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# UNIVERSITÀ DEGLI STUDI DI TORINO

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## **PDGFs and PDGFRs in canine osteosarcoma: New targets for innovative therapeutic strategies in comparative oncology**

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### **A B S T R A C T**

Platelet derived growth factor receptor (PDGFR)a and PDGFRp are tyrosine kinase receptors that are over-expressed in 70-80% of human osteosarcomas (OSAs) and may be suitable therapeutic targets for specific kinase inhibitors (TKIs). Canine OSA shows histopathological and clinical features similar to human OSA, and is considered an excellent model in comparative oncology. This study investigated PDGF-A, PDGF-B, PDGFRa and PDGFRp expression in 33 canine OSA samples by immunohistochemistry and in seven primary canine OSA cell lines by Western blot and quantitative PCR analysis.

Immunohistochemical data showed that PDGF-A and PDGF-B are expressed in 42% and 60% of the OSAs analysed, respectively, while PDGFRa and PDGFRp were expressed in 78% and 81% of cases, respectively. Quantitative PCR data showed that all canine OSA cell lines overexpressed PDGFRa, while 6/7 overexpressed PDGFRp and PDGF-A relative to a normal osteoblastic cell line. Moreover, in vitro treatment with a specific PDGFR inhibitor, AG1296, caused a dose- and time-dependent decrease in AKT phosphorylation. Collectively, these data show that PDGFRs/PDGFs are co-expressed in canine osteosarcomas, which suggests that an autocrine and/or paracrine loop is involved and that they play an important role in the aetiology of OSA. PDGFRs may be suitable targets for the treatment of canine OSA with a specific TKI.

## Introduction

Spontaneous tumours in dogs and cats are suitable models of human cancer (Withrow et al., 1991; Vail and MacEwen, 2000; Khanna et al., 2006). Canine osteosarcoma (OSA), in particular, represents an excellent model for OSA in humans due to the relatively high incidence of the tumour in dogs, similarities in the biological behaviour of the disease between the two species, common molecular features, the large body size of the breeds that are frequently affected and the similar physical environments of the disease (Mor-ello et al., 2011). In dogs, OSA represents the most common primary malignant tumour of bone (80% of all bone tumours) and is characterised by a locally aggressive and highly metastatic behaviour, mainly in large and giant breeds of dogs (Farese et al., 2009).

Similarities between dog and human OSA also exist at the molecular level: canine OSA cell lines and tumours, like their human counterparts (Freeman et al., 2008; Kaseta et al., 2008; Wu et al., 2011), frequently contain mutations that inactivate p53 (Johnson et al., 1998) and the PTEN family of tumour suppressor genes (Levine et al., 2002). Insulin and hepatocyte growth factor (HGF) influence tumour growth, invasion and malignant phenotype in canine and human OSA cell lines and tissues (Gorlik et al., 1999; MacEwen et al., 2003, 2004; Ferrari et al., 2004). Overexpression of the *erb-B2* gene, which encodes human epidermal growth factor receptor 2 (HER2), was observed in 86% and 40% of canine OSA cell lines and tissue samples, respectively (Flint et al., 2004).

Matrix metalloproteinases (MMPs) 2 and 9 and STAT3 are also expressed in canine and human OSA cell lines and tissues (Lana et al., 2000; Loukopoulos et al., 2004; Chen et al., 2007; Fossey et al., 2009; Korpi et al., 2011). Furthermore, we have recently demonstrated that (as in humans) 79% of canine OSA samples over-express the MET oncogene and that cell motility and invasiveness are MET-dependent, as they are abrogated by small interfering RNAs that are specific for MET (De Maria et al., 2009). These similarities between canine and human OSA suggest that the canine system could be a suitable model to study novel therapeutic approaches for humans (Ma et al., 2003).

Platelet-derived growth factors (PDGFs) play important roles during wound healing and embryonic development. Their expression has been linked to several diseases, including cancer, in which these factors promote angiogenesis and autocrine stimulation of tumour cells (Alvarez et al., 2006). PDGFR $\alpha$  and PDGFR $\beta$  are tyrosine kinase receptors that can activate many of the same major signal transduction pathways including the PI3K (phosphatidylinositol 3-kinase), Ras mitogen-activated protein kinase (MAPK), phospholipase C $\gamma$  pathways and p-AKT (Liu et al., 2011). AKT and MAPK are cytoplasmic proteins kinases activated by phosphorylation (p-AKT and p-MAPK) that play an important role in cell survival, proliferation and cell growth by controlling the expression of anti-apoptotic genes (Seger and Krebs, 1995; Nicholson and Anderson, 2002; Tokunaga et al., 2008).

