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Immunohistochemical Expression of Potential Therapeutic Targets in Canine Thyroid Carcinoma

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Background: Thyroid carcinoma is a common endocrine tumor in the dog. Local invasive growth frequently precludes surgical excision and, in up to 38% of dogs, the tumor has already metastasized by the time of diagnosis. Therefore, it is important to investigate new treatment modalities that may be useful for the large number of dogs with inoperable tumors or metastatic disease.

Hypothesis/Objectives: To investigate the immunohistochemical expression of potential therapeutic targets in canine thyroid tumors.

Animals: 74 dogs with thyroid neoplasia.

Methods: Immunohistochemistry was performed for thyroglobulin, calcitonin, vascular endothelial growth factor (VEGF), p53, cycloxygenase-2 (cox-2), and P-glycoprotein (P-gp).

Results: Fifty-four (73%) tumors were classified as follicular cell thyroid carcinomas (FTCs) and 20 (27%) as medullary thyroid carcinomas (MTCs). Eighty percent of FTCs and all MTCs had a high percentage (76–100%) of neoplastic cells immunopositive for VEGF. Thirteen percent of FTCs and 50% of MTCs expressed cox-2. Seven percent of FTCs and 70% of MTCs expressed P-gp. No tumor was immunopositive for p53 expression. Expression of VEGF ($P = .034$), cox-2 ($P = .013$), and P-gp ($P < .001$) was significantly higher in MTCs compared to FTCs.

Conclusions and Clinical Importance: VEGF is a potential therapeutic target in both FTC and MTC in dogs. Cox-2 and P-gp may be useful molecular targets in canine MTC.

Key words: Cyclooxygenase-2; p53; P-glycoprotein; VEGF.

Thyroid cancer represents 10–15% of all head and neck neoplasms in the dog, and 90% of thyroid tumors detected clinically are carcinomas.[1,2] Thyroid carcinomas can be classified as follicular cell thyroid carcinomas (FTCs), which arise from follicular thyroid cells, and medullary thyroid carcinomas (MTCs), which arise from the parafollicular C cells and have a neuroendocrine origin. Although thyroidectomy is the preferred treatment modality, invasive nonresectable thyroid tumors are common in dogs, and in up to 38% of dogs, the tumor has already metastasized by the time of diagnosis.[3,4] Furthermore, almost 50% of dogs undergoing thyroidectomy experience recurrence or metastatic disease within 2 years of surgery.[4] Therefore, it is important to investigate new treatment modalities for the large number of dogs with inoperable tumors or metastatic disease.

Vascular endothelial growth factor (VEGF) is the main stimulator of angiogenesis in the thyroid gland, and VEGF overexpression has been found in human thyroid cancer.[5] VEGF is secreted by cancer cells and binds to VEGF tyrosine kinase receptors on the surface of endothelial cells and thyrocytes. In people, vascular endothelial growth factor receptor-2 (VEGFR-2) inhibition with tyrosine kinase inhibitors (TKIs) is the most effective new therapeutic strategy developed to date in the treatment of advanced thyroid cancer.[6] VEGF, angiogenesis, and VEGF-induced pathway activation may play important roles in the progression of canine thyroid cancer and constitute important therapeutic targets.

Tumor suppressor gene p53 encodes a nuclear phosphoprotein that mediates cell cycle regulation and apoptosis in response to DNA damage.[7] Mutations that result in loss of normal p53 function lead to loss of cell cycle control and increased risk of malignancy. In the normal cell, p53 protein has a short half-life and is undetectable by immunohistochemistry (IHC). However, some p53 gene mutations lead to expression

Abbreviations:

ABC	ATP-binding cassette
ABCC1	multi-drug resistance-related protein 1
APES	3-aminopropyltriethoxysilane
cox	cyclooxygenase
FF-PE	formalin-fixed paraffin-embedded
FTC	follicular cell thyroid carcinoma
HE	hematoxylin and eosin
IHC	immunohistochemistry
MTC	medullary thyroid carcinoma
P-gp	P-glycoprotein
TCC	transitional cell carcinoma
TKI	tyrosine kinase inhibitor
VEGF	vascular endothelial growth factor
VEGFR-2	vascular endothelial growth factor receptor-2

