**Expression of VEGFR and PDGFR-α/-β in 187 canine nasal carcinomas.**

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**Introduction**

Canine nasal tumours account for approximately 1% of all neoplasms in dogs. Average age at presentation is 10 years, and medium to large breeds are more commonly affected (1,2). Intranasal carcinomas [adenocarcinoma, squamous cell carcinoma (SCC) and undifferentiated carcinoma] represent two-thirds of nasal neoplasms (3). Their biological behaviour is characterized by progressive local invasion and a generally low metastatic rate at the time of diagnosis, though metastases are evident in 40% to 50% of dogs at the time of death, with regional lymph nodes and lungs most commonly affected (2). The presence of regional lymph node metastasis at diagnosis is associated with a poor outcome (4).

A median survival time (MST) of 95 days has been reported for nasal carcinomas if no treatment is pursued (5). The main goal of therapy is typically to control local disease and treatment is most often radiotherapy (RT) alone, although surgery (rhinotomy), alone or in combination with RT is also reported (6–8). MSTs following surgery alone are approximately three to six months although the procedure is associated with a high rate of morbidity (1). Reported MSTs with curative intent RT range between eight and 19.7 months, with one- and two-year survival rates of 43% to 68% (9–12). A combination of surgical debulking and adjuvant RT has not been proven to increase MSTs (1). Combining RT with cyclooxygenase-2 (COX-2) inhibitors and chemotherapy has also been investigated, but no survival benefit (MST of 201 to 474 days) compared to RT alone was identified (6,7,13). Chemotherapy as a sole treatment is not routinely recommended, but is reported: one study showed some benefit for individual dogs with a clinical response rate of 27% using single-agent cisplatin treatment (14). Another small study reported an objective response rate of 75% with resolution of clinical signs in all patients using a combination treatment of chemotherapy with doxorubicin and carboplatin and the COX-2 inhibitor piroxicam (15). Tyrosine kinase inhibitors may offer an additional therapeutic approach: a recent study demonstrated a 71.4% response rate to toceranib phosphate in a small patient cohort with nasal carcinoma, in which the majority of patients received prior RT(16).

Despite most dogs experiencing a favourable clinical response to RT the long-term survival is poor, due to local recurrence. Understanding the molecular mechanisms associated with canine nasal carcinoma oncogenesis may provide additional targets for therapy. Studies have identified p53 accumulation in nasal adenocarcinomas (17) and expression of cyclooxygenase-2 (18,19), peroxisome proliferator-activated receptor γ (20) and either epithelial growth factor receptor (EGFR) or vascular endothelial growth factor receptor (VEGFR) in nasal carcinomas (21).

VEGFR has been shown to be responsible for vasculogenesis as well as angiogenesis. Stimulation of VEGFRs leads to endothelial proliferation, migration and survival (22). VEGF is closely related to the platelet-derived growth factor (PDGF) family and its receptor PDGFR has also been shown to be involved with angiogenesis. Signalling from these receptor tyrosine kinases (rTK) has the potential to produce a supportive microenvironment for neoplasms by providing vasculature and proliferative drive. These rTKs are expressed in a variety of canine neoplasms including mast cell tumours (MCT) (23), anal sac adenocarcinomas (ASAC) (16), T-cell lymphomas (24) and nasal carcinomas (21).

In veterinary medicine there are currently two licensed tyrosine kinase inhibitors (TKIs), masitinib and toceranib phosphate. Masitinib has inhibitory activity against KIT, PDGF and the fibroblast growth factor 3 receptor (25), whereas toceranib phosphate targets KIT, VEGF and PDGF receptors (26). These TKIs were primarily developed for the inhibition of KIT signalling in canine MCTs, but they have been shown to have a spectrum of activity including ASACs, thyroid carcinomas, pulmonary carcinomas, nasal carcinomas and metastatic osteosarcomas (16,27,28).

In this study we sought to characterise the expression of VEGFR, PDGFR-α and PDGFR-β in a large group of canine malignant intranasal carcinomas as a first step to a rational assessment of the potential efficacy of TKIs in this tumour type.

