Upregulation of the PI3K/Akt Pathway in the Tumorigenesis of Canine Thyroid Carcinoma

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Background: Information on the genetic events leading to thyroid cancer in dogs is lacking.

Hypothesis/Objectives: Upregulation of the PI3K/Akt pathway has an important role in the tumorigenesis of thyroid carcinoma in dogs.

Methods: Fifty-nine dogs with thyroid carcinoma and 10 healthy controls.

Results: Forty-three dogs (73%) had follicular cell thyroid carcinoma (FTC) and 16 dogs (27%) had medullary thyroid carcinoma (MTC). The relative mRNA expressions of VEGFR-1, VEGFR-2, EGFR, PIK3CA, PIK3CB, PDPK1, PTEN, AKT1, AKT2, COX-2, and CALCA. Mutation analysis was performed for known hotspots of RAS (N, K, H), PIK3CA, BRAF, RET, and for the entire coding region of PTEN.

Conclusions and Clinical Importance: The increased expression of several genes associated with PI3K/Akt signaling suggests the involvement of this pathway in the pathogenesis of thyroid carcinoma in dogs, warranting further research on pathway activation and gene amplification. The mutations most frequently associated with thyroid cancer in humans are rare in dogs.

Key words: C-cell; Dog; Follicular; Medullary; RAS.

Thyroid cancer represents 10–15% of all head and neck neoplasms in the dog. Ninety percent of thyroid tumors in dogs detected clinically are malignant and can be classified as either follicular cell thyroid carcinoma (FTC), which arises from thyroid follicular cells, or medullary thyroid carcinoma (MTC), which arises from the parafollicular cells (C-cells). Differentiated FTC (follicular, compact, follicular-compact, papillary) in dogs is remarkably similar in histology and biologic behavior to follicular thyroid carcinoma in humans. Likewise, the morphologic, cytologic, and immunohistochemical features of canine MTC also resemble human MTC.

Information on the genetic pathogenesis of thyroid cancer in dogs is scarce. Oncogenic gene amplification or copy number gain are prominent genetic mechanisms causing thyroid gland tumorigenesis in humans. 

Abbreviations:

- AKT1: v-akt murine thymoma viral oncogene homolog 1
- AKT2: v-akt murine thymoma viral oncogene homolog 2
- APES: 3-aminopropyltriethoxysilane
- bp: base pairs
- BRAF: v-raf murine sarcoma viral oncogene homolog B
- CALCA: calcitonin-related polypeptide alpha
- COX-2: cyclooxygenase-2
- CT: cycle threshold
- EGFR: epidermal growth factor receptor
- FF-PE: formalin-fixed paraffin-embedded
- FTC: follicular cell thyroid carcinoma
- Fw: forward
- GDP: guanosine diphosphate
- GTase: guanosine triphosphatase
- GTP: guanosine triphosphate
- HE: hematoxylin and eosin
- HPRT: hypoxanthine phosphoribosyltransferase 1
- H-RAS: Harvey rat sarcoma viral oncogene homolog
- IHC: immunohistochemistry
- K-RAS: Kirsten rat sarcoma viral oncogene homolog
- MAPK: mitogen-activated protein kinase
- MTC: medullary thyroid carcinoma
- N-RAS: neuroblastoma RAS viral (v-ras) oncogene homolog
- PDPK1: 3-phosphoinositide-dependent protein kinase-1
- PI3K: phosphatidylinositol-3-kinase
- PIK3CA: phosphatidylinositol-3,4,5-triphosphate 3-kinase
- PIK3CB: phosphatidylinositol-3,4,5-triphosphate 3-kinase catalytic subunit beta
- PTEN: phosphatase and tensin homolog
- qPCR: quantitative RT-PCR
- RET: rearranged during transfection
- RPS5: ribosomal protein S5
- RTK: receptor tyrosine kinase
- Rp: reverse

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This is especially true for genes involved in the phosphatidylinositol-3-kinase (PI3K)/Akt pathway (Fig 1). Prominent examples of such genes include EGFR, VEGFR-1, VEGFR-2, PIK3CA, PIK3CB, AKT1, AKT2, and PDPK1.6 Epidermal growth factor receptor, VEGFR-1 and VEGFR-2 are important regulators of both mitogen-activated protein kinase (MAPK) and PI3K/Akt signaling pathways. However, copy number gains in these receptor tyrosine kinase (RTK) genes in human thyroid cancer are particularly associated with PI3K/Akt pathway activation, which is the major pathway involved in the pathogenesis of follicular thyroid carcinoma in humans.6-8 An important and expected consequence of gene amplification or pathway activation is increased mRNA and protein expression and consequent aberrant activation of downstream signaling.6 The mRNA expression of the RTKs and downstream effectors involved in PI3K/Akt signaling may provide valuable information regarding gene amplification and pathway activation, and has not yet been investigated in thyroid tumors in dogs.