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of an altered p53 protein with longer half-life that is detectable by IHC.[8] In humans, p53 mutations have been described in 40–62% of undifferentiated thyroid carcinomas and in 5–10% of other thyroid carcinomas.[9] Research in human thyroid cancer shows that restoration of wild-type p53 expression by gene therapy is associated with inhibition of tumor cell growth and enhanced sensitivity to chemotherapy and radiation.[7] Earlier investigations of the p53 gene coding region identified a somatic mutation in 1 of 23 canine thyroid carcinomas.[10] Thus, the p53 tumor suppressor gene may be a potential molecular target in canine thyroid cancer.

Cyclooxygenases (cox), particularly cox-2, may play a critical role in tumor development and progression. In particular, epithelial neoplasms are prone to express large amounts of the inducible form of cox-2. In dogs, cox-2 overexpression has been described in transitional cell carcinoma (TCC) of the urinary bladder and in prostatic carcinoma.[11] Several studies have shown that cox-2 or cox-1/cox-2 inhibitors have antitumor and chemopreventive effects, presumably by induction of apoptosis, reduction in angiogenic growth factors, and suppression of regulatory T-cells.[12,13] Cox-2 is an appealing therapeutic target and its expression has not yet been investigated in canine thyroid tumors.

One study in 44 dogs with surgically excised thyroid carcinoma failed to demonstrate a clinical benefit of adjuvant chemotherapy.[14] Moreover, the reported survival times for dogs with unresectable thyroid tumors treated with chemotherapy are disappointing.[15] One of the major mechanisms for resistance to chemotherapy is high expression of ATP-binding cassette (ABC) transporter proteins such as P-glycoprotein (P-gp; ABCB1) and multi-drug resistance-related protein 1 (ABCC1).[16] These ATP-dependent membrane efflux pumps decrease the intracellular concentration of chemotherapeutic agents, thereby limiting cytotoxicity at their target site. Recent research shows that TKIs and cox-2 inhibitors can reverse multi-drug resistance by decreasing the expression and function of P-gp.[17,18] P-gp expression has been identified in several canine tumors and may constitute an attractive molecular target.[19]

The goal of this study was to evaluate the immunohistochemical expression of VEGF, p53 protein, cox-2 and P-gp in canine thyroid tumors and to assess their potential as therapeutic targets.

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 neoplasia from 1986 to 2012. Patients for which paraffin-embedded tumor samples were no longer available were excluded.

Tumor Specimens

Formalin-fixed paraffin-embedded (FF-PE) tissue blocks of each patient were collected. In some dogs, several blocks from 1 tumor site or blocks from multiple sites (local, regional node, distant metastases) were available. All samples were obtained at surgery or necropsy. In total, 304 FF-PE blocks from 74 patients (52 from Utrecht University, 22 from Ghent University) were available.

Histopathology

Five-μm sections from each FF-PE block were stained with hematoxylin and eosin (HE). All HE slides were reviewed by the same board-certified pathologist (R.D.) blinded to the clinical information and previous histopathology report.

When neoplastic thyroid tissue was identified, the section was classified as primary tumor, lymph node metastasis, or distant metastasis. The distinction between adenoma and carcinoma was based on histologic evidence of either capsular invasion, vascular invasion, or metastases. The histologic type of primary thyroid tumors was classified according to the World Health Organization classification as tumors of follicular-cell origin (follicular, compact, follicular-compact, papillary, poorly differentiated, undifferentiated, and carcinosarcoma) or C-cell (medullary) origin.[20] Classification of medullary thyroid tumors also was based on positive IHC for calcitonin, as previously described.[21]

