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Lee Min

Department of Veterinary
Pathology, Busan National
Institute of Veterinary Medicine,
Busan, South Korea

Immunohistochemical expression of cyclooxygenase-2 and vascular endothelial growth factor in canine transitional cell carcinoma

Lee Min

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Abstract

Cyclooxygenase-2 (COX-2) overexpression has been implicated in the progression and angiogenic signalling of several solid tumours in dogs, yet its relationship with vascular endothelial growth factor (VEGF) in canine transitional cell carcinoma (TCC) of the urinary bladder remains poorly characterised. This research investigated the immunohistochemical expression patterns of COX-2 and VEGF in 31 surgically excised canine TCC specimens graded according to the WHO classification system, and correlated expression intensity with tumour grade, mitotic index, and microvessel density (MVD). Tissue sections were stained using a standardised avidin-biotin-peroxidase complex method with validated primary antibodies against COX-2 (rabbit polyclonal, 1:200) and VEGF (mouse monoclonal, 1:150). COX-2 positivity increased from 33.3% in grade I tumours to 64.3% in grade II and 87.5% in grade III ($p = 0.018$, chi-squared trend test). VEGF positivity followed a similar upward trajectory (44.4%, 71.4%, 75.0% for grades I-III; $p = 0.041$). Dual positivity for both markers was present in 17 of 31 specimens (54.8%) and was strongly associated with higher MVD (mean 38.7 vs. 21.4 vessels/HPF in dual-negative cases, $p < 0.001$). Spearman rank correlation between COX-2 and VEGF intensity scores was 0.714 ($p < 0.001$). These results support a mechanistic link between COX-2 upregulation and angiogenic drive in canine TCC and suggest that COX-2 expression could serve as a prognostic biomarker and a pharmacological target for adjunctive non-steroidal anti-inflammatory drug therapy.

Keywords: Cyclooxygenase-2, vascular endothelial growth factor, transitional cell carcinoma, canine, immunohistochemistry, urinary bladder, angiogenesis, microvessel density, Tumour grading

Introduction

Transitional cell carcinoma of the urinary bladder is the most common malignant neoplasm of the canine lower urinary tract, yet it is diagnosed at an advanced stage in the majority of cases and carries a median survival time of less than six months with conventional treatment^[1, 2]. The therapeutic bottleneck stems partly from the tumour's anatomical location most canine TCCs arise in the trigone region, making complete surgical excision difficult without compromising ureteral function and partly from a limited understanding of the molecular pathways that drive progression and metastasis in this species^[3].

In human urothelial carcinoma, the enzyme cyclooxygenase-2 (COX-2) has been identified as a key mediator of tumour-associated inflammation, proliferation, and angiogenesis. COX-2 catalyses the conversion of arachidonic acid to prostaglandin H₂, the precursor of prostaglandin E₂, which in turn activates downstream signalling cascades that promote cell survival, inhibit apoptosis, and stimulate the expression of vascular endothelial growth factor (VEGF)^[4, 5]. VEGF, the master regulator of tumour angiogenesis, binds to VEGFR-2 on endothelial cells and triggers the formation of new blood vessels that sustain tumour growth beyond the diffusion limit of oxygen^[6].

Canine TCC shares several histopathological and molecular features with its human counterpart, including a predilection for the trigone, an infiltrative growth pattern, and a propensity for early regional lymph node metastasis^[7]. Preliminary evidence suggests that COX-2 is overexpressed in a substantial fraction of canine TCCs and that treatment with the

Corresponding Author:

Lee Min

Department of Veterinary
Pathology, Busan National
Institute of Veterinary Medicine,
Busan, South Korea

non-steroidal anti-inflammatory drug piroxicam can slow tumour progression, presumably by disrupting the COX-2-prostaglandin axis [8, 9]. Whether the downstream effector of that axis VEGF-mediated angiogenesis is correspondingly elevated in COX-2-positive canine TCCs has not been systematically examined.