Several reports have suggested that the PI3K/Akt signaling pathway regulates the expression of cyclooxygenase-2 (Cox-2).9 Cox-2 expression is increased in colorectal cancer, and increased Akt activity is crucial for COX-2 overexpression in apoptotic-resistant cells.9 Cox-2 mRNA expression could therefore also be used to infer the activity of the PI3K/Akt pathway.

One of the most important genetic events described in human follicular thyroid carcinoma is point mutations in 1 of the 3 RAS genes.6 The RAS genes (N, K, and H-RAS) encode membrane-bound intracellular proteins involved in cellular signal transduction. These proteins act as on/off switches, relaying extracellular signals to the cytoplasmic signaling cascades of the MAPK and PI3K/Akt pathways, which control cellular proliferation, differentiation, and survival (Fig 1).11 In many cancers in humans, point mutations in exon 1 (codons 12 and 13) or exon 2 (codons 59 and 61) fix RAS proteins in a permanently activated state, promoting uncontrolled cellular division and malignant transformation.12 Although RAS is a classical dual activator of both PI3K/Akt and MAPK signaling, RAS mutations seem to preferentially activate the PI3K/Akt pathway in thyroid gland tumorigenesis.6 Mutations in RAS genes have not yet been investigated in thyroid tumors in dogs.

Mutations in the tumor suppressor gene phosphatase and tensin homolog (PTEN) and in the phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) gene also are reported in human follicular thyroid carcinoma.5 Because PTEN has an inhibitory effect on the PI3K/Akt pathway, inactivating mutations or deletions in PTEN lead to PIK3/Akt pathway activation and promote tumorigenesis.13 Activating point mutations in the PIK3CA gene lead to a constitutively activated protein that also activates PI3K/Akt pathway. Mutations in PTEN or PIK3CA have not been evaluated in thyroid cancer in dogs.

The most important genetic alterations in papillary thyroid carcinoma in humans are activating point mutations of v-raf murine sarcoma viral oncogene homolog B (BRAF) which lead to activation of MAPK signaling pathway.14 Mutations in BRAF have not been investigated in thyroid tumors of dogs.

The principal molecular mechanism underlying human MTC is aberrant activation of RET (rearranged during transfection), a RTK which signals through the PI3K/Akt and MAPK pathways.15,16 Germ line RET mutations are responsible for the hereditary forms of human MTC, whereas somatic RET mutations are present in approximately 50% of patients with sporadic MTC.17 In addition, RAS mutations have been reported in up to 68% of human MTCs without RET mutations.18 In the only case report of canine familial MTC, no mutation was found after complete sequencing of RET.19 The genetic events underlying sporadic canine MTC have not yet been investigated.

Immunohistochemistry (IHC) for thyroglobulin, calcitonin, or markers of neuroendocrine tissue aids differentiation of canine FTC and MTC.4 mRNA expression of the gene encoding calcitonin (calcitonin-related polypeptide alpha—CALCA) has not yet been evaluated in dogs with thyroid tumors and also may help differentiate FTC from MTC.

We hypothesized that upregulation of the PI3K/Akt pathway has an important role in the tumorigenesis of thyroid carcinoma in dogs. The first aim of our study

Fig 1. Simplified schematic illustration of PI3K/Akt and mitogen-activated protein kinase (MAPK) signaling pathways in thyroid cancer. These pathways are involved in propagation of signals from various receptor tyrosine kinases into the nucleus, and they regulate multiple cell processes including proliferation, differentiation, and survival. Adapted by permission from Macmillan Publishers Ltd: [Nature Reviews Endocrinology], copyright (2011).40
was to determine if there is increased mRNA expression of selected genes involved in the PI3K/Akt signaling pathway by performing quantitative analysis of mRNA expression of those genes. The second aim of our study was to investigate hotspot mutations in selected genes involved in the PI3K/Akt and MAPK signaling pathways by sequencing selected regions of those genes that correspond to the human hotspots. The third aim of our study was to determine if mRNA expression of CALCA differs among FTC, MTC, and normal thyroid gland in dogs by performing quantitative analysis of CALCA gene expression and comparing it among the 3 groups.