**Material and methods**

*Patient collective and tissue selection*

The database at Bridge Pathology Ltd. in Bristol, United Kingdom, was searched for canine intranasal carcinomas. Tissue samples had been sent by first opinion practices and referral clinics in the United Kingdom. In the database tissue samples of 272 dogs with intranasal carcinoma were diagnosed between 2009 and 2014. All tissue was fixed in 10% neutral buffered formalin and subsequently paraffin embedded. Characterization of tumour subtype of all 272 tissue samples was confirmed in haematoxylin and eosin-stained sections by one of the board-certified pathologists at Bridge Pathology Ltd. according to established criteria (29). In all cases the original diagnosis was used. Eighty five samples were excluded for one or more of the following reasons: 1) the nasal carcinoma was associated with the nasal planum rather than intranasal, 2) no definitive diagnosis was achieved via histopathology alone and no immunohistochemistry was performed, 3) submitted sample volume was too small to perform further analysis. Therefore, a total of 187 archived tissue samples were included.

*Identification of tumour tissue and preparation of tissue microarrays*

For all 187 tissue blocks the corresponding slides were reviewed to identify the tumour tissue and to separate it from areas of normal tissue, inflammation or necrosis. The identified tumour tissue was punched out using a disposable punch biopsy (Punch biopsy, 2mm, Kai Medical). Twenty punches (one of each tissue sample) were re-embedded in one paraffin tissue block (Leica EG 1160 paraffin embedding station) along with two internal positive controls (canine ASAC [PDGFR], canine granulomatous tissue, [VEGFR]) for which positive expression of VEGFR, PDGFR-αand PDGFR-β has already been described (30,31).

*Immunohistochemical staining for VEGFR, PDGFR-α and PDGFR-β*

Antibody optimization was performed using a commercially available antibody diluent (Envision FLEX antibody diluent, DAKO). A dilution of 1:100 was suitable for VEGFR and PDGFR-β and a dilution of 1:50 was chosen for PDGFR-α. The optimal dilution factor was chosen based on expression intensity and pattern by a board-certified pathologist (Tim Scase). For each antibody optimization two tissue blocks (canine ASAC for PDGFR; canine granulomatous tissue for VEGFR) were used.

Per paraffin-embedded tissue block 3 μm sections were taken (Leica RM2235) and mounted on positively charged glass lysine slides (Superfrost Plus, Menzel). Two glass lysine slides were created per tissue block as an additional control during immunostaining. Sections were de-waxed and a heat mediated antigen retrieval was performed in a high (Tris/EDTA buffer, pH 9) pH solution (Envision FLEX Target Retrieval Solutions, DAKO) in a water bath (PT Link, DAKO) at 97°C for 30 minutes. Immunohistochemistry was performed using an automated immunohistochemical staining machine (DAKO Cytomation Autostainer Plus). Endogenous peroxidase activity was blocked by incubation in hydrogen peroxide solution for 30 minutes (EnVision FLEX Peroxidase-Blocking Reagent). The tissue sections were incubated with the diluted (Envision FLEX antibody diluent, DAKO) primary antibodies for 30 minutes at room temperature. Immunohistochemical staining was detected using a monoclonal mouse anti-human VEGFR (sc-393163 FLK-1 (D-8), Insight Biotechnology Ltd.) (32), a polyclonal rabbit anti-human PDGFR-α (sc-431 PDGFR-alpha (951), Insight Biotechnology Ltd.) (33) and a polyclonal rabbit anti-human PDGFR-β antibody (sc-432 PDGFR-beta (958), Insight Biotechnology Ltd.) (34) which have been previously validated for use with formalin fixed canine tissue (33,35,36). Staining was developed with 3,3’-diaminobenzidine tetrahydrochloride (EnVision FLEX DAB+ Chromogen, DAKO) and counterstained with haematoxylin. Replacing the primary antibody with antibody dilution buffer acted as a negative controls.