PDGFRs and PDGFs are known to play a crucial role in the pathogenesis, invasion and distant metastasis of human cancers, and recent studies have suggested their involvement in an autocrine or paracrine loop that causes tumour growth and progression in OSA (Sulzbacher et al., 2000; Uren et al., 2003; Ostman, 2004). In human OSA, the expression of PDGFRs does not appear to have a prognostic value, but they have been suggested as a therapeutic target for tyrosine kinase inhibitors (TKIs) (Sulzbacher et al., 2003, 2010; Kubo et al., 2008). In veterinary oncology, PDGFRs have been investigated only in spontaneous canine astrocytomas, where increased PDGFR $\alpha$  expression was observed (Higgins et al., 2010).

Dysfunction of tyrosine kinases occurs frequently in human cancers, and recent studies have indicated that a similar

pattern of dysfunction is observed in canine and feline cancers (Lachowicz et al., 2005; London, 2009). TKIs that are specific for the c-KIT receptor and others are currently used in the treatment of canine mast cell tumours with excellent results (London et al., 2009). Despite these results, further molecular characterisation is needed to test the biologic activity of TKIs in canine cancers and to investigate the effects of combining TKIs with standard therapeutics such as radiation therapy and chemotherapy. The main goal of this study was to evaluate the expression of PDGFRs and PDGFs in canine OSAs and to demonstrate their biological role in tumour progression.

## **Materials and methods**

### Sample collection and clinical follow-up

Tissue samples were examined from 33 cases of spontaneous canine appendicular OSA treated between 2005 and 2010 at the Department of Animal Pathology of the University of Turin. None of the dogs included in this study had evidence of macroscopic metastases at presentation, and appendicular OSA was confirmed by histological diagnosis. In all cases, the initial data collected included history, a physical examination, complete blood count, serum biochemical profile, urinalysis and abdominal ultrasound. Limb (lateral-lateral [LL] and anterior-posterior [AP] views) and chest (LL, right and left, and dorso-ventral [DV] views) radiographic evaluation was performed to examine the features and the extent of the tumour and the presence of lung metastasis. Computer tomography was performed for cases in which the lung radiographs were not conclusive. In cases where regional lymph nodes were enlarged, they were aspirated and examined cytologically.

All dogs included in this study were surgically treated (amputation or limb sparing) before receiving adjuvant chemotherapy using doxorubicin (30 mg/m<sup>2</sup>, 4-5 administrations, 21 days apart) or cisplatin (70 mg/m<sup>2</sup>, 4-5 administrations, 21 days apart) as single agents or in combination (4 cycles, 21 days apart, each cycle consisting of cisplatin 50 mg/m<sup>2</sup> at day 1 and doxorubicin 15 mg/m<sup>2</sup> at day 2). Surgical treatment was followed by chemotherapy administration because adjuvant chemotherapy has been shown to improve survival in dogs with appendicular OSA, although no specific protocol has been demonstrated to be superior (Dernell et al., 2007). Thus, the protocol was determined based on both the data in the literature and the owner's compliance (Straw et al., 1991; Berg et al., 1995; Chun et al., 2005).

Dogs were examined clinically and radiographically every 3 months during the first year after the conclusion of chemotherapy and then every 6 months for a minimum of 2 years. For animals that died from tumour-related causes within the 2-year period, overall survival (OS) was considered as the days between the surgery and death, while the disease-free interval (DFI) was considered as the number of days between surgery and tumour recurrence and/or evidence of metastatic disease.

Samples collected after tumour excision were fixed in formalin 10% for at least 24 h, embedded in paraffin, sectioned (4 µm) and stained with haematoxylin and eosin (HE). The histological diagnosis was established using HE-stained slides,

according to the WHO guidelines (Slayter et al., 1994), while the histological grade was determined according to the system proposed by Loukopoulos and Robinson (2007).

### Immunohistochemical analysis

Immunohistochemical (IHC) analysis was carried out on 4 $\mu$ m paraffin sections. 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 with 98 °C citric acid buffer (pH 6). The anti-human primary antibodies and the dilutions used are listed in Table 1. Antibodies were detected using the avidin-biotin peroxidase complex technique with the Vectastain Elite ABC Kit (Vector Laboratories). The following positive controls were used: canine lung tissue for PDGF-A and PDGF-B, canine skin for PDGFR $\alpha$  and canine prostatic carcinoma for PDGFR $\beta$ . Endothelial cells were used as an internal positive control. For negative controls, the sections were incubated without the primary antibodies.

Immunolabelled slides were randomised and masked for blind examination, which was performed independently by two observers (L.M., S.I.). When there was disagreement (<5% of the slides), a consensus between the two observers was reached using a multihead microscope. Cytoplasmic immunolabelling was evaluated in neoplastic cells and in stromal cells (fibroconnective tissue within and surrounding the tumour) separately using the scoring system adopted by Don-nem et al. (2008).

