Comparative studies on prognostic markers and signaling pathways for canine and human osteosarcoma

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Comparative studies on prognostic markers and signaling pathways for canine and human osteosarcoma

Vergelijkende studies naar prognostische merktekens en signaalpaden voor osteosarcomen bij hond en mens

(met een samenvatting in het Nederlands)

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For amma, appa, Shree and my late ammamah

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Chapter 1

General introduction

Prognosis, prognostication and prognosticators

According to the Oxford English Dictionary, "**prognosis"** can be defined in two contexts: medical and formal. By medical definition, prognosis is "an opinion, based on medical experience, of the likely development of a disease or an illness"; in the formal context it is "a judgment about how something is likely to develop in the future". The word "prognosis" is commonly used by medical practitioners and veterinarians in their recommendations to their patients or clients for the future management of a particular disease. For a veterinarian, one of the core skills is being able to provide **prognostication,** especially in the context of terminal and chronic diseases such as cancer in pet dogs and cats. It is necessary to fully inform clients on the expected disease outcome for their beloved pets; after consideration of the diseased animal's welfare, the stage of disease is of utmost importance in deciding whether to recommend euthanasia. To be able to define a prognosis and make recommendations for clients, a veterinarian needs to be experienced and well versed in the field's literature: published studies and reports on **prognosticators**. It is of particular concern that although numerous prognostic studies have been published to date on cancers in pet animals, few of these studies have led to a credible prognostic model.

Dogs with spontaneous cancer are suitable large animal models for human cancer

Dogs, as "man's best friend", live in our environment, are exposed to the same carcinogens, and share similar immunologic features; some even eat our food! Cancer is at least as common in dogs as in humans. Cancers occur naturally in dogs and develop, as in humans, in the context where the tumor, host and environment are synergistic (Paoloni and Khanna, 2008). Canine cancer as a model for human cancer has been widely accepted as a better option than the more commonly used murine models. The range of spontaneous cancers observed in dogs is similar to those in human patients: the former closely resemble the complex pathobiology and heterogeneity of the latter. Tumor initiation and progression are also influenced by many similar factors including genetics, sex, age, external exposures, hormonal and nutritional factors. Canine spontaneous cancer models are used in drug discovery and developmental pathways largely because of their similarities to human anatomy and physiology and because they have similar tumor growth characteristics in the setting of an intact immune system. The similarities include: duration and pathogenesis of tumor growth; the development of recurrent disease; resistance to conventional therapy; intra-tumoral heterogeneity; and metastasis to distant sites (Gorlick and Khanna, 2010; Khanna et al., 2006; Paoloni and Khanna, 2008; Paoloni et al., 2009; Paoloni et al., 2010; Withrow and Khanna, 2009). There are differences, however, which must be kept in mind when extrapolating data from one species to another.

Canine osteosarcoma: a relevant comparative model for osteosarcoma in humans

Of all naturally arising tumors of the dog, osteosarcoma has been said to be a good large animal model for the disease in humans (Khanna et al., 2006; Paoloni and Khanna, 2008; Paoloni et al., 2009; Withrow and Khanna, 2009). Osteosarcoma is the primary malignant type of bone tumor of mesenchymal origin; it commonly affects large to giant breed dogs of older age (>7 years old) (Dickerson et al., 2001; McNeill et al., 2007; Phillips et al., 2007; Rosenberger et al., 2007). This tumor is said to arise from primitive bone-forming mesenchymal cells, which typically produce osteoid and are highly metastatic compared to other types of primary bone tumors, including chondrosarcoma and fibrosarcoma (Cleton-Jansen et al., 2009; Gorlick and Khanna, 2010). In most cases, dogs present with clinical signs of lameness or a hard bony swelling arising from skeletal or even from extraskeletal locations (Carr et al., 2010; Jabara and McLeod, 1989; Kuntz et al., 1998; Langenbach et al., 1998; Miller et al., 2006; Patnaik et al., 1976; Patnaik, 1990; Ringenberg et al., 2000; Sato et al., 2004; Schena et al., 1989; Thomsen and Myers, 1999; Urbiztondo et al., 2010). Often there is no known history of trauma prior to presentation. Tumors are highly metastatic and metastases are predominantly observed in the lungs, though other soft tissue and skeletal sites has been reported. Diagnosis is often made based on radiographic appearance supported by cytology and histopathology findings (Berg et al., 1990; Britt et al., 2007; Lamb et al., 1990; LaRue et al., 1986; Straw et al., 1989). Evaluation for lung and bone metastasis is often made by 3-view thoracic radiographic survey and bone scintigraphy or long-bone radiographs. Radiographic lesions often represent a mixture of osteolytic and osteoproductive bony activity with or without sunburst appearance and presence of Codman's triangle. In some advanced cases a pathological fracture may be observed on bone survey radiographs. However, these findings are not pathognomonic for OS and it is essential to rule out the possibility of other bone malignancies, benign processes, bone infarctions, osteomyelitis or fungal infections (Boston et al., 2010). Histopathology is the gold standard for diagnosis of OS; this can be achieved by performing a bone biopsy or by examination of the whole lesion upon surgical removal. Histology grading and tumor staging then follow to determine the aggressive nature of the tumor; this may aid in the decision about follow-up therapies (Kirpensteijn et al., 2002; Loukopoulos and Robinson, 2007).

Two main therapy goals for dogs with OS are to control local pain and to eradicate micrometastasis (or slow down the process). Although evidence of metastasis primarily in the lungs is the generally accepted endpoint, it can vary from case to case due to the influence of several other factors including culture, client education, economy, facilities and expertise. In a few cases, the dog will have to be euthanized due to complications of the therapeutics given, or due to deteriorating quality of life caused by other factors. For instance, limb amputation is still not commonly accepted practice in some cultures (especially in several Asian and European nations). In these cases, then, the veterinarian may advise a limb amputation and additional therapies based on known prognosticators, yet it is still at the client's discretion to

proceed with the recommendations or to choose euthanasia. It gets even tougher when including consideration of the client's economic status, as this can affect the decision for euthanasia or lead to declining therapies offered by the veterinarian. In many states in the US, limb-sparing is a preferred choice of treatment for large breed dogs, aiming primarily to preserve the limb and locomotion. The limb is spared by removing the primary tumors with limited margins and replacing them with a bone allograft or an artificial device. This reduces the possibility of complications that may arise in amputation, especially in giant to large breed dogs (Boston et al., 2007; Lascelles et al., 2005; Liptak et al., 2006). This procedure is highly dependent on the local extent of the primary tumor and type of bone involved, and is not widely available.

Amputation and post-operative administration of chemotherapy remain the gold standard for most veterinary practices. Through the years, several different protocols of chemotherapy have been reported, varying according to drug delivery methods, cytotoxic agents and delivery protocols. Common chemotherapy agents used are platinum-based compounds (cisplatin, carboplatin) and doxorubicin. Radiotherapy (either external beam or radioisotopes) may be initiated at a neo-adjunctive setting prior to surgery to provide pain palliation for dogs with OS where pain control can be achieved up to 2.5 months (Boston et al., 2007; Fan et al., 2009; Mayer and Grier, 2006; Mueller et al., 2005). Stereostatic radiosurgery techniques or administration of bone-targeting radiopharmaceutical agent ^{153}Sm-EDTMP are among other therapies available at selected facilities with the intention of treating primary bone tumors with more than just palliation (Farese et al., 2004; Kvinnsland et al., 2002; Lattimer et al., 1990; Milner et al., 1998; Moe et al., 1996). Other targeted therapies, including the aforementioned, are under investigation. Some of the recent advances in anti-cancer therapies for canine OS are provided in Table 2.

As early as in the 1980s, researchers and clinicians alike have highlighted the importance of studying canine osteosarcoma as a model for humans in order to understand the disease pathogenesis. Several of the common characteristics between canine and human osteosarcoma includes the aggressive nature of the disease locally and highly metastatic potentials, appearance on gross morphology and histopathology; tumor primary sites and several molecular genetic alterations including mutations for common tumor suppressor and oncogenes; aberrant expression of growth factors and their receptors (De Maria et al., 2009; Kim et al., 2009; Kirpensteijn et al., 2008; Paoloni et al., 2009; van Leeuwen et al., 1997; Zhang et al., 2009). Table 1 lists comparative aspects for canine and human osteosarcoma.

Despite the many advances in therapy and molecular markers discovered for humans as compared to dogs with osteosarcoma, the prognosis still looks grim for both species. There is still no therapy available with curative intent, although in some cases both dogs and people appear to benefit from primary interventions (amputation or limb-sparing surgeries) combined with the current adjuvant therapies, including the use of cytotoxic agents (Bacon et al., 2008). Differences in metastatic rate exist: while most dogs develop metastasis rapidly, a smaller percentage of dogs have a tendency to develop late metastasis. Researchers are still

on the lookout for factors that may contribute to these differences. This is especially pertinent to metastatic disease, which is considered the primary endpoint in most cases. When there is metastasis, euthanasia is commonly recommended for dogs.

There is a need to stratify dogs into prognostic groups by using conventional and novel biomarkers in order to provide better therapeutic options for dog owners. Therefore, the studies described in this thesis attempt to unravel new markers for metastasis and prognosis for dogs with OS. Only in some cases is this relevant for the human disease; caution is advised in translating directly from dog to human unless research has been performed proving a similarity. This is especially true concerning prognosis.

Table 1: Comparative aspects for osteosarcoma of the dog and man

Characteristics	CANINE	HUMAN
Incidence in USA	>8000 cases/ year	600 cases/ year
Median age	Middle aged to older dogs Peak incidence 7-9 years Second small peak at 18-24 months Median peak age at 7 years	Adolescent disease Peak incidence at 10-20 years Median peak age at 16 years
Body weight	90% >20kg	Heavy
Breed / Size	Large/ giant breeds Familiar pattern in Saint Bernard, Rottweiler and Scottish Deerhound	Tall people
Sex	Males slightly more than females: ratio 1.1-1.5:1	Males more than females
Aetiology	Not completely known	Not completely known
Tumor sites	75% appendicular skeleton, metaphysis of long bones, mainly distal radius, proximal humerus, distal femur and proximal and distal tibia	Metaphysic or diaphysis of long bones (80-90%) Bones of the knee joint Proximal humerus (25%)
Predisposition	Implants, fractures, familial tendency, bone infarction, radiation, parasites (*Dirofilaria repens and Spirocerca lupi*)	Implants, fractures, familial tendency, bone infarction, radiation, Paget's disease
Clinical Signs	Pain Swelling Hard painful mass	Pain, swelling, hard painful mass, decreased joint mobility, localized erythema
Biochemistry profile	Increased serum alkaline phosphatase and lactate dehydrogenase enzyme levels (also as negative prognosticator)	Increased serum alkaline phosphatase and lactate dehydrogenase enzyme levels (also as negative prognosticator)
Diagnostic imaging	-Cranio-caudal and latero-medial radiographic views of the primary lesion, including the joint above and below the affected bone, are required - CT of the thorax is superior to radiography in detecting smaller lung lesions -Nuclear bone scintigraphy for detection of bone metastases -MRI for staging and planning of limb-sparing procedures	-at least two orthogonal radiographic views are required when a bone lesion is suspected -MRI represents the primary mode of evaluation of OS in humans and can clearly demonstrate the extent of tumour invasion of the surrounding soft tissue, neurovascular involvement, extent of bone marrow replacement and presence of discontinuous metastases -A CT scan of the chest and a nuclear scintigraphy bone scan are recommended to rule out metastasis to the lungs and bone - Use of positron-emission tomography (PET) for staging and monitoring treatment

Primary tumor radiographic features	Bone destruction, new bone formation, Codman's triangle, soft tissue swelling, sunburst appearance	Bone destruction, new bone formation, Codman's triangle, soft tissue swelling, sunburst appearance
Pathological fractures	Uncommon pathological fracture (3%)	Uncommon pathological fracture (5-10%)
Karyotype	-75% aneuploid, complex to chaotic, -Gains and losses identified in many autosomes - various centrometric translocations and rearrangements	75% aneuploid, complex to chaotic -Gains and losses identified in many autosomes and X chromosome, chromosome gains outnumber the losses by 20-30% -many centrometric rearrangements
Histological grade and features	High grade Predominantly osteoblastic, other histologies: chondroblastic, fibroblastic, telangiectic	High grade (central) Predominantly osteoblastic, other histologies: chondroblastic, fibroblastic, telangiectic, giant cell rich, Small cell osteosarcoma-(rare variant)
Metastatic sites	Lungs> bones> soft tissue 90% cases with metastasis at diagnosis	Lungs>bones> soft tissue 20% cases metastasis at diagnosis
Therapies	Amputation (Most common) Limb sparing techniques (at specialized centers) Adjuvant chemotherapy (Pre-operative protocol did not have significant increase in survival as compared to post-operative protocols)	Limb sparing techniques (90% of cases) Amputation (rare) Neoadjuvant chemotherapy
Surgical repair	Often with arthrodesis	Often with modular articulating devices
Common cytotoxic agents used for chemotherapy	Doxorubicin, cisplatin, lobaplatin, carboplatin -metronomic chemotherapy with doxycycline, piroxicam and cyclophosphamide	doxorubicin, cisplatin, methotrexate and ifosfamide
Duration of adjuvant chemotherapy	4-6 cycle of adjuvant chemotherapy	Up to 1 year of adjuvant chemotherapy
Regional lymph node metastasis	4.4-9% of cases Poor prognosis	<10% of cases Poor prognosis
Metastatic rate without chemotherapy	90% before 1 year	80% before 2 years
Survival	-60% survival at one year post operative chemotherapy	-70% survival at 5-year postoperative chemotherapy - 30–40% of OS patients still experience relapses within 3 years of treatment
Molecular genetic alterations	Refer to Chapter 3: Review on prognostic and predictive biomarkers for canine osteosarcoma (Selvarajah and Kirpensteijn, 2010)	
Prognostic and predictive markers		

This table above has been partially adapted from (Morello et al., 2010; Withrow and Wilkins, 2010)

Table 2: Recent advances (2008-2011) in new therapeutic approaches for canine osteosarcoma

Therapy or type of management	In vitro models/ clinical subjects	Mechanism/ target/ research conclusions	Reference(s)
Combination immune- and suicide gene therapy	Pilot study on 5 dogs with OS	Cytokine-enhanced vaccine and interferon-β plus suicide gene as combined therapy	(Finocchiaro et al., 2011)
Lycopene	In vitro models	Carotenoid synthesized from plant material (natural compound). Lycopene did not negatively or positively affect survival of osteosarcoma cells during doxorubicin treatment and independently induced apoptosis in the HMPOS cell line. These findings warrant further in vitro and in vivo studies into the use of this natural compound as an adjuvant antiproliferative, proapoptotic treatment in dogs with osteosarcoma.	(Wakshlag and Balkman, 2010)
Rapamycin	In vitro, dogs with OS	Targets mTOR pathway. Rapamycin may be safely administered to dogs and can yield therapeutic exposures.	(Paoloni et al., 2010b)
Gemcitabine	In vitro models, dogs with OS	Gemcitabine replaces cytidine, during DNA replication and targets enzyme ribonucleotide reductase (RNR) during cell replication. Gemcitabine exhibited biological activity against canine OSA cell lines in vitro, and a combination of gemcitabine and carboplatin exhibited synergistic activity at biologically relevant concentrations. Aerosol gemcitabine may be useful against pulmonary metastases of osteosarcoma.	(McMahon et al., 2010)(Rodriguez et al., 2010)
Histone deacetylase inhibitor valporic acid	Canine OS xenograft model	Treatment of canine and human OS cell lines with clinically achievable VPA concentrations resulted in increased histone acetylation but modest anti-proliferative effects.	(Wittenburg et al., 2010a)(Wittenburg et al., 2010b)
Sorafenib	In vitro models	Small molecular inhibitor of several tyrosine kinases : VEGFR, PDGFR and Raf	(Wolfesberger et al., 2010)

		A significant decrease of neoplastic cells was observed after incubation with 0.5-16 microM sorafenib or with 80-640 microM carboplatin	
Bisphosphanates	Dogs with OS, in vitro models	Single-agent pamidronate administered intravenously with NSAID therapy relieves pain and diminishes pathologic bone turnover associated with appendicular OSA in a subset of dogs. Upon pain control, adjuvant pamidronate appears to decrease focal bone resorption in the local tumor microenvironment.	(Fan et al., 2009b), (Fan et al., 2007; Fan et al., 2008; Poirier et al., 2003)
Thoracoscopy approach to resect pulmonary metastasis	Dogs with OS	Single case report- thoracoscopy by lateral approach to resect lung lesions	(Dhumeaux and Haudiquet, 2009)
HSP90 inhibitor (STA-1474)	In vitro models	Targets Heat shock protein 90 STA-1474 induced tumor regression, caspase-3 activation and downregulation of p-Met/Met and p-Akt/Akt in OSA xenografts, suggesting that HSP90 represents a relevant target for therapeutic intervention in OSA	(McCleese et al., 2009)

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Chapter 2

Thesis aims and chapter outline

This thesis aimed to further exploit the canine genome in order to discover new markers for prognosis and metastasis; highlighting several important signaling pathways, which may contribute to the aggressive nature of the tumor and to identify potential therapeutic targets for the clinical management of dogs with OS. Along with the studies performed primarily for identifying markers for the disease and its metastasis in dogs, some of the markers which are novel for human OS were investigated on human tumor specimens and *in vitro* models. Several therapeutic approaches, which are relatively new for canine OS, are further explored using *in vitro* models.

Several studies were performed in the course of identifying various molecular prognostic markers and exploring signal transduction pathways for canine OS and few for human OS. This thesis will discuss a couple of novel biomarkers which show in some cases relevance for human disease and in others not. Caution should be vested in translating directly from dog to human, if research has not been performed proving a similarity, especially when it concerns prognosis. The research objectives and brief outline of these studies are listed below:

CHAPTER 3:
This chapter reviews several of the known molecular prognostic and predictive biomarkers in canine OS. This chapter also explores how molecular investigations in affected dogs may assist in the multimodal management of this disease and addresses some of the progress and limitations of such investigations to date. A short list of molecular markers reported for canine OS is presented and their comparative aspects to human OS are further discussed.

CHAPTER 4:
This chapter describes the study that was aimed (1) to identify survival-associated genes and signaling pathways in a panel of canine OS primary tumors by means of cDNA microarray approach; (2) to compare gene expression profile of the tumors from dogs with short survival time to the published human OS dataset to determine the common signaling pathways involved in the advanced disease stage of both species; and (3) to identify functional clusters of genes which could be targeted for therapy.

CHAPTER 5:
The objectives of this chapter are (1) to identify genes and signaling pathways associated with high-metastatic potentials in canine OS *in vitro* models with different metastatic potential, using cDNA microarray technology; and (2) to identify transcription factors among the differentially expressed genes through gene network analysis.

CHAPTER 6:
In order to facilitate reliable gene expression data analysis for canine OS, this research undertook the identification of a panel of reference genes, which are useful for normalization of quantitative real-time PCR expression data.

CHAPTER 7:
Among the signaling pathways discussed in Chapters 4 and 5, which have been discovered to be important for survival and metastasis of canine OS, Wnt signaling appeared among the top pathways, also from comparative pathway analysis. Wnt signaling is a complex pathway which involves extremely diverse key players and interactions with other signaling pathways which is crucial for bone physiology, development and most importantly for cancer tumorigenesis, cell proliferation, differentiation and metastasis. Several studies have been published on human OS while information on this signaling status in canine OS is relatively scarce and deserves further exploration. Therefore, the goal of this Chapter was: (1) to characterize the Wnt activation status in canine OS cell lines and primary tumors and (2) to evaluate the prognostic value of nuclear beta catenin and axin2 for canine and human OS.

CHAPTER 8:
In addition to Wnt signaling, epidermal growth factor receptor (EGFR) signaling is another pathway commonly studied in human OS but has been understudied in the canine counterpart. This pathway was among the top pathways revealed from the metastasis associated gene expression profiling described in Chapter 5. The study presented in this chapter was aimed to determine the prognostic and clinicopathological relevance of mRNA and protein expression of epidermal growth factor receptor (EGFR) in canine OS primary tumors and cell lines.

CHAPTER 9:
This study was preceded by the findings described in Chapter 4 and 5 wherein several heat shock protein (HSP) members were found to be highly expressed in dogs with poor prognosis (short survivors) and highly expressed in the highly metastatic cell line. The objectives of this study are (1) to investigate the prognostic and clinicopathological relevance of several HSPs at mRNA and protein level (immunohistochemistry) in snap-frozen tumors and cell lines of canine OS; and (2) to evaluate the potential for *in vitro* targeting of selective HSP in canine OS.

CHAPTER 10:
The steroid hormone vitamin D3 exerts its biological actions through the vitamin D receptor (VDR) where the Vitamin D3 and VDR have a key function in bone formation and is known to exert anti-proliferative actions in human cancer. Although considerable work has been carried out in understanding the molecular mechanisms underlying the vitamin D-mediated differentiation of human OS cells, the prognostic relevance of this pathway is yet to be

explored for OS in both species. The aim of this study was to evaluate the expression status and prognostic significance of the primary molecular markers involved in Vitamin D signaling, including the vitamin D receptor (VDR), enzymes 24-hydroxylase (CYP24A1) and 1α-hydroxylase (CYP27B1) in both canine and human OS.

CHAPTER 11:
Receptor and non-receptor tyrosine kinase and several protein kinases are involved in multiple proliferative signaling pathways in malignant diseases. There is growing body of evidence which suggest that tyrosine kinase and general kinase inhibitors are useful for canine cancer therapies but the investigations on *in vitro* and *in vivo* canine OS models are still at its infancy and require further exploration. This chapter aimed at screening a kinase inhibitor compound library in 4 canine OS cell lines to identify several highly potential compounds, which could be used as molecular-targeted therapies for canine OS.

CHAPTER 12:
The prognostic markers and signal transduction pathways evaluated from the current research and markers reported by others that are important for canine OS pathogenesis and metastasis are further discussed. The use of the canine as a model for human OS is discussed with relevance to the findings of this thesis. New insights into therapeutic approaches for canine OS are mentioned: targeting the Vitamin D pathway, molecular chaperones (Heat shock protein), or even specific molecular targeted therapy (Wnt signaling, tyrosine kinase, protein kinases). The thesis concludes with a brief overview on the limitations and potential challenges for targeted therapies/individualized management of dogs with OS and recommendations for future prospective research strategies.

CHAPTER 13:
Key finding from each chapter is summarized in English and translated in Dutch (Samenvatting).

Chapter 3

Prognostic and predictive biomarkers of canine osteosarcoma

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Abstract

Canine osteosarcoma (OS) is an aggressive tumour that accounts for approximately 90% of primary bone tumours in the dog. Although the standard treatments (including limb amputation/sparing, chemotherapy and palliative radiotherapy) have significantly increased survival rates, almost 90% of animals will eventually develop predominantly pulmonary metastases. Despite advances in various therapies, prognosis remains poor, with median survival times ranging from 3 months to 1 year and <20% of dogs survive for >2 years following diagnosis. Various clinical and epidemiological markers have facilitated decision-making with respect to therapy but no single molecular biomarker has been shown to enhance prediction of disease progression. The publication of the canine genome in 2005 raised the possibility of increasing understanding of the genetic mechanisms underpinning canine OS. This review explores the use of biomarkers within the multi-disciplinary management of dogs with OS, and highlights the few known, potential prognostic/predictive molecular markers including their potential value as 'bridging biomarkers' for human OS. Although high-throughput profiling of canine OS remains in its infancy, research within the next decade using leading-edge screening technologies has the potential to identify biomarkers that may enhance diagnostic and prognostic accuracy and result in more effective, individually tailored, treatment and management protocols for affected dogs.

Introduction

Osteosarcoma (OS) is a malignant neoplasm of mesenchymal origin that produces osteoid and accounts for approximately 85% of all primary canine bone tumours. A heterogeneous tumour with regard to location, metastatic sites, radiological presentation, histopathological subtypes, progression and response to treatment, it is almost exclusively observed in large or giant breeds such as the Rottweiler, Great Dane, Greyhound, Saint Bernard and Doberman pinscher (Norrdin et al., 1989; Ru et al., 1998; McNeill et al.,2007). There is some evidence to suggest males are more predisposed to OS and the median age of onset of clinical signs ranges from 8 to 10 years (Spodnick et al., 1992; Boston et al., 2006), although a subset of tumours arises in younger dogs (Evans, 1983).

Dogs often present with a history of lameness or even fracture of the affected bone. Predilection sites are the weight-bearing regions of the long bones (humerus, femur, radius, tibia and ulna) (Liptak et al., 2004) with approximately 25% of tumours arising in the axial skeleton including the flat bones of the skull, ribs, vertebrae, sternum, and pelvis (Hammer et al., 1995; Dickerson et al.,2001). Intriguingly, given that OS is a 'sarcoma of bone', primary tumours arising at extra-skeletal sites have also been described (Kuntz et al., 1998; Langenbach et al., 1998). OS is an aggressive and invasive neoplasm that causes local skeletal destruction resulting in radiographic evidence of both osteoproductive and osteolytic lesions. It is highly metastatic, predominantly to the lungs with a lower frequency of spread to distant bones, regional lymph nodes (Hillers et al., 2005) and other soft tissues (Peremans et al., 2003; Gorman et al., 2006). A clinical diagnosis is made following assessment of case signalment and history and based on the radiographic appearance of the lesion.

The current diagnostic 'gold standard' for OS is histopathological examination with tumour classification based on the formation of osteoid matrix with osteoblastic, fibroblastic, chondroblastic and telangiectic subtypes (Kirpensteijn et al., 2002; Loukopoulos and Robinson, 2007). There can be considerable variation in the histological appearance both between and within individual neoplasms. Metastatic lesions usually appear histologically identical to the primary tumour, although they more frequently exhibit a greater degree of necrosis. The histopathological grading system employed by Kirpensteijn et al. (2002) demonstrated that grade III tumours, which account for 75% of cases, have a significantly poorer prognosis than grade I and II neoplasms. However, this grading system was not significantly prognostic for tumours from non-appendicular sites.

Historically, dogs with OS that were treated by amputation alone had poor overall survival times (ST), typically <3 months, with the majority dying or being euthanased due to metastatic disease (Brodey and Abt, 1976). Over the years, although there have been advances in disease management, amputation remains the basic standard-of-care for appendicular OS with 'limb-sparing' used only to selectively remove tumours located in the distal radius, ulna and tibia (Straw and Withrow, 1996; Boston et al.,2007). With primary

tumour removal, dogs will be pain-free for a short time, but 90% succumb to metastatic disease within 3–6 months.

Adjuvant therapy, such as multi-modal chemotherapy regimes, treatment with bisphosphonates or immune modulators and palliative radiation, is now provided at specialised veterinary practices (Tomlin et al., 2000; Dow et al., 2005; Walter et al., 2005; Fan et al.,2009). Combined therapy can contribute significantly to ST, with 40–50% of dogs surviving >1 year when treated by amputation in combination with chemotherapy (Bergman et al., 1996; Phillips et al., 2009), although <10–20% survive >2 years. Finally, although not widely practiced due to operative and post-operative complications, a subset of dogs with less than three pulmonary metastatic lesions are reported to benefit from metastasectomy (O'Brien et al.,1993).

Over the years, various compounds have been used in adjuvant chemotherapy including cisplatin, carboplatin and doxorubicin. These have been used in single and in multi-agent regimes and at varying dosage and treatment interval (Berg et al., 1995; Bergman et al., 1996; Chun et al., 2000; Bailey et al., 2003). No differences in survival are seen when these treatments are compared with pre- or post-operative chemotherapy (Berg et al., 1997) and no differences in the disease-free interval (DFI) have been reported for dogs treated using single- or multi-agent chemotherapeutic regimes. Prolonged, intense use of chemotherapy is often not an option due to adverse side-effects compromising any clinical benefits (Barabas et al., 2008), and to date, aggressive therapy has proven ineffective in restricting the growth of metastases. A small number of cases of canine OS that do not receive adjuvant chemotherapy do not succumb to metastatic disease once the primary tumour has been removed (Selvarajah et al., 2009). This suggests that genetic composition of both the host and tumour may contribute to differences in the metastatic potential. In this regard it is important to differentiate patients that respond to chemotherapy, from those that do not.

There are many documented prognostic indicators for canine OS (Table 1), and the majority of these are similar to those reported from large retrospective studies of the human neoplasm, including age, tumour size, location and histological subtypes (Owen, 1967; Kim et al., 2007; Bramer et al., 2009; Pakos et al., 2009). The accurate segregation of canine patients into distinct prognostic subgroups, based on such indicators, is key to the tailoring of appropriate treatment.

The potential use of biomarkers in the management of canine OS

Biomarkers are central to 'personalised' medicine. One definition of a biomarker is that it is a specific 'measure' of a biological/ pathological process or cellular response to a particular therapy or stage of the disease. Advances in our understanding of the canine genome have provided opportunities to enhance our knowledge of the molecular basis of pathogenesis and progression of dog cancers. Gene expression data allows us to further classify animals with OS and to use statistical analysis to enhance clinical decision-making. The evaluation of such patients based on clinical need rather than on other aspects of the disease state is vital. Many

proteins that could reflect disease are not released or do not leak from diseased tissue into the circulation, and biomarkers are typically those substances that can be detected in samples such as serum, plasma or urine.

Table 1: Summary of current clinical and histological predictors / prognosticators in canine OS

Study	**Prognostic factor**	**Summary / interpretation(s)**
(Boston et al., 2006)	Lung metastasis	Grave prognosis, most of the time euthanasia is offered
(Hillers et al., 2005)	Lymph node metastasis	Rare in dogs but those with lymph node metastasis have significantly poorer prognosis (both DFI and ST) compared to those without
(Kirpensteijn et al., 2002) (Hammer et al., 1995) (Moore et al., 2007)	Tumor mitotic index	Increased number of mitoses reflects higher hazard for DFI
(Lascelles et al., 2005)	Postoperative infection	Postoperative infections after limb-sparing have positive influence on survival
(Misdorp and Hart, 1979) (Forrest et al., 1992)	Larger tumor diameter and size	Larger tumor significantly associated with pulmonary metastasis & poor prognosis
(Misdorp and Hart, 1979)	Extension of tumor into the adjacent soft tissue	Poor prognosis
(Berg et al., 1995) (Powers et al., 1991)	% tumor necrosis	Significant direct correlation with ST Percent tumor necrosis was strongly predictive for local tumor control while no correlation between with time to metastasis (Powers et al., 1991)
(Misdorp and Hart, 1979) (Loukopoulos and Robinson, 2007)	Histological subtype	Fibrosarcomatous subtype bears a favorable prognosis
(Loukopoulos and Robinson, 2007; Moore et al., 2007; Spodnick et al., 1992)	Age	Dogs aged 5 years and below have shorter DFI compared to older dogs. Higher mitotic index in tumors from young dogs.
(Bergman et al., 1996)	Tumor location: humerus	Negative prognostic factor Humerus location has shorter DFI and ST than with OSA at other locations.
(Kirpensteijn et al., 2002)	Histological grade	Higher grades are associated with decreased ST and DFI. Grade 1 and 2 having significantly better prognosis compared to grade 3.
(Kirpensteijn et al., 2002) (Selvarajah et al., 2009) (Hillers et al., 2005) (Garzotto et al., 2000) (Vail et al., 2002) (Ehrhart et al., 1998) (Moore et al., 2007)	Serum alkaline phosphatase	An increased level of alkaline phosphatase was associated with a shorter DFI and ST
(Dickerson et al., 2001) (Straw et al., 1996)	Tumor location: Mandible	Favorable prognosis. Dogs treated with surgery alone had a one-year survival rate of 71%, which is higher than for dogs with appendicular OS
(Bergman et al., 1996) (Lascelles et al., 2005) (Moore et al., 2007)	Body weight	lower body weights (< 40 kg) had significantly longer DFI and ST than larger dogs
(Kirpensteijn et al., 2002)	Blood vessel invasion	Increased risk in terms of DFI

Over the last decade, increased molecular-based research has improved our understanding of the pathogenesis of both canine and human OS. Many investigations have been directed at identifying single gene alterations as predictors of metastasis, cell proliferation, drug resistance, bone turnover and other processes central to disease progression. In this context we propose a conceptual framework for the potential use of such biomarkers as diagnostic and prognostic indicators as well as their use in therapeutic decision-making in the management of dogs with OS (Fig. 1).

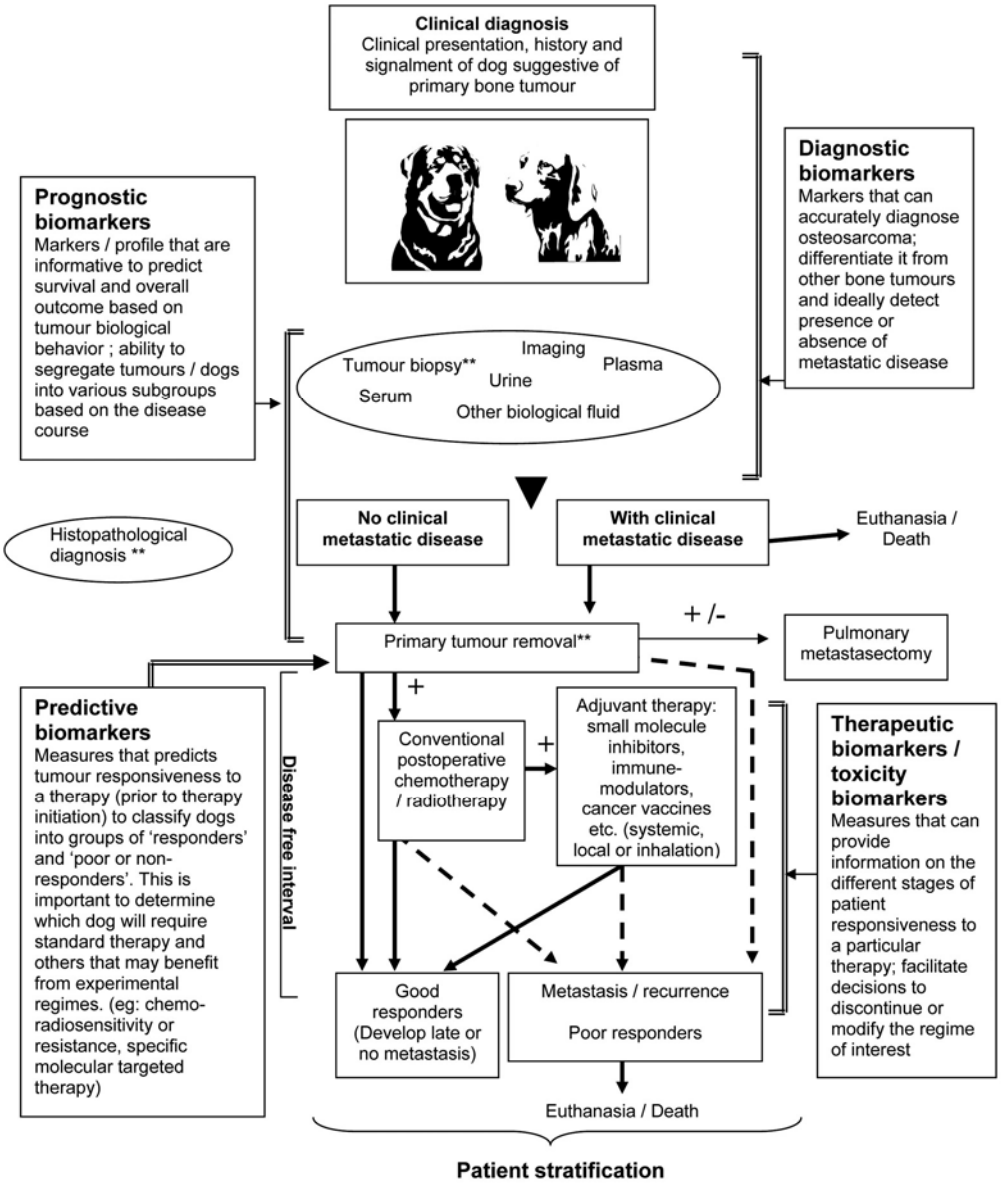
It is likely that reliable biomarkers will be those useful in multiple species, so-called translational or 'bridging' biomarkers. Indeed, canine OS provides an excellent model for studying this neoplasm in humans (Paoloni et al., 2009) since the tumour has features common to both species including clinical presentation, histopathological appearance, location and sites of metastases and prognosis. However, few compounds to date qualify as such 'bridging' biomarkers, largely because of the paucity of research on the canine form. Furthermore, while many biomarkers have potential translational applications, most of these have been based on functional, in vitro assays and have not been validated by retrospective or prospective studies.

Following a search of peer-reviewed publications from 1954 to 2009 using the National Center for Biotechnology Information (NCBI) Entrez Pubmed search engine1 and the Web of Knowledge2, and using the keywords 'CANINE', 'DOG', 'OSTEOSARCOMA' and

'OSTEOGENIC SARCOMA', retrieved articles were assessed for 'molecular' prognostic indicators/predictive markers for OS. The predictive and translational relevance/significance of the identified potential biomarkers is discussed below.

Diagnostic biomarkers for canine osteosarcoma

The differential diagnosis of OS has become increasingly reliant on molecular diagnostics and immunohistochemistry, although currently there are no biomarkers that can reliably classify the histopathological subtype or predict the malignant potential of canine bone tumours. Alkaline phosphatase is an unreliable diagnostic biomarker for OS as it cannot differentiate this tumour from other bone-forming tumours such as multilobular osteochondrosarcoma or from reactive bone lesions (Barger et al., 2005). Ezrin, a cytoskeletal linker protein, has recently been reported to have 100% specificity in differentiating human chondroblastic OS from chondrosarcoma. This form of OS expressed this protein regardless of histology grade (Salas et al., 2009) and ezrin is an example of how molecular-based markers can facilitate diagnostic precision. The use of this biomarker in differentiating the histological variants in canine OS remains to be determined.


Clinical diagnosis
Clinical presentation, history and signalment of dog suggestive of primary bone tumour
Prognostic biomarkers
Markers / profile that are informative to predict survival and overall outcome based on tumour biological behavior ; ability to segregate tumours / dogs into various subgroups based on the disease course
Diagnostic biomarkers
Markers that can accurately diagnose osteosarcoma; differentiate it from other bone tumours and ideally detect presence or absence of metastatic disease
Tumour biopsy**
Imaging
Plasma
Urine
Serum
Other biological fluid
Histopathological diagnosis **
No clinical metastatic disease
With clinical metastatic disease
Euthanasia / Death
+ /-
Primary tumour removal**
Pulmonary metastasectomy
+
Conventional postoperative chemotherapy / radiotherapy
+
Adjuvant therapy: small molecule inhibitors, immune-modulators, cancer vaccines etc. (systemic, local or inhalation)
Predictive biomarkers
Measures that predicts tumour responsiveness to a therapy (prior to therapy initiation) to classify dogs into groups of 'responders' and 'poor or non-responders'. This is important to determine which dog will require standard therapy and others that may benefit from experimental regimes. (eg: chemo-radiosensitivity or resistance, specific molecular targeted therapy)
Disease free interval
Therapeutic biomarkers / toxicity biomarkers
Measures that can provide information on the different stages of patient responsiveness to a particular therapy; facilitate decisions to discontinue or modify the regime of interest
Good responders (Develop late or no metastasis)
Metastasis / recurrence
Poor responders
Euthanasia / Death
Patient stratification

Prognostic and predictive biomarkers

Prognostic biomarkers help predict patient survival and overall clinical outcome. In consequence, these markers should reflect particular biological properties of the neoplasm such as their proliferative and metastatic capacities, and should also be able to segregate tumours / dogs into various subgroups based on likely disease-course. Predictive biomarkers are those that aid clinicians in assessing if a dog will respond favourably to a particular therapy (Gogas et al., 2009). The capacity to stratify dogs into prognostic and predictive subgroups thus greatly facilitates the delivery of optimal therapy. Although still at the developmental stage, gene expression profiles have been used in such patient stratification (Selvarajah et al., 2009).

Elevated total alkaline (TALP) and bone alkaline phosphatase (BALP) concentrations are known prognostic indicators for canine OS. Dogs with normal pre-treatment TALP and BALP levels survived significantly longer than did animals with increased pre-treatment levels of this enzyme (Garzotto et al., 2000). Although the preoperative elevation of alkaline phosphatase can be used as a prognostic biomarker in dogs, there are discrepancies as to its use postoperatively in predicting metastases or tumour recurrence. To date, the molecular markers that have been investigated for canine OS reflect the various genetic alterations of the tumour with respect to disease progression. Few appear prognostically useful because of lack of independent validation and significant further investigation is required in this area.

TP53 tumour suppressor

Tumour suppressor genes encode proteins that prevent or retard cell division, and their mutation contributes to the development and progression of cancer. Commonly mutated tumour suppressors found in canine OS include phosphatase and tensin homolog (PTEN) and tumour protein 53 (TP53). Although PTEN mutations and subsequent down-regulation of protein expression are present in a majority of canine cell lines and tumours, no prognostic significance to this event has been reported (Levine et al.,2002). However, TP53 mutational inactivation has been described in both in vitro models and in spontaneous OS in the dog (van Leeuwen et al., 1997; Johnson et al., 1998). Elevated levels of TP53 protein are commonly encountered in tissues harbouring cells containing the mutation. In 84% of appendicular and 56% of axial cases of canine OS, there is elevated TP53 expression (Sagartz et al., 1996; Loukopoulos et al., 2003). In one independent study population, TP53 had mutated in approximately 40% of tumours from both axial and appendicular sites; this mutation status had prognostic value as dogs with mutated TP53 had significantly shorter ST that correlated with elevated serum alkaline phosphatase concentration and tumour histological grade (Kirpensteijn et al., 2008). To date, it remains unclear if the TP53 mutation occurs in the metastases as well as in the primary tumour (van Leeuwen et al., 1997; Kirpensteijn et al., 2008), although it is quite possible that other events, rather than this mutation, contribute to tumour spread (Vousden, 2002). The TP53 mutation may be an independent prognostic

indicator and predictor of a more malignant phenotype of neoplasm which could be used in prospective patient evaluation.

Angiogenic markers: vascular endothelial growth factor

Plasma vascular endothelial growth factor (VEGF) is associated with more aggressive tumours in dogs (Wergin and Kaser-Hotz,2004) and for OS, VEGF concentrations significantly correlate withDFI (Thamm et al., 2008). Similarly, VEGF expression in resected human OS is an important negative prognostic factor (Bajpai et al., 2009), and strong predictor of metastasis and poor survival in the 'chemo-naïve' human neoplasm (Kaya et al., 2000). The VEGF pathway has a number of critical members, particularly receptors that play significant roles in angiogenesis and hence characterize the malignant tumour phenotype. VEGF receptor (R)-3 negatively correlates with ST and DFI in human OS (Abdeen et al., 2009), and VEGFR-2 links with candidate genes associated with poor prognosis in canine OS on pathway analysis (Selvarajah et al.,2009).

Mesenchymal–epithelial transition factor

Mesenchymal–epithelial transition factor (MET) is a protooncogene that encodes a protein also known as c-Met or hepatocyte growth factor receptor (HGFR). MET has many roles in neoplasia including an ability to activate oncogenic pathways and to participate in angiogenesis and metastasis. MET is expressed in canine OS including lung metastases (Ferracini et al., 2000), and expression of MET in primary tumours was found to predict for metastasis via lymphatics (Fieten et al., 2009). MET mRNA is constitutively expressed by both human and canine OS cell lines (De Maria et al., 2009), where stimulation with hepatocyte growth factor results in increased cellular proliferation and the formation of larger in vitro cell colonies (MacEwen et al., 2003). Small molecule inhibitors of MET have been shown in vitro to impair the invasive properties of canine OS cells and may represent a future treatment option (De Maria et al., 2009).

Cyclooxygenase-2

Cyclooxygenase (COX)-2 is an enzyme involved in apoptosis, immune surveillance and in angiogenesis and has been implicated in the production of prostaglandins in tumours including OS (Mohammed et al., 2004). The value of COX-2 as a biomarker in human OS is debatable as a predictor of metastasis and ST (Dickens et al., 2003; Rodriguez et al., 2008; Urakawa et al., 2009). In cases of canine appendicular OS, dogs with tumours with strong expression of COX-2 had a significantly decreased overall ST (86 days) relative to dogs with tumours with minimal expression of this enzyme (>300 days) (Mullins et al., 2004). However, since the number of dogs investigated in this study was small, more extensive studies will be required to validate this finding.

Ezrin

As outlined above, ezrin is a cytoskeletal linker protein, a member of the ezrin–radixin–moesin (ERM) protein family, which has a key role in the coordination of tumour metastasis (Hunter, 2004). An immunohistochemical study on canine OS revealed that high expression of this protein is associated with early metastases, and hence poorer clinical outcome. Consistent with this finding, a significant association between high ezrin expression and poor outcome is reported in paediatric OS (Khanna et al., 2004). In other studies of primary human OS, ezrin over-expression predicted lung metastasis (Xu-Dong et al., 2009) and, when associated with elevated alkaline phoshatase levels, predicted patients that responded to chemotherapy but had poor overall survival relative to patients with tumours that did not express this protein (Kim et al., 2009a).

Metalloproteinases

Metalloproteinases (MMP) are zinc-dependent enzymes commonly expressed in neoplasia and in inflammatory disease. In the context of cancer, MMP have been implicated as biomarkers of shorter DFI (Uchibori et al., 2006), and as predictors of survival following neo-adjuvant chemotherapy (Foukas et al., 2002). These enzymes also correlate with the invasive and metastatic forms of human OS, and in particular, MMP-2 and -9 have been shown to be highly expressed in three canine OS cell lines (Loukopoulos et al., 2004). Primary OS in 30 dogs had greater MMP expression in tumour than in stromal cells, suggesting a role for the enzyme in malignancy (Lana et al., 2000). Although an elevated plasma MMP-2 concentration has been linked with poor prognosis, its inhibition with BAY 12-9566, in combination with doxorubicin chemotherapy, did not improve ST in dogs with OS (Moore et al., 2007).

Chemoresistance markers

Survival for dogs with OS has remained static for the last decade despite advances in chemotherapy and has been attributed in part to the chemoresistance of the neoplasm. In treating human OS, two well-characterised molecular markers of multi-drug resistance are used: the drug resistance pump P-glycoprotein (P-gp), and the multi-drug resistance-related protein 1 (MRP1). P-gp, a substrate for MRP1, is expressed in canine OS cell lines resistant to doxorubicin (Page et al., 2000) and in 66% of cases of canine OS (Cagliero et al., 2004), but its prognostic or predictive value of ST has not been determined. Recently, gene expression profiling of canine OS revealed up-regulation of transcripts associated with drug resistance such as MGST1 (Selvarajah et al., 2009). Future elucidation of the chemoresistance mechanisms operational in canine OS will be key to circumventing this problem.

Therapeutic and toxicity biomarkers

Therapeutic and toxicity biomarkers monitor the ongoing effects of a compound on a patient. Haematology, serology, urine biochemistry, blood gas analysis and histopathology are currently used to monitor patients for adverse effects on cardiac, renal and hepatic function

(Barabas et al., 2008). Molecular approaches may be able to precisely determine the effect of particular therapies during OS progression. These could include markers of bone remodelling, such as the collagen breakdown products N- and Ctelopeptide, which could monitor dogs undergoing anti-resorptive therapies (Lacoste et al., 2006; Fan et al., 2007; Lucas et al., 2008).

Similarly, serum concentrations of cardiac troponin I (cTnI), a sensitive and specific marker of cardiomyocyte death, could be used to monitor dogs on post-operative chemotherapy, especially animals receiving the known cardiotoxin doxorubicin (Selting et al., 2004). Studies to discover novel therapeutic and toxicity biomarkers can be performed in parallel with clinical trials where the serial assessment of various molecular markers is being carried out.

Integrative platforms for biomarker discovery: pitfalls and prospects

State-of-the-art technologies will be required to carry out noninvasive, in vivo serial assessment of disease progression, therapeutic response and drug toxicity in dogs with OS. The advent of high throughput 'genomic' assessment has raised questions as to whether single or multiple markers are most appropriate. In most circumstances, given tumour heterogeneity and the variation in stage of development, single biomarkers are unlikely to be sufficiently sensitive and specific and it is likely that panels of biomarkers will be required.

We have recently published the novel correlation of gene expression profiling with prognosis for canine OS (Selvarajah et al., 2009). The differences in gene expression profile between two survival groups were minor (<100 genes) compared to similar studies of human OS, where large numbers of candidate genes were identified (Nakano et al., 2003; Srivastava et al., 2006; Walters et al., 2008). The genes that were found to have prognostic significance in the canine study had roles in cell proliferation, drug resistance and in metastasis, which, taken together, reflect tumour malignancy. The study also identified subgroups of animals that correlate highly with ST. Although the sample size was small, these findings provide initial insights that merit further investigation and cross-validation with studies in humans.

The immunohistochemical labelling of particular proteins in tumours provide information regarding tumour nature and behaviour. However, limitations in the numbers of specific, labeling antibodies that can be used in dogs restrict the usefulness of this methodology. Tissue arrays consisting of panels of >100 'outcome-linked' tumour tissue punctures on a single slide can be used to screen for novel biomarkers. Such an approach can be used to screen large numbers of patients and can determine if particular proteins are co-expressed. Multiple 'spots' within an OS tumour have to be assessed, because of the heterogeneous nature of this neoplasm and because expression of a protein marker in a single spot may not represent its expression in the tumour as a whole. Although prognostic biomarker discovery using immunohisto-chemistry is increasingly popular, standardised methods across veterinary laboratories including antigen retrieval methods, types of antibodies used (polyclonal or monoclonal), incubation periods and staining evaluation criteria have not been established.

Despite advances in diagnostic imaging, micrometastases present at the onset of disease are frequently not detected. Methods need to be developed to detect neoplastic cells in the circulation and thus predict metastatic disease based on the primary tumour protein expression 'signature'. In this context, in vitro metastatic sub-clone models are useful in elucidating key transcripts and signaling pathways important in the survival of metastatic cells. The gene profiling of such models of canine OS are underway and it is anticipated these will identify pathogenic mechanisms and targets for therapy. Although in vitro models are frequently used in the discovery of biomarkers for OS, different cell lines may contain different genetic alterations and activated signalling pathways, raising questions as to how closely these mimic the in vivo situation.

Comparative genomic hybridisation cytogenetic array analysis (Thomas et al., 2005), cDNA/oligonucleotide microarray gene expression profiling, protein profiling using mass spectrometry, and miRNA profiling are all currently used to investigate canine OS and in the future, cancer lipidomic (Wenk, 2005; Fernandis and Wenk, 2009) and metabolomic (Kim et al., 2009b) profiling approaches could be used in the pursuit of novel biomarkers.

Conclusions

The study of spontaneously occurring tumours of the dog, such as OS, provides invaluable translational opportunities for human medicine, particularly in the potential discovery of novel, 'bridging biomarkers'. The few molecular biomarkers with predictive and prognostic value so far identified in dogs require validation on a larger scale which can be achieved given appropriate collaboration between the pharmaceutical industry, biomedical and veterinary scientists within academia, and veterinarians working in clinical settings. An exciting decade of biomarker discovery using high throughput methodologies is now anticipated, and it is hoped that this will result in more effective, individually tailored, treatment and management protocols for affected dogs.

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Chapter 4

Gene expression profiling of canine osteosarcoma reveals genes associated with short and long survival times

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Abstract

Background

Gene expression profiling of spontaneous tumors in the dog offers a unique translational opportunity to identify prognostic biomarkers and signaling pathways that are common to both canine and human. Osteosarcoma (OS) accounts for approximately 80% of all malignant bone tumors in the dog. Canine OS are highly comparable with their human counterpart with respect to histology, high metastatic rate and poor long-term survival. This study investigates the prognostic gene profile among thirty-two primary canine OS using canine specific cDNA microarrays representing 20,313 genes to identify genes and cellular signaling pathways associated with survival. This, the first report of its kind in dogs with OS, also demonstrates the advantages of cross-species comparison with human OS.

Results

The 32 tumors were classified into two prognostic groups based on survival time (ST). They were defined as short survivors (dogs with poor prognosis: surviving fewer than 6 months) and long survivors (dogs with better prognosis: surviving 6 months or longer). Fifty-one transcripts were found to be differentially expressed, with common upregulation of these genes in the short survivors. The overexpressed genes in short survivors are associated with possible roles in proliferation, drug resistance or metastasis. Several deregulated pathways identified in the present study, including Wnt signaling, Integrin signaling and Chemokine / cytokine signaling are comparable to the pathway analysis conducted on human OS gene profiles, emphasizing the value of the dog as an excellent model for humans.

Discussion and Conclusion

A molecular-based method for discrimination of outcome for short and long survivors is useful for future prognostic stratification at initial diagnosis, where genes and pathways associated with cell cycle / proliferation, drug resistance and metastasis could be potential targets for diagnosis and therapy. The similarities between human and canine OS makes the dog a suitable pre-clinical model for future 'novel' therapeutic approaches where the current research has provided new insights on prognostic genes, molecular pathways and mechanisms involved in OS pathogenesis and disease progression.

Background

Naturally occurring cancer in the dog has been repeatedly emphasized as an excellent model for human, because similarities in histology, tumor biology, disease progression and response to conventional therapies offer a unique translational opportunity in the broader prospect of cancer research. Since the release of the canine genome in 2005, dog spontaneous tumors have been in the spotlight for 'state-of-the-art' linkage to preclinical human cancer research, where strong similarities in gene families were found when comparing the humans to dog than the classical mice and rodent models [1]. Among the tumors of the dog, osteosarcoma (OS), an aggressive malignant bone tumor that occurs spontaneously is one of the most outspoken cancers and invaluable for comparative oncology studies [2]. Commonly affected dog breeds include the large-to-giant breeds [3-6]. The median age of dogs affected with OS is around 7 to 10 years, with a subset of tumors arising in younger dogs (18-24 months). The appendicular skeleton is affected in 77% of the dogs, implying an association with rapid early bone growth [3, 7] as well as with increased stress on weight bearing areas of the limb. Affected dogs often present with progressive lameness, hard bony swelling or even pathological fracture of the affected bone [8, 9]. No strong sex predilection is noted, although males are overrepresented in most studies. Histologically, OS is a heterogeneous tumor that in addition to producing an osteoid matrix, can also present with a fibroblastic and cartilaginous matrix. OS is commonly subdivided into osteoblastic, fibroblastic, telangiectatic, chondroblastic and mixed forms classifications [10].

The prognosis of dogs with OS is unfortunately poor, mainly due to its fast spreading nature; by the time the tumor is found at the primary site, most have already metastasized [11], usually to the lungs, or less frequently in bone and other soft tissues [12]. The goal of therapy is to remove the primary tumor and detectable metastases as well as to initiate multimodal chemotherapy to eradicate micrometastases. The prognosis varies with the type of surgery and chemotherapy [13, 14]. The prognosis for dogs without surgery and adjuvant chemotherapy is poor, with a median survival time of 1-3 months. With amputation alone, median survival time can vary from 1 to 6 months [12, 15, 16] Some dogs develop metastases within 4 months regardless of the therapy modality, while others survive for longer periods of time [17]. One of the key factors contributing to intensified proliferative activity of the tumor in dogs leading to poor outcome is the deregulation of cellular signals, including growth factors and hormones [18, 19]. This poor prognosis feature is comparable to human OS, where there is still ample room for new therapeutics development, primarily to eradicate micrometastases and improve survival.

Recent advances in human cancer management have focused on molecular targeted therapies where high throughput screening technologies have been incorporated to identify novel markers for cancer pathogenesis and specific characterizations of tumors. Over the last decade, gene expression profiling has been able to identify key genes and cellular signaling pathways involved in development and progression of human OS. Microarray technology, a

robust method for analyzing global gene expression profiles has been incorporated in those studies. In line with this technology, human OS cell lines have been compared with normal human osteoblasts to provide insights into genes that are involved in OS tumorigenesis [20]. Aside from the conventional comparisons between tumor and normal tissues, other comparisons have been conducted in human OS, including the differential analysis of metastatic tumors compared with less aggressive tumor models [21], gene expression profiling that predicts response to chemotherapy [22] and molecular classification of chemotherapy resistant pediatric OS [23]. The use of these genetic markers for diagnosis and/or prognosis in canine OS is not completely understood and to date no such prognostic global gene profiling have been carried out for the dog.

Thus, for comparative pathobiology and new drug discovery arenas, it would be useful to stratify dogs at diagnosis into 'poor' and 'good' outcome groups based on tumor gene expression profiles. To address this issue, a canine-specific cDNA microarray representing 20,313 genes was used to differentiate the gene expression profile of tumors from dogs that survived less than 6 months from the profiles of those that survived longer. The survival-associated genes and cellular signaling pathways based on global gene expression profiles of thirty-two primary canine OS are identified. Molecular profiling of canine OS with known survival times will help define tumor biology pertaining to prognosis and facilitate future targeted therapies.

Results

Retrospective clinical - histopathological data analysis

The 32 dogs in this study varied in clinicopathological parameters (Table 1). The present experimental design hereby defines dogs with poor prognosis (short survivors, SS) as those surviving less than 6 months and favorable prognosis (long survivors, LS) as those dogs with survival of 6 months or longer. The overall survival times (ST) of these dogs were between 6 and 1752 days with a median value of 204 days. Dogs with poor prognosis (SS) had a ST of 6-169 days (<6 months); while the better prognosis group (LS) had a ST of 239-1752 days (>6 months). Beside the distinction in survival time between these two groups, the SS group also exhibited a significantly shorter disease free interval in a Kaplan-Meier survival analysis (Figure 1). Fisher's exact test revealed no significant differences in discrete data distribution between the 2 groups of survival with respect to sex, neuter status, histological grade and postoperative chemotherapy, the exception being metastatic disease status ($P<0.018$).

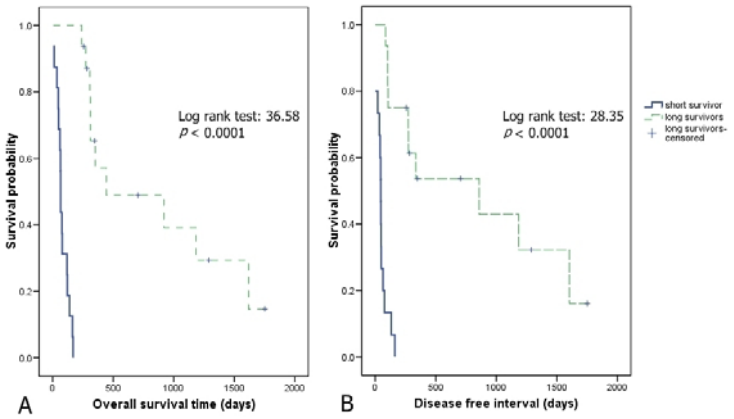

Figure 1. Kaplan-Meier survival analysis comparing long survivors (LS) and short survivors (SS) of dogs with OS.
(A) Survival time (ST) and (B) disease free interval (DFI) Kaplan-Meier product limit estimate revealed differences in ST with significance of P<0.0001 (log rank test of 36.58) and DFI with P<0.0001 (log rank test of 28.35).

This is because metastatic disease was seen in all dogs in the short survivor's group while 6 dogs from the long survivors' group did not develop metastasis before death (died due to other causes); this was censored for subsequent analyses (Table 2). Tumors were located mostly at appendicular sites, but other locations such as mandible, rib, scapula, metatarsus and extraskeletal were also present. Further potential confounders identified through a univariable Cox regression analysis of the whole population (n=32) included histological grade, postoperative chemotherapy, alkaline phosphatase (AP) measurement at diagnosis, sex, age and neuter status (Additional file 1). Alkaline phosphatase (ALP) level and age at presentation were found to have P values of less than 0.15 and were then further subjected to multivariate analysis. Elevation of serum ALP is a known negative prognosticator in dogs with OS and this is apparently also true here, multivariate analysis using the ALP data available for the dogs in this study (n=23) showed this confounder to be significantly associated with survival (HR=1.005; CI: 1.000-1.009 with corresponding P value of 0.035).

Although the small population size in the present study is unlikely to show significant differences, it is important to identify any possible confounding factors that may influence survival/ prognosis and should be considered when interpreting the expression data at biological levels. In both survival groups, dogs were subjected to a variety of single agent therapies, including lobaplatin, doxorubicin or carboplatin; or a combination of carboplatin and doxorubicin. Some were treated with postoperative chemotherapy and some not, and Kaplan Meier analysis showed that chemotherapy did not significantly prolong survival in the overall population (log rank score 1.353, P value 0.245), nor among short survivors (log rank score 0.460, P value of 0.498) nor among long survivors (log rank score 0.033, P value 0.857) separately (Figure 2).

Table 1

Clinical and pathological data from short survivors (SS) and long survivors (LS) of canine OS used in this study.

SURVIVAL GROUP	ARRAY	SURVIVAL TIME (days)	AGE (Year, Month)	SEX	BREED	TUMOR LOCATION	HISTO SUBTYPE	HISTOLOGY GRADE	POST OPERATIVE CHEMOTHERAPY
SHORT	1	36	9,2	F	boxer	rib	OB/CB/FB	highly malignant	no
SHORT	2	79	8,8	M	vizsla	distal radius	OB	highly malignant	yes
SHORT	3	13	2,6	F	labarador retriever	proximal humerus	OB	highly malignant	no
SHORT	4	169	4,8	M	great dane	distal radius	OB/FB	highly malignant	yes
SHORT	5	121	7,9	M	Rottweiler	proximal humerus	OB/CB/FB	medium malignant	yes
SHORT	6	66	9,8	M	dobberman	ulna	OB/TL	medium malignant	no
SHORT	7	119	7,1	F	Rottweiler	distal femur	OB	highly malignant	yes
SHORT	8	66	11,6	F	belgian shepherd (tervuren)	proximal humerus	OB	highly malignant	yes
SHORT	9	77	7,0	M	great dane	distal tibia	OB/FB	highly malignant	yes
SHORT	10	164	6,7	M	dobberman	distal tibia	CB	highly malignant	no
SHORT	11	140	7,4	M	Rottweiler	distal radius & ulna	OB/FB	highly malignant	no
SHORT	12	61	5,8	F	great dane	distal femur	OB	medium malignant	yes
SHORT	13	50	11,9	M	belgian shepherd (tervuren)	distal tibia & fibula	OB	highly malignant	no
SHORT	14	47	6,8	F	rhodesian ridgeback	distal femur	OB/FB	highly malignant	yes
SHORT	15	65	6,2	M	great dane	proximal humerus	OB/CB	highly malignant	yes
SHORT	16	6	6,1	F	Rottweiler	mandibular	OB/FB	highly malignant	no
LONG	17	352	9,8	M	dobberman	proximal tibia	OB/FB	medium malignant	yes
LONG	18	274	8,8	M	Cross	ulna	OB/FB/TL	medium malignant	no
LONG	19	1752	3,8	M	Stabyhoun	extraskeletal	OB/FB	highly malignant	yes
LONG	20	307	4,3	M	great dane	distal radius	CB	medium malignant	yes
LONG	21	1619	3,7	F	siberian husky	mandibular	OB/TL	highly malignant	no
LONG	22	1185	2,3	F	flatcoat retriever	rib	OB/TL	highly malignant	no
LONG	23	1289	5,0	F	Rottweiler	distal radius	OB/FB	highly malignant	yes
LONG	24	257	8,4	F	Bouvier	distal radius	OB/FB	highly malignant	yes
LONG	25	445	10,4	M	scottish collie	distal radius	OB	highly malignant	yes

LONG	26	284	7,0	F	Cross	distal radius	OB/CB	highly malignant	yes
LONG	27	239	8,4	M	Rottweiler	scapula	OB/FB	highly malignant	yes
LONG	28	705	8,3	F	Cross	metatarsus	FB	low malignant	yes
LONG	29	348	9,0	F	Mastiff	distal radius	OB	highly malignant	yes
LONG	30	312	5,8	M	Mastiff	scapula	OB/FB	highly malignant	yes
LONG	31	920	7,3	M	belgian shepherd (malinios)	mandibular	OB/CB/FB	highly malignant	no
LONG	32	312	10,2	M	Rottweiler	distal femur	OB/CB	highly malignant	yes

Abbreviations: M, male; F, female; CB, chondroblastic; OB, osteoblastic; FB, fibroblastic; TL, telangiectic

Table 2. Distribution of variables between the SS and LS groups by Fisher's exact test. Analysis on discrete variables comparing the short and long survivors revealed no significant differences in the distribution of variables assessed except for metastatic disease status. Significance defined as P<0.05.