*Immunohistochemical evaluation protocol*

Immunohistochemical evaluation was done via evaluation of staining intensity and percentage of rTK positive tumour cells. Staining intensity was graded as no immunostaining, weak immunostaining, moderate and intense immunostaining. Positivity of tumour cells was assessed in five separate fields at x40 magnification and the average of the five fields was given as percentage. Percentage positivity was graded as follows: 0%, 1–25%, 26-75% and 76-100%. Necrotic areas were avoided because inflammatory cells and stromal macrophages may express certain rTKs. Tumours were considered positive for rTK if at least a weak immunostaining was present in at least 1-25% of tumour cells. Immunohistochemical localization was defined as cytoplasmic, membranous, cytoplasmic-membranous, nuclear or stromal depending on the predominant (>50%) staining pattern (Table 1).

This study obtained ethics approval by the Veterinary Research Ethics Committee (University of Liverpool, School of Veterinary Science, Neston, UK) with the reference number VREC319.

**Results**

*Sample demographics*

Tissue samples were available for immunohistochemistry from 187 dogs. In 69 cases a definitive diagnosis was achieved via evaluation of histopathological features alone; in 111 cases a carcinoma was diagnosed based on histopathological features alone but a further subclassification was not possible and in seven cases immunohistochemistry was performed to achieve a definitive diagnosis. In four cases the sex was not specified at submission. Of the remaining 183, 100 dogs were male (male entire, n=36; male neutered, n=64) and 83 were female (female entire=24; female spayed=59). The female to male ratio was 1:1.2. Forty-three different breeds were presented of which Golden and Labrador Retriever (n=38), cross breeds (n=34) and Border Collies (n=18) were the most common. The median age was 9.9 years (range 2-16 years). Information on clinical signs, duration of clinical signs, tumour involvement of other structures and regional or distant metastasis was variably recorded on the submission forms; it was therefore decided not to include this information in this study.

*Histopathological findings and immunohistochemistry*

Of the 187 carcinomas diagnosed 36 (19.3%) were transitional cell carcinomas (TCC), 23 (12.3%) were squamous cell carcinomas (SCCs), eight (4.3%) were poorly differentiated carcinomas (PCA) and nine (4.8%) were other carcinomas (OCAs); (adenocarcinomas (ACA), n=3; neuroendocrine carcinomas (NCA), n=3; tubulopapillary carcinomas (TCA), n=3) (Figure 1)). Fifty nine point three percent (n=111) of the OCAs could not be definitively classified using histopathological features alone. In seven (3.7%) of these, immunohistochemistry was performed to achieve a definitive diagnosis (OCAs, n=2; SCCs, n=1; NCAs, n=1; PCAs, n=3). In the remaining 104 cases, the final diagnosis was simply recorded as carcinoma.

In 154 cases (82.3%) concurrent lymphocytic, plasmacytic and occasionally neutrophilic rhinitis was evident.

VEGFR was expressed in 158 (84.5%) of cases including one NCA, two ACAs, three TCAs, seven PCAs, 19 SCCs, 30 TCCs and 96 OCAs (Table 2). A weak expression pattern was identified in 22 cases (11.8%), a moderate expression in 69 (36.9%) and an intense expression in 67 (35.8%) cases (Figure 2). In nine cases between 1-25% of tumour cells expressed VEGFR, whereas in 28 dogs 26-75% of tumour cells and in 121 dogs >76% of tumour cells expressed VEGFR (Table 2). The cytoplasmic-membranous expression pattern was predominant in 111 cases (70.9%) followed by cytoplasmic expression in 41 dogs (21.9%); membranous expression in five cases and nuclear expression in one case (Figure 2).

