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PDGFRs expression in dogs affected by malignant oral melanomas: correlation with prognosis

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Abstract

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Canine malignant melanoma (CMM) is the most common canine oral tumour, and up to 70–75% of dogs in stage II–III die within 1 year after surgery. The purpose of this study was to evaluate the expression of platelet-derived growth factors receptors (PDGFR)-α and -β in stage II and III CMMs and to correlate it with prognosis. PDGFRs expression was evaluated by immunohistochemistry on 48 cases of formalin-fixed CMM samples and correlated with clinical–pathological findings and outcome after surgery. PDGFRs co-expression was observed in 37.5% of cases. Positivity for PDGFR-α and -β receptor was present in 54.2 and 47.9% of cases, respectively. Ki67 values >19.5% were ascertained in 66.7% of cases. Statistical analysis showed that PDGFRs co-expression and Ki67 values > 19.5% were both associated with worse prognosis. PDGFRs expression suggests a role in the pathogenesis and progression of CMM, and α and β co-expression appears to be associated to worse prognosis.

Keywords
canine malignant melanoma, PDGF receptors, prognostic factor, targeted therapy

Introduction

Malignant melanoma (MM) represents the most frequent oral neoplasm occurring in dogs.[1–3] Oral canine MM (CMM) has an aggressive behaviour, grows rapidly, is locally invasive, frequently metastasizes to regional lymph nodes (RLNs) and distant sites, and it may recur following surgical resection. Nuclear atypia, mitotic index and Ki67 index are the prognostic factors that are known to be most significant.[4] The molecular alterations involved in CMM arising from mucosal or digital sites have not been yet fully identified. Recently, Gillard *et al.*[5] used cDNA sequencing data from 95 dogs to detect somatic mutations in NRAS and PTEN genes at human hotspot sites, while no mutations were found in the analysis of BRAF Exon 15,[6] as frequently occurs in human melanomas.[7,8]

Platelet-derived growth factors receptors (PDGFR-α and PDGFR-β) are tyrosine kinases receptors that can activate many of the major signal transduction pathways, including phosphatidylinositol 3-kinase (PI3K), Ras, mitogen-activated protein kinase (MAPK) and phospholipase Cγ pathways.[7]

They are involved in physiological and pathological diseases mainly by paracrine mechanisms. In the physiological processes of adults, they stimulate fibroblast and endothelial cell proliferation and are involved in tissue regeneration and fibrotic processes; during embryogenesis they are responsible for tissue differentiation.[8,9] In human cancers PDGFRs can be activated by various genetic alterations,[10,11] and tumours of mesenchimal, glial and haematopoietic origin may show PDGFRs dysfunctions.[12] The most frequent alterations are over-expression, constitutive activation of the tyrosine kinase domain as well as post-transcriptional regulation by specific RNA sequences such as miRNA 34.[13] The dysfunction of tyrosine kinases occurs frequently in human cancers, and more studies indicate that a similar pattern of dysfunction may also be observed in canine and feline

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cancers.[14,15] In domestic animals PDGFRs have been studied in canine osteosarcoma, lymphoma, apocrine gland carcinoma, glioma and hemangiosarcoma but their expression has not been found to be correlated to prognosis.[16–20]

The aim of this research was to evaluate the expression of PDGFR-α and -β in CMM, in order to identify their role in the tumour pathogenesis and their possible correlation with prognosis.

Materials and methods

Sample collection and clinical follow-up

The tissue samples examined were from spontaneous oral CMMs, treated between 1998 and February 2014 at the Department of Veterinary Sciences of the University of Turin. In all cases, the initial data collected included patient history, physical examination, blood cell count, serum biochemistry and urinalysis. Fine needle aspiration of palpable RLNs, even if not enlarged (as size has not been considered sufficiently predictive)[21] and/or biopsy of the primary lesion were used for preoperative tumour diagnosis. A definitive and more objective staging was achieved in all cases via the surgical removal of all palpable RLNs at the time of primary tumour resection and their full histological evaluation. Full tumour staging included a skull and three-view chest radiographs and abdominal ultrasound examination; alternatively, a total body CT-scan was performed. Dogs without concurrent life-threatening diseases but with histologically confirmed stage II (2–4 cm diameter, negative RLN) or III (>4 cm diameter and negative RLN or any tumour size with regional-positive surgically resected RLN)[22] oral CMM were included in the study. All the animals were followed until the recurrence of the neoplasm, death or for a minimum of 12 months after surgery. Together with the regional lymphadenectomy, a primary tumour *en bloc* resection was performed, with the inclusion, when feasible, of at least 2 cm of macroscopically normal tissue around the tumour.