Parameter	Short survivors	Long survivors	(Fisher's exact test) P value
	n	n	
Gender			> 0.999
Male	9	9	
Female	7	7	
Neuter status			> 0.999
Neutered	5	6	
Intact	11	10	
Metastasis present at time of death			**0.018****
Yes	16	10	
No	0	6	
Postoperative chemotherapy			0.458
No	7	4	
Yes	9	12	
Histological grade			> 0.999
Med-low	3	4	
High	13	12	

Although these survival curves only represent bivariable analyses (treated vs. not treated) that do not include all confounding variables, a separate univariable and subsequent multivariable statistical model with inclusion of variables such as histological grade, alkaline phosphatase, neuter status, gender and age upon chemotherapy stratification revealed that none of the variables appeared to have influenced survival significantly (Additional file 2). An additional important insight from the long survival group is that all 4 dogs that were censored from the study (death due to other causes and no apparent metastatic disease until death) received postoperative chemotherapy, which suggests possibilities that these dogs had responded reasonably well to the chemotherapeutic regime. Finally, since both survival groups consist of comparable heterogeneous populations of dogs and poor survivors tend to have higher ALP measurements, the arbitrary but defendable approach to a binary distinction of 'good' and 'poor' outcome based on 6 month survival time is supported for further gene expression profiling.

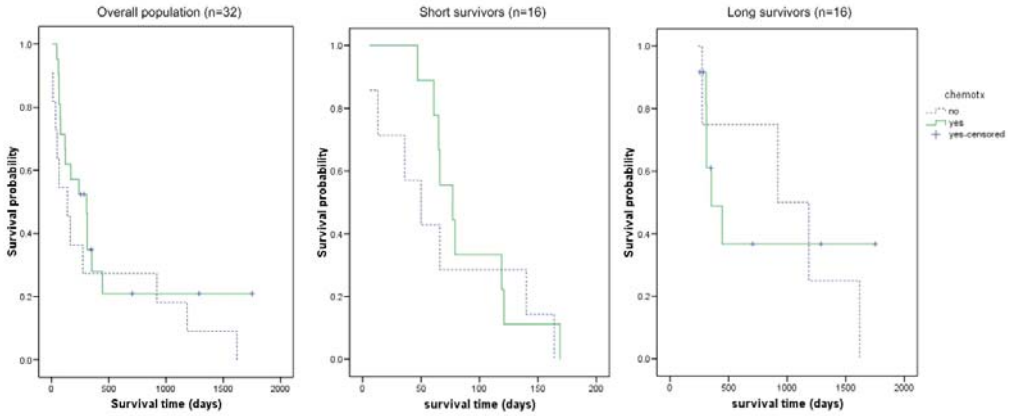

Figure 2. Kaplan-Meier survival curves of dogs treated with and without postoperative chemotherapy.
(A) Overall population (n=32 dogs); (B) Among short survivors group (n=16 dogs) and (C) long survivors (n=16 dogs). Chemotherapy did not significantly prolong life in the overall population or the subpopulations.

Microarray data analysis

Comparison of short and long survivors using two-class unpaired *Significance Analysis of Microarrays* (SAM) revealed fifty-one genes that were differentially regulated at a false discovery rate (FDR) of 10%. All these genes were found to be upregulated in the SS group where 37 of them were upregulated with a fold change of more than 1.4 (Table 3). Of the 37 genes, 8 were not functionally annotated for the dog and 5 others were unknown Expressed Sequence Tags (ESTs). To further visualize the gene expression data, hierarchical clustering was performed on all 32 arrays based on the differentially expressed genes (Figure 3). A gene tree dendogram revealed two distinct clusters, where Cluster 2 appears to distinguish long from short survivors or, in other words, this signature defines prognosis based on the 6 month survival binary outcome. Within Cluster 1, particular attention should also be given to a further separation into 2 different subgroups (A and B). Although Fisher's exact test revealed no significant differences among the variables assessed with respect to histological grade, sex, neuter status and postoperative chemotherapy (Additional file 3), this could primarily be due to the low number of subjects within each subgroup (n=8) which most likely will not allow statistical significance. We did find a significant difference in terms of overall survival time between the two subgroups, with subgroup A: having a shorter survival time than subgroup B. The log rank score was 5.82, with a corresponding P value of 0.0158 (Figure 4).

Gene Clone ID	Q-value (%)	Fold change	Gene description	Gene symbol
DG2-21g13	0.00	4.1	WD repeat and SOCS box containing protein 2	WSB2
DG2-23c15	0.00	3.3	cofilin 2	CFL2
DG2-72g4	0.00	2.7	ankyrin repeat domain protein 17 isoform a	ANKRD17
DG42-128j23	0.00	2.1	paraoxonase 1	PON1
DG2-112n11	0.00	2.1	Kinesin heavy chain (Ubiquitous kinesin heavy chain)	UKHC
DG32-161c11	0.00	2.0	WNK lysine deficient protein kinase 1	WNK1
DG2-63l7	0.00	1.7	nuclear receptor co-repressor 1	NCOR1
DG32-237k11	0.00	1.6	Ribosomal L1 domain containing protein 1(PBK1)	RSL1D1
DG11-239n21	0.00	1.6	cell-cycle and apoptosis regulatory protein 1	CCAR1
DG2-28n13	0.00	1.5	28S ribosomal protein S31, mitochondrial precursor	MRPS31
DG2-59p21	0.00	1.4	#N/A	
DG2-90b10	4.61	2.2	Heat shock protein HSP 90-alpha	HSP86
DG2-123a3	4.61	2.0	Microsomal glutathione S-transferase 1	MGST1
DG2-86b3	4.61	1.6	Canis familiaris similar to T06D8.1a	
DG8-102i3	4.61	1.5	#N/A	
DG2-100e22	4.61	1.5	COMM domain containing protein 8	COMMD8
DG2-106f2	4.61	1.4	Translocation protein SEC63 homolog	SEC63
DG2-130m14	4.61	1.4	Canis familiaris similar to CG1218-PA	
DG2-90c16	6.15	2.6	#N/A	
DG43-1a15	6.15	2.4	#N/A	
DG14-71c7	6.15	1.8	serine/arginine repetitive matrix 1	SRRM1
DG2-72p3	6.15	1.6	Vacuolar ATP synthase subunit C	V-ATPase C
DG32-216j13	6.15	1.6	Flavin reductase (NADPH-dependent diaphorase) (FLR)	BVRB
DG2-94j4	6.15	1.5	Canis lupus familiaris high-mobility group box 1	HMGB1
DG2-42j4	6.15	1.4	FRA10AC1 protein isoform FRA10AC1-1)	
DG42-89n19	6.15	1.4	#N/A	
DG2-19i16	9.70	7.2	#N/A	
DG2-18m22	9.70	4.6	plasma glutamate carboxypeptidase	PGCP
DG9-134g22	9.70	1.9	ankyrin repeat domain 11	ANKRD11
DG2-123m9	9.70	1.9	CG12795-PA	
DG14-14i19	9.70	1.7	Stress-70 protein, mitochondrial precursor (HSAP9) (Mortalin)	MOT
DG9-212f9	9.70	1.5	CG12795-PA	
DG2-60f11	9.70	1.5	#N/A	
DG2-25k5	9.70	1.4	SMC6 protein	SMC6
DG14-86l17	9.70	1.4	#N/A	
DG2-24i24	9.70	1.4	60 kDa heat shock protein, mitochondrial precursor	HSP60
DG11-243e16	9.70	1.4	splicing factor, arginine/serine-rich 2, interacting protein	SFRS2IP

Table 3

Thirty-seven differentially expressed genes between short survivors (SS) and long survivors (LS) of canine osteosarcoma from SAM analysis. All these genes were upregulated in short survivors. Genes were selected at a FDR <10% and of fold change of ≥ 1.4. * #N/A: Non-annotated

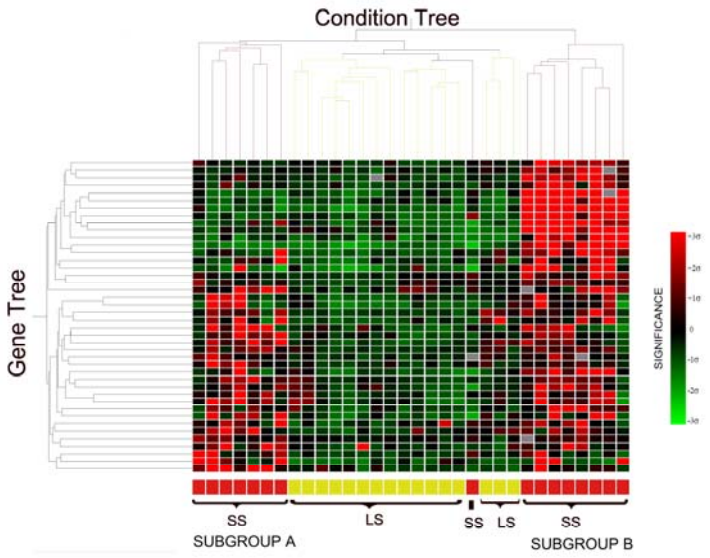

Figure 3. Dendogram and heat map generated by hierarchical clustering of differentially expressed genes between SS and LS. Differentially expressed genes were selected at false discovery rate of 10% between short survivors (SS) and long survivors (LS) of canine OS. Genes found upregulated are shown in red and downregulated genes are represented in green.

Quantitative real-time PCR analysis

To verify the microarray analysis, QPCR was performed on four candidate genes and two reference genes, HPRT and RPS19 that were used to normalize the expression data. The overall expression patterns of the candidate genes were comparable to the microarray analysis. Quantitative real-time PCR revealed common overexpression of ANKRD17, MGST1, MRPS31 and NCOR1 in short survivors compared to long survivors by 1.35, 1.46, 1.53 and 1.1 fold respectively.

Molecular functions and biological process analyses

Various molecular functions of the genes differentially expressed by short and long survivors of canine OS were defined into categories according to the Gene Ontology database,

including: DNA repair and integrity, cell cycle/proliferation, stress response, apoptosis regulation, protein modification and metabolism, and mRNA transcription regulation (Additional file 4). Several of these genes have been implicated in tumorigenesis and cancer biology for both humans and dogs. HSP70/MOT, HSP60 and NCOR1 have been implicated in human OS, while HMGB1, HSP90 and MGST1 have been associated with both human and canine OS. Generally, the overexpressed genes were linked to 3 main biological processes known in advanced cancer: mainly cell cycle and proliferation, followed by drug resistance and /or metastasis (Table 4).

Table 4

Upregulated genes in short survivors of canine osteosarcoma associated with 3 main biological process of interest: cell cycle/ proliferation, drug resistance and metastasis-associated.

Gene ID	Fold change	Gene description	Gene symbol
Metastasis- associated			
DG2-23c15	3.3	cofilin 2	CFL2
DG14-14i19	1.7	Stress-70 protein, mitochondrial precursor (HSAP9) (GRP 75) (Mortalin)	MOT
DG2-72p3	1.6	Vacuolar ATP synthase subunit C	V-ATPase C
Drug resistance			
DG2-112n11	2.1	Kinesin heavy chain (Ubiquitous kinesin heavy chain)	UKHC
DG2-123a3	2.0	Microsomal glutathione S-transferase 1	MGST1
DG2-72p3	1.6	Vacuolar ATP synthase subunit C	V-ATPase C
DG2-94j4	1.5	high-mobility group box 1	HMGB1
Cell cycle/ proliferation			
DG2-23c15	3.3	cofilin 2	CFL2
DG32-161c11	2.0	WNK lysine deficient protein kinase 1	WNK1
DG14-71c7	1.8	serine/arginine repetitive matrix 1	SRRM1
DG14-14i19	1.7	Stress-70 protein, mitochondrial precursor (HSAP9) (GRP 75) (Mortalin)	MOT
DG32-237k11	1.6	Ribosomal L1 domain containing protein 1(PBK1 protein)	RSL1D1
DG11-239n21	1.6	cell-cycle and apoptosis regulatory protein 1	CCAR1
DG2-106f2	1.4	Translocation protein SEC63 homolog	SEC63
DG2-25k5	1.4	SMC6 protein	SMC6

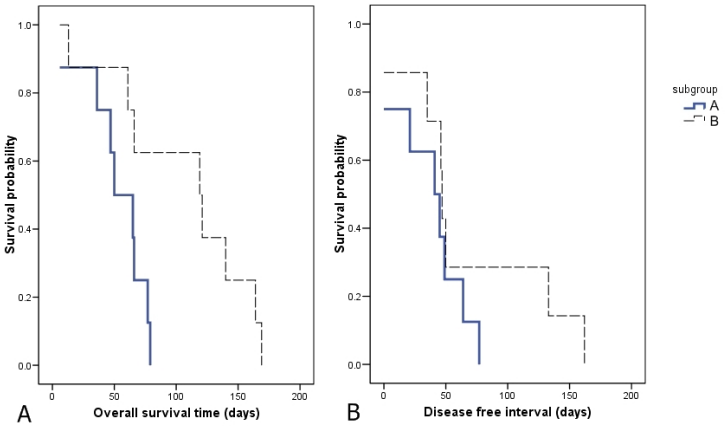

Figure 4. Kaplan-Meier curves of overall survival and disease free interval comparing subgroup A with subgroup B. All dogs that lived less than 6 months are characterized as poor survivors but among these dogs there were differences in gene expression which divides them into 2 different subgroups where they were subsequently found to significantly differ in survival time and not disease free interval.

Pathway analyses

The number of annotated genes in the present study is too low to form any relevant protein networks. Therefore, we used the 'Shortest path analyses' by MetaCore, which allows for one intermediate extra gene to be linked to the differential genes in order to form a more informative gene-protein network. These intermediate genes may be transcription factor, receptor or ligand. Genes with limited information available on network / associated pathways were excluded, leaving a final gene regulatory network generated based on 11 genes that were overexpressed in short survivors of canine OS with the addition of seven transcription factors, one receptor and a ligand (Figure 5). Transcription factors p53, c-myc, SP1 and NF-kB seemed to play roles as 'central hubs' connecting these transcripts. All three heat shock proteins (HSP90-alpha, HSP60 and MOT/HSAP9/GRP75) appeared to be linked to a common transcription factor c-myc.

Because the interaction among genes is definitely more complex, we performed a PANTHER® pathway analysis in which functional and pathway data available on human transcripts were used as reference to analyze the transcripts from the present study in the dog. PANTHER® analysis on the 51 differentially expressed genes by SAM yields too few genes associated with a cellular signaling pathway, undermining the power of pathway analysis. This is mainly because a majority of genes are not associated with a signaling pathway, but also because the dog lacks functional annotation at present. Therefore, we excluded the FDR correction for multiple testing and selected a longer list of genes with a threshold of $P<0.05$ from EDGE.

Table 5

Survival associated deregulated pathways in canine OS; comparison with the top 20 pathways from 3 published human OS studies. (▲: common pathways of present study to the human osteosarcoma published studies)

Reference	**Present study on canine osa**	Srivastava et al, 2006	Rajkumar, T. et al, 2008	Srivastava et al, 2006
study approach	**poor survival associated profile**	high metastatic profile	drug resistant profile	osteogenic tumor profile
samples used	**32 primary tumors**	human osa cell lines	cell line resistant to doxorubicin	10 tumors and 8 normal bones
Total differential expressed genes	1426	1999	485	194
Total Mapped IDs on PANTHER	533	1185	295	159
Huntington disease	12	▲	▲	
Wnt signaling pathway	**10**	▲	▲	▲
Chemokine / cytokine signaling pathway	**8**	▲	▲	▲
Integrin signalling pathway	7	▲		▲
Parkinson disease	6		▲	▲
Ubiquitin proteasome pathway	6		▲	
Alzheimer disease-presenilin pathway	**5**	▲	▲	▲
Cadherin signaling pathway	5		▲	
Endothelin signaling pathway	5			
Glycolysis	5			
Heterotrimeric G-protein signaling pathway	4	▲		
Angiogenesis	3			▲
Cytoskeletal regulation by Rho GTPase	3	▲		
EGF receptor signaling pathway	3	▲		
FGF signaling pathway	**3**	▲	▲	▲
p53 pathway	3		▲	
PDGF signaling pathway	**3**	▲	▲	▲
T cell activation	3	▲	▲	
TGF-beta signaling pathway	3	▲		▲
Androgen/estrogene/progesterone biosynthesis	2			
Apoptosis signaling pathway	**2**	▲	▲	▲

B cell activation	2			
Hedgehog signaling pathway	2			
Hypoxia response via HIF activation	2			
Interferon-gamma signaling pathway	2			
Interleukin signaling pathway	**2**	▲	▲	▲
Oxidative stress response	2			
p53 pathway feedback loops 2	2		▲	
PI3 kinase pathway	2	▲	▲	
Ras Pathway	2	▲		▲

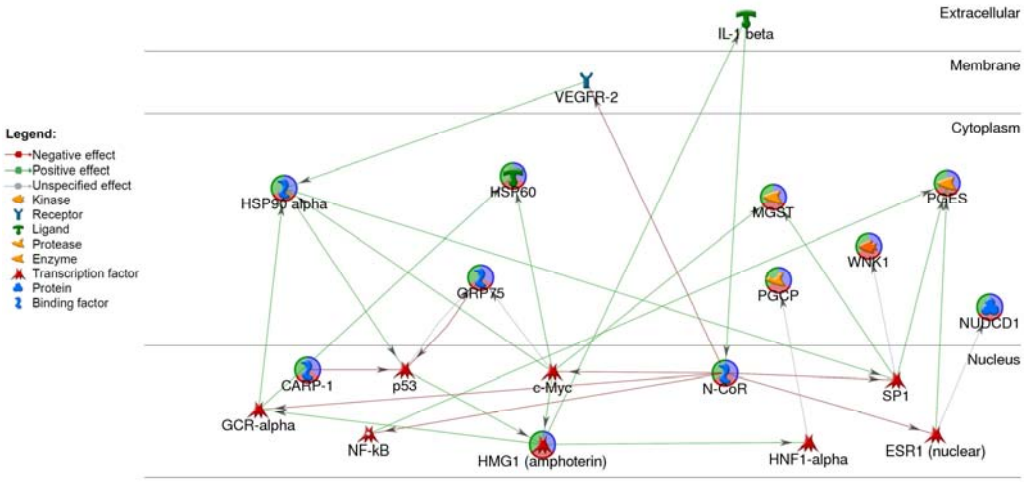

Figure 5. Gene regulatory network generated through MetaCore's 'shortest path analysis'.

After removal of non-annotated genes and transcript replicates, there were 533 genes mapped on PANTHER® which permitted further analysis. Identified pathways included the top four commonly associated with cancer and OS pathogenesis: Wnt signaling, inflammation mediated by chemokine and cytokine signaling, integrin signaling, and ubiquitin proteasome (Table 5). Our pathway list was then compared with three microarray datasets of human osteosarcoma [24] [25]. Two gene lists were obtained from Srivastava A., *et al*., 2006: one of genes overexpressed in human osteogenic sarcomas in comparison to normal bones, the other a partial list of genes upregulated in a highly metastatic human OS cell line, and a gene list from Rajkumar T., *et al*., 2008 identifying genes that were differentially expressed in a doxorubicin drug resistant human OS cell line. These gene lists, obtained either from the authors themselves or via the supplementary tables provided in their articles, were subsequently subjected to PANTHER® pathway analysis as similarly conducted in the present study to define pathways pertaining to drug resistance/ metastasis / tumorigenesis of OS. Comparison of the top 20 pathway hits from these 3 studies in human OS (Table 5) revealed seven pathways overlapping with the present study on canine OS, including Wnt, chemokine / cytokine, Alzheimer disease-presenilin pathway, fibroblast growth factor (FGF), platelet derived growth factor (PDGF), apoptosis and interleukin signaling pathways.

Discussion

Canine OS gene expression profiling as a model for human OS

Osteosarcoma (OS) is a devastating disease in both human and dogs. The clinical presentation, characteristics and disease progression is similar in people and dogs except for the age of clinical onset, where 75% of human cases affect young adolescents while onset is predominantly observed in middle-aged to older dogs. For dogs, the decision to initiate treatment is often made by the owners. The standard therapy for canine OS primarily involves amputation or limb sparing surgery, followed by adjuvant chemotherapy. Some dogs tend to develop metastases within 4 months regardless of the therapy modality, while others survive for longer periods of time [17]. Similarly, despite the advance standard of care for children with OS, only 60% reach a 5-year disease free interval and 20% will not survive beyond 5 years [26].

The similarities seen in OS disease progression and survival rates in humans and dogs provides a reasonable justification to compare gene expression profiles based on clinical outcomes once these are adjusted for comparative lifetimes. The similarities in the genetic expression and biological behavior of canine and human OS also makes the dog a suitable model to study this disease [27, 28]. Gene expression profiling of spontaneous tumors in the dog offers a unique translational opportunity to identify prognostic biomarkers and signaling pathways common to both species. This is further supported by a report on the strong similarity in the gene expression profile found in both canine and human pediatric OS, suggesting that specific genes and pathways are commonly involved in these two species (Paoloni, M. *et al*., 2005, personal communication).

Further, various clinical and pathological methods are being used as prognostic indicators for canine OS, but to our knowledge, this is the first research conducted using canine specific cDNA microarray to analyze gene expression profiles associated with survival in a panel of thirty-two primary canine OS. The ability to identify gene markers of tumor aggressiveness at the time of primary tumor removal would provide prognostic values for a tailored therapy which can be translated to human medicine.

Clinicopathological relevance for OS prognostic gene expression studies in dogs

Gene expression profiling on the basis of segregating and then identifying poor and favorable outcome markers is not novel, but this is the first study conducted in dogs with OS. Selection of appropriate tumor candidates based on clinicopathological relevance for gene profiling is crucial for the overall experimental design. Although advanced histological grade and postoperative chemotherapy are known confounders to influence survival and prognosis in dogs with OS [29], these factors lack to significantly influence survival in the present study population, mainly due to the small population size. Elevation of serum alkaline phosphatase is a known negative prognosticator in dogs with OS, which is true for the present study population where poor survivors had significantly higher alkaline phosphatase levels than

long survivors. Some dogs, despite receiving postoperative chemotherapy, did not live beyond 6 months and can be characterized as 'poor responders' to chemotherapy. On the other hand, the majority of dogs that received chemotherapy within the long survivors was censored which suggests that either these tumors responded to the chemotherapy or the primary tumor did not harbor the aggressive phenotype. These data further justify distinguishing between good and poor outcome: dogs can be assessed at the gene-biological level of primary tumors regardless of post-operative chemotherapy. In addition, the current gene profiling was carried out on tumors prior to chemotherapy, where 'clues' at gene expression levels will help to determine which animals will probably live longer regardless of either the type of regime or decision to include postoperative chemotherapy (Figure 6). To further improve the efficacy of therapy, it is necessary to identify dogs with OS with an increased risk of treatment failure, as well as to identify those that might not need aggressive chemotherapeutic protocols.

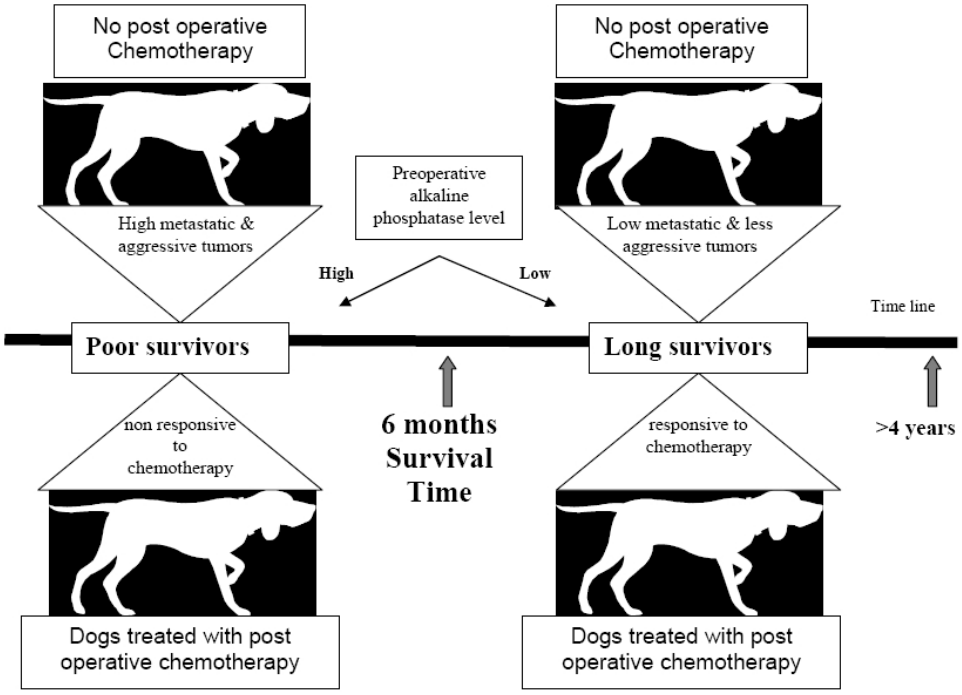

Figure 6. Classification of dogs: poor (SS) and long survivors (LS).

To address issues concerning applicability for tailored therapies, microarray technologies have identified new molecular subclassifications in various human tumors, but to date there are no

such reports for prognostic signatures in human and canine OS. The current research thus provides new insights into possible prognostic molecular subclassifications in canine OS. Hierarchical clustering of the differentially expressed genes revealed that long survivors have a similar gene expression profile, which is distinctly different from that of short survivors: these genes appear to be consistently downregulated among the long survivors. In contrast, clustering the genes based on expression was found to separate the short survivors into 2 subgroups. Cluster 1 genes were found to be overexpressed in subgroup B in comparison to the rest of the tumors, making this subgroup unique among the 32 tumors. Intriguingly, subgroup A appeared to have a significantly decreased survival compared to subgroup B where it shares common downregulation of genes with cluster 1. The mechanism behind this evident contradiction can only be speculated. Possibly, upregulation of both cluster 1 and cluster 2 genes provides a somewhat protective mechanism that improves survival as compared to the combination seen for subgroup A. On the other hand, the data can also be interpreted such that Cluster 1 genes are upregulated in 25% of canine OS, where its overexpression is related to poor prognosis. Cluster 2 genes appear to be true prognostic gene signature, defining the gene expression that segregates short (<6 months) from long survivors. The importance of these findings is they provide insights into possible existence of molecular subclassifications among short survivors that may be regulated by different signaling pathways whereby, due to the complex interaction among genes, these subgroups may respond differently to certain targeted therapies. These interpretations must be addressed cautiously given the small number of samples and differentially expressed genes selected for the generation of the present dendogram. As has been noted previously, “a replicable classification is not necessarily a useful one; but a useful one that characterizes some aspect of the population must be replicable” [30]. Therefore, insights into molecular sub-classifications among the canine OS from the present study require further validation on a larger and independent set of tumors.

Differentially expressed genes between short and long survivors: similarities with human OS

For a more biologically meaningful approach to data analysis, we looked at the level of common biological processes that differentiates short from long survivors. Genes highly expressed among short survivors of canine OS are likely to contribute to the aggressive nature of the disease in terms of increased cell cycle and proliferation, drug resistance and metastasis-associated properties. Among the transcripts that were differentially expressed between the two survival groups that have been implicated in human OS were HSP70/MOT, HSP60 and NCOR1, while HMGB1, HSP90 and MGST1 have been reported for both human and canine OS. Following discussion will focus on the relevance of these genes to human and canine OS pathogenesis and disease progression.

The main goal for discovering new therapeutic interventions for OS is to inhibit metastasis and eradicate micrometastases. The difference in growth patterns of metastases

may be detected by differences in gene expression of primary tumors at the point of amputation. Due to their gene expression differences, some dogs develop metastasis rapidly and others have delayed metastatic disease, which contributes significantly to their survival. In the overall clinical data comparison in this study, there were long survivors who did not develop metastases at all. We did not exclude these tumors from the analysis because they appear to be true survivors, where there was no metastatic disease detected after primary tumor removal. Among the differentially expressed genes between short and long survivors, CFL2, MOT and V-ATPase C have been associated with invasion and metastasis in various human tumors [31-33]. The metastatic potential of the stated genes in OS has not been reported previously; therefore additional research into their exact functions in OS metastasis is required.

Over the past decade, besides metastasis, there has been growing interest in the development of multidrug resistance in OS, where various genes have been reported to contribute to the drug resistance phenotype [24, 34, 35]. The two drug resistance related genes overexpressed in poor survivors found in the present research in canine OS include HMGB1 and MGST1. HMGB1 expression levels have been previously investigated in 5 canine OS where its expression was suggested to be a potential marker for cisplatin therapy based clinical outcome[36]. This gene may play a role in protecting OS cells, making them less susceptible to the cisplatin where it has been similarly associated with activation of p53 [37, 38] and drug resistance in other human tumors [37, 39]. Similarly, MGST1 was found to be overexpressed in several human malignant tissues where it was shown to protect cells from several cytotoxic drugs [40, 41] as well as by direct detoxification and downstream protection of tumor cells from oxidative stress [40]. Preliminary evidence suggests overexpression of another family member, glutathione S-transferase π, is significantly related to poor histological response to preoperative chemotherapy and poorer prognosis in human OS [41]. Similarly, an *in vitro* model of canine OS (COS31) with high glutathione-S-transferase activity was found to be resistant to cytotoxic effects of cisplatin [42]. Drug resistance issue is of interest in the present study population because despite given post operative chemotherapy there were dogs unable to extend survival beyond 6 months while others received no chemotherapy and yet lived much longer. This suggests that screening through gene profiles can help with therapeutic decisions by segregating dogs based on key targets for personalized chemotherapy, and hence ensure treatment success.

Genes associated with proliferation, cell cycle and differentiation revealed at present have not been described for OS pathogenesis except for Mortalin (MOT/HSP70), HSP90 and HSP60. MOT/HSP70 has been previously shown to form complexes with p53 tumor suppressor, causing inactivation of the wild type p53 leading to excessive proliferation [43]. Knockdown of MOT/HSP70 by shRNA expression plasmids have proven to cause growth arrest in human OS [44]. Since MOT has been found upregulated in short survivors in this study and since it is known that p53 mutations are common feature of poor prognosis in canine and human OS, extensive in depth investigation should be done to elucidate both the role of

MOT/HSP70 as a prognostic marker and its association with p53 in OS. The other two heat shock protein family members revealed by the present study include HSP90 and HSP60, both having been strongly implicated in multiple stages of tumorigenesis from proliferation to impaired apoptosis and angiogenesis, invasion and metastasis. High expressions of HSP90-beta have been reported in 3 human OS cell lines as well as in 3 primary tumors from microarray analysis in previous research [20]. In primary human OS removed at surgery, HSP90α was found to be 40% overexpressed and HSP60 60% [45]. In another, expression of HSP60 was detected in 83% of human OS biopsy specimens, and 43% of these OS patients had increased levels of anti-hsp60 antibodies in their serum [46]. Recently, *in vitro* studies have demonstrated that inhibition of HSP90 exhibits selective cytotoxicity in canine OS cells by downregulation of Met, Akt and p-STAT3, key players for multiple oncogenic signaling pathways [47]. A growing body of evidence suggests that heat shock proteins provide protection to a large group of client proteins: they help protect highly aggressive and metastatic cells from cellular stressors in the tumor microenvironment. The overexpression of all these HSP family members in poor survivors of canine OS makes them interesting in the context of drug development for the next generation of HSP inhibitors.

Another candidate gene of interest to both human and canine OS is NCOR1. We found that NCOR1 was overexpressed in 8 out of 32 (25%) of tumors, all from subgroup B (among the candidates from gene Cluster 1) and there was a clear downregulation among all long survivors. In human OS, NCOR1 was amplified in 22.6% of tumors where it did not correlate with disease free interval, and amplification status correlated significantly with tumor size [48]. The mechanistic role of NCOR1 as an independent negative prognosticator in both human and canine OS is currently unknown, although the overexpression of NCOR1 in 25% of canine tumors could be related to its possible amplification status, similar to what is seen for human OS. Here, we have confirmed the overexpression of MGST1 & NCOR1 in poor survivors as has been previously described for both canine and human OS, and identified two new candidates, ANKRD17 and MRPS31, choosing to validate expression data using quantitative real-time PCR. Those dogs that lived less than 6 months were found to highly express these genes in parallel to the microarray expression data, which suggests these transcripts could be attractive novel therapeutic targets and markers for future prognosis and disease progression for canine OS.

Pathway Analyses

Pathway analysis in dogs has its limitations because pathway identification relies heavily on existing functional annotation, which is still limited for the canine species. Our preliminary work networking a limited number of candidate genes identifies c-myc, SP1, p53 and NF-kB as the 'central hubs' connecting the candidate genes, where these transcription factors have been extensively described and associated with poor prognosis in various tumors, including OS [49-51]. Transcripts such as MOT/GRP75, HSP60, p53, HMGB1; VEGFR and NCOR1 from this network have been previously described for cancer pathogenesis. It has been proposed

that one or a few key genes can trigger a succession of events leading to abnormal expression of many genes in cancer. Pathway analysis provides a much more practical way to analyze expression data across species which may shed light into common pathways important for targeted therapies. A few pathways have been previously proposed to be of importance in either human or canine OS pathogenesis. Gene enrichment for two major pathways, namely Wnt signaling and chemokine / cytokine signaling, was found to be common among the studies conducted in human OS, and in the present study in dogs. Extensive investigations have been initiated to understand how Wnt signaling is involved in human OS pathogenesis and disease progression. Wnt signaling is not only important for cell proliferation and differentiation, but may also have possible autocrine or paracrine influence on the metastatic potential of OS [52-54]. Preliminary investigations on the roles of Wnt signaling in canine OS pathogenesis and disease progression are underway. Nevertheless, the importance of chemokine signaling has been described in canine OS where its receptor (CXCR4) was found to participate in directional migration *in vitro* [55]. Further research has shown that canine and human primary OS do express CXCR4, which may be involved in metastatic progression of this disease [56]. Molecular strategies targeting these 2 pathways may be beneficial for both human and dogs.

Use of microarrays for OS prognosis prediction

Clinical, histological and gene expression analysis in combination have the ability to improve the accuracy of clinical diagnostics and prognostics by precisely segregating individuals into meaningful groups, allowing for better decision making for therapeutics. However, in the past, these advanced approaches have, for most studies in human malignancies, failed to be reproduced; instead, parallel studies using distinct data sets frequently identify different gene sets as a result of variations in computational analysis methods to sample processing, array platforms and research questions [57, 58]. Other common barriers to reproducibility include differences in experimental design, small sample size and lack of assessment in secondary independent populations. A general problem of prognostic studies is that analyses are carried out on thousands of genes generated from small number of samples, which are often difficult to acquire from dogs where decisions to treat and follow up rely heavily on the owners. Although different human OS gene sets from different platforms and analysis techniques were identified from independent studies, they all seem to achieve agreement when it comes to pathway analysis. This insight is first described in the present study, where we performed gene-pathway enrichment analysis on distinct gene sets from several human OS studies and compared the result with our prognostic genes from the dog to reveal several common pathways conferring pathogenesis and the aggressive phenotype of OS. The conventional single biomarker discovery approach is being slowly overtaken by the idea that complex interactions among multiple genes are required to produce a disease phenotype which can be best described in terms of its deregulation of cellular signaling pathways. Preliminary studies have been initiated in an independent and larger set of canine and human OS samples to

further investigate and validate the roles of the main pathways for the aggressive phenotype revealed by the present analysis.

Conclusions

The premise that gene expression profiling can be used to segregate and then identify negative and favorable prognosis cancer patients is not novel; however this is the first report of its kind in canine OS and probably in any naturally occurring cancer in the dog. The present study has revealed candidate genes that can be followed up in prospective studies as negative prognostic markers and therapeutic targets for canine OS. A molecular-based method to discriminate between short and long survivors of canine OS may be useful for future prognostic stratification of dogs at initial diagnosis, since genes associated with cell cycle/ proliferation, drug resistance and metastasis are commonly overexpressed in short survivors of canine OS. In addition, we found that these survival-associated genes enrich prominent pathways such as Wnt and chemokine / cytokine signaling, which were also among the top pathways revealed by comparative pathway analysis with those of human OS gene profiling studies. These findings emphasize the excellent translational opportunity offered by canine expression studies of OS pathogenesis and disease progression that can be used for future therapeutic and prognostic strategies. Other approaches, such as protein profiling as well as *in vitro* studies, will be necessary to elucidate further the biological significance of these findings.

Methods

Patient and tumor data

Thirty-two histological confirmed canine OS, with available survival data, that were presented at the University Clinic for Companion Animals in Utrecht The Netherlands from 1996 – 2003 were selected in this study. These dogs were not subjected to any sort of therapy prior to harvesting of the tumor tissue. Tumor samples were harvested under sterile conditions during surgery (amputation / marginal resection / total resection). Samples were snap-frozen in liquid nitrogen and stored in sterile tubes at -70°C. An adjacent tumor specimen was fixed in 4% neutral buffered formalin, decalcified in 10% EDTA and embedded in paraffin. Four µm tissue sections were stained with hematoxylin and eosin and conformation of the diagnosis and histological grading was carried out by a board certified veterinary pathologist [59]. Various clinical and pathological parameters were evaluated retrospectively for the dogs included in this study.

Statistical and survival analysis

All 32 dogs in this study were followed up from the time of diagnosis until death, for up to a period of 5 years. Survival time (ST) was recorded for all dogs. ST was defined as the time period from initial diagnosis until death. Patients were censored if they died due to causes other than metastatic disease. The Kaplan-Meier method was used to draw disease free interval survival curves. Tests for comparison of groups of survival data were made using the Mantel-Cox log rank test (SPSS version 15.0). Univariate Cox proportional hazard analysis was conducted on the overall population of 32 dogs with and without stratification for chemotherapy. Variables found by univariate analysis to influence survival at a cut off of $P<0.15$ were further subjected to multivariate analysis using the Cox proportional hazard regression model (backward elimination using Newton Raphson algorithm) to determine whether the variables could independently influence survival in the whole and/or subpopulation of dogs in the present study. Hazard ratios (HR) and confidence intervals (CI) were calculated and reported using EGRET for Windows Version 2.013 (Cytel Software Corporation, Cambridge, MA, USA). Further analyses on discrete variables comparing the subpopulation of dogs were performed using the Fisher's exact test on SPSS version 15.0. Statistical significance was defined as $P<0.05$.

RNA isolation and amplification

Bone tumor samples were pulverized for 45 seconds at a speed of 2000 rounds per minute in the Mikro Dismembrator U® in ribonuclease free plastic containers. The procedure was repeated if samples were too big. Cooling in liquid nitrogen between cycles was performed. Bone tumor powder was stored in sterile tubes at -70ºC. Total RNA isolation and purification was carried out with 600mU/ml Proteinase K® as an additional pretreatment step using the RNeasy mini kit (Qiagen, The Netherlands) following the manufacturer's protocol. The RNA samples were treated with DNase-I (Qiagen Rnase-free DNase kit). Total RNA was quantified using the Nanodrop® spectrophotometer. cRNA synthesis was performed using the protocol described [70]. Quality of total and amplified cRNA was analyzed using a bioanalyzer (Agilent Technologies, The Netherlands).

Microarray hybridization and data normalization

Thirty-two tumors were sorted into two prognosis groups based on survival time (ST). They were defined as short-term survivors (SS; 16 dogs with a negative prognosis that survived less than 6 months) and long-term survivors (LS; 16 dogs with better prognosis that survived 6 months or longer). Microarray hybridization, scanning and image analysis were conducted according to the protocol described. A common reference cRNA pool consisting of all 32 tumors was used to hybridize against each tumor on the array. Dyes were swapped as described in previous literatures [61], where equal numbers of samples within each tumor group were subjected to dye swap to avoid dye bias effect. Defective spots were flagged and normalization was carried out using the Lowess print-tip normalization technique. Log

transformation was applied to the microarray raw data after normalization and data from dye swap arrays were switched before they were subjected for further statistical analysis. The microarray data files were deposited in the public database (GEO: GSE14033).

Microarray data analysis

Microarray data analysis and calculations were performed using a two class unpaired approach to compare the normalized and log-transformed expression data between the two groups. Calculations were done using *Significance Analysis of Microarray* (SAM) [62], using 100 permutations to the k-nearest neighbor with 10 neighbors and newly initialized random seeds for each analysis. The gene list for this analysis was generated at a false discovery rate (FDR) of 10%. Genes with fold changes of 1.4 and above were selected for further analyses [63]. Hierarchical clustering of genes that were differentially regulated between the two groups of tumors was performed. A two dimensional dendogram of both gene tree and condition tree based on standard correlation was generated using the GeneSpring® software [64]. The Gene Ontology (GO) database was used to cross check the gene molecular and biological functions (http://www.ncbi.nlm.nih.gov).

Quantitative real-time PCR

Total tumor RNA was isolated using the protocol mentioned above. Synthesis of cDNA was carried out from 1.0µg total RNA in 40µl reaction volumes using the iScript™ cDNA synthesis kit as described by the manufacturer's protocol (Bio-Rad, The Netherlands). Quantitative real-time PCR (Q-PCR) was performed on four candidate genes from the list of differential expressed genes, as well as the ribosomal protein S19 (RPS19) and hypoxanthine phosphoribosyl transferase (HPRT) as endogenous reference gene for normalizations. Primer sets (Table 6) were designed using the software Primer3 [65]. Q-PCR was carried out using the SYBR® green fluorescent dye method, which was further analyzed by the Bio-Rad MyIQ software (BioRad, The Netherlands). The Q-PCR products were sequenced to verify the specificity of the primer sets. Data was analyzed on REST-XL (pair wise fixed reallocation and randomization test) software [66] that estimates a relative normalized fold change of gene expression between the two groups which is then compared to the microarray fold change data.

Pathway analyses

Analysis of gene regulatory networks present among the differentially expressed genes identified by SAM was performed using the 'Shortest Path Analysis' of MetaCore (GeneGo Inc) [67]. In addition, for further insight into overall pathway discovery, we applied a less stringent rule where correction for multiple testing method was not applied in obtaining differential expressed genes [68]. EDGE (Extraction of Differential Gene Expression) software (http://faculty.washington.edu/jstorey/edge) was used to generate the genes differentially expressed between the short and long survivors of canine OS at a cutoff threshold of $P < 0.05$

[69]. The differential expressed genes identified by SAM and EDGE from the present study in canine were compared with the human homologues to obtain a list of gene symbols or human RefSeq ID that was later subjected for pathway analysis using the program PANTHER® (**P**rotein **AN**alysis **TH**rough **E**volutionary **R**elationships) (http://www.pantherdb.org/) [60, 68]. Pathways revealed by this analysis were sorted according to number of gene hits and compared with the top 20 pathways from human OS studies.

Table 6
Primers used for quantitative real-time PCR

Gene symbol	Forward and Reverse Primers	Temp (°C)
ANKRD17	FW: 5'-AAGTAGCGCACCACCTTCAC-3' RW: 5'-CTAGCAGCAAATGGTGGACA-3'	60.0
MRPS31	FW: 5'-GAATTGGTCCTTGCTTTGGA-3' RW: 5'-ATCCAGTGGACGAAAGATGG-3'	60.0
NCOR1	FW: 5'-TCTTCCTCTGCGTTTTCCAT-3' RW: 5'-GCATCCCAAAAACTTTGGAC-3'	59.6
MGST1	FW: 5'-CGGACAGATGATAGGGTGG -3' RW: 5'-GATTTGGCTGGGGAAGG -3'	62.0
RPS19	FW: 5'-CCTTCCTCAAAAAGTCTGGG-3' RW: 5'-GTTCTCATCGTAGGGAGCAAG-3'	61.0
HPRT	FW: 5'-AGCTTGCTGGTGAAAAGGAC-3' RW: 5'-TTATAGTCAAGGGCATATCC-3'	56.0

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Additional files

Additional file 1

Univariate analysis of specific variable influences on survival time (ST) among dogs from the in the entire population of study. Variables with P<0.15 from univariate analysis that were subsequently forced into multivariate model identifies elevation of serum alkaline phosphatase with significantly increased HR for a shorter ST.

§ Missing data, * Category which was used as baseline reference, ‡ Continuous variables

Univariate analysis

Parameter	No of dogs (n)	Hazard Ratio	Lower CI	Upper CI	P value
Age	32	1.137	0.956	1.353	0.147
Gender		1.438	0.631	3.273	0.387
Male*	18				
Female	14				
Neuter status		1.325	0.551	3.191	0.53
Neutered*	11				
Non neutered	21				
AP §	23	1.003	1	1.007	0.078
Histo grade		1.185	0.466	3.012	0.722
Low and medium*	7				
High	25				
Postoperative Chemotherapy		1.590	0.720	3.511	0.251
Non treated*	11				
Treated	21				

Multivariate analysis

Variable	Hazard ratio	95% CI	P value
AP § ‡	1.005	1.000-1.009	**0.035**
Age ‡	1.18	0.915-1.522	0.202

Additional file 2

Cox proportional hazard analysis (univariate) upon stratification for postoperative chemotherapy revealed no significant influence of the variables assessed on survival time of the dogs in the total population of study (n=32).

		with chemotherapy stratification			
Parameter	**No of dogs (n)**	**Hazard Ratio**	**Lower CI**	**Upper CI**	**P value**
Age	32	1.118	0.9393	1.3320	0.200
Gender		0.623	0.2554	1.5200	0.298
Male	18				
Female	14				
Neuter status		0.724	0.2747	1.9090	0.514
Neutered	11				
Non neutered	21				
AP [§]	23	1.003	0.9994	1.0070	0.153
Histo grade		0.928	0.3638	2.3680	0.877
Low and medium	7				
High	25				

§ missing data, CI; confidence interval

Additional file 3

Fisher's exact test and univariate Cox proportional hazard analysis carried out on the new subgroups. Both groups did not differ in frequency distribution of the variables assessed and none of the variables assessed were found to significantly influence survival.

	Univariate Cox regression analysis				
	8A subgroup	HR (CI) for subgroup 8A	8B subgroup	HR (CI) for subgroup 8B	(Fisher's exact test)
Variable	**(n)**	**P value**	**(n)**	**P value**	**P value**
Gender		0.003 (0.000-646.96)		0.006 (0.00- 39.93)	> 0.999
Male	5	0.351	4	0.256	
Female	3		4		
Neuter status		0.355 (0.070-1.810)		3.499 (0.485-25.272)	> 0.999
Neutered	3	0.213	2	0.214	
Intact	5		6		
Postoperative Chemotherapy		3.465 (0.620-19.354)		0.963 (0.206-4.503)	0.614
No	4	0.157	3	0.962	
Yes	4		5		
Histological grade		0.780 (0.088 - 6.875)		2.248 (0.371-13.622)	> 0.999
Low and medium	1	0.823	2	0.378	
High	7		6		
AP	5	0.995 (0.984 - 1.007)	5	0.999 (0.994-1.004)	-
		0.441		0.749	

NA, non applicable; HR, hazard ratio; CI, confidence interval

Additional file 4

Differential expressed genes categorized based on their molecular functions.

Gene ID	Fold change	Gene description	Gene symbol
DNA repair and integrity			
DG2-18m22	4.6	plasma glutamate carboxypeptidase	PGCP
DG2-72g4	2.7	ankyrin repeat domain protein 17 isoform a	ANKRD17
DG2-112n11	2.1	Kinesin heavy chain (Ubiquitous kinesin heavy chain)	UKHC
DG2-123a3	2.0	Microsomal glutathione S-transferase 1	MGST1
DG32-161c11	2.0	WNK lysine deficient protein kinase 1	WNK1
DG14-71c7	1.8	serine/arginine repetitive matrix 1	SRRM1
DG14-14i19	1.7	Stress-70 protein, mitochondrial precursor (HSAP9) (GRP 75) (Mortalin)	MOT
DG2-63l7	1.7	nuclear receptor co-repressor 1	NCOR1
DG32-237k11	1.6	Ribosomal L1 domain containing protein 1(PBK1 protein)	RSL1D1
DG2-72p3	1.6	Vacuolar ATP synthase subunit C	V-ATPase C
DG11-239n21	1.6	cell-cycle and apoptosis regulatory protein 1	CCAR1
DG2-94j4	1.5	high-mobility group box 1	HMGB1
DG2-106f2	1.4	Translocation protein SEC63 homolog	SEC63
DG2-25k5	1.4	SMC6 protein	SMC6
DG11-243e16	1.4	splicing factor, arginine/serine-rich 2, interacting protein	SFRS2IP
Cell cycle/ proliferation			
DG2-23c15	3.3	cofilin 2	CFL2
DG32-161c11	2.0	WNK lysine deficient protein kinase 1	WNK1
DG14-71c7	1.8	serine/arginine repetitive matrix 1	SRRM1
DG14-14i19	1.7	Stress-70 protein, mitochondrial precursor (HSAP9) (GRP 75) (Mortalin)	MOT
DG32-237k11	1.6	Ribosomal L1 domain containing protein 1(PBK1 protein)	RSL1D1
DG11-239n21	1.6	cell-cycle and apoptosis regulatory protein 1	CCAR1
DG2-106f2	1.4	Translocation protein SEC63 homolog	SEC63
DG2-25k5	1.4	SMC6 protein	SMC6
Stress response			
DG2-90b10	2.2	Heat shock protein HSP 90-alpha	HSP86
DG42-128j23	2.1	paraoxonase 1	PON1
DG2-123a3	2.0	Microsomal glutathione S-transferase 1	MGST1
DG32-161c11	2.0	WNK lysine deficient protein kinase 1	WNK1
DG14-14i19	1.7	Stress-70 protein, mitochondrial precursor (HSAP9) (GRP 75) (Mortalin)	MOT
DG32-216j13	1.6	Flavin reductase (FR) (NADPH-dependent diaphorase) (FLR)	BVRB
DG2-24i24	1.4	60 kDa heat shock protein, mitochondrial precursor	HSP60

Apoptosis regulation			
DG2-90b10	2.2	Heat shock protein HSP 90-alpha	HSP86
DG14-14i19	1.7	Stress-70 protein, mitochondrial precursor (HSAP9) (GRP 75) (Mortalin)	MOT
DG2-72p3	1.6	Vacuolar ATP synthase subunit C	V-ATPase C
DG11-239n21	1.6	cell-cycle and apoptosis regulatory protein 1	CCAR1
DG2-94j4	1.5	high-mobility group box 1	HMGB1
DG2-24i24	1.4	60 kDa heat shock protein, mitochondrial precursor	HSP60
Protein modification and metabolism			
DG2-18m22	4.6	plasma glutamate carboxypeptidase	PGCP
DG2-21g13	4.1	WD repeat and SOCS box containing protein 2	WSB2
DG2-72g4	2.7	ankyrin repeat domain protein 17 isoform a	ANKRD17
DG2-90b10	2.2	Heat shock protein HSP 90-alpha	HSP86
DG2-123a3	2.0	Microsomal glutathione S-transferase 1	MGST1
DG14-71c7	1.8	serine/arginine repetitive matrix 1	SRRM1
DG14-14i19	1.7	Stress-70 protein, mitochondrial precursor (HSAP9) (GRP 75) (Mortalin)	MOT
DG2-63l7	1.7	nuclear receptor co-repressor 1	NCOR1
DG32-237k11	1.6	Ribosomal L1 domain containing protein 1(PBK1 protein)	RSL1D1
DG2-72p3	1.6	Vacuolar ATP synthase subunit C	V-ATPase C
DG32-216j13	1.6	Flavin reductase (FR) (NADPH-dependent diaphorase) (FLR)	BVRB
DG2-94j4	1.5	high-mobility group box 1	HMGB1
DG2-106f2	1.4	Translocation protein SEC63 homolog	SEC63
DG2-25k5	1.4	SMC6 protein	SMC6
DG2-24i24	1.4	60 kDa heat shock protein, mitochondrial precursor	HSP60
DG11-243e16	1.4	splicing factor, arginine/serine-rich 2, interacting protein	SFRS2IP
mRNA transcription regulation			
DG2-18m22	4.6	plasma glutamate carboxypeptidase	PGCP
DG2-21g13	4.1	WD repeat and SOCS box containing protein 2	WSB2
DG2-72g4	2.7	ankyrin repeat domain protein 17 isoform a	ANKRD17
DG2-90b10	2.2	Heat shock protein HSP 90-alpha	HSP86
DG32-161c11	2.0	WNK lysine deficient protein kinase 1	WNK1
DG14-71c7	1.8	serine/arginine repetitive matrix 1	SRRM1
DG2-63l7	1.7	nuclear receptor co-repressor 1	NCOR1
DG32-237k11	1.6	Ribosomal L1 domain containing protein 1(PBK1 protein)	RSL1D1
DG2-94j4	1.5	high-mobility group box 1	HMGB1
DG2-106f2	1.4	Translocation protein SEC63 homolog	SEC63
DG2-24i24	1.4	60 kDa heat shock protein, mitochondrial precursor	HSP60

Chapter 5

Gene expression profiling and pathways associated with high metastatic potential in canine osteosarcoma

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Abstract

Osteosarcoma (OS) is one of the naturally occurring cancers in the dog which share many characteristics of those seen in human OS especially with respect to disease progression, histology and metastatic disease. Both bear poor prognosis that renders local control of the primary tumor inadequate whether alone or in combination with adjuvant chemotherapy. To gain more insights into OS metastatic processes, cDNA microarray consisting of 20,313 transcripts were used to profile a canine OS cell line with high metastatic capacity to the lungs (HMPOS) and its primary parental line (POS) with low metastatic behavior. Microarray analysis revealed 278 differentially expressed genes with enrichment for several deregulated pathways including integrin and Wnt signaling. When compared with published human and mouse microarray datasets, 9 pathways were common to all species. Among the candidate genes, decorin mRNA was significantly overexpressed in HMPOS and high decorin mRNA expression was observed in dogs with shorter disease-free interval. Decorin protein expression was observed in both human and canine OS tissues while immunohistochemistry evaluation of a human OS tissue microarray revealed decorin expression to be significantly associated with poor prognosis. Selective functional investigation of the identified targets and pathways '*in vitro*' are essential before dogs can be used for translational studies aiming at inhibition of OS cell proliferation and metastasis in a spontaneous tumor model for humans.

Introduction

A wide variety of translational 'corridors' for cancer research have become available since the release of the canine genome in 2005 showed pronounced genetic similarities between humans and dogs in a majority of the gene families. Among the spontaneous tumors of the dog that are highly invaluable for comparative oncology and drug discovery is osteosarcoma (OS) (Vail and MacEwen, 2000). Canine OS, is a malignant bone tumor, affects predominantly large to giant breeds and closely resemble human OS especially in its clinical presentation, histology, disease progression, response to conventional therapy and prognosis (Bielack et al., 2002). Distant metastasis remains the greatest challenge in the management of both human and canine patients. In human OS, despite advances in standard care including amputation or limb sparing followed by adjuvant chemotherapy, only 60% of patients will reach a-5 year disease-free interval. Similarly, 90% of dogs with OS will develop metastatic pulmonary disease; with one year survival rate of approximately 50% for those received post operative chemotherapy. Although various chemotherapy protocols have been shown to significantly prolong survival of both human and canine patients, there appears to be a limit to what these agents can achieve in the control of distant metastatic disease, making the identification of new avenues for detection and markers for metastasis essential.

To further understand OS metastasis, it is critical to define the molecular signature which can describe the metastatic characteristic of malignant cells. Microarray analyses have been conducted using human and murine OS *in vitro* models to uncover the metastasis associated genes and processes (Khanna et al., 2001; Lisle et al., 2008; Nakano et al., 2003; Zucchini et al., 2008). Several subpopulations of cells with high metastatic and invasive abilities derived from a primary tumor have been described and characterized. The first few microarray analyses performed on human and murine OS models focused on identifying genes and functional enrichment of genes attributed to metastasis. These include identification of five genes differentially expressed between three high and three low metastatic human OS sublines (Nakano et al., 2003). In a similar approach, metastatic murine OS lines revealed 53 differentially expressed genes where genes with functions pertaining to proliferation and apoptosis, cytoskeleton regulation and motility, invasion, immune surveillance, adherence and angiogenesis were associated with high metastatic potentials. Another study on human OS *in vitro* models with differing metastatic capacity revealed regulation of the NF-κB cell survival pathway and apoptotic markers as a major determinant for metastasis (Zucchini et al., 2008).

Global gene expression profiling of canine OS metastatic models has not been carried out, although various genes and pathways essential for metastasis have been described. One similar *in vitro* model to those described for murine and human OS is available for the canine: POS (a primary cell line with low metastatic potential) and HMPOS (a highly metastasizing POS sub-line). The POS line was established from a canine OS of the proximal femur (Kadosawa et al., 1994) and HMPOS was isolated from lung metastatic tumor deposits formed by

subcutaneous growth of the POS cell lines in nude mice. *In vivo* biological characterizations performed indicate that HMPOS have a higher growth rate and a high colonization potential to the lungs in nude mice.

Because differentiating metastatic potentials *in vitro* may help highlight differences in genes and pathways important for the multistep processes of metastasis, the present study used canine specific cDNA microarrays consisting of 20,313 genes to analyze variations in gene expression between HMPOS and POS. Our main objectives were (a) to define a gene profile that would distinguish the two cell lines, (b) to identify involvement of important transcription factors among the differentially regulated genes and (c) to identify and compare signaling pathways important for metastasis from the present study in canine cell lines with those published for human and mouse models. One of the genes with differential expression between the two cell lines was selected for further investigation on human and canine OS tissue materials where the expression was compared between lung metastasis-primary tumor pairs from canine OS; and subsequent prognostic evaluation on a panel of outcome-linked primary canine and human OS tissues.

Materials and Methods

Cell lines and culture conditions

Two canine OS cell lines with different metastatic potentials (HMPOS and POS) (Barroga et al., 1999; Kadosawa et al., 1994) were maintained on RPMI medium, supplemented with 2mM L-glutamine (Sigma-Aldrich, St. Louis, MO) and 10% heat inactivated fetal calf serum at 37ºC in a humidified 5% CO2 atmosphere. Cells were passed every 4-5 days.

Tritium-thymidine incorporation assay

Cells were seeded at a density of 1x104 cells/ 3mL culture medium in 6-well Multiwell™ Primaria plates (Falcon®, USA) for 72 h. To each well, 0.1µCi/mL tritium-thymidine (GE Healthcare, UK) was added and incubated for 4.5 h. After 4.5h, culture medium was removed and cells were washed prior to adding 0.2 mL of 0.2% SDS. Cells were further incubated for 15 min and 300µl RNAse free water was added thereafter. Three mL of scintillation fluid was added to 400µl cell lysate and tritium-thymidine incorporation was measured by a liquid scintillation analyzer (TRI-CARB 2900TR, Packard, USA). DNA content from 6 independent wells for each cell line was measured using a previously described protocol (Labarca and Paigen, 1980) Fluorescence was measured and DNA contents were read using the LS 50 Fluorescence Spectrometer (PerkinElmer®, U.S.A.). Experiments were performed in 6 well plates in triplicate and repeated 3 times. Differences in tritium-thymidine incorporation after normalization for DNA content was calculated using the student's t-test and $P<0.05$ was considered significant.

Colony formation assay

Anchorage independent growth ability was determined using a semisolid agarose bilayered system. Six well plates were coated with 1.5 mL of base agar consisting of 2.0% Low Melting Point (LMP) Agarose (Promega, USA) in media as described above. Base agar was allowed to solidify for 1 hr before adding the top layer of 0.75% agarose with cell suspension. Cells were trypsinized, counted using a hemacytometer and resuspended at low density (3x104 cells in1.5 mL) in 0.75% agarose in media enriched with 10% FCS. Cells were grown for 14 days. Medium was replaced every 4-5 days. Experiments were performed in 6 well triplicates. Enumeration of colonies was carried out manually by 3 independent assessors on 7 random focus areas where the mean number of colonies bigger than 100 µm at 10X magnification from each cell line were calculated and compared for significant differences using the student's t-test. Significance was defined as $P<0.05$. Colonies were photographed at 4X and 20X objective magnifications using an IMT-2 phase contrast microscope with attached E-330 digital camera (Olympus, The Netherlands).

RNA isolation and amplification

Cells grown to 80% confluence in 75 mL flasks were washed once with Hank's Balanced Salt Solution and lysed using 1mL RLT lysis buffer (Qiagen, The Netherlands). RNA isolation and purification was performed with the RNeasy mini kit (Qiagen, The Netherlands) according to the manufacturer's protocol. The RNA samples were treated with DNase-I (Qiagen RNase-free DNase kit). Total RNA was quantified using the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies), and cRNA synthesized as described previously (Rao et al., 2008; Selvarajah et al., 2009). Quality of total and amplified cRNA was analyzed using a bioanalyzer (Agilent Technologies, The Netherlands).

Microarray hybridization and data normalization

Microarray hybridization, scanning and image analysis were conducted according to the protocol described (Rao et al., 2008). A reference pool consisting of cRNA from both cell lines was used to hybridize against each cell line on the array. In total 4 arrays were hybridized for each cell line, which represents RNA samples from 2 independent culture passages with dye swaps against the reference pool. Defective spots were flagged and normalization was carried out using the Lowess print-tip normalization technique. Log transformation was applied to the microarray raw data after normalization and data from dye swap arrays were switched before they were subjected for further statistical analysis. The microarray data are MIAME compliant and files have been deposited in the public database (GEO: GSE15517).

Microarray data analysis

Significance Analysis of Microarray (SAM) (Stanford University, CA, USA) (Tusher et al., 2001) was used to compare the normalized and log-transformed expression data between HMPOS and POS, using 100 permutations to the k-nearest neighbor with 10 neighbors. A gene list for

this analysis was generated at a false discovery rate (FDR) of 10%. Hierarchical clustering of differentially regulated transcripts between the two cell lines was carried out. A two-dimensional dendrogram of both gene tree and condition tree based on standard correlation on the differentially expressed genes was generated using the GeneSpring® (Agilent Technologies, The Netherlands) software (Slonim, 2002).

Quantitative real-time PCR for microarray validation

Total RNA was isolated from 7 independent, serial culture passages for each cell line (biological replicates) using the protocol mentioned above. Synthesis of cDNA was carried out from 1.0 µg total RNA in 40 µl reaction volumes using the iScript™ cDNA synthesis kit according to the manufacturer's protocol (Bio-Rad, The Netherlands). Quantitative real-time PCR (qPCR) was performed on six candidate genes, as well as Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta-glucuronidase (GUSB) and heterogeneous nuclear ribonucleoprotein H (hnRNPH) as reference genes for normalization. Primer sets were designed (located on different exons) using the software Primer3 (Rozen and Skaletsky, 2000). A gradient qPCR was carried out to determine the ideal temperature range for each primer set, and the products were sequenced to verify the specificity of the primer sets. Quantitative real-time PCR was carried out in duplicate using the SYBR® green fluorescent dye method and further analyzed using the Bio-Rad MyIQ software (Bio-Rad, The Netherlands). Statistical analysis was carried out using REST-XL (pairwise fixed reallocation and randomization test) software (Pfaffl et al., 2002) that estimates a relative normalized fold change of gene expression between the two cell lines. A $P<0.05$ was considered statistically significant.