Materials and Methods

Case Selection

The medical record databases of the Companion Animal Clinics of Ghent and Utrecht Universities were searched for dogs diagnosed with thyroid carcinoma from 1986 to 2013. Patients from which frozen (–80°C) tumor samples were not available were excluded.

Thyroid Specimens

In total, 59 thyroid tumors (43 FTCs, 16 MTCs) and 10 normal thyroid glands (whole tissue explants) were analyzed. Tumor samples were collected from the Departments of Pathology of Ghent and Utrecht Universities. Samples were collected immediately after surgical or necropsy removal, part formalin-fixed paraffin-embedded (FF-PE), and part snap-frozen in liquid nitrogen and conserved at –80°C until total RNA extraction.

Histopathology

All HE-stained slides were reviewed by a board-certified pathologist (RD). All tumors were classified according to World Health Organization classification of canine thyroid tumors.20 The distinction between adenoma and carcinoma was based on histologic evidence of capsular invasion, vascular invasion, or metastases.

Immunohistochemistry

Five-μm sections from each FF-PE block were prepared on 3-aminopropyltriethoxysilane-coated slides. IHC was performed as previously described.21 For calcitonin IHC, sections were incubated overnight with the primary antibody (rabbit polyclonal antibody A0576a diluted 1 : 400) in a humidity chamber at 4°C. IHC was performed in 2 batches. All sections were examined by the same investigator (MC). Thyroid tumors positive for calcitonin were classified as MTC and thyroid tumors negative for calcitonin were classified as FTC.4 To verify the accuracy of our classification, the subset of tumors positive for calcitonin also was stained for thyroglobulin and normal thyroid gland in dogs by performing RT-PCR. However, in no tumor section were neoplastic cells observed to be immunopositive for both antibodies.

RNA Isolation and Reverse Transcription

Frozen tissue samples were disrupted and homogenized with a rotator-stator homogenizer and total RNA was isolated by using an RNAeasy kit3 according to the manufacturer’s instructions. A DNase step was performed to avoid genomic DNA contamination. Purified RNA was quantified on a NanoDrop ND-1000 Spectrophotometer and, in 12 samples (first 10 patients of inclusion period and 2 normal thyroid glands), its integrity was assessed on a Bioanalyzer Micro RNA Chip.21 Exactly, 1,000 ng of total RNA of each sample were reverse transcribed into cDNA by using the iScript cDNA synthesis kit2 according to the manufacturer’s instructions.

 Primer Design

All PCR amplification primers (Table 1) were designed with Perl-primer v1.1.21 according to the parameters of the Bio-Rad iCycler manual and were ordered from Eurogentec.3 All PCR primers also were used as sequence primers. When the region of interest could not be amplified in 1 stretch, overlapping primer pairs were used (Table 2). For quantitative RT-PCR (qPCR), temperature gradients were performed to determine the optimal annealing temperature of each primer pair and primer specificity was confirmed by melting curve analysis and sequence analysis of the PCR products (Table 3).

PCR Amplification

PCR amplification was performed by using the Phusion Hot Start Flex DNA Polymerase6 on a C-1000 Touch thermal cycler.5 PCR products were evaluated by agarose gel electrophoresis to confirm expected product length. PCR products were amplified for sequencing by using the BigDye Terminator version 3.1 Cycle Sequencing Kit8 and filtrated using Sephadex G-50 Superfine.7 Sequencing was performed with the ABI3130XL Genetic Analyzer according to the manufacturer’s instructions. The obtained sequences were compared to the consensus mRNA sequence using DNAstar Lasergene core suite SeqMan Pro.18 All mutations affecting the amino acid sequence were confirmed by repeating RNA extraction, reverse transcription, and sequencing.