Histopathology

Formalin-fixed and paraffin-embedded sections were subjected to haematoxylin/eosin staining and histopathological examination was performed by two independent pathologists (S. I.–L. M.), recording mitotic index, degree of nuclear atypia and amount of pigmentation.[4,23,24] In order to determine the melanocytic origin of the tumours, each sample was tested for PNL-2 expression.[2,4]

Immunohistochemical analysis

Immunohistochemical (IHC) analysis was carried out on 4 µm sections of formalin-fixed, paraffin-embedded samples. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 30 min at room temperature. Sections underwent high-temperature antigen unmasking by incubation at 98 °C with citric acid buffer (pH 6.0). Samples were immunohistochemically tested for Ki67, PNL-2, PDGFR-α and β expression. The details of primary antibodies employed and the dilutions used are summarized in Table 1. Antibodies were detected using the avidin–biotin–peroxidase complex technique with the Vectastain Elite ABC Kit (Vector Laboratoires). The following external positive controls were used: canine skin for PDGFR-α and canine prostatic carcinoma for PDGFR-β. As an internal positive control, endothelial cells of the normal blood vessels were used. For negative controls, the sections were incubated in the absence of the primary antibodies. Immunolabelled slides were randomized and masked for blinded examination, which was performed independently by two observers (L. M. and S. I.); in case of disagreement, a consensus was reached using a multi-head microscope. Cytoplasmic positivity was evaluated in both tumour and stromal cells, located separately using the scoring system adopted by Donnem *et al.* (2008).[25] Immunostaining at the stromal level was

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Table 1. Source and conditions of the antibodies employed

Antibody	Type	Source	IHC
PDGFR-α	Rabbit polyclonal	Santa Cruz Biotechnology	1:200
PDGFR-β	Rabbit polyclonal	Santa Cruz Biotechnology	1:200
Ki67	Rabbit polyclonal	Dako	1:25
PNL-2	Rabbit polyclonal	Santa Cruz Biotechnology	1:25

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considered as cytoplasmic labelling in the fibroconnective tissue that forms bundles within and surrounding the tumour.

Ki67 was evaluated considering the cut-off of 19.5 positive cells in five ×400 fields.[24]

Statistical analysis

IHC results and clinical–pathological findings were grouped into contingency tables and analysed using Pearson's Chi-squared test with Yates' continuity correction. Survival curves were computed using the Kaplan–Meier method and tests for differences in survival, considering all known prognostic factors for CMM, were performed using the log-rank test. Co-expression and presence of Ki67 values greater than 19.5% were evaluated in interaction using a Cox proportional hazard regression model. Overall survival (OS) was considered as the number of days between surgery and death, while the disease-free interval (DFI) as the number of days between surgery and tumour recurrence and/or evidence of metastasis. Cases that were still alive or that did not present tumour recurrence or metastasis at the end of the monitoring period (minimum 12 months), or that died for unrelated causes, were considered as censored. Data were analysed with R (R Core Team (2014). R: R Foundation for Statistical Computing, Vienna, Austria); *P* values less than 0.05 were considered statistically significant.

Results

Epidemiologic and clinical data

The data presented here come from 48 cases of oral CMMs. The mean age of dogs was 11.4 years (range: 5–14 years); 64.6% of the dogs (31/48) were males and 35.4% (17/48) females. Fifty percent of dogs were mixed breed, while 50% were pure breeds. The latter included: five dachshunds (10.4%), four Cockers (8.3%), three German Shepherds (6.3%), three Golden Retrievers (6.3%) and one each of (2.1%) Syberian Husky, Beagle, Dogue de Bordeaux, Greyhound, Yorkshire terrier, Schnauzer, Miniature Schnauzer, West Highland White Terrier and Labrador Retriever.