Transcription factor, gene function and pathway analyses

Analysis of gene regulatory networks among the differentially expressed genes identified by SAM was performed using the 'Shortest Path Analysis' of MetaCore™ Analytical Suite (GeneGo Inc., MI, USA) (Kang et al., 2009). MetaCore™ generates a network based on available information from published databases regarding a gene of interest and its interaction with other genes. 'Shortest path analyses' allows for one intermediate additional gene, either a transcription factor, receptor or a ligand to be linked to the candidate genes in investigation in order to form an informative gene network. In addition, EDGE (Extraction of Differential Gene Expression) software (http://faculty.washington.edu/jstorey/edge) was used to generate the differentially expressed genes between the two cell lines at a cutoff threshold of $P<0.05$ (Leek et al., 2006; Selvarajah et al., 2009). The differentially expressed genes were compared with their human homologue to obtain a list of gene symbols or human RefSeq ID that were later subjected for pathway analysis (Rao et al., 2008; Selvarajah et al., 2009) on PANTHER® (**P**rotein **AN**alysis **TH**rough **E**volutionary **R**elationships) (http://www.pantherdb.org/). PANTHER® software was subsequently used to categorize the candidate genes based on their involvement in various biological processes and molecular functions. Two previously published human and mouse OS metastasis-associated gene

datasets were selected for PANTHER® pathway analysis to generate pathways for comparative analyses (Srivastava et al., 2006; Zucchini et al., 2008). Top 20 pathways from each of those human and mouse OS dataset were compared with the pathways generated from the present study on canine OS cell lines to discover and reveal common pathways for metastasis across species. Pathway lists were compared using the interactive tool: Venn Diagrams, VENNY (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

TP53 mutation analyses

Canine specific TP53 primers were designed (FW: 5-ATGGAGGAGTCGCAGTCAGAG-3' and RW: 5'-TTTTATGGCGAGAGGTAGATTGC-3') and cDNA samples of two different passages from each cell line were incorporated for the analysis. PCR was performed in a 20µl reaction using the Phusion™ Hot Start DNA polymerase and 5x Phusion™ HF buffer (Finnzymes, Finland) according to the manufacturer's protocol. The protocol includes denaturation at 95°C for 4 min, followed by 35 cycles comprising 95°C for 30 seconds; annealing at 50°C for 1 min followed by extension for 1 min at 72°C. The final extension was accomplished through incubation at 72°C for 10 min and further cooling down to 20°C. PCR product size of 1108 base pairs was further verified by gel electrophoresis. Sequencing was performed using the Big Dye Terminator Ready reaction kit (Applied Biosystems, USA) with slight modification to the manufacturer's protocol which includes using PCR product that was diluted ten times before a volume of 3µl was used as template for amplification. Amplified PCR product sequences were analyzed by ABI Prism® 3130xl genetic analyzer (Applied Biosystems, USA). Sequences from HMPOS and POS were aligned and compared with each other and to the wild type canine TP53 (NM_001003210) sequence obtained from NCBI database.

Independent validation study on canine OS tissues

Fifteen snap frozen canine primary OS tissues with available clinical data from the University Clinic for Companion Animals in Utrecht, The Netherlands, was selected for the follow-up study. Clinical and pathological parameters were evaluated retrospectively. Total RNA isolation and cDNA synthesis was carried out as described previously (Selvarajah et al., 2009). Quantitative real-time PCR was carried out in duplo using the SYBR green fluorescent dye method to investigate the expression in these tumors. Decorin expressions in terms of starting quantity (SQ) were normalized against a reference gene, GAPDH for further statistical analysis. The Kaplan-Meier method was used to draw survival curves of tumors with high and low decorin mRNA expression where tests for group comparisons were made using the Mantel-Cox log rank test using SPSS version 16.0. Additionally a two-sided Fisher's exact test was performed to compare the proportions of clinicopathological parameters between the two groups. Known prognostic factors such as histology grade, tumor subtype, serum alkaline phosphatase levels together with decorin mRNA expression (as a continuous variable) were investigated for their unadjusted association with survival in univariate analysis. Variables with $P<0.15$ were subjected for further multivariate analysis using Cox-regression model to

estimate the hazard ratios and confidence intervals for death due to disease among these 15 dogs. All statistical analysis was performed using SPSS 16.0 and P values <0.05 were considered statistically significant.

Decorin immunohistochemistry and scoring method

Four µm paraffin-embedded decalcified sections from canine OS tissues (5 primary-lung metastasis pairs) were cut and mounted on Superfrost+ slides (Fisher Scientific). Immunohistochemistry was performed as described previously (Spee et al., 2005) with slight modifications to the antigen retrieval method: 850W boiling in microwave oven for 2 min; 5 min cooling; 850W boiling for 2 min followed by 25 min cooling at bench top. Sections were incubated overnight at 4°C with polyclonal rabbit anti-DCN antibody (HPA003315, SIGMA® Prestige Antibodies, USA) diluted 1:100 in PBS with 1% bovine serum albumin. Sections were washed in PBST (0.1% tween) and incubated with secondary anti-rabbit antibody (EnVision®-HRP detection system, DakoCytomation, USA) for 1 hr at room temperature. Staining was visualized with DAB substrate chromogen system (K3468, Dako, USA) and counterstained with hematoxylin. Negative control tissue sections were incubated in the absence of the primary antibody, substituted with normal rabbit serum with the same concentration.

Identical protocol was applied on a human tissue microarray (Abdeen et al., 2009) obtained from the National Cancer Institute, Bestheda, USA. Human OS tissue microarray consist of primary biopsies, definitive resected primary tumors, metastasis and normal tissues were stained for decorin and scored by manual inspection at 20-40X objective magnification by 3 independent observers who were blind to clinical data. Tissue cores with <10% of tumor cells present for analysis were excluded. Tissue cores were scored as 0 or 1 for corresponding negative or positive staining (in >25% of tissue) for decorin. Tissues positive for decorin were further scored for intensity: 1=weak, 2=moderate and 3= strong. Univariate Cox- regression analysis was carried out using SPSS version 16.0 to determine the prognostic impact of decorin expression on survival. Images were captured using a CCD camera and Olympus BX41 microscope linked to a Cell^B imaging software (Soft Imaging Solutions GmbH, Germany).

Results

Cell line characteristics

Two different assays were performed to differentiate the growth abilities of HMPOS and POS cells. Tritium-thymidine incorporation after normalization for DNA content revealed significantly higher number of HMPOS cells showing advanced growth velocity as compared to POS. HMPOS had higher anchorage independent growth ability, with significantly larger and increased number of colonies formed in soft agar. Colony appearance differed remarkably where HMPOS tend to form more colonies of irregular shape with ‘projections’ and ‘satellite-like’ colonies surrounding the main colony (Figure 1). These features were seen

occasionally in colonies formed for the POS cell line, but at much lower incidence compared to HMPOS.

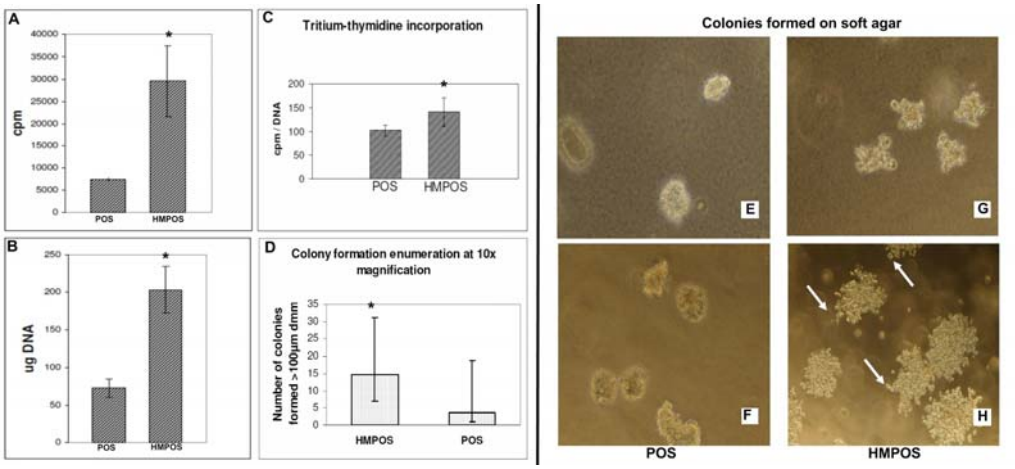

Figure 1: Enhanced proliferation rate seen in HMPOS as compared to POS: A- Counts per mL (cpm) detected after 4.5 hr of tritium incubation; B- DNA (µg) content of parallel wells not treated with tritium (for normalization purpose); C- Tritium-thymidine incorporation/ µg DNA content mean ratio; D- HMPOS produced significantly higher number of colonies with diameter > 100µm at 10x magnifications; Colony formation on soft agar: POS: E- Colonies at Day 10 appear more spherical and densely clumped; G- Colonies at Day 14. HMPOS: F- polygonal shaped colonies at day 10; H- large colonies with prominent 'branching appearance" and 'satellite-like' colonies surrounding the main colonies as shown by arrows at Day 14 (400X magnification) (*significance at $P<0.05$).

Differentially expressed genes between POS and its highly metastatic clone HMPOS

The approach to microarray data analysis is depicted in Figure 2. *Significance Analysis of Microarrays* (SAM) generated a list of 278 genes that were differentially expressed between POS and HMPOS at a False Discovery Rate of 10%. A dendrogram generated by hierarchical clustering of all 278 differentially expressed genes between HMPOS and POS revealed clear clustering of replicate experiments (Figure 3). A minimum gene list consisting of both down and upregulated genes in HMPOS with a fold change of 2.0 is listed in Table 1 while the complete gene list is available as supplementary Table S1.

Quantitative real-time PCR was performed on cDNA from 7 independent serial culture passages from each cell line including the passages used for the microarray hybridizations. Validated primer sets are provided in Table 2. Expression data were normalized using three reference genes, GAPDH, GUSB and hnRNPH. Among the 4 candidate genes analyzed using qPCR, four genes: DCN and GSTmu5 were validated for their comparable expression to the microarray analysis, while 2 others DTD1 and GCAP14 were found to be false positives (Table 3).

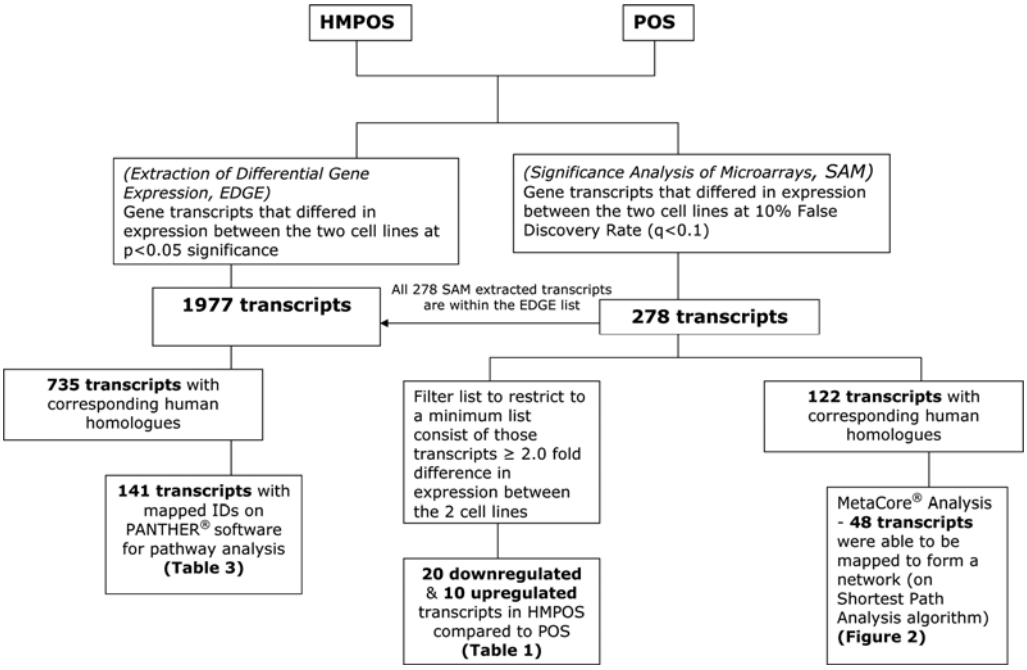

Figure 2: Flow chart on the algorithm used to extract and define the differentially expressed genes between the two cell lines which were also used for pathway analysis and generation of the gene regulatory network.

Regulatory gene network, functional and pathway analysis

Among the SAM generated genes, 122 had a corresponding human homologue (RefSeq IDs) and these were further categorized by biological process and molecular function. Biologically, the majority of transcripts were important for protein metabolism and modification, followed by nucleoside, nucleotide and nucleic acid metabolism. Important biological processes for metastasis such as protein metabolism and modification, cell communication; immunity, cell structure and motility were also noted (Table 4). Next, a gene network was generated based on 48 genes using the 'shortest path analyses' algorithm from MetaCore™ (Figure 4). The generated network revealed that 3 transcription factors, namely TP53, SP1 and c-myc, appear as a 'central hub', connecting the differentially expressed genes found in this study. Further TP53 mutation analysis revealed that both cell lines had an identical single nucleotide polymorphism (SNP) in codon 77 (normal sequence: ccc**TCG**tgg, in cell lines: ccc**TCC**tgg) which did not cause a change in the encoding amino acid (serine), and a point mutation in codon 162 causing an amino acid change from arginine to histidine (wild type: cgg**CGC**tgc, cell lines: cgg**CAC**tgc).

Table 1: List of genes differentially expressed between HMPOS and POS with a minimum fold change of 2.0, #N/A, non-annotated for *Canis familiaris*.

Gene accession	Expression in HMPOS	Fold change	Gene symbol	Transcript name (description)
CO713802	Up	15.38	GCAP14	granule cell antiserum positive 14
CO606032	Up	12.22	Slc39a13	solute carrier family 39 (zinc transporter), member 13
CO664643	Up	5.63	S100P	S-100P protein
CO655222	Up	5.24		#NA
CO608762	Up	4.98	GSTmu5	glutathione-S-transferase, mu 5
CO717608	Up	4.16	DCN	Canis lupus familiaris decorin
CO588648	Up	4.09	RBM17	RNA binding motif protein 17
CO591194	Up	2.78		#N/A
CO692221	Up	2.67	ACAA1	3-ketoacyl-CoA thiolase, peroxisomal precursor (Beta-ketothiolase)
CO689888	Up	2.52		#NA
CO607706	Down	35.48	DTD1	Probable D-tyrosyl-tRNA(Tyr) deacylase
CO630565	Down	25.50		#NA
CO656541	Down	20.39	SLC17A3	Na/Pi cotransporter 4
CO697932	Down	12.67		#NA
CO598005	Down	12.21		#NA
CO617943	Down	10.94		#NA
CO612781	Down	10.71		#NA
CO632112	Down	8.11		Similar to CG11141-PB, isoform B (LOC490864)
CO659846	Down	7.70	RPS23	ribosomal protein S23
CO615002	Down	7.49		#N/A
CO586195	Down	6.66	VLDLR	Very low-density lipoprotein receptor precursor (VLDL receptor)
CO614419	Down	5.93		#NA
CO587490	Down	5.11		#NA
CO689455	Down	4.91	PIGG	GPI7 protein, Phosphatidylinositol glycan anchor biosynthesis, class G
CO585908	Down	3.99		#NA
CO685697	Down	3.75		Canis Familiaris chromosome 10, clone XX-162K3
CO664309	Down	3.22	PRSS23	Serine protease 23 precursor (Putative secreted protein ZSIG13)
CO597068	Down	3.04		CG6878-PA (LOC477215)
CO656046	Down	2.75		#NA
CO585397	Down	2.67		#NA

Table 2. Validated primer sets used for quantitative real-time PCR.

Gene name	Gene symbol	Forward and Reverse Primers	Annealing Temperature (°C)
Decorin	DCN	FW: 5'-CTGTCAGTGCCATCTCCGAG-3' RW: 5'-AGCGTAGTGTCAGGGGGAAG-3'	60
Gluthathione S-transferase µ5	GSTmu5	FW: 5'- GTTTGAGCCCAAGTGCCTG-3' RW: 5'- CAGATCAAGTCCACCTCCTGC-3'	65
Granule cell antiserum protein 14	GCAP14	FW: 5'-TTGATGTGGATCTGCCTGAG-3' RW: 5'-CGAACATTTCTGTCTGACCG-3'	61
Probable D-tyrosyl-tRNA (Tyr) deacylase	DTD1	FW: 5'-CATCTGTGTGTTGCTGGG-3' RW: 5'-TGGAGTGTGAACTGGCTG-3'	61.5
Heterogeneous nuclear ribonucleoprotein H	hnRNPH	FW: 5'-CTCACTATGATCCACCACG-3' RW: 5'-TAGCCTCCATAACCTCCAC-3'	61.2
Beta-glucuronidase	GUSB	FW: 5'-AGACGCTTCCAAGTACCCC-3' RW: 5'-AGGTGTGGTGTAGAGGAGCAC-3'	62
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	FW: 5'-TGTCCCCACCCCCAATGTATC-3' RW: 5'-CTCCGATGCCTGCTTCACTACCTT-3'	58

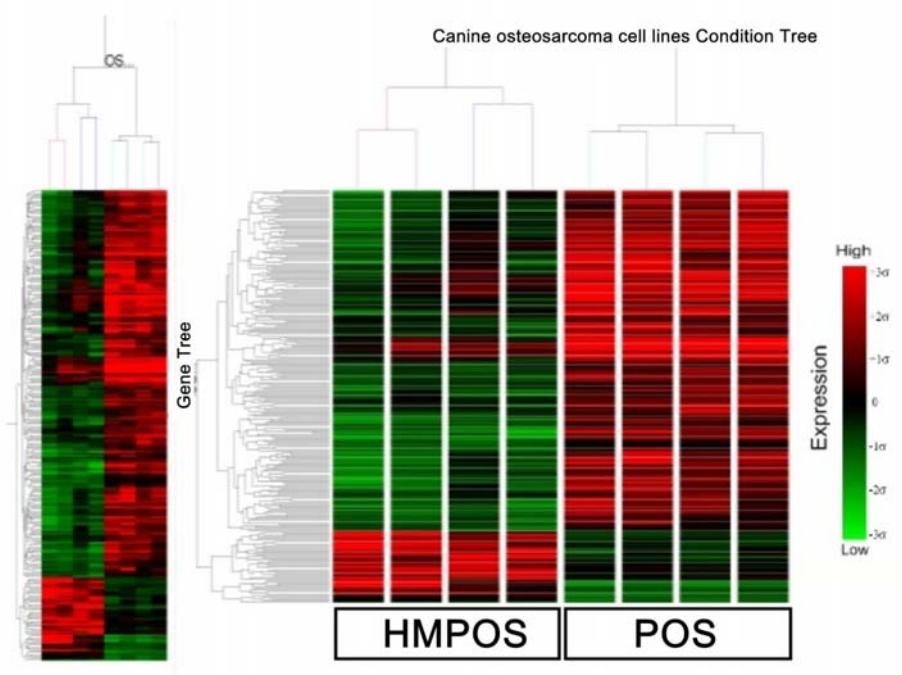

Figure 3: Dendrogram of differentially expressed genes between HMPOS and POS. Dendrogram generated by 2 dimensional hierarchical clustering of the 278 differentially expressed genes found from SAM analysis. Majority of genes were downregulated in HMPOS as compared to POS. There is clear clustering of replicate samples and their dye swaps. Red indicates genes with high expression and green are those with low expression.

Table 3: Quantitative real-time PCR analysis comparing mRNA expression of 4 selected candidate genes between the two cell lines. Expression level represents those quantified for the HMPOS compared to the POS cell line. mRNA expression of 2 transcripts validated with *P<0.001 had comparable expression level to the microarray data analysis; while two others (DTD1 and GCAP14) were falsely positive while being non-significantly different between the two cell lines (NS).

Gene Symbol	**DTD1**	**GCAP14**	**GSTmu5**	**DCN**
Regulation Level	UP	DOWN	UP	UP
Target gene is UP regulated by factor	1.07		21.71	44.41
Target gene is DOWN regulated by factor		-1.79		
Absolute gene regulation (QPCR) (2-log)	**0.10**	**-0,84**	**4.44**	**5.47**
Absolute gene regulation (2-log Standard Error)	+/- 2.549	+/- 3.597	+/- 2.408	+/- 4.824
Fold Change from SAM analysis	**-35.48**	**+15.38**	**+4.98**	**+4.16**
P value (significance)	NS	NS	P<0.001	P<0.001

Table 4: The differentially expressed genes between HMPOS and POS involved in several biological processes pertaining to high invasiveness and metastasis.

Gene ID	Expression in HMPOS	Fold Change	Gene Symbol	Gene description
nucleoside, mucleotide and nucleic acid metabolism				
DG11-32b19	**Down**	**2.13**	ANT 3	ADP/ATP translocase 3
DG11-253f7	**Down**	**1.72**	TCF4	transcription factor 4 (TCF4)
DG2-87n13	**Down**	**1.49**	AHCY	Adenosylhomocysteinase
DG2-130i11	**Down**	**1.39**		3(2),5-bisphosphate nucleotidase 1
DG2-120o6	**Down**	**1.88**	TOP2B	DNA topoisomerase II, beta isozyme
DG2-102p11	**Up**	**1.59**		transcription elongation factor B (SIII), polypeptide 1
DG14-41g14	**Down**	**1.50**		Dynein intermediate chain 2, cytosolic
DG2-59c21	**Down**	**1.39**		transcription elongation factor A (SII)-like 1
DG8-119p24	**Down**	**1.34**	ADA	adenosine deaminase; adenosine aminohydrolase; ADA
DG9-20n13	**Up**	**1.68**	NFIL3	nuclear factor, interleukin 3 regulated
DG2-19i19	**Down**	**1.51**	OSCP	ATP synthase O subunit (Oligomycin sensitivity conferral protein
DG2-110b1	**Down**	**1.31**	DDX1	DEAD (Asp-Glu-Ala-Asp) box polypeptide 1
DG2-136e3	**Down**	**1.37**		general transcription factor Iia 2
DG8-115g4	**Down**	**1.20**	PTTG1	Securin (Pituitary tumor-transforming protein 1)
DG2-2g12	**Up**	**1.50**		eukaryotic translation initiation factor 4A2
DG8-137i11	**Down**	**2.40**		small nuclear ribonucleoprotein E
DG2-43d14	**Down**	**1.35**		transmembrane protein 62
DG2-22o24	**Up**	**4.09**	RBM17	RNA binding motif protein 17
DG14-94e23	**Up**	**1.41**		U11/U12 snRNP 35K isoform a
protein metabolism and modification				
DG2-102d6	**Down**	**1.39**		dual specificity phosphatase 12
DG32-251a13	**Down**	**1.52**	SCPEP1	serine carboxypeptidase 1 precursor protein
DG14-95i14	**Down**	**1.49**	MRPL9	mitochondrial ribosomal protein L9
DG2-56b12	**Down**	**1.40**	USP48	ubiquitin specific protease 48
DG8-108k7	**Down**	**1.35**	PSMA6	Proteasome subunit alpha type 6
DG32-227c5	**Down**	**1.50**	LAMP2	Lysosome-associated membrane glycoprotein 2 precursor
DG2-8n23	**Up**	**2.06**	RPL22	ribosomal protein L22 like 1
DG8-2m8	**Down**	**1.47**	ICT1	immature colon carcinoma transcript 1
DG32-262l17	**Down**	**1.36**	C1S	Complement C1s subcomponent precursor (C1 estrase)
DG11-180h9	**Up**	**1.74**		adaptor-related protein complex 2, mu 1 subunit isoform b
DG2-127o22	**Down**	**1.26**	HEMK1	HemK methyltransferase family member 1 (M.HsaHemKP)
DG40-118d2	**Down**	**3.22**	PRSS23	Serine protease 23 precursor (Putative secreted protein ZSIG13)

DG2-66f24	**Down**	**2.25**		glutamyl-prolyl tRNA synthetase, transcript variant 3
DG2-99i16	**Down**	**1.55**	PCMT1	Protein-L-isoaspartate(D-aspartate) O-methyltransferase
DG16-1o14	**Down**	**1.46**	RPL4	ribosomal protein L4
DG11-44b7	**Up**	**2.67**	ACAA1	3-ketoacyl-CoA thiolase, peroxisomal precursor
DG11-103g6	**Down**	**1.51**	LONP1	peroxisomal lon protease
DG2-20l7	**Up**	**1.89**	MRPL15	mitochondrial ribosomal protein L15
DG8-166o2	**Down**	**1.86**	TSP50	testes-specific protease 50
DG32-109e21	**Down**	**1.67**		sorting nexin 1 isoform c
DG9-22h15	**Down**	**2.37**	TIMP3	tissue inhibitor of metalloproteinase 3
DG11-233k17	**Down**	**1.57**	TIMP3	tissue inhibitor of metalloproteinase 3
DG2-24i24	**Up**	**1.98**	HSP60	60 kDa heat shock protein, mitochondrial precursor
DG2-2a7	**Up**	**1.74**	PMP41	Programmed cell death protein 6
DG20-2h2	**Up**	**1.64**	RPL7	60S ribosomal protein L7, transcript variant 1
DG2-58i8	**Up**	**1.38**	CARS	cysteinyl-tRNA synthetase isoform c, transcript variant 1
DG2-51e20	**Down**	**1.65**	UCH37	ubiquitin C-terminal hydrolase UCH37
DG42-38n20	**Down**	**1.58**	GSTK1	Glutathione S-transferase kappa 1 (GST 13-13)
immunity and defense				
DG8-77g15	**Up**	**4.98**	GSTM5	glutathione-S-transferase, mu 5
DG32-109e21	**Down**	**1.67**		sorting nexin 1 isoform c
DG11-277k12	**Down**	**1.59**	PRDX1	peroxiredoxin 1
DG9-20n13	**Up**	**1.68**	NFIL3	nuclear factor, interleukin 3 regulated
DG32-262l17	**Down**	**1.36**	C1S	Complement C1s subcomponent precursor (C1 estrase)
cell communication				
DG2-119l15	**Up**	**2.38**	E-FABP	Fatty acid-binding protein, epidermal (E-FABP)
DG2-27a1	**Up**	**1.41**	STMN1	Stathmin (Phosphoprotein p19) (pp19) (Oncoprotein 18)
DG14-52p4	**Up**	**4.16**	DCN	Canis lupus familiaris decorin (DCN)
DG33-2h3	**Down**	**1.61**	VCL	Vinculin (Metavinculin)
cell cycle / proliferation				
DG8-70j15	**Up**	**1.41**	BUB3a	BUB3 budding uninhibited by benzimidazoles 3 isoform a
DG32-100b10	**Down**	**1.45**	MOB1	Mob4B protein (MATS1)
DG2-120o6	**Down**	**1.88**	TOP2B	DNA topoisomerase II, beta isozyme
DG14-41g14	**Down**	**1.50**	DH IC-2	Dynein intermediate chain 2, cytosolic
DG33-101c10	**Down**	**1.25**	CCNG1	Cyclin G1 (Cyclin G)
DG8-115g4	**Down**	**1.20**	PTTG1	Securin (Pituitary tumor-transforming protein 1)
cell adhesion				
DG14-52p4	**Up**	**4.16**	DCN	Canis lupus familiaris decorin (DCN)
DG33-2h3	**Down**	**1.61**	VCL	Vinculin (Metavinculin)
cell structure & motility				
DG2-9i8	**Down**	**1.98**	TPM1	tropomyosin, transcript variant 1
DG2-122j7	**Down**	**1.59**	ABLIM1	actin binding LIM protein 1

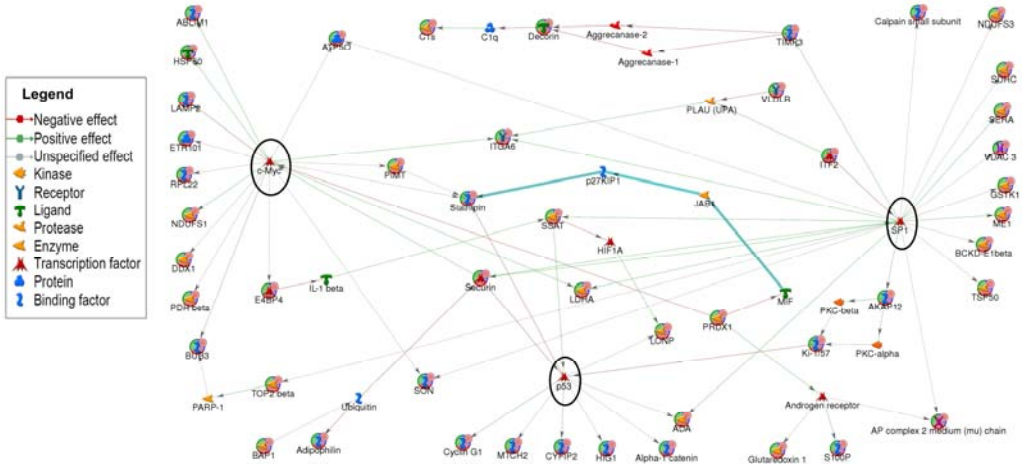

Figure 4: MetaCore™ gene regulatory network. Three transcription factors p53, SP1 and c-myc were found as 'central hubs' connecting the differential expressed genes found in the present study.

PANTHER® pathway analysis of the differentially expressed genes from SAM revealed too few genes linked to a certain pathway, mainly because the majority of genes were not associated with a particular pathway. Next, the software EDGE was used to generate a final list of 1977 genes that were differentially expressed between the two cell lines at a cut off of $P<0.05$. As expected, all 278 genes of SAM appeared to be a subset of those from EDGE analysis. Among these transcripts, 735 genes were found to be homologous with *Homo sapiens*. After removal of replicates and non-annotated gene transcripts from this list, a PANTHER® pathway analysis was performed on a final list of 141 gene transcripts that were mapped to several signaling pathways. The genes were found to have enrichment for several pathways including integrin (16 transcripts) and Wnt (13 transcripts) signaling. Two other datasets from previously published research on identification of genes associated with metastasis in human and mouse *in vitro* models with similar approach as our study were used for comparative pathway analysis. Gene lists from Zuchini C. *et al*., 2008 and Srivastava A. *et al*., 2006 publications were subjected to PANTHER® pathway analysis and compared to the present study. Further comparison between the pathways generated from the present study in canine models and the top 20 pathways from the above studies revealed 9 overlapping pathways (Table 5).

Table 5: Top 20 pathway hits revealed by PANTHER® analysis on differentially expressed genes between the two cell lines of different metastatic potentials generated by EDGE at cut off of P<0.05.

Top 20 pathway hits from the present study	Gene hits
Integrin signaling pathway*	16
Wnt signaling*	13
Huntington disease*	12
Ubiquitin proteasome pathway	11
Parkinson disease	10
Angiogenesis	10
Inflammation mediated by chemokine and cytokine signaling*	10
Cytoskeletal regulation by Rho GTPase	7
Apoptosis signaling	7
FGF signaling	6
Cadherin signaling	6
General transcription regulation	5
Alzheimer disease-presenilin pathway	5
Transcription regulation by bZIP transcription factor	5
Heterotrimeric G-protein signaling pathway: Gi alpha and Gs alpha mediated pathway	5
Blood coagulation	4
5HT2 type receptor mediated signaling	4
P13 kinase pathway*	4
EGF receptor signaling*	4
Heterotrimeric G-protein signaling pathway: Gq alpha and G0 alpha mediated pathway	4

Pathways sorted according to number of associated gene hits. **Bold pathways***: Gene enrichment seen for 9 signaling pathways common to the (a) present study in canine OS cells, (b) Srivastava *et al*, 2004 (comparing SaOS2, low metastatic human OS cell line with LM8 and 143B mouse OS cells which have higher metastatic abilities) and (c) Zucchini *et al.*, 2008 (comparing low metastatic L/B/K ALP and CD99 transfected clones of U2OS with the original U2OS cells with high metastatic potentials).

Decorin mRNA expression in canine OS

Among the candidate genes further analyzed by qPCR, decorin appeared to have the highest expression difference between HMPOS and POS cells; in which higher expression was observed for the high metastatic cell line. Therefore we selected decorin for further investigation where the mRNA expression of decorin was determined for 15 primary canine OS snap-frozen tissues. These tissues were selected based on the following criteria: (a) snap-frozen samples with good quality and quantity RNA, (b) availability of clinical follow up (DFI censored data) (c) dogs all treated primarily with amputation or total resection of an appendicular sites for OS followed by postoperative chemotherapy and (d) tumors with histological grading data available (assessed by a board certified veterinary pathologist M.K.).

Table 6: Univariate analysis of possible confounders influencing survival among the 15 dogs tested for decorin mRNA expression.

Variable	Sub-category	N	Hazard ratio (HR)	95% Confidence interval (CI)		P value
				Lower	Upper	
Histology grade	Medium (Grade 2)	8	0.549	0.165	1.830	0.329
	High (Grade 3)	7				
Histology subtype	Osteoblastic	7	2.029	0.637	6.464	0.231
	Osteoblastic + mixed histology	8				
Alkaline phosphatase*		15	1.005	1.003	1.009	0.167
Decorin mRNA expression*		15	1.999	1.007	3.967	**0.048**

*Continuous variable

Dogs were followed up from the time of diagnosis till death for up to approximate period of 2 years. Disease-free interval (DFI) ranged from 35 to 705 days where 12 out of 15 dogs were observed developed metastasis while 3 dogs were free from metastatic disease during the follow up period (these 3 dogs died due to other causes and were censored for survival analysis). Primary tumors were from medium to large dog breeds, appendicular sites and consist of osteoblastic (n=7) and mixed histological subtypes (combination of osteoblastic with chondroblastic- fibroblastic-telangiectic subtypes) (n=8). Canine OS primary tumors with

decorin mRNA expression above the median calculated expression value =1.23 (range: 0.361-3.132) were compared to tumors with lower expression. Univariate Cox-regression analysis revealed decorin mRNA as an independent prognosticator compared to other known variables for canine OS including tumor subtype, histology grade and alkaline phosphatase measurement (Table 6). Similarly, Kaplan-Meier curves demonstrated significantly poorer DFI in dogs with tumors with high decorin mRNA expression compared to those with lower expression (Figure 5).

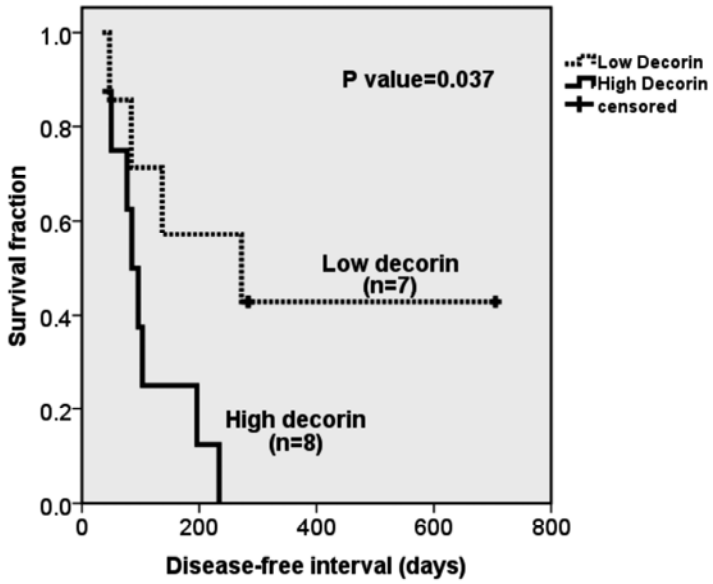

Figure 5: Kaplan-Meier curves comparing canine OS tumors with higher mRNA decorin expression (n=8) to those with lower expression (n=7). Dogs with higher decorin expression (above the qPCR calculated median value) in primary tumors have a significantly lower DFI (P value=0.037, Mantel-Cox log rank score of 4.340).

No significant association was found between tumors expressing high or low decorin mRNA expression with regards to other factors including histological grades, tumor subtypes and metastasis locations. This could be due to the low number of tumors investigated in the present study. However, all 3 dogs that did not develop metastasis had low decorin expression while 2 of the dogs that developed bone metastases have primary tumors expressing higher decorin mRNA (Table 7).

Table 7: Association of clinicopathological parameters with regards to decorin mRNA expression in primary canine OS

Variable	Sub-category	Total (n)	Low decorin mRNA expression (below median)	High decorin mRNA expression (above median)	Fisher's exact test score (Chi-square) P value
Histology grade	Low&medium (Grade 1&2)	8	5	3	0.315
	High (Grade 3)	7	2	5	
Histology subtype	Osteoblastic	7	3	4	1.000
	Mixed histologies*	8	4	4	
Metastasis location	No metastases	3	3	0	0.058
	Lungs	10	4	6	
	Bone	2	0	2	
Death due to metastatic disease	Censored (died due to other causes)	3	3	0	0.077
	Death due to metastatic disease	12	4	8	

*Mixed histologies refer to osteosarcoma with other histological subtype (chondroblastic, telangiectatic, fibroblastic)

Decorin protein expression in canine and human OS

Decorin protein expression and localization were found aberrant throughout whole paraffin embedded tissues from canine primary tumors and 4/5 lung metastasis. Among the 4 lung metastasis stained positive, 2 demonstrated strong expression towards the border of the tumor while the central zones were weak to absent in expression (Figure 6). Sixty primary human OS tissue cores in a tissue microarray were assessed for decorin expression in which only 12 was positive (20%). Disease-free interval (DFI) of patients with OS absent for decorin expression ranged from 10.87 to 229.3 months and for those with positive expression ranged from 7.97 to 111.23 months. Kaplan-Meier survival analysis (for group comparison) revealed human patients with OS positive (regardless of intensity) for decorin (n=12) have significantly shorter DFI compared to those with negative expression (n=48) (P=0.029, Log Rank Mantel-Cox test score= 4.756) (Figure 7). Among the positive tumors, 8 demonstrated moderate intensity for decorin while 4 had weak expression (compared to positive control: normal muscle tissue). Univariate Cox-regression analysis revealed decorin expression as a negative prognosticator with a P value=0.039 (HR=3.172, 95% CI: 1.061 - 9.478).

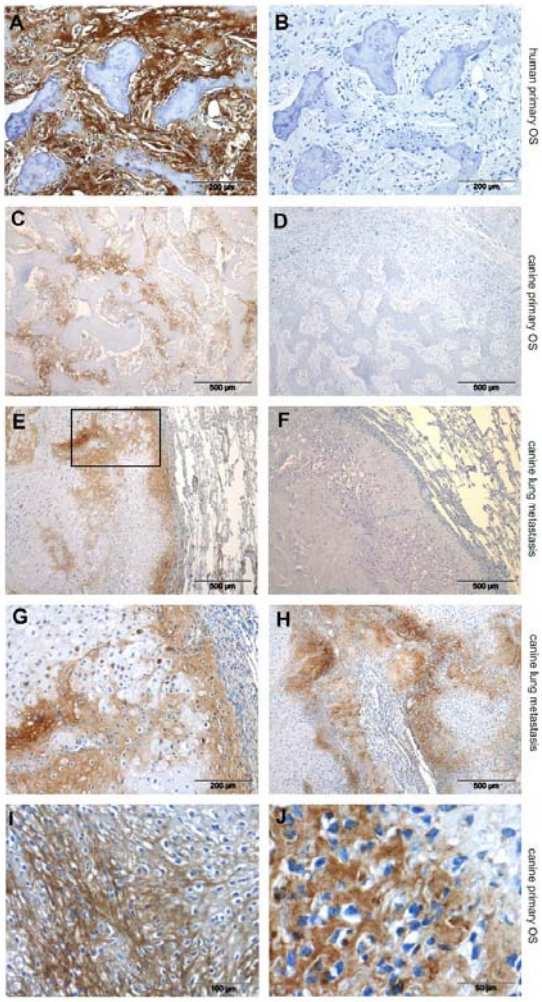

Figure 6: Immunohistochemistry evaluation of decorin expression in canine and human OS. A- High grade human OS, showing high intensity and widespread positivity for decorin within the extracellular matrix. Trabecular bone and osteocytes is negative for decorin expression; C- Canine OS primary tumor with variable decorin expression; B & D- human OS and canine OS control slides stained upon omission of the primary antibody; E- Canine OS lung metastasis with high decorin expression at the borders with low to negative expression towards the center of the tumor; F- corresponding negative control tissue for canine lung metastasis from E; G- higher magnification of a tissue section positive for decorin expression from E; H- osteoblastic cells towards the peripheral margins of the canine OS lung metastasis tissue have positive decorin expression; I & J- randomly selected areas of primary canine OS tissue sections stained for decorin observed at higher magnifications (X20) and (X40) respectively.

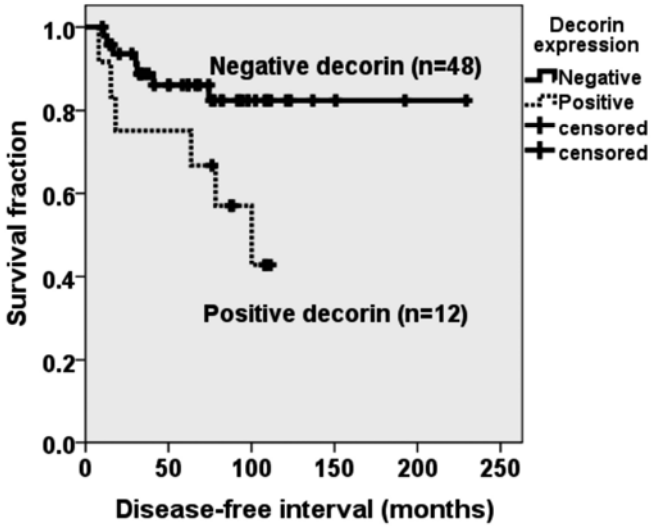

Figure 7: Decorin expression predicts poor clinical-outcome in OS human patients based on tissue microarray analysis. Patients with decorin expression in their primary tumors have significantly shorter disease-free interval compared to those patients without decorin expression (P=0.029, Log rank Cox-Mantel Score= 4.756).

Discussion

Human and dogs with OS share many common features especially in terms of tumor characteristics, metastatic disease progression and overall prognosis. It becomes more evident that various gene alterations, mutations as well as the intricate interactions between proteins in a cellular signaling cascade definitely needs more attention especially those related to OS disease development, progression and most importantly metastasis. The complex multi-step metastatic molecular mechanism have been proposed and investigated on various *in vivo* and *in vitro* human cancer models but the level of agreement and integration of all these knowledge and evidence is poorly understood and requires further exploration. In the present study, gene expression profiling was carried out on two clonally related canine OS cell lines to eliminate possible irrelevant genetic variability between cell lines from different origin. HMPOS is a highly metastatic sub-line of the parent cell line POS. *In vitro*, HMPOS and POS cellular growth patterns were comparable to that of their *in vivo* growth patterns in nude mice with a slightly higher doubling time for HMPOS in culture (Barroga et al., 1999), in addition to having enhanced directional migration potential in serum supplemented media (Fan et al., 2008). The present study confirmed previous observations that HMPOS cell line has advanced proliferation and anchorage independent growth ability exceeding that of its parental POS cells (Barroga et al., 1999; Kadosawa et al., 1994). The

genes differentially expressed between POS and HMPOS were found to be classified into several biological processes attributed to metastatic potentials (Khanna et al., 2001; Khanna and Hunter, 2005).

Microarray analyses using different platforms and computational analyses may result in different gene signatures. However, genes identified using various methodologies are in most cases part of identical pathways. Moreover, cross species comparison of the pathways involved may identify important common gene denominators or transcription factors for tumorigenesis and metastasis. Using this assumption, comparing the canine OS metastasis pathways with those studies published for murine and human OS revealed 9 overlapping pathways which includes the integrin and Wnt signaling. Extensive investigations have been initiated for human OS to understand how these signaling pathways are involved in OS pathogenesis and disease progression. Loss of cell adhesion molecules can be considered a characteristic of the metastatic phenotype (Luu et al., 2005). The integrin signaling is notably important for cell survival during tumor progression and metastatic dissemination. This pathway is considered essential at various levels in the metastasis cascade, where dynamic interactions and cross-talks between this pathway and various growth factors, cytoskeleton protein, proteases, angiogenic factors and intracellular signaling molecules take place (Felding-Habermann, 2003). Wnt signaling, too, has recently caught the attention of researchers in the field of human OS, where expression of a few Wnt ligands, receptors and inhibitors have been associated with aggressive phenotype and poor prognosis (Chen et al., 2008; Hoang et al., 2004a; Hoang et al., 2004b) while others noted inactivation of this pathway (Cai et al., 2010). This pathway plays important roles in cell proliferation and differentiation. Investigations to further elucidate the roles of this pathway in canine OS pathogenesis and metastasis are currently underway.

Identification of the central transcription factors present upstream of a signaling pathway may contribute to further understanding of the multistep process of metastasis (Inoue and Shiramizu, 1999; Yang, 2006). MetaCore™ analysis revealed 3 major transcription factors (TP53, SP1 and c-myc) as common denominator among the differentially expressed genes between the two cell lines. The expression of these transcription factors was not different, but they may play an important role in interaction with other proteins or depend on phosphorylation rather than expression per se for their activity. In the case of TP53, the question was raised as to whether the HMPOS line was selected based on an acquired TP53 mutation. However, both HMPOS and the parent line POS appeared to have the same hotspot for mutation at codon 162, which corresponds to codon 175 for the human TP53 gene, the third most common somatic and germ line mutation reported in human cancers (including OS) involving substitution of arginine to histidine (McIntyre et al., 1994; Olivier et al., 2002). Point mutations were frequently observed among tumors from dogs with OS and dogs with mutations of TP53 have significantly shorter survival time than those with wild type TP53 (Kirpensteijn et al., 2008). However, the TP53 mutation alone may not be sufficient for metastatic behavior and TP53 interacting proteins play a role as well. Both TP53 and c-myc

are involved in the regulation of genome stability while Sp1 is involved in the regulation of thousands of genes that regulate cellular processes such as cell growth, differentiation, and apoptosis. The present gene network suggests that TP53, Sp1 and c-myc are master regulators being the 'central hubs' linking the differential expressed genes that may be causally associated with OS metastasis where the observed changes in gene expression can be useful to model those metastatic OS with a TP53 gene mutation.

Genes revealed here are novel and have not been previously investigated in canine OS tumorigenesis. Three of them have been previously described for the pathogenesis of human OS including tissue inhibitor of metalloproteinase 3 (TIMP3) (Hou et al., 2006), Decorin (DCN) (Zafiropoulos et al., 2008) and heat shock protein 60kda (HSP60) (Trieb et al., 2000). TIMP is involved in restraining tumor growth by their protein properties and matrix metalloproteinases (MMPs) inhibition whereby downregulation of this gene may contribute to the enhanced growth and metastatic potentials. HSP60 has inconsistent expression data regarding to prognosis across various cancers including OS, although two studies have suggested that HSP60 can be a marker for therapeutic response and poor prognostic marker in human and canine OS (Selvarajah et al., 2009; Trieb et al., 2000). Nevertheless few of these transcripts have been reported to play important roles in the malignant phenotype of various human cancers. These include the colorectal mutant cancer protein (MCC) which strongly described its role as a tumour suppressor in colorectal carcinogenesis (Fukuyama et al., 2008); Very low-density lipoprotein receptor precursor (VLDLR) where epigenetic or genetic silencing of VLDLR have been reported to increase cell motility (Takada et al., 2006), and may influence colony forming ability; Dipeptidyl peptidase 4 (DPP IV) being overexpressed in thyroid malignant phenotypes (Umeki et al., 1996), Vinculin (VCL) a cytoskeletal protein that forms cell to cell interactions in synergy with other cytoskeletal and integrins to regulate transitions of cell migration (Weller et al., 1990). Although these seem attractive candidates to follow up, we chose decorin and 5 other candidate genes which are notable novel for canine and human OS for subsequent quantitative real-time PCR validation.

Decorin is a small leucine-rich proteoglycan; commonly altered in various human tumor tissues and cell lines (De Luca et al., 1996; Nash et al., 2002; Santra et al., 1995). There has been contrasting data on the roles of decorin in human soft tissue tumors from overexpression being associated with poor prognosis and poor response to chemotherapy (Crnogorac-Jurcevic et al., 2001; Newton et al., 2006) to its ability to induce cytostatic effect *in vitro* and as a putative tumor suppressor (Goldoni et al., 2008; Reed et al., 2005; Santra et al., 1995). Among the candidate genes verified using quantitative real-time PCR; decorin mRNA expression was significantly higher in the high metastatic canine OS cell line which was further selected for protein investigations in an independent set of canine OS paraffin-embedded tissues and human OS tissue microarray. Here, for the first time, we report that a high metastatic clone of a canine OS cell line expressing higher decorin mRNA and dogs with decorin mRNA overexpression in their primary tumors have significantly shorter DFI than those with lower expression. Similarly, decorin protein expression on a panel of human OS

primary tumor predicts poorer outcome, indicating that decorin could be a marker for poor prognosis. Decorin protein expression was observed to be variable in the extracellular matrix surrounding osteoblastic cells in both human and canine primary OS; consistent with a previous report on human OS where decorin expression was found at the vicinity of tumor osteoid (Bosse et al., 1993). In addition to that we also demonstrated decorin protein in lung metastasis of canine OS which intriguingly, stroma and tumor cells towards the periphery of lung metastasis have detectable expression while low to absence in other regions which is also observed similarly for early stage human prostate cancers in comparison to non-malignant hyperplastic tissues (Ricciardelli et al., 1998). Early evidence have shown that human OS cells SaOS-2 do express decorin mRNA (McQuillan et al., 1995) but not the protein (Zafiropoulos et al., 2008) while the MG63 cells constitutively expressed decorin protein and these cells were not sensitive to exogenous decorin induced growth arrest (Zafiropoulos et al., 2008). In fact, knockdown of endogenous decorin in MG63 cells significantly reduced motility. In contrast to these reports from human OS and the present study in dogs, investigations from murine models revealed downregulation of decorin in a highly aggressive K7M2 compared to a less aggressive line K12 (Khanna et al., 2001) while LM8 cells revealed no difference in morphology and growth rates instead motility and invasion of these cells were inhibited by exogenous decorin (Shintani et al., 2008). OS cells usually produce more decorin which is the essential extracellular matrix protein for producing woven bone and is involved in multiple signaling pathways important for tumorigenesis. Although the number of canine OS tumors incorporated in the present study is too small, 2 of the dogs that developed bone metastasis were found to exhibit high decorin expression which suggest possible alternative mechanism for bone-metastasizing OS cells. Although only 20% of human OS tumors exhibited decorin expression, it was observed to be an independent prognosticator which warrants further validation and investigation.

Conclusion

Differences in gene expression profiles between the two canine OS cell lines have provided us with new candidate genes; among them, decorin to be associated with aggressive nature of canine and human OS which negatively correlated with disease-free events in both species. Future functional approach is required to validate these genes and signaling pathways to increase our understanding on the pathogenesis of OS metastasis and discover new therapeutic targets to combat metastasis entities across species.

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Additional file

Additional File 1a: Complete gene list of those genes down-regulated in HMPOS compared to POS generated by SAM. Genes shown here are those of and above 1.5 fold change, within 10% false discovery rate and sorted according to ascending q-values. #NA indicates non-annotated genes for the dog at present.

DOWNREGULATED GENES IN HMPOS COMPARED TO POS				
Accession	**Fold change**	**q-value(%)**	**Symbol**	**Gene name (description)**
CO585908	**3.99**	**0.00**		#N/A
CO685697	**3.75**	**0.00**		Canis Familiaris chromosome 10, clone XX-162K3, complete sequence (1)
CO656046	**2.75**	**0.00**		#N/A
CO585397	**2.67**	**0.00**		#N/A
CO594681	**2.38**	**0.00**		#N/A
CO622467	**2.37**	**0.00**	TIMP3	tissue inhibitor of metalloproteinase 3
CO687142	**2.36**	**0.00**	DPP IV	Dipeptidyl peptidase 4 (Dipeptidyl peptidase IV)
CO592844	**2.25**	**0.00**		glutamyl-prolyl tRNA synthetase
CO700011	**2.14**	**0.00**		#N/A
CO594856	**1.98**	**0.00**	TPM1	Tropomyosin
CO667361	**1.96**	**0.00**		NADH-ubiquinone oxidoreductase 49 kDa subunit, mitochondrial precursor
CO717124	**1.80**	**0.00**	ME1	malic enzyme 1, NADP(+)-dependent, cytosolic
CO667936	**1.75**	**0.00**		#N/A
CO688495	**1.75**	**0.00**		#N/A
CO690557	**1.54**	**0.00**		#N/A
CO617943	**10.94**	**3.24**		#N/A
CO587490	**5.11**	**3.24**		#N/A
CO596896	**2.40**	**3.24**		small nuclear ribonucleoprotein E

Genes downregulated in HMPOS (continued)				
CO685809	**2.25**	**3.24**		hypothetical protein LOC610171
CO590835	**2.15**	**3.24**		#N/A
CO587228	**2.11**	**3.24**		#N/A
CO610848	**2.07**	**3.24**	GLRX2	glutaredoxin 2 isoform 1
CO689570	**1.98**	**3.24**		hypothetical LOC475625
CO586896	**1.95**	**3.24**		#N/A
CO694401	**1.92**	**3.24**		Canis Familiaris chromosome 10, clone XX-162K3, complete sequence (1)
CO694357	**1.83**	**3.24**		succinate dehydrogenase complex, subunit C precursor
CO589374	**1.79**	**3.24**	NDUFS3	NADH dehydrogenase (ubiquinone) Fe-S protein 3, 30kDa (NADH-coenzyme Q reductase)
CO591267	**1.78**	**3.24**		Tropomyosin
CO670745	**1.68**	**3.24**		#N/A
CO677590	**1.63**	**3.24**		Ufm1-conjugating enzyme 1
CO713681	**1.63**	**3.24**		NADH-ubiquinone oxidoreductase 49 kDa subunit, mitochondrial precursor (Complex I-49KD)
CO667740	**1.61**	**3.24**	VCL	Vinculin (Metavinculin)
CO687572	**1.57**	**3.24**	TIMP3	tissue inhibitor of metalloproteinase 3
CO598005	**12.21**	**3.83**		#N/A
CO689850	**2.22**	**3.83**		#N/A
CO591550	**1.65**	**3.83**	UCH37	ubiquitin C-terminal hydrolase UCH37
CO714204	**1.62**	**3.83**		#N/A
CO615002	**7.49**	**4.68**		#N/A
CO595300	**2.16**	**4.68**		hypothetical protein LOC610171
CO679994	**2.15**	**4.68**		#N/A
CO591992	**1.91**	**4.68**		#N/A
CO598522	**1.86**	**4.68**	TSP50	testes-specific protease 50

Genes downregulated in HMPOS (continued)				
CO590437	**1.86**	**4.68**		#N/A
CO679590	**1.51**	**4.68**	LONP1	peroxisomal lon protease
CO612781	**10.71**	**4.81**		#N/A
CO617072	**1.97**	**4.81**	SCPEP1	serine carboxypeptidase 1 precursor protein
CO591635	**1.94**	**4.81**		#N/A
CO590315	**1.92**	**4.81**		#N/A
CO711759	**1.91**	**4.81**		#N/A
CO585821	**1.88**	**4.81**	TOP2B	DNA topoisomerase II, beta isozyme
CO591671	**1.88**	**4.81**		#N/A
CO605755	**1.87**	**4.81**		#N/A
CO586520	**1.72**	**4.81**		#N/A
CO675523	**1.65**	**4.81**	PDE4DIP	phosphodiesterase 4D interacting protein isoform 2
CO584485	**1.63**	**4.81**		#N/A
CO593908	**1.59**	**4.81**	COMMD3	COMM domain containing 3
CO594695	**1.55**	**4.81**	PCMT1	Protein-L-isoaspartate(D-aspartate) O-methyltransferase (Protein-beta-aspartate methyltransferase)
CO590488	**1.54**	**4.81**	BCKDHB	2-oxoisovalerate dehydrogenase beta subunit, (Branched-chain alpha-keto acid dehydrogenase E1 component beta chain)
CO633991	**1.51**	**4.81**		#N/A
CO594825	**1.50**	**4.81**		WD repeat, SAM and U-box domain containing 1
CO717105	**1.50**	**4.81**		Dynein intermediate chain 2, cytosolic (DH IC-2) (Cytoplasmic dynein intermediate chain 2)
CO664309	**3.22**	**5.61**	PRSS23	Serine protease 23 precursor (Putative secreted protein ZSIG13)
CO691446	**2.13**	**5.61**	ANT 3	ADP/ATP translocase 3 (Adenine nucleotide translocator 2)(Solute carrier family 25, member 6)
CO592095	**2.01**	**5.61**		#N/A
CO634450	**1.82**	**5.61**		#N/A

Genes downregulated in HMPOS (continued)				
Accession	**Fold change**	**q-value(%)**	**Symbol**	**Gene name (description)**
CO593252	**1.82**	**5.61**		preimplantation protein 3
CO590926	**1.75**	**5.61**	DDHD1	DDHD domain containing 1
CO715441	**1.74**	**5.61**		#N/A
CO689013	**1.72**	**5.61**	TCF4	Canis lupus familiaris transcription factor 4. Dog mRNA for putative transcription factor recognizing thyroglobulin promoter
CO590268	**1.69**	**5.61**		#N/A
CO586956	**1.67**	**5.61**		#N/A
CO666356	**1.65**	**5.61**	BCKDHB	2-oxoisovalerate dehydrogenase beta subunit, (Branched-chain alpha-keto acid dehydrogenase E1 component beta chain)
CO688698	**1.63**	**5.61**		#N/A
CO655370	**1.59**	**5.61**	TIMP3	tissue inhibitor of metalloproteinase 3
CO588786	**1.59**	**5.61**		#N/A
CO677190	**1.58**	**5.61**	GSTK1	Glutathione S-transferase kappa 1 (GST 13-13) (Glutathione S-transferase subunit 13)
CO584251	**1.53**	**5.61**	GRHPR	Glyoxylate reductase/hydroxypyruvate reductase
CO587566	**1.53**	**5.61**		Canis Familiaris chromosome 26, clone XX-497F13, complete sequence (-1)
CO716523	**1.52**	**5.61**	VDAC3	Voltage-dependent anion-selective channel protein 3
CO589868	**1.73**	**6.11**		#N/A
CO588377	**1.72**	**6.11**		NADH-ubiquinone oxidoreductase 49 kDa subunit, mitochondrial precursor (Complex I-49KD) (CI-49KD)
CO702298	**1.50**	**6.11**	LAMP-2	Lysosome-associated membrane glycoprotein 2 precursor
CO597068	**3.04**	**6.83**		CG6878-PA (LOC477215)
CO600939	**1.88**	**6.83**	CUTA	divalent cation tolerant protein CUTA isoform 1
CO585422	**1.86**	**6.83**		#N/A
CO697113	**1.67**	**6.83**		sorting nexin 1 isoform c
CO676587	**1.60**	**6.83**		Alcohol dehydrogenase class III chi chain (Glutathione-dependent formaldehyde dehydrogenase) (FDH)
CO585920	**1.59**	**6.83**		#N/A
CO599518	**1.58**	**6.83**	PDHB	pyruvate dehydrogenase (lipoamide) beta

Genes downregulated in HMPOS (continued)				
CO591129	**1.56**	**6.83**	HEPCOP	Coatomer alpha subunit (Alpha-coat protein) (Alpha-COP)
CO587925	**1.55**	**6.83**		Canis familiaris mitochondrion, complete genome (-1)
CO630565	**25.50**	**6.84**		#N/A
CO655342	**1.85**	**6.84**		#N/A
CO605977	**1.61**	**6.84**		#N/A
CO584669	**1.50**	**6.84**		#N/A
CO607706	**35.48**	**7.95**	DTD1	Probable D-tyrosyl-tRNA(Tyr) deacylase
CO593167	**2.00**	**7.95**		hypothetical LOC475923
CO593134	**1.84**	**7.95**		#N/A
CO588080	**1.84**	**7.95**		Canis lupus isolate 1 from Spain mitochondrion, complete genome (-1)
CO587858	**1.70**	**7.95**		CG6432-PA (LOC475414)
CO603616	**1.61**	**7.95**	MCC	Colorectal mutant cancer protein (MCC protein)
CO656541	**20.39**	**9.14**		Na/Pi cotransporter 4
CO697932	**12.67**	**9.14**		#N/A
CO632112	**8.11**	**9.14**		#N/A
CO659846	**7.70**	**9.14**	RPS23	ribosomal protein S23
CO586195	**6.66**	**9.14**	VLDLR	Very low-density lipoprotein receptor precursor (VLDL receptor)
CO614419	**5.93**	**9.14**		#N/A
CO589436	**2.30**	**9.14**		STE20/SPS1-related proline-alanine rich protein kinase (Ste-20 related kinase) (DCHT)
CO617136	**2.19**	**9.14**		#NA
CO679564	**2.12**	**9.14**		#N/A
CO634934	**2.10**	**9.14**		#N/A
CO626390	**1.84**	**9.14**	SLC25A6	Canis familiaris similar to ADP/ATP translocase 3, (Solute carrier family 25, member 6)
CO592976	**1.73**	**9.14**	CUTA	divalent cation tolerant protein CUTA isoform 1
CO593044	**1.72**	**9.14**		#N/A

Genes downregulated in HMPOS (continued)				
CO585419	**1.70**	**9.14**	ADAM10	a disintegrin and metalloprotease domain 10
CO694964	**1.65**	**9.14**		#N/A
CO671994	**1.63**	**9.14**		Tetraspanin-3 (Tspan-3) (Transmembrane 4 superfamily member 8) (Tetraspanin TM4-A)
CO586277	**1.60**	**9.14**		#N/A
CO589941	**1.60**	**9.14**	ETFB	electron-transfer-flavoprotein, beta polypeptide isoform 1
CO585516	**1.58**	**9.14**		#N/A
CO671884	**1.57**	**9.14**		Tetraspanin-3 (Tspan-3) (Transmembrane 4 superfamily member 8) (Tetraspanin TM4-A)
CO591812	**1.53**	**9.14**		dehydrogenase/reductase (SDR family) member 6
CO593758	**1.52**	**9.14**	FTO	fatso (LOC478125)
CO681312	**1.52**	**9.14**	RPL4	ribosomal protein L4
CO703127	**1.52**	**9.14**	SCPEP1	serine carboxypeptidase 1 precursor protein (LOC480566)
CO624062	**1.50**	**9.14**		#N/A
CO689455	**4.91**	**9.69**	PIGG	GPI7 protein
CO593735	**2.34**	**9.69**		Canis Familiaris chromosome 31, clone XX-265A5, complete sequence (-1)
CO586324	**1.87**	**9.69**		Canis familiaris isolate 1 breed Miniature Schnauzer mitochondrion, complete genome (-1)
CO690607	**1.59**	**9.69**	PRDX1	peroxiredoxin 1
CO660179	**1.57**	**9.69**	RPL4	ribosomal protein L4
CO588122	**1.51**	**9.69**	OSCP	ATP synthase O subunit, mitochondrial precursor (Oligomycin sensitivity conferral protein)

Additional File 1b: Gene upregulated in HMPOS compared to POS. Genes sorted according to ascending q-values and filtered to show only those genes with 1.5 fold and above. #NA: non-annotated genes for the *Canis familiaris*

Accession	Fold change	q-value(%)	Symbol	Gene name (description)
CO591194	**2.78**	**0.00**		#N/A
CO588610	**1.73**	**0.00**		#N/A
CO655222	**5.24**	**3.83**		#N/A
CO585619	**2.38**	**3.83**	E-FABP	Fatty acid-binding protein, epidermal (Psoriasis-associated fatty acid-binding protein homolog)
CO585777	**2.14**	**3.83**		#N/A
CO591632	**1.95**	**3.83**		solute carrier family 38, member 2
CO665984	**1.84**	**3.83**		adaptor-related protein complex 2, mu 1 subunit isoform b
CO592784	**1.69**	**3.83**		#N/A
CO584403	**1.59**	**3.83**		transcription elongation factor B (SIII), polypeptide 1
CO717608	**4.16**	**4.21**	DCN	Canis lupus familiaris decorin
CO698950	**1.87**	**4.21**		Canis familiaris hypothetical protein LOC610900
CO593128	**1.63**	**4.21**		reticulon 3 isoform b
CO712701	**1.56**	**4.21**		#N/A
CO589664	**1.50**	**4.21**		eukaryotic translation initiation factor 4A2
CO713802	**15.38**	**4.63**	GCAP14	granule cell antiserum positive 14
CO594065	**2.06**	**4.63**	RPL22	ribosomal protein L22 like 1
CO656405	**1.83**	**4.63**	ADRP / ADFP	Adipophilin (Adipose differentiation-related protein)
CO598788	**1.68**	**4.63**		PDZ and LIM domain 1 (elfin)
CO660196	**1.64**	**4.63**	PRPL7	60S ribosomal protein L7, transcript variant 1
CO707019	**1.55**	**4.63**		eukaryotic translation initiation factor 4A2
CO664643	**5.63**	**5.61**	S100P	S-100P protein
CO589629	**1.74**	**5.61**		Programmed cell death protein 6
CO588371	**1.89**	**6.11**		mitochondrial ribosomal protein L15
CO606032	**12.22**	**7.95**		solute carrier family 39 (zinc transporter), member 13
CO620702	**1.68**	**7.95**	NFIL3	nuclear factor, interleukin 3 regulated
CO585015	**1.51**	**7.95**		Canis Familiaris chromosome 12
CO588648	**4.09**	**8.76**	RBM17	RNA binding motif protein 17
CO689888	**2.52**	**8.76**		#N/A
CO588775	**1.91**	**8.76**		#N/A
CO617136	**1.77**	**8.76**		#N/A
CO684257	**1.74**	**8.76**		adaptor-related protein complex 2, mu 1 subunit isoform b
CO689881	**1.58**	**8.76**	STX5	Syntaxin-5
CO589587	**1.53**	**8.76**	IER2	immediate early response 2
CO701623	**1.52**	**8.76**	BI-1	Bax inhibitor-1 (BI-1) (Testis enhanced gene transcript)
CO588865	**1.98**	**9.14**	HSP60	60 kDa heat shock protein, mitochondrial precursor (Hsp60) (60 kDa chaperonin) (
CO608762	**4.98**	**9.69**	GSTM5	glutathione-S-transferase, mu 5
CO692221	**2.67**	**9.69**	ACAA1	3-ketoacyl-CoA thiolase, peroxisomal precursor (Acetyl-CoA acyltransferase)
CO589331	**1.74**	**9.69**	MRPS28	Mitochondrial 28S ribosomal protein S28 (S28mt) (MRP-S28) (MRP-S35)

Chapter 6

Reference gene validation for gene expression normalization in canine osteosarcoma: a geNorm algorithm approach

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Abstract

Quantitative real-time polymerase chain reaction (qPCR) is used to quantify mRNA and gene expression. Given the heterogeneity of gene expression present in tumour tissues, target gene mRNA data need to be normalized using appropriate reference genes. We used the geNorm algorithm to validate reference genes commonly used for normalizing canine gene expression data. Quantitative real-time PCR analysis of nine genes was performed on 40 snap-frozen primary OS tumours with varying clinical and pathological characteristics and seven cell lines. Gene expression stability and the optimal number of reference genes were calculated. RPS5 and HNRNPH were highly stable among OS tumours, while RPS5 and RPS19 were the best combinations for the cell lines. Pair-wise variation analysis identified four reference genes for normalizing the expression data for canine OS tumours and two for the cell lines. This information may facilitate target gene expression quantification essential for investigating genes involved in OS metastasis and comparative biomarker discovery.

