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Osteosarcoma tissues and cell lines from patients with differing serum alkaline phosphatase concentrations display minimal differences in gene expression patterns

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Abstract

Serum alkaline phosphatase (ALP) concentration is a prognostic factor for osteosarcoma in multiple studies, although its biological significance remains incompletely understood. To determine whether gene expression patterns differed in osteosarcoma from patients with differing serum ALP concentrations, microarray analysis was performed on 18 primary osteosarcoma samples and six osteosarcoma cell lines from dogs with normal and increased serum ALP concentration. No differences in gene expression patterns were noted between tumours or cell lines with differing serum ALP concentration using a gene-specific two-sample *t*-test. Using a more sensitive empirical Bayes procedure, defective in cullin neddylation 1 domain containing 1 (DCUN1D1) was increased in both the tissue and cell lines of the normal ALP group. Using quantitative PCR (qPCR), differences in DCUN1D1 expression between the two groups failed to reach significance. The homogeneity of gene expression patterns of osteosarcoma associated differing serum ALP concentrations are consistent with previous studies suggesting serum ALP concentration is not associated with intrinsic differences of osteosarcoma cells.

Keywords
cell signalling, comparative oncology, genetics, oncology, tumour biology

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Osteosarcoma is the most frequent primary bone malignancy in dogs.[1] The disease is associated with a high metastatic rate and most disease-related mortalities are because of pulmonary metastatic disease. Osteosarcoma most frequently affects large breed dogs and arises in the metaphyseal region of long bones, specifically the proximal humerus, proximal tibia and distal femur. Prognostic factors that have been identified for osteosarcoma include tumour location, tumour size, presence of metastatic disease at diagnosis, response to chemotherapy, various genetic alterations, and serum alkaline phosphatase (ALP) concentration.[2–10]

The prognostic significance of serum ALP concentration in dogs with osteosarcoma was initially reported by Ehrhart and subsequently confirmed in

multiple other veterinary studies.[3–8,11,12] Similarly, serum ALP concentration is a negative prognostic factor in humans with osteosarcoma.[13–19] Whereas prognostic significance of serum ALP concentration has been widely accepted, other studies have failed to find a prognostic significance of increased serum ALP.[20] Until recently, relatively little was known regarding the biological or mechanistic relevance of increased serum ALP concentration in osteosarcoma.

ALP is an enzyme that catalyses the hydrolysis of phosphate esters at an alkaline pH.[21] In dogs, there are two distinct ALP iso-enzymes which are encoded by two genes, the intestinal and tissue non-specific genes. The tissue non-specific iso-enzyme gives rise to three isoforms: bone, liver and kidney.[21,22] The ALP enzymes are linked to the outer surface of the cell membrane via a glycophosphytidylinositol (GPI) linkage protein. Bone-specific ALP (bALP) is frequently used as a marker of cells from the osteoblastic lineage and osteoblastic activity.[22,23] Additionally, immunocytochemical or immunohistochemical staining for the presence of ALP has been used to differentiate osteosarcomas from other sarcoma types during cytologic and histopathologic examination, respectively.[24] The function of bALP is poorly understood, although it is likely be involved with skeletal mineralization.[22,23]

As previously noted, a subset of osteosarcoma patients have an increased serum ALP concentration at the time of diagnosis, which is associated with a worse prognosis.[3–8,11,12] Recently, studies have attempted to better define the biological or mechanistic significance of increased serum ALP in canine osteosarcoma. Initial studies attempting to correlate ALP concentration with tumour size or volume found no definitive association.[3,25,26] However, a recent study by Sternberg et al. indicated that tumour burden is a determinant of ALP concentration, although it may not be the sole determinant.[27] Additionally, the behaviour of primary osteosarcoma cell lines derived from patients of differing serum ALP status were found to be no different between cell lines derived from patients with normal or increased serum ALP.[28] Surprisingly, there was no difference in the ALP activity of these cell lines despite the difference in serum ALP concentration. These findings would be consistent with those of Sternberg et al. in that differences in ALP activity would result from differences in cell number, or tumour burden, as opposed to intrinsic differences between cells comprising the individual tumours. However, it is conceivable that these tumours may differ in ways that were not assessed by the *in vitro* assays. Also, the generation of primary osteosarcoma cell lines selects for the clonal expansion of cells that may not be representative of the entire tumour.

Therefore, the aim of this study was to determine if the gene expression pattern of osteosarcoma differed between osteosarcoma-bearing dogs having different serum ALP concentrations (i.e., normal versus increased). We hypothesized that the gene expression profile of osteosarcoma tissue from dogs associated with increased serum ALP concentration would be distinct from osteosarcoma tissue associated with normal serum ALP.

