Increased expression of insulin-like growth factor-1 receptor is correlated with worse survival in canine appendicular osteosarcoma

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ABSTRACT

Insulin-like growth factor 1 receptor (IGF-1R) is a cell membrane receptor widely expressed in tissues and involved in different cancers in humans. IGF-1R expression in human osteosarcoma has been associated with the development of tumour metastasis and with prognosis, and represents an attractive therapeutic target. The goal of this study was to investigate the expression of IGF-1R in canine osteosarcoma tissues and cell lines and assess its role and prognostic value. Samples from 34 dogs were examined by immunohistochemistry for IGF-1R expression. IGF-1R/AKT/MAPK signalling was evaluated by western blot and quantitative polymerase chain reaction in the cell lines. In addition, the in vitro inhibition of IGF-1R with pyrrotopodophillin (PPP) was used to evaluate molecular and biological effects.

Immunohistochemical data showed that IGF-1R was expressed in 71% of the analysed osteosarcoma samples and that dogs with higher levels of IGF-IR expression (47% of cases) had decreased survival (P < 0.05) when compared to dogs with lower IGF-IR expression. Molecular studies demonstrated that in canine osteosarcoma IGF-1R is activated by IGF-1 mostly in a paracrine or endocrine (rather than autocrine) manner, leading to activation of AKT/MAPK signalling. PPP caused p-IGF-1R dephosphorylation with partial blocking of p-MAPK and p-AKT, as well as apoptosis. It was concluded that IGF-1R is expressed and plays a role in canine osteosarcoma and that its expression is correlated with a poor prognosis. As in humans, IGF-1R may represent a good therapeutic target and a prognostic factor for canine osteosarcoma.

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Introduction

Canine and feline spontaneous tumours are suitable models for human cancers due to strong molecular, histopathological and clinical similarities. As such, dogs and cats represent useful alternative models to rodents for human cancer biology and translational therapy (Withrow et al., 1991; Morello et al., 2011). Osteosarcoma (OSA), the most common primary malignant bone tumour in dogs, is locally aggressive, and has a high metastatic potential and a poor prognosis (Farese et al., 2009; Morello et al., 2011; Casteleyn et al., 2013).

Several studies have demonstrated similarities between canine and human OSA at the molecular level by showing comparable expression of different proteins. Canine OSA cell lines and tumours frequently contain mutations that inactivate p53 (Johnson et al., 1998) and the phosphatase and tensin homologue (PTEN) family of tumour suppressor genes (Levine et al., 2002). Insulin and hepatocyte growth factors influence tumour growth, invasion and malignant phenotype in canine OSA cell lines (MacEwen et al., 2003, 2004). Matrix metalloproteinase 2 (MMP-2) expression has been detected only in high-grade OSAs, while pro-MMP-9 production correlated with the histological grade of OSA, suggesting a potential role of MMPs in the pathogenesis of canine OSA growth and metastasis (Loukopoulos et al., 2003). STAT3 activation has also been shown to contribute to the survival and proliferation of human and canine OSA cell lines (Lana et al., 2000; Loukopoulos and Robinson, 2007; Fossey et al., 2009).

Recently, the role and expression of the tyrosine kinase receptors (TKRs) have been investigated in veterinary oncology. Overexpression of the erb-B2 gene, encoding for 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), suggesting an involvement in the pathogenesis of this tumour like in humans (Rainusso et al., 2012). Furthermore, as in humans, 79% of canine OSA samples overexpress the MET oncogenes; cell motility and invasiveness appear to be MET-dependent since they can be inhibited by small interfering RNAs (siRNAs) that are specific for MET (De Maria et al., 2009). Finally, we have recently reported...
that PDGF receptors are overexpressed in canine OSA, adding a new potential therapeutic target (Maniscalco et al., 2013). Insulin-like growth factor-1 receptor (IGF-1R) is a transmembrane TKR consisting of two extracellular α-subunits responsible for ligand binding, two β-subunits with a transmembrane domain and an intracellular tyrosine kinase COOH-terminal domain (Werner et al., 2008). The specific interaction between IGF-1 and IGF-1R induces the phosphorylation of intracellular tyrosine residue (β-subunit) and results in activation of the receptor. The activated form of IGF-1R is able to activate the PI3K/AKT and mitogen-activated kinase signalling (MAPK) pathways (Girin et al., 2004). In humans, the IGF pathway is involved in bone homeostasis, since it regulates the proliferation of osteoblasts (Giustina et al., 2008; Zhang et al., 2012; Kubota et al., 2013), but it is also able to promote tumour initiation and progression (Baserga, 1995; Avnet et al., 2009) and is implicated in radio- and chemoresistance (Bohula et al., 2003).