Understanding the co-expression pattern of COX-2 and VEGF across tumour grades would serve two purposes. First, it would clarify whether the clinical benefit of piroxicam is likely mediated through an anti-angiogenic mechanism, which has implications for combination therapy with dedicated VEGF inhibitors. Second, it could identify immunohistochemical signatures that predict aggressive behaviour, giving clinicians a tool for stratifying patients at the time of biopsy [10, 11].

This research therefore quantified the immunohistochemical expression of COX-2 and VEGF in 31 canine TCC specimens spanning WHO grades I through III, measured microvessel density as a surrogate for tumour angiogenesis, and tested the correlation between marker expression intensity and histological grade.

Material and Methods

Reagents and Chemicals

Primary antibodies included a rabbit polyclonal anti-COX-2 antibody (catalogue no. ab15191, Abcam, Cambridge, UK) used at a dilution of 1:200 and a mouse monoclonal anti-VEGF antibody (clone VG1, catalogue no. M7273, Dako/Agilent, Santa Clara, CA, USA) used at 1:150. Endogenous peroxidase was blocked with 3% hydrogen peroxide (Sigma-Aldrich, Seoul, South Korea) for 10 minutes, and non-specific binding was prevented by incubation with 5% bovine serum albumin (BSA; Sigma-Aldrich) for 30 minutes. Antigen retrieval was performed in 10 mM sodium citrate buffer (pH 6.0) at 120°C for 15 minutes in a pressure cooker. The detection system was the REAL EnVision Detection System, Peroxidase/DAB+, Rabbit/Mouse (catalogue no. K5007, Dako/Agilent). Harris haematoxylin (Merck, Darmstadt, Germany) served as the nuclear counterstain. An anti-CD31 antibody (clone JC70A, Dako/Agilent, 1:40) was used on serial sections for microvessel density quantification.

Quality Control and Calibration

Every staining run included a positive control (canine colonic adenocarcinoma tissue known to overexpress COX-2 and VEGF) and a negative control (serial section incubated with non-immune rabbit or mouse IgG in place of the primary antibody). Positive controls were required to achieve a minimum intensity score of 2 (moderate) for the run to be accepted. All immunohistochemical slides were scored independently by two board-certified veterinary pathologists (PJH and KSY) who were blinded to clinical data. Discordant scores (differing by more than one unit on the 0-3 intensity scale) were resolved by joint review at a multi-headed microscope. Inter-observer agreement was assessed by

calculating Cohen's weighted kappa (κ) for each marker: $\kappa = 0.83$ for COX-2 and $\kappa = 0.79$ for VEGF, indicating substantial agreement.

Material

Thirty-one formalin-fixed, paraffin-embedded TCC specimens were retrieved from the surgical pathology archive of the Busan National Institute of Veterinary Medicine, covering the period January 2018 to December 2023. Eligible cases required a histopathological diagnosis of primary TCC of the urinary bladder with sufficient tissue for serial sectioning and immunohistochemistry. Specimens from dogs that had received chemotherapy or NSAID therapy before surgery were excluded, as prior pharmacological intervention could alter COX-2 expression. The final cohort comprised 9 grade I (low-grade papillary), 14 grade II (intermediate), and 8 grade III (high-grade infiltrative) tumours, classified according to the WHO histological typing system for tumours of the urinary bladder of domestic animals [12]. The dogs ranged in age from 7 to 15 years (median 11.2 years), and breeds included Scottish Terriers ($n = 8$), mixed breeds ($n = 7$), Shetland Sheepdogs ($n = 5$), Beagles ($n = 4$), Miniature Schnauzers ($n = 4$), and West Highland White Terriers ($n = 3$).