#### Isolation and characterisation of primary canine osteosarcoma cell lines

Immediately following surgery, specimens of canine OSA and normal canine bone were collected in culture medium, washed in sterile phosphate buffered saline (PBS) and minced into small fragments (<1 mm<sup>3</sup>) in a sterile environment. Tissue fragments were then digested at 37 °C for 30-60 min in PBS containing 0.25 mg/mL collagenase type IA (Sigma-Aldrich). After digestion, the lysate was centrifuged at 184 g for 1 min and the resulting pellet was suspended in Iscove's standard medium supplemented with 10% foetal bovine serum (FBS), 1% glutamine, 100  $\mu$ g/mL penicillin and 100  $\mu$ g/mL streptomycin. Cells were plated in 25 cm<sup>2</sup> tissue culture flasks, were then cultured at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>, and the medium was renewed twice weekly.

To characterise the primary OSA cell lines, each line was tested for alkaline phosphatase activity. Cells grown in 6-well chamber slides were fixed in PBS containing 0.5% glutaraldehyde, washed twice in PBS and incubated overnight with NBT/BCIP solution (Sigma-Aldrich). After incubation, the slides were mounted with glycerol and observed under a microscope. The normal osteoblastic cell lines (OSB1 and OSB2) were isolated from healthy dogs using the procedure described above (De Maria et al., 2009). To confirm their normal osteoblastic origin, part of the tissue was analysed histologically to exclude any pathological processes affecting the bone and any malignant origin (Table 3). After cellular isolation, normal phenotype of OSB1 and OSB2 was confirmed by cytological features and by the lack of the expression of Ki67, p53 and PCNA evaluated by immunocytochemistry (data not shown).

#### Western blot analysis

Western blot (WB) analysis was carried out on samples from the primary OSA cell lines and from a normal osteoblastic cell line (OSB). Total protein was obtained with boiling lysis buffer containing 1% SDS and 0.1 M Tris-HCl (pH 6.8). Total protein from each sample (20  $\mu$ g) was separated on an 8% SDS-polyacrylamide (PAGE) gel, transferred onto a Hybond-C Extra membrane (Amersham Biosciences) and incubated overnight at 4 °C with the primary antibodies listed in Table 1. After incubating with a horseradish peroxidase (HRP)-linked secondary

antibody diluted 1:2000 in PBS-Tween, membranes were washed in PBS-Tween for 30min and incubated with an enhanced chemiluminescence reagent (Super Signal West Pico Mouse IgG Detection Kit, Thermo Scientific

).

Antibody	Type	Source	IHC	WB
PDGF-A	Mouse monoclonal	Santa Cruz Biotechnology	1:400	1:1000
PDGF-B	Rabbit polyclonal	Santa Cruz Biotechnology	1:400	1:1000
PDGFR $\alpha$	Rabbit polyclonal	Cell Signaling Technology	1:100	1:1000
PDGFR $\beta$	Rabbit polyclonal	Santa Cruz Biotechnology	1:200	/
PDGFR $\beta$	Rabbit polyclonal	Cell Signaling Technology	/	1:1000
p-AKT	Rabbit polyclonal	Cell Signaling Technology	/	1:1000
p-MAPK	Rabbit polyclonal	Cell Signaling Technology	/	1:1000
$\alpha$ -Tubulin	Rabbit polyclonal	Cell Signaling Technology	/	1:1000

Table 1 Source and conditions of the antibodies used for immunohistochemistry (IHC) and Western blot (WB).

Table 2

Primer	Gene	Sequence
Forward	<i>PDGF-A</i>	5'-CCCCCGGAGGTGATTGAGA-3'
Reverse	<i>PDGF-A</i>	5'-TCCTCGGCCCCTACGGAGTC-3'
Forward	<i>PDGF B</i>	5'-CAAACCTCGGGTGACCATTTCG-3'
Reverse	<i>PDGF B</i>	5'-TGCTTGAACITCCGGTGCTT-3'
Forward	<i>PDGFR <math>\alpha</math></i>	5'-CATCCCCCTGCCGACATCG-3'
Reverse	<i>PDGFR <math>\alpha</math></i>	5'-TGAGCTGTGTCTGTTCTCTTGCC-3'
Forward	<i>PDGFR <math>\beta</math></i>	5'-GTCCTCAAAGGCCAGGCACTGTGG-3'
Reverse	<i>PDGFR <math>\beta</math></i>	5'-CCCCGGGGGTGTGATGACCAG-3'
Forward	<i>GAPDH</i>	5'-GGCACAGTCAAGGCTGAGAAC-3'
Reverse	<i>GAPDH</i>	5'-CCAGCATCACCCATTGAT-3'