Materials and methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the UW-Madison's School of Veterinary Medicine Animal Care and Use Committee (Protocol: V01391-0-09-08). Owner consent was obtained prior to the collection of tumour tissue.

Clinical sample selection

Patient requirements for tumour tissue samples to be collected for this study included a histopathologic diagnosis of osteosarcoma and no previous treatment with any cytotoxic chemotherapy agent or radiation therapy. The determination of bALP isoform concentration was not performed, although attempts were made to be as stringent as possible regarding patient selection for the collection of tumour samples associated with increased serum ALP concentration. Patients could not have received any corticosteroids for a period of at least 2 weeks preceding the identification of an increased serum ALP concentration and tissue collection.

In addition, with the exception of serum ALP, all renal and hepatic enzyme values were required to be within normal limits of the reporting clinical pathology laboratory. The determination of serum ALP concentration for patient samples utilized in this study occurred at multiple clinical pathology laboratories and at different times, resulting in differences in the reference range for ALP. For this reason each patient was classified as having normal or increased serum ALP according to the normal reference range for the reporting clinical pathology laboratory. Canine osteosarcoma tissue was collected at the time of diagnostic biopsy, surgical amputation or necropsy at either the University of Wisconsin-Madison Veterinary Medical Teaching Hospital or the Flint Animal Cancer Center at Colorado State University. All tumour tissue was snap frozen in liquid nitrogen and stored at −80 F until used for RNA isolation. Samples shipped from CSU to UW-Madison for processing were sent on dry ice and all samples remained frozen upon inspection at the time of receipt. For the canine osteosarcoma tissue collected at UW-Madison, a portion of tumour tissue was placed into phosphate-buffered saline (PBS) for generation of canine primary osteosarcoma cell lines and another portion was snap frozen in liquid nitrogen.

Generation of canine primary osteosarcoma cell lines

Primary cell lines were generated from clinical tissue samples using previously described procedures.[28] Briefly, the tumour tissue collected in PBS with PenStrepFungizone (Invitrogen, CA, USA) underwent enzymatic and mechanical digestion using collagenase I (200 U mL^{-1}) (Worthington Biochemical, Lakewood, NJ, USA) and DNase I (100 U mL^{-1}) (Sigma Aldrich, St. Louis, MO, USA) in association with scalpel and scissors mincing, followed by filtration through a 40 mesh sieve until a single cell suspension was created. The resulting cell suspension was centrifuged for 7 min at 1400 rpm. The pellet was washed with sterile saline and re-centrifuged with the same conditions. To the pellet, 3 mL of complete modified eagle media (CMEM) was added, and the mixture was incubated in a flask with an additional 9 mL of CMEM. All cells were maintained in CMEM supplemented with 10% heat-inactivated cosmic calf serum (Thermo Scientific, Waltham, MA, USA), sodium pyruvate (Corning, Manassa, VA, USA), L-glutamine (Corning), modified eagle medium (MEM; Corning) vitamins, non-essential amino acids, and 1% Pen/Strep (Corning) at 37 °C in a humidified incubator with 5% CO_2. All cell lines used in this experiment were beyond the 15th passage.

Microarray experiments

Total RNA was isolated from clinical samples using Trizol (Invitrogen), and purified by PureLink RNA Mini Kit (Ambion, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. All RNA was isolated from tumour tissue samples and cell lines by one individual at UW-Madison. When isolating RNA from the tumour samples and cell lines care was taken to always pair a normal serum ALP and increased ALP sample at the same time. The quantity and purity of RNA was assessed by UV Spectroscopy. The absorbance of RNA samples was measured at wavelengths of 260 and 280 nm using a NanoDrop ND-100 spectrophotometer (Isogen Life Science NV, Sint-Pieters-Leeuw, Belgium). RNA integrity was assessed by running all samples on a denaturing agarose gel. Array analysis with GeneChip Canine 2.0 Genome Arrays (Affymetrix, Santa Clara, CA, USA) was performed for all tumour samples. The oneCycle Target Labeling and Control Reagents package (Afftmetrix) was used to synthesize cDNA from total RNA spiked with prokaryotic Poly-A RNA as a control. The Sample Cleanup Module (Affymetrix) was used to purify the cDNA which was then used for synthesis of biotin-labelled cRNA. cRNA was purified, quantified and fragmented before hybridization with the GeneChips. Hybridized chips were washed, stained using streptavidin-conjugated phycoerythrin fye, Invitrogen) and enhanced with biotinylated goat anti-streptavidin antibody (Vector Laboratories, Burlingame, CA, USA) using an Affymetrix GeneChip Fluidics Station 450 and GeneChip Operating Software. The Affymetrix GeneChip scanner 3000 was used to acquire images. All Affymetrix GeneChip processing was carried out

at the UW-Madison Gene Expression Center. The canine osteosarcoma tissue and cell line (GSEarray data from this study are available through the Gene Expression Omnibus (GEO) website as accession numbers: GSE63476 and GSE57884, respectively.