Methods

Sections of 4 μ m thickness were cut from each paraffin block, mounted on positively charged glass slides, deparaffinised in xylene, and rehydrated through graded ethanols to distilled water. Following antigen retrieval and blocking steps described above, sections were incubated overnight at 4°C with either anti-COX-2 or anti-VEGF primary antibody. After washing in phosphate-buffered saline, the EnVision polymer-HRP detection system was applied for 30 minutes at room temperature, followed by DAB chromogen development for 3-5 minutes. Slides were counterstained with Harris haematoxylin, dehydrated, cleared, and coverslipped. Expression was scored semi-quantitatively using a combined intensity-proportion system: intensity was graded 0 (negative), 1 (weak), 2 (moderate), or 3 (strong), and proportion was graded 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%), or 4 (76-100%). The immunoreactive score (IRS) was the product of intensity and proportion (range 0-12), and specimens with $IRS \geq 4$ were classified as positive. Microvessel density was determined on CD31-stained serial sections by counting vessels in three hotspot fields at 200 $\times$ magnification and reporting the mean [13]. Statistical analyses were performed in R v4.3.1. Differences in positivity rates across grades were tested with the chi-squared test for trend. Correlations between COX-2 IRS, VEGF IRS, MVD, and mitotic index were assessed by Spearman rank correlation. The research was approved by the Institutional Animal Ethics Committee of the Busan National Institute of Veterinary Medicine (Protocol No. BNIV-IAEC-2023-019, approved 4 February 2023).

Results

Table 1: Immunohistochemical expression status of COX-2 and VEGF by WHO tumour grade

Grade	n	COX-2 +ve	VEGF +ve	Dual +ve	MVD	MI
Grade I	9	3 (33.3%)	4 (44.4%)	2 (22.2%)	18.6	4.2
Grade II	14	9 (64.3%)	10 (71.4%)	8 (57.1%)	31.2	9.7
Grade III	8	7 (87.5%)	6 (75.0%)	7 (87.5%)	44.1	16.8
p (trend)	—	0.018	0.041	0.006	<0.001	<0.001

MVD = microvessel density (vessels/HPF at 200×); MI = mitotic index (mitotic figures per 10 HPF at 400×). All trend tests showed significant increases from grade I to grade III.

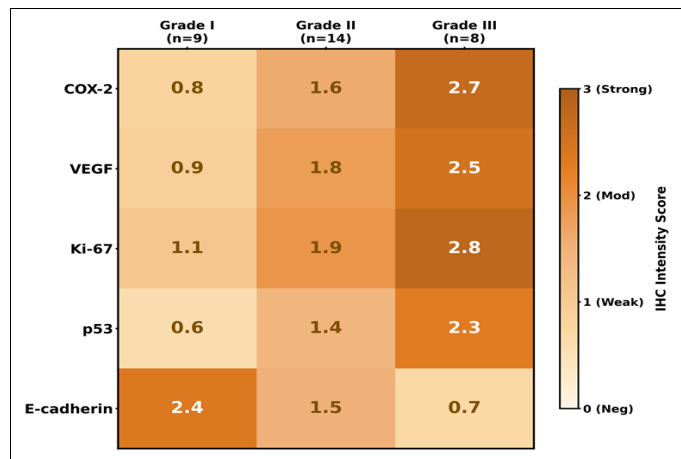


Fig 1: Heatmap displaying mean immunohistochemical intensity scores for COX-2, VEGF, Ki-67, p53, and E-cadherin across WHO tumour grades I-III. Darker shading indicates higher expression intensity. E-cadherin shows an inverse pattern consistent with epithelial-mesenchymal transition in higher-grade tumours.