### Total RNA extraction and quantitative PCR expression analysis

Total RNA was obtained from all OSA cell lines and cDNA was synthesised from 1  $\mu$ g of total RNA using the QuantiTect Reverse Transcription kit (Qiagen). To determine the relative amounts of specific PDGF-A, PDGF-B, PDGFR $\alpha$  and PDGFR $\beta$  transcripts, the cDNA was subjected to quantitative PCR using the IQ SYBR Green Supermix (BioRad) and the IQ.5 detection system (BioRad). Primer sequences were designed using Primer Express v. 1.5 and are listed in Table 2. GAPDH was used as housekeeping gene. The level of gene expression was calculated using a relative quantification assay corresponding to the comparative cycle threshold (Ct) method: the

amount of target, normalised to the endogenous housekeeping gene (CAPDH) and relative to the calibrator (control sample), was then transformed by  $2^{-\Delta\Delta Ct}$  (fold increase), where  $\Delta\Delta Ct = \Delta Ct(\text{sample}) - \Delta Ct(\text{control})$  and  $\Delta Ct$  is the  $Ct$  of the target gene subtracted from the  $Ct$  of the housekeeping gene.

#### PDGFR inhibition in vitro

The 'Penny, cell line was treated with the specific PDGFR inhibitor AG1296 (Cal-biochem) at 1  $\mu\text{M}$ , 2.5  $\mu\text{M}$  or 5  $\mu\text{M}$  for 12, 24 or 48 h. Cytotoxicity was evaluated using the MTT method (Dacasto et al., 1994). Flow cytometric analysis of cell cycle and apoptotic activity were carried out after propidium iodide (PI) and PI versus An-nexin V-FTTC staining, respectively. All experiments were performed in triplicate. WB analysis of p-AKT was performed on cellular lysates obtained from Penny cells treated at different times and concentrations.

#### Statistical analysis

IHC results were grouped into contingency tables and analysed using Fisher's exact test. The analysis of OS and DFI was performed using the Kaplan Meier method with a log rank test.  $P < 0.05$  was considered statistically significant. Data were analysed with GraphPad Prism Software v. 4.0.

## RESULTS

#### Epidemiologic and clinical data

Data were collected from 33 cases of appendicular OSA removed from dogs with a mean age of  $7.17 \pm 2.62$  years (range, 2-12 years), which were predominantly males (60.6%). Eight dogs (24.24%) were mixed breed, and 25 (75.76%) were pure breed. The pure breed dogs included 7 Boxers, 3 Rottweilers, 3 Great Danes, 2 St. Bernards, 2 Newfoundlands and 1 each of the following breeds: Beauceron, Siberian Husky, Maremma Shepherd, Malinois, German Shepherd, Greyhound, Doberman and Golden Retriever. The mean DFI observed was  $311.85 \pm 263.01$  days (median, 251), and the mean OS was  $359.06 \pm 392.22$  days (median, 261).

The data collected from the seven dogs from which the primary cell lines were derived are reported in Table 3. Three of them (Wall, Pedro and Lord) were still alive at the time of writing, while the others died from tumour-related causes after a mean of 74.75 days (median, 68.4) after surgery. Two of the seven dogs (Wall and Lord) had not shown metastases at the time of this report, while the mean DFI observed in the others was 94.4 days (median, 76.5).

#### Histopathology

The following distribution was observed: 19 cases (57.58%) of osteoblastic productive OSA, seven cases of (21.21%) chondroblas-tic OSA, four cases (12.12%) of fibroblastic OSA, two cases (6.06%) of unproductive osteoblastic OSA and one case (3.03%) of giant cell OSA. The cases were categorized into 8 (24.24%) grade I, 13 (39.40%) grade II and 12 (36.36%) grade III OSAs. Cell lines were collected from seven OSA and from one lymph nodal metastasis (Dark). Histopathological examination was used to diagnose and grade the samples as listed in Table 3.



## Immunohistochemistry

The results of IHC staining for PDGF-A, PDGF-B, PDGFRa and PDGFRp in OSAs of different grades are summarised in Table 4. PDGF-A and PDGF-B immunolabelling was observed in the cytoplasm mainly at the tumour border. In particular, PDGF A was detected in 42.42% (14/33) of cases in neoplastic cells (Fig. 1) and in 48.48% (16/33) of cases in stromal cells (Fig. 2), while PDGF B was observed in 60.61% (20/33) of cases in neoplastic cells (Fig. 3) and in 18.18% (6/33) of cases in stromal cells.

Staining for PDGFRa and PDGFRp was observed with a uniform distribution within the neoplastic tissue, mainly in the cytoplasm and in some cases with a brighter membrane labelling (Appendix A, Supplementary file). PDGFRa immunostaining was observed in 78.79% (26/33) of cases in neoplastic cells (Fig. 4) and in 21.21% of cases (7/33) in stromal cells. PDGFRp was observed in neoplastic cells in 81.81% (27/33) of cases and in stromal cells in 24.24% (8/33) of cases (Fig. 5). Immunolabelling of PDGFs and PDGFRs was also detected in the cytoplasm of endothelial cells of normal capillaries and blood vessels.