Immunohistochemistry

Five-μm sections from each FF-PE block were prepared on 3-aminopropyltriethoxysilane-coated (APES) slides. After dewaxing and rehydration, antigen retrieval was performed by immersion in citrate-buffered (0.01 M, pH 6) distilled water and microwaving in a pressure cooker for 15 min at 850 W and 15 min at 300 W. Slides then were allowed to cool for 20 min. Endogenous peroxidase was blocked with 0.03% hydrogen peroxide for 5 minutes followed by rinsing with water and phosphate-buffered saline (PBS pH 7.4). Sections were incubated overnight with the primary antibodies (Table 1) in a humidity chamber at 4°C. Validation and evaluation of the optimal concentration of each primary antibody were performed with serial antibody dilutions using the respective positive controls (Table 1); normal canine thyroid tissue was used as negative control. Incubation with a polymer-based secondary antibody[a] was performed at room temperature for 30 min. After each incubation step, sections were rinsed with PBS. 3,3′ Diaminobenzidine[b] in substrate buffer solution served as chromogen and was allowed to react for 5 min (10 min for p53 antibody). The sections then were counterstained with hematoxylin, rinsed in tap water, dehydrated, and mounted with cover slips. Immunohistochemistry for each antibody was performed in 2 batches, given the large number of slides to be stained.

The subset of tumors positive for calcitonin also was stained for thyroglobulin in an automated immunostainer.[c]

Quantification of Immunoreactive Cells

All sections were examined by the same investigator (M.C.) who was blinded to the clinical information and outcome of each patient. For each marker, immunohistochemistry also was performed on normal canine thyroid gland to allow comparison with neoplastic tissue. Quantification of VEGF immunolabeling was performed by evaluating the entire section at 200× magnification and estimating the percentage of neoplastic cells positive for VEGF. Only neoplastic cells with cytoplasmic granular immunolabeling were considered positive. The tumors were classified according to the immunolabeling as 0, 1–25, 26–50, 51–75, and 76–100% positive cells, as previously described.[22] In each batch, endothelial cells were used as internal positive controls and normal thyroid gland was used as negative control.

Table 1. Antibodies used for immunohistochemistry.

Antibody	Antibody Name	Antibody Type	Dilution	Positive Control
VEGF	SPM225[a]	Mouse monoclonal	1 : 25	Canine mammary carcinoma
P53	Clone PAb 240[b]	Mouse monoclonal	1 : 50	Canine mammary carcinoma known to harbor p53 gene mutation
Cox-2	Clone 33[c]	Mouse monoclonal	1 : 800	Normal canine kidney (macula densa)
P-gp	C494[d]	Mouse monoclonal	1 : 200	Normal canine liver
P-gp	JSB-1[e]	Mouse monoclonal	1 : 100	Normal canine liver
Calcitonin	A0576[f]	Rabbit polyclonal	1 : 400	Normal canine thyroid gland
Thyroglobulin	A0251[f]	Rabbit polyclonal	1 : 800	Normal canine thyroid gland

[a]Santa Cruz Biotechnology, Inc, Dallas, TX.
[b]Thermo Fischer Scientific, Loughborough, UK.
[c]BD Transduction Laboratories, San Jose, CA.
[d]Enzo Life Sciences, Inc., Farmingdale, NY.
[e]Covance, Princeton, NJ.
[f]Dako, Glostrup, Denmark.

Quantification of p53 immunolabeling was performed by evaluating each section using an optical grid at 400× magnification. The fields were chosen randomly with a minimum of 10 fields per section and counting at least 500 cells per section. Only neoplastic cells with nuclear staining were recorded as positive. The labeling index was calculated as the number of positive cells divided by the number of positive plus negative cells. Only sections with a labeling index ≥5% of positive neoplastic cells were considered positive for p53 expression, as previously described.[23] In each batch, normal thyroid gland was used as negative control.

Quantification of cox-2 immunolabeling was performed by calculating the labeling index as described for p53. Only neoplastic cells with cytoplasmic granular staining were recorded as positive. Based on the labeling index and staining intensity (absent, weak, moderate or strong), an overall IHC score (0–12) was calculated as previously described.[24] In each batch, normal kidney was used as positive control and normal thyroid gland was used as negative control.