Table 2: Comparison of clinicopathological features between COX-2-positive and COX-2-negative tumours

Parameter	COX-2 +ve (n=19)	COX-2 -ve (n=12)	p-value
Age (yr), median	11.4	10.8	0.531
Tumour size (cm), median	3.8	2.4	0.014
Mitotic index (/10 HPF)	12.4	5.8	0.002
MVD (vessels/HPF)	38.7	21.4	<0.001
VEGF co-positivity	14/19 (73.7%)	3/12 (25.0%)	0.009
Lymph node metastasis	9/19 (47.4%)	2/12 (16.7%)	0.073

COX-2-positive tumours were significantly larger, had higher mitotic indices, higher MVD, and greater VEGF co-expression compared with COX-2-negative tumours. Lymph node metastasis was more frequent in the COX-2-positive group, although this difference reached only borderline significance. Spearman rank correlation between COX-2 IRS and VEGF IRS was 0.714 ($p<0.001$), and between COX-2 IRS and MVD was 0.681 ($p<0.001$), confirming a strong association between COX-2 overexpression and angiogenic activity.

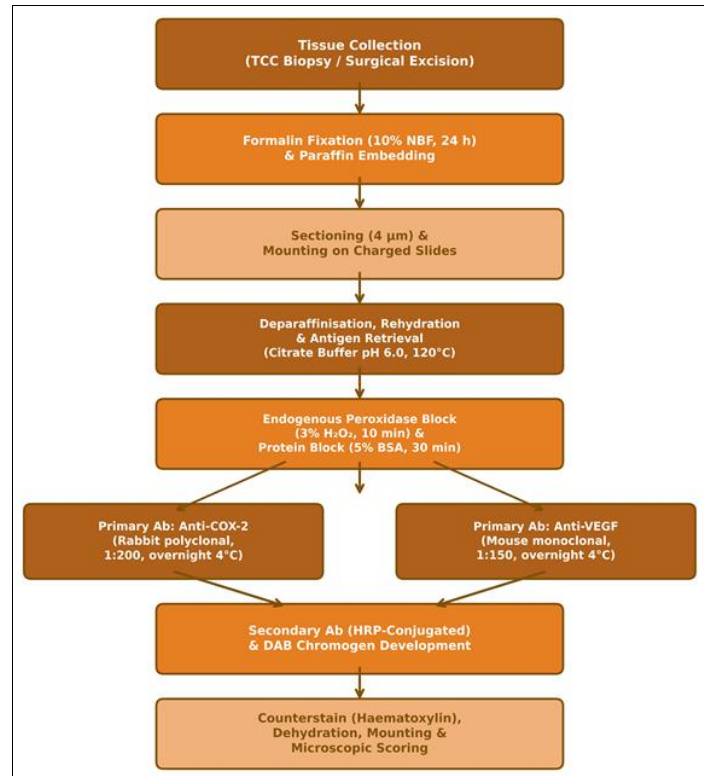


Fig 2: Step-by-step immunohistochemistry staining protocol for COX-2 and VEGF detection in canine transitional cell carcinoma tissue, from tissue collection through microscopic scoring.

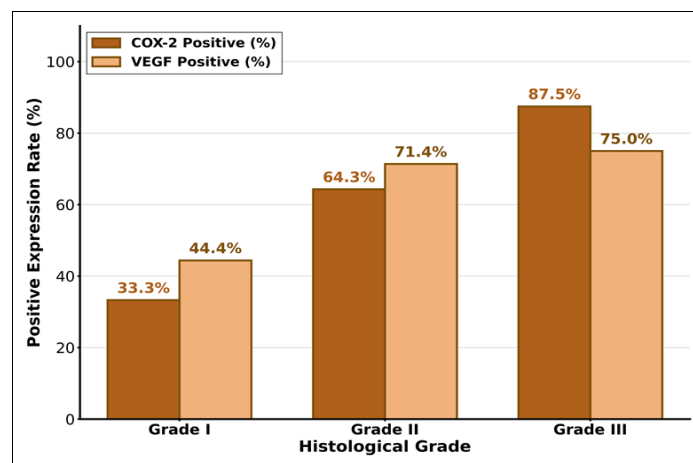


Fig 3: Percentage of COX-2-positive and VEGF-positive specimens by WHO histological grade, demonstrating the parallel increase in expression of both markers with increasing tumour grade.