Introduction

Osteosarcoma (OS) is the most common malignant bone tumour in dogs. Apart from having complex metastatic characteristics, it also has a complex histology, which is predominantly osteoblastic with a mixture of fibroblastic and chondroblastic cells with varying degrees of necrosis and tumour matrix present (Kirpensteijn et al., 2002; Loukopoulos and Robinson, 2007). Gene expression studies in canine OS are valuable because dogs develop OS spontaneously and have many common clinical and molecular characteristics which are an invaluable resource for biomarker discovery and translational opportunities (Paoloni et al., 2009; Selvarajah et al., 2009). Furthermore, the publication of the human and canine genomes along with the advent of quantitative real-time polymerase chain reaction (qPCR) and other high-throughput technologies have facilitated studies addressed at investigating key genes involved in OS metastasis and disease progression.

Quantitative real-time PCR is a sensitive method for quantifying mRNA gene transcripts in which the two most commonly used real-time assays includes the SYBR green fluorescent dye and Taqman assay. Many studies have demonstrated the importance of studying gene expression at mRNA transcription levels, using snap-frozen tissues, micro-dissected tumours from paraffin-embedded blocks (Drury et al., 2009), cellular content from fine-needle aspirates of primary tumours and various cell culture models (Jacobs et al., 2007; Kang et al., 2007; Park et al., 2006; Park et al., 2008). The quantification of gene expression using qPCR requires appropriate standardization from initial tissue sampling, RNA extraction protocols, cDNA synthesis, assay characteristics and reference gene validation. It is important to incorporate internal standards such as reference genes to normalize mRNA levels between different samples for an exact comparison of mRNA transcription levels. Ideally a reference gene should be stably expressed in tissues or cells regardless of histology, pathological condition, or cellular physiological or metabolic state.

Reference gene expression validation studies have been done with normal, diseased (Wood et al., 2008) and tumour canine tissues (Etschmann et al., 2006). Although there are discrepancies in the selection of type and the optimal number of reference genes suggested by these studies, most researchers agree that no single reference gene is ideal for the normalization of a given gene's expression data. These studies have also suggested that genes that have stable expression can differ by tissue origin and disease condition, especially with cancer.

The majority of gene expression studies performed on canine OS from 2005 to 2009 used one reference gene as an internal control for data normalization. Given the biological and pathological diversity seen among OS tumours, it is crucial to determine the stability of reference genes and their suitability for normalization for accurate quantification of gene expression data. To date, no study has validated various reference genes or determined the appropriate number of references to be included within an experimental setup for gene expression investigations of either canine or human OS. Therefore, in the present study,

mRNA expression of nine commonly used canine reference genes was quantified using the SYBR green fluorescent dye qPCR assay on canine OS snap-frozen tissues and cell lines. The geNorm algorithm approach was used to determine the reference genes with stable expression for normalization of canine OS mRNA gene expression data.

Materials and methods

Tissue specimens and clinicopathological data

From 40 medium- to large-breed dogs with OS presented to the University Clinic for Companion Animals in Utrecht, The Netherlands, 40 histologically confirmed primary tumours were selected for this study. Tissue samples were harvested under sterile conditions during surgery (amputation, marginal resection or total resection), snap-frozen in liquid nitrogen and stored in sterile tubes at −70°C. A sample of each tumour was fixed in 4% formalin, decalcified and embedded in paraffin for histological diagnosis. The histological diagnosis and grading (Kirpensteijn et al., 2002) were done by a certified veterinary pathologist. The medical records were reviewed retrospectively.

Cell lines and culture conditions

Seven well-characterized canine OS cell lines were used for this study. Cell lines COS31(Shoieb et al., 1998), HMPOS (Barroga et al., 1999) and POS (Kadosawa et al., 1994) were provided through a collaboration with University of Florida, USA; MCKOS, CSKOS, BWKOS and SKKOS were kind gifts from the National Cancer Institute, National Institutes of Health, Bethesda, USA. All cell lines were tested and found negative for mycoplasma by using a mycosensor qPCR assay kit according to the manufacturer's protocol (Agilent Technologies, USA). Cells were maintaines in sub-confluent monolayer in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (PAA Laboratories GmbH) at 37°C in a humidified atmosphere with 5% CO_2.

RNA isolation and cDNA synthesis

RNA isolation from snap-frozen OS tumour materials were carried out according to the method described previously (Fieten et al., 2009)(Selvarajah et al., 2009). In brief, frozen bone tumour materials were pulverized to form bone powder from which RNA was isolated. For cells grown in culture, 1 mL of RLT lysis buffer (Qiagen) was used to lyse 75% to 90% confluent cells grown in 75-mL flasks, after first washing them once with Hank's balanced salt solution (PAA Laboratories GmbH). Cells grown in three different passages were selected to represent biological replicates for the analysis. RNA isolation and purification were carried out using the RNeasy mini kit according to the manufacturer's protocol (Qiagen). The RNA samples were treated with Qiagen RNase-free DNase kit (DNase-I) and subsequently eluted in purified water. The total RNA was quantified by using the Nanodrop ND-1000 spectrophotometer

(Isogen Life Sciences). RNA quality was evaluated using the Agilent 2100 bioanalyzer (Agilent Technologies). The synthesis of cDNA was performed using 0.5 µg total RNA into a total reaction volume of 20 µL from each sample using the iScript kit cDNA Synthesis Kit according to the manufacturer's protocol (I-Script cDNA Synthesis Kit, Bio-Rad).

Quantitative real-time PCR

Primers were designed and qPCR products were sequenced for specificity as previously described (Brinkhof et al., 2006; Schlotter et al., 2009). Pooled cDNA was serially diluted with purified water to generate the qPCR standard line. Independent assays were carried out for the OS cell lines and tumours. Each individual sample cDNA was diluted twofold prior to qPCR using the SYBR green fluorescent dye method. Initial screening for genomic DNA contamination was performed on all samples using a non–reversed-transcribed RNA template. Quantitative real-time PCR runs were carried out using the MyIQ software (Bio-Rad). Reactions were performed in duplicate, involving two-step reaction protocols, except for hypoxanthine phosphoribosyltransferase (HPRT) which involved a three-step reaction protocol up to 40 qPCR cycles (Brinkhof et al., 2006; Schlotter et al., 2009).

Data analysis

Individual reaction data were corrected for qPCR efficiencies and analyzed using the IQ5 software (Bio-Rad). A box-plot was generated from the absolute Cq values (Bustin et al., 2009) referring to the RNA transcription of the tested reference genes in OS tissues and cell lines using the statistical software SPSS version 16.0. The cases with values between 1.5 and 3.0 box length, from the upper or lower edges of the box, are presented as outliers and indicated with a dark dot. The expression stability of each reference gene in tumours and cell lines was calculated independently, along with their average values being recalculated using the step-wise exclusion and pair-wise variation analyses, all of which were analysed using the geNorm (version 3.5) software (http://medgen.ugent.be/~jvdesomp/genorm/) (Vandesompele et al., 2002).

Results

Canine OS samples and reference gene selection

Clinical and pathological data associated with the primary canine OS tissues used for this study are summarized in Table 1. The tissues were obtained upon amputation or tumour resection prior to chemotherapy, and tumours showed mixed histological characteristics (percentage of necrosis, histology subtype, and grade) with variable clinical properties (primary site, dog breed, neuter status and sex). Seven canine OS cell lines with varying characteristics, based on morphology, cell proliferation, colony-forming abilities, migration and apoptotic rates, were selected. Sub-confluent cells from three independent passages

were lysed for RNA isolation as representatives for biological replicates for each cell line. The reference genes selected for this study had previously been used in studies of canine OS (e.g. RPS19, HPRT, GAPDH) (Fieten et al., 2009; Selvarajah et al., 2009) or investigations of other canine tissues (e.g. SRPR, HNRNPH, GUSB, RPL8, RPS5)(Brinkhof et al., 2006; Schlotter et al., 2009). These genes represent different functional groups, thus avoiding having a cluster of genes which may be co-regulated with regards to a particular cellular mechanism (Table 2).

Table 1. Characteristics of canine OS tissues (*n* = 40) used for this study.

Parameter	*n*	%
Histological subtype[a]		
OB + FB	12	30
OB + TL	5	12.5
OB + CB + FB	7	17.5
OB + FB + TL	2	5
OB	14	35
Histological grade		
High	28	70
Medium-low	12	30
Necrosis		
<50% (low)	12	30
>50% (high)	28	70
Sex		
Female	14	35
Male	26	65
Neuter status		
Intact	22	55
Neutered	18	45
Location of primary tumor		
Extraskeletal	1	2.5
Femur	1	2.5
Humerus	8	20
Mandible/maxilla	3	7.5
Radius/ulna	14	35
Rib	2	5
Scapula	3	7.5
Tibia/fibula/metatarsus	8	20

[a] CB, chondroblastic; FB, fibroblastic; OB, osteoblastic; TL, telangiectic.

Table 2. Reference genes investigated for canine OS and their cellular function(s).

Gene symbol	Name	Function
RPS5	Ribosomal protein S5	Ribosomal protein that is a component of the 40S subunit, belongs to the S7P family of ribosomal proteins
RPS19	Ribosomal protein S19	Ribosomal protein that is a component of the 40S subunit, belongs to the S19E family of ribosomal proteins
HPRT	Hypoxanthine guanine phosphoribosyl transferase	Purine metabolism, salvage of purines from degraded RNA
HNRNPH	Heterogeneous nuclear ribonucleoprotein H	RNA-binding protein that forms a complex with heterogeneous nuclear RNA (hnRNA). These proteins are associated with pre-mRNAs in the nucleus and appear to influence pre-mRNA processing and other aspects of mRNA metabolism and transport
RPL8	Ribosomal protein L8	Ribosomal protein that is a component of the 60S subunit which catalyzes protein synthesis
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Enzyme in glycolysis and gluconeogenesis pathway
B2M	β-2-Microglobulin	Beta chain of MHC class I molecules
SRPR	Signal recognition particle receptor	Ensures, in conjunction with the signal recognition particle, the correct targeting of the nascent secretory proteins to the endoplasmic reticulum membrane system
GUSB / BGLR	β-glucuronidase	Role in degradation of dermatan and keratin sulphates

Pre-qPCR quality control measures and qPCR efficiencies

RNA quantity in tumours ranged from 173.0 ng/µL to 2399.3 ng/µL while the RNA quality of all samples was acceptable with a 260/280 ratio of 1.97 to 2.11. The RNA integrity number (RIN) values ranged from 9.5 to 10.0 for all cell lines and above 6.5 for the snap-frozen tumours. Primer sequences, product size and optimal annealing temperature for each reference gene were previously verified (Brinkhof et al., 2006; Schlotter et al., 2009) and are summarized in Table 3. Quantitative real-time PCR was performed in duplicate for each sample in which separate assays for cell lines and tumours were carried out. Both the non–reversed-transcribed template and template-absent (purified water) control samples were below detection limits (up to 45 cycles) in each qPCR reaction. Quantitative real-time PCR efficiencies were between 91.1% and 103.1% for the cell lines and between 94.9% and 104.1% for the tumours. All qPCR amplifications exhibited a single melting curve representing a specific product.

Table 3. Details of primers and qPCR conditions for the putative reference genes used in this study

Reference gene	Accession number	Forward primer 5' to 3'	Reverse primer 5' to 3'	Product length (bp)	T_a (°C)
RPS5	XM_533568	TCACTGGTGAG/AACCCCCT	CCTGATTCACACGGCGTAG	141	62.5
RPS19	XM_533657	CCTTCCTCAAAAA/GTCTGGG	GTTCTCATCGTAGGGAGCAAG	95	61
HPRT	AY_283372	AG/CTTGCTGGTGAAAAGGAC	TTATAGTCAAGGGCATATCC	114	56
HNRNPH	XM_53857	CTCACTATGATCCACCACG	TAGCCTCCATAAC/CTCCAC	151	61.2
RPL8	XM_532360	CCATGAAT/CCTGTGGAGC	GTAGAGGGTTTGCCGATG	64	55
GAPDH	NM_001003142	TGTCCCCACCCCCAATGTATC	CTCCGATGCCTGCTTCACTACCTT	100	58
B2M	XM_535458	TCCTCATCCTCCTCGCT	TTCTCTGCTGGGTGTCG	85	61.2
SRPR	XM_03184	GCTTCAGGATCTGGACTGC	GTTCCCTTGGTAGCACTGG	81	61.2
GUSB	NM_001003191	AGACGTTCCAA/GTACCCC	AGGTGTGGTGTAGAGGAGCAC	103	62

[a] T_a: annealing temperature; bp: base pair

Reference gene expression variation in OS tumours and cell lines

Reference genes that were highly expressed in both OS tumours and cell lines based on average quantitative real-time PCR cycle threshold (Cq) values were GAPDH followed by ribosomal RNA genes RPS19, RPS5 and RPL8. SRPR was observed to have the lowest expression. Although the absolute Cq range differed slightly between the tumour and cell line assays, a common expression pattern was observed. The expression range and average Cq values for each reference gene in OS tumours and cell lines are shown in Figure 1.

Expression stability of reference genes in canine OS tumours and cell lines

The average reference gene expression stability (M value) upon step-wise exclusion method and the pair-wise variation (V value) were calculated using the geNorm algorithm approach for the tumours and cell lines separately. A higher absolute M value indicates lower expression stability (Table 4). Among the reference genes tested in the canine OS cell lines, HNRNPH was the most stable gene with an M value of 0.420, while SRPR appeared to be the least stably expressed gene with an M value of 0.588; however, all reference genes had acceptable M values. For the OS tumours, the absolute M values ranged from 0.790 for RPS19 (most stable) to 1.210 for B2M (least stable) compared to the other reference genes. The average expression stability for the nine reference genes tested among cell lines and tumours

based upon the step-wise exclusion algorithm is depicted in Figure 2. The HNRNPH and RPS5 expression, in combination, produced the lowest variability for the tumours while RPS19 and RPS5 were the best combination for the cell lines.

The V value, which reflects the optimal number of reference genes for normalization in tumours and cell lines, was also calculated. Optimally, normalization of gene expression data among 40 OS tumours required a combination of four genes, while a combination of two genes was sufficient for all cell lines (Figure 3). These values were determined according to a cut-off V value of 0.15 per published recommendations (Vandesompele et al., 2002).

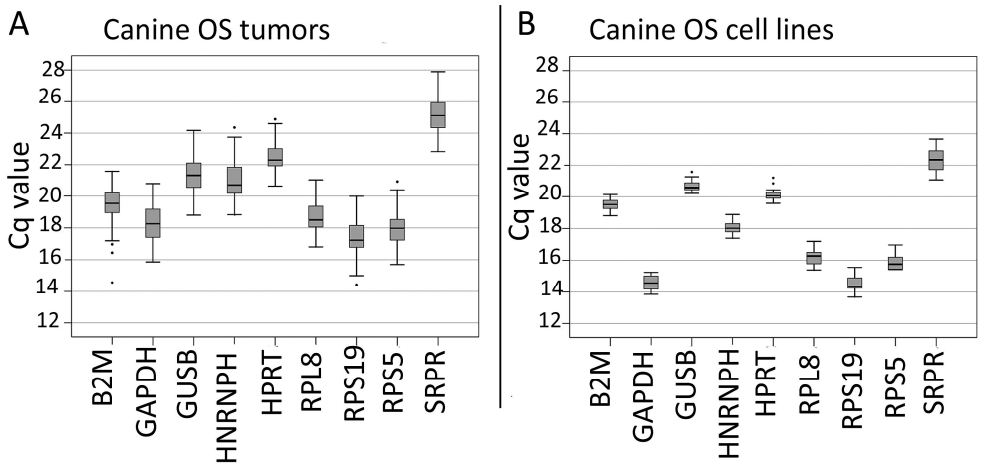

Figure 1. Box-plots demonstrating the absolute Cq values, the 25th and 75th percentiles and outliers (indicated by dark dots) for mRNA transcription quantified for the putative reference genes in (A) canine OS snap-frozen primary tumours and (B) canine OS cell lines.

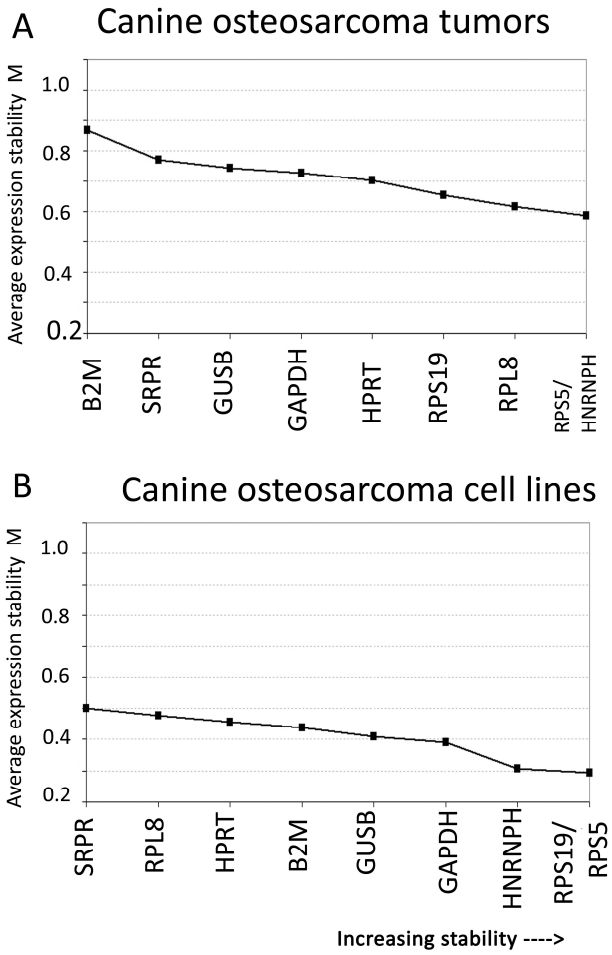

Figure 2. Expression plots generated by geNorm for (A) canine OS primary tumours and (B) canine OS cell lines for the average expression stability (M values) for the nine tested genes with the step-wise exclusion method. Less stable genes are eliminated by the step-wise exclusion method in which the average M value is re-calculated among the remaining candidate genes. The two most stable genes for OS primary tumours include RPS5 and HNRNPH, while RPS5 with RPS19 were the most stable combination among cell lines.

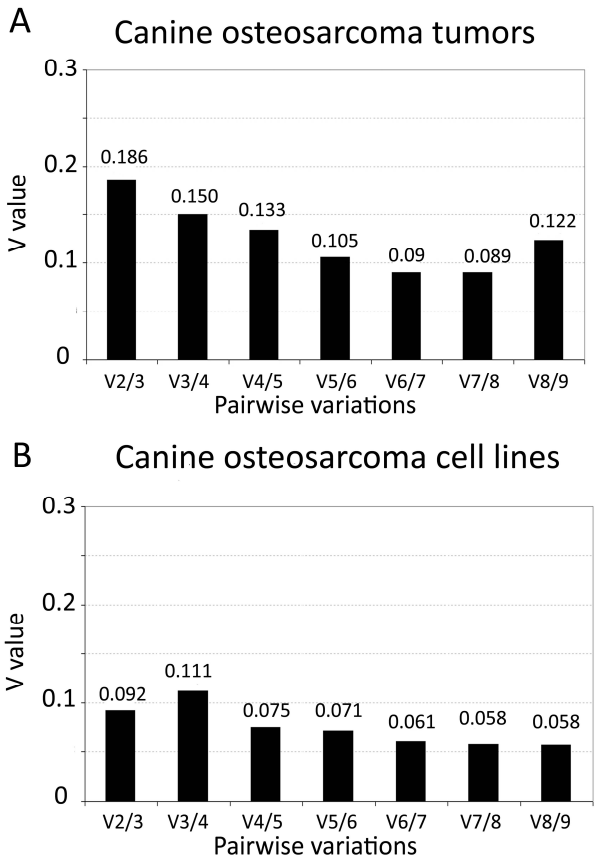

Figure 3. Pair-wise variation plots for the nine reference genes provide the minimum to the optimal number of reference genes required for normalization in (A) canine OS primary tumours and (B) canine OS cell lines. The geNorm software calculates the pair-wise variation V value which illustrates the variation generated by incorporating various number of reference genes for normalization based on the individual absolute M values. The lower the V value, the lower the variation between the selected combinations of reference genes.

Discussion

Importance of using suitable reference genes for qPCR data normalization

For dogs, OS is one of the most common spontaneous tumours and thus offers an important and unique translational advantage in studying disease pathogenesis and discovering new molecular mechanisms which can benefit both humans and dogs (Khanna et al., 2006; Paoloni and Khanna, 2008; Paoloni et al., 2009; Selvarajah et al., 2009). An increasing number of

studies, using various high-throughput approaches and biomolecular techniques, are aimed at exploring the canine genome in order to unravel genes associated with metastasis and disease progression for dogs with OS. Quantitative real-time PCR is one of the methods used for rapid and reliable quantification of mRNA gene transcription and selection of suitable reference genes is crucial for the interpretation of the data (Rubie et al., 2005; Vandesompele et al., 2002). Many quality control measures, from initial sample collection to data analysis, should be evaluated critically prior to gene expression data analysis (Becker et al., ; Botling et al., 2009).Reference genes, previously known as 'housekeeping genes', are essential not only for normalizing mRNA expression of target genes, but also for correcting variations in the samples' initial RNA input, extraction methods and reaction efficiencies (Peters et al., 2007). Failure to normalize gene expression data will most likely yield less accurate interpretations and promote a false perception of target gene expression.

Numerous studies have already been carried out to validate panels of reference genes in different tissues from a range of species (Figueiredo et al., 2009; Nygard et al., 2007; Olsvik et al., 2008; Penning et al., 2007) including dogs (Ayers et al., 2007; Brinkhof et al., 2006; Etschmann et al., 2006; Schlotter et al., 2009; Wood et al., 2008) and humans (Drury et al., 2009; Gao et al., 2008; Gur-Dedeoglu et al., 2009; Jung et al., 2007; Kheirelseid et al., ; Lallemant et al., 2009; Li et al., 2009; Neuvians et al., 2005; Ohl et al., 2005; Ohl et al., 2006; Rubie et al., 2005). Studies on canine and human OS tissues and cell lines commonly utilize one (minimum) or two (maximum) references for normalization of qPCR expression data. The present study validates nine reference genes commonly used in dog tissues for qPCR investigations. Although this was not the first study to demonstrate the need for reference gene validation in tumour tissues from dogs and humans, this was the first study to be carried out on OS tissues. Additionally, it incorporated the largest number of snap-frozen canine tumour tissues and cell lines in a given canine reference gene validation study thus far. The widely used and established statistical tool geNorm (version 3.5) was used to calculate the reference gene expression stability. With regards to technical considerations, most of the 'essential' criteria outlined in the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) standards were employed in the current investigation in canine OS tissues(Bustin et al., 2009).

Reference gene expression stability in canine OS

All nine reference genes tested on canine OS snap-frozen tumours and cell lines had acceptable expression stability, with M values below 1.5. Overall, reference genes were much more stably expressed in cell lines (M values from 0.420 to 0.588) compared with tumour tissues (M values from 0.790 to 1.210), which clearly reflects the greater homogeneity among cell populations in cultured systems.

Ribosomal protein genes (both subunit 40S and 60S components) are highly expressed across various tissues and have been shown as the preferred references for normalization in various models (Brinkhof et al., 2006; Penning et al., 2007; Schlotter et al., 2009; Wood et al.,

2008) including the present study in canine OS. Although there are slight differences in the ranking of genes (according to absolute M values) between those tested for the cell lines and the tumours, RPS5 was observed to be the most stable gene in both model systems. RPS5 in combination with RPS19 (for cell lines) or HNRNPH (for tumours) had the highest expression stability compared to other genes such as B2M and GAPDH, which have been the most commonly used reference genes for many human and canine OS studies (Fieten et al., 2009; Flint et al., 2004; Miyajima et al., 2006). GAPDH expression did not appear to differ remarkably between the OS samples, but its expression stability was much lower compared with the other reference genes investigated in the present study, which is in accordance with several other reports (Lallemant et al., 2009; Nguewa et al., 2008). GAPDH is an enzyme involved in several metabolic pathways essential for cell growth and proliferation and its expression has been shown to differ based on tissue type and environmental conditions (Greer et al., 2010; Rubie et al., 2005). In an investigation on canine articular connective tissue, GAPDH and B2M were found to be highly stable (Ayers et al., 2007) while, for canine mammary tumours, GAPDH was less stable (Etschmann et al., 2006). Furthermore, a recent study indicated that GAPDH protein expression in cultured cells may change depending upon cell density (Greer et al., 2010) and it was also found to be differentially expressed between tumours of epithelial origin compared with normal counterparts (Rubie et al., 2005). Among canine OS tumours, B2M had the lowest expression stability in comparison with the other eight candidate genes investigated in this study. It is not recommended, therefore, to rely on B2M or GAPDH as a sole reference gene to normalize expression data.

Upon pair-wise analysis of a combination of genes which can be used for normalization, four reference genes for canine OS tumours and two for the cell lines were essential based on a recommended cut-off point of 0.15. Because more genes were incorporated for normalization, the V value decreased to an optimal seven reference genes, which would ideally be considered when normalizing, given expression data across canine OS tumours. Whenever sample availability and RNA yield are limited, especially from OS tumour materials, four reference genes would be appropriate for normalization. OS is most often presented with a complex heterogeneous phenotype, which is why the present study recommends including multiple reference genes for the normalization of mRNA gene expression data.

Study limitations and future recommendations

First, the current study use OS tumours that were chemo-naïve and thus we cannot exclude therapeutic modalities employed in clinical and experimental settings possibly inducing changes in reference gene stability in tumours. If gene expression quantification comparing the effects of a given therapy is required, the screening of a panel of reference genes may be essential prior to data normalization. Second, based on an assumption that the RNA isolated from a particular tissue section will represent the overall pooled expression of the tumour, RNA transcription for canine OS tumour tissues was quantified from a single tissue section from an individual OS tumour. Several other studies have recommended incorporating

different parts of the same tumour in order to include separate biological replicates for a more accurate quantification of gene expression. Although this would be ideal, it is frequently unfeasible due to limited tissue availability. Future investigations are necessary to test other potential or novel reference genes discovered by global gene expression profiling methods and subsequently validated with other statistical algorithms. Since canine spontaneous OS is a clinically and biologically relevant model for human OS, we propose incorporating multiple reference genes for normalization of gene expression data for both species in order to facilitate accurate and reliable gene expression quantification. Ongoing experimental and target gene investigations at our laboratory and others are expected to generate more informative prospects for inter-assay and inter-laboratory variability which can be used to re-evaluate the use and suitability of these reference genes in canine OS.

Conclusions

This study was carried out in agreement with the consensus opinion that no single reference gene could ideally normalize given expression data. Hence, gene transcription studies for canine OS have to be carefully normalized by using appropriate reference genes. A combination of reference genes is recommended to normalize OS tumour and cell line gene expression data, with preference for RPS5 as a highly stable reference gene in OS. Accurate normalization and quantification of various target genes for canine OS metastasis and disease progression is necessary before such data can be interpreted and used for comparative biomarker discoveries and translational studies.

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Chapter 7

Characterization of the Wnt canonical signaling in canine osteosarcoma: comparative prognostic evaluation of ß-catenin and Axin2 expression in man and dogs

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Abstract

Introduction: Osteosarcoma (OS) is the primary bone tumor found in dogs and humans. It is heterogeneous in nature, and its pathogenesis is not completely understood. Wnt/β-catenin signaling has important roles in skeletal development, differentiation and various oncogenic processes. The objectives of this study are to characterize Wnt/β-catenin signaling in canine OS and to determine the prognostic value of β-catenin or axin2 expression in canine and human OS.

Method: The transcriptional activation of Wnt/β-catenin signaling in 8 canine OS cell lines was determined using a TCF-responsive *luciferase* reporter assay. Wnt/β-catenin pathway activity was stimulated using lithium chloride (LiCl) or CHIR99021, two inhibitors of GSK3β. Beta catenin expression was quantified by western blots, and its localization was observed using confocal fluorescence microscopy. The gene expression of several target genes was also assessed using quantitative real-time PCR. In parallel to pathway stimulation, dominant-negative TCF4 (dn-TCF4) luciferase reporter constructs were transfected in canine and human OS cell lines to assess the effects of the downregulation of this pathway on TCF transcription, target gene expression and cell proliferation. Canine and human OS tissue microarrays were assessed by immunohistochemistry for nuclear β-catenin or axin2 expression alone, or in combination, to determine their value in predicting prognosis.

Results: Active Wnt/β-catenin signaling was observed when the pathway was stimulated in the different cell lines. Cytoplasmic β-catenin was observed in all cell lines, whereas nuclear localization was only detected in two cell lines with autocrine activity. Upon stimulation, β-catenin translocation to the nucleus in 7/8 cell lines was observed. Axin2 mRNA expression did not correlate with basal canonical Wnt activation in canine OS cell lines; however, upon pathway stimulation in all cell lines, there was a consistent upregulation of axin2 mRNA. Transfection with dn-TCF4 significantly decreased pathway activity, but only four cell lines had a significant reduction in axin2 expression, and five had a significant decrease in cell proliferation. Aberrant expression for both β-catenin and axin2 was observed in tissue microarrays. Beta catenin nuclear localization or axin2 expression alone was not prognostic in either species, although the co-expression of these proteins did have a tendency for poorer prognosis in human OS ($p=0.055$, Log score=3.685).

Conclusion: Canine OS cell lines have low basal levels of Wnt signaling, and axin2 and nuclear β-catenin are reliable markers of Wnt signaling activation. There was variability in cell proliferation when the pathway was either stimulated or inhibited in human and canine cells, suggesting that targeting canonical Wnt signaling may only be useful for a subset of OS. The aberrant expression of nuclear β-catenin and axin2 was not prognostic in either human or canine OS. The findings from this study suggest that spontaneous canine OS with regards to axin2 and ß-catenin expression is a relevant model for human OS where Wnt/β-catenin signaling activation and regulation are similar.

Introduction

Osteosarcoma (OS) is an osteoid producing, highly malignant primary bone tumor that is mesenchymal in origin. Canine OS is a good comparative model for human OS. This is largely due to the fact that this tumor occurs spontaneously in a diverse outbred dog population with many similar to human OS including tumor histology, patient prognosis and response to conventional therapies. Disease progression is similar between these two species, and metastasis is the major cause of death aside from the high recurrence rate of partially resected tumors (Morello et al., 2010; Mueller et al., 2007b). Although the prognosis has remarkably improved over the past decade, there have been many new insights into the various molecular alterations involved in the pathobiology of this disease in both humans and dogs. Several signaling pathways are involved in the development and progression of OS, including Wnt signaling (Cleton-Jansen et al., 2009; Kansara and Thomas, 2007; O'Donoghue et al., 2010; Selvarajah et al., 2009).

Wnt signaling comprises a large family of secreted, lipid-modified glycoproteins that have many important functions during embryonic development, such as the regulation of cell fate determination, cell proliferation, differentiation, invasion and cell polarity (Galli et al., 2010; Gordon and Nusse, 2006; Kim et al., 2007; Reya and Clevers, 2005; Schinner et al., 2009; Thomas, 2010; Williams and Insogna, 2009). The canonical Wnt/β-catenin pathway is activated when Wnt ligands binds to both a seven-pass membrane receptor from the Frizzled family and to a low-density lipoprotein receptor-related protein 5 or 6. These interactions result in the disruption of a complex that normally functions to facilitate β-catenin phosphorylation, targeting it for ubiquitination and rapid proteasomal degradation. Consequently, β-catenin accumulates in the cytoplasm and is ultimately localized to the nucleus, where it interacts with members of the T cell factor (TCF) family of DNA-binding proteins as well as other transcriptional co-factors. This interaction leads to specific target gene expression (Holsken et al., 2009; Jho et al., 2002; Leung et al., 2002; Pradeep et al., 2004; Rohrs et al., 2009). The dysregulation of Wnt signaling has been observed in several diseases, including cancer. Beta-catenin (Armadillo) generally activates TCF-regulated genes, including c-myc, cyclinD1 (CCND1), survivin, MMP-7 and axin2 (Conductin/Axis inhibitor protein 2). The activation of canonical Wnt signaling is common in many types of cancer, with a particularly high prevalence in colorectal carcinoma (Fukuyama et al., 2008; Kaur et al., 2010; van de Wetering et al., 2002). The loss of function mutations in tumor suppressors, such as the Adenomatous Polyposis Coli protein (APC), or activating mutations in effectors, such as β-catenin, can constitutively activate this pathway (Korinek et al., 1997). Given the diverse roles of Wnt signaling in oncogenesis and the pathobiology of metastasis, as well as osteoblast differentiation and bone physiology (Monaghan et al., 2001), this pathway may have an important role in OS, a highly malignant bone tumor found in humans and dogs (a relevant, spontaneous-large animal model for this disease).

Several studies have demonstrated that β-catenin is expressed in human and murine OS models, primary tumors and metastasis, however, there has only been a single report addressing Wnt/β-catenin-induced transcriptional activation in OS using reporter assays (Cai et al., 2010; Dieudonne et al., 2010; Guimaraes et al., 2010; Haydon et al., 2002; Iwaya et al., 2003; Iwaya et al., 2003; Leow et al., 2009). Gene expression profiling using cDNA microarray techniques has shown that there is enrichment of genes for Wnt signaling in profiles associated with prognosis and metastasis of canine OS, however, β-catenin was not identified as one of the key markers in these profiles (Selvarajah et al., 2009)(unpublished data, GT, Selvarajah). Recently, independent studies examining β-catenin expression by immunohistochemistry failed to demonstrate prognostic value in human and canine OS (Cai et al., 2010; Guimaraes et al., 2010; Stein et al., 2011). There have been discrepancies in tumor classifications based on β-catenin expression and its cellular localization for the prognostic evaluation of these tumors. The target gene axin2 expression was observed to correlate with Wnt signaling activation in several human OS cell lines (Cai et al., 2010), but the prognostic value of this protein in OS has not been investigated. Although there are several other Wnt-related proteins that have promising prognostic values for human OS (Chen et al., 2008; Hoang et al., 2004), the prognostic value of these proteins have not been determined for canine OS.

Before investigating the role and prognostic value of Wnt/β-catenin signaling activation in canine OS, it is essential to select reliable activation markers. Therefore, the aim of our research is to characterize canonical Wnt signaling in canine OS. We mean to accomplish this by investigating the reliability of nuclear β-catenin expression as a 'hallmark' of pathway activation in canine OS and by subsequently evaluating the prognostic value of this marker in primary tumors. In this study, 8 canine OS cell lines were evaluated for autocrine and stimulated activation levels of canonical Wnt signaling using luciferase reporter assays in addition to β-catenin expression and localization. Furthermore, several canonical Wnt signaling target genes were also evaluated after pathway activation or inhibition. The prognostic values for β-catenin sub-cellular localization alone or in combination with axin2 expression observed in primary canine and human OS were evaluated using tissue microarray. The capacity of this pathway to regulate cell proliferation and its effects on target genes were investigated *in vitro*. The comparative characterization and evaluation of Wnt signaling activation in canine and human OS are presented and discussed here.

Materials and methods

Cell lines and culture conditions

Eight canine OS cell lines [COS31, HMPOS, POS, D17 (ATCC: Cat.no.CRL-6248), MC-KOS, CS-KOS, BW-KOS and SK-KOS and two human OS cell lines [SaOS2 and MG-63] were used in this study (Barroga et al., 1999; Kadosawa et al., 1994; Shoieb et al., 1998a; Shoieb et al., 1998b).

Cells were maintained on DMEM (Invitrogen®, the Netherlands) supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories GmbH, Austria) at 37°C with 5% CO_2.

TOP and FOP Flash reporter assays

Canine cell lines from passages 4 to 12 were used for *in vitro* experiments. Cells were seeded at a density of 100,000 cells /well into 24-well plates (Primaria, BD, USA). Twenty-four hours after seeding, attached cells were transiently transfected with the TOP-Flash or FOP-Flash luciferase reporter plasmids (gift from H. Clevers, Hubrecht Institute, Utrecht, The Netherlands) at a concentration of 0.8µg per transfection reaction. Transfections were optimized across the cell lines prior to the experiments to determine the optimal plasmid concentrations, renilla luciferase and transfection reagent. After optimization, cells were transfected with 3µl of Lipofectamine-2000 transfection reagent (Invitrogen, The Netherlands) in 500µl of serum-free media. Transfection efficiencies were normalized by co-transfection using 50ng of the Renilla luciferase reporter (Promega, The Netherlands) and incubated for 5h, followed by the replacement of transfection media with 10% fetal bovine serum media. Luciferase activity was determined 42h later according to the manufacturer's protocol (Dual Luciferase Assay System, Promega, The Netherlands): cells were lysed using 100µl of passive cell lysis buffer and luciferase measurements were made on 25µl of cell lysates in 96-well plates using a luminometer (Centro LB 960, Berthold Technologies, Belgium). TOP-Flash and FOP-Flash luciferase activities were normalized to Renilla luciferase values, and the TOP/FOP ratios were determined for each cell line. The mean and standard deviation were calculated from triplicate well measurements in four independent transfection experiments.

Canonical Wnt signaling stimulation assays

Canine OS cells were transfected with the TOP- and FOP-Flash reporter constructs as described above. Twenty-four hours post transfection, cells were treated with lithium chloride (LiCl) or CHIR99021 (Stemgent, San Diego, USA) for 18h before luciferase activity was measured according to the method described above (Ring et al., 2003)(Sinha et al., 2005; Yang et al., 2010).

Inhibition of canonical Wnt signaling

Cells were seeded into 24-multiwell plates (Primaria, BD, USA) at a density of 100,000 cells/ well and allowed to adhere overnight. Co-transfection with either 10ng of dn-TCF4 or the 4TO empty vector construct (kind gifts from Hubrecht Institute, The Netherlands) (van de Wetering et al., 2003), in combination with TOPFlash or FOPFlash reporter constructs, were performed using 3 µl of Lipofectamine-2000 reagent in 500ul of serum-free media. After 5h of incubation, the media was replaced with normal culture media. After 42h, cells were washed with Hank's Balanced Salt Solution (HBSS) and lysed using passive lysis buffer before

luciferase measurements were made. Experiments were performed twice with 6-well replicates in antibiotic-free conditions.

Cell line RNA isolation and cDNA synthesis

Cells grown in 24-multiwell plates subjected to a specific treatment or transfection protocol (pathway stimulation or inhibition) were lysed with 200µl of RLT lysis buffer preceding the RNA extraction. RNA extraction and purification were carried out using the RNeasy mini kit (Qiagen, The Netherlands) and treated with DNase-I (Qiagen RNase-free DNase kit) according to the manufacturer's protocol. Final RNA was eluted in 30 µl of purified water. The Nanodrop ND-1000 spectrophotometer (Isogen Life Sciences, The Netherlands) was then used to quantify total RNA and its quality was evaluated using the Agilent 2100 bioanalyzer (Agilent Technologies, The Netherlands). Samples with RIN (RNA Integrity Number) values above 8.5 were selected for cDNA synthesis. cDNA was synthesized using 1µg of total RNA in a reaction volume of 40 µl for each sample using the iScript kit (iScript cDNA Synthesis Kit, Bio-Rad, The Netherlands). The cDNA samples from cell line experiments were diluted 3-fold in RNase-free water before they were subjected to quantitative real-time PCR.

Protein isolation and western blot analyses

Cells treated with CHIR99021 or 0.03% dimethyl sulfoxide, (DMSO) (control) for 18h were washed once with Hank's Balanced Salt Solution (HBSS) and incubated with RIPA buffer containing 1% Igepal, 0.6mM phenylmethylsulfonylfluoride, 15µg/ml aprotinin and 1mM sodium-orthovanadate (Sigma-Aldrich Chemie BV, The Netherlands) for 30 min on ice. Cells were collected, homogenized and centrifuged at 10,000 rpm at 4°C, and the supernatant was collected to determine protein concentration using the Bradford-based assay with DC Protein Assay Reagents (Bio-Rad, The Netherlands). Approximately 30 µg of protein per sample was denatured at 95°C for 3 min and separated by electrophoresis on a 10% SDS-polyacrylamide gel. Proteins were transferred onto Hybond-C Extra Nitrocellulose membranes (Amersham, UK) and incubated in 4% ECL blocking powder diluted in 0.1% TBST for 1 h. Membranes were then probed with primary antibody: either β-catenin or axin2 at a dilution of 1:500 and 1:1000, respectively, in 4% BSA. Blots were incubated overnight at 4°C and washed 3 times with 0.1% TBST, followed by incubation with an anti-rabbit horseradish peroxidase-conjugated secondary antibody (R&D systems, Minneapolis, USA) at a dilution of 1:20,000. Subsequently, membranes were washed for 15 min in 0.3% TBST. Immunodetection was performed according to the manufacturer's protocol (ECL™ Advance Western Blotting Detection Kit, Amersham, UK). After images were processed for β-catenin or axin2, membranes were washed three times in 0.3% TBST and stripped using the Restore™ Western Blot stripping buffer (Thermo Scientific, IL, USA) according to the company's protocol. Stripped membranes were blocked (as described above) and incubated with a mouse monoclonal anti-beta-actin for 1h (as the loading control) at a dilution of 1:2000 in 0.1% TBST with 1% bovine serum albumin (BSA). Blots were subsequently washed and incubated with an

anti-mouse horseradish peroxidise-conjugated secondary antibody (R&D systems, Minneapolis) for 1h at a dilution of 1:20,000. Immunodetection and image processing were performed as described above using the Chemi-Doc imager (Bio-Rad, The Netherlands). Images were processed using Quantity One Analysis Software (Bio-Rad) and Image J 1.43u (National Institute of Health, USA).

Immunofluorescence and confocal microscopy

Canine OS cells seeded onto cover slips in 24-well plates were allowed to attach overnight. Cells were treated with 2μM CHIR99021 (Stemgent, San Diego, USA), a GSK-3β inhibitor, or 0.03% DMSO, as a vehicle control, for 18h at 37°C in a humidified chamber with 5% CO_2. Cells were then rinsed twice in cold PBS (137mM NaCl, 2.7mM KCl, 6. mM Na2HPO4 and 1.5mM KH2PO4 pH 7.4) and subsequently fixed in 4% paraformaldehyde for 30 min at room temperature. Cells were then washed twice with PBS, followed by incubation in 0.1% Triton X-100 for 15 min to allow cell permeabilization. For immunostaining, cells were washed in PBS supplemented with 0.05% v/v Tween-20 (three times for 5 min) and subsequently incubated in blocking buffer (PBS, 0.05% v/v Tween-20, 1% w/v BSA and 5% v/v normal goat serum) for 1h. Next, cells were incubated with a primary antibody against β-catenin (rabbit polyclonal, Abcam) diluted at 1:100 in blocking buffer for 1.5h at room temperature. For the negative controls, cells were incubated in PBS without primary antibody. After extensive rinsing, cells were incubated with Alexa 488-conjugated secondary anti-rabbit antibody (1:100) in blocking buffer for 1.5h at room temperature. Coverslips were rinsed twice in PBS and incubated with ToPro-3 iodide (Invitrogen, The Netherlands) for 30 min to counter stain the nucleus. Finally, coverslips were rinsed in PBS, followed by one final rinse in dH_2O and subsequently mounted onto glass slides in FluorSave (Calbiochem, San Diego, CA, USA). Procedures for secondary antibody incubation steps were performed in the dark. All slides were imaged with a Leica TCS-SP inverted confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany) (488 nm argon and 633 nm HeNe excitation lines). Merged images were made using ImageJ 1.43u (National Institute of Health, USA).

Cell proliferation assays

To assess pathway inhibition, 10,000 cells transfected with 2ng or 10ng of the dn-TCF4 construct and the empty vector 4TO in 24-well plates (as described above) were trypsinized 24h post-transfection and seeded into 96-well flat bottom plates (Greiner). Cell proliferation was measured after 24h (48h post-dn-TCF4 transfection) using the WST-1 reagent (Roche, Mannheim, Germany) according to the manufacturer's protocol. To assess pathway stimulation, cells grown to subconfluent monolayers in normal culture media (75 ml flasks) were trypsinized and seeded at a density of 10,000 cells /well into 96-well plates (Primaria) and allowed to grow for 48h. Next, cells were treated with either LiCl or CHIR99021 (Stemgent, USA) in complete culture media with 10% FBS. Cell proliferation was measured as stated above at an absorbance of 450 nm (a 620 nm reference) using an ELISA reader (DTX

880). Data were analyzed using the Software for Anthos Multimode Detectors. Experiments were performed in 6-well replicates and repeated twice (for the dn-TCF4 transfection experiments) or three times (for the pathway stimulation experiments). All data are calculated and presented as a mean ± SD. Statistical significance was determined using a 2-tailed Student's *t* test, with a P value less than 0.05 considered significant.

Quantitative real-time PCR (qPCR) and data analysis

The optimal annealing temperature (Tm) of all primers was determined with a qPCR gradient assay using the iQSYBR green SuperMix (Bio-Rad, The Netherlands) according to the manufacturer's protocol. The amplification product of each primer pair was cycle-sequenced using the MJ MiniCycler^{Tm} (BioRad, The Netherlands) and analyzed using the 3130 XI Genetic Analyzer (Applied Biosystems, California, USA) to determine primer specificity. qPCR runs were carried out using the Bio-Rad MyIQ detection system (Bio-Rad, The Netherlands) using the following protocol: denaturation (95°C, 2 min), followed by 40 cycles of amplification (95°C for 20 sec), annealing temperature according to Table 1 for specific primer sets (for 20 sec) and elongation (72°C for 20 sec). Reactions were performed in technical duplicate. Individual reactions were corrected for qPCR efficiencies and analyzed using the IQ5 software (Bio-Rad, The Netherlands).

Table 1: Primer sequences used for quantitative real-time PCR assays.

Gene symbol / name	Acc. number	Primer sequences (5'- 3')	Ta(°C)
GAPDH (glyceraldehyde-3-phosphate dehydrogenase	NM_001003142	FW: TGTCCCCACCCCCAATGTATC RW: CTCCGATGCCTGCTTCACTACCTT	58
RPS19	XM_533657	FW:CCTTCCTCAAAAAGTCTGGG RW: GTTCTCATCGTAGGGAGCAAG	61
Axin2	XM_548025.2	FW: GGACAAATGCGTGGATACCT RW: TGCTTGGAGACAATGCTGTT	60
Survivin	AY741504	FW: CCTGGCAGCTCTACCTCAAG RW: TCAGTGGGACAGTGGATGAA	58
CyclinD1	NM_001005757.1	FW: GCCTCGAAGATGAAGGAGAC RW: CAGTTTGTTCACCAGGAGCA	60

*3 step protocol; Ta: annealing temperature; FW: forward primer sequence; RW: reverse primer sequence

Tissue immunohistochemistry

Clinical outcome-linked canine and human OS tissue microarray slides were obtained from the Comparative Oncology Program, Center for Cancer Research, National Cancer Institute, Bethesda, USA. Slides were deparaffinized in xylene and rehydrated through a series of alcohol dilutions (from 96% to 60%) for 5 min per dilution. Antigen retrieval was performed by

microwaving slides (850 W) in 10 mM sodium citrate (pH 6.0) for 2 min, followed by further incubation for 5 min before slides were microwave treated for another 1 min at 850 W. Slides were cooled on a table top for 20 min before proceeding with the subsequent steps. Endogenous peroxidase was blocked using 0.3% methanol in hydrogen peroxide for 15 min and washed with PBS-0.1%Tween (DAKO, The Netherlands) before blocking the tissues with 20% normal goat serum (DAKO, Denmark) for 1h at room temperature. One slide was incubated overnight at 4°C in a 1:100 dilution of anti β-catenin or anti-Axin2 rabbit polyclonal primary antibodies (Abcam) (in 4% BSA and PBS-0.1% Tween), whereas negative control slides were incubated with the same concentration of normal rabbit serum (Sigma). After an overnight incubation, sections were washed in phosphate buffer saline with 0.1% Tween (PBST) and incubated with a secondary anti-rabbit antibody (EnVision® -HRP detection system, DakoCytomation, USA) for 1h at room temperature. Staining was visualized using a DAB substrate chromogen system (K3468, Dako, USA), rinsed in distilled water and counterstained with hematoxyline. Finally, sections were rinsed in running tap water, dehydrated in increasing alcohol dilutions and mounted in VectaMount (Vector laboratories, Canada). This protocol was also used to evaluate β-catenin expression in 30 paraffin-embedded primary canine OS tissue sections available at the Department of Clinical Sciences of Companion Animals, Utrecht University. Images were captured using a CCD camera and Olympus BX41 microscope linked to a Cell^B imaging software (Soft Imaging Solutions GmbH, Germany).

Tissue immunohistochemistry analysis

Tissue microarray was scored for β-catenin and axin2 protein expression and localization through manual inspection of 20X objective magnifications by three independent observers that were blind to the clinical outcome data. Higher objective magnifications (40X) were applied to assess the sub-cellular immunopositivity. The tissue cores were scored as follows: (1) positive if more than 10% of the tumor cells were immunopositive for β-catenin/axin2 (otherwise negative); (2) localization of positive immunoreactivity noted as membranous, cytoplasmic, nuclear or a combination of both and (3) overall percentage of positive cells <25% (low) or ≥25% (high) by assessment on a single field at 20X magnification. The final score given to a particular tissue core using these criteria were of the highest consensus among the three observers. Poor-quality tissue cores (<25% tissue availability for assessment) were excluded from further analysis.

Statistical analysis

The cell proliferation index was calculated by comparing fluorescence intensity in treated to control cells (after subtracting for background intensity). Ratios below 1.0 were indicative of a decrease in cell number, and ratios above 1.0 were indicative of an increase in cell number. Data are presented as the average of ratios from 6 replicates, and the ±SD was calculated from 6 replicate paired-samples. A paired-t test analysis was performed for these comparisons. Quantitative real-time PCR gene expression data were normalized against the

geometric mean of two reference genes (RPS19 and GAPDH). A Kaplan-Meier survival curve was used to compare the disease-free interval (DFI) among the tumors expressing different levels of β-catenin and axin2 in canine and human OS. These comparisons were assessed using the Mantel-Cox log rank test within a confidence interval of 95%. All statistical analyses were performed using the PASW Statistics version 18.0 software (IBM, USA), where a two-sided test with statistical significance at $P<0.05$ was considered significant.

Results

Autocrine and induced transcription activity of Wnt/β-catenin signaling in canine OS

The autocrine activity of Wnt/β-catenin signaling was assessed in 8 canine OS cell lines using a TOP-Flash/FOP-Flash luciferase reporter assay. Mean fluorescence intensity was normalized to that of renilla luciferase, and ratios above 1 indicate pathway activation (Figure 1).

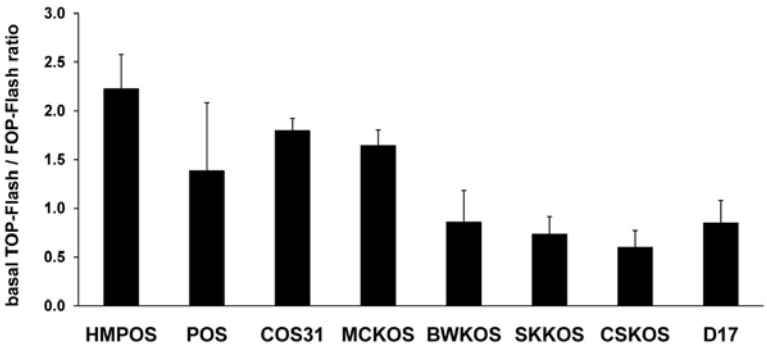

Figure 1: Autocrine or basal Wnt activation levels in 8 canine OS cell lines evaluated using TOP-Flash / FOP-Flash luciferase reporter ratios. Bars represent the average of ratios ±SEM from six-independent experiments. In cell lines with TOP/FOP ratios above 1.0, Wnt signaling is considered to be activated. HMPOS had the highest autocrine activation level. Four cell lines had TOP/FOP ratios below 1.0.

Although four canine cell lines had autocrine activity, the activation levels of canine cells were relatively low compared to the three human OS cell lines tested and were much lower compared to the SW480 colon carcinoma cell line containing an APC mutation (where Wnt signaling is constitutively active) (data not shown).

Active Wnt/β-catenin signaling was observed after stimulation of the pathway in 7 out of 8 cell lines observed using the luciferase reporter assays. Stimulation of the pathway was performed using two different compounds: LiCl, a non-specific inhibitor and CHIR99021 a specific GSK3β inhibitor. Although both compounds activated the pathway in a dose-

dependent manner, there was a clear difference between the two in the degree of activation. Treatment with CHIR99021 resulted in higher activation in 7 of the cell lines, whereas activation in one cell line (COS31) was comparable to that of LiCl treatment (Figure 2a). Although the luciferase assay indicated that the Wnt signaling pathway was activated, total β-catenin protein levels were not remarkably different in the majority of the cell lines. However, HMPOS and POS had moderate increases in expression after treatment with 2μM CHIR99021 (Figure 2b).

Nuclear β-catenin expression was enhanced in two of the canine OS cell lines (HMPOS and POS), whereas the other two cell lines with autocrine activity had enhanced cytoplasmic expression without apparent nuclear localization (COS31 and MCKOS). Upon the addition of 2 μM CHIR99021, 7 of the 8 cell lines showed nuclear localization (Figure 3). Nuclear localization was accompanied by an increase in cytoplasmic β-catenin, suggesting that there is an increase in the free pool of β-catenin that is not degraded. The D17 canine OS cell line, which has an autocrine luciferase reporter activity ratio below 1.0, did not respond to both concentrations of LiCl; however, the highest concentration of CHIR99021 (5 μM) resulted in transient activation. The D17 TOP/FOP reporter corresponded with very low cytoplasmic β-catenin expression, which did not translocate to the nucleus after stimulation.

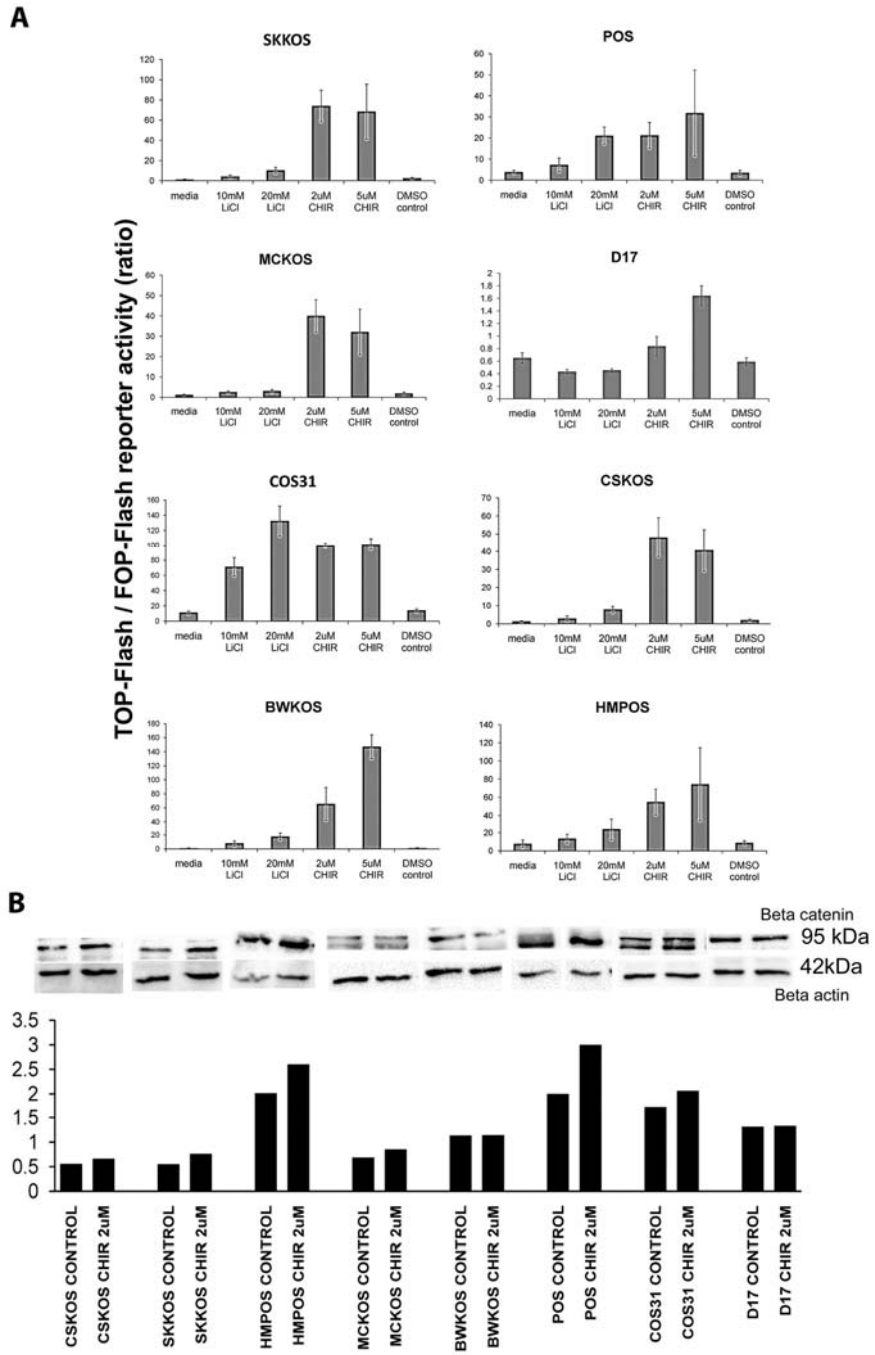
A
SKKOS
POS
MCKOS
D17
COS31
CSKOS
BWKOS
HMPOS
TOP-Flash / FOP-Flash reporter activity (ratio)
media
10mM LiCl
20mM LiCl
2uM CHIR
5uM CHIR
DMSO control
B
Beta catenin
95 kDa
42kDa
Beta actin
CSKOS CONTROL
CSKOS CHIR 2uM
SKKOS CONTROL
SKKOS CHIR 2uM
HMPOS CONTROL
HMPOS CHIR 2uM
MCKOS CONTROL
MCKOS CHIR 2uM
BWKOS CONTROL
BWKOS CHIR 2uM
POS CONTROL
POS CHIR 2uM
COS31 CONTROL
COS31 CHIR 2uM
D17 CONTROL
D17 CHIR 2uM

Figure 2

Figure 2: (**A**) TOP/FOP ratios for canine OS cell lines after the stimulation of Wnt signaling. Cells were treated with media alone (control for LiCl) or with dimethylsulfoxide (DMSO) at relevant concentrations (control for CHIR99021). Treatments were performed for 18h. Two concentrations were evaluated for both compounds. Bars represent average values from four-independent experimental wells from three separate experiments (±SEM is presented). (**B)** Western blot analyses for ß-catenin in the canine OS cell lines. The expression of total cell protein cell content is depicted, which did not show an increase in expression after treatment with 2µM CHIR99021. Band intensities were normalized against the loading control (ß-actin), and the ratios are presented in the bars below.