Statistical analyses comparing the protein expression of PDGF-A, PDGF-B, PDGFRa and PDGFRp and their locations (tumoural or stromal) as well as histological diagnosis and grading showed no statistical associations. Long rank tests performed to compare the

IHC results and the OS and DFI times showed no statistical correlation between PDGFs and PDGFRs staining and survival

**Table 3**

Detailed data for the dogs from which primary osteosarcoma cell lines were generated.

Cell line	Age (years)	Sex	Diagnosis	Grade	DFI (days)	OS (days)
Wall	0.5	m	Osteoblastic productive osteosarcoma	III	No relapse	Still alive
Penny	8	f	Chondroblastic osteosarcoma	III	70	70
Dark	10	m	Lymphnodal metastasis of osteoblastic productive osteosarcoma	III	64	64
Sky	9	m	Osteoblastic productive osteosarcoma	III	83	98
Pedro	9	m	Chondroblastic osteosarcoma	III	188	Still alive
Lord	8	m	Osteoblastic productive osteosarcoma	II	No relapse	Still alive
Desmond	4	m	Osteoblastic productive osteosarcoma	III	67	67
OSB1	8	f	Normal bone			
OSB2	5	m	Normal bone	/	ND	ND.

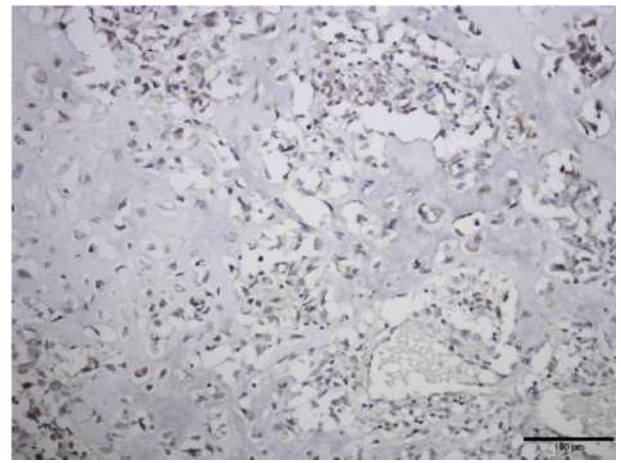
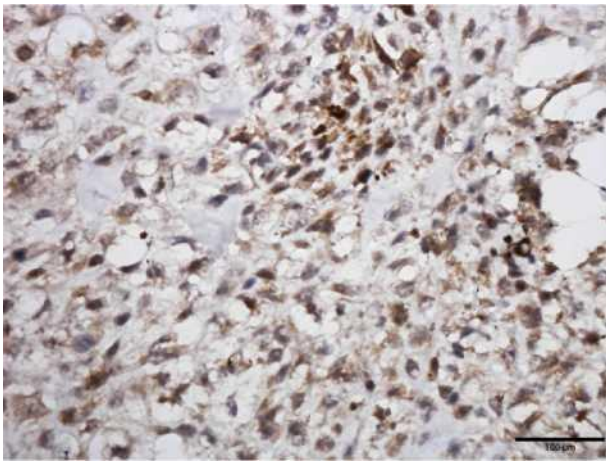
DFI, disease free interval; OS, overall survival; ND, not determined.

**Table 4**

Immunohistochemical results for canine osteosarcomas (OSA) of different grades.