Quantitative PCR (qPCR)

Total RNA was isolated from clinical samples using Trizol (Invitrogen) and purified by PureLink RNA Mini Kit (Ambion, Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The PureLink RNA Mini Kit includes treatment of RNA with DNAase to reduce genomic DNA contamination. The quantity and quality of isolated RNA was carried out as described in the microarray analysis section. qPCR was performed using TaqMan Gene Expression Master Mix with TaqMan Gene Expression Assays (Applied Biosystems) according to the manufacturers protocol on a Bio-Rad iQ5 Multicolor Real-Time PCR Detection System with Bio-Rad iCycler machine and iQ5 software. qPCR was performed on canine Frizzled-6 (Cf02625614_m1, Applied Biosystems), ABCC1 (Cf02624363_m1, Applied Biosystems), ABCG2 (Cf02627536_m1, Applied Biosystems) and ERCC1 (Cf02660663_g1, Applied Biosystems) to validate the results of our microarray data. The expression of defective in cullin neddylation 1 domain containing 1 (DCUN1D1) was evaluated amongst cell lines generated from tumour tissue associated with normal ($n = 3$) and increased ($n = 3$) serum ALP concentration using the following assay: canine DCUN1D1 (Cf02662796_m1, Applied Biosystems). Ct values were normalized to 18S expression (4352930E, Applied Biosystems). The qPCR primers utilized are designed to cross exon boundaries to exclude the amplification of genomic DNA. Relative difference in mRNA expression of canine OSA cell lines were compared with normal canine osteoblasts (Cell Applications, San Diego, CA) using the $\Delta\Delta$Ct method. Gene expression of samples was measured in triplicate.

Statistical analysis

Microarrays were checked for outliers and preprocessed using robust multiarray averaging (RMA). The effect of serum ALP was assessed using a two-way analysis of variance per gene. A two-way ANOVA was performed to account for potential batch effects. The differential expression of genes between high and low ALP status was tested using gene-specific two-sample *t*-tests coupled with Benjamini–Hochberg *p*-value adjustment. Empirical Bayes for Microarrays (EBarrays)-Lognormal Normal with Modified Variances Model (LNNMV), a more sensitive empirical Bayes procedure was also applied after the first test.[29] A two-tailed *t*-test was used to detect differences in DCUN1D1 expression, as determined by QPCR, between normal and increased ALP cell lines. Results were considered significant at $P < 0.05$.

To evaluate the relationship between serum ALP concentration and outcome for cases used in this study, cases of appendicular osteosarcoma who had undergone surgical excision of the primary tumour and received adjuvant chemotherapy were divided into two sub-groups; those having normal serum ALP concentration and those having increased serum ALP concentration. Descriptive and comparative statistics were performed for the overall study population, as well as the two main sub-groups. Fisher's exact test was used to compare categorical non-numerical data, such as gender and tumour location between groups. The Wilcoxon Rank-Sum Test was used to compare continuous numerical data between groups. Results were considered significant at $P < 0.05$.

Owing to the lack of routine follow-up, progression free survival (PFS) was not able to be determined. Overall survival (OS) was defined as the time (in days) from the date of surgery until the date of death as a result of osteosarcoma or another cause. Data was considered right-censored at the time of last veterinary contact if the dogs remained alive at the conclusion of the study period, or if they were ultimately lost to follow-up. Kaplan–Meier survival curves were estimated for patients with normal and increased serum ALP concentration. The median OS was compared between patients with normal and increased serum ALP concentration using the Log-Rank Test. Results were considered significant at $P < 0.05$.

All statistical analyses were performed utilizing two commercially available software packages:

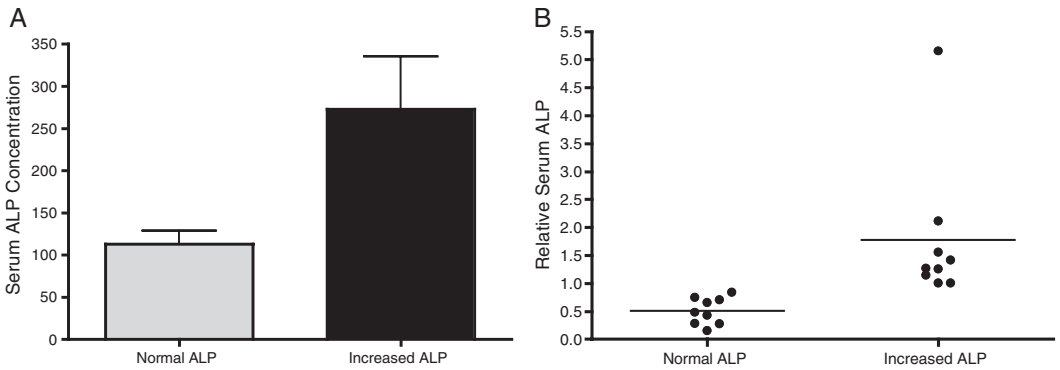

Figure 1. (A) The serum ALP concentration (mean ± SEM) for the normal (112.8 ± 16.1 U L^{-1}) and increased (273 ± 62.6 U L^{-1}) serum ALP samples used in this study. (B) Dot-plot representing the serum ALP concentration relative to the upper-limit of normal for individual samples from the normal and increased serum ALP groups.

Microsoft® Excel® for Mac 2011 (version 14.2.5) and R for Mac OS X GUI 2012 (version 2.15.2).

Results

Osteosarcoma tissue was collected from 18 canine patients at the University of Wisconsin-Madison Veterinary Medical Teaching Hospital ($n = 8$) and Colorado State University's Animal Cancer Center ($n = 10$). Of the 18 dogs from which osteosarcoma tumour tissue was collected, nine dogs had normal serum ALP concentration and nine dogs had an increased serum ALP concentration at the time of diagnosis (Table 1). The serum ALP concentration [mean ± standard error mean (SEM)] was 112.8 ± 16.1 and 273 ± 62.6 U L^{-1} for the normal and increased serum ALP groups, respectively (Fig. 1A). As the serum ALP concentration was evaluated at different institutions we normalized values to the upper-limit of the reference range provided by the testing laboratory. The relative serum ALP value (mean ± SEM) was 0.51 ± 0.08 and 1.78 ± 0.44 for the normal and increased serum ALP groups, respectively (Fig. 1B).

The average age of the nine dogs forming the normal serum ALP concentration group (8.7 years ± 0.8) was not significantly different from the age of the increased serum ALP concentration group (9.2 years ± 0.6) ($P = 0.73$). The group of dogs with normal serum ALP was comprised of five castrated males, three spayed females and one intact male; similarly, the group of dogs with increased serum ALP was comprised of four castrated males, four spayed females and 1 intact male. The tumour locations for dogs with normal serum ALP concentration included the humerus in three (two proximal and one diaphyseal), radius in three, femur in one, tibia in one and calcaneous in one. The locations of tumours for dogs with increased serum ALP concentration consisted of the humerus in four cases, radius in four, and tibia in one. Finally, the histologic sub-type of osteosarcomas present in the normal serum ALP group consisted of seven osteoblastic, one chondroblastic/osteoblastic, and one that was not defined. The osteosarcomas comprising the increased serum ALP group included seven osteoblastic, one chondroblastic and one with no description of histologic subtype.

Of the 18 patients from whom samples were collected, 13 were known to have received adjuvant chemotherapy following amputation. Following amputation, dogs received adjuvant chemotherapy protocols consisting of carboplatin, adriamycin, gemcitabine or liposomal-encapsulated muramyl tripeptide ethanolamine (L-MTP-PE). The chemotherapeutics were given as single agents or in combination. Of the remaining five dogs, two underwent amputation for treatment of osteosarcoma but information regarding adjuvant chemotherapy was not available, one dog received only metronomic chemotherapy following amputation, and one dog was euthanized at the time of tissue collection. The median OS time for all dogs contributing tissue to this study was 217 days. The median OS time for dogs with normal and increased

Table 1. Summary of clinical data from 18 dogs used in this study

Institute	Group	ALP	Age (years)	Breed	Sex	Tumour site	Histology description
UW	Normal	218	10	Rottweiler	Spayed female	Right Proximal Humerus	None
UW	Normal	124	10	Mastiff	Castrated male	Left Proximal Radius	Chond/Osteoid
UW	Normal	104	9.5	Irish Terrie	M	Left Diaphyseal Humerus	Osteoblastic
UW	Normal	45	5	Great Dane	Castrated male	Right Distal Radius	Osteoblastic
UW	Normal	81	11	Eng. Springer Spaniel	Castrated male	Calcaneus	Osteoblastic
UW	Normal	82	5	Doberman	Castrated male	Right Proximal Radius	Osteoblastic
CSU	Normal	101	7	Greyhound	Spayed female	Left Distal Femur	Osteoblastic
UW	Normal	140	10	Boxer	Castrated male	Right Proximal Humerus	Osteoblastic
CSU	Normal	120	11	Mix	Spayed female	Tibia	Osteoblastic
CSU	Increased	164	8.5	Rottweiler	Castrated male	Left Proximal Humerus	Intermediate
CSU	Increased	733	7	Rottweiler	Castrated male	Right Distal Radius	Osteoblastic
CSU	Increased	301	7.5	Rottweiler	Spayed female	Left Proximal Humerus	Osteoblastic
CSU	Increased	181	7	Great Pyrenees	Castrated male	Right Distal Radius	Osteoblastic
CSU	Increased	144	10	Golden Retrivier	Spayed female	Right Distal Radius	Chondroblastic
UW	Increased	365	11.5	Labrador Retrivier	Castrated male	Right Distal Radius	Osteoblastic
CSU	Increased	144	11	Irish Setter	Male	Right Proximal Humerus	Osteoblastic
CSU	Increased	222	9	Great Dane	Spayed female	Right Proximal Tibia	Osteoblastic
CSU	Increased	202	11	Labrador Retrivier	Spayed female	Right Proximal Humerus	Osteoblastic