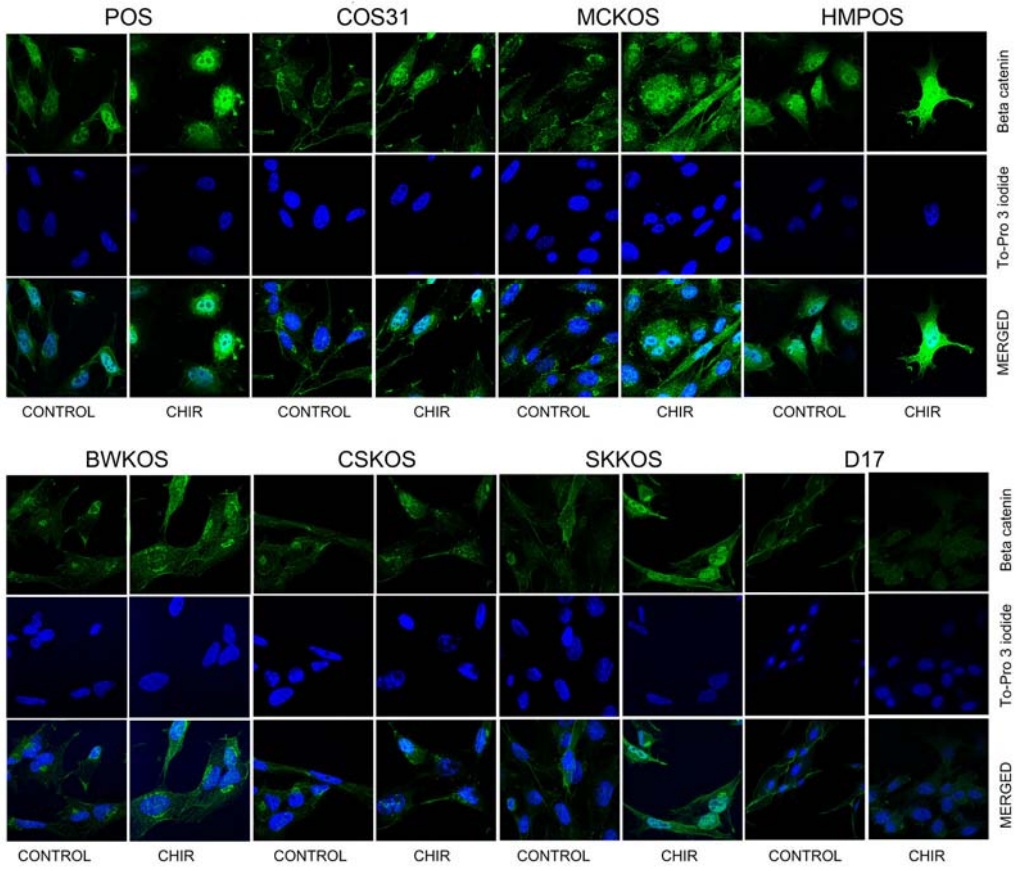

Figure 3: Beta catenin expression demonstrated by confocal imaging of canine OS cells. Two cell lines (HMPOS and POS) have basal nuclear β-catenin expression, which increases after treatment with 2µM CHIR99021. Beta-catenin is not localized to nuclei in two of the cell lines, CSKOS and D17; even after Wnt signaling is stimulated. There was prominent peri-nuclear staining in BWKOS cells upon stimulation. Nuclei were counterstained with TO-Pro3 iodide.

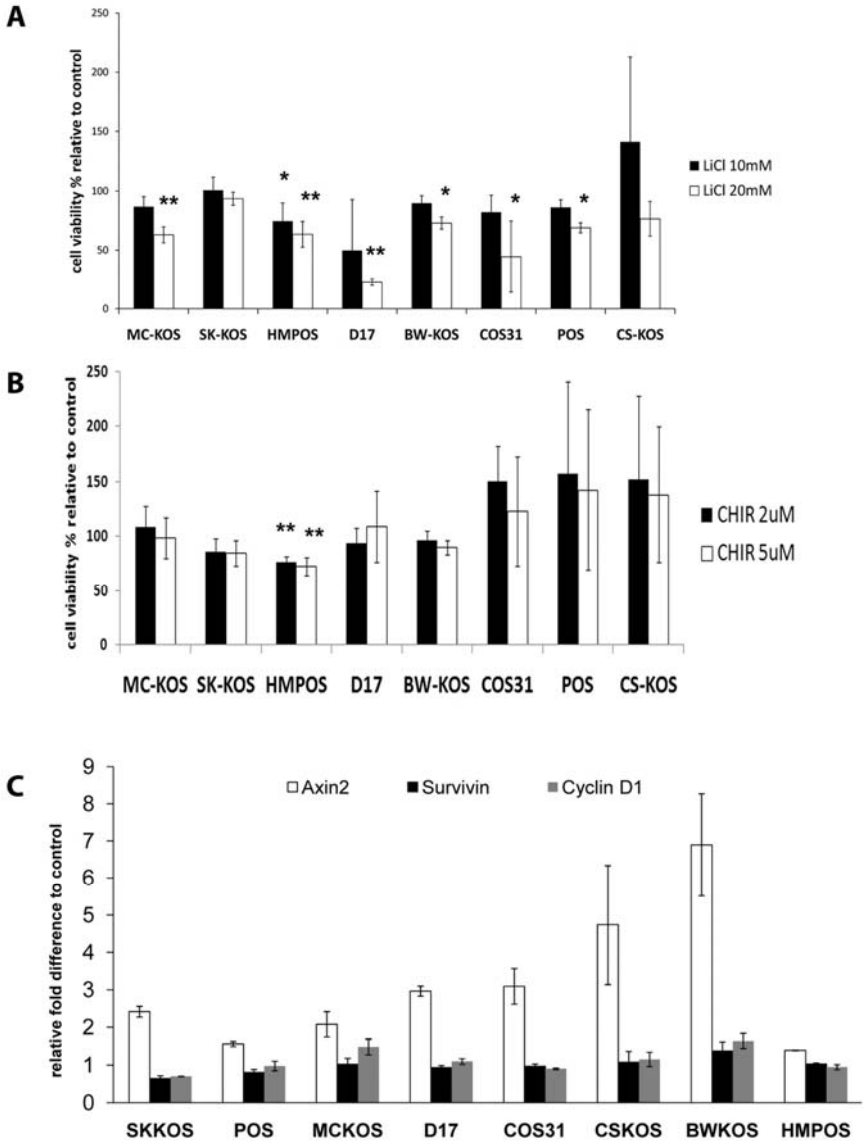

Figure 4: Cell proliferation assay for canine OS cell lines treated with **(A)**10 mM and 20 mM LiCl, **(B)** 2 µM and 5 µM CHIR99021, **(C**) Gene expression in 2 µM CHIR99021-treated cells normalized against the geometric mean of 2 reference genes and calculated relative to control (cells treated with DMSO) *P<0.05; **P<0.001.

Wnt pathway induced activation: effects on OS cell proliferation

Stimulation of the Wnt signaling pathway through the inhibition of GSK3β was first performed using two different compounds: LiCl (a non-specific inhibitor) and CHIR99021 (which specifically inhibits GSK3β). OS cells were treated with these compounds for 18h before cell proliferation was assessed for two different concentrations of each compound. In HMPOS cells, there was a decrease in cell proliferation in 10mM and 20 mM LiCl. However, 6 of the 8 cell lines showed a significant decrease in cell proliferation in 20 mM LiCl. Two cell lines, SKKOS and CSKOS, did not show significant differences in cell proliferation. On the contrary, there was enhanced proliferation in CSKOS at 10 mM LiCl (Figure 4A).

Treatment with CHIR99021 at both concentrations did not significantly affect cell proliferation in 7 of the 8 cell lines; however, there was a decrease in cell proliferation in HMPOS with an autocrine Wnt activity. In fact, treatment with CHIR99021 moderately increased cell proliferation in five of the cell lines, although this increase was not statistically significant (Figure 4B).

Wnt pathway activation: target gene expression analysis

Basal and pathway-stimulated expression of three known Wnt/β-catenin signaling target genes (survivin, cyclinD1 and axin2) was assessed. Axin2 mRNA expression did not correlate with basal canonical Wnt activity in the canine OS cell lines; however, upon LiCl treatment, there was a consistent upregulation of axin2 mRNA in all cell lines, which appeared to be both time and LiCl concentration-dependent (data not shown). There was a similar response in CHIR99021-treated cells for all cell lines (Figure 4C).

dn-TCF4 inhibition of canonical Wnt canonical signaling: effects on reporter activity and target gene expression

Inhibition of the autocrine Wnt canonical signaling was performed by stable transfection using the dominant negative TCF4 (dn-TCF4) at two concentrations (2ng and 10ng). After transfection with 10ng of dn-TCF4, luciferase reporter activity significantly decreased in 7/8 of the canine OS cell lines ($p=0.055$ for SKKOS) and two of the human OS cell lines (U2OS and MG63). Cell proliferation was significantly inhibited in 5/8 of the canine and only one of the human OS cell lines, suggesting that there are differences in sensitivity to TCF4 regulation of target genes in the different canine and human OS cell lines (Figure 5). Canine OS cells transfected with dn-TCF4 were further assessed for cyclinD1, axin2 and survivin expression. Cells transfected with 10ng of dn-TCF4 did not show significant differences in any of the target genes assessed. However, the transfection of 100ng of dn-TCF4 caused a significant downregulation of axin2 in four of the eight canine cell lines, whereas the downregulation of survivin and cyclinD1 was significant in only one and two cell lines respectively.

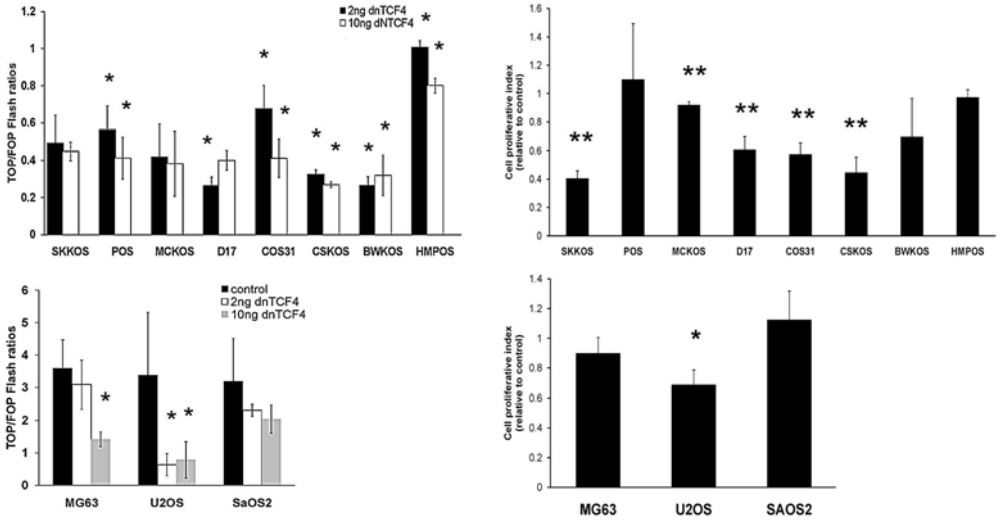

Figure 5: Downregulation of TOP/FOP ratios caused by dn-TCF4 mediated Wnt signaling inhibition in **(A)** canine and **(C)** human OS cells (y axis represents dn-TCF4 TOP/FOP ratios relative to the 4TO empty vector). Cell proliferation index (ratio of absorbance of dn-TCF4 transfected cells relative to 4TO vector-transfected control cells) for **(B)** canine OS and **(D)** human OS cells. Bars are averages from six experimental wells. Paired Student's t-test analyses were performed to compare the differences in the TOP/FOP ratio or cell proliferation between dn-TCF4 transfected cells and 4TO empty vector control cells, *P<0.05 and **P<0.001.

β-catenin and axin2 prognostic evaluation in human and canine OS tissues

After excluding tissue cores of poor quality, a total of 58 human OS tissues were available for both axin2 and β-catenin immunohistochemistry (Figure 6). The canine OS tissue microarray assessment was made on 83 and 67 tissue specimens for β-catenin and axin2 expression, respectively. In canine OS, 39% of specimens were positive for β-catenin, in which 53% had nuclear +/- cytoplasmic localization. In human OS, 67% of specimens were positive for β-catenin expression, in which 41% had nuclear +/- cytoplasmic localization. *Axin2* expression was observed in 74% of human and 58% of canine OS. Nuclear localization of either β-catenin or axin2 alone was not prognostic in either species (Figure 7). Co-expression of these proteins had more of a tendency to be significant in human OS (p=0.055, Log score=3.685), which was not observed in canine OS (p=0.954) (Figure 8).

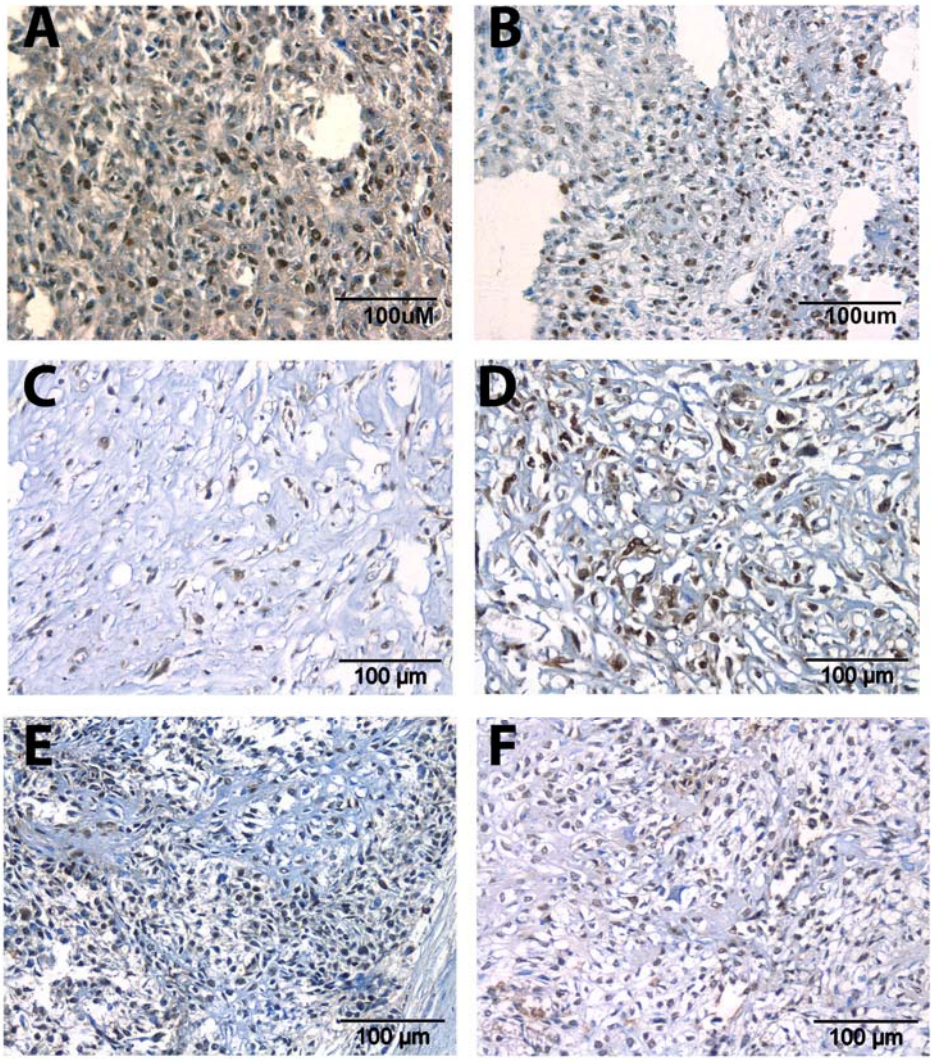

Figure 6: Immunohistochemistry of β-catenin or axin2 in OS tissue sections. **(A)** β-catenin nuclear and cytoplasmic expression with an abundance of protein observed in canine OS; **(B)** low levels of β-catenin localized to the nuclei, with poor staining scattered throughout the cytoplasm in canine OS; **(C)** cytoplasmic β-catenin expression in human OS, which occasionally had staining in the nuclei; **(D)** high expression of β-catenin in human OS, with prominent nuclear and cytoplasmic staining; nuclear and cytoplasmic axin2 were observed in **(E)** human and **(F)** canine OS.

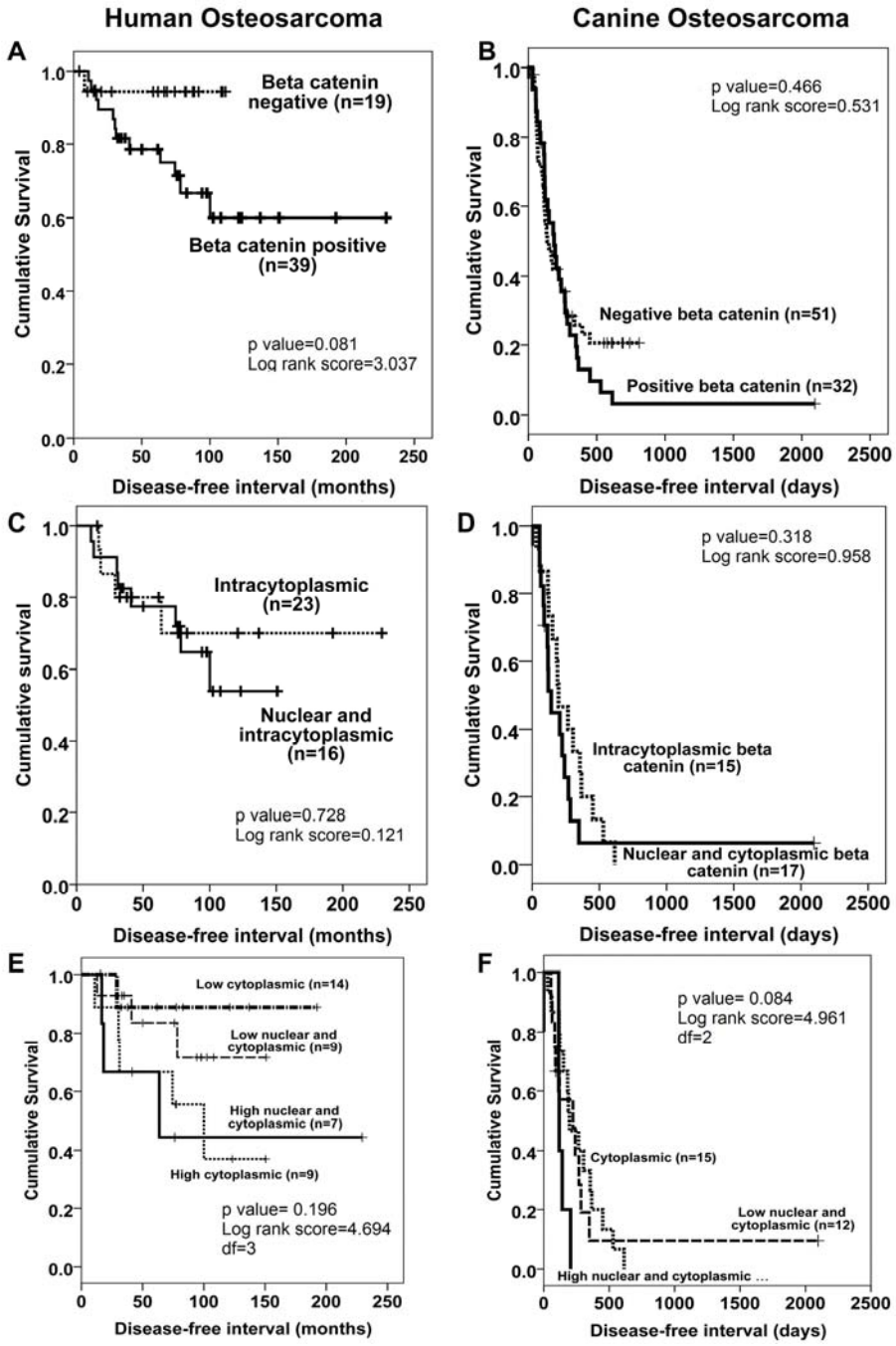

Figure 7: Kaplan-Meier disease-free interval curves following surgical removal of primary canine and human OS grouped on the basis of β-catenin expression and localization.

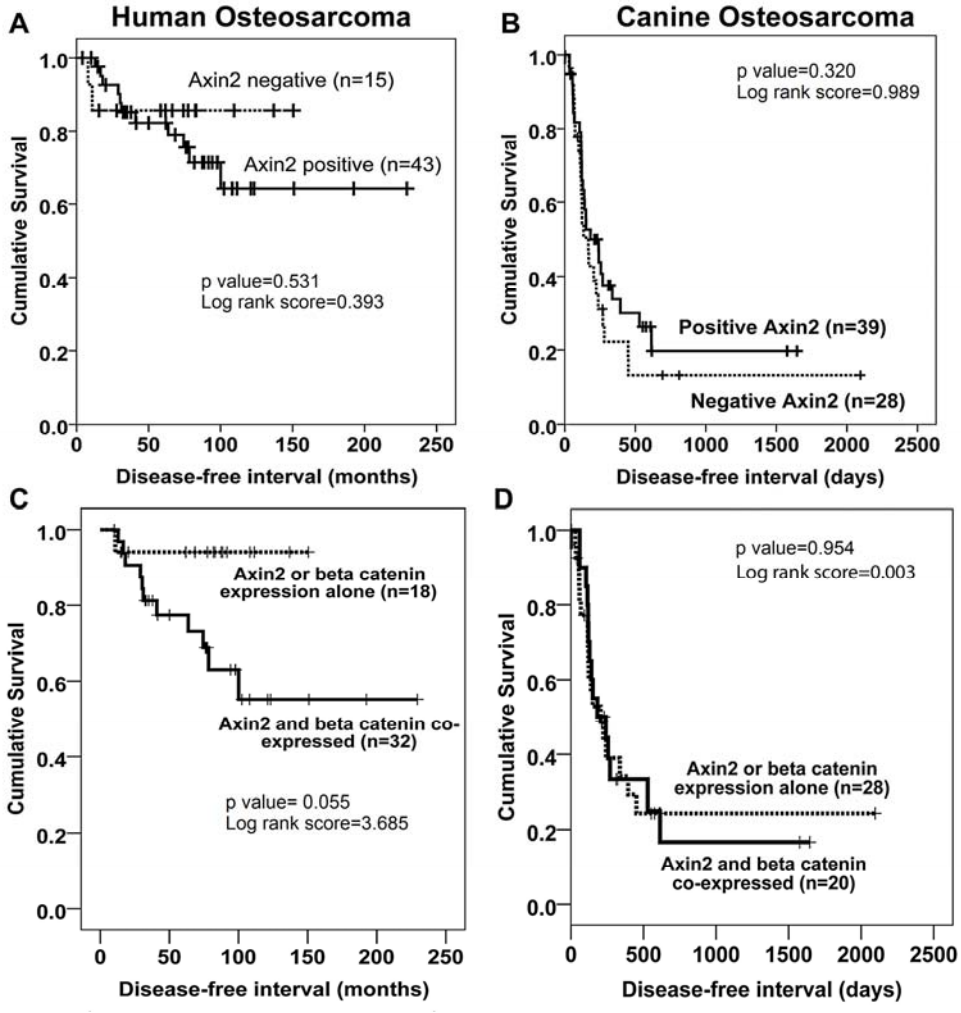

Figure 8: Kaplan-Meier disease-free interval curves for human and canine OS grouped on the basis of axin2 expression **(A, B)** and in those cases with β-catenin and axin2 coexpression **(C, D**).

Discussion

Canine OS is a spontaneous cancer that is found in dogs, a relevant large animal model for humans. The similarities and differences observed in OS between these two species with regards to tumor histology, clinical presentation, local disease progression, metastasis, gene expression and prognosis have been well documented. Osteosarcoma is locally aggressive and is highly metastatic (predominantly in the lungs) (De Maria et al., 2009; Mueller et al., 2007a; Paoloni and Khanna, 2008; Paoloni et al., 2009). Recent clinical outcomes based on global gene expression profiling of canine OS have demonstrated that there are several pathways important in OS disease progression, among which is Wnt signaling (Selvarajah et al., 2009).

Canonical Wnt signaling mediated by β-catenin regulation drives the activation and transcription of specific genes involved in the cell cycle, proliferation, epithelial-mesenchymal transformation and differentiation. This pathway has been well characterized over the years, and it is generally considered important in cancer pathogenesis (Reya and Clevers, 2005). Before investigating the role and prognostic value of Wnt/β-catenin signaling activation in canine OS, it is essential to select reliable markers that allow us to determine whether this pathway is activated. First, the present study utilized 8 well-characterized canine OS cell lines to evaluate basal canonical Wnt activation. Luciferase reporter assays revealed that four cell lines have enhanced basal signaling (TOP/FOP ratios >1). However, the level of activation is much lower compared to both the human OS and the human colon adenocarcinoma cell line (constitutively active due to presence of an APC mutation) (data not shown).

Cell lines with low or absent Wnt autocrine activation have weak cytoplasmic β-catenin expression. Nuclear β-catenin expression was only observed in two of the cell lines with higher basal activity. A similar study on human OS cell lines suggested that the Wnt signaling pathway is inactive in the panel of human OS cell lines and that the exogenous stimulation for activation of Wnt signaling further inhibited growth and promoted osteogenic differentiation. In the latter study, GIN, a specific GSK3β inhibitor, was used to stimulate Wnt signaling evident by reporter assays and the translocation of β-catenin into nuclei (Cai et al., 2010). In the present study, CHIR99021 and LiCl were able to stimulate Wnt signaling in the canine cell lines (increased reporter activity) which resulted in the translocation of ß-catenin to the nucleus. However, the degree of activation demonstrated by LiCl was much lower compared to CHIR99021. Intriguingly, LiCl treatment led to a decrease in cell proliferation in many of the cell lines, which was not the case for CHIR99021 treatment. This may be due to a cytotoxic effect caused by LiCl and other off-target effect involving interactions between LiCl with other genes and signal transduction pathways. Due to the diverse roles of Wnt signaling and the cross-talk with other pathways (Dao et al., 2007; Gordon and Nusse, 2006; Pendas-Franco et al., 2008), it is not possible to firmly conclude that the observed effects on proliferation are due solely to the activation of Wnt canonical signaling at the level of GSK3β. Of the canonical Wnt signaling target genes, axin2 appears to be the most specific target gene in agreement with other reports (Cai et al., 2010; Jho et al., 2002; Leung et al., 2002; Wang et al., 2007). In

this study, axin2 was also the most consistently upregulated gene, and was a much better Wnt activity reporter then cyclinD1 or survivin.

Other reports have shown that the dn-TCF4 transfections inhibited cell proliferation in cancer cell lines (van de Wetering et al., 2003). However, it has also been reported that there was no effect on cell proliferation in selected human gastric carcinoma cell lines (Asciutti et al., 2010). Wnt signaling inhibition, at the level of the TCF transcription factor, allowed us to modulate and evaluate the effects that inhibition of canonical Wnt signaling has on cell proliferation. When human and canine OS cells are transfected with dn-TCF4, there is a decrease in reporter transcriptional activation. The TOP/FOP ratios decreased to values as low as 0.3, indicating that at ratios near 1, residual Wnt signaling may still be possible. Consistent with previous reports (Asciutti et al., 2010), we saw differences in the inhibition of cell proliferation among the cell lines. Among the three target genes examined, only axin2 was downregulated after Wnt signaling was inhibited, which is in agreement with others (Jho et al., 2002)**.**

Nuclear β-catenin and upregulation of axin2 mRNA expression were found to be reliable markers of Wnt pathway activation in canine OS cells *in vitro*, a result similar to a previous report in human OS cells and other types of cells (Cai et al., 2010; Couffinhal et al., 2006; Leung et al., 2002). Hence, the prognostic value of the sub-cellular localization of β-catenin or axin2 expression alone or in combination in primary canine and human OS was evaluated using tissue microarrays. Beta catenin expression in both human and canine tumors did not reveal any prognostic significance; however, there was a tendency for ß-catenin immunopositivity to be associated with poor prognosis in both dogs and humans (which fail to reach statistical significance). Nuclear β-catenin nor axin2 expression were not prognostic in either canine or human OS, in contrast to reports on other human and canine cancers. This is in agreement with all of the previous prognostic studies in human OS, where β-catenin expression and localization were not significant for the clinical outcome (Cai et al., 2010; Guimaraes et al., 2010; Haydon et al., 2002; Stein et al., 2011). It is interesting to note that a subset of canine and human OS have strong axin2 expression in combination with nuclear β-catenin where the possibilities for mutations of axin2 in subset of osteosarcoma with active signaling awaits further exploration.

In conclusion, this is the first study to characterize canonical Wnt signaling in canine OS, demonstrating the reliability of the nuclear β-catenin expression as a 'hallmark' for pathway activation *in vitro*, along with expression of axin2. However, the prognostic value of nuclear β-catenin expression and the increase in axin2 expression was low in canine OS primary tumors, as illustrated by tissue microarrays. The findings from this study suggest that spontaneous canine OS is a relevant model for human OS where Wnt/β-catenin signaling activation and regulation with regards to axin2 and ß-catenin expression are similar.

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Chapter 8

Expression of epidermal growth factor receptor in canine osteosarcoma: association with clinicopathological parameters and prognosis

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Abstract

Expression of epidermal growth factor receptor (EGFR) is associated with aggressive growth and metastasis of a range of tumours, including osteosarcoma (OS), although some studies have reported no relevance to clinicopathological events or prognosis. The present study evaluated EGFR mRNA and protein expression in a panel of OS cell lines, normal bones, snap-frozen primary OS and tissue microarrays. EGFR expression was significantly elevated in primary OS compared to normal bones and in metastases of OS to the lungs compared to extrapulmonary sites. However, no clinical and pathological associations were found with mRNA expression levels in snap-frozen tumours. Tissue microarray analysis demonstrated that a subset of canine OS with high EGFR expression was associated with significantly shorter survival times and disease-free intervals. Cytoplasmic expression of EGFR was present in 75% of metastases and was similar to expression in primary tumours. EGFR expression alone is not a reliable predictor of outcome and other markers are necessary for further prognostic stratification of dogs with OS. However, these findings suggest that a subset of dogs may benefit from anti-EGFR adjuvant therapies.

Introduction

Canine osteosarcoma (OS), the primary bone tumour of large and giant breed dogs, is highly aggressive and metastatic disease is almost inevitable. Despite using adjuvant chemotherapy over the past decade, the median survival time of dogs remains in the range of 6 months to 1 year (Bacon et al., 2008; Chun et al., 2000). There is a need to develop new strategies that can stratify dogs into prognostic groups to create specific therapies that would benefit a subset of dogs with specific molecular alterations, namely, aberrant expression of growth factors, tumour suppressors or oncogenes and other signalling molecules important for cancer cell survival and metastasis.

Over-expression of growth factors and their receptors provides growth advantage to tumour cells insofar as regulating cell proliferation, promoting angiogenesis and increasing metastatic potential. The most widely studied growth factor receptor in human cancers, including OS, is the family of ErbB receptor proteins, which consist of four members: ErbB1- 4 (HER1-4). Epidermal growth factor receptor (EGFR), also known as ErbB1 or HER1, is a transmembrane receptor with an extracellular ligand-binding domain and an intracellular domain with tyrosine kinase activity. The receptor is involved in signal transduction under normal physiological conditions and deregulated signalling, such as in cancer growth and metastasis.

Over-expression of EGFR has been associated with an aggressive phenotype and poor prognosis in human cancers (Hadzisejdic et al., 2010; Rego et al., 2010; Rimawi et al., 2010), whereas others studies have reported no association with prognosis (Duran et al., 2010; Gaafar et al., 2010). EGFR is also reported to be a poor prognosticator for feline squamous cell carcinoma (Sabattini et al., 2010), whereas a study on canine mammary carcinomas found that EGFR had no prognostic value (Gama et al., 2009).

Aberrant expression of EGFR has been reported in human OS due to amplification (Oda et al., 1995; Freeman et al., 2008; Kersting et al., 2008; Do et al., 2009), but information on the EGFR expression status in canine OS is lacking. The prognostic and clinicopathological significance of EGFR expression in canine OS and its relevance for targeted therapies has yet to be fully explored. Therefore, this study investigated EGFR expression in two independent sets of canine OS samples using: (1) snap-frozen tumour materials linked with clinicopathological data for EGFR mRNA expression analysis and (2) an outcome-linked tissue microarray approach to investigate EGFR protein expression and localization for prognosis. Several canine OS cell lines and paraffin-embedded metastases were also incorporated to demonstrate the expression and localization of EGFR.

Materials and methods

Tissue specimens and clinicopathological data

Forty snap-frozen primary tumours and five lung metastases from dogs with histologically confirmed OS (Kirpensteijn et al., 2002) from the University Clinic for Companion Animals, Utrecht, from 1993 to 2004 were selected for this study. Normal canine bones (as control specimen) were snap-frozen from 14 healthy mature dogs euthanased for other purposes at the department (approved by the Dutch ethics committee. Clinical and pathological data were reviewed retrospectively.

Cell lines and culture conditions

Eight established and well-characterised canine OS cell lines were used for this study: COS31, HMPOS, POS, D17 (American Type Culture Collection CRL-6248), MC-KOS, CS-KOS, BW-KOS and SK-KOS (Shoieb et al., 1998; Barroga et al., 1999). The Madin-Darby canine kidney (MDCK) cell line was used as a set-point reference for gene expression analysis. A human osteosarcoma cell line MG63 and canine mammary carcinoma cell line (CMT9) was incorporated as controls for investigations on antibody specificity. Cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (PAA Laboratories) at 37 °C in a humidified atmosphere with 5% CO_2.

RNA isolation and cDNA synthesis

RNA isolation from snap-frozen OS tumour material was performed according to Selvarajah et al. (2009). Two independent passages from each cell line were lysed with RLT buffer and subjected to RNA isolation using the RNeasy Mini Kit (Qiagen). Samples were treated with DNase-I (Qiagen RNase-free DNase kit) and eluted in purified water. Synthesis of cDNA was performed using 0.5 µg total RNA in a reaction volume of 20 µL for each sample with the iScript cDNA Synthesis Kit (Bio-Rad).

Quantitative real-time PCR

Primers were designed on the EGFR canis familiaris predicted mRNA sequence XM_533073 in which the forward and reverse primers were designed on separate exons (intron spanning) using the Primer 3 software version 0.4.0 and Mfold version 3.2 (http://mfold.bioinfo.rpi.edu). The optimal annealing temperature (Ta) of all primers was determined by a qPCR gradient protocol, using the iQ SYBR green SuperMix (Bio-Rad, The Netherlands) according to the manufacturers' protocol. To confirm the specificity of the primers, the amplification product of each primer pair was cycle-sequenced using the MJ MiniCycler^{Tm} (BioRad, The Netherlands) and the 3130 XI Genetic Analyzer (Applied Biosystems, California, USA), followed by a nucleotide BLAST-search. Primers for the reference genes were designed and sequenced for specificity as described previously by Brinkoff et al. 2006. The optimal annealing temperature of all primers is provided in Table 1.

Each canine osteosarcoma sample cDNA was diluted 2-fold before being subjected to qPCR using the SYBR green fluorescent dye method. Quantitative real-time PCR runs were performed in duplicate up to 40 PCR cycles using the Bio-Rad MyIQ software. Individual reaction data were corrected for qPCR efficiencies and analysed using the IQ5 software (Bio-Rad).

Table 1
Primer details used for quantitative real-time PCR.

Gene name and symbol	Accession number	Primer sequence	Annealing temperature (°C)
Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	NM_001003142	FW: 5'-TGTCCCCACCCCCAATGTATC-3' RW: 5'-CTCCGATGCCTGCTTCACTACCTT-3'	58
Ribosomal protein S19 (*RPS19*)	XM_533657	FW: 5'-CCTTCCTCAAAAAGTCTGGG-3' RW: 5'-GTTCTCATCGTAGGGAGCAAG-3'	61
Epidermal growth factor receptor (*EGFR*)	XM_533073	FW: 5'-CTGGAGCATTCGGCA-3' RW: 5'-TGGCTTTGGGAGACG-3'	53

Protein isolation and western blot analyses

Cell grown under normal culture conditions in sub-confluent layers in 75ml culture flasks (Primaria) were washed once with HBSS and incubated with 1ml of RIPA buffer containing 1% Igepal, 0.6 mM phenylmethylsulfonylfluoride, 15 µg/ml aprotinin and 1 mM sodium-orthovanadate (Sigma-Aldrich Chemie BV) for 30 min on ice. Normal canine kidney and duodenum were pulverized, and protein extraction was performed according to a previously published protocol (Spee et al., 2005) Cell lysate were collected and homogenized at 10,000 rpm and 4°C, and the supernatants were collected for protein determination using the Bradford-based assay with DC Protein Assay Reagents (Bio-Rad). Protein acquired from all samples was further diluted in PBS to 1µg/µl. The protein samples were diluted 1:1 with 2 x sample buffer containing DTT (Sigma). Afterwards, the samples were heated for 2 min at 95°C to denature the proteins. Aliquots of protein (30µg) were applied to designated slots within a 5% SDS-polyacrylamide gel, and SDS-PAGE was carried-out for approximately 1h at 100V. The proteins were further blotted for 1h at 100V on a Hybond-C Membrane (Amersham, UK). Subsequently, a 60 min blocking of the membrane was performed in 4% ECL blocking solution in 0.1% TBST (Amersham, UK, ECL™ Advance Western Blotting Detection Kit). The membrane was incubated overnight at 4°C with anti-EGFR primary antibody (Abcam 2430, Cambridge, UK) in 0.1% BSA in TBST at a dilution of 1:200. Next the blots were washed 15 min (3 cycles of 5 min) in 0.1% TBST and further incubated for 60 min with anti-rabbit HRP-conjugated secondary antibody at a dilution of 1:10,000 on table top. The membrane was washed and the ECL™ Advance Western Blotting Detection Kit (Amersham) was used to facilitate

detection of the desired protein bands on the Chemi-Doc imager (Bio-Rad, The Netherlands) and Quantity One Analysis software. Images were processed on Image J 1.43u (National Institute of Health, USA).

Tissue microarray immunohistochemistry

Clinical outcome-linked canine OS tissue microarray slides were obtained from the US National Cancer Institute (Comparative Oncology Program, Centre for Cancer Research). Antigen retrieval was performed by microwave oven treatment of slides at 850 W in 10 mM sodium citrate (pH 6.0) for 2 min and followed by further incubation for 5 min before a second treatment of 1 min at 850 W. Endogenous peroxidase was blocked with Dako peroxidase blocking reagent for 15 min and washed with phosphate-buffered saline containing 0.1% Tween (PBST) before blocking the tissues with 20% normal goat serum (Dako) for 1 h at room temperature. One slide was incubated overnight at 4 °C with 1:100 dilution of anti-EGFR rabbit polyclonal primary antibody (Abcam 2430, Cambridge, UK); the negative control was incubated with normal rabbit serum (Sigma) at the same concentration. Slides were washed with PBST and incubated with secondary anti-rabbit antibody (EnVision-HRP Detection System, DakoCytomation) for 1 h at room temperature. Staining was visualised using the DAB substrate chromogen system (Dako), rinsed in distilled water and counterstained with haematoxylin. Slides were rinsed in tap water, dehydrated in increasing alcohol dilutions and mounted with VectaMount, (Vector Laboratories). Canine normal skin tissue was included as a positive control.

Tissue immunohistochemistry scoring method

Tissue microarray was scored for EGFR protein expression and localization by two independent observers who were blind to clinical outcome data. The whole tissue core from a tumor on the tissue microarray was assessed for % of cells positivity for EGFR expression at 20X objective magnification, which involves 1 field of observation. Higher magnifications, 40 X objectives were used to assess for localization specificity. Tissue cores were considered positive when ≥10% of cells demonstrated EGFR expression, as described previously (Pinter et al., 2008), with slight modifications where subsequent EGFR expression was scored as low (+1: 10-50% cells positive) or high (+2: >50% cells positive). The localization of positive immunoreactivity was noted as membranous, cytoplasmic, nuclear or a combination. In cases of scoring discrepancies, a third independent observer scored the particular core and the final score was determined by consensus among the three observers. Tissue cores with poor quality were excluded. Images were captured using a CCD camera and Olympus BX41 microscope linked to a Cell^B imaging software (Soft Imaging Solutions).

Cell lines and immunocytochemistry

Cultured cells in 8-well chambers (LabTek II) were fixed in 4% paraformaldehyde and permeabilised with triton-x 100 (0.25%) (Sigma, Germany). Next, cells were blocked with 10%

bovine serum albumin and subsequently incubated overnight with anti-EGFR at a dilution of 1:100 (Abcam 2430, Cambridge, UK) or PBS for negative control. Next, the cells were incubated with polymer anti-rabbit secondary antibody using the Envision system (Dako); peroxidase was visualised using diaminobenzidine and cells were counterstained with haematoxylin. Slides were rinsed with tap water, mounted using aqueous mounting medium and visualised under a light microscope.

Statistical analysis

RNA transcription of the target gene was normalised against the geometric mean of two reference genes *RPS19* and *GAPDH*. Gene expression analysis was performed using the $\Delta\Delta^{CT}$ method as previously described (Pfaffl, 2001) and gene expression values were subjected to Mann-Whitney *U* nonparametric tests. The Kolmogorov-Smirnov test was used to determine the distribution of EGFR mRNA expression across the categories of variables assessed. A Pearson correlation analysis was also performed to investigate the degree of correlation between continuous variables like tumour mitotic index, age and presurgical serum alkaline phosphatase measurements with EGFR mRNA expression. Cox regression analysis was performed where appropriate to determine the confounding factors for survival in the stated population. The Kaplan-Meier survival method was used to compare overall survival time (ST) and disease-free interval (DFI) among the tumours expressing different levels of EGFR on the tissue microarray. These comparisons were assessed using the Mantel-Cox log-rank test within a confidence interval of 95%. Statistical analysis was performed using PASW Statistics version 18.0 (IBM) where a two-sided test with $P < 0.05$ was considered to be significant.

Results

Clinical and pathological relevance of EGFR mRNA expression

Forty canine OS primary tumours (Table 2) were harvested from chemo-naive dogs from several large and giant breeds during a biopsy procedure or primary tumour removal either by amputation ($n = 20$), total resection ($n = 8$), or euthanasia shortly after diagnosis ($n = 12$). Dogs that had amputation or total resection were free from metastatic disease on thoracic radiography; 20 received postoperative chemotherapy. Dogs euthanased on diagnosis were mainly those with clinical metastatic disease, whereas the others were euthanased once clients declined therapies. A univariate Cox regression analysis was performed for 19 dogs with appendicular OS (with survival and clinical follow-up after amputation and postoperative chemotherapy); none of the assessed factors (age, tumour subtype, histology grade, neuter status nor alkaline phosphatase) was found to have a predictive value for survival among these dogs.

Table 2
Clinical and pathological characteristics of the 40 canine OS tumours for EGFR mRNA gene expression study

Variable	Number of dogs	Percentage
Sex		
Male	25	62.5%
Female	15	37.5%
Neuter status		
Intact	23	57.5%
Spayed/castrated	17	42.5%
Histological grade		
High malignant (grade 3)	29	72.5%
Medium malignant (grade 2)	11	27.5%
% tumour necrosis		
Low (<50%)	18	45%
High (≥50%)	22	55%
Histology subtype		
Osteoblastic	14	35%
Mixed histologies [a]	26	65%
Postoperative chemotherapy		
Yes [b]	20	50%
No	20	50%
Metastatic location		
Lungs	29	72.5%
Bones	4	10%
No metastasis during duration of follow-up	2	5%
Other sites [c]	2	5%
Lost to follow-up	3	7.5%
Primary tumour location		
Axial	5	12.5%
Appendicular	35	87.5%

[a] Mixed histologies include a heterogeneous appearance of tumour consisting of osteoblasts, chondroblasts, fibroblasts and some with telangiectatic subtypes.
[b] Chemotherapy with either lobaplatin, doxorubicin, or carboplatin, or a combination.
[c] Subcutaneous and renal metastases.

Canine OS primary tumours (n = 40) had significantly higher EGFR mRNA expression compared with normal canine bones (Fig. 1A). EGFR expression between primary tumours arising from different appendicular and axial sites showed no remarkable differences (Fig. 1B). In all 40 dogs, no statistical correlation was found between EGFR mRNA and mitotic index (n = 39; P = 0.852) or presurgical serum alkaline phosphatase measurements (n = 27; P = 0.999), nor for the analysis involving only the 19 dogs with appendicular OS. No significant difference was found in expression when comparing several categorical prognostic variables for canine OS, such as histological grade, tumour subtypes and degree of tumour necrosis. Interestingly, statistically significant higher EGFR expressions were observed for canine primary OS that metastasised to the lungs (n = 27) compared to OS that metastasised to extrapulmonary sites (P = 0.041), as shown in Fig. 2.

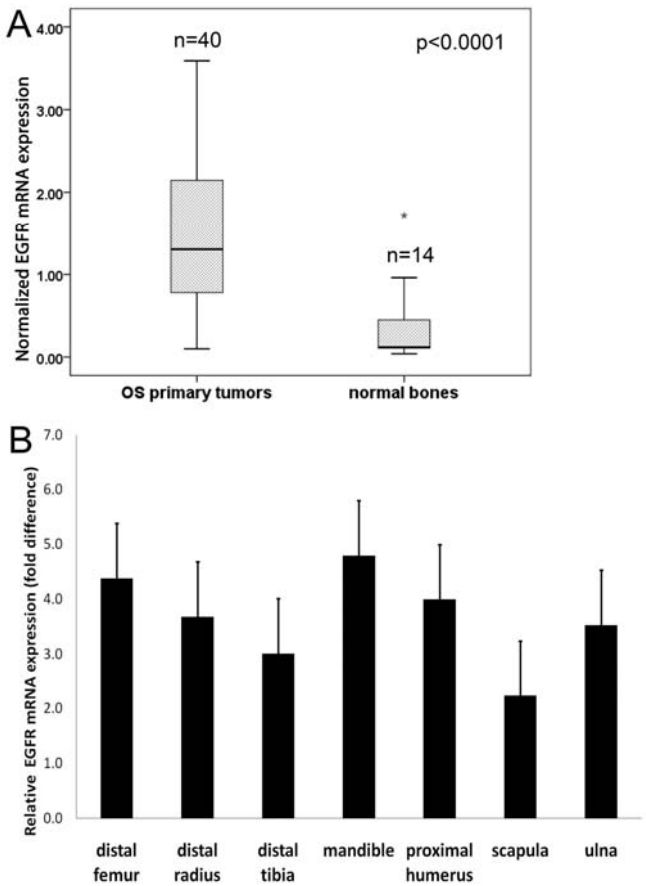

Fig. 1. Epidermal growth factor receptor (EGFR) mRNA expression in canine osteosarcoma (OS) and normal canine bones. (A) Significantly higher EGFR expression (Δ^{CT}: target gene normalised against the geometric mean of two reference genes) in 40 canine OS primary tumours compared to 14 normal bones (P <0.001). (B) EGFR mRNA expression levels in primary tumours arising from several appendicular and axial sites reveal no differences with regard to primary sites.

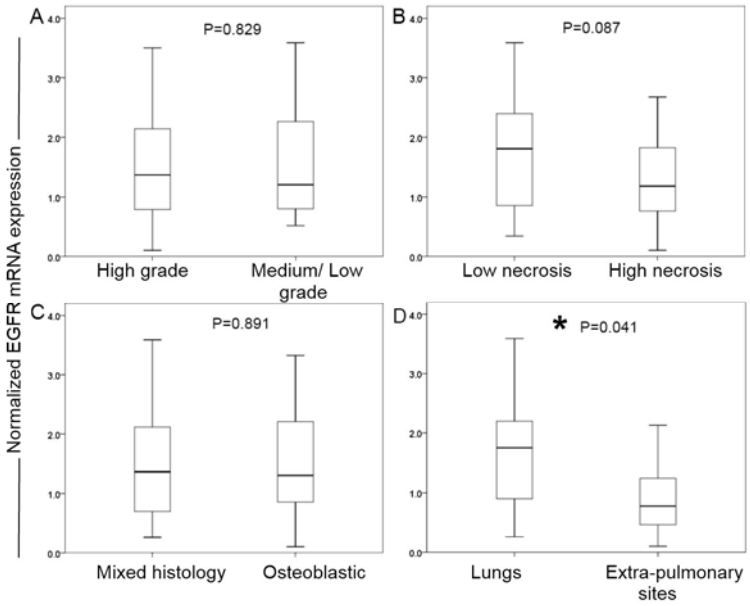

Fig. 2. Clinical and pathological relevance of epidermal growth factor receptor (EGFR) mRNA expression for canine osteosarcoma (OS). Corresponding box plots represent the normalised mRNA expression for EGFR (CT value for EGFR/ geometric mean CT of two reference genes), calculated average and whisker plots representing the mean, 25th and 75th percentiles with standard deviations. Nonparametric independent *t* test with Mann-Whitney *P* <0.05 are considered significant. 'Low' necrosis refers to <50% necrosis, whereas 'High' refers to cases with ≥50% necrosis.

Prognostic evaluation of EGFR protein expression

Of the primary OS cores assessed on the tissue microarray (from 75 dogs), 65.3% were negative and 34.7% of tumours were positive for EGFR expression with predominant cytoplasmic-membranous localization (73%); the remaining demonstrated scattered nuclear expression (27%). From various appendicular sites, all primary OS cores were chemo-naive and were subjected to amputation or a limb-sparing procedure with different postoperative chemotherapy protocols, depending on the veterinary institution where the dog was treated. Tumours were reported to metastasise to lungs (n = 35), bone (n = 12) and soft-tissue sites (n = 8); the rest of the animals were either lost to follow-up or did not die of metastatic disease during follow-up (censored for survival analysis). ST for these 75 dogs ranged from 51 to 1646 days and the DFI was 30 to 1646 days. The median DFI and ST is 167 and 223 respectively.

Survival curves in Fig. 3 demonstrate the prognostic value of EGFR expression in these tumours, showing no difference in ST and DFI in dogs with tumours positive for EGFR compared to those that were negative. However, the 16 dogs with tumours with low expression and 49 dogs negative for EGFR had significantly better survival compared to the 10 with tumours with high expression.

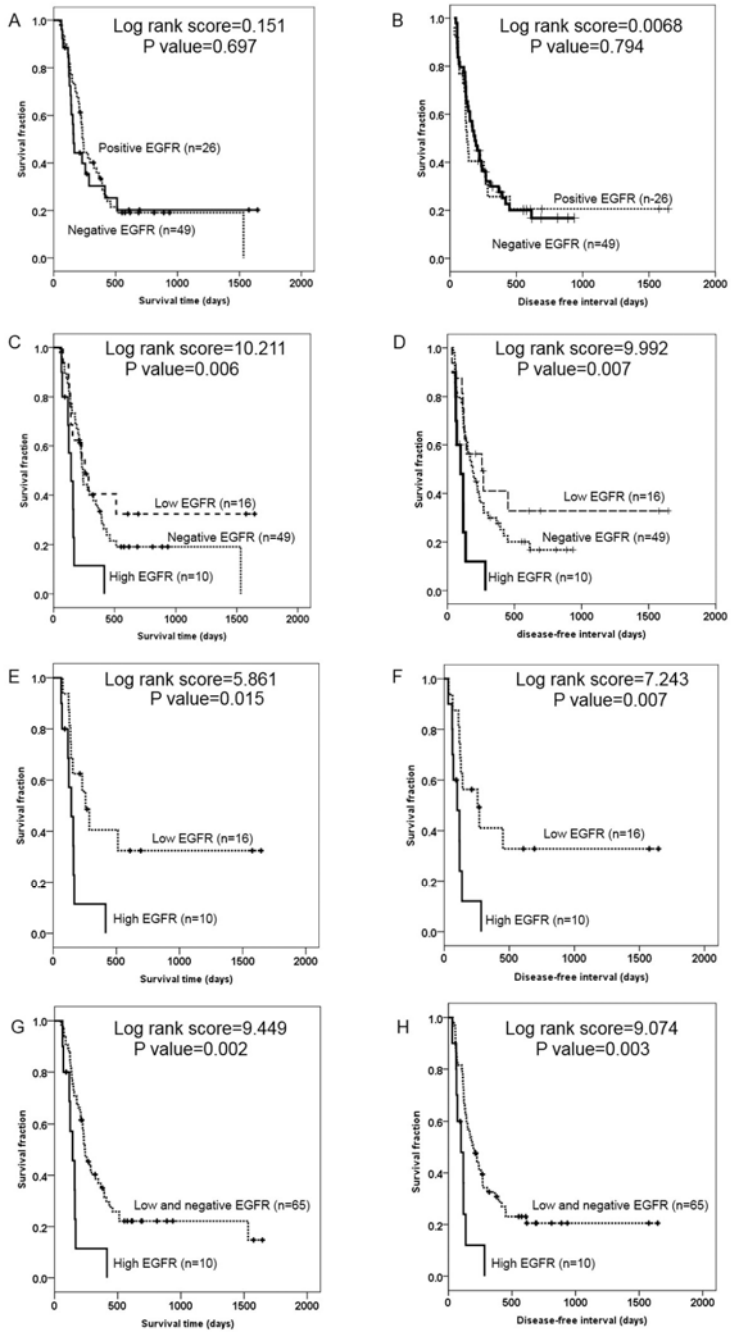

Fig. 3. Kaplan-Meier survival curves for epidermal growth factor receptor (EGFR) expression in canine osteosarcoma (OS) primary tumours. (A and B) Kaplan-Meier survival curves for overall survival time (ST) and disease-free interval (DFI) for dogs with OS based on EGFR expression in their primary tumours. No difference in ST and DFI was found for dogs with tumours positive for EGFR compared to those whose tumours were negative. (C-F) On further subclassification of tumours into groups with high or low EGFR expression based on the scoring criteria mentioned above, tumours with low expression (n = 16) and those negative (n = 49) for EGFR had significantly better survival compared to tumours with high expression (n = 10). ST and DFI for dogs with tumours negative for EGFR expression were not different from those with low expression with P = 0.355 and P = 0.313, respectively. (G and H) Collectively, tumours with negative and low expression had significantly better DFI (P = 0.003) and ST (P = 0.002) compared to dogs with high EGFR expression.

Survival analysis comparing tumours with cytoplasmic-membranous expression alone to those with scattered nuclear positive cells did not reveal any prognostic significance with a P value=0.6 (for ST) and P value= 0.728 (for DFI). Additionally, no significant associations were observed for EGFR expression and metastasis location. Expression and localization of EGFR in canine OS primary tumours are shown in Fig. 4A-C.

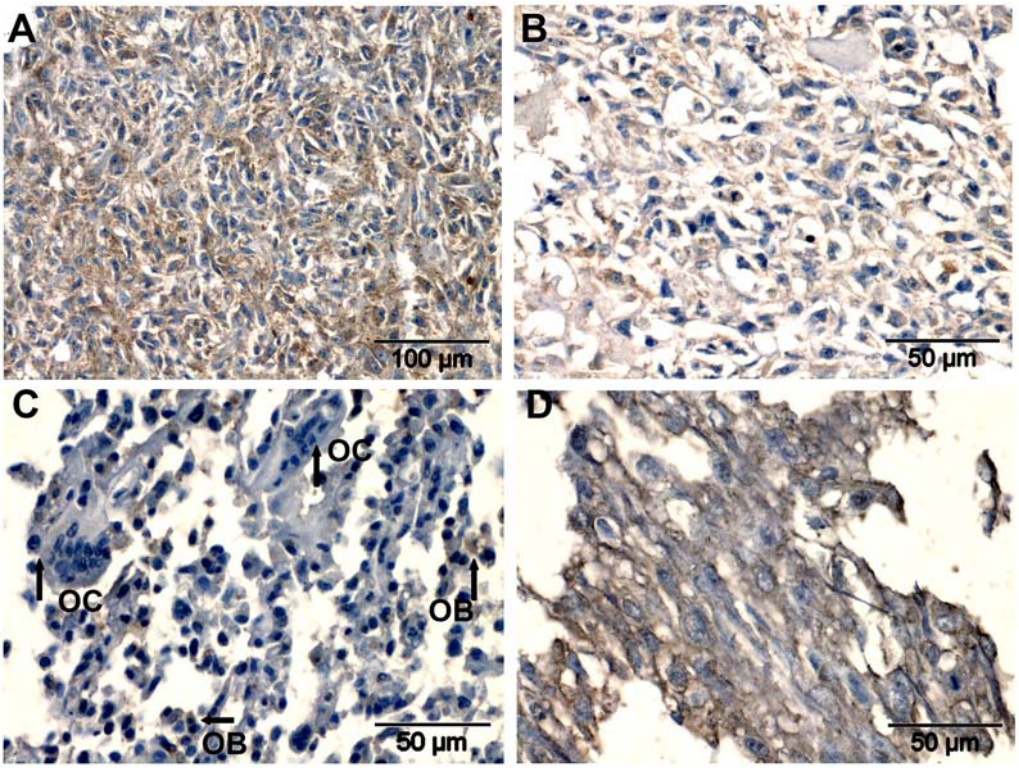

Fig. 4. Epidermal growth factor receptor (EGFR) protein expression and localization in canine osteosarcoma (OS) primary tumours and metastasis. (A and B) High EGFR expression in two different canine primary OS. (C) Low EGFR expression in a primary tumour with positive expression in osteoblasts (OB) scattered throughout the tumour (arrows), whereas the osteoclast (OC) is absent for EGFR expression. (D) Canine OS metastasis strongly positive for EGFR with cytoplasmic-membranous localization.

EGFR expression in canine OS metastasis

Five paraffin-embedded canine OS lung metastatic tissues from the archives of Utrecht University and three metastases on the tissue microarray were assessed for EGFR immunoreactivity (lung, *n* = 2; liver, *n* = 1). Of the metastases examined, six demonstrated cytoplasmic expressions, whereas two were negative. Among the six that were positive, four

exhibited strong high expression throughout the tissue section and the other two displayed lower expression.

EGFR protein expression and localization in canine OS cell lines

In order to verify the specificity of the antibody for canine tissue samples, a western blot assay was performed. Positive bands corresponding to the expected molecular weight of EGFR were observed in canine tissue samples and human osteosarcoma cell lines (control) (Fig. 5). Immunocytochemistry was performed on eight canine OS cell lines. Negative control cells were absent for staining. Cytoplasmic-membranous EGFR immunoreactivity was present with varying degrees of intensity across all eight canine OS cell lines (Fig. 6), reflected by the quantified EGFR mRNA expression (Fig. 7).

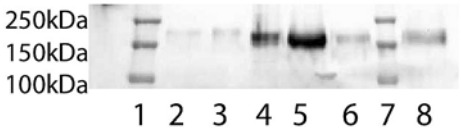

Fig. 5. Western blot analysis demonstrating EGFR expression. Lane 1, molecular weight marker; lane 2, canine OS cell line (HMPOS); lane 3, canine OS cell line (COS31); lane 4, human OS cell line (MG63); lane 5, canine normal kidney tissue; lane 6, canine normal duodenum; lane 7, molecular weight marker; lane 8, canine mammary carcinoma cell line (CMT-9). Expected molecular weight for EGFR is approximately 180 kDa which was clearly demonstrated by all the canine and human samples except that the 2 canine OS cell lines were observed to have slightly higher molecular weight (~190 kDa) which could be due to presence of glycosylation.

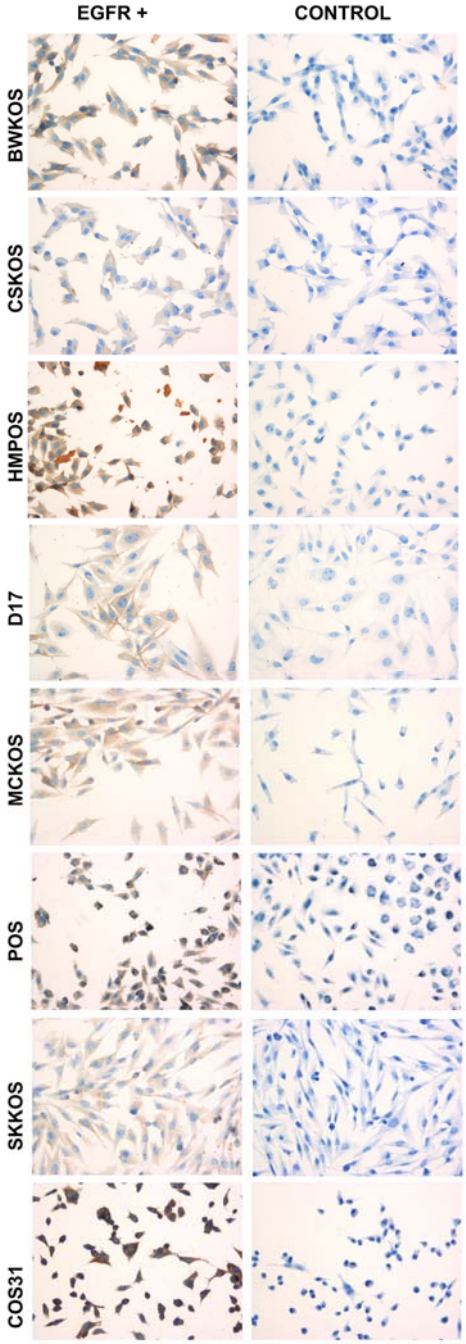

Fig. 6. Immunocytochemistry demonstrates EGFR expression in eight canine OS cell lines. All cell lines have cytoplasmic immunopositivity with varying degrees of intensity across cell lines. Staining was absent in the negative controls.

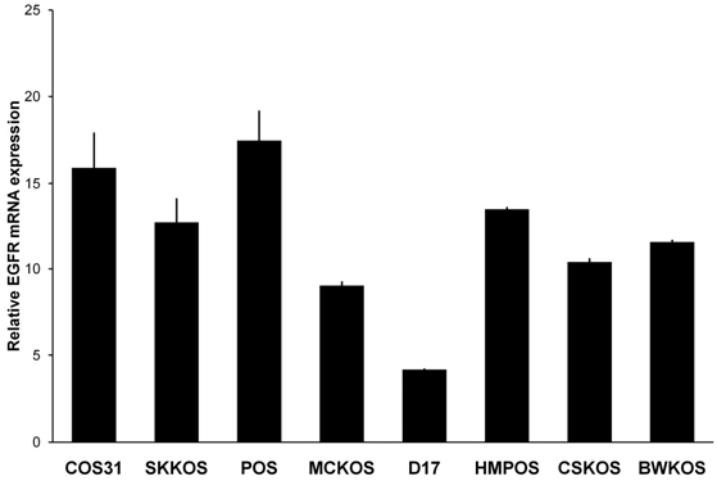

Fig. 7. Epidermal growth factor receptor mRNA expression in a panel of canine osteosarcoma (OS) cell lines. The normal canine kidney cell line (MDCK) was used as a reference set point for the calculations using the $\Delta\Delta^{CT}$ method.

Discussion

This study demonstrated aberrant genetic expression of EGFR in canine primary OS and their metastases in two distinct tumour groups, shown by immunohistochemistry and quantitative real-time PCR assays. Primary canine OS were observed to have higher EGFR mRNA expression compared to normal canine bones, but expression levels were not different among various primary tumour sites (both axial and appendicular sites). This result is not surprising, since tumours are known to have an increased expression of growth factors, which promotes cell proliferation and survival through autocrine or paracrine mechanisms. Similar qPCR investigations on human OS primary tumours demonstrated the presence of EGFR expression (Hughes et al., 2004). A panel of human OS primary tumours showed predominantly higher expression for EGFR relative to its ligand EGF (Oda et al., 1995), suggesting that other ligands could also activate this receptor. We have demonstrated that EGFR over-expression is not limited to epithelial-type cancers, such as non-small cell lung carcinomas, breast cancer or glioblastomas (Ruano et al., 2009; Monaco et al., 2010; Rimawi et al., 2010), but is also evident in a subset of OS.

No significant association with clinical or pathological parameters was noted for EGFR mRNA expression in canine OS. The six primary OS tumours that metastasised to extrapulmonary sites had lower EGFR mRNA expressions compared to those that metastasised to the lungs, suggesting that EGFR over-expression could activate different downstream pathways with preferences for different metastatic sites. However, this

association was not observed in immunohistochemistry studies on the tissue microarrays. This might be due to differences in techniques, since it appears that immunohistochemistry is less sensitive in detecting EGFR (35% of the samples were stained positive), whereas mRNA expression was detected by qPCR in all samples. Canine OS metastases showed cytoplasmic-membranous EGFR expression, similar to observations in human OS (Witlox et al., 2002; Wen et al., 2007). From these data we suggest that, despite the small sample size in our mRNA study, EGFR is over-expressed in primary tumours and metastases, which may serve as a relevant therapeutic target for canine OS where additional research is required to determine the pathway activity.