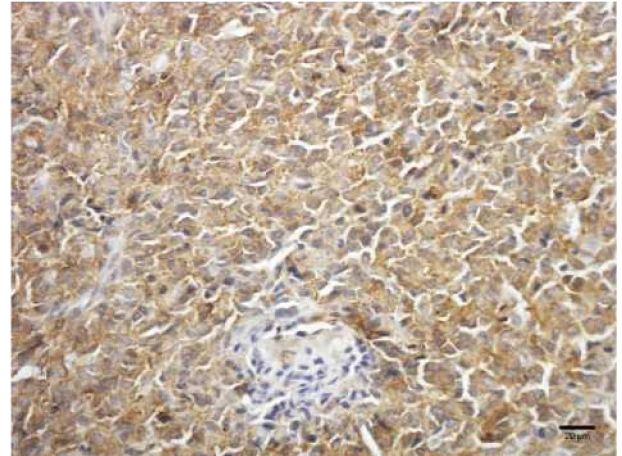
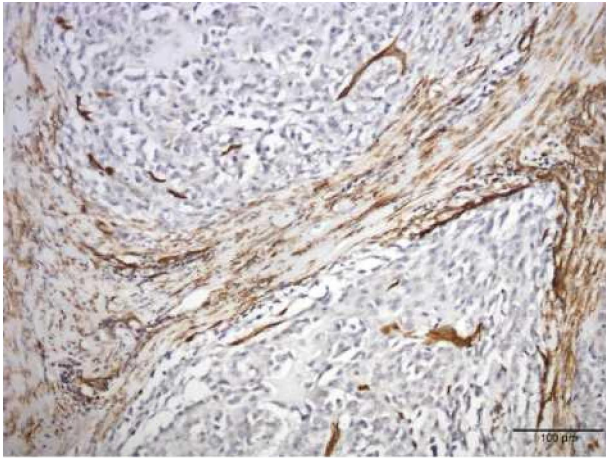
	PDGF-A		PDGF-B		PDGFR $\alpha$		PDGFR $\beta$	
	Tumour +/tot (%)	Stroma +/tot (%)	Tumour +/tot (%)	Stroma +/tot (%)	Tumour +/tot (%)	Stroma +/tot (%)	Tumour +/tot (%)	Stroma +/tot (%)
I grade	4/8 (50.00%)	6/8 (75.00%)	5/8 (62.50%)	2/8 (25.00%)	6/8 (75.00%)	2/8 (25.00%)	8/8 (100%)	3/8 (37.5%)
II grade	4/13 (30.77%)	6/13 (46.15%)	7/13 (53.85%)	2/13 (15.38%)	9/13 (69.23)	4/13 (30.77%)	10/13 (76.92%)	3/13 23.08%
III grade	6/12 (50.00%)	4/12 (33.33%)	8/12 (66.67%)	2/12 (16.67%)	11/12 (91.66%)	1/12 (8.3%)	9/12 (75.00%)	2/12 (16.67)
Total OSA +/tot (%)	14/33 (42.42%)	16/33 (48.48%)	20/33 (60.61%)	6/33 (18.18%)	26/33 (78.79%)	7/33 (21.21%)	27/33 (81.81%)	8/33 (24.24%)

+/tot, positive cases/total cases.



**Fig. 1.** Osteoblastic productive osteosarcoma. Neoplastic cells are characterised by diffuse and strong cytoplasmic immunolabelling for PDGF-A. Streptavidin-biotin-peroxidase method. Mayer's haematoxylin counterstain.

**Fig. 2.** Chondroblastic osteosarcoma. Stromal cells characterised by strong cytoplasmic immunolabelling for PDGF-A and neoplastic cells PDGF-A negative. Streptavidin-biotin-peroxidase method. Mayer's haematoxylin counterstain.



**Fig. 4.** Chondroblastic osteosarcoma. Neoplastic cells are characterised by diffuse and strong cytoplasmic positivity for PDGFRot. Streptavidin-biotin-peroxidase method. Mayer's haematoxylin counterstain.

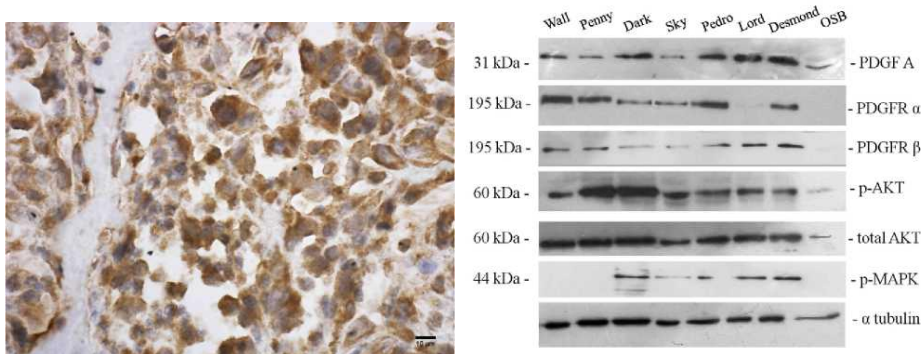
**Fig. 3.** Osteoblastic productive osteosarcoma. Neoplastic cells are characterised by diffuse and weak cytoplasmic immunolabelling for PDGF-B. Streptavidin-biotin-peroxidase method. Mayer's haematoxylin counterstain.

### *Molecular investigations*

WB analyses of canine PDGFR $\alpha$  and  $\beta$ , PDGF-A, total AKT, p-AKT and p-MAPK were performed. Specific bands corresponding to canine PDGFR  $\alpha$  (195 kDa) and  $\beta$  (195 kDa) and to PDGF-A (31 kDa) were found in all OSA cell lines except for the Lord cell line, which did not express PDGFR $\alpha$  (Fig. 6). Higher expression of PDGFR $\alpha$  was observed in the Wall and Penny cell lines, while PDGFR $\beta$  was detected at a very low level in all of the OSA cell lines.