PDGFR-α was detected in 133 (71.1%) of cases (Table 2). Amongst these, two NCAs and TCAs, six PCAs, 17 SCCs, 23 TCCs and 83 OCAs were identified; no PDGFR-α expression was evident in ACAs. A weak expression pattern was present in 77 cases (57.9%), a moderate expression in 55 of cases (41.3%) and an intense one in one (0.5%) patient. In ten cases between 1-25% of tumour cells expressed PDGFR-α, in 17 dogs 26-75% of tumour cells and in 106 dogs >76% of tumour cells expressed PDGFR-α (Table 2). The predominant expression pattern was cytoplasmic in 117 cases (87.9%) followed by a cytoplasmic-membranous expression in 15 dogs (8.0%) and a membranous expression in one (0.5%) case (Figure 2).

PDGFR-β was identified in 74 (39.6%) patients including one NCA, three TCAs and PCAs, 12 SCCs, 13 TCCs and 42 OCAs; none of the ACAs expressed PDGFR-β (Table 2). PDGFR-β showed a weak expression in 28 cases (15.0%), a moderate expression in 33 (17.6%) and an intense expression in 13 dogs (7.0%). In ten cases between 1-25% of tumour cells expressed PDGFR-β, whereas in 27 dogs 26-75% of tumour cells and in 37 dogs >76% of tumour cells expressed PDGFR-β (Table 2). The cytoplasmic expression pattern was most predominant in 47 patients (63.5%) followed by a cytoplasmic-membranous pattern in 26 (13.9%) and a membranous pattern in one (0.5%) case (Figure 1).

Co-expression of rTKs was common with 70 dogs (37.2%) expressing two and 63 (33.5%) expressing all three rTKs; in 36 (19.1%) cases only one rTK was expressed. One hundred twenty five cases (94.0%) with PDGFR-α expression showed co-expression of VEGFR and 69 cases (93.2%) with PDGFR-β expression exhibited simultaneous expression of VEGFR. Stromal expression of VEGFR was seen in 16 (8.5%), PDGFR-α in 29 (15.5%) and PDGFR-β in 114 (60.9%) cases (Figure 2).

**Discussion**

It is well recognized that rTKs are important factors in the development of malignant neoplasms in veterinary medicine. This is currently best characterized in canine MCTs (37). After approval of TKIs for the treatment of canine MCTs, further studies have been conducted to evaluate their use in other solid tumours, e. g. canine ASACs (16,38). Despite reported use of TKIs in nasal tumours, to date, molecular markers, particularly VEGFR, PDGFR-α and PDGFR-β, have not been evaluated in a large cohort of canine nasal carcinomas.

We have attempted to characterize these tumours using tissue immunohistochemistry to determine the strength and pattern of VEGFR, PDGFR-α and PDGFR-β expression, as they represent targets for toceranib phosphate as well as masitinib (25,26). Seven different subtypes of canine intranasal carcinoma, OCA, ACA, PCA, SCC, TCC, NCA and TCA were evaluated. In this study, 84.5% of canine nasal carcinomas were positive for VEGFR staining, 71.1% for PDGFR-α and 39.6% for PDGFR-β.

VEGFR expression was relatively intense with >76% of tumour cells expressing the rTK in the majority of dogs (in 121 of 187). This is also represented in the relatively high median expression score of 134.7. The predominant VEGFR expression pattern in this study was cytoplasmic-membranous (70.9%). This is an interesting finding as tyrosine kinases (TKs) move away from the membrane once they have been activated (39). A similar staining pattern has already been demonstrated for KIT in canine MCTs and was associated with their grade. Diffuse cytoplasmic staining was associated with higher grade MCTs whereas low grade tumours more commonly had membranous staining (40). The expression pattern of VEGFR may therefore suggest activation of this TK and thus might be a relevant consideration in predicting a nasal carcinoma’s biological behaviour. In previous reports of canine oral melanoma and canine mammary tumours, a predominant cytoplasmic pattern was similarly demonstrated (41,42). Another study demonstrated a nuclear staining pattern (two cases), which was observed in only one case in this study (21). Nuclear staining may represent aberrant localization of VEGFR but its relationship to tumour behaviour is unclear. Given the strong expression of VEGFR in canine nasal carcinomas, this rTK may play some role in the development or progression of this cancer and may therefore give possible indication for the use of TKIs. Considering that RT currently plays an important role in the treatment of canine intranasal carcinomas, the concurrent use of TKIs could be considered to enhance the effect of irradiation, leading to improved outcomes. As abnormal tumour vessel morphology contributes to intratumoral hypoxia stimulating angiogenesis via VEGFR, TKIs may normalize tumour blood vessels leading to improved tissue oxygenation and thereby increased sensitivity to radiotherapy (43–46). Although there is currently no data on radiosensitization in canine intranasal carcinomas, inhibition of VEGFR in combination with RT has shown promise in treatment improvement in human clinical trials (47,48).