As recommended, 2 monoclonal antibodies recognizing 2 different epitopes of P-gp were used to improve the reliability of IHC (Table 1).[25] Quantification of P-gp immunolabeling was performed by evaluating the entire section at 200× magnification and estimating the percentage of neoplastic cells with membranous labeling of P-gp. The tumors were classified as 0–10, 11–25, 26–50, 51–75, and 76–100% positive cells, as previously described.[26] In addition, the intensity of the staining was recorded as absent, weak, moderate, or strong. Only sections with immunolabeling of ≥11% of neoplastic cells with both antibodies (C494 and JSB-1) were considered positive for P-gp expression.[25,26] In each batch, normal canine liver was used as a positive control and normal thyroid gland was used as negative control.

Thyroid tumors positive for calcitonin were classified as MTCs and thyroid tumors negative for calcitonin were classified as FTCs.[21] To ensure the accuracy of this classification, the subset of tumors positive for calcitonin also was stained for thyroglobulin. Calcitonin and thyroglobulin immunolabeling were not quantified. The tumor was considered positive when the cytoplasm of neoplastic cells exhibited a fine granular staining pattern with cell-to-cell variation. Normal thyroid gland was used as control in each batch.

Statistical Analysis

Expression of VEGF, cox-2 labeling index, cox-2 IHC score, and expression of P-gp were compared between FTCs and MTCs. For VEGF, cox-2 labeling index, and cox-2 IHC score, the analysis was based on the Wilcoxon rank sum test. For P-gp expression, the analysis was based on the Fisher exact test.[d] Significance level was set at .05.

Results

Thyroid tumor tissues from 74 dogs, with a mean age of 9.3 years (range, 4–16 years) were reviewed. All thyroid tumors were classified as carcinomas. From the 74 patients included in this study, 54 (73%) had FTC and 20 (27%) had MTC. Histologic subtypes of FTC included follicular (n = 13, 18%), follicular-compact (n = 17, 23%), compact (n = 19, 26%), follicular-papillary (n = 1, 1%), undifferentiated (n = 1, 1%), and carcinosarcoma (n = 3, 4%).

Twelve dogs with FTC (22% of FTCs) and 3 dogs with MTC (15% of MTCs), or 20% of all patients, had evidence of distant metastases at the time of diagnosis. Eight dogs with FTC (15% of FTCs) and 4 dogs with MTC (20% of MTCs), or 16% of all patients, had evidence of regional lymph node metastases at the time of diagnosis.

VEGF Expression

Immunohistochemistry for VEGF in normal canine thyroid gland identified staining of endothelial cells and clusters of parafollicular cells, whereas follicular cells were negative with rare exceptions. In contrast, 85% of all tumors (80% FTCs, 100% MTCs) exhibited a high percentage of VEGF-positive tumor cells (76–100%). Expression of VEGF was significantly higher in MTCs than in FTCs ($P = .034$; Table 2; Fig 1).

P53 Expression

Immunohistochemistry for p53 in normal canine thyroid tissue did not identify positive nuclei. Likewise, all sections of thyroid carcinoma tissue had <5% positive nuclei, and therefore no tumor was considered positive for p53 expression. Eighty-five percent of all thyroid tumors (87% FTCs, 80% MTCs) had <1% positive nuclei.

Table 2. Immunohistochemical expression of VEGF, cox-2, and P-gp (C494, JSB-1) in primary thyroid tumors of 74 dogs.

	FTC	MTC	Total
	Count (%)	Count (%)	Count (%)
VEGF			
0	0	0	0
1–25	1 (2)	0	1 (1)
26–50	2 (4)	0	2 (3)
51–75	8 (14)	0	8 (11)
76–100	43 (80)	20 (100)	63 (85)
Cox-2 IHC score			
0	47 (87)	10 (50)	57 (77)
1	2 (4)	2 (10)	4 (5)
2	3 (5)	5 (25)	8 (11)
3	2 (4)	0	2 (3)
6	0	3 (15)	3 (4)
P-gp (C494 & JSB-1)			
Negative	50 (93)	6 (30)	56 (76)
Positive	4 (7)	14 (70)	18 (24)
N	54 (73)	20 (27)	74 (100)

Cox-2, cyclooxygenase-2; FTC, follicular cell thyroid carcinoma; IHC, immunohistochemistry; MTC, medullary thyroid carcinoma; P-gp, P-glycoprotein; VEGF, vascular endothelial growth factor.