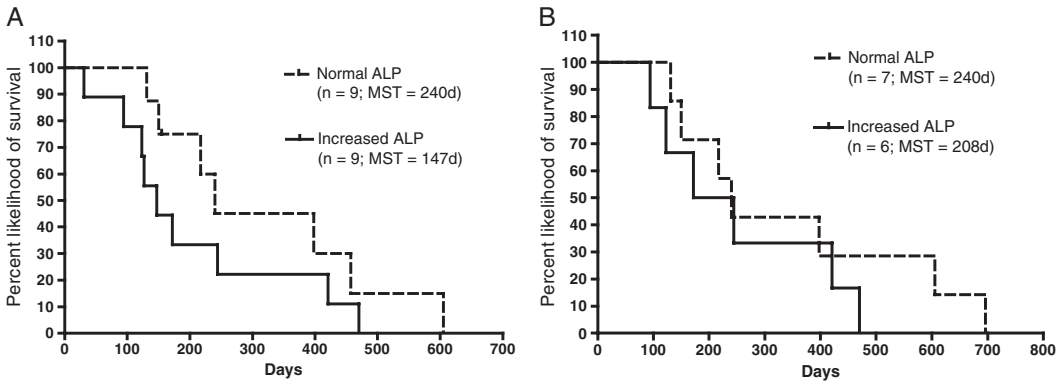

Figure 2. (A) The median overall survival time for dogs with osteosarcoma associated with normal ($n = 9$) and increased ($n = 9$) serum ALP concentration was 240 and 147 days, respectively ($P = 0.2$). (B) The median survival time for dogs with osteosarcoma associated with normal ($n = 7$) and increased ($n = 6$) serum ALP concentration undergoing amputation and adjuvant chemotherapy was 240 and 208 days, respectively ($P = 0.4$).

serum ALP concentration was 240 and 147 days ($P = 0.2$), respectively (Fig. 2A). If limited to only the 13 dogs undergoing amputation and receiving cytotoxic chemotherapy, the overall median survival time was 240 days. When assessing the OS time for these dogs according to serum ALP concentration, the median OS time for dogs with normal and increased serum ALP concentration was 240 and 208 days ($P = 0.4$), respectively (Fig. 2B).

Gene expression profiling was performed using RNA isolated from tumour tissue of the 18 cases of primary canine osteosarcoma, 9 samples were from patients with normal serum ALP and 9 from patients with increased serum ALP concentration at diagnosis. A comparison of gene expression level indicates a homogeneity gene expression between groups. The microarray probed 43 035 transcripts and there were no differences noted in the gene expression pattern of tumour tissue isolated from patients with normal and increased serum ALP concentration when analysed using a gene-specific two-tailed *t*-test (minimum adjusted *p*-value 0.05). When gene expression patterns were assessed using a more sensitive empirical Bayes procedure,