Quantitative RT-PCR

After reverse transcription, qPCR analyses were performed on cDNA to determine and compare the levels of expression of VEGFR-1, VEGFR-2, EGFR, PIK3CA, PIK3CB, PDK1, PTEN, AKT1, AKT2, COX-2, and CALCA in thyroid tumors and normal thyroid glands. To correct for differences in sample input, the expression levels were normalized to the expression of the reference genes ribosomal protein S5 (RPS5) and hypoxanthine phosphoribosyltransferase (HPRT), already proven to be stable in other canine tissues.22 Furthermore, the stability (M-value) of the reference genes was verified.

Quantitative RT-PCR was performed on a CFX384 real-time PCR detection system.5 Each qPCR reaction mixture consisted of 4-μL cDNA (diluted 50x), 0.4 μL of both the forward and the reverse primers, 5-μL iQ SYBR Green Supermix and 0.2-μL MilliQ, for a final reaction volume of 10 μL. The thermal cycles were performed as previously described.21 A 4-fold reference
standard dilution series using 10x diluted cDNA was included in every plate to assess reaction efficiency, and negative controls were used to assess the specificity of the reaction and check for contamination. Data were analyzed with CFX manager version 3.0. Relative mRNA expression levels were calculated by relative quantitation and the fold-expression changes were determined by means of the 2^(-ΔΔCT) method. The maximum allowed cycle threshold (CT) value for calculations was 45.

### Table 1. PCR primers for amplification of canine RAS (K, N, H), BRAF, PIK3CA, RET, and PTEN. All positions are based on the mRNA sequence published on NCBI.

| Primer | Sequence (5’–3’) | Location Exons | T_a (°C) | Product Length (bp) |
|--------|------------------|----------------|----------|---------------------|
| K-RAS Fw18 | ATAAACTTGTGTTAGTTGGAGC | 18/39 | 1–3 | 62 | 463 |
| K-RAS Rv480 | GTATAGAAGGCATCGTCACAC | 459/480 | | | |
| N-RAS Fw12 | GGTCCTCAACCTTTTCTCC | 12/29 | 1–5 | 55 | 660 |
| N-RAS Rv672 | AGTGCTTTGTTACATCAAC | 653/672 | | | |
| H-RAS Fw54 | CCATGACGGAGTTAAGCTG | 54/73 | 1–2 | | 253 |
| H-RAS Rv306 | ATGGAATAACACAGAAGAAGA | 286/306 | | | |
| BRAF Fw155 | CGACAGACTGCACAGGAG | 1,555/1,573 | 13–16 | 55 | 372 |
| BRAF Rv1926 | CCGTACCTTACTGAGATCTGGAG | 1,904/1,926 | | | |

K-RAS, Kirsten rat sarcoma viral oncogene homolog; N-RAS, neuroblastoma RAS viral (v-ras) oncogene homolog; H-RAS, Harvey rat sarcoma viral oncogene homolog; BRAF, v-raf murine sarcoma viral oncogene homolog B; PIK3CA, phosphatidylinositol-4,5-biphosphate 3-kinase, catalytic subunit alpha; RET, ret proto-oncogene; PTEN, phosphatase and tensin homolog; Fw, forward; Rv, reverse; T_a, optimal annealing temperature; bp: base pairs.

Accession numbers: K-RAS: XM_003433561.2, N-RAS: NM_001287065.1, H-RAS: NM_001287069.1, BRAF: XM_532749, PIK3CA: XM_545208.4, RET: NM_001197099.1, PTEN: NM_001003192.1.

### Table 2. Sequencing primers for canine RAS (K, N, H), BRAF, PIK3CA, RET, and PTEN. All positions are based on the mRNA sequence published on NCBI.

