genome sequence (Table III, Fig. 4), showing 86% identity in the nucleotide level. Alignment of cDNA *RET* orthologues of both species showed that the putative exons 5-7 and 9-24 in the dog *Ret* correspond to exons 2-4 and 5-20 in the human *RET* respectively. A 1236 aa dog *Ret* protein was predicted (Dog isoform A) and was

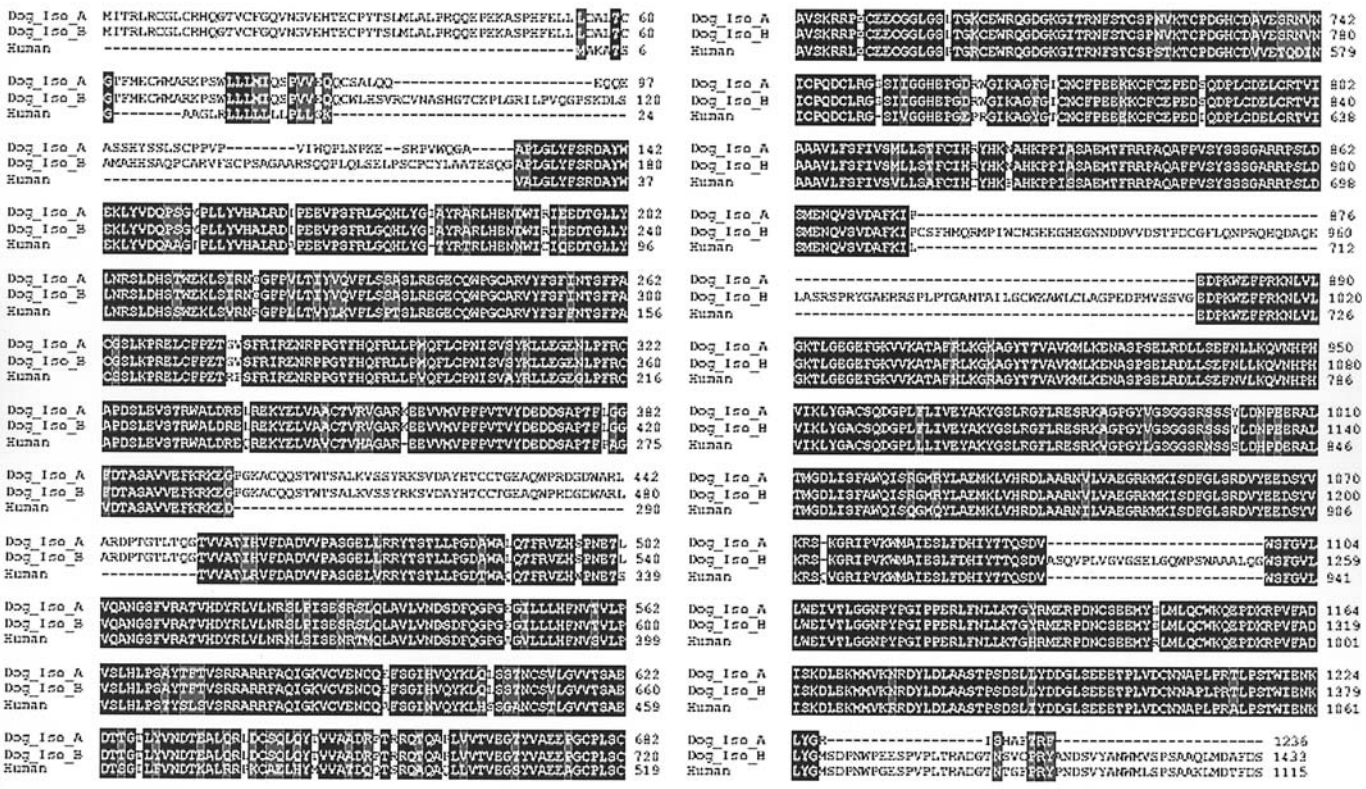

Figure 5. Alignment of the predicted dog Ret proteins (two isoforms) with human RET protein. Identical amino acids are highlighted in black and similarity amino acids are highlighted in grey. Dog_Iso_A denotes dog Ret isoform A and Dog_Iso_B denotes dog Ret isoform B.

aligned with its human counterpart (1115 aa), showing 83% identity (Fig. 5).