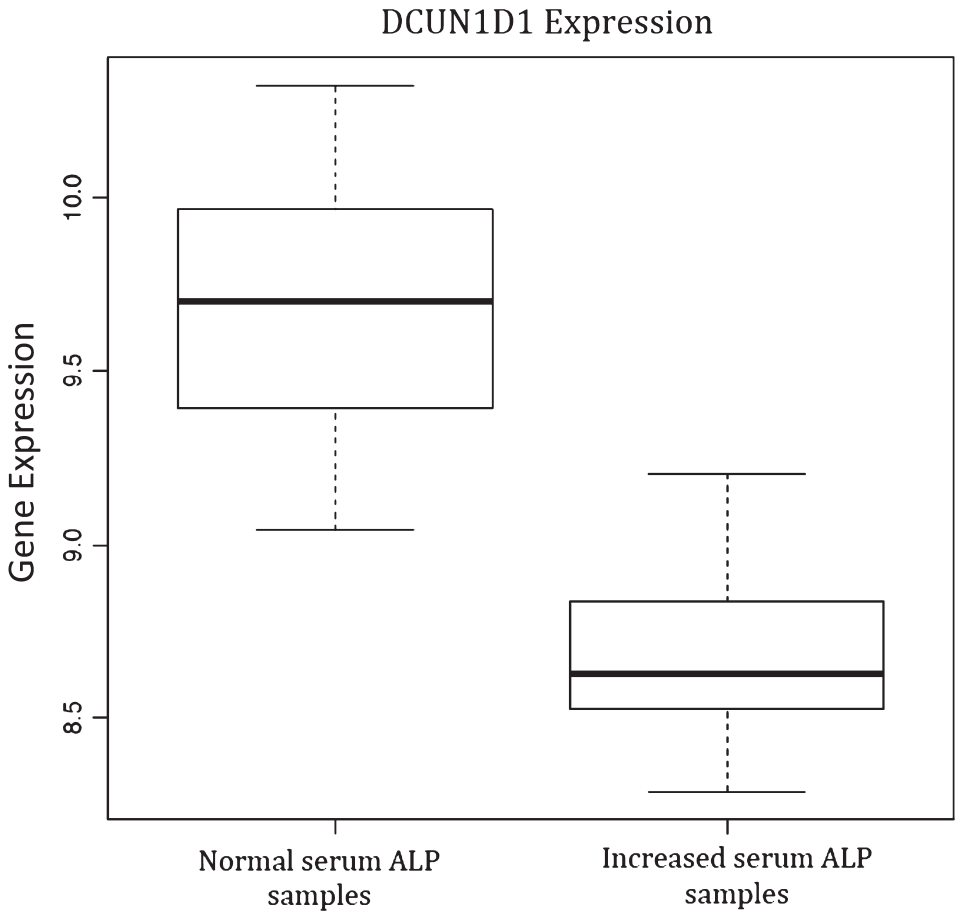

Figure 3. Microarray analysis identifies DCUN1D1 as being differentially expressed between canine osteosarcoma primary tumour in dogs with normal and increased serum ALP concentration at diagnosis. Data presented as the mean (solid bar in box), inter-quartile range (box), and overall range (whiskers) of DCUN1D1 expression on microarray analysis.

EBarrays (posterior probability of differential expression 0.98) (2% FDR), the expression of DCUN1D1 was found to be increased in normal serum ALP group compared to the increased serum ALP group (Fig. 3). Although the difference in DCUN1D1 expression between groups reached statistical significance, the relative difference in DCUN1D1 expression between the two groups was very slight, just less than one-fold.

To ensure the lack of differences between the gene expression patterns of osteosarcoma tissues associated with differing serum ALP concentration was not due to non-tumoural tissue, which may also be present in the biopsy samples, drowning out any potential difference in tumour tissue we also performed microarray analysis on primary osteosarcoma cell lines generated from a subset of these tumours. All six primary osteosarcoma cell lines were previously noted to express ALP, thereby confirming their osteoblast-lineage origin.[28] The gene expression patterns of six canine primary osteosarcoma cell lines (three normal ALP and three increased ALP) were compared first using the gene-specific two-tailed *t*-test. Similar to the results obtained using tumour tissue samples, there were no significant differences identified in gene expression between cell lines associated with normal or increased serum ALP. Therefore, we used the more sensitive EBarrays to detect any differences in gene expression between the two groups. Again, the expression of DCUN1D1 was greater in normal serum ALP cell lines compared with increased serum ALP cell lines. No other genes were identified as being differentially expressed

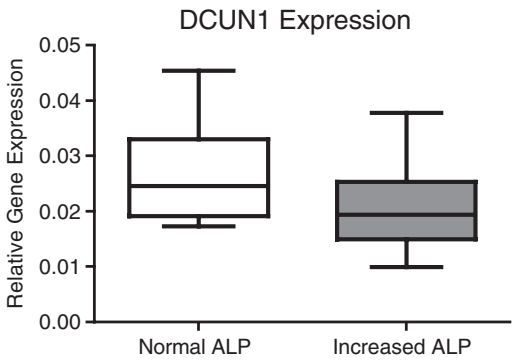

Figure 4. The difference in relative gene expression of DCUN1D1 between primary canine osteosarcoma cell lines associated with normal ($n = 3$) and increased ($n = 3$) serum ALP concentration fails to reach significance ($P = 0.16$). Data presented as the mean (solid bar in box), inter-quartile range (box), and overall range (whiskers) of relative expression of DCUN1D1 with qPCR.

between the cell lines associated with normal and increased serum ALP concentration.

Quantitative reverse-transcription polymerase chain reaction (qPCR) was used to validate the increased expression of DCUN1D1 noted in the osteosarcoma samples associated with normal serum ALP relative to samples of osteosarcoma associated with increased serum ALP. The increase in DCUN1D1 expression in cell lines from patients with normal serum ALP concentration relative to cell lines from patients with increased serum ALP concentration failed to reach significance ($P = 0.16$) (Fig. 4).