Cox-2 Expression

Immunohistochemistry for cox-2 in normal canine thyroid gland did not identify positive cells. Twenty-three percent of all primary tumors (13% FTCs, 50% MTCs) exhibited cox-2 expression (Table 2, Fig 2). Cox-2 labeling index in MTCs (median, 0.5%; range, 0–22.4%) was significantly higher ($P = .002$) than in FTCs (median, 0%; range, 0–7%). Likewise, the cox-2 IHC score of MTCs (median, 1; range, 0–6) was significantly higher ($P = .013$) than in FTCs (median, 0; range, 0–3).

P-gp Expression

Normal canine thyroid tissue did not have cells with membranous immunolabeling using monoclonal antibody JSB-1. With C494, weak membranous immunolabeling was observed in follicular cells (especially apical membrane), endothelial cells, and occasionally parafollicular cells.

Twenty-four percent of all primary thyroid tumors (7% FTCs, 70% MTCs) were positive for C494 and JSB-1 (Table 2, Fig 3) and therefore were considered truly positive for P-gp expression. The proportion of MTCs expressing P-gp was significantly higher ($P < .001$) than that of FTCs. The membranous staining intensity for C494 was considered mild in 4 (5%), moderate in 7 (9%), and strong in 7 (9%) primary tumors. The membranous staining intensity for JSB-1 was mild in 11 (15%), moderate in 4 (5%), and strong in 3 (4%) cases.

Discussion

Local invasive growth frequently precludes surgical excision of canine thyroid tumors and in up to 38% of dogs, the tumor has already metastasized by the time diagnosis.[3,4] Furthermore, approximately half of the patients treated with thyroidectomy experience local recurrence or metastatic disease within 2 years of surgery. Therefore, it is important to search for new treatment modalities. In this study, we found high expression of VEGF in both FTC and MTC, and common expression of cox-2 and P-gp in canine MTC, indicating that these may represent valuable therapeutic targets for dogs with inoperable thyroid tumors or metastatic disease.

In this study, 85% of all thyroid tumors (80% FTCs, 100% MTCs) expressed VEGF in ≥76% of tumor cells. In human thyroid carcinoma, VEGF expression also is up-regulated.[5] The high expression of VEGF observed in this study suggests it may play an important role in the progression of canine thyroid cancer. Consequently, the VEGF system seems to be an attractive target for the treatment of both FTC and MTC in dogs. In a preliminary study in dogs with solid tumors, a multitargeted TKI, targeting VEGFR-2, induced partial remission in 4 of 15 dogs and stable disease in 8 of the 15 dogs with thyroid carcinoma.[27]

Loss of function mutation of p53 often leads to accumulation of p53 in the nucleus, which becomes readily detectable by IHC staining.[8] In this study, no

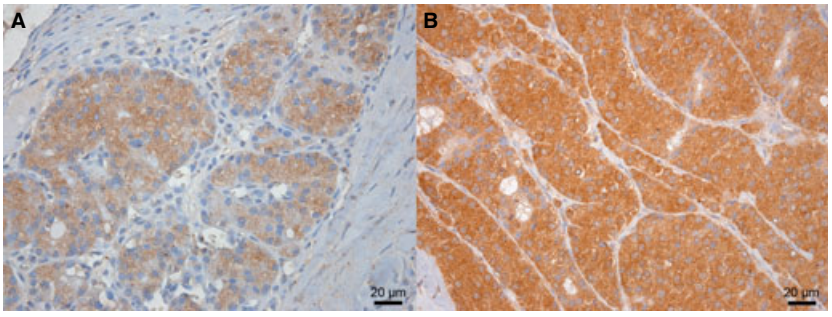

Fig 1. (A) Immunohistochemical expression of VEGF in an FTC of compact type with 76–100% of positive neoplastic cells (400×). (B) Immunohistochemical expression of VEGF in an MTC with 76–100% of positive neoplastic cells (400×).