A total of 20 dogs had a stage II oral CMM and 28 a stage III oral CMM. All dogs underwent surgical excision. Histology revealed incomplete excision margins in 13 dogs (27.1%). The median DFI recorded was 196 days (range 30–992 days) and the median OS was 258 days (range 70–992 days). Five censored cases were included in this study: two died for unrelated causes (euthanasia for orthopedical problems in one dog and *ab ingestis* pneumonia caused by idiopathic megaesophagus in the second dog) while three dogs were still alive at the end of the monitoring period.

Histopathology

Histopathology revealed that 30/48 cases (62.5%) of CMMs were characterized by the presence of melanin, while 18/48 (37.5%) were amelanotic. Regarding the histotype, 14 CMM were spindle-shaped (29.2%), 12 epithelioid (25%) and 22 mixed (45.8%).

IHC analysis

All samples analysed showed positivity to PNL-2 antigen, confirming the diagnosis of melanoma.[4] Positivity for Ki67 was <19.5% in 16 cases (33.3%) and >19.5% in 32 cases (66.7%).

Immunolabelling for both PDGFR-α and -β receptors was observed at cytoplasmic level and diffusely within the tumour (Figs. 1 and 2). PDGFR-α and β expression was observed in 26/48 (54.2%) cases and 23/48 (47.9%), respectively. Among the 48 cases analysed, 15 (31.2%) were negative for both the PDGF receptors, 18 samples (37.5%) were positive to both PDGFR-α and -β, 8 (16.7%) were positive to PDGFR-α and negative to PDGFR-β, while seven (14.6%) were positive to PDGFR-β only. Regarding the positivity in the stromal cells compartment, PDGFR-α was present in 13/48 cases (27.1%), PDGFR-β in 8/48 (16.7%); of those, 5/48 (10.4%) were positive for both receptors.

Statistical analysis

Dogs with oral CMM expressing both PDGFR-α and -β had a statistically significant lower DFI (median 159 days versus 239 days, $P < 0.05$) and a lower OS (median 183 days vs. 335 days, $P < 0.05$) compared with dogs with CMM not co-expressing these receptors (Fig. 3). Also, a high Ki67 index

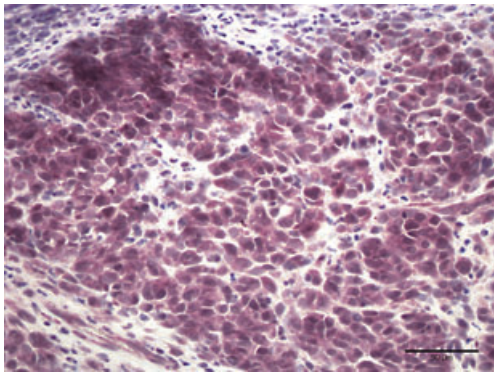

Figure 1. Malignant melanoma. Neoplastic cells with a diffuse and strong cytoplasmic immunolabelling for PDGFR α (purple staining) streptavidin – biotin – peroxidase method. Mayer's haematoxylin counterstaining. Scale bar: 50 µM.

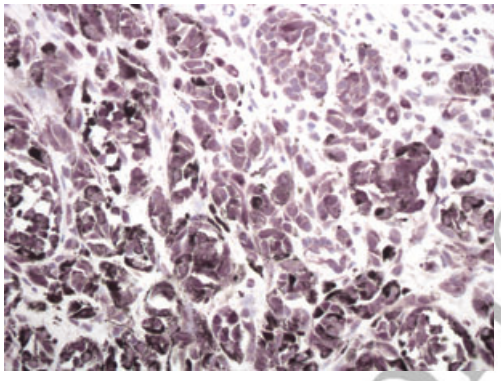

Figure 2. Malignant melanoma. Neoplastic cells with a diffuse and strong cytoplasmic immunolabelling for PDGFR β (purple staining) streptavidin – biotin – peroxidase method. Mayer's haematoxylin counterstaining. Scale bar: 50 µM.