Discussion

Serum ALP concentration has been correlated with OS time and disease-free interval (DFI) in osteosarcoma patients, and is considered a negative prognostic factor when increased at diagnosis in humans and dogs with osteosarcoma.[5,14] The findings from a recent meta-analysis of prognostic factors for canine osteosarcoma performed by Boerman et al. supports serum ALP concentration as a prognostic factor in dogs with osteosarcoma. In the meta-analysis, the association of serum ALP and survival time was assessed using data from seven previous studies and dogs with an increased serum ALP concentration had a shorter survival time compared with dogs with normal ALP level within reference range, with a hazard ratio of 1.62 (95% confidence interval [CI]: 1.21–2.17).[7] Additionally, the meta-analysis examined the association between serum ALP and DFI using data from 10 previous studies and found a hazard ratio of 1.96 for dogs with an increased serum ALP concentration. These findings were supported by another meta-analysis of individual patient data, which reported a hazard ratio of 1.34 for metastatic development and 1.43 for mortality in dogs with increased serum ALP concentration.[8] In the current study, the association between serum ALP concentration and DFI was not assessed due to the use of archived tissue and lack of standardized clinical follow-up on all patients. Whereas a numeric difference existed in the median OS time between dogs with normal (240 days) and increased (208 days) serum ALP concentration that were treated similarly, this difference did not reach statistical significance ($P = 0.4$). The lack of significance is likely due to the small sample size used for the study. Additionally, the use of survival time as an endpoint in veterinary medicine is fraught with potential biases that limit its usefulness.

Relatively little is known regarding the biological or mechanistic relevance of increased serum ALP concentration as a negative prognostic factor. ALP is considered an important marker of osteoblast differentiation and activity.[30] Serum ALP concentration is frequently used as a biomarker of osteoblastic activity and is known to be increased in non-neoplastic, orthopeadic conditions including bone growth. Our laboratory has reported on the lack of differences in behaviour of cell lines generated from patients with differing serum ALP concentration, suggesting the prognostic significance of ALP is not necessarily due to intrinsic differences between the neoplastic cells comprising the tumour. However, our previous findings were based on the results of *in vitro* assays carried out with osteosarcoma cell lines. As noted in the introduction, the use of cell lines has its limitations when attempting to address questions relating to the behaviour of disease processes which arise *in vivo* amongst a complex interaction between tumour cells, tumour microenvironment, and the immune system. Therefore, in attempts to improve upon our previous *in vitro* study, we evaluated the gene expression of

osteosarcoma tumour tissue in dogs with normal and increased serum ALP concentration. The aim of this study was to determine whether differences in gene expression pattern existed between osteosarcoma tissue from patients with normal or increased serum ALP. The identification of unique gene expression patterns or differentially regulated pathways could be used to address differences in prognosis. We hypothesized that there would be differences in the gene expression profiles between osteosarcoma tissues derived from dogs with normal and increased serum ALP concentration.

Surprisingly, osteosarcoma tumour tissue from patients with normal and increased serum ALP concentration did not show any consistent differences in gene expression patterns when evaluated using a two-tailed *t*-test. Similarly, when the gene expression patterns of canine primary osteosarcoma cell lines were evaluated, there were still no differences noted based on serum ALP concentration. These findings suggest the differences in serum ALP concentration in osteosarcoma-bearing dogs is not due to intrinsic differences within the osteosarcoma cells themselves. Although it is possible there could be differences in the translation of RNA or proteins that are involved with the post-translational modification of ALP that may result in differences in ALP stability and subsequently serum ALP concentration. The current findings are consistent with the results of our previous study in which there were no differences in the behaviour of osteosarcoma cell lines based on the serum ALP concentration of patients from which they were derived.[28] Similarly, Sternberg et al. showed tumour burden as being a determinant of serum ALP concentration, but did not rule out other potential factors influencing serum ALP concentration in osteosarcoma patients.[27]