**Fig. 5.** Osteoblastic productive osteosarcoma. Neoplastic cells are characterised strong cytoplasmic immunopositivity for PDGFR $\beta$ . Streptavidin-biotin-peroxidase method.

Maver's haematoxylin counterstain



To evaluate which molecular pathways were activated by PDGFRs, p-AKT and p-MAPK levels were evaluated. p-AKT was overexpressed in all OSA cell lines, particularly the Penny and Dark cell lines. However, MAPK phosphorylation was observed in only 5/ 7 cell lines. The primary osteoblastic cell line (OSB) had low levels of PDGFA and p-AKT and did not express PDGFRs a or p or p-MAPK. The specificity of the antibodies for the canine species was evaluated using the human OSA cell line MG63 as a positive control (data not shown).

Quantitative PCR results (Fig. 7) showed that 4/7 cell lines had high expression of PDGFRa mRNA with the greatest levels observed in the Penny and Wall cell lines. PDGFRp mRNA was highly expressed in all OSAs, with the greatest levels observed in the Pedro and Sky cell lines. The expression of PDGFA and PDGFB was slightly higher in all cell lines compared to the normal OSB cell line.

#### *Proliferation and apoptosis assays*

The effects of AG1296 on apoptosis and cell proliferation were evaluated in the Penny cell line, which has high expression of

PDGFRs and shows activation of AKT. No significant differences in apoptotic activity and in cell cycle analysis at the doses and times evaluated were found (data not shown). Furthermore, the MTT assay did not show evidence of cytotoxicity. WB analysis indicated a reduction in p-AKT in the Penny cell line in a dose- and time-dependent manner after treatment for 24 h with 5.0  $\mu$ M and for 48 h with 2.5  $\mu$ M and 5  $\mu$ M AG1296 (Fig 8).

## Discussion

OSA is the most frequently diagnosed cancer in the bone of dogs. Several authors have demonstrated that this tumour shows many similarities with human OSA (De Maria et al., 2009; Morello et al., 2011), which is one of the most frequent tumours of childhood and adolescence (Kempf-Bielack et al., 2005; Ottaviani and Jaffe, 2009). Despite the use of aggressive treatments such as adjuvant chemotherapy and wide tumour resection, OSA remains a major cause of fatality in both species; therefore, new therapeutic strategies based on targeted therapy may improve the treatment and prognosis of OSA.

In the current study, we demonstrated that PDGFRs and their specific ligands, PDGF-A and -B, are overexpressed in canine OSA and that they may represent suitable targets for specific (targeted) therapy. Because PDGFs and PDGFRs are physiologically expressed in a variety of cell types, such as fibroblasts, vascular smooth muscle cells and endothelial cells (Alvarez et al., 2006), immunolabeling of PDGFs and PDGFRs was detected not only in tumour tissue but also in the stromal compartment (fibroblasts and endothelial cells). PDGFRA and PDGFRp were expressed in 78.79% (26/33) and 81.22% (27/33) of tumour cells, respectively, while expression in the stromal compartment was 21.21% and 24.24%, respectively. These data are similar to those previously reported for humans (Kubo et al., 2008), where PDGFRA and PDGFRp were present in 79.8% and in 86% of tumours, respectively.

A difference in PDGF-A expression was observed between canine (42%) and the reported value for human (80.4%) samples. In contrast, PDGF-B is expressed in 60.61% of canine OSAs and in 75.4% of human OSAs. No significant correlation was observed between the expression of these molecules and survival, histological grading or age. These results are different from data in humans, since one study has demonstrated that expression of PDGFRA and PDGF-A are correlated with inferior event-free survival (Kubo et al., 2008) and another study showed that PDGF-A expression was associated with tumour progression (Sulzbacher et al., 2003). The co-expression of PDGFRs and their specific ligands in our samples suggests a functional autocrine and/or paracrine stimulation loop that promotes both tumour growth and angiogenesis, as suggested by Ostman (2004).

We found that 24% of OSA expressed PDGFRp in the stromal compartment. This is in accordance with humans, in which a series of IHC studies have shown prominent PDGFRp expression in the stroma of several solid tumours (Sundberg et al., 1993; Ponten et al., 1994; Fjallskog et al., 2003); however, a significant correlation between PDGFRA and PDGF-A and B has not been previously reported. On the basis of the IHC results, we can determine that PDGFR and PDGF expression does not have a prognostic value in canine OSA, but based on their high expression, they may represent important therapeutic targets.