was statistically associated with both a shorter DFI (188 days versus 484 days – $P < 0.05$) and a shorter OS (median 224 days versus 484 days – $P < 0.05$) (Fig. 4). The number of the samples available did not allow the evaluation of the prognostic value of the single expression of PDGFR-α or -β. However, the expression of PDGFR-α was statistically associated with the expression of PDGFR-β (Chi square test, $P < 0.05$). Other evaluations comparing the IHC results with all the clinical or pathological data available did not show any statistical association. Besides, in this series of cases, no statistical differences in survival were found comparing patients of different clinical stage or those with complete and incomplete surgical excision. The Cox regression model for proportional hazard assessment of PDGFR co-expression in interaction with high values of Ki67 was statistically significant ($P < 0.05$) and yielded odds ratio of 1.65 for the co-expression and 1.96 for the Ki67, respectively ($R^2 = 0.159$, log-rank test $P < 0.05$). However, the confidence intervals for the coefficients were quite wide owing to the limited sample size (0.77 – 3.57 for co-expression and 0.78 – 4.81 for Ki67).

Discussion

CMM is the most common oral malignancy in dogs and is generally locally aggressive and highly metastatic. Primary tumour *en bloc* resection and regional lymphadenectomy, with or without adjuvant radiotherapy, are the preferred methods of treatment and results in loco-regional control in up to 75% of CMMs. Disappointingly, the 1-year survival rate is less than 30%, even after adjuvant treatment; in particular, adjuvant chemotherapy does not result in a significant increase of the disease-free period.[26–28] It may also be argued that the post-surgical outcome may be influenced by the clinical stage, but the authors of this paper did not reach any conclusion from the present data.[29] Immunotherapy against specific tumour associated antigens[30] has been employed in an adjuvant setting in an attempt to improve the life expectancy in case of CMM and results appear encouraging.[31,32]

PDGFRs are physiologically expressed in a variety of cell types, such as fibroblasts, vascular smooth muscle cells and endothelial cells,[10] suggesting a role also in the interaction between neoplastic cells and stromal compartment during tumour progression and invasion.[29] PDGFR-α and -β receptors are activated by specific soluble factors known as PDGF-A and -B that act as dimeric isoforms (PDGF-AA, -AB, and -BB) as well as the newly discovered protease activated isoforms PDGF-C and PDGF-D. PDGF-AA binds selectively to PDGFR-α, while PDGF-B chain isoforms bind and dimerize both PDGFR-α and PDGFR-β. In humans, several studies demonstrated the ability of PDGF ligands to interact with PDGFR-α and -β and induce homodimerization and/or heterodimerization of the receptors.[33,34]

As shown previously, in our samples we found that the co-expression of both isoforms was higher

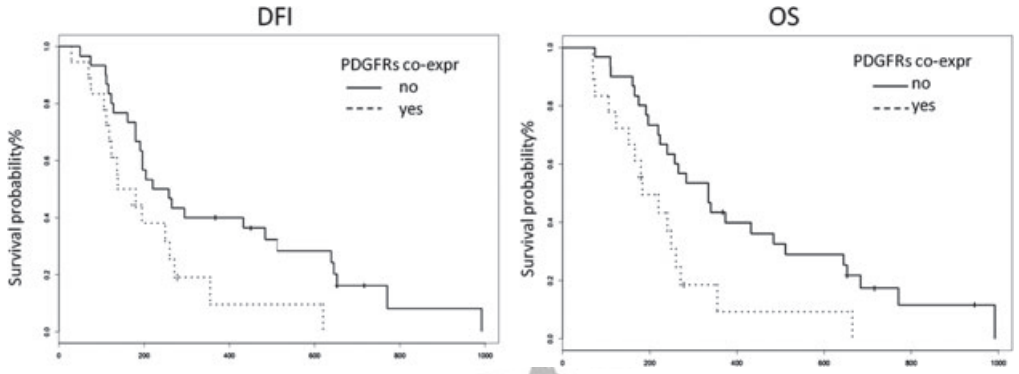

Figure 3. Kaplan–Meier curve of DFI (left box) in patients with melanoma co-expressing both PDGFR-α and -β (median 159 days) and not co-expressing PDGFR-α and -β (median 239 days – log-rank test: $P < 0.05$) and Kaplan–Meier curve of OS (right box) in patient co-expressing both PDGFR-α and -β (median 183 days) and not co-expressing PDGFR-α and -β (median 335 days – log-rank test: $P < 0.05$).