| Primer | Sequence (5’–3’) | Location |
|--------|------------------|----------|
| K-RAS Rv355 | ATTTCTACTAGGACCATTAGGT | 334/355 |
| N-RAS Fw217 | AAACAGTGTTGTTATAGAGG | 217/236 |
| N-RAS Rv524 | GTTCTCAAGGATGGAATCCC | 505/524 |
| H-RAS Fw167 | GACCTCTACGGAGAAGAAG | 167/185 |
| H-RAS Rv230 | CTGTTGCTCAGGATGTCCAG | 212/230 |
| PIK3CA Fw1549 | CTCATCTTTAGGATTTGGAG | 1,549/1,568 |
| PIK3CA Rv1815 | CACATCTTGTCTGTGGCTC | 1,796/1,815 |
| PIK3CA Rv3029 | GATTATGAAAAGGAGCAGAAG | 3,029/3,048 |
| PIK3CA Rv3336 | CATGCTGTGTTAATGGTGTTG | 3,317/3,336 |
| RET Fw1906 | GTGCCTTTCTCTCCTCATGT | 1,906/1,925 |
| RET Fw2221 | AAAGGCAAAGCGGATACAC | 2,221/2,240 |
| RET Rv1958 | GTGGGCAATCTGGTGTAGAC | 1,977/1,995 |
| RET Rv2278 | GGGAACCATTCTCTTCTGAG | 2,259/2,278 |
| PTEN Fw475 | CACTGTAAAGCGGATACAC | 475/494 |
| PTEN Rv249 | CCTGTATACGGCCTGTCAGTC | 230/249 |
| PTEN Rv595 | TTGGAACTGTTTCTCTTCTCC | 576/595 |
| PTEN Fw1158 | TGTagggagggagcataac | 1,158/1,177 |
| PTEN Rv1245 | CATGCTGGTAACTGTTG | 1,266/1,285 |

K-RAS, Kirsten rat sarcoma viral oncogene homolog; N-RAS, neuroblastoma RAS viral (v-ras) oncogene homolog; H-RAS, Harvey rat sarcoma viral oncogene homolog; PIK3CA, phosphatidylinositol-4,5-biphosphate 3-kinase, catalytic subunit alpha; RET, ret proto-oncogene; PTEN, phosphatase and tensin homolog; Fw, forward; Rv, reverse.

Accession numbers: K-RAS: XM_003433561.2, N-RAS: NM_001287065.1, H-RAS: NM_001287069.1, BRAF: XM_532749, PIK3CA: XM_545208.4, RET: NM_001197099.1, PTEN: NM_001003192.1.
Table 3. Quantitative RT-PCR primers for canine VEGFR-1, VEGFR-2, EGFR, PIK3CA, PIK3CB, PDPK1, PTEN, AKT1, AKT2, COX-2, CALCA, RPS5, and HPRT. All positions are based on the mRNA sequence published on NCBI.

| qPCR Primers | Sequence (5’-3’) | Location | $T_m$ (°C) | Product Length (bp) |
|--------------|------------------|----------|------------|---------------------|
| VEGFR-1 Fw 189 | GGCTCAGGCAAACACAC | 189/206 | 63 | 190 |
| VEGFR-1 Rv 378 | CCGGCAGGGGATGAGCAT | 378/361 | 59 | 90 |
| VEGFR-2 Fw 3606 | GGAAGAGAAGTGTTGACCCCC | 606/3,627 | 64 | 181 |
| VEGFR-2 Rv 3786 | GACATACACTGTCCCTGTTG | 3,786/3,786 | 64 | 180 |
| EGFR Fw 2078 | CTGAGGACATTCGGCA | 2,078/2,092 | 53 | 108 |
| EGFR Rv 2185 | TGGCTTTGGGAGAACGC | 2,171/2,185 | 59 | 104 |
| PIK3CA Fw 1269 | CCTGTATGAACACAGGTG | 1,269/1,288 | 58.5 | 134 |
| PIK3CA Rv 1402 | GGACATGTTCTTCTTACACG | 3,099/3,119 | 64.5 | 142 |
| PIK3CB Fw 2978 | CTTCAACAAGATGCCCC | 2,978/2,995 | 62.5 | 142 |
| PIK3CB Rv 3119 | CTATGCTATCAAACTACCA | 3,099/3,119 | 64.5 | 142 |
| PDPK1 Fw 667 | AGGGTCGAACCTCACCGC | 667/667 | 55.5 | 199 |
| PDPK1 Rv 865 | AGGGCTACATTACGGAGG | 846/865 | 63 | 142 |
| PTEN Fw 1209 | AGATGTAGTTGACAAATGAACCT | 1,209/1,230 | 62 | 102 |
| PTEN Rv 1310 | GTGATTTGTGTGCTGATC | 1,291/1,310 | 63 | 142 |
| AKT1 Fw 71 | GGACCTTCCACGTAGACTC | 71/89 | 60.5 | 195 |
| AKT1 Rv 799 | TTCTCCTTGACCAGGATCACC | 779/799 | 64 | 182 |
| AKT2 Fw 71 | GGACCTTCCACGTAGACTC | 71/89 | 60.5 | 195 |
| AKT2 Rv 265 | CATTCTAGTTGACCTTTCGGC | 247/265 | 63 | 142 |
| COX-2 Fw 971 | TTCCAGACGAGCCAGCTATA | 971/990 | 60 | 112 |
| COX-2 Rv 1082 | GCAGCTCTGGGTCAAACTTC | 1,063/1,082 | 65 | 142 |
| CALCA Fw 157 | ATCATGGGCTTGTGGAAGTC | 157/176 | 58.5 | 98 |
| CALCA Rv 254 | AGAGCGGACCTGAATGGTC | 237/254 | 64 | 142 |
| RPS5 Fw 405 | TCACTGGTGAGAACCCCT | 405/423 | 62.5 | 142 |
| RPS5 Rv 545 | CCTGATTCACACGGCGTAG | 527/545 | 64 | 142 |
| HPRT Fw 484 | AGCTTGCTGGTGAAAAGGAC | 484/503 | 58 | 104 |
| HPRT Rv 587 | TTATAGTCAAGGGCATATCC | 568/587 | 64 | 142 |