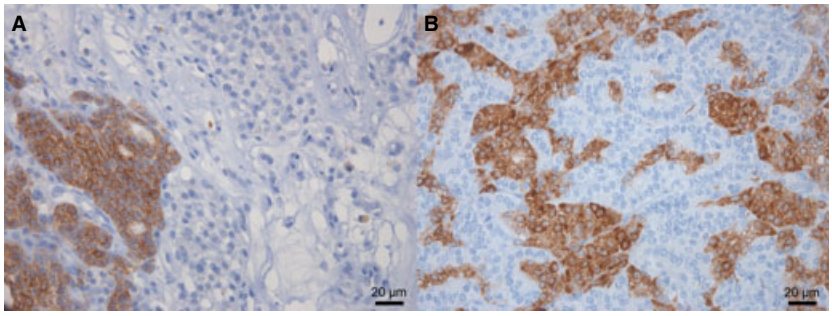

Fig 2. (A) Immunohistochemical expression of cox-2 in an FTC of follicular-compact type with labeling index of 6.8% (400×). (B) Immunohistochemical expression of cox-2 in an MTC with a labeling index of 22.4% (400×).

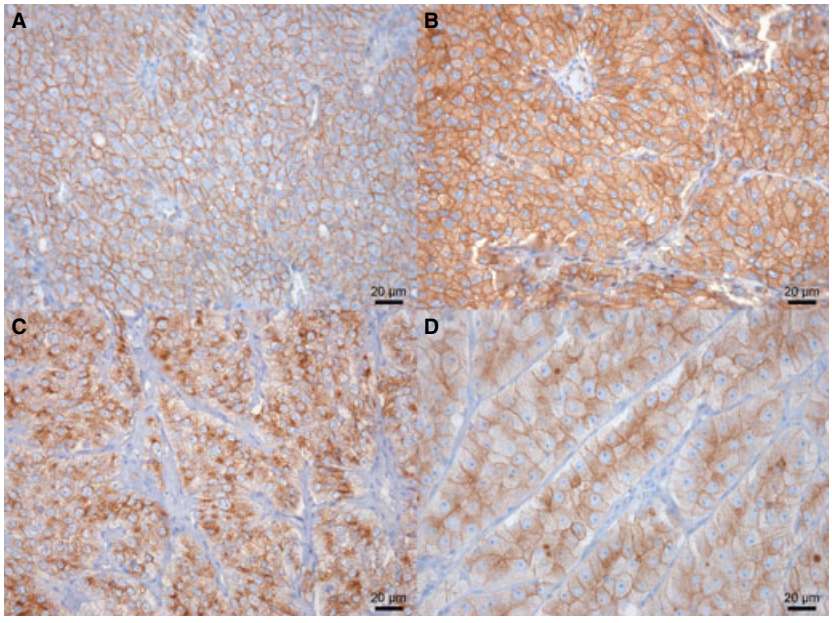

Fig 3. (A) Immunohistochemical expression of P-gp (C494) in an FTC of compact type (400×). (B) Immunohistochemical expression of P-gp (C494) in an MTC (400×). (C) Immunohistochemical expression of P-gp (JSB-1) in an FTC of compact type (400×). (D) Immunohistochemical expression of P-gp (JSB-1) in an MTC (400×).

section was considered positive for p53 protein. A low prevalence of p53 expression was expected because p53 mutation only was found in 1 of 23 canine thyroid tumors in a previous study examining part of the coding region of the p53 gene.[10] In humans, p53 mutations have been described in 40–62% of undifferentiated thyroid carcinomas and in 5–10% of other thyroid carcinomas.[9] A study investigating the immunohistochemical expression of p53 in canine malignant tumors with and without p53 mutations indicated that antibody PAb240 (used in this study) had a sensitivity of 36% and a specificity of 94% for detection of p53 mutations on FF-PE sections.[8] Although in that study another antibody (CM1) was reported to have the highest sensitivity (55%) for detection of p53 mutations in FF-PE tumor samples, in our laboratory it repeatedly stained nuclei of the negative control (normal thyroid gland) showing lack of specificity. Given the reported low sensitivity of IHC with PAb240, our results may underestimate the true prevalence of p53 mutation in canine thyroid carcinoma. Nevertheless, taking our results and results from previous studies into account, the p53 tumor suppressor gene does not seem to be a realistic therapeutic target for most cases of canine thyroid carcinoma.