Ret mutational screening. We screened individuals lll:1, lll:2, lll:3 and lll:4 for mutations in exons 12-21 of the dog *Ret* gene (corresponding to human exons 8-17), where all the known MEN2 and MTC related mutations are located by sequencing analysis. The screening of other exons that corresponded to other human *RET* exons was carried out by SSCP. Sequencing was performed on all variations observed by SSCP. Three variations were present in these individuals of the family but did not segregate with the disease. Four other variations were only observed in an unrelated dog (Fig. 4), which was included in the screening as comparison.

Discussion

We report a dog family where four members developed thyroid carcinomas in a pattern suggesting dominant inheritance of autosomal or X-linked type. From the clinical, histopathological and biochemical examinations, the affected individuals demonstrated symptoms including calcitonin positive thyroid cancer, hypothyroidism, chronic dermatitis, and in one case elevated serum calcium levels. These findings suggest a familial form of medullary thyroid carcinoma similar to human FMTC. However, the observation of multinodular hyperplasia in the adrenal cortex of dog lll:3 at autopsy would suggest a form of hereditary multiple endocrine neoplasia (MEN) not restricted to FMTC. On the other hand, this dog suffered from the most progressed and long-standing MTC. Therefore the observed changes in the adrenal cortex could also represent secondary alterations due to physical and hormonal stress.

The finding of familial MTC prompted the screening for constitutional *Ret* mutation. To date, only a partial dog *Ret* cDNA sequence spanning 2 exons was reported (GenBank AF364316) (16). In this study, we have predicted the complete dog *Ret* genomic sequence (BK005621), which is ~33 kb long giving a 3711-bp of ORF (23 exons) and a predicted 1236 aa protein (isoform A). According to the human Ret protein sequence, there are two major isoforms, with a smaller isoform encoded from exons 2 to 19 and a longer isoform encoded from exons 2 to 20 of a different transcript. From the latest completed first draft dog genome sequence, we found another isoform (isoform B), predicted using GenScan based on the draft genomic sequence (UCSC genome browser database of dog). Isoform B was predicted to span 1433 aa encoded from 4302 bp of cDNA (24 exons). In addition recently there was a predicted full length dog *Ret* cDNA in the Ensembl Database (ENSCAFG00000007076), this predicted sequence spans 1944 bp (12 exons) with 413 aa translated. This annotated sequence (ENSCAFG00000007076) was aligned and revealed to be within our predicted sequence (data not shown). The dog isoforms A and B share high similarity and both have a human counterpart. Notably the entire coding parts of the hot spot exons were entirely screened.

We explored the possible involvement of *Ret* in this dog family due to the established causative role of *RET* in inherited MTC in human. All exons of the annotated dog *Ret* gene were explored either by SSCP or/and sequencing, except exons 1-4 and 8 as these exons were found not to be corresponding to

human *RET*. The variations found did not segregate with the disease in this family, and we therefore conclude that these are polymorphisms of no clinical relevance to the phenotype. From a comparative genetic point of view, the familial MTC in this family does not share the genetic etiology commonly underlying phenotype in human. Therefore, mutations in the same dog *Ret* outside the human hotspots as well as mutations in other genes should be considered.