To better understand the role of PDGFRs and PDGFs in canine OSA, seven primary cell lines were characterised, and the molecular pathways associated with PDGFR investigated. Six cell lines overexpressed PDGFRA compared to normal OSBs, with the exception of the Lord cell line. PDGFRp and PDGF-A were expressed in all cell lines including OSB, the normal cell line. PDGF-A expression is consistent with what has been demonstrated in humans, where a

low level of PDGF-A is physiologically expressed in osteoblasts during osteogenesis (Horner et al., 1996), while overexpression is observed in OSA (Sulzbacher et al., 2000, 2003).

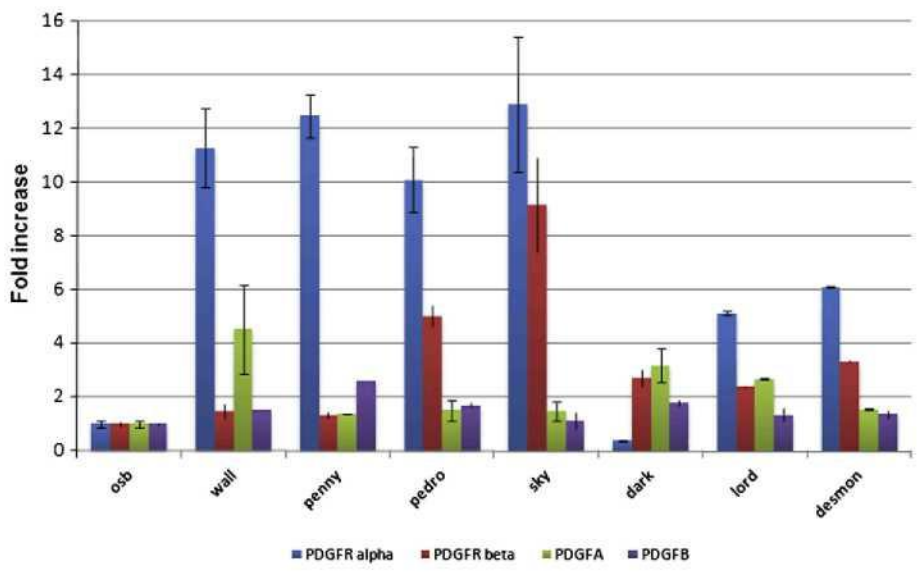
All cell lines expressing PDGFRs show activation of p-AKT, and five cell lines showed activation of p-MAPK. These results confirm the data available in the human literature, in which the major signal transduction pathways activated by PDGFR $\alpha$  and PDGFR $\beta$  are the PI3K (phosphatidylinositol 3-kinase), Ras mitogen-activated protein kinase and phospholipase C $\gamma$  pathways (Liu et al., 2011). On the basis of these results, we investigated the inhibitory effect of AG1296 on AKT phosphorylation in the Penny cell line, which overexpresses PDGFRs and has a high level of p-AKT. We demonstrated that AG1296 can decrease p-AKT in a dose- and time-dependent manner (Fig. 8). These data are similar to the effects observed in human OSA cell lines, where imatinib mesylate (ST1571) *in vitro* decreased p-AKT and p-MAPK levels (Kubo et al., 2008). However, AG1296 did not induce apoptosis or inhibit proliferation. Apoptosis and proliferation are not solely p-AKT-dependent and other cellular pathways that sustain tumour growth and anti-apoptotic pathways exist, including MET (De Maria et al., 2009), the IGF-1 and IGF1R pathways (MacEwen et al., 2004) or the STAT 3 pathway (Fossey et al., 2009).

#### **Conflict of interest statement**

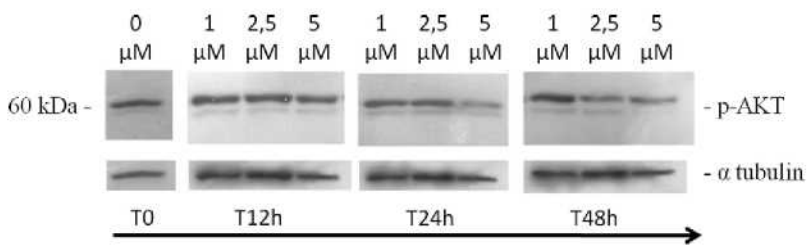
None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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**Fig. 7.** Expression by quantitative PCR of PDGF-A, PDGF-B, PDGFRot and PDGFRp in primary osteosarcoma (OSA) cell lines. PDGFRs mRNAs were expressed at higher levels in OSA cell lines while PDGFs transcripts were expressed at slightly higher levels compared to the normal cell line (OSB). The fold increase of each specific mRNA was normalised with normal OSB cell line and the error bars indicate the standard deviation of experimental triplicates.



**Fig. 8.** Evaluation of p-AKT expression in the 'Penny' cell line after PDGFR inhibition with AG1296 at different times and concentrations. AG1296 induced downregulation of p-AKT at 2.5 μM (48 h) and 5 μM (24 and 48 h).





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