This study is the first to have used an immunohistochemical tissue microarray approach to investigate EGFR expression in canine OS in association with prognosis. Although EGFR immunopositivity on tissue microarray appeared not to be a reliable predictor for ST or DFI alone, further subclassification of tumours based on overall percentage of tumour cell positivity showed that tumours with high EGFR expression had a significantly worse clinical outcome. EGFR immunohistochemistry investigations on human OS primary tumours showed discrepancies ranging from no prognostic value to good clinical outcome (Kersting et al., 2007; Do et al., 2009). In our study, we observed high EGFR expression in only 13.3% of primary tumours. Whether this is due to EGFR mutation or amplification is unknown. In human OS, the frequency of EGFR amplification is low (Ozaki et al., 2002; Kersting et al., 2007). Similarly, the frequency of mutations appeared to be low (Wen et al., 2007; Kersting et al., 2008). Although several other studies on canine cancer have reported high expression for EGFR in a subset of tumours (Gama et al., 2009; Shiomitsu et al., 2009; Higgins et al., 2010), no studies have reported the amplification or mutation status of EGFR in canine OS.

It was not possible to distinguish tumours with distinct membranous staining from those with cytoplasmic staining by immunohistochemistry, an observation also found by others (Kersting et al., 2008). Nuclear expression was seen occasionally, but the majority of tumours had cytoplasmic-membranous immunostaining, in accordance with findings in human OS. All canine OS cell lines showed cytoplasmic-membranous expression, although the intensity varied across different cell lines. This is similar to results reported for human primary cultured OS cells which had moderate to high expression of EGFR, with predominantly membranous expression, compared to primary tumours, which manifested cytoplasmic expression, consistent with ligand binding, activation and internalisation of the receptor (Hughes et al., 2004; Wen et al., 2007; Do et al., 2009).

Currently, several therapeutic approaches targeting EGFR signalling pathways are under investigation, including strategies to block the extracellular receptor domain using monoclonal antibodies and inhibition of intracellular tyrosine kinase activity using specific small molecule inhibitors. Examples include CI-1033 (Hughes et al., 2006) and gefitinib (Freeman et al., 2006). Preclinical pharmacodynamic and pharmacokinetic data showed that disposition of gefitinib in dogs is similar to that in man, supporting and justifying the use of

dogs in safety evaluation studies and clinical trials on OS targeting the EGFR (McKillop et al., 2004a and b).

Conclusions

EGFR is over expressed in subset of canine primary OS, the majority of OS metastases and all canine OS cell lines investigated. A subset of OS with higher EGFR expression by immunohistochemistry has a significantly poorer prognosis compared to tumours with low and negative EGFR expression, indicating that EGFR expression may help stratify dogs with OS for anti-EGFR-based adjuvant therapies. Other molecular mechanisms, such as amplification, mutation and transactivation of other pathways causing EGFR signalling activation and aggressive biological behaviour in canine OS remain to be elucidated.

Acknowledgments

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Chapter 9

Gene expression analysis and functional RNAi reveal Hsp60 as a relevant therapeutic target for a subset of canine osteosarcoma

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Abstract

Heat shock proteins (HSP) are highly conserved across eukaryotic and prokaryotic species. These proteins play a role in response to cellular stressors, protecting cells from damage and facilitating recovery. In tumor cells, HSPs can have cytoprotective effects and interfere with apoptotic cascades. This study was performed to assess the prognostic and predictive values of the gene expression of several HSP family members in canine osteosarcoma (OS) and their potential for targeted therapy. Gene expression for HSP10, -22, -27, -40, -60, -70 (mortalin), -90α and -90β were assessed using quantitative real-time PCR (qPCR) for 58 snap-frozen primary canine OS tumors and 8 cell lines. The expression of HSPs was assessed for their association with several clinical-pathological factors, including survival. HSP60 expression in cell lines and tissues was assessed by western blots and immunohistochemistry, and siRNA-mediated gene silencing was performed in two canine OS cell lines with differential expression of HSP60 mRNA. Functional assays to assess cell proliferation, cell cycle and apoptosis were also performed. Gene expression of survivin and cyclin D1 was determined using qPCR to assess the consequence of HSP60 gene silencing. Highly necrotic tumors displayed a significant down-regulation of HSP40 expression, while HSP10 and HSP60 overexpression was observed among tumors with predominantly osteoblastic histology. Significant overexpression of HSP22 was observed in canine OS that responded poorly to chemotherapy, and HSP60 was associated with shorter overall survival. Using immunohistochemistry, HSP60 was observed to be expressed in tumor osteoblasts and showed intense expression in osteoclasts. Both HSP60 mRNA and protein were constitutively expressed across all canine OS cell lines. Canine-specific HSP60 siRNA inhibited cell proliferation and showed early induction of apoptosis in both cell lines. Cyclin D1 was down-regulated in both cell lines, but the magnitude of the survivin decrease was delayed in one cell line compared to the other, suggesting the possible involvement of other intermediate mechanisms or proteins in the induction of apoptosis by siRNA-mediated HSP60 ablation in canine OS. The present study has shown that the mRNA expression of several HSPs, including HSP60, has predictive and prognostic value for canine OS. Hsp60 expression inhibition using synthetic siRNA or compounds specifically targeting this protein *in vivo* may offer a novel therapeutic approach for a subset of canine OS.

Introduction

The primary bone tumor, osteosarcoma (OS) is the most common type of bone tumor in large and giant dog breeds (Norrdin et al., 1989; Ru et al., 1998). Most often, dogs present with clinical signs of lameness or painful swelling of the affected bone. At an advanced stage, some dogs present with evidence of pathological fractures. However, almost 90% of dogs have metastatic disease at the time of clinical presentation. Metastasis, predominantly to the lungs and with fewer incidences in other skeletal and soft tissue locations, is a major end-point for this disease in both humans and dogs (Morello et al., 2010). There are many causes and contributing factors for the development and pathogenesis of OS in dogs (Kirpensteijn et al., 2008; Levine et al., 2002; Misdorp and Hart, 1979b). Although improvements in diagnosis and therapy have been made over the past 20 years, the prognosis for dogs with OS is still grim. Elevated levels of several genetic markers and proteins have been found in human and canine OS (Selvarajah and Kirpensteijn, 2010). However, very few of these markers have been independently validated in a large cohort of samples for their prognostic values, especially among dogs with OS.

Heat shock proteins (HSPs) are highly conserved proteins that are expressed in both prokaryotic and eukaryotic organisms. They are classified into separate families according to their molecular weight (kDa). Each family may consist of several molecules that show great resemblance in primary structure and perform similar functions in different sub-cellular compartments. In times of chemical or physical stress, these HSPs prevent protein mis-folding and aggregation by refolding denatured proteins or by assisting in their degradation (Burel et al., 1992; Calderwood et al., 2006; Hartl and Hayer-Hartl, 2002; Helmbrecht et al., 2000; Hendrick and Hartl, 1993; Romanucci et al., 2008). In addition to their role during cellular stress, HSPs are also involved in tumor development, with roles in apoptotic pathways, cell cycle regulation and immune modulation.

HSP expression has been explored in various tumors and has been observed to have diagnostic, prognostic and therapeutic predictive values in both humans and dogs (Cappello et al., 2008; Faried et al., 2004; Kumaraguruparan et al., 2006; Romanucci et al., 2005). Heat shock proteins have long been examined in human OS, and some valuable insights have been made. A positive correlation has been found between the presence of anti-HSP70 serum antibodies and lung metastases in OS patients at the time of diagnosis, but no difference in anti-HSP70 antibodies could be observed between sera from patients and healthy controls. Anti-HSP60 antibodies are significantly increased in human patients with OS compared to a healthy control group. This increase in anti-HSP60 antibodies could be used as a potential diagnostic marker (Trieb et al., 2000b). Meanwhile, others have found no prognostic association for HSP60 expression in primary human OS tumors (Moon et al., 2010). In human OS a correlation has been detected between the presence of anti-HSP90 antibodies and a better response to neo-adjuvant chemotherapy, whereas the absence of anti-HSP90 antibodies correlates with the occurrence of metastases (Trieb et al., 2000a). Additionally,

HSP72 protein expression seems to be a predictive immunohistochemical marker for OS. HSP72-positive tumors show a significantly better response to chemotherapy than HSP72-negative tumors (Trieb et al., 1998). At the biopsy and surgical level, immunohistochemical classification has revealed a correlation between HSP27 overexpression and poorer prognosis. Therefore, HSP27 is a potential negative prognostic marker of OS (Moon et al., 2010) (Uozaki et al., 2000). A cDNA microarray study on human OS cell lines observed an increased expression level of HSP90beta in OS cell lines compared to normal human osteoblasts, indicating that HSP90beta may play a role in the development or progression of OS (Wolf et al., 2000). To date, the value of HSP10, HSP22 and HSP40 expression has not been reported for human OS, although their implications in other types of cancer have been reported (Akyol et al., 2006; Cappello et al., 2003; Cappello, 2003; Cappello et al., 2005; Mitra et al., 2009; Sun et al., 2007).

The expression of HSPs has also been reported in canine cancers, including mammary carcinomas, transmissible venereal tumors, intracutaneous cornifying epitheliomas and squamous cell carcinomas (Chu et al., 2001; Romanucci et al., 2005; Romanucci et al., 2008). In a previous study on the cDNA microarray gene expression profiling of canine OS tissues, members of different heat shock protein (HSP) families, including HSP 90alpha, HSP70 (Mortalin) and HSP60, were associated with poor survival (Selvarajah et al., 2009). In another study, HSP90 gene inhibition was performed in both canine and human OS cell lines and resulted in loss of cell viability, inhibition of cell proliferation and induction of apoptosis, demonstrating a value for targeting HSP90 in OS *in vitro* models (McCleese et al., 2009). Although later gene expression profiling studies have suggested important roles for HSPs in the prognosis of canine OS, these findings are preliminary and deserve further validation using independent samples and other molecular approaches. The present research, therefore, aimed to evaluate the mRNA expression of 8 different HSPs and their association with several clinicopathological parameters and survival in dogs with OS. Several human OS cell lines and tumor specimens were included to demonstrate the cross-species and comparative expression of the HSP60 protein. Based on mRNA analyses, HSP60 expression was observed to be negatively correlated with survival. Hence, HSP60 was selected for gene silencing using RNAi in 2 metastatic canine OS *in vitro* models.

Material and methods

Canine OS tissue specimens and clinicopathological data

Fifty-eight histology-confirmed, primary canine OS tissue samples were selected from the bone tumor bank. These dogs were presented to the Veterinary Teaching Hospital of Utrecht University, The Netherlands, between 1994 and 2007. No dogs had therapy prior to harvesting the tumor tissue. Dogs included in the study had amputation or primary tumor resection and were followed until death. During surgery, all tumor samples were harvested

under sterile conditions, snap-frozen in liquid nitrogen and stored in sterile tubes at -70 °C. Diagnosis and histological grading of OS was determined by a board-certified veterinary pathologist according to a previously described histological grading system (Kirpensteijn et al., 2002). Clinical data and histological data were reviewed retrospectively.

Cell lines and culture conditions

Eight well characterized canine OS cell lines (D17, COS31, HMPOS, POS, MCKOS, CSKOS, BWKOS and SKKOS) and three human OS cell lines (SaoS2, MG63 and U2OS) were used in this study. The mycosensor qPCR assay kit (Agilent Technologies, US) was used to ensure that all cell lines were negative for mycoplasma. Cells were maintained in a sub-confluent monolayer in DMEM (Invitrogen®, The Netherlands) supplemented with 10% fetal bovine serum (PAA Laboratories GmbH, Austria) at 37°C in a humidified atmosphere with 5% CO_2. The cells were trypsinized and passaged every 4 days.

RNA isolation and cDNA synthesis

RNA isolation and purification from the OS tumors were performed as previously described (Selvarajah and Kirpensteijn, 2010) (Selvarajah et al., 2009) with the RNeasy® Mini kit (Qiagen, The Netherlands) according to the manufacturer's protocol. Cells grown in culture under normal or experimental conditions were washed once with Hank's Balance Salt Solution (PAA Laboratories GmbH, Austria), followed by cell lysis using the appropriate volume of RLT cell lysis buffer (Qiagen, The Netherlands) before extraction of RNA. In both the bone tumor and the cell line RNA isolation protocols, an additional DNA digestion step was included to assure genomic DNA removal. Total RNA was quantified using a NanoDrop® ND-1000 spectrophotometer (Isogen Life sciences, The Netherlands) and subsequently assessed for quality using a 2100 Bioanalyzer (Agilent Technologies, The Netherlands). Synthesis of cDNA was carried out with 0.5 µg total RNA of each sample in a 20 µl reaction volume using the iScript cDNA Synthesis Kit (Bio-Rad, The Netherlands) according to the manufacturer's instructions. To ensure that DNA contamination did not occur during the process of RNA and cDNA synthesis, several reactions were also performed without the reverse transcriptase enzyme.

Quantitative real-time PCR (qPCR) assays

Primers for the target genes were designed using Primer3 version 0.4.0 software and Mfold version 3.2 (http://mfold.bioinfo.rpi.edu). The optimal annealing temperature (Ta) of all primers was determined by a qPCR gradient protocol, using the iQ SYBR green SuperMix (Bio-Rad, The Netherlands) according to the manufacturers' protocol. To confirm the specificity of the primers, the amplification product of each primer pair was cycle-sequenced using the MJ MiniCycler™ (BioRad, The Netherlands) and the 3130 XI Genetic Analyzer (Applied Biosystems, California, USA), followed by a nucleotide BLAST-search. Primers for the reference genes were designed and sequenced for specificity as described above. The

geNorm algorithm analysis was performed to determine the number and type of reference genes appropriate for normalization of gene expression data.

Independent assays were performed for the bone tumors and cell lines. The standard dilution was prepared from a pool of cDNA (a mix from all samples) within an experimental setup. The standard concentration for qPCR analyses was generated using a 4-fold dilution series. Each sample cDNA was diluted 3-fold with RNAse-free water, and aliquots of 2 µl diluted cDNA were further subjected for qPCR reactions. All qPCR reactions were performed in duplicate using the Bio-Rad MyiQ™ Single Color Real-Time PCR Detection System. A 2-step qPCR protocol was performed for the target genes HSP27, -40, -60 and 90beta as well as for the reference genes GAPDH, GUSB, HNRNPH, RPS19 and RPS5. The thermal cycling conditions were 95 °C for 3 min, 45 cycles of 95 °C for 10 sec and Ta °C for 30 sec, followed by 24 cycles of 72°C for 30 sec. A 3-step qPCR protocol was performed for the target genes HSP10, -22, -70, and -90alpha as well as for the reference genes HPRT and RPL8. The thermal cycling conditions for this protocol were 95°C for 3 min, 45 cycles of 95°C for 10 sec, Ta °C for 30 sec and 72°C for 30 sec, followed by 24 cycles of 72°C for 30 sec. The annealing temperature was customized for each target and reference gene using a gradient qPCR assay prior to sample analyses.

Immunohistochemistry

Forty-seven formalin-fixed, paraffin-embedded canine OS tissue sections were stained for anti-HSP60 (working dilution 0.2 ug/ul) (LK-1, mouse monoclonal, Stress, Canada). In addition, 4 human OS and canine normal liver (positive control for HSP60) tissue sections were also included. A negative control immunostaining was performed for all tissue samples by replacing the primary antibody with isotype-specific mouse serum IgG, using the identical concentration as the primary antibody. Tissue sections were deparaffined in xylene, rehydrated in decreasing alcohol dilutions and rinsed in PBS-Tween 0.1% (PBST) for 10 min (2 cycles of 5 min). Antigen retrieval was performed by heat-treating the sections in 10 mM sodium citrate buffer at pH6 in a water bath. Heat-treatment was initiated at 37°C, and the temperature was increased to 72°C. Thereafter, the tissues were kept immersed in the water bath for 1 hour, followed by cooling of the slides within the heated citrate buffer for an additional 30 min until the temperature dropped to 30°C. The slides were then rinsed twice in PBS and incubated in 10% normal goat serum in 1% bovine serum albumin (BSA) in PBST (DAKO, The Netherlands) for 30 min at room temperature to reduce non-specific binding. Incubation with the primary antibody was performed overnight in a humidified chamber at 4°C. After rinsing with PBST, the sections were incubated with 0.35% hydrogen peroxide in PBS for 15 min to block endogenous peroxidase activity. Another round of rinsing with PBST was performed before the sections were incubated with the secondary HRP-conjugated antibody (1:10000, goat anti-mouse, DAKO, The Netherlands) for 30 min at room temperature. Afterwards, the sections were rinsed in PBS and incubated with DAB-chromogen mix (Sigma, The Netherlands) for 5 min, followed by a counterstaining with

hematoxylin (Vector laboratories, The Netherlands). Finally, the sections were rinsed in running water, dehydrated in increasing alcohol dilutions and mounted with Vecta Mount (Vector laboratories; Burlingame, Canada).

Immunohistochemistry scoring method

A semi-quantitative immunohistochemistry assessment was performed by two assessors who evaluated the slides and scored them based on their consensus agreement of the observed expression. Scoring for HSP60 protein expression was performed using a four-scale grading method (0: no positive cells; 1: <10% positive cells; 2: <10-50% of positive cells; and 3: >50% of positive cells). Due to the low number of tissue sections used for this assessment, the four-scale grading was reduced to a two-scale grading: low % of positive cells (grades 0 and 1) and high % of positive cells (grades 2 and 3). Immunopositivity was also assessed for localization (cytoplasmic or nuclear) and for intensity (high or low).

Protein isolation and western blot analyses

Cells grown under normal culture or experimental conditions in 24-well plates were washed once with cold HBSS and incubated with RIPA buffer containing 1% Igepal, 0.6 mM phenylmethylsulfonylfluoride, 15 µg/ml aprotinin and 1 mM sodium-orthovanadate (Sigma-Aldrich Chemie BV, The Netherlands) for 30 min on ice. The lysates were collected and homogenized at 10,000 rpm at 4°C, and the supernatants were collected for protein determination using the Bradford-based assay with DC Protein Assay Reagents (Bio-Rad, The Netherlands). Protein acquired from the 8 canine and 3 human OS cell lines was further diluted in PBS to 1 µg/µl. The protein samples were diluted 1:1 with 2 x sample buffer containing DTT (Sigma, The Netherlands). Afterwards the samples were heated for 2 min at 95°C to denature the proteins. Aliquots of protein were applied to an 8% SDS-polyacrylamide gel, and SDS-PAGE was carried out for approximately 1.5 h at 100 V. The proteins were further blotted for 1h at 100 V on a Hybond-C Membrane (Amersham, UK). Subsequently, 60 minute blocking of the membrane was performed using 4% ECL blocking powder diluted in 0.1% TBST (Amersham, UK, ECL™ Advance Western Blotting Detection Kit). The membrane was incubated overnight at 4°C with primary antibody (HSP60, LK-1, 1:100 in 0.1%TBST), followed by 15 min (3 cycles of 5 min) of washing in 0.1% TBST. Next, the membrane was incubated for 60 min with HRP-conjugated secondary antibody (1:20000, goat anti-mouse, DAKO) and subsequently washed for 15 min (3 cycles of 5 min) in 0.3% TBST. After adding the ECL™ Advance Western Blotting Detection Kit (Amersham, UK), the appropriate band corresponding to the expected molecular weight was detected using the Chemi-Doc imager (Bio-Rad, The Netherlands) and Quantity One Analysis software (Bio-Rad, The Netherlands). The blots were stripped using the Restore™ Western Blot stripping buffer (Thermo Scientific, IL, USA) and the same protocol was applied to incubate the antibody for beta-actin (for loading control). Blot images were processed in Image J 1.43u (National Institute of Health,

USA), band intensity was semi-quantified, and the expression of HSP60 was normalized against beta-actin expression.

siRNA design and synthesis

Canine sequence-specific HSP60 (synonym: HSPD1) (GenBank: XM_536016) siRNA was designed on the website http://www.dharmacon.com/designcenter/designcenterpage.aspx (Dharmacon RNAi Technologies, ThermoScientific, USA). Universal mock siRNA was used as the negative control for siRNA experiments. The designed siRNA sequences were blasted against the canine genome database to ensure there was no cross-silencing of non-target genes. The sequence of the canine–specific HSP60 siRNA duplex is as follows: sense- GUGAAUACGAAAAGGAAAAUU and antisense- UUUUCCUUUUCGUAUUCACUU. Cell transfections were optimized using siGlo (Dharmacon, Colorado USA) in 24-well plates (Primaria, BD, The Netherlands) using the transfection reagent Dharmafect 1 (Dharmacon, Colorado, USA). A concentration curve of 0-100nM siGLO was tested together with a Dharmafect range of 0.5µl to 2.5µl per well at different cell densities for several incubation periods ranging from 24 to 96h. The transfection efficiencies were assessed by determining the ratio between the fluorescent (transfected) cells and the non-fluorescent (untransfected) cells. The highest number of cells transfected with high cell viability was observed for transfections performed on 40,000 cells / well seeded in a 24-well plate with 50nM siGLO and 2µl Dharmafect 1 transfection reagent.

siRNA in vitro transfections and RNA isolation

Two canine OS cell lines, HMPOS and MCKOS, were selected for the siRNA-based functional assays. Cells were cultured in antibiotic-free media, and passages from 5 to 12 were used for the transfection protocols and cell culture experiments. Transfections were performed in 24-well plates (Primaria,BD The Netherlands) or 96-well plates (Cell Bind, Corning The Netherlands), depending on the type of cell-based experiment that was performed. Cells were seeded at a density of either 40,000 or 6,400 cells/well for the 24-wells or 96-wells plate setup. Transfections were carried out according to the siGLO-optimized conditions described above. Briefly, cells grown in antibiotic-free media were seeded in culture medium with FBS and allowed to attach over 12h. Next, 70% confluent cells were washed once with Hank's Balance Salt Solution (HBSS) and replaced with media without FBS. The cells were transfected with 50nM HSP60 siRNA or mock (non-targeting siRNA) and 2µl DharmaFECT 1 and diluted in RNase- and serum-free Dulbecco's Modified Essential Media (DMEM) according to the manufacturer's instructions for 12h at 37°C. Next, the transfection media was removed and replaced with complete DMEM media with 10% FBS, and the cells were further incubated for 24 to 96h. The same protocol was employed for the 96-well plate transfections with a ratio of 1:5 compared to the 24-well plate set ups. Cells were lysed for RNA from 24-well plates at several time points, including 18, 24, 30, 36, 48, 60, 72 and 84h post-transfection. Cell lysis was performed by adding 250µl RLT buffer into each well, and total RNA was isolated using

the RNeasy mini kit according to the manufacturer's protocol (Qiagen, The Netherlands). Synthesis of cDNA was performed using 0.5µg total RNA in a 20µl reaction volume using the iScript™ cDNA synthesis kit (Bio-Rad, The Netherlands).

MTT cell proliferation assay

Cell proliferation was determined by means of the colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay (MTT) (Sigma, The Netherlands). Briefly, cells seeded in 24-well plates were transfected according to experimental setup, and cell viability was determined by incubating 100 µl of MTT solution (5 mg/ml) in each well with 500 µl media at several time points (24, 48, 72 and 96 h). After 2 h of incubation, the culture media was removed, and 200 µl of dimethylsulfoxide (DMSO) was added to each well. The dissolved blue formazan crystals were transferred to a 96-well plate, and absorbance was measured at 595 nm with a reference reading at 650 nm. The background absorbance intensity was subtracted, and cell viability was calculated as a percentage of absorbance of the non-treated cells. The measurements are represented as the average and standard deviation of 2 independent experiments that were performed in triplicate.

DNA content measurement

Total DNA content was measured at several time points after siRNA transfections using the PicoGreen® dsDNA Quantification Reagent (Molecular Probes, Inc., USA). Briefly, the cells were washed once with Hanks Balanced Salt Solution (PAA, Germany) and freeze-thawed at -70°C thrice for a duration of 1 hour per cycle. The DNA standard curve was prepared according to the manufacturer's protocol, and DNA content measurements were carried out in polypropylene microplates with 96 round v-bottomed wells (Greiner). The fluorescence signal, measured as relative fluorescence units, was quantified at 485 nm excitation and 535 nm emission. The relative fluorescence units were exported through Anthos Multimode Detector version 2.0.0.13 software.

Tritium-thymidine incorporation assay

Cells were seeded at a density of 40,000 cells/well and siRNA transfection protocols were performed in 24-well Multiwell™ Primaria plates (Falcon®, USA) according to the protocol described above. The assay was performed on cells at 72 h post-transfection with the siRNA protocol. To each well, 0.1 µCi/mL tritium-thymidine (GE Healthcare, UK) was added and incubated for 4.5 h. After 4.5 h, the culture medium was removed, and the cells were washed prior to adding 0.1 mL of 0.2% SDS. The cells were further incubated for 15 min, and then 400 µl RNAse-free water was added. Three milliliters of scintillation fluid was added to 400 µl cell lysate, and tritium-thymidine incorporation was measured by a liquid scintillation analyzer (TRI-CARB 2900TR, Packard, USA). DNA content from 6 independent wells for each cell line was measured in parallel with the tritium-thymidine incorporation assay. Experiments were performed in 6-well plates in triplicate and repeated twice. Differences in tritium-thymidine

incorporation after normalization for DNA content (siRNA treated / mock cells) were calculated using the student's t-test and $P<0.05$ was considered significant.

Apoptosis assay

Cells transfected with siRNA for 24, 48 and 72 h in opaque 96-well plates (CellBind, Corning) were incubated with 20 µl/well viability/cytotoxicity reagent (ApoTox-Glo™ Triplex assay kit, Promega Benelux, The Netherlands) for 2 h. The fluorescence measurement was performed using the Infinite 200, Tecan (Belgium) software according to the manufacturer's recommendations. Next, 100 µl Caspase-Glo 3/7 reagent was added to measure apoptosis and incubated for 1 h before measuring the luminescence (Centro, Berthold, Belgium) according to the manufacturer's instructions.

Statistical analyses

Differences in gene expression between groups of tumors with regard to clinical or pathological parameters were analyzed using REST2008 software. Univariate and multivariate survival analyses (Cox's proportional hazard model) were performed to determine the prognostic value of the various HSPs. Kaplan-Meier survival curves were drawn to show differences in survival between groups. The corresponding hazard ratio (HR) and 95% confidence interval (CI) were reported, with statistical significance defined as $P<0.05$. SPSS16 software (IBM Company, Chicago, USA) was used for most of the statistical analyses. Statistical significance was defined as $P<0.05$.

Results

Retrospective analysis of clinical and histological data

The 58 dogs included for qPCR analyses varied in clinicopathological parameters (Table 1). The median disease-free interval (DFI) was 111 days, and the median survival time (ST) was 161 days. ST was defined as days from initial diagnosis until death, and DFI was defined as days from initial diagnosis until the observed occurrence of metastasis or the recurrence of the tumor. The samples represent several large to medium-sized breeds and were collected from the predilection sites, including the proximal humerus, distal femur and distal radius. Multiple chemotherapeutic protocols were employed across the dogs. When performing group comparison analyses, the histological subtypes were divided into purely osteoblastic tumors and mixed tumors that were purely chondroblastic, fibroblastic or telangiectatic or a combination of these subtypes. Most of the tumors were of high histological grade (Grade 3) and therefore considered highly malignant. As there were fewer tumors with medium or low histological grades, these tumors were combined to address a lower malignant class of OS for further statistical analyses.

Table 1**:** Clinical and pathological characteristics of the 58 canine OS included for qPCR analyses.

Parameter	**N**
Gender	
Female	25
Male	33
Neuter status	
Intact	30
Neutered	28
Postoperative chemotherapy*§*	
Yes	36
No	19
Histological subtype*§*	
Chondroblastic	1
Fibroblastic	1
Osteoblastic	19
Mixed	35
Histological grade	
High	40
Low/Medium	15
Location of primary tumor	
Appendicular	
Femur	6
Humerus	8
Metatarsus	1
Radius / Ulna	22
Scapula	5
Tibia / Fibula	9
Axial	
Extraskeletal	1
Mandible / Maxilla	4
Rib	2

Dogs that were diagnosed with OS but did not undergo amputation or primary tumor resection due to the presence of metastatic disease or owner refusal of further therapy were only included for the assessment of gene expression correlation with few clinicopathological parameters and were excluded from survival analysis. Dogs that died for reasons other than tumor burden or metastatic disease were censored for survival analysis. Dogs that were lost

to follow-up were censored as well, but the ST and DFI were recorded until the time they were lost to follow-up.

Univariate analysis of the variables (age, pre-surgical serum alkaline phosphatase levels, tumor histology grade, initiation of post-operative chemotherapy and tumor histology subtype) was performed. There was a positive impact of post-operative chemotherapy on survival; dogs without postoperative chemotherapy had a higher risk (HR 1.844, p = 0.048) of death than dogs who did receive postoperative chemotherapy. Because postoperative chemotherapy has a recognized influence on survival time, univariate analyses were repeated upon stratification for postoperative chemotherapy. Clinicopathological parameters including elevated pre-surgical serum alkaline phosphatase (AP) levels (p = 0.043) and histological-osteoblastic subtype (p = 0.005) were significantly associated with poorer survival on univariate analysis upon stratification for postoperative chemotherapy (Table 2). Multivariate analysis, stratified for postoperative chemotherapy, showed statistical significance for tumor subtype (HR = 0.341, 95% CI = 0.169-0.688, p = 0.003). Therefore, the histology subtype was an independent prognosticator for the present dataset of canine OS.

Table 2: Univariate analyses including stratification for postoperative chemotherapy treatment: Clinicopathological parameters and their association with survival time.

Parameter	No. of dogs (n)	Hazard ratio	Lower Confidence Limit (95%)	Upper Confidence Limit (95%)	P value
Age §	55	1.038	0.922	1.168	0.541
Sex Male* Female	55	0.713	0.380	1.340	0.293
Histological grade Low/medium* High	53	1.005	0.511	1.976	0.989
Subtype Osteoblastic* Mixed	53	0.362	0.179	0.734	**0.005**
Alkaline phosphatase §	38	1.003	1.000	1.006	**0.043**

§ Continuous comparison; * Category used as the baseline reference

Clinical and pathological association of the mRNA expression of various HSPs

A geNorm algorithm approach was first used to test for the optimal number of reference genes to be included in the expression analysis; a combination of five reference genes (RPS5, RPL8, RPS19, HNRNPH and GAPDH) in the present dataset resulted in the optimal number of reference genes for normalization. Quantitative real-time PCR primers and their corresponding annealing temperatures are provided in Table 3.

Table 3: Quantitative real-time PCR primer sequence details

Gene	Accession ID	Primer	Sequence (5′ to 3′)	Ta (°C)
HSP10	XM_536017	Forward	TGG AGG CAC CAA AGT AGT CC	60
		Reverse	TTC GCT TCA TGC CGT TTT A	
HSP22	NM_001003029	Forward	AGC CCT GGA AAG TGT GTG TC	58.5
		Reverse	ATG CCA CCT TCT TGT TGC TT	
HSP27	NM_001003295	Forward	GGT GGA GAT AAC TGG CAA GC	59
		Reverse	AGG AGG AGA CCA GGG TAG GA	
HSP40	XM_531970	Forward	TCG TCA TCA CCT CTC ATC CA	59
		Reverse	AGC CAT TCT CGG GGA AGT T	
HSP60	XM_536016.2	Forward	GCA GAG TTC CTC AGA AGT TGG	60
		Reverse	CAG CAG CAT CCA ATA AAG CA	
HSP70	XM_858735.1	Forward	CTT TGA CCA GGC CTT GCT AC	62
		Reverse	CAC CTG CAC AGA TGA GGA GA	
HSP90alpha	XM_843690	Forward	CTT GAC CGA TCC CAG TAA GC	59
		Reverse	TAT TGA TCA GGT CGG CCT TC	
HSP90beta	NM_001003327	Forward	AGA AAG AAT GCT TCG CCT CA	62
		Reverse	TCA TCG TCC TGC TCT GTG TC	
Survivin	AY741504.1	Forward	CCTGGCAGCTCTACCTCAAG	58
		Reverse	TCAGTGGGACAGTGGATGAA	
Cyclin D1	NM_001005757.1	Forward	GCCTCGAAGATGAAGGAGAC	60
		Reverse	CAGTTTGTTCACCAGGAGCA	

Ta: annealing temperature

Associations of the relative gene expression of HSPs with the various clinicopathological parameters are presented in Table 4. HSP60 and HSP10 were upregulated in osteoblastic tumors compared to mixed subtype tumors. Downregulation of HSP90beta and HSP40 was observed for age and necrosis, respectively; HSP90beta was downregulated in older dogs (> 5 years of age) and HSP40 was downregulated in highly necrotic (>50%) tumors compared to low necrotic (<50%) tumors. Additionally, HSP22 was found to be augmented in appendicular tumors of dogs that did not respond well to post-operative chemotherapy and that lived less than 6 months compared to those dogs that survived longer than 6 months. This overexpression remained significant even upon inclusion of the 7 dogs with OSs in axial locations (n=58, HR=1.694, p=0.024). No significant differences in HSP gene expression were observed with regard to histological grade. HSP27, -70 and -90alpha did not display significant associations with any of the clinicopathological parameters assessed (Table 4a). The panel of HSP mRNA expression was observed in all of the 8 canine OS cell lines except MCKOS, which was observed to have below levels of HSP27 only (Figure 1).

Table 4a: Association of HSP mRNA expression and clinicopathological parameters in 58 canine OS assessed in this study.

Parameter	HSP	Expression ratio	95% Confidence Interval	P value	Regulation*
Histological subtype					
Mixed versus Osteoblastic*	10	1.321	0.321 - 4.505	0.053	
Mixed versus Osteoblastic*	60	1.311	0.481 - 5.073	**0.037**	UP
Survival time					
Long survivors^{-} versus short survivors^{+*}	60	1.621	0.869 – 2.617	**0.003**	UP
Long survivors versus short survivors^{+*}	60	1.330	0.470 - 3.562	**0.007**	UP
Age					
≤ 5 years versus > 5 years*	90B	0.689	0.214 - 2.389	**0.020**	DOWN
Necrosis					
<50% versus > 50%*	40	0.649	0.209 - 1.954	**0.005**	DOWN
Response to post-operative chemotherapy					
Poor responders* versus good responders	22	1.730	0.229- 21.811	**0.026**	UP

Survivors^{+}: Short survivors: dogs that received postoperative chemotherapy treatment but lived less than 6 months due to high metastasis rate; Long survivors^{-}: dogs that did not receive postoperative chemotherapy and lived more than 6 months post-limb amputation. *Expression regulation is depicted for this group

Table 4b: Univariate analyses of HSP mRNA expression in tumors upon stratification for postoperative chemotherapy (association with survival time).

HSP	No. of dogs (n)*	Hazard ratio	95% Confidence interval	P value
HSP10	55	1.278	0.779 – 2.097	0.331
HSP22	55	1.041	0.830 – 1.306	0.729
HSP27	55	1.036	0.782 - 1.372	0.807
HSP40	55	0.928	0.583 – 1.477	0.752
HSP60	55	**1.699**	**1.045 - 2.763**	**0.032**
HSP70	55	1.109	0.804 – 1.531	0.528
HSP90alpha	55	1.128	0.851 - 1.494	0.404
HSP90beta	55	1.297	0.783 – 2.149	0.313

* For three dogs, no data were available on postoperative chemotherapy. Therefore, survival analyses including stratification for chemotherapy was performed on 55 dogs.

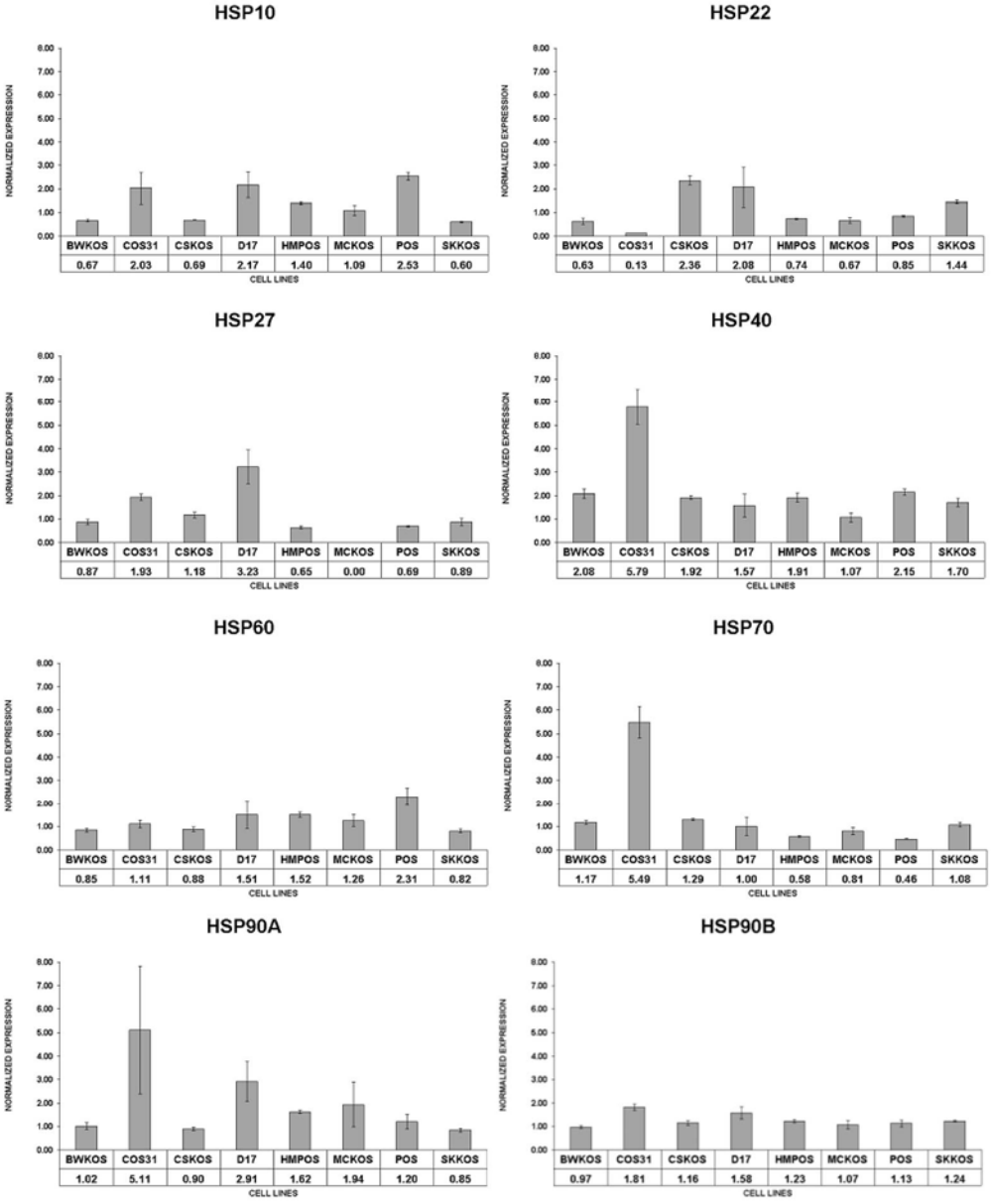


Figure 1: Heat shock protein mRNA expression in canine osteosarcoma cell lines
Target gene mRNA expression was calculated based on the standard line calculated efficiencies (MyIQ software) and normalized with reference genes (geometric mean of GAPDH and RPS19). The normalized expression from 3 serial RNA samples of cells grown under a sub-confluent monolayer in normal culture conditions was averaged, and the data are presented with ±SD.

Prognostic value for heat shock protein mRNA expression in tumors

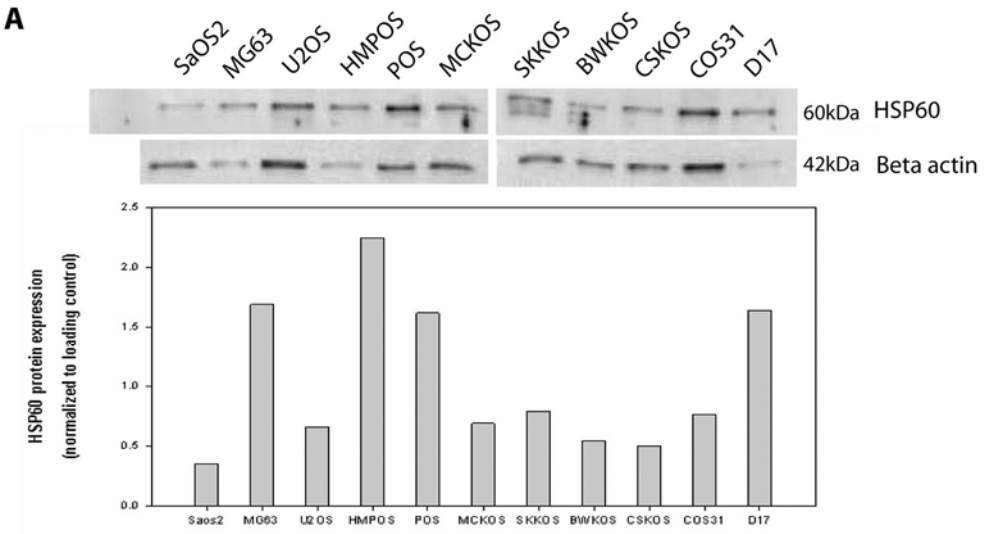
HSP10, -22, -27, -40, -70, -90alpha and -90beta mRNA expression (continuous data) did not show significant associations with survival time on univariate Cox regression analysis. However, a significant negative association between HSP60 and survival time was noted. Stratification for postoperative chemotherapy did not result in a significant change to the other HSPs, while the HSP60 association retained its significance (Table 4b). However, when all parameters with a P value <0.15 were analyzed by multivariate methods (backward stepwise LR), HSP60 did not retain its significance, and only tumor histology subtype was a strong predictor for survival among the 58 dogs with OS.

HSP60 protein expression and localization in tumors and cell lines

Because HSP60 appears to be significantly associated with poor survival based on tumor mRNA expression data, this protein was further selected for immunohistochemical analysis. A western blot analysis was carried out using total protein lysate isolated from 8 canine OSs and 3 human OS cell lines. This analysis was performed to demonstrate the cross-species reactivity and specificity of the antibody used for subsequent immunohistochemistry and to quantify expression across the cell lines. A band corresponding to 60 kDa was detected by western blot in all OS cell lines (Figure 2A). Immunohistochemistry for HSP60 was performed on 32 of the histological sections available from the tumors used for the mRNA expression analysis. HSP60 immunoreactivity was detected as granular cytoplasmic staining in the canine OS tissues, with higher expression and intensity found within the multinucleated giant cells and less expression in tumor osteoblasts (Figure 2B). A heterogeneous immunopositivity was observed within the tumor. Survival analyses revealed a trend for HSP60 protein expression comparable to the results of mRNA expression; medium / high HSP60 protein expression was detected for the 32 tumor sections. The comparison of tumors with immunopositivity based on the aforementioned classification of the 32 tumors did not reach significance for either survival (log rank score= 1.857, p=0.173) or the disease-free interval (log rank score = 1.928, p=0.165) (Figure 3). However, a trend toward poor prognosis for tumors expressing high levels of HSP60 was demonstrated on Kaplan-Meier survival analysis, with a more than 1.8-fold higher risk for death. These tests revealed no significant difference in data distribution between the two groups (high vs. low HSP60 expression) with respect to sex, neuter status, postoperative chemotherapy, body weight, histological grade and subtype (Table 5).

Figure 2: Immunohistochemistry and western blot analyses for HSP60 in canine and human OS

(A) HSP60 protein expression was normalized to loading control beta-actin expression. Variable HSP60 expression levels were detected for eleven OS cell lines (3 human cell lines, U2OS, MG63, and SaOS2, and 8 canine cell lines). All cell lines demonstrated a clear single band at 60kDa for the HSP60 antibody LK-1; (B) Immunohistochemistry of HSP60 in primary canine and human OS: a- human OS with high expression in osteoblasts and osteoclasts; b- high expression in canine OS; c- canine OS tumor negative for expression of HSP60; d- granular, cytoplasmic expression in multinucleated cells and tumor osteoblasts of canine OS; e-moderate to high expression in canine OS; f-heterogeneous expression within the same tissue section of a canine OS; g & h- scattered, weak intensity in osteoblasts; i- canine liver tissue (positive control)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U2OS
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HSP60 protein expression
(normalized to loading control)
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2.0
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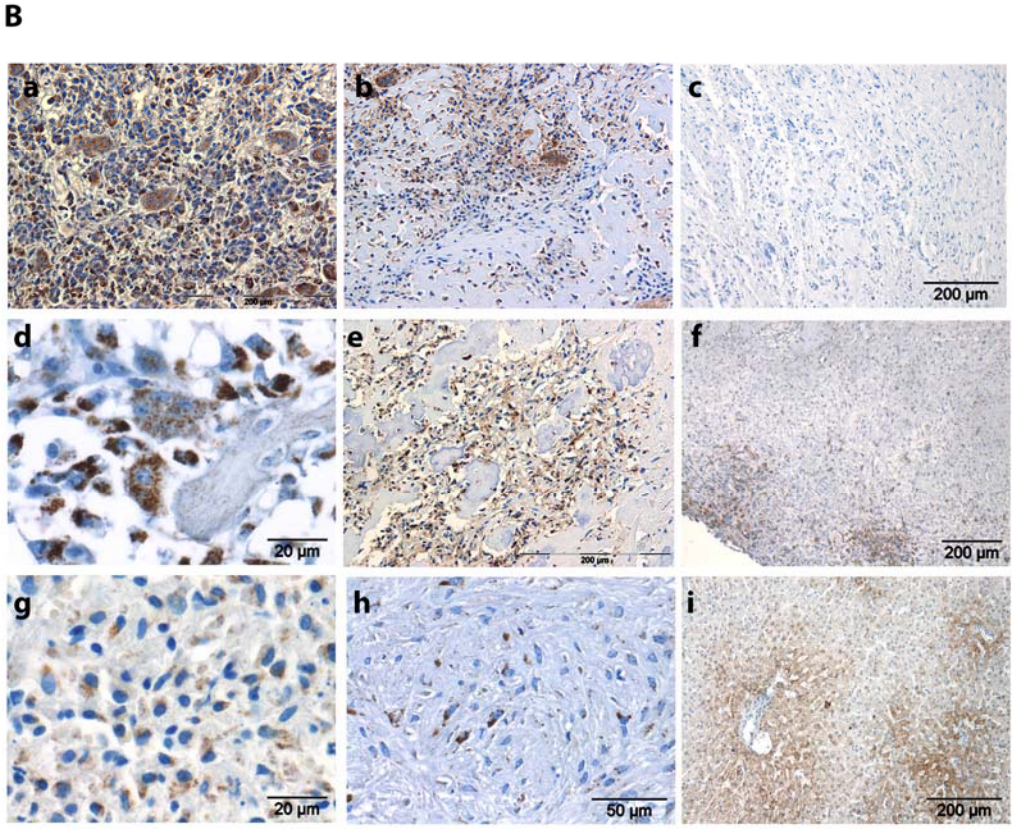
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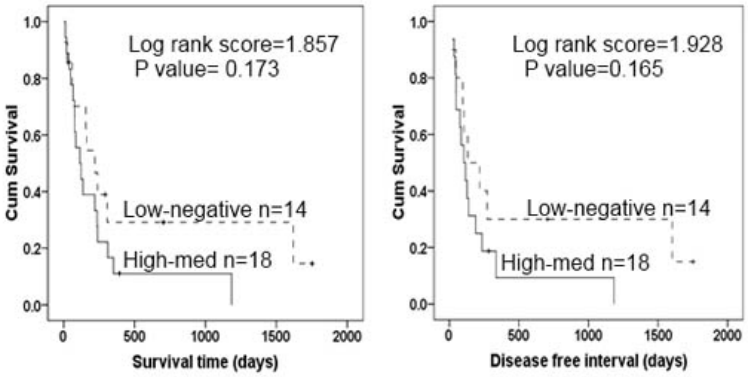

Figure 3: Kaplan-Meier survival curves for HSP60 expression in 32 canine OS primary tumors.

Table 5: Distribution of variables between the medium/high and absent/low HSP60-stained tissue sections by Fisher's exact test.

Parameter	**Absent/low HSP60 staining**	**Medium/high HSP60 staining**	**(Fisher's exact test) P value**
	n = 14	**n = 18**	
Gender			
Female	5	8	0.725
Male	9	10	
Neuter status			
Neutered	8	6	0.283
Intact	6	12	
Postoperative chemotherapy			
Yes	8	11	1.000
No	6	7	
Weight			
≤ 40 kg	6	9	0.735
> 40 kg	8	9	
Histological grade *§*			
High	8	13	0.441
Medium / Low	6	4	
Subtype *§*			
Osteoblastic	5	9	0.473
Mixed	9	8	
Median survival time (days)	191 (3-1752)	118 (13-1185)	
Median disease free interval (days) *§*	106 (0-1752)	103 (0-1185)	

§ Missing data

Down-regulation of HSP60 expression by transfection of canine–specific siRNA

To investigate the biological roles of HSP60 in canine OS, canine-specific siRNA from predicted HSP60 sequences were custom designed to selectively inhibit gene expression. Two canine OS cell lines, HMPOS and MCKOS, with metastatic characteristics were used. HSP60 siRNA-transfected cells clearly demonstrated down-regulation of HSP60 mRNA from as early as 12 h to 60 h post-transfection (Figure 4A). MCKOS was observed to have a maximum of approximately 70% HSP60 mRNA down-regulation compared to 90% in HMPOS (Figure 4B), and obvious protein down-regulation was observed from 48 to 96h for both cell lines on western blot assay (Figure 4C)

Effect of HSP60 siRNA on canine OS cell cycle and proliferation

Cell proliferation of both canine OS cell lines was significantly inhibited by HSP60 siRNA, as demonstrated by decreased total DNA content at 72h and MTT cell proliferation assays (Figure 5). However, HMPOS showed more profound growth inhibition compared to MCKOS across the 24 to 96h time points. Cells transfected with siRNA were subjected to cell cycle analysis after 72h of transfection. Tritium-thymidine incorporation after normalization for DNA content revealed significantly lower numbers of siRNA-treated HMPOS cells in the S phase of the cell cycle after 4.5h of incubation, suggesting that the growth velocity of HMPOS was inhibited. Intriguingly, MCKOS cells did not show a significant difference in tritium-thymidine incorporation.

Targeted down-regulation of HSP60 siRNA induced early apoptosis

Both cell lines demonstrated enhanced caspase-3/7 activity after siRNA transfections, suggesting that down-regulation of HSP60 in these two OS cells promotes apoptosis. The most significant activation of caspase-3/7 was observed at 24h post-transfection ($p<0.05$) for both cell lines; however, the activity was observed to decrease over time in both HMPOS and MCKOS (Figure 6A, B).

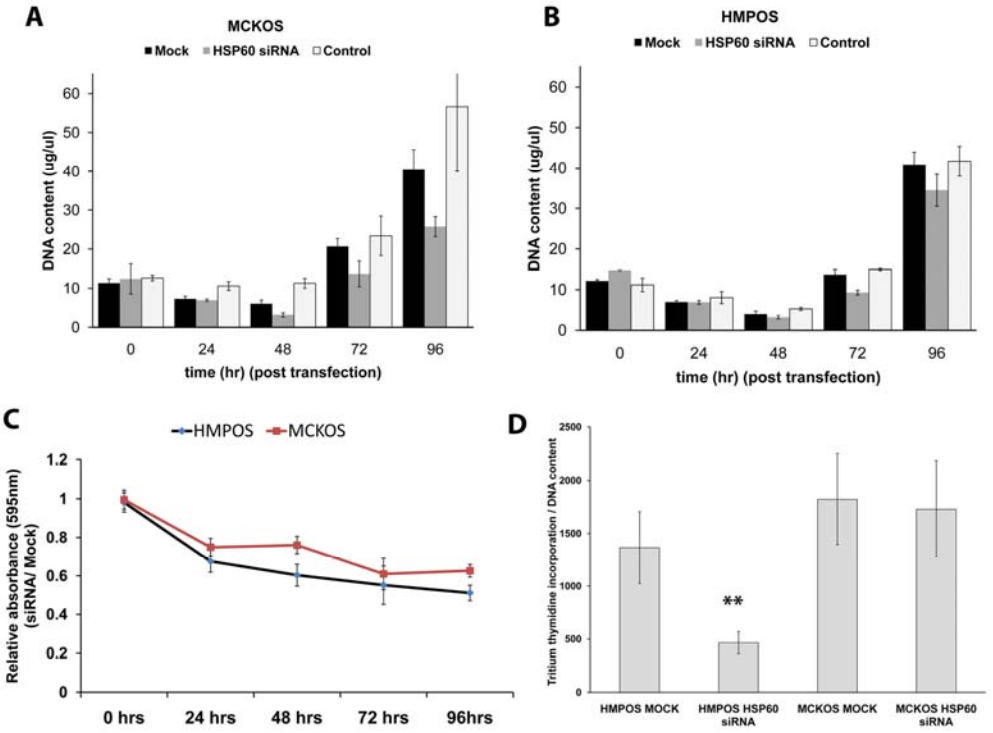

Figure 5: HSP60 siRNA inhibition of cell proliferation observed from different assays. **A, B**- Pico green DNA content assay. A significant decrease in DNA content was measured from 72 -96h post-transfection (bars represent triplicate measurements (±) SD). **C-** MTT assay demonstrates significant cell growth inhibition in both cell lines over time. **D**- Tritium-thymidine incorporation was significantly decreased in HMPOS ($P<0.001$), but no differences were observed in MCKOS cells treated with siRNA compared to mock controls (bars represent average of 6 experimental wells repeated twice, and the ratios of tritium incorporation (cpm) to the total DNA content (µg/ml) are averaged and presented with ±SD.

Effect of HSP60 siRNA on gene expression of apoptosis and cell cycle regulators

Gene expression of a cell cycle regulator (cyclin D1) and an apoptosis marker (survivin) was assessed as a consequence for HSP60 siRNA down-regulation. HSP60 down-regulation in HMPOS induced moderate survivin expression for the first 30 h, and thereafter, the expression decreased relative to the mock-transfected cells. However, the expression of survivin was consistently down-regulated in MCKOS across at all time points assessed relative to mock controls. Cyclin D1 expression ratios decreased at all time points in both cell lines. This decrease relates to the growth inhibition effect observed by HSP60 knock-down and

suggests that cyclin D1 could be one of the proteins involved directly or indirectly in the molecular chaperone functions of HSP60 (Figure 6C, D).

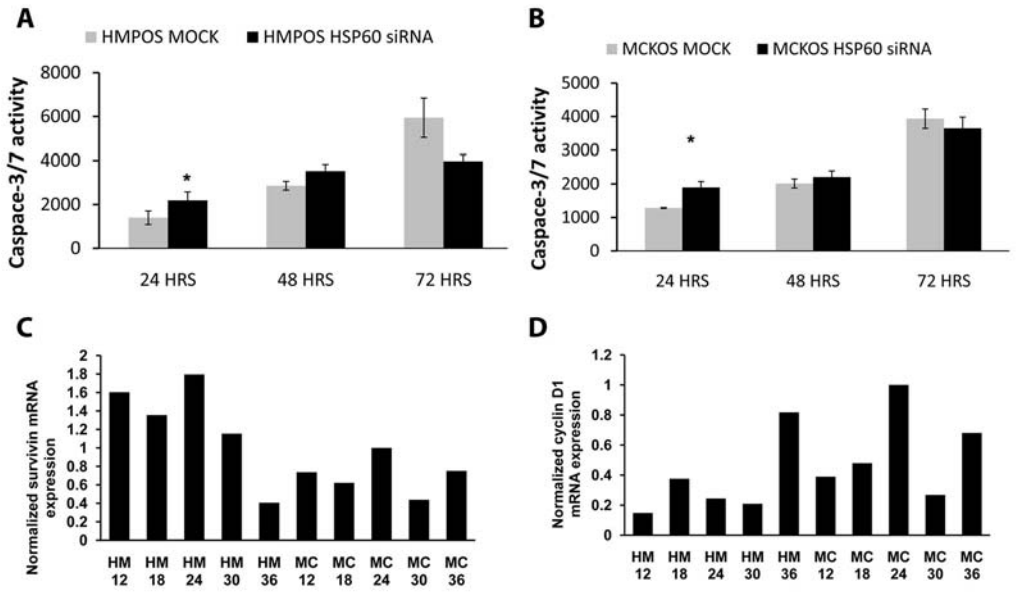

Figure 6: A, B- Apoptosis was increased significantly as observed by induction of caspase-3/7 in both MCKOS (48% increase relative to mock) and HMPOS (57% relative to mock) cells at 24 h post-transfection. A subsequent decline in caspase-3/7 activity was observed at 48 and 72h. (*p<0.05, 2-sided unpaired student's t-test). The caspase activity was calculated after subtracting background luminescence. **C, D-** Bar charts represent the relative mRNA expression in cells transfected with siRNA relative to mock-transfected cells (non-targeting RNA duplex) after normalization with reference genes (RPS5 and GAPDH). Ratios >1.2 are considered upregulated, 0.8≥ x ≥1.2 are considered as no change, and <0.8 are considered down-regulated. Bars represent the pooled mRNA gene expression from 4 experimental wells with averaged duplicate measurements from the qPCR assay.

Discussion

Clinical and pathological relevance for HSP expression in canine OS

In the present study, we evaluated the expression of several heat shock proteins in a cohort of 58 canine primary appendicular tumors and 8 OS cell lines. In accordance with previous clinicopathological studies (Garzotto et al., 2000; Kirpensteijn et al., 2002; Misdorp and Hart, 1979a; Selvarajah et al., 2009), the osteoblastic subtype and increased serum alkaline phosphatase were associated with poor survival in dogs with OS in this dataset. The relative mRNA expression of HSP60 and -10 had significant overexpression in canine OS with

predominant osteoblastic histology compared to mixed histologies (osteoblastic with chondroblastic, fibroblastic or telangiectatic combinations). In reports examining human OS, a strong correlation between HSP27 and histological subtype has been found (Moon et al., 2010). Studies on canine malignant mammary tumors have revealed enhanced HSP27 and -90 expressions compared to normal mammary gland tissues, while HSP27 is associated with invasive stages and correlates with shorter post-surgical survival in dogs. HSP27 expression in canine OS was not associated with clinicopathological parameters.

One of the major challenges of managing dogs with OS is to inhibit metastasis, as metastasis is almost inevitable and is commonly associated with resistance to conventional chemotherapies. In the canine OS tumors in this study, HSP22 was found to be significantly over-expressed in tumors from dogs that responded poorly to post-operative chemotherapy regimes. Because limited investigations have focused on the roles of HSP22 in cancer and resistance to chemotherapy, this finding deserves further exploration. However, HSP27, which functions closely with HSP22 (a small heat shock protein) (Benndorf et al., 2001), was observed to be associated with resistance to chemotherapy and poor prognosis in human OS (Uozaki et al., 1997), while inhibition of this protein was found to enhance the response to zoledronic therapy (Morii et al., 2010).

Of the 8 HSPs investigated for prognosis, only HSP60 mRNA expression was significantly associated with survival time. In a previous microarray study on canine OS tissues, HSP60, -70 and -90α mRNA expression was found to be associated with poor survival. Although we found an overexpression of HSP70 or HSP90α in poor survivors in the present study, these associations were not statistically significant. Further immunohistochemical staining for HSP70 and HSP90 on canine tumor tissue sections should clarify the prognostic value of these HSPs in canine OS.

Apart from the HSP expression in canine OS tumors, we have shown that the various HSP mRNAs are also expressed in canine OS cell lines. Additionally, among the clinically relevant HSPs from the mRNA screening in canine tumors, HSP60 was selected for further protein expression and functional analyses. HSP60 expression in all canine OS cell lines was comparable to the 3 human OS cell lines by western blot. This examination was essential to prove the specificity of the antibody for canine tissues. HSP60 protein expression was observed in osteoblast-type tumor cells, and it was highly expressed in giant cell types (osteoclasts), as demonstrated by immunohistochemistry performed on both dog and human OS tumor sections. However, not all tumors were found to express this protein, and due to the heterogeneity of expression within individual tumor sections, the expression alone did not reveal statistical significance upon prognostic evaluations. There was a clear trend of higher expression correlating with poorer outcome in dogs with OS. Unfortunately, the small number of cases made statistical analyses of the complicated data cumbersome. Although HSP60 was associated with survival time, this result should be confirmed using a larger study. Analysis of large-scale prognosis-associated gene expression is limited because of heterogeneous histology characteristics, the variable HSP60 protein expression within each

tumor, the limited clinical data available, and the non-standardized chemotherapy protocols and follow-up times. Prognostic studies for HSP60 protein expression have been performed on several human tumors, including OS (Cappello et al., 2003; Cappello, 2003; Cappello et al., 2005; Faried et al., 2004; Moon et al., 2010), and have been associated with discrepancies in the prognostic evaluation of HSP60 on human OS tissue sections by means of immunohistochemistry (Moon et al., 2010; Uozaki et al., 2000). However, the great similarity in tumor histology, characteristics and gene expression found between dog and human OS (Paoloni et al., 2009) suggests that the molecular function of HSP60 in OS pathogenesis contributes to the aggressive phenotype and deserves further exploration.

HSP60 as a relevant therapeutic target for subset of canine OS

Among the different approaches for cancer therapy, RNA interference offers a mechanism that facilitates the sequence-specific gene expression silencing of a protein that is highly expressed in tumors. This approach is normally evaluated on cancer cells *in vitro*, and the specificity for targeting a tumor cell type from the surrounding normal cells needs to be determined before they can be further translated into the clinical setting. In this study, we used small interfering RNA (siRNA) to investigate the cellular effects of HSP60 downregulation in 2 canine OS cell lines. The hypothesis was that HSP60 is essential for cell cycle and proliferation and that down-regulation would elicit anti-apoptotic effects in OS. Inhibiting HSP60 in canine cells is novel, but this approach has already shown promising effects on human cancer cells, including OS. A study on human OS cells has demonstrated that short hairpin RNA (shRNA- stable lentiviral transfection) that targets HSP60 results in cell growth arrest (Kaul et al., 2006).

By downregulating this protein *in vitro*, both canine OS cell lines demonstrated increased apoptosis for the first 24 h, which declined over time. Although cell proliferation of both MCKOS and HMPOS was significantly inhibited by down-regulation of HSP60 siRNA, tritium-thymidine incorporation was observed to be different between the two cells lines, suggesting that a different HSP60-interacting protein may be involved in cell cycle regulation. Consistent with the growth inhibition observed in the cells, gene expression analysis revealed down-regulation of cyclin D1 mRNA in both cell lines by up to 50% compared to control cells. The mRNA expression of survivin increased in HMPOS cells at early time points after transfection and declined after 36 h, although these cells demonstrates a significant increase in caspase-3/7 activity. MCKOS induction of caspase activity was consistent with a decrease in survivin mRNA expression. Acute HSP60 ablation has been shown to result in destabilization of the anti-apoptotic protein survivin and to result in increased expression of p53 and activation of p53-dependent apoptosis in tumor cells (Ghosh et al., 2008). The potential role for cytosolic HSP60 in carcinogenesis has been suggested; however, its role in tumor cell survival is controversial because both pro-apoptotic and anti-apoptotic (pro-survival) functions have been reported. HSP60 is involved in forming a complex with HSP10 and the apoptotic protein pro-caspase-3 (Samali et al., 1999). *In vitro* experiments have demonstrated a stimulatory

effect of HSP60 on pro-caspase-3 activation by cytochrome c and dATP. Therefore, HSP60 may be involved in activating pro-caspase-3 by folding/maintaining this protein in a protease-sensitive conformation. Other investigations have revealed that cytosolic HSP60 accumulation is commonly seen during apoptosis induction. However, this accumulation occurs with or without apparent mitochondrial HSP60 release. It seems that HSP60 possesses a pro-apoptotic role when a significant mitochondrial HSP60 release is present. In contrast, a pro-survival role is present when HSP60 accumulates without mitochondrial release. These variations are the consequence of differential interactions with caspase-3 (Chandra et al., 2007). The canine OS cells demonstrated early caspase-3/7 induction, which was apparent within 24 h of siRNA targeting. However, this induction decreased over time. The ratio and composition of HSP60 within the mitochondrial or cytosolic compartments in these canine cells was not investigated in this study, and further exploration is needed to determine whether the differences in caspase activity and cell cycle are due to differences in the role of HSP60 resulting from the different cellular localization.