Until date ~5% of human MEN2 kindreds have been reported to be without the *RET* genetic alterations (17). It was recently suggested that alterations in the succinate dehydrogenase D gene (*SDHD*) was responsible for familial non-*RET* C-cell hyperplasia, which is associated with MTC (18). While it is presently unclear if *SDH* genes are causative genes for non-*RET* C-cell hyperplasia, the possibility of other genes in the development of tumourigenesis in non-*RET* familial MEN2 syndromes could not be excluded (19). The absence of demonstratable *Ret* mutation in this study, further suggests the possibility of additional genes involved in the tumourigenesis of MTC.

All three examined dogs exhibited similar pathology of their thyroid cancers. By immunohistochemistry calcitonin (C-cells) were found expressed in the tumour tissues of all three cases, in agreement with a diagnosis of medullary thyroid carcinoma. Serum measurement of calcitonin levels would be optimal to confirm the diagnosis. However, no such assay is presently available for dog species (20). Given the prominent tumour vascularity, the thyroglobulin positivity in two cases and the single case of dog with hypercalcemia, we have also considered the following differential diagnoses: thyroid paraganglioma, parathyroid carcinoma and follicular thyroid carcinoma. However, these alternative diagnoses were considered highly unlikely because of the negative staining results for S-100 protein (tumour marker for paraganglioma) and PTH (tumour marker for parathyroid) and positive staining for calcitonin (a tumour marker for MTC).

Although thyroglobulin was found to be expressed in two tumour tissues (lll:1 and lll:2), no thyroglobulin was expressed in the tumour tissues of lll:3. This picture would exclude both follicular thyroid carcinoma and follicular carcinoma of mixed follicular-medullary subtype as differentiated diagnosis. It is likely that the timing of the tissue preservation attributed to the positive staining for thyroglobulin. In this case, the tumour tissues from lll:3 were well preserved due to immediate fixation after autopsy, whereas there was a delayed in fixing the tumour tissues from lll:1 and lll:2. It has also been previously demonstrated in human tumour tissues that thyroglobulin staining could be easily diffused and thus creating a false positive staining in immunohistochemistry in less well-preserved tissues (unpublished data). The mixed staining of thyroglobulin in medullary carcinomas in dogs has been previously observed (12). It was suggested that the expression of thyroglobulin observed was due to the pre-existing thyroid tissues included in the tumour and not from the tumour tissue itself. Two studies in human have reported MTCs concurrently producing calcitonin and thyroglobulin (21,22).

Although most of the genetic basis of MEN syndromes has been elucidated in humans, the molecular pathogenesis of new familial MEN-type tumours remain to be identified. There are ongoing studies using rat as a model to define the genetic mechanisms of a newly identified MEN-like syndrome called MEN X (23,24). MEN X is characterised by inborn cataract and multiple tumours affecting the neuroendocrine system and the disease gene has been mapped and the data demonstrated has excluded the involvement of *Ret*. The unique occurrence of familial MTC makes this potentially an important model in further defining the genetic basis of MTC.