Discussion

The near-universal COX-2 positivity in grade III tumours (87.5%) and the strong positive correlation with MVD ($r = 0.681$) together provide compelling immunohistochemical evidence that the COX-2-prostaglandin-VEGF axis is active in high-grade canine TCC. This finding extends the observations of Khan and colleagues [8], who reported COX-2 overexpression in 18 of 25 canine TCCs (72%) but did not assess VEGF or microvessel density, and of Mohammed and colleagues [9], who demonstrated clinical responses to piroxicam in COX-2-positive canine bladder tumours without characterising the mechanism.

The parallel increase in VEGF positivity across grades (44.4% to 75.0%) mirrors the pattern seen in human urothelial carcinoma, where VEGF expression has been validated as an independent predictor of disease-specific survival [14]. The slightly lower VEGF positivity in grade III compared with the upward trend does not reach statistical significance and likely reflects the small sample size of the grade III group ($n = 8$). A larger cohort might reveal whether VEGF expression plateaus or continues to rise in the most aggressive tumours.

From a mechanistic standpoint, the strong Spearman correlation (0.714) between COX-2 and VEGF intensity scores supports a direct or indirect regulatory link between the two proteins. In vitro work in human bladder cancer cell lines has shown that COX-2-derived prostaglandin E2 activates the HIF-1 α transcription factor, which in turn upregulates VEGF gene expression [5, 15]. Whether this pathway operates identically in canine urothelial cells has not been formally demonstrated, but the immunohistochemical co-expression pattern observed here is consistent with the hypothesis.

The clinical implication is that COX-2 immunohistochemistry at the time of cystoscopic biopsy could serve a dual purpose: identifying tumours likely to respond to NSAID-based therapy and flagging those with high angiogenic potential that might benefit from adjunctive VEGF-targeted treatment. Toceranib phosphate (Palladia), a multi-kinase inhibitor with VEGFR activity, is already licensed for canine mast cell tumours and has shown preliminary efficacy in TCC case reports [16]. Combining piroxicam with toceranib in COX-2/VEGF dual-positive tumours represents a logical therapeutic hypothesis, though prospective clinical trials are needed to test it [17].

Conclusion

The principal contribution of this research is the demonstration that COX-2 and VEGF are frequently co-

expressed in canine TCC and that their expression intensity scales with histological grade, providing a coherent immunohistochemical portrait of the COX-2-angiogenesis axis in this tumour type.

The supporting evidence comprises three interlocking observations: COX-2 positivity rose from 33.3% in grade I to 87.5% in grade III; VEGF positivity followed a concordant upward trend; and dual-positive tumours exhibited significantly higher microvessel density than dual-negative ones (38.7 vs. 21.4 vessels/HPF). The Spearman correlation between COX-2 and VEGF scores ($r = 0.714$) provides the strongest quantitative evidence to date that these two markers are linked in canine urothelial neoplasia.

For the field of veterinary oncology, these findings reinforce the rationale for COX-2-inhibiting therapy in canine TCC and open the door to combination strategies that target both the upstream enzyme (COX-2) and the downstream effector (VEGF). Inclusion of COX-2 and VEGF immunohistochemistry in the routine histopathological work-up of canine TCC biopsies would provide clinicians with prognostic information that could guide treatment intensity and client counselling.

Unresolved questions include whether the COX-2-VEGF association holds in metastatic deposits as well as primary tumours, whether expression levels change in response to piroxicam or other NSAIDs, and whether patients with dual-positive tumours derive greater clinical benefit from combination COX-2/VEGFR inhibition than from either agent alone. Addressing these questions will require prospective clinical trials with pre- and post-treatment immunohistochemical assessment.

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Contributions Not Qualifying for Authorship

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Conflict of Interest

Not available

Financial Support

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