In conclusion, canine OS primary tumors and cell lines express several HSP members. HSP60 mRNA was associated with poor clinical outcome. Heterogeneous protein expression was observed in OS of both humans and dogs. Gene silencing using siRNA approaches against HSP60 promotes apoptosis and inhibits cell proliferation of canine OS cells *in vitro,* with concomitant down-regulation of cyclin D1. Synthetic HSP60 canine-specific siRNA or compounds specifically targeting this protein *in vivo* may offer a novel therapeutic approach for canine OS and could provide an invaluable comparative spontaneous large tumor model for human OS studies.

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Chapter 10

Vitamin D 24-hydroxylase (CYP24A1) expression predicts poor clinical-outcome in people and dogs with osteosarcoma

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Abstract

The active form of vitamin D (1 alpha, 25-dihydroxycholecalciferol; calcitriol) has potent anti-proliferative activity in the management of a number of human and canine malignancies. Calcitriol functions primarily through the nuclear receptor for vitamin D (VDR), while its synthesis is mediated by CYP27B1 (1α-hydroxylase) and its degradation is mediated by CYP24A1 (24-hydroxylase). Overexpression of VDR, CYP27B1 and/or CYP24A1 has been reported in several tumors, but the prognostic relevance of these proteins has not been investigated in canine and human osteosarcoma. The objective of this study is to assess the protein expression and to evaluate their prognostic value of VDR, CYP27B1 and CYP24A1in canine and human osteosarcoma. Immunohistochemistry for VDR, CYP27B1 and CYP24A1 was performed and Kaplan-Meier survival analyses were used to evaluate the prognostic significance of these proteins in canine and human osteosarcoma using a tissue microarray approach. Aberrant expression of these proteins was observed in both human and canine osteosarcoma. The expression of VDR and its regulatory enzyme CYP27B1 are not prognosticators for either canine or human osteosarcoma. however, dogs with osteosarcoma expressing CYP24A1 had significantly poorer survival (p=0.044), while human patients expressing high levels of CYP24A1 in their tumors had a significantly shorter disease-free interval (p=0.042). Increased CYP24A1 expression was associated with poor prognosis and could serve as a predictive marker for the efficacy of vitamin D therapy in human patients and dogs with osteosarcoma. The increased expression of CYP24A1 is thought to abrogate the local anti-cancer effects of calcitriol in osteosarcoma cells. Therefore, approaches targeting CYP24A1 may enhance the anti-proliferative effects of calcitriol therapy in osteosarcoma.

Introduction

Canine osteosarcoma (OS) is a malignant tumor of bone cells that accounts for approximately 90% of primary bone tumors in the dog. These tumors are locally aggressive and highly metastatic. In dogs with no evidence of metastatic disease, surgery alone is associated with a median survival time of 3 to 6 months. Postoperative chemotherapy has proven to be beneficial in a subset of dogs, but chemotherapy-related toxicities often precede its clinical benefits. The similarities between human and canine OS make the dog a suitable pre-clinical model to study the pathogenesis of OS and response to therapies.

Vitamin D metabolism involves a series of hydroxylation reactions catalyzed by members of the cytochrome P450 family in the liver and kidneys. The active molecular form of vitamin D, 1α,25-Dihydroxyvitamin D_3 (1α,25$(OH)_2$ D_3) or calcitriol, has many physiological roles including maintenance of calcium/ phosphate homeostasis, immune cell regulation and hormone secretion. Calcitriol functions primarily through the receptor for nuclear vitamin D (VDR) while its synthesis is mediated by CYP27B1 (1α-hydroxylase) and its degradation is mediated by CYP24A1 (24-hydroxylase). The ligand 1,25-dihyroxyvitamin D_3 binds to the nuclear VDR and transcriptionally regulates the differentiation and proliferation of a wide variety of cell types (Ribeiro et al., 1995)(Pike and Meyer, 2010; St-Arnaud, 2008). Notably, calcitriol is locally produced and modulates osteoblastic proliferation and differentiation in a paracrine and autocrine manner (Anderson and Atkins, 2008).

Calcitriol has direct cytostatic and cytotoxic effects on cancer cell lines of various origins, as well as demonstrated anti-cancer activities *in vivo*. Furthermore, recent advances have shown that calcitriol enhances the anti-tumor effects of chemotherapeutic agents and other molecular-based therapies in multiple tumor types (Ribeiro et al., 1995) in both human and dogs (Akhter et al., 2001; Barroga et al., 1999; Gruber and Anuszewska, 2002; Hansen et al., 2001; Kovalenko et al., 2010). Overexpression of VDR, CYP27B1 and/or CYP24A1 has been reported for several tumors (Anderson et al., 2006; Evans et al., 1998; Horvath et al., 2010; King et al., 2010; Luo et al., 2010; Mimori et al., 2004), but the prognostic relevance of the expression of these proteins has not been investigated in canine or human OS. The objective of this study is to evaluate the prognostic values of VDR, CYP27B1 and CYP24A1 in canine and human OS primary tumors using a tissue microarray approach.

Materials and methods

Cell lines and culture conditions

Four canine cell lines (COS31, HMPOS, MCKOS and SKKOS) and two human OS cell lines (MG63 and U2OS) were maintained in a sub-confluent monolayer in Dulbecco's Modified Eagle's Medium DMEM (Invitrogen®, The Netherlands) supplemented with 10% heat

inactivated Fetal Bovine Serum (FBS) (PAA Laboratories GmbH) at 37°C in a humidified atmosphere with 5% CO_2. The cells were trypsinized and sub-cultured every 4 days.

Protein isolation and western blot analyses

Cell grown under normal culture conditions in sub-confluent layers in 6-well plastic plates (Primaria) were washed once with HBSS and incubated with RIPA buffer containing 1% Igepal, 0.6 mM phenylmethylsulfonylfluoride, 15 µg/ml aprotinin and 1 mM sodium-orthovanadate (Sigma-Aldrich Chemie BV) for 30 min on ice. Normal canine kidney and duodenum were pulverized, and protein extraction was performed according to a previously published protocol (Spee et al., 2005) Cell lysate were collected and homogenized at 10,000 rpm and 4°C, and the supernatants were collected for protein determination using the Bradford-based assay with DC Protein Assay Reagents (Bio-Rad). Protein acquired from all samples was further diluted in PBS to 1 µg/µl. The protein samples were diluted 1:1 with 2 x sample buffer containing DTT (Sigma). Afterwards, the samples were heated for 2 min at 95°C to denature the proteins. Aliquots of protein were applied to designated slots within a 12% SDS-polyacrylamide gel, and SDS-PAGE was carried-out for approximately 1.5 hrs at 100 V. The proteins were further blotted for 1 hr at 100 V on a Hybond-C Membrane (Amersham, UK). Subsequently, a 60 min blocking of the membrane was performed in 0.1% TBST (Amersham, UK, ECL™ Advance Western Blotting Detection Kit) or in 1% BSA for the VDR antibody (refer to Table 1). Primary and secondary antibody details and antibody-specific protocols are listed in Table 1. The membrane was incubated overnight at 4°C with primary antibody in 0.1% BSA in TBST followed by 15 min (3 cycles of 5 min) of washing in 0.1% TBST. Next, the membrane was incubated for 60 min with HRP-conjugated secondary antibody and was washed for 15 min (3 cycles of 5 min) in 0.3% TBST. The ECL™ Advance Western Blotting Detection Kit (Amersham) was used to facilitate detection of the desired protein bands on the Chemi-Doc imager (Bio-Rad, The Netherlands) and Quantity One Analysis software. The blots were next stripped using the Restore™ Western Blot stripping buffer (Thermo Scientific, USA) according to the manufacturer's protocol. Stripped membranes were blocked with 4% ECL in 0.1% TBST and incubated with 1:2000 dilution of mouse monoclonal anti beta actin (for loading control). Blots were further incubated in 1:20,000 dilution of anti-mouse HRP-conjugated secondary antibody. Images were processed on Image J 1.43u (National Institute of Health, USA).

Tissue microarray immunohistochemistry

Clinical outcome-linked canine and human OS tissue microarray slides were obtained from the Comparative Oncology Program, Center for Cancer Research, National Cancer Institute, Bethesda, MD. Briefly, tissue microarray slides were deparaffinized in xylene, rehydrated in decreasing alcohol dilutions and rinsed in PBS-Tween 0.1% (PBST) for 10 min (2 cycles of 5 min). Antigen retrieval was performed by heat-treating the sections in 10 mM sodium citrate buffer at pH 6 in a water bath to preserve optimal tissue architecture. Heat-treatment was initiated at 37°C, and the temperature was increased to 72°C.

Table 1: Antibodies and protocol details for immunohistochemistry and western blot analyses

Primary antibody and species raised in	**IHC (Blocking reagent)**	**IHC (Secondary antibody)**	**IHC (Primary antibody dilution)**	**WB (Primary antibody dilution)**	**WB (Blocking reagent and dilution)**	**WB (Secondary antibody)**
Anti-CYP24A1 Sigma Prestige HPA022261 (rabbit polyclonal)	20% normal goat serum in 0.1% TBST (Dako Cytomation, Denmark)	Secondary antibody: polymer DAKO anti-rabbit (Envision Polymer, K4003)	1:100 (diluted in 0.1%BSA in TBST)	1: 1000	4% ECL blocking solution powder (Amersham ECL™ Advance Western Blotting Detection Kit, RPN2108)	Goat anti-rabbit IgG, R&D systems, Mineapolis HFA008 1:20,000
Anti-CYP27B1 Santa Cruz Biotechnology sc-67260 (rabbit polyclonal IgG)	20% normal goat serum in 0.1% TBST (Dako Cytomation, Denmark)	Secondary antibody: polymer anti-rabbit DAKO (Envision Polymer, K4003)	1:300 (diluted in 0.1%BSA in TBST)	1:500	4% ECL blocking solution powder (Amersham ECL™ Advance Western Blotting Detection Kit, RPN2108)	Goat anti-rabbit IgG, R&D systems,Mineapolis HFA008 1:20,000
Anti-VDR MAI-710 (rat monoclonal)	20% normal goat serum in 0.1% TBST (Dako Cytomation, Denmark)	Secondary antibody: MILIPORE (IHCSelect®) 21543 readily used goat anti-rat IgG	1:50 (diluted in 0.1%BSA in TBST)	1:400	1% albumin from bovine serum, Sigma, A3059	Goat anti-rat IgG, AP183P Temecula, California 1:10,000

IHC: immunohistochemistry ; WB: Western Blot

Thereafter, the tissues remained immersed in the water bath for 1 h, followed by cooling of the slides within the heated citrate buffer for an additional 30 min until the temperature dropped to 30°C. The slides were then rinsed twice in PBS and incubated in blocking solution for 30 min at room temperature to reduce non-specific binding. Incubation with the primary antibody was performed overnight in a humidified chamber at 4°C (refer to Table 1 for antibody details and associated protocols). After rinsing with PBST, the sections were incubated for 15 min at room temperature with Peroxidase Blocking Reagent (S2001, DAKO) to block the endogenous peroxidase activity. Another round of rinsing with PBST was performed, and the sections were incubated with the secondary antibody for 1 hr at room temperature. Afterwards, the sections were rinsed in PBS and incubated with a DAB-chromogen mix (Sigma) for 5 min, followed by counterstaining with hematoxylin (Vector Laboratories). Finally, the sections were rinsed in running water, dehydrated in increasing alcohol dilutions and mounted with VectaMount (Vector Laboratories). Images were captured using a CCD camera mounted on an Olympus BX41 microscope linked to Cell^B imaging software (Soft Imaging Solutions GmbH, Germany). Negative control tissue sections were included for the analysis where the primary antibody was omitted and substituted with the specified concentration of the serum from the species the primary antibody was raised in or with BSA in TBST. Canine normal kidney tissues were incorporated as positive control.

Immunohistochemistry scoring method

Human and canine OS tissue microarrays were scored for VDR, CYP27B1 and CYP24A1 protein expression and localization by manual inspection at 40X objective magnifications by three independent observers who were blind to the clinical outcome data. Tissue cores were assessed according to the following criteria: Negative (0), Positive (% of positive cells [low <25%, moderate 25-50%, high >50%]; intensity [low-1 and high-2]; and localization (nuclear, cytoplasmic or both). In cases in which there were discrepancies in the scores among the 3 observers, scores from a fourth independent observer were obtained and the score with the highest consensus was forwarded for further assessment. Tissue cores with poor quality (<10% tissue availability or totally absent) were excluded from further analysis. For statistical analyses, the scores were regrouped as follows: (a) NEGATIVE, (b) LOW (<25% positive cells x low intensity) and (c) HIGH (≥25% positive cells regardless of intensity). Survival analyses were performed to compare the following groups of tumors: (1) positive versus negative immunopositivity, (2) LOW versus HIGH expression and (3) localization (where indicated). Tissues from intestines and kidney were used as positive controls.

Statistical analysis

Kaplan-Meier survival curves were drawn using SPSS 18.0 (SPSS, Inc. IBM Statistics, Chicago, USA) for group comparisons; a Mantel-Cox Log rank test was calculated with a corresponding P value (two-sided analysis with statistical significance defined at $P<0.05$).

Results

Characterization of canine and human osteosarcoma tissue microarrays

The canine OS tissue microarray utilized in this study consisted of 126 tissue cores made up of primary bone tumors, cell lines, normal tissue materials and few OS metastases. These tumors were collected from several different institutions across the United States and monitored for clinical outcome. The disease-free interval range for the canine dataset varied from 3 to 1646 days. Some cores had incomplete data regarding the primary tumor or metastasis sites, and most cores had no histology data available. Therefore, the tissue microarray was assessed only with regards to disease-free intervals and survival time and not to other clinical or pathological variables. After excluding poor quality cores (those with <10% tissue availability), 89 cores were available for assessment. Several primary tumor cores were excluded because of lack of follow up data and existing metastases (1 dog had lung metastasis at presentation). In total, 76 cores were included in the prognostic study. All cores were from appendicular sites for OS, were collected prior to chemotherapy, had undergone amputation or limb-sparing procedures and had received post-operative adjuvant therapies either with carboplatin / cisplatin / adriamycin or with other experimental therapies.

The human OS tissue microarrays consisted of primary biopsies and definitive resected primary tumors (Abdeen et al., 2009). The disease-free interval in this tumor dataset ranged from 4.1 to 230 months. Patients were censored if they died due to other causes or were lost to follow up. Briefly, majority of the tumors were from appendicular sites while there were also from craniofacial bones (n=4), pelvis (n=5) and extraskeletal (n=2) locations. On histology, tumors were mainly of osteoblastic subtype, to a lesser extent of chondroblastic, fibroblastic, giant cell rich and mixed subtypes. In total there were 60 cores available, however few cores were excluded due to poor quality from each of the array used for the different antibodies and analysis was performed on the remaining tissues available. In total, 60 cores were available for analysis; however, several cores were excluded due to poor quality from each of the array used for the different antibodies and analysis was performed on the remaining tissues available.

Verification of antibody specificity

The antibodies used in this study were validated on a panel of human and canine OS cell lines; canine normal kidney and duodenum were used to verify the specific bands corresponding to the respective molecular weights of these proteins (Additional file 1). The localization and expression of these proteins were also verified by means of immunohistochemistry (discussed in the following paragraphs).

Nuclear VDR expression and prognostic insignificance in canine and human osteosarcoma

Sixty primary canine OS tissue cores were assessed for VDR expression, in which 60% of the tumors were negative, 32% were positive with nuclear expression and 8% had weak

cytoplasmic expression with no evidence of nuclear immunopositivity. Comparatively, the 76 human OS tissues that were assessed demonstrated 33% with nuclear positivity, 63% of the tumors were negative and 4% had weak cytoplasmic expression. Immunohistochemistry on representative tumor sections from the dog and human are shown in Figure 1.

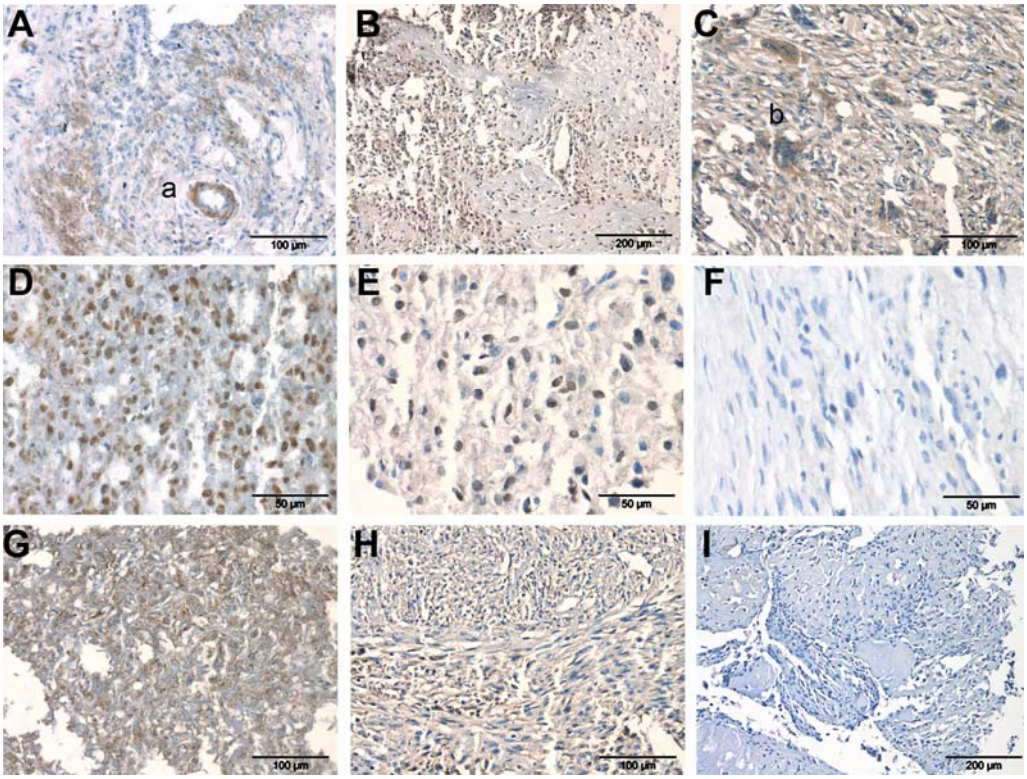

Figure 1: Immunohistochemistry for VDR, CYP24A1 and CYP27B1 in canine and human osteosarcoma. A- Canine OS: CYP24A1 with low intracytoplasmic expression, (a- blood vessels within the tumor observed to have increased expression); B- Canine OS: CYP24A1 high and intracytoplasmic expression; C- Human OS: CYP24A1- cytoplasmic expression and osteoclasts (b-multinucleated giant cells) have strong expression; D- Canine OS: nuclear VDR expression with occasional cytoplasmic expression; E- Human OS: nuclear VDR expression, scattered throughout the tumor; F- Human OS: Negative for VDR expression; G- Canine OS: Widespread intracytoplasmic expression with high intensity of CYP27B1; H- Human OS: High expression of CYP27B1 with occasional nuclear positivity; I-Canine OS: negative for CYP27B1 expression.

Kaplan-Meier analysis comparing tumors positive and negative for VDR expression in both species did not show any prognostic significance of VDR expression (Figure 2A, B). The same scenario was true when the tumors were regrouped based on their total score (intensity x %

of positive cells), as described in the “Materials and methods” section. However, a small subset of tumors with high VDR expression (15% of human OS and 10.5% of canine OS) had a tendency for poorer prognosis (Figure 2C, D).

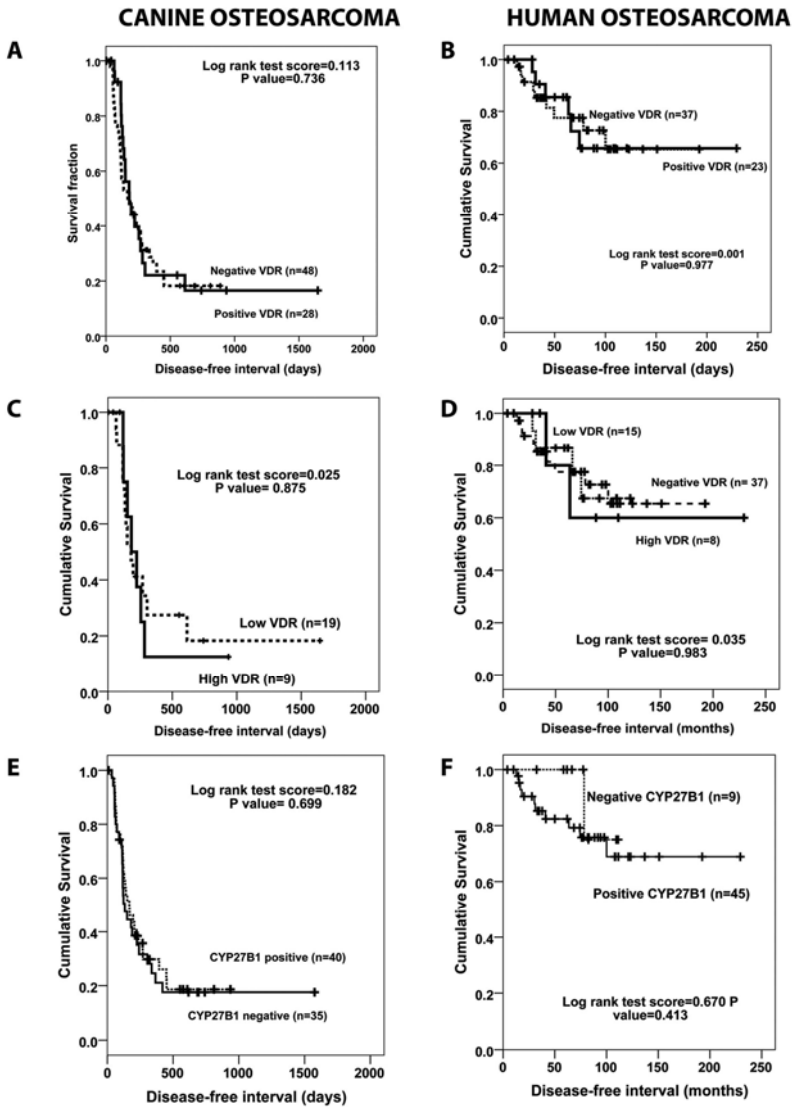

Figure 2: Kaplan-Meier survival curves show no significant prognostic value for VDR expression (A-D) and CYP27B1 expression (E-F) in canine and human OS.

High expression of CYP27B1 is more frequent in human than in canine osteosarcoma

Expression of CYP27B1 was assessed in a total of 75 tumor cores from the canine and 54 cores from the human tissue microarrays. CYP27B1 was expressed within the cytoplasm for both species, although scattered nuclear expression was detected in 3 of the human tumor cores and 4 of the canine tissue cores. Eighty-three percent of the human OS tissue cores were observed to have immunopositivity for CYP27B1, while only 53% of the canine tissue cores had detectable expression. Among the tumors positive for CYP27B1, 69% of the human OS had high expression in contrast to the dog, in which only 35% had high protein expression. The Kaplan-Meier survival analyses comparing tumors with positive expression versus negative expression did not reveal any statistically significant difference (Figure 2E, F).

CYP24A1 expression is a poor prognosticator for canine and human osteosarcoma

For CYP24A1, a total of 62 cores from the canine tissue array and 58 cores from the human OS tissue array were available for evaluation. CYP24A1 was expressed in 69% of canine and 41.4% of human OS. All positive tumors demonstrated cytoplasmic expression; however, intense nuclear staining was occasionally observed among the tumors with high CYP24A1 expression in both the human and dog OSs. The Kaplan-Meier curves generated for the canine tumors expressing CYP24A1 showed that CYP24A1 was a predictor for poor survival (P=0.044, Log rank score=4.055); however, this significance dropped upon analyzing the data in terms of the disease-free interval (P=0.072, Log rank score= 3.226) (Figure 3A, B). Segregating the canine tumors based on high versus low expression scores did not reveal any prognostic relevance for either survival time or disease-free interval (Figure 3C, D).

Comparatively, human OS were observed to be positive for CYP24A1 expression with a relatively lower frequency compared to the canine tumors, and the disease-free interval comparison for patients with or without CYP24A1 expression alone had a tendency for poorer outcome but was not statistically significant (P value=0.143, Log rank score=2.147). However, upon segregating the tumors positive for CYP24A1 into high or low expression, the human OS with high CYP24A1 had a significantly poorer prognosis (P value=0.042, Log rank score=4.146). Subsequent inclusion of negative tumors within the model resulted in an even more highly significant association of high CYP24A1 expression with poorer prognosis (P value =0.030, Log rank test score=6.989, degree of freedom=2) (Figure 3E, F).

Co-expression of nuclear VDR and CYP24A1 is not prognostic in osteosarcoma

A co-expression analysis for nuclear VDR and cytoplasmic CYP24A1 was performed for those tissue cores in which expression data were available for both proteins. In total, 50 and 57 cores were available for the co-expression analysis from human and canine OS, respectively. Of the 57 canine OS tissues, 18 were negative for both nuclear VDR and cytoplasmic CYP24A1, 22 tissues were positive for cytoplasmic CYP24A1 alone and 16 were observed to co-express nuclear VDR and cytoplasmic CYP24A1. A single canine tumor appeared to express nuclear VDR, while it was negative for CYP24A1 expression.

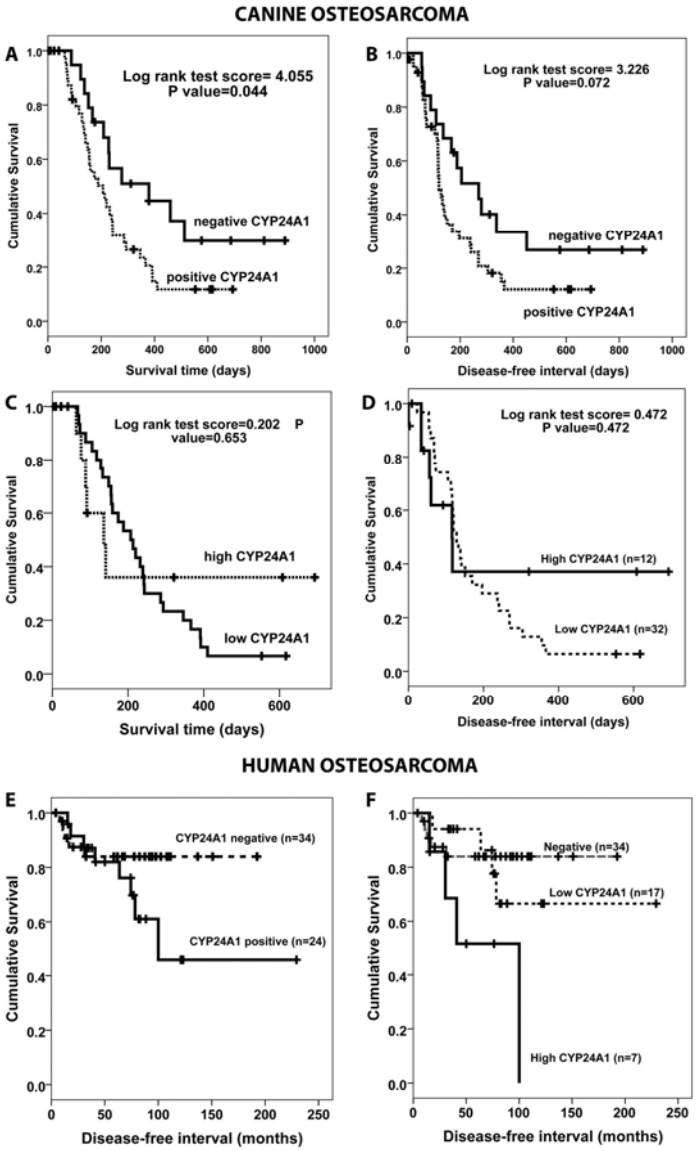

Figure 3: A-B: Kaplan-Meier survival and disease-free interval curves for canine OS comparing tumors positive for CYP24A1 expression to those without expression (expression below detection limits). CYP24A1 expression was higher in tumors of dogs with shorter survival time and disease free-interval; C-D: Canine OS tumors positive for CYP24A1 did not show any significance upon further classification of tumors into high versus low CYP24A1 expression groups. E-F: High CYP24A1 expression in human OS predicts poor clinical-outcome with shorter disease-free interval (P=0.042). Higher CYP24A1 appears to harbor a worse prognosis, but this comparison among tumors with low expression and those negative for expression did not reach statistical significance.

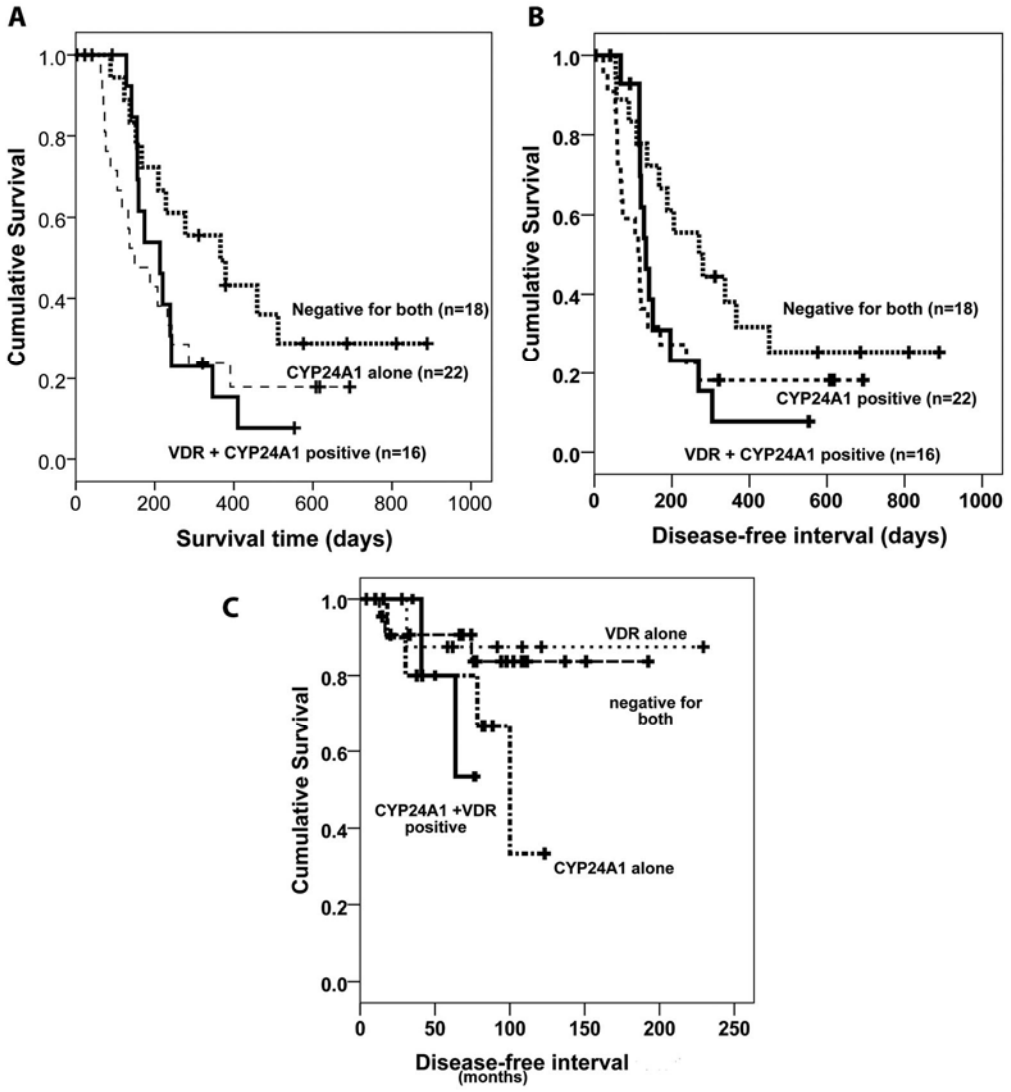

Figure 4: A-B: Canine OS with CYP24A1 and VDR co-expression did not show prognostic significance demonstrated by the Kaplan-Meier survival and disease-free interval curves. C: Human OS expressing CYP24A1 alone or in combination with VDR bears poorer prognosis compared to tumors having absence for CYP24A1 regardless of VDR expression.

A Kaplan-Meier survival analysis was performed to compare survival among the tumor groups expressing CYP24A1 alone, negative for both or co-expressing nuclear VDR and cytoplasmic CYP24A1. Canine tumors that co-expressed VDR and cytoplasmic CYP24A1 did not harbor a

statistically worse prognosis compared to the other classifications (Figure 4A, B) with P value=0.134, log rank score=4.021 for survival time and a P value=0.126, Log rank score=4.136 for disease-free interval. Like the canine OS, human tumors that co-expressed VDR and CYP24A1 did not harbor a worse outcome as compared to tumors expressing CYP24A1 or VDR alone or negative for both (P value=0.308, degrees of freedom=2, Log rank test score=3.603). However, among the human OS, only a few tumors showed nuclear VDR and cytoplasmic CYP24A1 co-expression (n=6), compared to the incidence of co-expression observed in the dog (n=16).

Discussion

Osteosarcoma (OS) is a locally aggressive tumor which has a high propensity to metastasize to distant sites both in human and canine patients. Given the similarities in histology, disease progression, response to conventional therapies and prognosis, canine OS is a relevant model for human cancer (Hansen and Khanna, 2004; Khanna et al., 2006; Paoloni and Khanna, 2008; Paoloni et al., 2009b). Recent advances have shown that both human and canine OS share strong similarities in gene expression and cellular signaling pathways for disease progression and metastasis (Khanna et al., 2004; Paoloni et al., 2009a; Selvarajah et al., 2009). Understanding the differences and similarities in the pathogenesis and protein expression in this aggressive bone tumor will allow for the development of adjuvant therapies that will benefit both species.

The study reported here was undertaken to comparatively evaluate the protein expression and prognostic value of the expression of VDR, CYP27B1 and CYP24A1 in canine and human OS using a tissue microarray approach. Active vitamin D_3 ($1,25(OH)_2D_3$; calcitriol) and its analogs are potential agents for anti-cancer therapy in various tumors. Calcitriol has potent anti-proliferative activity in the management of a number of human and canine malignancies (Akhter et al., 2001; Barroga et al., 1999; Barroga et al., 1998; Jimenez-Lara, 2007; Shany et al., 2001). The anti-proliferative effects of calcitriol are mediated through a genomic signaling pathway by binding to a specific nuclear VDR.

In the present study, immunohistochemistry for VDR in both canine and human OS showed nuclear localization; however, cytoplasmic expression was also observed, in accord with the reported nuclear and cytoplasmic VDR protein localization in osteoblasts and osteoclasts in canine and human bone (Langub et al., 2000b; Tryfonidou et al., 2010). The VDR mRNA and protein is expressed in several human and canine tumors (Anderson et al., 2006; Evans et al., 1998; Kivineva et al., 1998; Miller et al., 1997). Previous immunohistochemical analysis of a human OS cell line with osteoblastic characteristics has demonstrated the expression of nuclear VDR (Langub et al., 2000a). The cross-species comparison of VDR expression in the present study revealed that both dog and human OS have a relatively low frequency of nuclear VDR expression, which did not reveal any prognostic significance. Low

VDR expression has been observed to be a poor prognosticator for selected human cancers, such as esophageal and renal carcinoma (Blomberg Jensen et al., 2010; Mimori et al., 2004). Mutations in VDR (in which the function of VDR was not altered) have been reported in 2/57 human OS primary tumors by direct DNA sequencing (Miller et al., 1997).

In contrast to VDR expression, CYP27B1 expression was augmented in the majority (83%) of human OS, while approximately 53% of canine tumors revealed high CYP27B1 expression. This discrepancy could explain one of the reasons why canine patients with OS may have advanced disease progression relative to human patients. Dogs with osteosarcoma have a tendency to develop metastasis within 3 to 6 months and the 1 year survival is only 60%. The increased CYP27B1 expression in human tumors suggests that the tumors are actively producing the active form of vitamin D, which is in accord with the skeleton being an intracrine organ for vitamin D metabolism (Anderson 2008). Recent studies in human OS cells have shown that CYP27B1 mRNA silencing by RNAi results in the suppression of calcitriol production and subsequent reduction of vitamin D pathway target genes such as osteocalcin and CYP24A1 mRNA expression (Anderson et al., 2007). The value of inhibiting CYP27B1 expression as an approach for targeted therapy for osteosarcoma still warrants research.

CYP24A1, which encodes the enzyme 24-hydroxylase that degrades/deactivates calcitriol, is expressed in a subset of canine and human OS and is associated with poor prognosis. This observation has been reported for several other human cancer models such as esophageal cancer and colorectal carcinoma (Horvath et al., 2010; King et al., 2010; Mimori et al., 2004). In preliminary studies, canine OS cell lines treated with calcitriol show augmented expression of CYP24A1 mRNA, which is a specific and sensitive target gene for vitamin D pathway activation (unpublished data from our group) and therefore degradation of vitamin D metabolites towards less bioactive compounds. Similar observations have also been reported in human OS cell lines. Altogether, increased CYP24A1 expression by tumor cells allows a mechanism whereby CYP24A1 abrogates locally produced active vitamin D_3 and may thus reduce the anti-proliferative effects of exogenous calcitriol therapy *in vivo*. Inhibition of CYP24A1 activity using RNA interference or compounds selectively targeting this protein has shown to potentiate the anti-tumor activity of calcitriol (Muindi et al., 2010). Therefore, CYP24A1 expression may be increased in advanced or highly metastatic canine and human OS, and it not only serves as a marker for poor prognosis, but also as a relevant target for therapeutic strategies involving the use of calcitriol as an adjuvant to conventional chemotherapy.

Conclusion

The expression of VDR and CYP27B1 was not prognostic for either canine or human OS. However, increased CYP24A1 expression was associated with poor prognosis. Based on the above data, CYP24A1 could represent a predictive marker for the efficacy of vitamin D

therapy, and approaches targeting this enzyme may enhance the anti-proliferative effects of calcitriol therapy in both human and canine OS. Identification of canine and human OS patients that may respond to vitamin D therapy is necessary before it could be included as an adjuvant therapy in the management of this disease.

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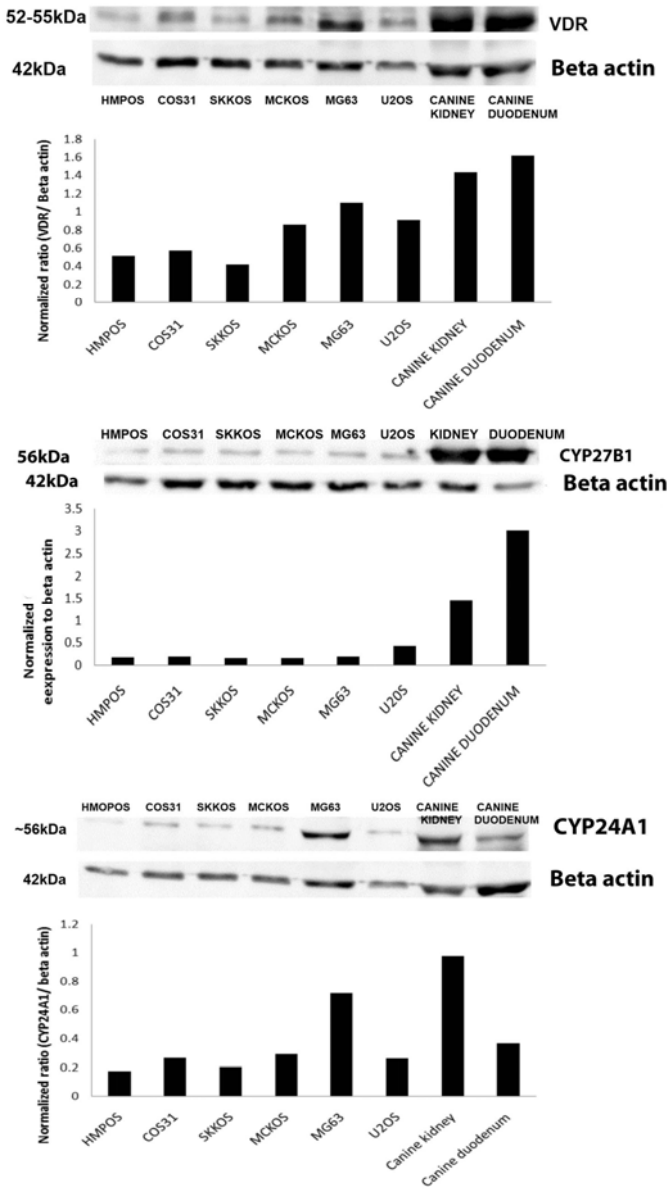
Additional file



Additional File 1: Western blot analyses identified the relevant bands of the specific molecular weight for protein expression in all canine OS cell lines comparable to the human OS cell lines. Canine normal kidney and duodenum demonstrated high total protein expression for VDR, CYP24A1 and CYP27B1 on western blot analyses.

Chapter 11

Screening for anti-proliferative effects of kinase inhibitors in four canine osteosarcoma cell lines

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Abstract

Osteosarcoma is the most common primary bone cancer in the dog and is highly malignant in nature. Dogs most often present with metastatic disease at the time of diagnosis. Various forms of therapy, from primary removal of tumors to adjuvant chemotherapy and palliative radiation, have not significantly improved the clinical outcome in dogs. Therefore, a need exists to identify novel therapeutic targets and approaches to improve clinical therapeutic benefit and outcome while reducing toxicity. Several signal transduction pathways have been suggested to be important for osteosarcoma metastasis, some of which represent attractive molecular targets for therapy. The present study utilized four well-characterized canine osteosarcoma cell lines to survey a panel of kinase inhibitors from a compound library for their capacity to cause growth inhibition in one or more of these cell lines. Cells were treated with 2 different concentrations in 96 well plates. The WST-1 cell proliferation assay was used to assess cell proliferation after exposure to the kinase inhibitors for 72h. This screening identified 22 different kinase inhibitors demonstrating significant anti-proliferative effects across the 4 canine osteosarcoma cell lines. Among them, 4 compounds showed potent and significant cell growth inhibitory effects at 10 μM with $P<0.05$. These compounds are known to inhibit signaling mediated by the EGFR, NF-κB, PKC, PKA and ERK2. Based on the preliminary assessment, more studies on toxicity and anti-metastatic potential of selected inhibitors are required before in vivo anti-cancer therapy for dogs with osteosarcoma can be initiated.

Introduction

Osteosarcoma (OS) is the most common bone sarcoma of the dog, which arises spontaneously in various skeletal locations and is highly metastatic. Despite the advancement in diagnostics for disease detection and monitoring, as well as the availability of multiple platforms for therapy, the prognosis and survival rates of diseased dogs have not changed dramatically over the past decade (Bergman et al., 1996). The major concerns for managing dogs with this malignant disease are to the ability to control micrometastasis and local recurrence. Ninety percent of dogs have metastatic disease at the time of diagnosis, most often to the lungs, and this is usually the primary end-point in managing the disease in dogs because euthanasia is then commonly recommended. Although the exact pathogenesis for OS development and progression is still poorly understood, several signaling pathways and random molecular alterations, including increased expression, mutations, deletions or gene amplifications, have been suggested. Identifying key molecular signaling mechanisms involved in OS metastasis and resistance will not only increase our understanding of the disease but will also lead to the discovery of potential targets for newer therapies.

Since the discovery of v-Src (an oncogene encoding a kinase) in 1978, kinases have been of interest as pharmacological targets across a broad spectrum of pathological conditions, including cancer, due to their pivotal roles in signal transduction and diverse cellular processes. The interest in kinases as drug targets was fuelled in recent years by the success of several kinase inhibitors in both human and veterinary clinical trials (Hahn et al., 2008; Houghton et al., 2008; Larkin et al., 2010; London et al., 2009; Perotti et al., 2010; Schlumberger, 2010). The investigations in dogs with spontaneous tumors show that they are good models for researchers to evaluate the therapeutic index of targeted therapeutics in a clinical setting (McKillop et al., 2004; Paoloni et al., 2010; Yancey et al., 2010).

Several kinases have been found to be overexpressed in canine and human OS tumors and cell lines (Gordon et al., 2008; Yang et al., 2010). Inhibitors against kinases have been developed for human research for more than a decade, and some have been approved for clinical applications in OS for humans (Keir et al., 2010; Perotti et al., 2010); however, their use and application in dogs with OS are still limited and in their infancy. Several of these kinase inhibitors have been reported to be efficacious in vitro, and very few of these have already been submitted for pharmacodynamic and pharmacokinetic studies for dogs with and without clinical metastatic OS and other solid tumors (London et al., 2009; Paoloni et al., 2010; Yancey et al., 2010). The present study utilized 4 well-characterized canine OS cell lines to survey 78 kinase inhibitors for their capacity to cause growth inhibition in one or more of these cell lines. Based on the preliminary screening assessment, the feasibility of using selected kinase inhibitors as anti-cancer therapy for dogs with OS is further discussed.

Methods

Cell lines and culture conditions

Four canine OS cell lines HMPOS, D17, MC-KOS and SK-KOS were used in this preliminary study (Barroga et al., 1999; Shoieb et al., 1998). All cells were grown in Dulbecco's minimal essential medium DMEM (Invitrogen®, The Netherlands) supplemented with 10% fetal bovine serum and maintained at 37°C in a 5% CO_2 humidified chamber. Cells were tested to be free from mycoplasma according to standard laboratory procedures. Cells were trypsinized and subcultured every 4 days.

Kinase inhibitor compound library

The kinase inhibitor compound library was kindly provided by E. de Vries from the Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands. These compounds were obtained from BIOMOL International, Plymouth Meeting, PA, USA (Enzo Life Sciences). Compounds were provided in 96-well plates at a 100-µM concentration.

Cell viability assay

A total of 3000 cells from each cell line was seeded in 96-well plates (Primaria, Falcon) and allowed to attach overnight. The cells were subsequently incubated with the kinase inhibitors to a final concentration of 1 µM or 10 µM. After 72 h of treatment, cell proliferation was established using the WST-1 cell proliferation/viability assay (Roche Diagnostics GmbH, Mannheim, Germany). In brief, 10% WST-1 reagent was added to the culture medium, and cells were incubated for 45 min. The optical density at 450 nm was measured in an ELISA microplate reader (BioTek, Beun de Ronde, Abcoude, The Netherlands). The viability of the compound-treated cells was calculated relative to that of the vehicle control wells containing 1% dimethylsulfoxide DMSO (v/v). Positive control wells contained the pan inhibitor, staurosporine in 1% DMSO (v/v). Experiments were performed in triplicate.

Statistical analysis

One-way ANOVA was performed for each individual cell line to determine whether there was a significant difference in cell viability between the compound- and the control-treated wells. A total of 80 compounds were assessed: 78 kinase inhibitors, staurosporine and DMSO-treated control cells. Post hoc analyses were performed using Games-Howell tests to determine which compounds statistically differed from the control ($p<=0.05$ or $p<=0.005$ where indicated).

Results

Cell line characteristics

Four previously established canine OS cell lines with different morphologies, cell proliferation rates and anchorage independent growth abilities (unpublished data) were used for the preliminary screening of 78 different kinase inhibitors. Among these cell lines, HMPOS, MC-KOS and D17 are highly metastatic cell lines that form large colonies in anchorage independent growth conditions, whereas SK-KOS forms very few and small colonies, in comparison to the other cell lines; however, this cell line was observed to have a much higher proliferation rate by the MTT and [H^3]-thymidine incorporation assays when grown on plastic (data not shown). Morphologically, HMPOS appears as epithelial-like polygonal cells; SK-KOS and MC-KOS have elongated, fibroblastic-like features while D17 resembles polygonal stretched-type cells.

Anti-proliferative effects of kinase inhibitors in canine OS cell lines

A total of 82 compounds were available in the kinase inhibitor library. Three compounds served as specific negative controls and staurosporine, a broadly selective and potent kinase inhibitor was included as a positive control. In total, 78 different compounds inhibiting different kinases were screened in this preliminary study. The complete list of compounds available in the library and their targeted kinase and/or signaling pathways is listed in Table 1. The percentage of viable cells after treatment with 10 μM of each drug relative to the DMSO control was calculated (Figure 1). One-way ANOVA showed that there was a significant difference between the cell viability for control wells and the compounds for MCKOS ($F(80,162) = 35.30$, $p < 0.05$), HMPOS ($F(80,162) = 11.00$, $p < 0.05$), SKKOS ($F(80,162) = 9.36$, $p < 0.05$), and D17 ($F(80,162) = 17.04$, $p < 0.05$) by post-hoc analysis.

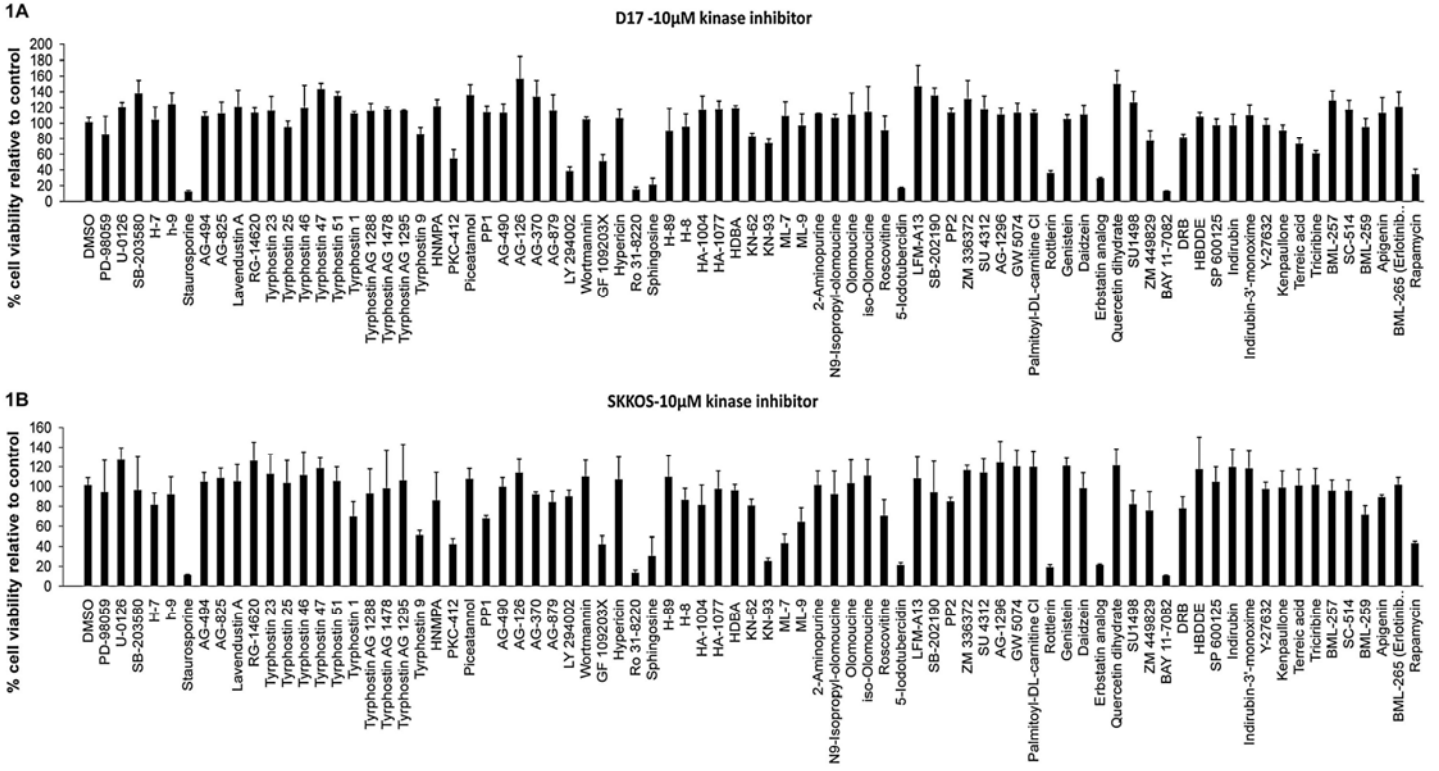
In Figure 2, those compounds that caused cell growth inhibition with $P<0.05$, as determined by post-hoc analysis across the 4 cell lines at a concentration of 10 μM, are depicted. Their corresponding P values are listed in Table 2. In total, 22 out of the 78 kinase inhibitors tested exhibited anti-proliferative effects with more than 50% growth inhibition with varying degree of sensitivity across the cell lines. Among the 4 cell lines investigated, MCKOS was observed to be the most sensitive to the anti-proliferative effects of kinase inhibitors, and more compounds exhibited >50% growth inhibition in this cell line, compared to the remainder: MC-KOS was sensitive to 18/78 compounds, D17 was sensitive to 12/78 compounds, SKKOS was sensitive to 11/78 compounds and HMPOS was sensitive to 6/78 compounds. Among the 22 compounds, 10 exhibited significant anti-proliferative effects unique for a particular cell line, 8 compounds in at least 3 cell lines and only 4 compounds were observed to have potent anti-proliferative effects across all 4 cell lines (Erbstatin, RO 31-8220, BAY 11-7082 and 5-iodotubericidin). Three other kinase inhibitors (Rottlerin, PKC-412 and Rapamycin) demonstrated significant anti-proliferative effects in 3 of the 4 cell lines investigated.

Table 1: Kinase inhibitors within the compound library tested on canine OS cells.

COMPOUND	TARGET / PATHWAY	COMPOUND	TARGET/ PATHWAY
PD-98059	MEK	ML-7	MLCK
U-0126	MEK	ML-9	MLCK
SB-203580	p38 MAPK	2-Aminopurine	p58 PITSLRE beta1
H-7	PKA, PKG, MLCK, and PKC.	N9-Isopropyl-olomoucine	CDK
H-9	PKA, PKG, MLCK, and PKC.	Olomoucine	CDK
Staurosporine	**Pan-specific**	iso-Olomoucine	**Negative control for olomoucine.**
AG-494	EGFRK, PDGFRK	Roscovitine	CDK
AG-825	HER1-2	5-Iodotubercidin	ERK2, adenosine kinase, CK1, CK2,
Lavendustin A	EGFRK	LFM-A13	BTK
RG-14620	EGFRK	SB-202190	p38 MAPK
Tyrphostin 23	EGFRK	PP2	Src family
Tyrphostin 25	EGFRK	ZM 336372	Craf
Tyrphostin 46	EGFRK, PDGFRK	SU 4312	Flk1
Tyrphostin 47	EGFRK	AG-1296	PDGFRK
Tyrphostin 51	EGFRK	GW 5074	cRAF
Tyrphostin 1	**Negative control**	Palmitoyl-DL-carnitine Cl	PKC
Tyrphostin AG 1288	Tyrosine kinases	Rottlerin	PKC delta
Tyrphostin AG 1478	EGFRK	Genistein	Tyrosine Kinases
Tyrphostin AG 1295	Tyrosine kinases	Daidzein	**Negative control for Genistein.**
Tyrphostin 9	PDGFRK	Erbstatin analog	EGFRK
HNMPA	IRK	Quercetin dehydrate	PI 3-K
PKC-412	PKC inhibitor	SU1498	Flk1
Piceatannol	Syk	ZM 449829	JAK-3
PP1	Src family	BAY 11-7082	IKK pathway
AG-490	JAK-2	DRB	CK II
AG-126	IRAK	HBDDE	PKC alpha, PKC gamma
AG-370	PDGFRK	SP 600125	JNK
AG-879	NGFRK	Indirubin	GSK-3beta, CDK5
LY 294002	PI 3-K	Indirubin-3'-monoxime	GSK-3beta
Wortmannin	PI 3-K	Y-27632	ROCK
GF 109203X	PKC	Kenpaullone	GSK-3beta
Hypericin	PKC	Terreic acid	BTK

Ro 31-8220	PKC	Triciribine	Akt signaling pathway
Sphingosine	PKC	BML-257	Akt
H-89	PKA	SC-514	IKK2
H-8	PKA, PKG	BML-259	Cdk5/p25
HA-1004	PKA, PKG	Apigenin	CK-II
HA-1077	PKA, PKG	BML-265 (Erlotinib analog)	EGFRK
HDBA	EGFRK, CaMK II	Rapamycin	mTOR
KN-62	CaMK II		
KN-93	CaMK II		

Figure 1: Bar chart represents the % cell viability observed upon treatment with the various kinase inhibitors at 10-µM concentration in canine OS cell lines: (a) D17; (b) SKKOS; (c) HMPOS and (d) MCKOS. Several kinase inhibitors caused increases in cell proliferation, whereas others showed marginal cell growth inhibition. A small number of compounds were found to have significant anti-proliferative effects. Calculated % is the average of cell viability from treated cells relative to control wells (treated with DMSO) ±SD and were based on average of 3 experiments.



1C
HMPOS-10μM kinase inhibitor
% cell viability relative to control
0
20
40
60
80
100
120
140
160
180
200
DMSO
PD-98059
U-0126
SB-203580
H-7
h-9
Staurosporine
AG-494
AG-825
Lavendustin A
RG-14620
Tyrphostin 23
Tyrphostin 25
Tyrphostin 46
Tyrphostin 47
Tyrphostin 51
Tyrphostin 1
Tyrphostin AG 1288
Tyrphostin AG 1478
Tyrphostin AG 1295
Tyrphostin 9
HNMPA
PKC-412
Piceatannol
PP1
AG-490
AG-126
AG-370
AG-879
LY 294002
Wortmannin
GF 109203X
Hypericin
Ro 31-8220
Sphingosine
H-89
H-8
HA-1004
HA-1077
HDBA
KN-62
KN-93
ML-7
ML-9
2-Aminopurine
N9-Isopropyl-olomoucine
Olomoucine
iso-Olomoucine
Roscovitine
5-Iodotubercidin
LFM-A13
SB-202190
PP2
ZM 336372
SU 4312
AG-1296
GW 5074
Palmitoyl-DL-carnitine Cl
Rottlerin
Genistein
Daidzein
Erbstatin analog
Quercetin dihydrate
SU1498
ZM 449829
BAY 11-7082
DRB
HBDDE
SP 600125
Indirubin
Indirubin-3'-monoxime
Y-27632
Kenpaullone
Terreic acid
Tricirbine
BML-257
SC-514
BML-259
Apigenin
BML-265 (Erlotinib..
Rapamycin
1D
MCKOS-10μM kinase inhibitor
% cell viability relative to control
0
20
40
60
80
100
120
140
160
180
200
DMSO
PD-98059
U-0126
SB-203580
H-7
h-9
Staurosporine
AG-494
AG-825
Lavendustin A
RG-14620
Tyrphostin 23
Tyrphostin 25
Tyrphostin 46
Tyrphostin 47
Tyrphostin 51
Tyrphostin 1
Tyrphostin AG 1288
Tyrphostin AG 1478
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AG-879
LY 294002
Wortmannin
GF 109203X
Hypericin
Ro 31-8220
Sphingosine
H-89
H-8
HA-1004
HA-1077
HDBA
KN-62
KN-93
ML-7
ML-9
2-Aminopurine
N9-Isopropyl-olomoucine
Olomoucine
iso-Olomoucine
Roscovitine
5-Iodotubercidin
LFM-A13
SB-202190
PP2
ZM 336372
SU 4312
AG-1296
GW 5074
Palmitoyl-DL-carnitine Cl
Rottlerin
Genistein
Daidzein
Erbstatin analog
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Y-27632
Kenpaullone
Terreic acid
Tricirbine
BML-257
SC-514
BML-259
Apigenin
BML-265 (Erlotinib..
Rapamycin

Table 2: Twenty-two kinase inhibitors demonstrated anti-proliferative effects in 4 canine OS cells. P values are depicted in ** for those with P≤0.005 and in * for those with P>0.005 – P<0.05 significance in cell growth inhibition. Those left blank did not show significant anti-proliferative effects

Kinase inhibitor/ compound	**MCKOS**	**HMPOS**	**SKKOS**	**D17**
PD-98059	p=0.030*			
Staurosporine	p=0.002**	p=0.008*	p=0.000**	p=0.001**
Tyrphostin AG 1295				p=0.021*
Tyrphostin 9			p=0.035*	
PKC-412	p=0.011*	p=0.014*	p=0.034*	
PP1	p=0.024*		p=0.033*	
AG-370	p=0.008*			
LY 294002				p=0.029*
Ro 31-8220	p=0.002**	p=0.003**	p=0.004**	p=0.005**
Sphingosine	p=0.01*			p=0.041*
KN-93	p=0.035*		p=0.008*	
ML-7	p=0.02*			
ML-9	p=0.037*			
GW 5074	p=0.048*			
2-Aminopurine	p=0.038*			p=0.033*
5-Iodotubercidin	p=0.004**	p=0.005**	p=0.005**	p=0.001**
Palmitoyl-DL-carnitine Cl	p=0.004**			
Rottlerin	p=0.016*		p=0.005**	p=0.011*
Erbstatin analog	p=0.004**	p=0.015*	p=0.001**	p=0.002**
BAY 11-7082	p=0.001**	p=0.002**	p=0.000**	p=0.000**
Triciribine				p=0.039*
Rapamycin	p=0.03*		p=0.007*	p=0.038*

Figure 2: Kinase inhibitors that demonstrated significance of P<0.05 as determined by post hoc analysis are depicted in **black boxes**. The anti-proliferative effect of each compound was calculated relative to the control wells treated with 1% dimethylsulfoxide (DMSO) (v/v).

Discussion

This study provides preliminary insights for potential use of protein kinase inhibitors in canine OS by screening their anti-proliferative effects in a panel of established cell lines. Twenty-two of 78 kinase inhibitors demonstrated a significant anti-proliferative effect in canine OS cells at a concentration of 10 μM. The lower concentration of drug exposures most often did not elicit significant anti-proliferative effect across the cell lines and some even caused increased cell proliferation compared to the controls. This finding further suggests that the variable response to the anti-proliferative effects of kinase inhibitors measured across the cells may be due to the range of diverse kinase expression, molecular alterations and the degree of various signal transduction pathway activation, all which are cell line specific. Several kinase inhibitors in this library were expected to increase cell proliferation, including inhibitors against glycogen synthase kinase 3β (GSK3-β). The inhibition of this kinase triggers a cascade of cellular events involving different signaling pathways, which among them promotes destruction of the β-catenin degradation complex and, hence, activates the Wnt/β-catenin canonical signaling, which is a pathway that is important for cell proliferation and differentiation. Based on the preliminary screening, 8 of the compounds exhibited profound anti-proliferative effects on at least 3 of 4 canine OS cell lines. The potential use and current preclinical / clinical status of these kinase inhibitors for canine OS therapy is discussed in the following paragraphs.