PDGFR-α expression was relatively weak to moderate with >76% of tumour cells expressing the rTK in the majority of dogs (n=106). This is also reflected in the relatively lower median expression score of 97.8 compared to VEGFR expression. PDGFR-β expression was also relatively weak to moderate compared to PDGFR-α with 26-75% (n=27) and >76% of tumour cells (n=37) expressing the rTK. This is again reflected in the relatively lower median expression score of 54.6 compared to VEGFR expression. The predominant PDGFR expression pattern in the current study was cytoplasmic (PDGFR-α, 87.9%; PDGFR-β, 63.5%) as already reported in previous studies with canine vascular tumours (49). Interestingly, this study also demonstrated a strong stromal expression for both PDGFRs (PDGFR-α in 15.5%; PDGFR-β in 60.9%) as compared to VEGFR (8.5%). This has already been previously described in human breast cancer (50). While VEGFR exerts important cellular functions, such as driving angiogenesis in tumour development, through a predominant cytoplasmic expression in tumur cells (following TK activation), PDGFRs may play a more important control function in the tumour’s microenvironment – hence its strong stromal expression. The tumour microenvironment is involved with increased vessel function and can thereby also lead to increased tumor growth (51). Inhibition of PDGFRs may therefore lead to alterations in the tumour microenvironment (stromal expression) as well as at tumour cells (cytoplasmic expression). Targeting stromal tissue has been documented in human solid tumours (52).

This study had some limitations including possible heterogeneity within individual tumours and small sample size within the microarray. In addition, only one sample per tumour was assessed; as these samples were small an individual sample may not have reflected heterogenous rTK expression. However, the large number of individual tumour samples should give sufficient power to limit this particular limitation across tumour populations. However, for individual patients, tumour heterogeneity could be very important as it could limit the effectiveness of a particular targetted therapy chosen on the basis of a small sample. If only a small subpopulation of tumour/stroma cells express the target, then a significant treatment response is less likely. We were also unable to correlate expression with tumour subtype, because of the high percentage of carcinomas without further sub classification and the small numbers of specified subgroups.

In the current study, expression of the three evaluated rTKs was predominantly intense with >76% of tumour cells expressing VEGFR, PDGFR-α and PDGFR-β. Interestingly, none of the ACAs in this study expressed PDGFRs, but they exhibited a predominantly intense VEGFR expression with >76% of tumour cells expressing the rTK. In summary, we can state that the majority of canine intranasal carcinoma subtypes expressed at least one rTK intensely in >76% of tumour cells. We therefore conclude that further investigation of the clinical utility of TKI treatment for canine intranasal carcinomas with should be pursued.

**Conflict of interest statement**

Prof. Laura Blackwood, was a member of the ACEE panel funded by Pfizer (2009-2012).

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**Table 1: rTK scoring protocol**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Staining intensity**  | **Parameter on x20 magnification** | **Positive tumour cells** | **Parameter on x40 magnification** | **Staining localization** | **Parameter on x40 magnification** |
|  | no staining |  | 0% |  | cytoplasm |
|  | weak |  | 1-25% |  | membranous |
|  | moderate |  | 26-75% |  | cytoplasm-membranous |
|  | intense |  | 76-100% |  | nuclear |