In human OSA, IGF-1R is correlated with tumour metastasis and a molecule, a potent and selective IGF-1R inhibitor, is currently under development (Klopfleisch et al., 2010; Dolka et al., 2011), in melanomas (Thamm et al., 2010) and in insulinomas (Buishand et al., 2012) as well as in OSA cell lines (MacEwen et al., 2004), however, there are no data about its role in canine OSA as a prognostic factor and as a potential specific target for TKIs. The aim of this study was to investigate IGF-1R expression in canine OSA tissues and cell lines, to evaluate its prognostic value in relation to clinical outcome, and to test the in vitro effects of IGF-1R inhibition in canine OSA cell lines.

**Materials and methods**

**Study population**

Thirty-four dogs diagnosed with canine appendicular OSA and treated in the period 2007–2011 at the Department of Veterinary Sciences (University of Turin) were included in the study (Table 1). Data for the dogs included history, physical examination, complete blood and urine work-up, and abdominal ultrasonography. In addition, limb and chest (two and three views, respectively) radiographic evaluation to examine features and extent of the tumour and to exclude lung metastases was performed; computed tomography (CT) was additionally available and used during the latter 1.5 years of the study.

Dogs with pulmonary or lymph node metastases were excluded and in case of regional lymph node enlargement, aspiration and cytology were performed to exclude metastases. All dogs included were treated surgically (amputation or limb sparing) before receiving adjuvant chemotherapy using doxorubicin (30 mg/m², four to five administrations, 21 days apart) or cisplatin (70 mg/m², four to five administrations, 21 days apart) as single agents or in combination (4 cycles, 21 days apart) as single agents or in combination (4 cycles, 21 days apart) as single agents or in combination (4 cycles, 21 days apart).

**Immunohistochemical analysis**

In all cases immunohistochemical (IHC) analysis was carried out on 4-μm-thick paraffin sections. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 30 min at room temperature. Sections were subjected to high temperature antigen unmasking by incubation for 30 min with citric acid buffer (pH 6) at 98 °C and all sections were processed in an automated immunostainer (Dako) using an immunohistochemical detection substrate (Dako REAL EnVision Detection System, Peroxidase/DAB+, Rabbit/Mouse, Dako). A rabbit polyclonal anti-human IGF-1R antibody was used (Table 2). Canine normal kidney and adipose tissue were used as positive and negative controls, respectively. A ‘no primary antibody control’ on canine kidney was also employed. Specificity of the primary antibody was evaluated by incubating the primary antibody with excess of the specific peptide used to immobilise rabbit.

Immunolabelled slides were randomised and masked for blind examination, which was performed independently by two observers (LM, SI). In case of disagreement (2/34 cases; 6%), a consensus was reached using a multi-head microscope. Cytoplasmic immunolabelling was evaluated in neoplastic cells with a semi-quantitative method (Wang et al., 2012).