PKC inhibitors

Among the 8 kinase inhibitors observed to have potent activity against cell proliferation, 4 of these compounds target PKC including RO 31-8220, PKC-412, Rottlerin and Staurosporine. Although it was noted that Staurosporine was incorporated as a positive control for this kinase screening due to its potential as a pan inhibitor, its potent anti-proliferative properties is mainly through its PKC inhibitory activity and has been shown to inhibit metastasis in cancer models (Schwartz et al., 1990). Several PKC isoforms are expressed in canine OS cell lines, and RO 31-8220 has been shown to inhibit cell migration of canine OS cell lines in vitro (Hong SY et al. personal communication) by targeting PKC and, hence, suppressing the C-terminal phosphorylation of ezrin/ radixin/ moesin (ERM) proteins. These ERM proteins and PKC are commonly expressed by cells with metastatic phenotypes, including human and canine OS (Ren et al., 2009). Among the cell lines investigated in the present study, cell proliferation of MCKOS and SKKOS was shown to be significantly inhibited by 10 μM RO 31-8220, consistent with the results reported by Hong S.Y (personal communication). In addition, studies on PKC-412 have shown anti proliferative effects in 3 human OS cell lines by down regulation of phosphorylation of PKC-α (Kawamoto et al., 2008), which was found to be phosphorylated and expressed in all of the cell lines studied. From the current studies on 4 canine OS cells, PKC-412 was able to inhibit proliferation significantly at both 10-μM and 1-μM concentrations, in agreement with the aforementioned study on human OS cell lines.

5-iodotubercidin

In addition to PKC, the compound 5-iodotubercidin, which has multiple kinase targets, showed pronounced anti-proliferative effects in all the 4 canine cell lines. This compound was discovered in the early 1990s as a potent inhibitor of adenosine kinase and Ser/Thr-specific kinases, including casein kinase 1/2 (CK1/2) and protein kinase A (PKA) (Massillon et al., 1994). This compound also competitively inhibits the mitogen-activated protein kinase (MAPK), ERK2 (extracellular regulated-signal kinase 2). Although the expression of the CK1 and MAP kinase pathway related proteins in canine OS is unknown at present, studies have shown that human OS and, to a lesser extent, benign bone tumors express MAPK and ERK proteins (Li et al., 2009) (Abdeen et al., 2009a) and represent attractive targets for therapy. Because 5-iodotubercidin has a broad spectrum of targets, this compound is still under in vitro investigation and has not successfully translated into clinical settings.

BAY 11-7082

Nuclear factor-kappaB (NF-kappaB) is constitutively activated in many types of tumors and plays a key role in promoting cancer cell proliferation, survival, and invasion. A pivotal regulator of all inducible NF-kappaB signaling pathways is the I-kappaB kinase (IKK) complex which is the specific target of BAY 11-7082. This kinase inhibitor showed profound cell growth inhibition at a concentration of 10 µM across all 4 canine OS cell lines. Limited studies have evaluated the potential for this kinase inhibitor in OS or other canine tumors, although there are an increasing number of related compounds that target IKK through the NF-kappa beta signaling that are emerging as anti-cancer therapies for human cancers. Gene expression profiling of human OS cell lines revealed that high metastatic clones have distinct enrichment for genes associated with the NF-kappaB signaling pathway, which may have a strong anti-apoptotic function.

Rapamycin, mTOR inhibitor

Rapamycin is a macrocyclic lactone antibiotic that was originally investigated as an anticancer agent in the 1980s. It has been shown to inhibit the development of OS in murine models and pediatric OS xenografts (Ren et al., 2009). As a serine/ threonine kinase, mTOR controls mRNA translation and regulates cell growth and proliferation. Signaling molecules upstream and downstream of the mTOR pathway were found to be deregulated with aberrant expression in several types of human cancer including OS (Gordon et al., 2008). In vitro investigations on human OS and other solid tumor cell lines showed that rapamycin potently inhibited cell cycle but is a less potent inducer of apoptosis with variable response across the different cell lines tested (Houghton et al., 2008)(Gazitt et al., 2009). In the present study, rapamycin significantly inhibited cell proliferation in 3 canine OS cell lines. This further supports several in vivo findings in dogs with metastatic OS. Pharmacodynamic and pharmacokinetic of rapamycin in dogs with OS demonstrated modulation of the mTOR pathway in their tumors,

which may yield therapeutic efficacy (Paoloni et al., 2010). The results highlight canine OS as an invaluable model for translational cancer medicine.

Erbstatin analog

Erbstatin is among the earliest discovered tyrosine kinase inhibitors that target the epidermal growth factor receptor tyrosine kinase (EGFR). Epidermal growth factor receptors are expressed in both canine (Chapter 8 of this thesis) and human OS primary tumors, metastatic tissues and cell lines (Abdeen et al., 2009b; Do et al., 2009; Kersting et al., 2007). In total, 13 different inhibitors for EGFR were present within the compound library tested in this study. However, only Erbstatin demonstrated significant cell growth inhibition among all 4 cell lines. Recent studies by our research group found that EGFR protein expression was aberrantly expressed in the canine OS primary tumor. However, expression alone was not prognostic, with discrepancies from poor to good prognosis in studies on human OS (Do et al., 2009; Kersting et al., 2007). An exclusive subset of dogs with high EGFR expression in their tumors was found to have poorer prognosis, making EGFR a relevant target for therapy for a subset of aggressive canine OS. We have also demonstrated that all four canine OS cell lines used in this study do express EGFR mRNA. The selectivity of Erbstatin to cause growth inhibition more potently, compared to the other EGFR kinase inhibitors, alone or in combination with other kinase inhibitors, is not fully understood and requires further exploration.

Previous studies on tyrosine and other protein kinase inhibitors have utilized one to two cell lines for in vitro preclinical assessment (Wan et al., 2005; Wolfesberger et al., 2010). However, here we demonstrate that variability in anti-proliferative and cytotoxicity does exist among in vitro models of the same tumor type and hence a larger screening panel of cell lines is required for in vitro canine pre-clinical studies. The 4 cell lines exhibited variable anti-proliferative effects across the different compounds, which can be explained by the variation in protein kinase expression and molecular alterations (phosphorylated status or activating mutations) that may exist in these cell lines leading to differences in targeting sensitivity. The variation seen in vitro may mimic the situation in vivo, where a subset of dogs will respond to a specific kinase inhibitor depending on the signaling pathways activated at a particular stage of the disease, whereas others may not respond.

Screening of a large compound library in vitro helps narrow down the large panel of anti-cancer drugs to the most potent candidates for further validation and eventual translation into a clinical setting. Although the present kinase inhibitor library screen revealed only 22 compounds with anti proliferative effects across the canine OS cell lines at a concentration of 10 μM, the remainder of the compounds may still elicit this effect at much higher concentrations. However, increased concentration may have also profound toxic effects that may not be only specific for targeted cancer cells. The variability in growth inhibition of these cell lines in response to treatment with inhibitors for protein kinases may be partly due to off-target effects that are independent of a particular pathway of a target. The anti-proliferative effects of these compounds in synergy with the common chemotherapy drugs used in

practice e.g., anthracyclines or platinum-based cytotoxic drugs or by dual combination with other protein kinase inhibitors warrants further research for canine OS. Targeted anti-cancer therapy may show greater promise if directed to molecularly defined subsets of OS. Ultimately, prospective profiling of individual OS tissues may allocate signature-directed therapy based on molecular defects identified at the bench.

Conclusions

The present study highlights several potent kinase inhibitors targeting PKC, CK1, PKA, EGFR, mTOR and NF-κB pathways for canine OS that warrant dose response and other *in vitro* molecular and cell based investigations before including dogs for clinical investigations. Several challenges remain before inhibitors of protein kinase can be incorporated in the therapeutic management of dogs with OS, including defining those dogs that are most likely to respond based on the tumor molecular signatures, establishing and evaluating their biologic activity in the phase of clinical and / or microscopic disease, and investigating strategies to combine them with the current standard therapeutics, such as chemotherapy and radiation.

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Chapter 12

General discussion

The contemporary therapeutic approach for dogs with osteosarcoma (OS) is comprised of initial extirpation of the primary tumor in the affected bone, followed by post-operative chemotherapy with palliative radiation therapy for those with incomplete surgical margins. Primary canine OS pathogenesis and disease progression are facilitated by diverse molecular alterations and activation or deactivation of signal transduction pathways. This thesis explores several of the molecular targets which can be used as prognostic markers and potential therapeutic targets for canine OS. Several of the cellular pathways which could contribute to OS disease progression and the aggressive nature of the disease were also investigated and discussed. A few of the markers identified for canine OS are also investigated in human OS; these could serve as a common marker across species.

New insights on gene expression profiling studies in canine OS

Canine genome data has been published and readily available for researchers to have the opportunity to understand the genomics of canine diseases. There are, however, still some limitations with regards to the annotation of the canine genes to date. The cDNA microarray technology used for two of the gene expression profiling studies in this thesis was among the first approaches to profiling gene transcripts for canine OS, when canine oligo microarrays were not yet commercially available. The first of the two gene expression profiling studies was performed to identify differences in transcript profiles between groups of primary canine OS based on clinical-outcome data (survival time). Tumors from dogs with survival time less than 6 months can be distinguished from tumors of dogs with better survival (>6 months) by expression of a 51 gene signature. This signature further sub-classifies short-survival dogs with 2 distinct gene profiles which are strongly associated with a different survival and disease-free interval in this study. The second profiling study was performed to identify gene transcripts that differentiate two canine OS cell lines with differential metastatic potentials. A high-metastatic cell line, which is a sub-line of a primary canine OS, has a distinct molecular profile compared to its less metastatic primary line.

A gene profile which differentiates dogs based on prognosis is made up of candidate genes reported to be involved in drug resistance, metastasis and cell proliferation/cell cycle. Although the genes were classified into functional groups based on reports from other types of tumors and diseases across a variety of species, these need to be verified and validated on independent samples of OS tissues to determine if the roles are conserved across species and tissue type. Because OS is a bone tumor, some of the genes and pathways may be regulated differently from other types of solid or blood tumors. This makes continuous validation necessary for individual candidate or groups of genes that are differentially expressed or regulated in the same manner. Such validation will provide more insights on the different levels of gene involvement and interaction in the pathobiology of this disease.

Gene expression studies may generate massive amounts of data with the help of various technologies and bioinformatics. These data may be analyzed and re-analyzed using many different methods to increase the understanding and biological significance of the profiles generated for a particular disease -- in this case, for canine OS. Gene profiles allow us not only

to identify individual genes or groups of genes that are co-regulated, but also to map genes based on their basic interactions among the different cellular components and to study profiles based on enrichment for a particular signal transduction pathway. Several of the new prognostic markers revealed by the studies described in this thesis are further discussed, along with important signaling pathways for canine OS and a few for human OS.

New prognostic markers and potential therapeutic strategies for canine and human OS

Several candidate genes from the cDNA microarray gene expression profiling studies were selected for further investigation in independent studies described in this thesis. The candidate markers for aggressive phenotypes in canine OS were evaluated on an independent group of canine OS tissues that were either snap-frozen or formalin fixed paraffin-embedded materials. Canine and human OS tissue microarrays were incorporated to evaluate the prognostic value of a few of these markers by means of immunohistochemistry. A number of the attractive markers for prognosis and metastasis are discussed below.

Decorin (DCN), an extracellular matrix protein, was observed to be highly expressed in the high metastatic canine OS cell line. Its expression in primary tumors was found to correlate with survival in dogs. Decorin protein expression was found to be high in both primary tumors and metastases of the dog and in 25% of human primary OS; remarkably, this had the worst clinical outcome. One of the examples given for this scenario included the identification of decorin, a bone proteoglycan over-expressed in the high metastatic canine OS cell line and expressed in a subset of human OS tumors where this small subset corresponded to poorer clinical outcome. In several other tumor types (Matsumine et al., 2007; Reed et al., 2005; Ricciardelli et al., 1998), decorin has been described as a tumor suppressor where it is commonly downregulated in cancer as compared to normal tissue counterparts. This finding is based solely on immunohistochemistry evaluation on tissue microarrays. However, another report on *in vitro* human OS models revealed that decorin is expressed in one of the cell lines in which, upon gene manipulation using siRNA techniques, it appears to interfere with cell migratory abilities of that particular cell line. This observation was not consistent with investigations from another human OS cell line and murine OS cell lines. This suggests that the decorin expression may be increased in a subset of OS, also reported by a gene profiling study on human OS addressing different subtypes (Kubista et al., 2010); the expression may indicate poorer outcome, but absence of decorin expression may not be of value for prognosis.

Epidermal growth factor receptor (EGFR) is a member of the tyrosine kinase receptor family, which is expressed in 35% of primary canine OS and produced a predominantly cytoplasmic-membranous expression. Although stratifying tumors based on expression “yes” or “no”

alone did not reveal any prognostic value, a small subset of these positive tumors with widespread positivity had a significantly poorer prognosis (both in DFI and ST) when compared to tumors expressing low or absence for EGFR expression. EGFR expression was also found to be significantly overexpressed in primary snap-frozen canine OS compared to normal canine bones and the majority of the metastases. EGFR expression has been closely associated with other prognostic molecular markers such as PTEN and p53 tumor suppressor genes. It was described for human gliomas that EGFR expression may be regulated at transcriptional level by p53 (Zheng et al., 2008). Another study showed p53 mutant prostate cancer cells were able to increase ligands for EGFR through a feedback loop involving transactivation of the ERK1/2 pathway, which activates the EGFR signaling pathway. Investigations of EGFR expression in human OS samples did not correlate with P53 expression on immunohistochemistry (Oda et al., 1995). Mutations for p53 and PTEN have been reported for canine OS and are commonly associated with poorer prognosis (Kirpensteijn et al., 2008; Levine et al., 2002; van Leeuwen et al., 1997). Since mutations for these two tumor suppressors are a common event in OS leading to poor outcome, the possibilities for p53 and PTEN mutation causing increased expression of EGFR in a subset of tumors with worst outcome is an area to be explored. The high metastasis-associated gene expression profile did reveal some enrichment of genes for EGFR signaling; however, EGFR was not among the genes that were differentially expressed between the two cell lines.

Several heat shock proteins (HSP60, HSP70-mortalin, HSP90-α) were identified among the genes that were differentially expressed between the two groups of tumors based on survival (outcome). The expression of these three heat shock proteins (HSPs) and several other HSP family members described for cancer pathogenesis was selected for further evaluation on an independent group of canine primary OS. Of the eight **Heat Shock Proteins** investigated, only **HSP60** mRNA expression was significantly related to survival time. In Chapter Four, HSP60 mRNA expression was found to be associated with poor survival. Apart from the HSP expression in canine OS tumors, we have shown that HSP60 expression in all canine OS cell lines was comparable to the three human OS cell lines on western blot. Although HSP60 mRNA was associated with survival time, this should be confirmed in a larger scale study. A subset of the tumors used for the RNA expression studies was selected to evaluate the protein expression of HSP. However, due to the small number of samples included and due to the heterogeneous expression observed across the tumors, a definitive prognostic value for HSP60 expression in canine OS was not able to be concluded. Although HSP60 has been reported expressed in human OS, inconsistency in the reports to date limits its value for prognosis: some studies show that HSP60 is prognostic, while others do not (Moon et al., 2010; Trieb et al., 2000; Uozaki et al., 2000). However, the study described in this thesis has explored the effects of HSP downregulation using a canine-specific small interfering RNA (siRNA). HSP60 gene product was efficiently downregulated in 2 canine OS cell lines, which resulted in decreased cell cycle and proliferation and elicited anti-apoptotic effects in the cell

lines investigated. Few may argue the value of targeting HSP60 due to its diverse roles even under normal physiological conditions. Recent reports have proven that HSP60 in cancer cells may have differing roles and interaction with proteins. HSP60 was observed to be secreted by tumor cells and directly associated with cyclophilin D (CypD), a component of the mitochondrial permeability transition pore. This interaction occurs in a multichaperone complex comprising HSP60, HSP90, and tumor necrosis factor receptor-associated protein-1, selectively assembled in tumor but not in normal mitochondria (Cappello et al., 2008; Ghosh et al., 2010; Merendino et al., 2010). In addition, stable transfection using HSP60-shRNA (short hairpin) in human OS cell lines resulted in growth arrest (Kaul et al., 2006); this further strengthens the potential for targeting HSP60 in canine and human OS. At present there are limited compounds targeting HSP60 available for *in vivo* studies; this could be an attractive approach for future OS research strategies.

The prognostic value for **VDR, CYP27B1 and CYP24A1** expression was evaluated in canine and human OS using a tissue microarray approach. Active Vitamin D_3 ($1,25(OH)_2D_3$; calcitriol) and its analogs are potential agents for anti-cancer therapy due to a potent anti-proliferative activity mediated through the genomic signaling pathway by binding to a specific nuclear VDR (Fleet, 2008; Pike and Meyer, 2010; St-Arnaud, 2008). Cross-species comparison for VDR expression in the present study revealed that both dog and human OS have a relatively low frequency for nuclear VDR expression (~30%) with no prognostic significance. **CYP24A1** or the enzyme 24-hydroxylase, that degrades/deactivates calcitriol, is expressed in a subset of canine and human OS and predicts poor clinical outcome. We have also performed additional studies on *in vitro* models of OS, where four canine and two human cell lines treated with calcitriol showed augmented expression for CYP24A1 mRNA, a specific and sensitive target gene for Vitamin D pathway activation (unpublished data). Increased CYP24A1 expression by tumor cells allows a mechanism whereby it abrogates locally produced active Vitamin D_3 and thus may reduce the anti-proliferative effects of exogenous calcitriol therapy *in vivo*. We will test this hypothesis in the near future by inhibiting the expression of CYP24A1 using RNA interference or compounds selectively targeting this protein to assess the anti-proliferative effects of calcitriol *in vitro* and *in vivo* (Muindi et al., 2010). CYP24A1 may be a relevant target for gene therapy in dogs and humans with OS.

Clinical outcome-based global gene expression profiling on canine OS observed the **Wnt signaling pathway** important for OS (O'Donoghue et al., 2010; Selvarajah et al., 2009). The **canonical signaling mediated by β-catenin** regulation drives the activation and transcription of specific genes involved in cell cycle, proliferation, epithelial-mesenchymal transformation and differentiation. The basal canonical Wnt activation status of eight well-characterized canine OS cell lines was evaluated using luciferase reporter assays, revealing enhanced basal signaling in four of them, although the transcriptional activity observed was lower than in a human OS cell line. **Nuclear β-catenin** expression was observed in only two of the cell lines

with higher basal activity, suggesting that the pathway was inactive in a majority of the canine OS cell lines. Of the target genes investigated, **Axin2** appears to be the most specific, much better than both cyclinD1 and survivin.

Inhibiting the Wnt canonical pathway using the dominant-negative TCF4 approach in canine and human OS cells demonstrated a decrease in transcriptional activity by reporter assays. There were differences in cell proliferation inhibition among the cell lines. Among the target genes, Axin2 was found to be down-regulated upon inhibition of the pathway in a majority of the cell lines. Nuclear β-catenin and up-regulation of Axin2 mRNA expression were found to be a reliable marker for Wnt pathway activation; this was similar to a previous report on a human OS study (Cai et al., 2010). However, the prognostic value of nuclear β-catenin expression and the presence of increased axin2 immunoreactivity in canine OS primary tumors using a tissue microarray approach were low. Our findings demonstrate that, although Wnt signaling is present in canine OS, its role in tumor proliferation and metastasis is limited, as it is in human OS.

The survival-associated gene expression profiling study in Chapter Four and metastasis-associated gene expression profiling from *in vitro* models in Chapter Five revealed gene enrichment for Wnt signaling among the top pathways. This was also the case when comparative analysis was performed with published gene sets for human OS. However, β-catenin and axin2 were not among the differentially expressed genes. This also means that even upstream players in the Wnt signaling may be involved in cross-talks with other pathways that may promote metastasis, cell migration or proliferation. As knowledge about the roles of proteins involved in Wnt signaling keeps expanding, further exploration will yield more new insights into the role of Wnt signaling and cross-talks with other pathways (e.g. **EGFR/ Vitamin D/ PI3K-Ak**t) involved in pathogenesis of OS.

Several forms of kinase have been found to be overexpressed in canine and human OS tumors and cell lines (Gordon et al., 2008; Yang et al., 2010). Inhibitors of kinases have been developed for human research for more than a decade now, and some have passed for clinical applications in OS for people (Keir et al., 2010; Perotti et al., 2010). However, their application in spontaneous canine OS models is still limited. This preliminary screening approach identified 22 different kinase inhibitors demonstrating significant anti-proliferative effects across the four canine OS cell lines. Among them, four compounds showed potent cell growth inhibitory effects at 10μM with $P<0.05$. These kinase inhibitors are known to inhibit targets such as **EGFR, NF-κB, PKC, PKA and ERK2.** This research, in fact, potentiates the use of EGFR inhibitors in canine OS *in vitro* models as described in Chapter Eight, where EGFR was found to be expressed in canine OS *in vitro* models, and a subset of dogs with high expression have poorer clinical outcome. Based on the preliminary assessment, biochemical and genetic studies are required to clarify the use of selected kinase inhibitors as anti-cancer therapy for dogs with OS.

Limitations of the current and reported prognostic studies

Several different statistical methods are used to evaluate prognosticators in the many canine OS studies published from the 1970s to 2010. These mainly include the log rank test scores calculated in univariate fashion and displayed as Kaplan-Meier survival curves. This method is widely accepted to assess an individual marker as a prognosticator in a defined population of subjects for which censored survival analysis is carried out. This is a less biased method to evaluate survival because it takes into consideration every single subject followed in a defined duration, even those who may have died due to other causes or dropped out of the study before the end of the evaluation period. Univariate analyses should be followed by multivariate analysis to analyze the factors that may naturally affect prognosis in concert for a population of study.

Not only the various statistical approaches, but also the number of variables assessed and number of subjects included in the prognostic models varied from study to study. In addition, most of the prognostic studies on canine OS were carried out in the US and a few others in selected European countries, yet there were none on other continents. Could these prognostic studies be repeated, and would they result in the same outcome in other independent studies? One other major variable which may influence the outcome of any given subject (in this case a pet dog) is the cooperation and willingness of dog owners to proceed with the recommended therapies and be diligent with the follow-up protocols. Another limitation needing to be addressed is the greater availability of marker assessments to academic institutions than to general veterinary practitioners. For these reasons, prognostic studies are evaluated for a selected population of dogs with OS with an assumption that this subset represents the entire population, which may not be true in some circumstances.

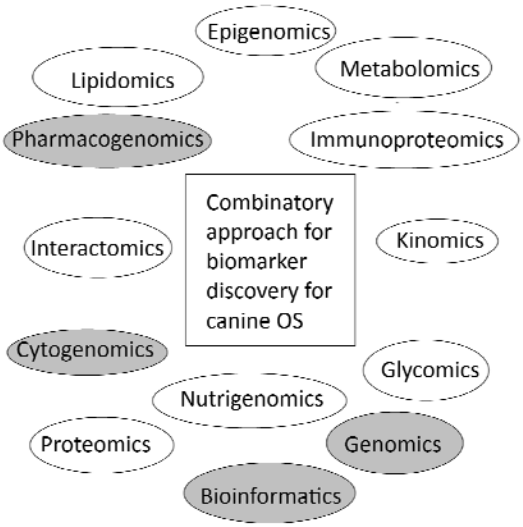
One of the drawbacks of the majority of the prognostic studies carried out for canine OS to date is that most are retrospective in nature and very few are randomized and placebo controlled. It is highly recommended to test and validate the “prognosticators” for applicability to different populations of dogs, not only by independent studies but also by independent investigators, at different times, in different geographical locations and with different follow-up periods. For this to be possible, many similar studies have to be undertaken, which will require that the common sort of “duplicate/ replicate” studies normally shunned by journal editors and reviewers as ”not novel” and of low priority for publication be given more attention.

Meta-analysis is a popular statistical method for evaluating prognosticators from several published studies. This method is highly valuable in assessing outcomes from several prospective and randomized studies. However, the publication of such studies for canine OS to date is too limited to allow for a meta-analysis. Non-randomized retrospective studies can be evaluated in the same way, but this approach may be biased towards a selected population, patient selection criteria or therapy. Given the current limitations for studies involving prognostic markers for canine OS, at present most of the prognosticators are based

on single- or bipartite studies. One exception involves a few markers such as alkaline phosphatase, which has been evaluated as a valid prognosticator in almost all the prognostic studies. Pre-surgical serum alkaline phosphatase level was also a prognosticator in a few of the studies described in this thesis. Over time, a meta-analysis of these independent studies should be performed to re-evaluate the status and strength of the prognostic variables assessed. Given the current limitations with respect to prognostic evaluation methods, future studies are likely to be aided by better designed experimental setups with better defined patient populations, research questions and therapeutic protocols.

Perspectives for future research strategies

1. Continuous validation of candidate genes revealed by the gene expression profiling studies. Continuous annotation of the non-annotated canine-specific transcripts is necessary. Many more novel markers await further validation and co-expression analysis should be performed among the reliable prognostic markers so to establish a multi-marker panel valuable for stratification of dogs into a more realistic prognostic groups. In vitro and in vivo models may be utilized to further elucidate the mechanisms by which the candidate genes may be upregulated or downregulated. Genes may also be evaluated for their amplification or mutation status in canine OS. Other profiling studies aided by bioinformatics may increase our understanding of OS pathogenesis and facilitate the discovery of novel and more useful biomarkers for the disease in humans and dogs (Figure 1).



2. Investigating genes associated with drug resistance in OS.
Several genes correlated with poor prognosis in the cDNA microarray study on canine OS were associated with drug resistance as reported in other types of tumor or disease conditions. Identification of novel, and validation of current, gene markers for drug resistance may be valuable for stratifying dogs with OS into groups of responders to a particular drug. This approach may enhance therapeutic efficacy in the selected subset of canine OS.

3. Addressing immunological components present in dogs and human patients with OS.
There have been interesting insights on the association of post-operative infections and increased survival in dogs undergoing limb-sparing procedures. The studies in this thesis evaluated novel markers on tumors from dogs that had undergone limb amputation or total resection of tumors, and none of them were subjected to limb-sparing protocols. The post-operative infection as a prognostic factor was not able to be evaluated for this study population. However, we did attempt to evaluate the prognostic value for presence of inflammatory cells within the stromal and/or the perivascular compartments of canine OS primary tumors. It was observed that few of the tumors showed the presence of inflammatory cells and further evaluation is underway (unpublished data). Furthermore, as chemokine and cytokine signaling was among the important pathways for prognosis in canine OS (Chapter Four), and hence it would be interesting to evaluate the different chemokine receptors and cytokines for their association with prognosis and target for therapy in canine OS.

4. Understanding the kinome of OS.
The preliminary study on kinase inhibitor compounds need to be further validated. However, it is also important to profile the various kinases in OS to try to identify a more relevant kinase that could be used for targeted therapy. The cell line studies dictate a need to acknowledge that the expression of selected kinases can differ from one cell line to another, similarly to the *in vivo* situation. Profiling of kinases from tumors by *in vivo* and *in vitro* functional assays, then, may provide more insights into new markers and targets for therapy. In recent years, several of the serine-threonine protein kinases such as the Mirk kinase, Aurora proteins or Polo-like kinases (PLK1) have received increased attention as putative cancer targets for human OS (Yang et al., 2010). Other less-studied mitotic kinases such Thymidin kinase 1, TTK, BUB and NEK proteins might also be relevant candidates as new targets of interest in cancer therapy for OS, since they play relevant roles in mitotic progression. The value of these kinase expressions in canine OS is yet to be explored.

5. Targeted therapies for canine OS.
The present study puts forward HSP60, CYP24A1, EGFR and decorin as potential targets for therapy for a subset of canine OS. CYP24A1 and decorin may be valuable for human OS as well. The conventional chemotherapeutic approach using cytotoxic agents in combination as

pre- or postoperative to extirpation of the tumor may be evaluated with or without the presence of adjuvant therapies such as the kinase inhibitors or compounds targeting the markers named above. RNA interference techniques may be employed to investigate the effects of CYP24A1 downregulation as an adjunct to Vitamin D therapy on in vitro models of OS before further studies can be initiated on dogs with spontaneous OS or on experimental animals.

6. Research on other relevant signal transduction pathways revealed by the pathway analyses from the studies described in this thesis. These other pathways include the integrin, fibroblast growth factor, chemokine and cytokine signaling, angiogenesis, ubiquitin proteasome degradation pathway as well as others such as the Huntington and Parkinson disease pathways. Although many may find the latter two pathways less attractive to explore, the genes related to these pathways are probably regulated differently in OS disease pathogenesis and progression. These pathways are known for two common genetic diseases in humans; as OS also has a genetic component among the causes for its occurrence in dogs and human, further evaluation of these two pathways is warranted. Several genes from the integrin and fibroblast growth factor signaling have been evaluated for their roles in prognosis and metastasis for human OS; however, these pathways are yet to be explored for canine OS.

7. Investigating developmental pathways.
Several of the developmental pathways for cancer such as Wnt, Notch and Hedgehog may be important for the pathogenesis of OS. However, it is still unclear at which level these pathways interact and which targets of these pathways are essential for metastasis and survival of OS cells. The study on Wnt signaling described in this thesis evaluated only two of the molecular markers for this pathway: beta catenin and axin2. Other Wnt ligands, receptors and inhibitors have been described as involved in OS metastasis in studies on murine models and human OS. Several of these Wnt ligands, receptors and inhibitors have already been profiled for the eight canine OS cell lines and await further analysis. Preliminary studies on Hedgehog and Notch signaling have been evaluated in human OS however their roles in metastasis and determining prognosis are yet to be clarified.

8. Investigating cross-talks between pathways
The present thesis has evaluated few of the molecular markers for pathways such as Vitamin D signaling, Wnt and EGFR signaling. It would be valuable to investigate the degree of cross talk among these pathways and the involvement in OS metastasis and disease progression. Studies on cross-talks between EGFR and Wnt signaling, and Wnt and Vitamin D signaling are under progress.

9. Cancer stem cells in canine OS.

The fact that there were genes enriched for drug resistance in the gene expression profiling studies makes it interesting to evaluate the co-expression of putative cancer stem cell markers to determine if the cells expressing high drug resistant phenotype are actual cancer stem cells. This is because one of the typical characteristics of a cancer stem cell is the ability for it to transport drugs out of the cell efficiently and thus display a phenotype resistant to most drugs including cytotoxic agents commonly used for chemotherapy protocols. In addition, as recent studies have shown that nuclear beta catenin is co-expressed with LRG5, one of the putative stem cell markers in human colorectal carcinomas, it is interesting to evaluate the co-expression of nuclear beta catenin with known markers for stem cells in canine and human OS. Issues pertaining to tumor dormancy, drug resistance, recurrence and metastasis are commonly associated with stem cell theory. Further identification, isolation and profiling of putative cancer stem cells in OS primary and metastasis cells may hold great promise for future stem cell targeted therapies.

Generalized conclusions

Spontaneous canine OS is known as a suitable large animal model for the human counterpart (Gorlick and Khanna, 2010; Hansen and Khanna, 2004; Khanna et al., 2006; Paoloni and Khanna, 2008; Paoloni et al., 2009; Withrow and Khanna, 2009); however, it is cautioned that some of the findings in dogs cannot be directly extrapolated to human tumors. This thesis discusses the importance of three of the major cellular signaling pathways for canine OS disease progression: EGFR, Wnt and Vitamin D signaling. These pathways may have important involvement in modulating OS cells to become highly invasive with metastatic potentials. Several genes were also highlighted as markers for poor prognosis and metastasis, addressing the aggressive phenotype of canine OS and a few for the disease counterpart in humans. It is becoming more evident that various levels of gene alterations, as well as the intricate interaction between genes in a cellular signaling cascade, definitely need more attention. This is especially true for those related to canine OS disease development, progression and, most importantly, metastasis processes. The identification of selected molecular markers and cellular pathways may hold great promise in understanding further mechanisms involved in metastasis. This understanding may lead to better prognostics and therapeutics not only for OS, but also for other solid tumors. Ultimately, the strategy of a combinatory therapeutic approach targeting several pathways at appropriate intervals for eradicating metastasis holds a promise for a wide array of future studies in both animal models and humans. One fact that everyone should acknowledge is that even the best prognosticator will probably fail in certain conditions and be inaccurate for a significant number of dogs and people with osteosarcoma. Hence, continuous reporting and reassessment of prognostication and prognosticators is warranted.

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Chapter 13

Summary

Samenvatting

Summary

Among the spontaneously arising tumors found in dogs, osteosarcoma has been named on numerous occasions as a relevant model for the disease in people. Canine OS provides an excellent model for studying this neoplasm in people since the tumor has features common to both species including clinical presentation, histopathology, location, sites of metastases and prognosis. There is still no curative therapy available, although in some cases for both dogs and man there are apparent benefits from primary interventions in conjunction with current adjuvant therapies, including the use of cytotoxic agents. There are also differences in metastatic rate, as few dogs develop metastasis rapidly, and a smaller percentage has a tendency to develop late metastasis. Despite the greater number of advances in therapies and molecular marker discoveries for people as compared to dogs with osteosarcoma, the prognosis still looks grim for both species. Many investigations have been directed at identifying single gene alterations as predictors of metastasis, cell proliferation, drug resistance, bone turnover and other processes central to disease progression. It is likely that reliable markers will be those that are useful in multiple species, and studying osteosarcoma in dogs provides good opportunity to discover newer strategies to combat this disease that are not only useful for dogs, but some may have valuable translational value for people.

Numerous prognostic studies have been conducted over the past three decades to identify factors for poor clinical-outcome in dogs and people diagnosed with osteosarcoma. Prognosticators or prognostic markers are useful as 'guidelines' for veterinarians to assess the disease state in the dog and provide reasonable recommendations to dog owners. Studies described in this thesis highlight several new molecular markers for poor clinical outcome in dogs with osteosarcoma, as well as a few with relevance for people. Various investigations were undertaken in the course of this thesis which involved: global gene expression profiling using cDNA microarray technology, tissue microarrays for immunohistochemistry, quantitative real-time PCR assays to quantify mRNA expression, use of a panel of osteosarcoma cell lines from both human and canine origin for *in vitro* studies, DNA sequencing for mutation analyses, various microscopy and cell imaging techniques, cell culture based functional assays and gene silencing using RNA interference and bioinformatics. Several genes and signal transduction pathways important for osteosarcoma pathogenesis, disease progression and survival are discussed in this thesis. The chapters are summarized below:

Chapter 3 reviews the predictive and prognostic molecular markers for canine osteosarcoma. This review highlights a few of the proposed markers available from research presented over

the past three decades. Many of the markers suggested were not independently validated and few qualified as relevant prognosticators for dogs with osteosarcoma, including pre-operative serum alkaline phosphatase levels and post-operative chemotherapy. This review also presents an algorithm on how molecular markers can be incorporated into the multimodal management of this disease in dogs.

Chapter 4 explores the gene expression profiles of short and long survivors of canine osteosarcoma, using a 6-month survival time as a gauge. Fifty-one candidate genes were observed to be regulated differentially between these two groups. However, there was also a distinct molecular sub-classification of poor survivors into two groups which displayed apparent contrast in the disease-free interval. This finding, although preliminary and requiring more validation, provides indication that canine osteosarcoma can be differentiated by molecular signatures, which may be informative for prognosis or even for therapeutic intervention. Further comparative pathway analyses revealed that cellular signaling pathways - such as Intergin, Wnt, fibroblast growth factor (FGF) and ubiquitin proteasome degradation pathways - are common among the gene profiles found in canine and human osteosarcoma.

Chapter 5 discusses the gene expression profiling of two metastatic sub-clones of canine osteosarcoma with differing metastasizing propensities, revealing that they have a distinct profile which shares several signaling pathways found in human and murine osteosarcoma metastasis gene signatures. Decorin, among the candidate genes listed, was selected for further validation because it is a bone proteoglycan and because of its role as a tumor suppressor in several other cancer models. What is intriguing here is that decorin was upregulated in the high metastatic cell line. And, equally intriguing, mRNA gene expression quantified from snap-frozen canine tumor specimens as well as tissue microarray immunohistochemistry on osteosarcoma from people demonstrated decorin expression associated with poorer clinical-outcome.

In **Chapter 6**, nine reference genes for canine tissues were validated on a panel of 40 canine osteosarcoma primary tumors and 7 cell lines. RPS5 and HNRNPH were the best combination reference genes for OS primary tumors, while RPS5 and RPS19 were for the cell lines. However, all of the tested reference genes were found to have good stability and are appropriate for normalizing gene expression data. Pair-wise variation analysis recommends 4 reference genes in normalizing the expression data for canine OS tumors, and 2 for the cell lines. However, this combination may be appropriate for chemo-naive snap frozen material and needs to be continuously validated for each gene expression analysis for osteosarcoma.

Chapter 7 characterizes the Wnt signaling activation status in canine osteosarcoma and describes some new findings for osteosarcoma in man. This chapter made use of several *in vitro* models of canine osteosarcoma and tissue microarrays in order to determine the

prognostic values for beta catenin and Axin2, a specific target gene for Wnt canonical signaling in both human and canine osteosarcoma. Canine osteosarcoma cell lines have low autocrine Wnt activity, with Axin2 and nuclear β-catenin as reliable markers for pathway activation. Stimulation and inhibition of the pathway resulted in variable cell proliferative response across the human and canine cells, suggesting that targeting the Wnt canonical pathway may only be useful for a subset of osteosarcoma. Aberrant expression for the nuclear β-catenin and Axin2 was not prognostic in either people or dogs with osteosarcoma. The comparative findings from this study suggest that spontaneous canine osteosarcoma has similar Axin2 and beta catenin expression and is a relevant model for human osteosarcoma with similar Wnt/β-catenin activation and regulation. This marks the first time that comparative research has been carried out, evaluating two markers independently and in combination on a large number of tumors for both man and dog osteosarcoma.

Chapter 8 assesses the epidermal growth factor receptor (EGFR) expression in canine osteosarcoma primary tumors, metastasis and cell lines. EGFR is the first member of the tyrosine kinase transmembrane receptor proteins; among the group of growth factor receptors expressed in an array of tumor types including osteosarcoma in man. Although EGFR expression was significantly elevated in primary tumors as compared to normal bones and had relatively higher expression in primary tumors metastasized to lungs than extra-pulmonary sites, no clinical or pathological association was found with mRNA expression levels in snap-frozen tumors. Tissue microarray analysis revealed that a subset of canine OS with high EGFR expression has significantly shorter survival time and disease-free intervals. Like the primary tumors, 75% of metastasis demonstrated cytoplasmic expression for EGFR. While the presence of EGFR expression alone is not a strong predictor of outcome and other markers are necessary for further prognostic stratification of dogs with OS, these findings suggest that a subset of dogs with high EGFR expression in their primary tumors may benefit from anti-EGFR adjuvant therapies.

Chapter 9 addresses the several heat shock protein (HSP) members that were found to be highly expressed in dogs with poor prognosis (short survivors) and highly expressed in the highly metastatic cell line as described in Chapters 4 and 5. Several HSP expression analyses at the mRNA and protein levels (immunohistochemistry) were determined for snap-frozen tumors and cell lines. HSP60 mRNA expression was found to be a poor prognosticator, however, the protein expression in tumors did not have enough statistical significance to make it a strong marker for survival. This is mainly due to the heterogeneous expression of HSP60 present within the tumors and low number of tissues assessed. However, a further gene silencing approach using siRNA for canine specific HSP60 showed that through down-regulation of this protein in two canine osteosarcoma cell lines, cell proliferation can be remarkably inhibited with induction of moderate apoptosis and with consistent down-regulation of cyclinD1, the gene involved in cell cycle regulation. Differences in sensitivity to

siRNA between the two cell lines were observed, warranting further research on possible HSP60 interacting proteins for osteosarcoma cell proliferation and metastasis.

Chapter 10 discusses the prognostic value of CYP24A1, which is a specific and sensitive target gene for Vitamin D pathway activation. CYP24A1 encodes the enzyme 24-hydroxylase which degrades the active form of Vitamin D in a negative feedback mechanism, abrogating its anti-cancer properties in bone cancer cells. For this reason, increased doses of Vitamin D are most often necessary to potentiate anti-cancer properties *in vivo*. However, the toxicities observed, including hypercalcemia and excessive mineralization in major organs such as the kidney and liver, have resulted in undesirable outcomes. The up regulation of CYP24A1 as a response to activated Vitamin D signaling observation has been reported in several human cancer *in vitro* models including osteosarcoma. The Vitamin D receptor (VDR) and CYP27B1, the gene that encodes the enzyme 1α-hydroxylase which converts the native form of Vitamin D to the active molecular form, did not show any prognostic value. However, CYP24A1 was upregulated and appeared to be a poor prognosticator for osteosarcoma in both dogs and man. Therefore, in order to potentiate therapies using calcitriol for osteosarcoma, future studies *in vitro* and *in vivo* should incorporate compounds targeting CYP24A1.

Chapter 11 reviews the results from screening a kinase inhibitor compound library in four canine osteosarcoma cell lines in order to identify potentially useful compounds that could be used for molecular targeted based therapies for canine osteosarcoma. In total, 80 compounds were investigated and the differences in sensitivity of these compounds to inhibit cell proliferation were analyzed. This screening identified 22 different kinase inhibitors, demonstrating significant anti-proliferative effects across the 4 canine osteosarcoma cell lines. Among them, 4 compounds showed potent and significant cell growth inhibitory effects which include compounds known to inhibit signaling mediated by the EGFR, NF-κB, PKC, PKA and ERK2. Based on the preliminary assessment, more studies on toxicity and anti-metastatic potential of the selective kinase inhibitors are required before *in vivo* anti-cancer therapy for dogs with osteosarcoma can be undertaken.

Chapter 12 describes the key findings from the 9 research chapters of this thesis and discusses the relevance of the dog model for osteosarcoma with concluding remarks and statements for future perspectives of prognostic research for canine osteosarcoma.

The past 5 years have shown several important advances in the biological understanding of canine osteosarcoma, including the findings presented in this thesis. These have indeed opened up more opportunities to battle the disease with newer therapeutic interventions and, most importantly, to be able to stratify these dogs into appropriate prognostic groups and thereby facilitate more individualized management of the disease. There is, however, still much to be done, and future well-designed prognostic studies on populations of dogs

undergoing new interventional therapies alone or in combination with current therapies are crucial. One fact that everyone should acknowledge is that even the best prognosticator will probably fail in certain conditions and be inaccurate for a significant number of dogs and people with osteosarcoma. Hence, continuous reporting and reassessment of prognostication and prognosticators is warranted.

Key findings from this thesis:

1. This thesis highlights several new molecular markers that have potential to predict poor clinical-outcome in canine osteosarcoma, including Decorin, EGFR, CYP24A1 and HSP60. Decorin and CYP24A1 were also observed to be poor prognosticators for osteosarcoma in people.
2. Canine osteosarcoma can be differentiated by molecular signatures, which may be informative for prognosis, predicting metastasis advancement or even for therapeutic intervention.
3. Gene expression profiling and subsequent pathway analyses on tumor specimens and *in vitro* models identified a few more relevant and important signal transduction pathways for prognosis and metastasis of canine osteosarcoma such as Integrin, Wnt, fibroblast growth factor (FGF), epidermal growth factor receptor (EGFR) and ubiquitin proteasome degradation pathways.
4. Canine and human osteosarcoma shares a few common signal transduction pathways for metastasis. These deserve more investigation.
5. Two prognostic markers, HSP60 and EGFR, were observed to be justified and relevant new targets for therapy in dogs with osteosarcoma. Targeting these markers on *in vitro* models of canine osteosarcoma lead to decreased cell proliferation, increased apoptosis and reduced cell viability.
6. The present study is the first to characterize the Wnt canonical signaling in canine osteosarcoma by demonstrating the reliability of the nuclear β-catenin expression as a 'hallmark' for pathway activation *in vitro* together with expression of Axin2. Spontaneous canine osteosarcoma has similar Axin2 and beta catenin expression and is a relevant model for human osteosarcoma with similar Wnt/β-catenin activation and regulation. Inhibition of this pathway lead to reduced cell proliferation in the majority of the cell lines, and may be useful for a subset of osteosarcoma in people and dogs.
7. Selective kinase inhibitors targeting cellular signaling mediated by the EGFR, NF-κB, PKC, PKA and ERK2 can be useful anti-cancer therapies for dogs with metastatic osteosarcoma.
8. Multiple reference genes are required to normalize canine osteosarcoma quantitative real-time PCR gene expression data. RPS5 and HNRNPH were the best combination reference genes for OS primary tumors, while RPS5 and RPS19 were for the cell lines.

Samenvatting

Het osteosarcoom bij de hond (OS) wordt vaak genoemd als een relevant spontaan tumormodel voor de ziekte bij de mens. Naast de klinische verschijnselen, zijn de histologische kenmerken, het voorkomen, de locatie van uitzaaiingen en de prognose na behandeling vergelijkbaar tussen de twee species. Ook is er vaak geen curatieve therapie mogelijk ondanks de verbeterde prognose die wordt verkregen door primaire interventies en adjuvant therapieën zoals het gebruik van cytotoxische middelen.
Er zijn verschillen in mate van metastasering tussen de hond en de mens, de meeste honden ontwikkelen metastasen vroeg in de ziekte terwijl een kleiner percentage pas in een laat stadium uitzaaiingen ontwikkelt. Ondanks dat er meer ontwikkelingen zijn in de humane OS therapie en vaker humane moleculaire markers worden ontdekt dan bij de hond, ziet de prognose er nog steeds somber uit voor mens en dier. Het humane onderzoek wordt vaak geconcentreerd op alteraties van een geïsoleerd gen, waarbij een voorspellende waarde wordt verwacht met betrekking tot metastasevorming, celproliferatie, medicijn ineffectiviteit, botregeneratie en andere essentiële processen die de progressie van de ziekte bepalen. Betrouwbare factoren bij de mens zijn hoogstwaarschijnlijk bruikbaar in andere species zoals de hond en kunnen gebruikt worden om nieuwe strategieën te ontwikkelen. Anderzijds kan onderzoek bij de hond bijdragen tot verbeterde behandeling van de mens en zo dienen als een valide translationeel model.

In de afgelopen 3 decennia zijn tal van prognostische studies uitgevoerd die risicofactoren beschrijven die een slechte klinische uitkomst voorspellen bij honden en de mens. Prognosticatoren of prognostische markers kunnen gebruikt worden als richtlijn voor dierenartsen om de status van de ziekte te beoordelen en redelijke aanbevelingen te kunnen doen aan hondeneigenaren. Dit proefschrift beschrijft diverse nieuwe moleculaire markers die prognose voorspellend zijn in het hondenosteosarcoom en enkele die relevant zijn voor de ziekte bij de mens. Het onderzoek betrof het bepalen van gen expressie profielen met behulp van cDNA microarray technieken, immunohistochemische weefsel microarrays, mRNA expressie profielen door middel van quantitatieve rt-PCR technieken, het gebruik van een panel of gevalideerde OS cellijnen voor *in vitro* studies, mutatie analyses door middel van DNA sequentie technieken, diverse microscopie en cel beeldvormende technieken, functionele assays voor celkweken, gen silencing door middel van RNA interferentie en bioinformatica. Meerdere genen en signaal transductie pathways die belangrijk zijn voor de pathogenese van het osteosarcoom, de progressie van de ziekte en de prognose worden ter discussie gesteld in dit proefschrift. De hoofdstukken worden als volgt kort samengevat:

Hoofdstuk 3 geeft een overzicht van nu bekende of ziekteverloop voorspellende of na therapie prognostische moleculaire markers voor OS bij de hond. Dit overzicht benoemt een aantal van de beschikbare markers die zijn beschreven in de afgelopen 3 decennia veterinaire literatuur. Echter, veel van de markers zijn niet onafhankelijk gevalideerd en weinig zijn echt gekwalificeerd als relevante prognostische factoren, met inbegrip van prechirurgisch plasma alkalische fosfatase concentraties en het gebruik van postoperatieve chemotherapie. Dit hoofdstuk bevat ook een algoritme dat verklaart hoe deze moleculaire markers gebruikt kunnen worden gedurende de multimodale behandeling van het OS bij de hond

Hoofdstuk 4 beschrijft onderzoek van de genexpressie profielen van OS-honden met korte en lange overlevingstijden met 6-maanden overleving als graadmeter. Eenenvijftig kandidaatgenen kwamen differentieel tot expressie als we beide groepen vergeleken. Naast dit was er een duidelijke subclassificatie tussen de slechte responders met een duidelijk verschil in ziektevrije periode. Ondanks dat deze bevinding validatie behoeft, is er een duidelijke indicatie dat het OS van de hond kan worden onderscheiden via moleculaire handtekeningen die prognose en behandeling kunnen voorspellen. Door vergelijkend onderzoek naar signaal-transductie paden bleek dat cellulaire signaalwegen - zoals Integrine, Wnt, fibroblast groeifactor (FGF) en ubiquitine proteasomale afbraak routes beide bij mens en hond van belang zijn bij het OS.

Hoofdstuk 5 bespreekt de profilering van genexpressie van twee gemetastaseerde subklonen van een honden OS cellijn die een verschillend metastaserend karakter hebben. Uit dit onderzoek blijkt dat zij een verschillend profiel hebben dat overeenkomt met verschillende signaalwegen gevonden in samenhang met metastasering van humaan en muizen OS. Decorine, was onder de genoemde kandidaat-genen en werd geselecteerd voor verdere validatie omdat het een bot proteoglycaan is. Ook heeft het een rol als een tumorsuppressorgen in verschillende andere kankersoorten. Opmerkelijk was dat decorine expressie verhoogd was in snel metastaserende cellijn. En, even boeiend, kwantitatieve mRNA expressie van direct ingevroren honden tumormonsters alsmede een immuunhistochemie microarray van menselijke OS toonde aan dat 25% van de tumoren decorine tot expressie brengen en dat deze expressie is geassocieerd met slechtere prognose.

In **hoofdstuk 6** werden negen referentie genen voor honden weefsels gevalideerd bij 40 primaire OS en 7 OS cellijnen van de hond. RPS5 en HNRNPH vormden de beste combinatie van referentie genen voor primaire OS, terwijl RPS5 en RPS19 de beste combinatie voor de cellijnen vormden. Echter alle van de geteste referentie genen hebben een goede stabiliteit en zijn geschikt voor het normaliseren van OS genexpressie. Paarsgewijze variatie analyse wees vier referentie genen aan voor het normaliseren van de expressie gegevens voor primaire OS, en 2 genen voor de cellijnen. Echter, alhoewel deze combinaties geschikt zijn

voor chemotherapie-naïeve, direct ingevroren OS, moeten andere typen OS opnieuw worden gevalideerd.

Hoofdstuk 7 karakteriseert de Wnt signalering activatie status van het OS bij de hond en belicht een aantal nieuwe bevindingen voor humane OS. In dit hoofdstuk wordt gebruik gemaakt van verschillende *in vitro* modellen van het OS van de hond en weefsel microarrays om de prognostische waarde van de expressie van β-catenine en Axine-2 te bepalen. Axine-2 is een specifiek target gen voor de canonieke Wnt signalering, in het OS van zowel de mens als de hond. Cellijnen van honden-OS hebben een lage autocriene Wnt activiteit, terwijl Axine-2 en nucleair ß-catenine betrouwbare markers bleken voor activering van het Wnt pad. Stimulatie en remming van het WNT pathway resulteerde in variabele respons in cel proliferatie in zowel de humane als de hondencellen. Dit suggereert dat beïnvloeding van de canonieke Wnt signalering alleen nuttig is voor een beperkt aantal OS. Een afwijkende expressie van nucleair β-catenine en van Axine-2 was niet prognostisch bij de mens en de hond. De resultaten van deze studie suggereren dat honden met OS vergelijkbare Axin2 en β-catenine expressie vertonen met de mens en dat het een relevant model is voor humaan OS met vergelijkbare Wnt/β-catenine activering en regulering. Dit is de eerste keer dat een dergelijk vergelijkend onderzoek is uitgevoerd met gelijktijdige evaluatie van een dubbele marker, individueel en in combinatie in een relatief groot aantal humane en honden OS.

Hoofdstuk 8 stelt de expressie vast van de epidermale groeifactor receptor (EGFR) in primaire tumoren, metastasen en cellijnen van honden OS. EGFR maakt deel uit van de transmembraan-receptor eiwitten die tyrosine kinase activiteit vertonen. Deze groep van groeifactorreceptoren worden tot expressie gebracht in een reeks van tumoren inclusief het humaan OS. Hoewel EGFR expressie significant verhoogd was bij primaire tumoren in vergelijking met normale botten en relatief hoger tot expressie kwam in primaire tumoren uitgezaaid naar de longen dan extra-pulmonaire lokaties, werd geen klinische of pathologische verband gevonden met mRNA expressie niveaus. Hoge EGFR expressie op een hondenweefsel microarray was geassocieerd met aanzienlijk kortere overleving en ziektevrije periode na behandeling. Net als de primaire tumoren, vertoonden 75% van de metastasen een cytoplasmatische aankleuring voor EGFR. Ondanks dat EGFR-expressie alleen geen betrouwbare voorspeller is van het klinische verloop en andere markers nodig zijn voor de verdere prognostische stratificatie, suggereren deze bevindingen dat een beperkt aantal honden mogelijk kunnen profiteren van anti-EGFR therapie.

Hoofdstuk 9 behandelt de verschillende leden van de heat shock eiwit familie (HSP) die hoog tot expressie kwamen in honden met een slechte prognose (korte overlevingstijd). Hiernaast kwamen zij hoog tot expressie in een snel metastaserende cellijn (Hoofdstuk 4 en 5). Verschillen in HSP expressie werd geanalyseerd op mRNA en eiwit niveau (via immuunhistochemie) in tumoren en cellijnen. HSP60 mRNA expressie werd geassocieerd met

een kortere overleving, echter HSP60 eiwit expressie had niet genoeg statistische significantie om te kunnen dienen als sterke biomarker voor overleving. Dit is voornamelijk te wijten aan de heterogene expressie van HSP60 in OS en het lage aantal beoordeelde weefsels. Echter, via een siRNA experiment waarbij HSP mRNA expressie duidelijk werd verminderd in twee honden OS cellijnen, werd een opmerkelijke remming van celproliferatie met matige inductie van apoptose verkregen. Ook was er een inhibitie van CyclinD1 expressie, een gen betrokken bij de regulering van de celcyclus. Er werd een duidelijk verschil in gevoeligheid voor siRNA tussen de twee cellijnen waargenomen wat verder onderzoek naar mogelijke HSP60 remmende eiwitten voor OS celproliferatie en metastasering rechtvaardigt.

Hoofdstuk 10 bespreekt de prognostische waarde van CYP24A1, een specifiek en gevoelig target gen voor activering van het vitamine D pathway. CYP24A1 codeert voor het enzym 24-hydroxylase die de actieve vorm van vitamine D via een negatieve feedback afbreekt waardoor de antikanker eigenschappen van vitamine D bij OS wordt teniet gedaan. Verhoogde doses van vitamine D zijn daardoor nodig om de antikanker eigenschappen van vitamine D *in vivo* te versterken. Echter de resultaten zijn tegen gevallen vanwege de waargenomen toxische effecten zoals hypercalciëmie en pathologische mineralisatie in belangrijke organen zoals de nieren en de lever. De waargenomen verhoogde expressie van CYP24A1 als reactie op geactiveerde vitamine D signalering is ook gevonden in andere humane tumoren inclusief het OS. Echter, de studie beschreven in dit proefschrift laat zien dat CYP24A1 expressie in zowel mensen- als honden OS samengaat met een slechte prognose. De vitamine D receptor (VDR) en CYP27B1, het gen dat codeert voor het enzym 1α-hydroxylase dat vitamine D in een actieve vorm omzet, vertoonden geen enkele voorspellende waarde. Daarom moet bij het gebruik van therapieën met calcitriol tegen OS, rekening houden met het feit dat CYP24A1 geremd moet worden.

Hoofdstuk 11 wordt de screening van een bibliotheek van kinase-remmers beschreven in vier honden OS cellijnen om potentieel nuttige stoffen te identificeren die gebruikt kunnen worden als alternatieve therapie voor het honden OS. In totaal werden 80 verbindingen onderzocht en de verschillen in gevoeligheid van deze verbindingen met betrekking tot remming van celproliferatie werd geanalyseerd. Deze screening identificeerde 22 verschillende kinase-remmers die belangrijke celdeling remmende effecten lieten zien in de 4 honden OS cellijnen. Onder hen, vertoonden 4 verbindingen krachtige en belangrijke celgroei remmende effecten bij een concentratie van 10μM ($P < 0,05$). Van deze verbindingen is bekend dat de signalering via de EGFR, NF-kB, PKC, PKA en ERK2 routes worden geremd. Meer onderzoek is nodig naar de toxiciteit en het metastase remmende potentieel van de geselecteerde stoffen voordat *in vivo* anti-kanker therapie voor honden met OS kan worden gestart.

De belangrijkste bevindingen uit het onderzoek van de 9 hoofdstukken beschreven in dit proefschrift en de relevantie van het hondenmodel voor het osteosarcoom bij de mens

worden besproken in **hoofdstuk 12** met een slotbeschouwing en een voorstel voor toekomstige onderzoek voor het honden OS.

Het onderzoek van de afgelopen 5 jaar, met in begrip van dit proefschrift, heeft geleid tot een aantal belangrijke ontwikkelingen in de pathophysiologische kennis van honden OS. Deze ontwikkelingen zullen meer mogelijkheden gegeven om de ziekte met nieuwere therapeutische interventies te bestrijden. Hiernaast kunnen deze honden gestratificeerd worden naar geschikte prognostische groepen, waardoor meer geïndividualiseerde behandeling van de ziekte gegeven kan worden. Er is echter nog veel te doen en goed ontworpen prognostische studies op populaties van honden met nieuwe interventionele therapieën, alleen of in combinatie met de huidige therapieën zijn van cruciaal belang.

Een feit is dat zelfs de beste biomarker niet zal werken onder bepaalde omstandigheden en onnauwkeurig zal zijn voor een groot aantal honden met OS en eventueel de mens. Continue verslaglegging en herbeoordeling van deze biomarkers is dus gerechtvaardigd.

De belangrijkste bevindingen uit dit proefschrift:

1. Dit proefschrift belicht enkele nieuwe moleculaire biomarkers die het potentieel hebben om klinisch-resultaten in honden OS te voorspellen. Hieronder behoren: decorine, EGFR, CYP24A1 en HSP60. Decorine en CYP24A1 hebben ook een voorspellende waarde bij de mens
2. Het honden OS kan worden gedifferentieerd met behulp van moleculaire handtekeningen en deze verstrekken informative over de prognose, heteventueel voorkomen van metastasen en het eventuele succes van therapeutische interventie.
3. Gen expressie profielen en de daaropvolgende pathway analyses van tumor monsters en *in vitro* modellen heeft een aantal relevante en belangrijke signaaltransductiepaden voor de uiteindelijke overlevingskans en eventuele uitzaaiingen van honden OS, zoals integrine, Wnt, fibroblast groeifactor (FGF), epidermale groeifactor receptor (EGFR) en ubiquitine proteasoom omzettingsroutes geïdentificeerd.
4. Het osteosarcoom van de mens en van de hond delen enkele algemene signaal transductiepaden die interessant zijn voor verder onderzoek
5. De prognostische factoren HSP60 and EGFR kunnen dienen voor nieuwe therapie doelen bij honden met OS. Als we deze markers remmen bij *in vitro* modellen van het honden OS dan leidt dit tot een verminderde cel proliferatie, verhoogde apoptosis and verminderde cel overleving.
6. De huidige studie heeft voor het eerst de canonieke Wnt-signalering bij het honden OS aangetoond via een betrouwbare expressiepatroon van nucleaire β-catenine in combinatie met Axine-2. Het spontane honden OS heeft vergelijkbare Axine-2 en bèta catenine expressie en is een relevant model voor het humane OS met soortgelijke Wnt / β-catenine activering en regulering. Remming van deze route leidt tot een verminderde celproliferatie in de meerderheid van de cellijnen en dit zou nuttig kunnen zijn voor een beperkt aantal honden en humane OS.
7. Selectieve kinase remmers van de EGFR, NF-kB, PKC, PKA en ERK2 signalering zouden gebruikt kunnen worden als potentiele nieuwe anti-kanker therapieën voor honden met gemetastaseerde OS.
8. Meerdere referentie-genen zijn nodig om kwantitatieve real-time PCR genexpressie data te normaliseren voor het honden OS. RPS5 en HNRNPH vormden de beste combinatie referentie-genen voor primaire OS en RPS5 en RPS19 voor OS cellijnen.

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Curriculum Vitae

ABOUT THE AUTHOR

Gayathri Thevi Selvarajah (Gaya) was born in Kuala Lumpur, Malaysia on August 7th 1980. She received the Malaysian Higher School Certificate (STPM - equivalent to GCE-A Levels) in 1999 and started her undergraduate veterinary education at the Faculty of Veterinary Medicine, University Putra Malaysia (UPM) in May 2000. Gaya was entitled for the 'Dean's list' for several semesters and was also active in extra-curricular activities. Being the Vice President of VETERNAK, the faculty's veterinary student association, she participated in organizing many activities including leadership camps, *Dogathon*, national exotic rooster competition and educational symposiums for veterinary students. In July 2005, Gaya was awarded the Doctor of Veterinary Medicine (DVM), and was honored with the Vice Chancellor Award of University Putra Malaysia and Best DVM undergraduate in Small Animal Medicine by Friskies, Purina. In April 2005 she joined the University Veterinary Hospital (UVH) as an intern and in November the same year she was offered a tutorship position at the Department of Veterinary Clinical Studies of the Faculty of Veterinary Medicine (UPM). She officially embarked on studies in canine osteosarcoma from February 2007 onwards at the Department of Clinical Sciences of Companion Animals, Utrecht University. She received a full scholarship from the Ministry of Higher Education (MOHE) and University Putra Malaysia to support her research in the Netherlands. The research that she performed as part of her PhD doctoral studies are described in this thesis, which will be publically defended at the *Academiegebouw* on the 29th of March 2011. Upon receiving her doctorate degree, Gaya will return to Malaysia to continue working as a lecturer in companion animal medicine at the Faculty of Veterinary Medicine in UPM and live in Nilai with her husband Shree and pets *Tottot* (17 yo read-eared slider) & *Mylo* (4 yo male Rottweiler).

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List of abbreviations

OS	OS
SS	short survivors
LS	long survivors
ST	overall survival time
DFI	disease free interval
FDR	false discovery rate
SAM	Significance Analysis of Microarray
GEO	Gene Expression Omnibus
GSE	GEO Series
QPCR / qPCR	Quantitative real-time PCR
B2M	β-2-Microglobulin
GAPDH	glyceraldehyde-3-phosphatedehydrogenase
GUSB	β-glucuronidase
HNRNPH /hnRNPH	heterogeneous nuclear ribonucleoprotein H
HPRT	hypoxanthine phosphoribosyltransferase
RPL8	ribosomal protein L8
RPS5	ribosomal protein S5
RPS19	ribosomal protein S19
SRPR	signal recognition particle receptor
18sRNA	18S ribosomal RNA
mRNA	messenger RNA
DCN	Decorin
ANKRD17	Ankyrin repeat domain 17
MGST1	Microsomal gluthathione s-transferase 1
NCOR1	Nuclear receptor co-repressor 1
HSP	Heat shock protein
VDR	Vitamin D Receptor
CYP24A1	Vitamin D 24-hydroxylase
CYP27B1	Vitamin D 1α-hydroxylase
TBST	Triphosphate buffer saline- tween
PBS	Phosphate buffer saline
HBSS	Hank's Balanced Salt Solution
BSA	bovine serum albumin
EGFR	Epidermal growth factor receptor
PKA	Protein kinase A
PKC	Protein kinase C
h	hour
min	minute
sec	seconds
HR	hazard ratio
CI	confidence interval
OB	osteoblastic
CB	chondroblastic
TL	telangiectic
FB	fibroblastic
LiCl	Lithium chloride
DMSO	dimethyl sulfoxide
MRI	magnetic resonance imaging
CT	computed tomography