In this study, cox-2 expression was observed in 50% of MTCs and in only 13% of FTCs. The higher prevalence of cox-2 expression in MTC is in agreement with reports in humans, where 26–41% of FTCs and 75–82% of MTCs have been shown to express cox-2.[28] In a study investigating cox-2 expression in canine invasive TCC of the urinary bladder, the percentage of positive tumor cells at diagnosis ranged from 1 to 22%, comparable to that found in our study.[29] Interestingly, although in that study all tumors were positive for cox-2 expression, no significant association was found between the level of cox-2 immunolabeling and tumor remission with piroxicam, observed in 12 of

18 dogs. This suggests that clinical benefit may be observed even in cases of low cox-2 expression. Our results suggest that cox-2 is an interesting molecular target in canine thyroid carcinoma, particularly in MTC.

In our study, P-gp expression with both antibodies was observed in 7% of FTCs and 70% of MTCs. These results suggest that expression of P-gp in canine MTC is common and could explain multi-drug resistance in these patients. Literature on the expression of P-gp in human thyroid carcinoma is scarce. In vitro studies have shown expression of the ABCB1 gene in tumor cells from 12 patients with MTC and in several MTC cell lines.[18,30] In humans, MTC is refractory to conventional chemotherapy which yields partial responses in only 10 to 20% of patients.[31] Experimental evidence suggests that multi-drug resistance is one of the mechanisms for this highly chemoresistant phenotype and that by targeting P-gp, chemoresistance can be reversed.[18,30] Our study suggests that P-gp is an interesting molecular target for the treatment of canine MTC. Inhibition of P-gp using specific P-gp inhibitors (eg, verapamil) or TKIs could increase tumor sensitivity to chemotherapy and improve outcome.

Medullary thyroid carcinoma may be difficult to distinguish from compact FTC by light microscopy alone.[3] The different expression of potential molecular targets between these tumor types observed in our study underlines the importance of their adequate differentiation using routine IHC. The higher expression of cox-2 and P-gp in MTC is in agreement with clinical and experimental studies in human thyroid cancer. In fact, there is experimental evidence of a direct causal relationship between cox-2 expression and P-gp regulation. Overexpression of cox-2 leads to increased expression and function of P-gp in a dose-dependent manner and this effect can be blocked by specific cox-2 inhibitors.[32] Furthermore, in vitro studies in MTC cells have shown the ability of cox-2 inhibitors to reverse multi-drug resistance in these cells by inhibiting the expression of P-gp.[18] In an in vivo model of human colorectal cancer, cox-2 expression was correlated with chemoresistant phenotype, and the most tumor regression was achieved with a combination of cox-2 inhibitors and chemotherapy.[33]

An estimation of the percentage of immunopositive tumor cells in each section was performed for VEGF and P-gp because often all tumor cells in each section were either positive or negative, unlike for cox-2 or p53. We therefore considered that it would be more time-efficient to provide an estimation of the immunopositivity in the entire section, as previously described, instead of counting cellular fields with identical immunopositivity.[22,26] Limitations of our study include the relative low number of MTCs and the fact that review of IHC was performed by only 1 observer. Although this may decrease the accuracy of scoring, it maximizes consistency of comparative scoring among sections. Additional studies are needed to confirm if protein expression, as identified by IHC, correlates with in vivo protein function.

In conclusion, this study shows that the VEGF system is a potential therapeutic target in both FTC and MTC in dogs. Cox-2 and P-gp seem to be attractive molecular targets in canine MTC. p53 does not seem to be a potential molecular target for canine thyroid carcinoma.

Footnotes

[a] EnVision Dako, Glostrup, Denmark
[b] DAB, Dako
[c] S/N S38-7410-01, Dako
[d] SAS 9.3, Cary, NC

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