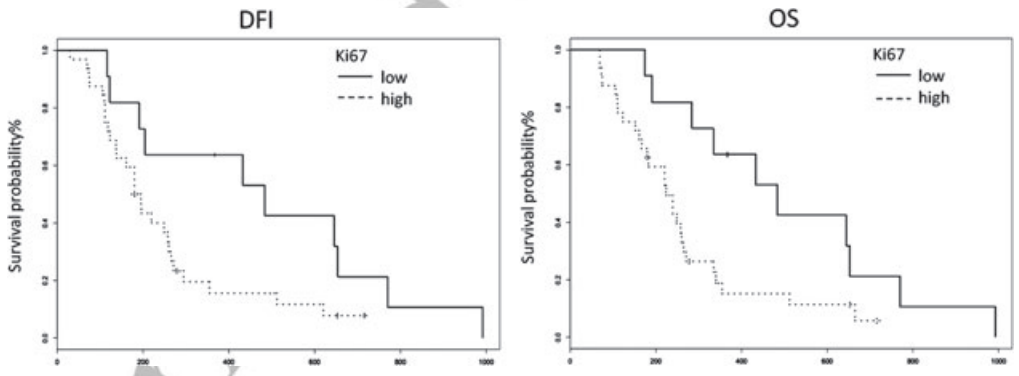

Figure 4. Kaplan–Meier curve of DFI (left box) in patient with Ki67 > 19.5% (median 188) and Ki67 < 19.5% (median 484 – log-rank test: $P > 0.05$) and Kaplan–Meier curve of OS (right box) in patient with melanoma positive (median 224 days) and negative for PDGFR-α (median 484 days – log-rank test: $P < 0.05$).

(37.5%) than the presence of PDGFR-α or -β alone (16.7 and 14.6%, respectively). This finding may suggest that these receptors can act independently by homodimerization as well as by heterodimerization.[35]

A study on PDGFs and PDGFRs in human cutaneous melanomas[36] demonstrated that, at IHC, both the primary and metastatic melanoma exhibited significant expression of PDGF-AA, PDGF-BB and PDGF-α receptor when compared with normal skin, while no expression for PDGF-β receptor was recorded. These results have been recently confirmed by a study where PDGFR-α resulted overexpressed in a small population of human melanomas (4.6%) and an increased copy number was found.[37–40] Contrary to human melanoma, in our sample PDGFR-β was expressed in 37.5% of samples, thus suggesting a different role. In the present study, PDGFRs were detected not only in tumour tissue but also in the stromal compartment, suggesting a potential role in matrix remodelling and tumour invasion.[12]

In domestic animal tumours, the IHC expression of PDGFRs has been investigated in astrocytoma,[19] lymphoma,[17] osteosarcoma,[16] anal sac adenocarcinoma,[18] thyroid carcinoma[41] and haemangiosarcoma,[20] highlighting the importance of these receptors also in the tumour biology of animals, as it occurs in humans.[42] However, for none of these tumours a prognostic relevance has been demonstrated.

One important limitation of this study is its retrospective nature. Nevertheless, results show that the co-expression of PDGFR-α and PDGFR-β (37.5% of all CMMs of this series) is statistically associated to both DFI and OS ($P < 0.05$) and could therefore

be considered as a negative prognostic factor. This study also confirms the prognostic importance of Ki67,[24] whereas results regarding free versus infiltrated surgical margins, in the face of an *en bloc* surgery (CMM considered inoperable, i.e. with no chance to get clean margins at surgery, were not included here), and clinical stage failed to correlate with survival. Although this is an unexpected result, it should be considered that, as shown in another study dealing with a greater number of dogs with CMM, other factors such as the old age of the dogs and the size of the tumour may act as negative prognosticators.[27] It should also be noted that, in this study, no stage I CMMs was included and only stage II and III CMM were considered. Collectively, the data obtained from this study suggest that PDGFRs may play a role in the pathogenesis of CMM and the co-expression of both PDGFRs-α and -β should be taken in account as a negative prognostic marker. Further prospective studies on a greater number of cases are warranted to confirm this finding.

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Conflict of interest

None of the authors have financial or personal relationships that could inappropriately influence or bias the content of the paper.

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