**Cell lines**

Seven primary canine OSA cell lines (Wall, Penny, Dark, Sky, Pedro, Lord and Desmon) (Maniscalco et al., 2013) and normal osteoblasts (OSB1) were cultured in Iscove’s standard medium supplemented with 10% fetal bovine serum (FBS), 1% glutamine, 100 μg/mL penicillin and 100 μg/mL streptomycin. NMuMG cells were purchased from American Type Culture Collection (ATCC) and cultured in DMEM medium containing 10% FBS (HyClone), 10 μg/mL insulin (Sigma-Aldrich), and 100 IU/mL penicillin.

**Total RNA extraction and quantitative PCR expression analysis**

Total RNA was obtained from all OSA cell lines and cDNA was synthesised from 1 μg of total RNA using the QuantiTect Reverse Transcription kit (Qiagen) including genomic DNA removal reagent. To determine the relative amounts of specific IGF-1R and IGF-1 transcripts, the cDNA was subjected to quantitative PCR using the IQ SYBR Green Supermix (BioRad) and the iQ5 detection system (BioRad). Forward and reverse primers employed were: IGF-1: 5′-GCCAAGCTGAGGAATGCTAC-3′, 5′-CCATCAATCGAGAGAGTC-3′, IGF-1R: 5′-CCACAGGCTGGAATGCTAC-3′, 5′-GGGATGCCTCCGTATC-3′, 5′-CCACAGGCTGGAATGCTAC-3′, 5′-CCACAGGCTGGAATGCTAC-3′, 5′-CCACAGGCTGGAATGCTAC-3′. 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 (GAPDH) and relative to the calibrator (control sample), was then transformed by 2ΔΔCt (fold increase), where ΔΔCt = ΔCt (sample) – ΔCt (control) and ΔCt is the Ct of the target gene subtracted from the Ct of the housekeeping gene. The vehicle control consisted of the Penny cell line with DMSO alone seeded at 3 × 10^3 cells/well. After incubation with a horseradish peroxidase (HRP)-linked secondary antibody diluted 1:2000 in PBS-Tween, membranes were washed in PBS-Tween for 30 min and incubated with an enhanced chemiluminescence reagent (Super Signal West Pico Mouse IgG Detection Kit, Thermo Scientific), Murine NMuMG cell line was used as positive control for IGF-1R and p-IGF-1R expression.

**Western blot analysis**

Total proteins were obtained from primary canine OSA cell lines, OSB1 (De Maria et al., 2009; Maniscalco et al., 2013) and NMuMG cell lines by boiling lysis buffer containing 1% SDS and 0.1 M Tris-HCl (pH 6.8). Total proteins from each sample (40 μg) were separated on an 8% sodium dodecyl sulphate (SDS)–polyacrylamide (PAGE) gel, transferred onto a Hybond-C Extra membrane (Amersham Biosciences) and incubated overnight at 4°C with the primary antibodies (Table 2). After incubation with a horseradish peroxidase (HRP)-linked secondary antibody diluted 1:2000 in PBS-Tween, membranes were washed in PBS-Tween for 30 min and incubated with an enhanced chemiluminescence reagent (Super Signal West Pico Mouse IgG Detection Kit, Thermo Scientific). Murine NMuMG cell line was used as positive control for IGF-1R and p-IGF-1R expression.
Western blot analysis of total IGF-1R, p-AKT and p-MAPK was performed on protein lysate obtained from Penny cells treated with PPP at 0.01 μM, 0.1 μM, 0.25 μM or 0.5 μM for 24 and 48 h. In addition, IGF-1R, p-IGF-1R, p-AKT, p-MAPK were evaluated after treatment with and without IGF-1 (1 μM) and PPP (0.5 μM) after 2 h of treatment in Penny and Sky cell lines cultured in medium supplemented with bovine serum albumin (BSA, 0.4%).

### Statistical analysis

IHC results and clinicopathological findings were grouped into contingency tables and analysed using Fisher’s exact test or Chi² test. Survival curves were computed using the Kaplan–Meier method and tests for differences in survival considering all known prognostic factors for canine OSA were performed using the log rank test.