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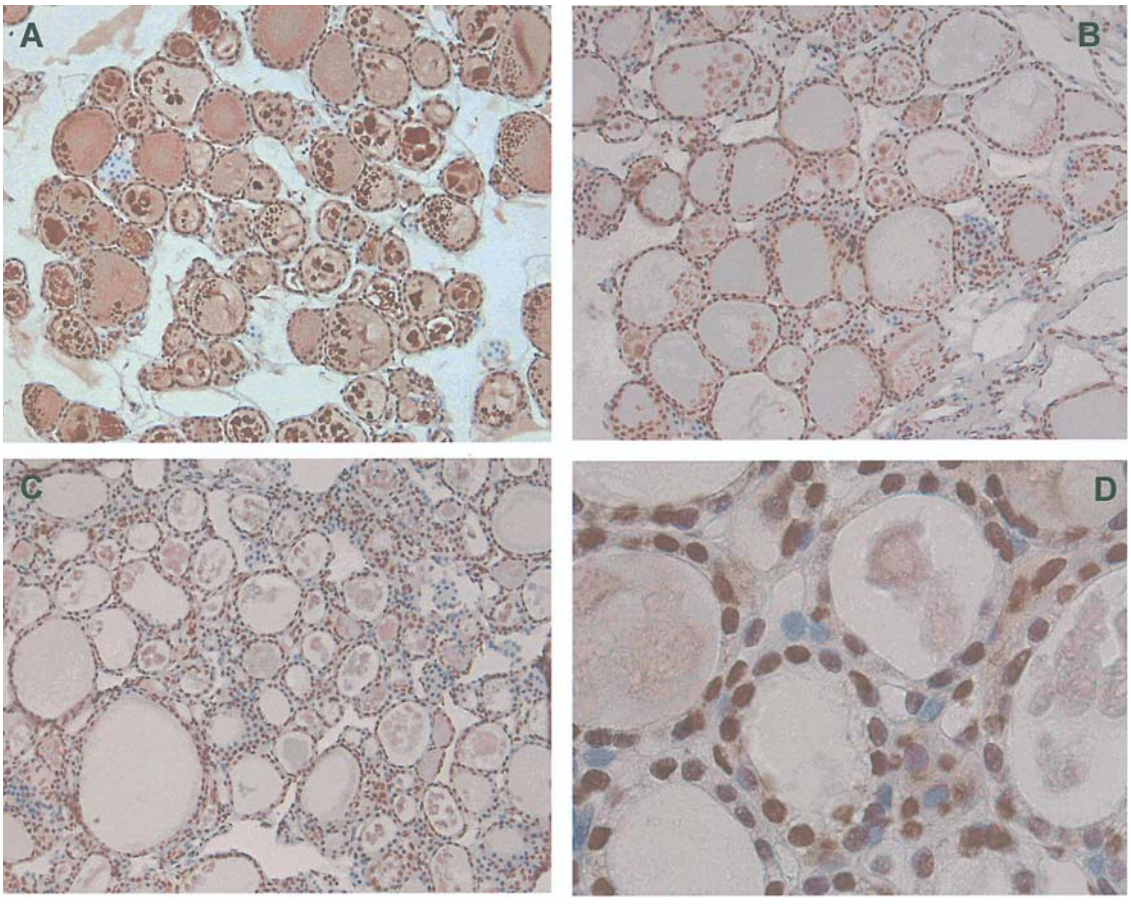
Supplementary

We have read the article by Sako *et al* (J Comp Pathol 133: 155-163, 2005) about chromogranin A in neuroendocrine carcinomas of dogs. The antibody used in that article was purchased from a company in Japan (Yanaihara Institute, Shizuoka, Japan). This antibody was tested on normal dog thyroid with an abundance of calcitonin cells (Supplementary Fig. 1A). Pretreatment and dilution of the antibody was the same as in the article by Sato *et al*. The dilution 1:100 gave an enormous background staining and the antibody stained the nuclei of the cells (Supplementary Fig. 1B). Nuclear staining by chromogranin A is never seen with other chromogranin antibodies (for instance the well known Chr A antibody clone LK2H10 produced by Ricardo Lloyd). There was still background staining and the nuclei were stained despite using two higher dilutions [1:1500 (Supplementary Fig. 1C)] and 1:3000 (Supplementary Fig. 1D) of the antibody. In conclusion, we still lack a chromogranin A antibody that is immunoreactive to dog Chr A.



Supplementary Figure 1. Immunohistochemistry (IHC) staining for calcitonin and chromogranin A of normal dog thyroid. (A), IHC expression of calcitonin in a normal dog thyroid, x20. (B), IHC expression of chromogranin A in normal dog thyroid using 1:100 dilution of the antibody, x20. (C and D), IHC expression of chromogranin A in normal dog thyroid using 1:1500 dilution, x20. (D), IHC expression of chromogranin A in normal dog thyroid using 1:3000 dilution, x20 and x100. There was nuclei staining at this high dilution.