Sequenceing

Sequenceing was performed for human mutation hotspots of K-, N-, and H-RAS (exons 1 and 2),$^{25}$ BRAF (exon 15),$^{26}$ PIK3CA (exons 9 and 20),$^{27}$ RET (exons 8, 10, 11, 13–16),$^{19}$ and for the entire coding region of PTEN (hotspot exons 5–8)$^{28}$ on all 59 tumor samples and 2 normal thyroid glands.

Statistical Analysis

Relative expression levels of VEGFR-1, VEGFR-2, EGFR, PIK3CA, PIK3CB, PDPK1, PTEN, AKT1, AKT2, COX-2, and CALCA were among thyroid tumors (FTC and MTC) and normal thyroid glands with the nonparametric independent-sample Kruskal-Wallis test because the data were not normally distributed.$^9$ The significance level was set at 5%. Dunn’s test was used for multiple comparisons (adjusted P values are shown in the results section). The same procedure was used to compare the relative expression of reference genes between tumor samples and normal thyroid glands.

According to the mRNA expression of target genes, patients were grouped by means of unsupervised hierarchical clustering.$^1$ Unsupervised clustering was implemented by Pearson correlation for genes, and by Spearman correlation for samples.

Results

Fifty-nine thyroid carcinomas were analyzed. Forty-three (73%) were FTC and 16 (27%) were MTC. Histologic subtypes of FTC included follicular (n = 8, 13%), follicular-compact (n = 11, 19%), compact (n = 18, 30%), papillary (n = 1, 2%), follicular-papillary (n = 1, 2%), and carcinomas (n = 4, 7%).

mRNA Expression of PI3K/Akt Pathway-Related Genes

Relative mRNA expression was evaluated in 41 FTC, 15 MTC, and 10 normal thyroid glands (Fig 2). Relative expression levels of reference genes were not significantly different between thyroid tumors and normal thyroid glands.

The relative expression levels of VEGFR-1 ($P < .001$), VEGFR-2 ($P = .002$), PDPK1 ($P < .001$), AKT1 ($P = .009$), and AKT2 ($P < .001$) were significantly higher in FTC than in normal thyroid glands. The relative expression levels of EGFR ($P < .001$), VEGFR-1...
Fig 2. Dot plots representing the relative mRNA expression levels of VEGFR-1, VEGFR-2, EGFR, PIK3CA, PIK3CB, PDPK1, PTEN, AKT1, AKT2, COX-2, and CALCA in canine normal thyroid gland (n = 10), FTC (n = 41) and MTC (n = 15). Significant differences between tumors and normal thyroid gland tissue are indicated with asterisks. FTC, follicular cell thyroid carcinoma; MTC, medullary thyroid carcinoma; EGFR, epidermal growth factor receptor; VEGFR-1, vascular endothelial growth factor receptor-1; VEGFR-2, vascular endothelial growth factor receptor-2; PDPK1, 3-phosphoinositide dependent protein kinase-1; AKT1, v-akt murine thymoma viral oncogene homolog 1; AKT2, v-akt murine thymoma viral oncogene homolog 2; PTEN, phosphatase and tensin homolog; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha; PIK3CB, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit beta; COX-2, cyclooxygenase-2; CALCA: calcitonin-related polypeptide alpha; *P < .05; **P < .01; ***P < .001.
were significantly higher in MTC than in normal thyroid glands. Relative expression levels of PTEN, PIK3CB, and COX-2 were not significantly different between thyroid tumors and normal thyroid glands. The relative expression levels of CALCA did not overlap between FTC (range, 0–3.6) and MTC (range, 3.9–156.1).