### Table 1

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<th>Weight</th>
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<th>Grade</th>
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<th>IV invasion</th>
<th>DFI (days)</th>
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ALP, serum alkaline phosphatase; PM, pulmonary metastases; LR, local recurrence; LN, lymph node metastases.

* Cause not related to OSA (censored data).
All parameters with $P < 0.15$ on univariate analysis (log rank test) were subjected to multivariate analysis using Cox proportional hazard regression model. Overall survival (OS) was the number of days between surgery and death and the disease-free interval (DFI) was the number of days between surgery and tumour recurrence and/or evidence of metastasis. Censored data were considered those cases that were still alive or that did not present tumour recurrence at the end of the monitoring period or that died for unrelated causes. Caspase 3/7 activity and q-PCR data were analysed using Student’s t test. Data were analyzed with MedCalc Statistical Software v13.3 (MedCalc Software bvba) and $P < 0.05$ was considered statistically significant.

### Results

#### Epidemiological and clinical data

Data were collected from 34 cases of appendicular OSA surgically removed from dogs with a mean age of $7 \pm 2$ years (range, 2–12 years), predominantly males (59%). Seven dogs (21%) were of mixed breed and 27 (78%) were of pure breed. The represented breeds were Boxers (seven dogs) and Rottweilers (three dogs). The mean DFI was 298 $\pm$ 258 days (median, 228), and the mean OS was 264 $\pm$ 285 days (median, 274). Clinicopathological findings are listed in Table 1 and details regarding surgical therapy and chemotherapy for each subject are listed in Supplementary Material S1 (Appendix).

#### Histopathology

Of the 34 cases, 19 (56%) were osteoblastic productive OSA, 7 (21%) were chondroblastic OSA, 3 (9%) were fibroblastic OSA, 3 (9%) were unproductive osteoblastic OSA and 2 (6%) were giant cell OSA. Ten OSAs (29%) were classified as grade I, 14 (41%) as grade II and 10 (29%) as grade III. Five cases had histological evidence of intra-osseous neoplastic invasion.

#### Immunohistochemistry

In 24 cases (71%) a cytoplasmatic expression for IGF-1R in the neoplastic cells was observed. The number of positive cells in all cases was >76% of the neoplastic cells in the section. Eight (24%) cases showed mild cytoplasmic immunolabelling (low-IGF-1R expression group; Figs. 1a and b), while 16 (47%) cases displayed strong cytoplasmic immunolabelling (high-IGF-1R expression group; Figs. 1c and d). In 10 (29%) cases immunolabelling was not observed.

### Statistical analysis

Animals with tumours with high IGF-1R expression had a lower survival (mean, 279.5 days) compared to those with OSAs with low IGF-1R ($423.8$ days, log-rank test $P < 0.05$; Fig. 2) in both multivariate and univariate analyses, while DFI was reduced but not statistically different ($246.8$ vs. $347.7$ days, respectively; Fig. 2). Moreover, dogs with low IGF-1R expression survived more than 200 days longer (Fisher’s exact test: $P < 0.05$) than those with high IGF-1R expression.

Statistical analyses comparing the immunohistochemical IGF-1R expression with all clinicopathological data listed in Table 1 did not show any statistical association. Univariate analysis indicated that high IGF-1R expression was a significant prognostic indicator for survival (log-rank test $P < 0.05$; odds ratio [OR], 2.2; 95% confidence interval [CI], 1.0–4.9), but was not an indicator for DFI (log-rank test $P < 0.05$; Fig. 2). The other histopathological criteria examined demonstrated that poorly differentiated OSA (grade III) was a good indicator of both survival (log-rank test $P < 0.00$; OR, 4.2; 95% CI, 1.1–15.7) and DFI (log-rank test $P < 0.001$; OR, 3.5; 95% CI, 1.0–12.7). On multivariate analysis, both histological grade III ($P < 0.05$; OR, 4.1; 95% CI, 1.3–12.6) and high IGF-1R expression ($P < 0.05$; OR, 2.9; 95% CI, 1.1–8.0) had predictive power with regard to OS. Multivariate analysis indicated that only histological grade III ($P < 0.05$; OR, 4.3; 95% CI, 1.3–14.0) had predictive power for DFI, while serum alkaline phosphatase (ALP) values did not have a prognostic significance ($P = 0.054$).