**Table2: rTK expression in 187 canine nasal carcinomas**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Expression of rTK** | ***Expression (patient no.)*** | **CA**(n=111) | **ACA**(n=3) | **PCA**(n=8) | **TCC**(n=36) | **SCC**(n=23) | **NCA**(n=3) | **TCA**(n=3) |
| **VEGFR** |  | *Expression (total)* | *96* | *2* | *7* | *30* | *19* | *1* | *3* |
| *Intensity**immunostaining* | *weak* | 14 | 0 | 0 | 3 | 4 | 0 | 1 |
| *moderate* | 39 | 2 | 3 | 13 | 12 | 0 | 0 |
| *intense* | 43 | 0 | 4 | 14 | 3 | 1 | 2 |
| *Positivity tumour cells* | *1-25%* | 4 | 0 | 0 | 2 | 1 | 0 | 2 |
| *26-75%* | 18 | 1 | 3 | 3 | 3 | 0 | 0 |
| *76-100%* | 74 | 1 | 4 | 25 | 15 | 1 | 1 |
| **PDGFR-α** |  | *Expression (total)* | *83* | *0* | *6* | *23* | *17* | *2* | *2* |
| *Intensity**immunostaining* | *weak* | 50 | 0 | 5 | 11 | 13 | 2 | 0 |
| *moderate* | 32 | 0 | 1 | 12 | 4 | 0 | 2 |
| *intense* | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Positivity tumour cells* | *1-25%* | 7 | 0 | 0 | 1 | 2 | 0 | 0 |
| *26-75%* | 12 | 0 | 0 | 3 | 1 | 1 | 0 |
| *76-100%* | 64 | 0 | 6 | 19 | 14 | 1 | 2 |
| **PDGFR-β** |  | *Expression (total)* | *42* | *0* | *3* | *13* | *12* | *1* | *3* |
| *Intensity**immunostaining* | *weak*  | 15 | 0 | 0 | 6 | 6 | 0 | 1 |
| *moderate* | 24 | 0 | 2 | 4 | 3 | 0 | 0 |
| *intense* | 3 | 0 | 1 | 3 | 3 | 1 | 2 |
| *Positivity tumour cells* | *1-25%* | 7 | 0 | 1 | 1 | 1 | 0 | 0 |
| *26-75%* | 16 | 0 | 1 | 3 | 5 | 1 | 1 |
| *76-100%* | 19 | 0 | 1 | 9 | 6 | 0 | 2 |

**Figure 1: Histopathological features nasal carcinomas**

Figure 2 with pictures a-c demonstrates the different histopathological features of canine nasal carcinomas. Picture a shows a transitional cell carcinoma which is in general a poorly differentiated neoplasm of the nasal surface epithelium. The tumour cells are closely packed and do not form intercellular bridges. Tumour cells are polyhedric and often arranged in solid sheets (here) or cords. Picture b demonstrates a squamous cell carcinoma of the non-keratinizing subtype with small pseudolumina containing eosinophilic material. Tumour cells form solid islets with intercellular bridges. Picture c shows a poorly differentiated carcinoma with small round to pleomorphic cells. Tumour cells do not resemble features of glandular or squamous differentiation. Abundant mitotic figures and areas of necrosis indicate a high degree of malignancy.

**Figure 2: Expression of VEGFR, PDGFR-α and PDGFR-β**

The expression pattern of VEGFR, PDGFR-α and PDGFR-β in different subtypes of canine nasal carcinomas are depicted in pictures a-i. Picture a and b show an adenocarcinoma, picture c, d and f demonstrate a poorly differentiated carcinoma whereas pictures e and g-i show carcinomas.

*a-c* VEGFR immunostaining: *a-b* - moderate to intense staining with cytoplasmic-membranous expression pattern, *c* - intense membranous and nuclear pattern;

*d-f* PDGFR-α immunostaining: *d* - weak cytoplasmic expression pattern, *e* - moderate to intense cytoplasmic-membranous expression pattern, *f* - intense cytoplasmic-membranous expression pattern;

*g-i* PDGFR-β immunostaining: *g* - no staining, *h* - stromal staining, *i* - weak to moderate cytoplasmic-membranous expression pattern