Unsupervised hierarchical clustering of the samples showed an almost perfect branching of normal thyroid glands, FTC, and MTC when all genes were included in the analysis (Fig 3). After omitting the relative expression of CALCA, clustering based on the remaining genes showed a more elaborate branching with normal thyroid gland enrichment in 1 branch, MTC enrichment in subsequent branching, and a final branch enriched in FTC.

Unsupervised hierarchical clustering of the genes showed separate branching for CALCA, COX-2, EGFR, and PTEN, whereas VEGFR-1 and VEGFR-2 were grouped together and closely positioned to all effectors of the PI3K/Akt pathway (PIK3CA, PIK3CB, PDK1, AKT1, and AKT2).

**Mutation Analysis**

Mutation analysis of K-RAS identified 2 amino acid-changing (missense) point mutations in 2 different tumors and a splice variant in all thyroid samples, including the normal thyroid glands. A G12R substitution (GGT > CGT) was present in 1 FTC of compact type and an E63K substitution (GAG > AAG) was observed in 1 MTC (Fig 4). In the K-RAS splice variant, exon 2 was missing.

Mutation analysis of N-RAS showed 1 silent mutation in codon 138 (GGG > GGA) in 2 FTC of compact type, occurring in heterozygous form.

Mutation analysis of H-RAS showed 1 silent mutation in codon 47 (GAC > GAT) in 21 FTC and 5 MTC occurring in both homozygous and heterozygous forms.

Mutation analysis of BRAF did not identify any point mutations. However, a splice variant in which exons 13 and 14 were missing was present in all samples, including normal thyroid glands.

Mutation analysis of PIK3CA and RET did not show any abnormalities in the sequenced region.

Mutation analysis of the entire coding region of PTEN showed 1 silent mutation in codon 325 (CTC > CTT) in 5 FTC and 3 MTC, occurring in both homozygous and heterozygous form.

**Discussion**

Information on the genetic events leading to thyroid cancer in dogs is scarce. In our study, mRNA expression of several genes involved in the PI3K/Akt signaling pathway was increased in canine FTC and MTC.
when compared to normal thyroid gland, and missense mutations of K-RAS were found in 1 FTC and 1 MTC. These findings support the role of the PI3K/Akt signaling pathway in the tumorigenesis of thyroid carcinoma in dogs.

In humans, gene amplification can lead to activation of cancer-related signaling pathways and plays an important role in thyroid gland tumorigenesis. In our study, overexpression of VEGFR-1, VEGFR-2, PDPK-1, AKTI, and AKT2 in canine FTC and overexpression of VEGFR-1, EGFR, and PIK3CA in canine MTC suggest activation of PI3K/Akt pathway, particularly in FTC. The PI3K/Akt pathway therefore could play an important role in canine thyroid tumorigenesis promoting cell proliferation, resistance to apoptosis, and malignant transformation. Mechanisms responsible for the upregulation of the above mentioned genes include gene amplification and altered promoter activity.

The two missense mutations in K-RAS identified in our study also have been reported in thyroid cancer of humans with a similar prevalence. The G12R substitution observed in a compact FTC has been described in 1 of 24 follicular thyroid carcinomas and in 3 of 108 sporadic MTC without RET mutation in humans. The RAS protein function occurs through intrinsic guanosine triphosphatase (GTPase) activity, which in the wild-type RAS switches the protein from an active (guanosine triphosphate [GTP]-bound) to an inactive (guanosine diphosphate [GDP]-bound) state. The substitution of an amino acid without a side chain (glycine) in position 12 by another amino acid with a side chain (arginine), interferes with the geometry of the protein, impeding hydrolysis of GTP by GTPase. Such mutations in RAS lead to a permanently activated protein and downstream signaling, facilitating uncontrolled cell division and tumor growth.