### Molecular investigations

Quantitative data obtained from q-PCR, showed a significant increase ($P < 0.05$) of IGF-1R transcript in Wall, Penny, Sky, Dark, Pedro and Lord cell lines compared to OSB1, while IGF-1 transcript levels were significantly decreased ($P < 0.05$) in Wall, Penny, Dark, Desmond, Pedro and Lord cell lines (Fig. 3). IGF-1 and IGF-1R proteins were evaluated by western blot analysis on the seven primary OSA cell lines and on OSB1. IGF-1 protein was not detectable (data not shown), while a specific band corresponding to canine IGF-1R (130 kDa) was found in all OSA cell lines except in Sky, with a higher expression in the Wall, Penny and Desmond and OSB cell lines (Fig. 4).

In order to investigate which molecular pathways were correlated to IGF-1R expression, total AKT, p-AKT and p-MAPK were also investigated by western blot. Specific bands corresponding to the total form of AKT (60 kDa) were present in all OSA cell lines with similar intensity, while the activated form of AKT (p-AKT) was expressed at higher levels in the Penny and Dark cell lines and at lower levels in the OSB1 cell line. The double band corresponding to the activated form of MAPK (p-MAPK – 44 kDa) was visible in all cell lines.

### Biological effects of pycropodophillin on canine osteosarcoma cell lines

In vitro treatment of OSA cell lines with increasing concentration of PPP induced cell death in a dose-dependent manner. IC50s were 0.25 μM at 48 h and 0.5 μM for both 24 and 48 h (data not shown). A statistically significant increase of caspase 3/7 activity with 0.5 μM PPP after both 24 and 48 h was observed (Fig. 5) ($P < 0.05$), while after 48 h of incubation with 0.25 μM PPP (value corresponding to IC50), caspase activity did not significantly increase.
In order to investigate the biochemical effects of PPP on the Penny cell line, the expressions of IGF-1R, p-AKT and p-MAPK were measured by western blot after 24 and 48 h of treatment. In these conditions no variations in IGF-1R and p-AKT expression were detected, while a dose- and time-dependent increased expression of p-MAPK, more evident at 0.5μM after 24h and 0.5μM after 48h, was observed (Fig. 6).

In addition, down-regulation of p-AKT and p-MAP was observed in the Penny cell line treated with PPP (Fig. 8).

**Discussion**

OSA is the most frequently diagnosed canine bone tumour and, despite the use of aggressive treatments such as wide tumour resection and adjuvant chemotherapy, it remains a major cause of death in dogs as in humans (Morello et al., 2011). Therefore, new therapeutic strategies based on targeted therapy could improve prognosis and therapeutic success. Among the potential targeted therapies, TKIs represent an attractive approach to treat cancer in humans and animals. Specific inhibitors against PDGFRs, C-KIT, and VEGFRs have shown (London, 2009; Rankin et al., 2012; Casteleyn et al., 2013) that TKRs, when expressed, can be prognostic factors in tumours and important molecular therapeutic targets (Yang and Zhang, 2013).

This is the first study investigating IGF-1R expression in canine OSA tissues, although a previous study demonstrated IGF-1R expression in OSA cell lines (MacEwen et al., 2004). In the current study, IGF-1R was expressed in 24/34 OSAs (71%) similar to what has been described in human OSAs (86%) (Wang et al., 2012). To better understand the prognostic value of IGF-1R expression, canine OSAs with high and low IGF-1R expressions were compared in terms of OS and DFI. Dogs with high IGF-1R expression were characterised by a shorter survival when compared to those with low IGF-1R expression in both univariate and multivariate analyses, but no difference were observed for DFI.
The multivariate analysis also demonstrated that a poorly differentiated histological grade was the most valuable prognostic variable in this study, while ALP serum values, a well-known predictor of outcome (Garzotto et al., 2000), did not reach statistical difference, probably due to the small number of samples. Dogs with low IGF-1R expression survived more than 200 days longer than dogs with high IGF-R expression. This prognostic value is comparable to the findings observed by Wang et al. (2012), in which humans with OSA characterised by high IGF-1R expression had a lower 5-year OS rate than those with low IGF-1R.

In the current study, IGF-1 mRNA and protein expression were not observed in cell lines and tumour samples, except for the Sky cells. This may suggest that in canine OSA, IGF-1R is activated by IGF-1 mostly in a paracrine or endocrine, rather than autocrine manner (Werner et al., 2008; Werner and Bruchim, 2009). IGF-1R signalling plays a key role in bone growth and development, but aberrant signalling of this pathway has been implicated in various human cancer types, including breast (Yang and Yee, 2012), prostate (Price et al., 2012), lung (Tsuta et al., 2013) and sarcomas (Asmane et al., 2012). Moreover, its expression induces...
chemoresistance and anti-apoptotic processes (Grothey et al., 1999). In veterinary oncology, IGF receptors have been studied only in canine mammary tumours (Dolka et al., 2011; Yang and Yee, 2012), insulinoma (Buishand et al., 2012) and canine melanomas (Thamm et al., 2010). In canine mammary tumours, the high level of IGF-1R expression was correlated to high oestrogen receptor (ER) alpha and progesterone receptor (PR) expression and to the expression of anti-apoptotic molecules such as p53 and Bax (Dolka et al., 2011).

The Wall, Penny and Desmon cell lines overexpressed IGF-1R when compared to the OSB1 line and IGF1-R expression correlated with activation of both AKT (two cell lines) and MAPK (all cell lines). IGF-1R was not detected in Sky cells and was only marginally expressed in Dark cells. The high level of p-MAPK expression in Dark cells suggests that MAPK can be phosphorylated through IGF-1R-independent signalling. When comparing protein and mRNA data, lower mRNA expression of IGF-1R was found in Desmon cell line compared to the OSB1 line. This disagreement can be explained by the fact that protein levels do not always mirror mRNA levels: low protein degradation and turnover can result in high protein levels even in the presence of low mRNA levels.

The molecular and immunohistochemical findings in the current study suggest that IGF-1R signalling could play an important role in the pathogenesis of canine OSA. Recently, PPP (Girinita et al., 2004) and a monoclonal antibody against IGF-1R (Asmane et al., 2012) have been used to specifically inhibit IGF-1R. Several studies in humans have demonstrated the ability of PPP to block proliferation and induce apoptosis in multidrug resistant OSA cell lines (Duan et al., 2009).

PPP has several advantages in that it does not react with the insulin receptor and induces very limited tumour resistance. In our study, PPP at 0.5 μM for 24 and 48 h induced caspase 3/7 activation, but not at 0.25 μM, the concentration associated with the IC50 for cytotoxicity. Likewise, PPP treatment led to a significant increase in MAPK activation, which has been shown to be involved in cell death (Martin and Pognonec, 2010; Subramaniam and Unsicker, 2010; Teixeiro and Daniels, 2010) and to represent a pro-apoptotic factor through ERK and caspase 3 activation (Cagnol and Chambard, 2010). This is supported by recent results, showing that neoplastic cell lines treated in vitro with TKIs show specific activation of MAPK (Schnetzke et al., 2014). Finally, PPP treatment also caused p-IGF-1R dephosphorylation with a partial blocking of p-MAPK and p-AKT, as it has been shown in humans with specific inhibition of IGF-1R.

Conclusions

This study demonstrated that IGF-1R most likely plays a role in the pathogenesis of canine OSA and that its expression is...
Conflict of interest statement

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

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.tvjl.2014.09.005.

References