The E63K substitution observed in MTC also has been described in 1 of 16 MTC without RET mutation in humans. This mutation affects an evolutionarily conserved amino acid residue identical in all RAS proteins. Similarly to what is described for G12R substitution, the change of glutamic acid in position 63 to lysine also has been demonstrated to abolish GTPase activity, leading to constitutive activation of RAS and potentiating cellular transformation.

No amino acid-changing mutations were found in the sequenced regions of H-RAS, N-RAS, BRAF, PIK3CA, and RET nor in the entire coding sequence of PTEN. Hence, the mutations most commonly involved in thyroid tumorigenesis in humans are rare and do not play a major role in the pathogenesis of thyroid carcinoma in dogs.

The absence of mutations in the sequenced region of RET in 16 MTC is in agreement with a case report of familial MTC in 3 dogs in which no mutations were found after complete sequencing of RET. Additional research is needed to investigate the genetic events involved in the pathogenesis of canine MTC.

Splice variants of K-RAS and BRAF were observed in all thyroid tumors. However, given their presence in normal thyroid gland tissue, these are unlikely to play a role in thyroid gland tumorigenesis in dogs.

Involvement of the PI3K/Akt pathway in the pathogenesis of thyroid carcinoma in dogs suggests this pathway may constitute a promising therapeutic target. The importance of PI3K/Akt pathway activation and the value of targeting this pathway recently have been demonstrated in several canine cancer cell lines. Furthermore, a preliminary study in dogs with solid tumors showed that toceranib phosphate, a multitargeted TKI which targets VEGFR-2, was associated with a clinical benefit rate of 80% in 15 dogs with thyroid carcinoma. In humans with unresectable, radioiodine-refractory thyroid cancer, TKIs and inhibitors of PI3K/Akt signaling have shown encouraging results in recent clinical trials.

Despite the overexpression of many PI3K/Akt-related genes suggesting pathway activation, the relative expression of COX-2 was not increased in thyroid tumors in dogs. Similar findings have been reported in human follicular thyroid carcinoma, in which PI3K/Akt pathway activation is of major importance. This suggests that mRNA expression of COX-2 may not reflect activation of PI3K/Akt signaling in thyroid cancer.

As expected, canine FTC and MTC showed distinct mRNA expression profiles of PI3K/Akt pathway-related genes. These differences were confirmed by unsupervised clustering and suggest that these tumor types probably arise by different molecular mechanisms. The fact that mRNA expression of CALCA did not overlap between FTC and MTC confirms the accuracy of tumor classification based on IHC for calcitonin.

In our study, sequencing of human mutation hotspots was performed to investigate the presence in dogs of the most common mutations involved in thyroid cancer in humans. This approach was adopted over sequencing the entire coding region and regulatory regions of each gene because of its efficiency to achieve our goal.

Limitations of our study include the lack of protein data when interpreting mRNA expression levels because mRNA and protein expression do not always correlate. Furthermore, our findings of increased expression of RTKs and intracellular effectors involved in PI3K/Akt signaling warrant further research on phosphorylation of Akt (pAkt) and other members of this pathway to verify pathway activation.

In conclusion, overexpression of VEGFR-1, VEGFR-2, PDPK-1, AKTI, and AKT2 in canine FTC and VEGFR-1, EGFR, and PIK3CA in canine MTC suggests that the PI3K/Akt signaling pathway is activated and likely involved in the pathogenesis of thyroid cancer in dogs, especially in FTC. The mRNA expression of CALCA did not overlap between canine FTC and MTC. Two missense mutations in K-RAS were identified in an FTC and an MTC which are likely to be relevant for thyroid gland tumorigenesis. The mutations most frequently associated with thyroid cancer in humans are rare in dogs.
Footnotes

a Dako, Glostrup, Denmark
b Qiagen, Hilden, Germany
c NanoDrop Technologies, Wilmington, DE
d Agilent Technologies, Santa Clara, CA
e Bio-Rad, Hercules, CA
f Eurogentec, Maastricht, The Netherlands
g New England BioLabs Inc, Ipswich, MA
h Applied Biosystems, Carlsbad, CA
i Amersham, Buckinghamshire, UK
j DNASTAR, Madison, WI
k GraphPad Prism 6.03, GraphPad Software Inc, La Jolla, CA
l R 3.0.2, R Foundation for Statistical Computing, Vienna, Austria

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