Using a more sensitive test, the EBarrays, to analyze the microarray results, the DCUN1D1 gene was found increased in osteosarcoma tissue from dogs with normal serum ALP group compared with osteosarcoma tissue from dogs with increased serum ALP. The DCUN1D, or squamous cell carcinoma related oncogene (SCCRO), gene was initially identified as a result of investigation of 3q amplification in head and neck squamous cell carcinoma.[31] DCUN1D1 augments neddylation, a posttranslational modification of cullins, which is a regulatory step in cullin RING ligase-mediated protein ubiquitination. Several studies suggest DCUN1D1 could be an oncogene as neddylation is an established pathway in cancer pathogenesis.[32–34] The expression of DCUN1D1 has been associated with non-small cell lung carcinoma (NSCLC) tumour stage. Approximately 25% of patients with NSCLC will development brain metastasis, and almost all of these tumours express DCUN1D1 identify by immunohistochemistry evaluation.[35] Over expression of DCUN1D1 mRNA and protein was also seen in primary gliomas relative to normal brain tissue, and shows ability to facilitate malignant transformation and carcinogenic progression *in vivo*.[34] The relevance of DCUN1D1 in osteosarcoma has yet to be determined. To our knowledge, DCUN1D1 has not been reported on previously in association with osteosarcoma. In this study, DCUN1D1 expression was identified via microarray analysis as being increased in osteosarcoma tissue and cell lines associated with normal ALP. Given its previous categorization as an oncogene and association with more aggressive squamous cell carcinomas in humans it is somewhat surprising that DCUN1D1 expression is increased in a population of tumours associated with a better prognosis. The counter-intuitive nature of this preliminary finding may be due to this gene having distinct functions in tumours of differing histologies, however additional studies are necessary to determine the significance of this finding. An additional reason for pause in the interpretation of this result is due to the difference in DCUN1D1 expression between groups was less than one-fold on microarray analysis and the difference in expression when assessed by QPCR failed to reach significance. However, the lack of significance in the QPCR may be due to the small sample size that was evaluated in both the normal ($n = 3$) and increased ($n = 3$) serum ALP cell lines.

Microarray analysis has been used in numerous studies of human osteosarcoma tissue and cell lines to identify genes involved chemotherapeutic resistance, the metastatic process and in attempts to identify the cell of origin for osteosarcoma.[36–40] The results of these, and other, studies, underscore the highly complex and heterogeneous nature of

osteosarcoma in which the molecular basis of malignant transformation and metastasis remains poorly understood.[9,41,42] More recently, microarray analyses have been used to better characterize altered gene expression in canine osteosarcoma in attempts to improve our understanding of genes and signalling pathways that may contribute to a more aggressive phenotype. Paoloni et al. initially described the use of canine osteosarcoma tissue in microarray experiments and highlighted the significant similarities between canine and human osteosarcoma gene expression patterns, thereby validating spontaneously arising canine osteosarcoma as a highly relevant model for the human disease.[2] In a study by Selvarajah et al. microarray analysis was performed on canine osteosarcoma tissue to identify differentially expressed genes, and subsequently signalling pathways, associated with survival.[9] In this study, the patients were separated in two groups based on survival time (>6 versus < 6 months), and using a false discovery rate of 10%, 51 genes were found to be differentially expressed between the two groups. Using a similar approach, O'Donoghue et al. performed gene expression profiling on osteosarcoma samples from patients who had undergone surgery and adjuvant chemotherapy but differed in their DFI (DFI < 100 versus DFI > 300 days).[42] The homogeneity of gene expression found in our study is not characteristic of canine osteosarcoma and should not be interpreted that osteosarcoma is a homogeneous disease as our results were concerned only in regards to differences in gene expression patterns based on the ALP tiers status. It is possible the homogeneity within our study is a reflection of the relative homogeneity, aside from serum ALP status, in the patient population that was assessed. Kubista et al. were able to use microarray analysis to differentiate osteosarcoma samples into the histologic subtypes of osteoblastic versus non-osteoblastic.[43] In our study the vast majority of histologic subtypes in both groups was osteoblastic, therefore the histologic subtype was highly homogeneous making it less likely that we would observe highly heterogeneous gene expression patterns. As noted previously, studies have identified differences in gene expression patterns associated with differences in outcomes in canine osteosarcoma,[9,41] however in this study there was no significant difference in the median survival time between the two groups. Different breakdowns in the current study population could show more diversity in gene expression comparing groups with different prognosis as in other studies.[9,36–38,42] It is conceivable that our study may have produced different results had we utilized a patient population in which the serum ALP concentration was associated with a poorer outcome; however, our study should have been able to detect a difference in the gene expression pattern between the tumours associated with normal and increased serum ALP concentration should a true difference have existed based upon this classification, as the serum ALP concentration was significantly different between the two groups.

The similarity in gene expression between groups with normal and increased serum ALP concentration at diagnosis identified in this study supports the theory arising from the results of the Sternberg et al. and Holmes et al. studies that increased serum ALP concentration might not be linked to enhanced tumourigenic or metastatic cellular behaviour.[27,28] Although gene expression analysis did not show or indicate pathways or genes involved in this process, these results could benefit further investigations regarding the role of ALP in tumourigenicity and/or osteogenic process in osteosarcoma, and its relation with prognosis.

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References

1. Ehrhart NP, Stewart SD and Fan TM. Tumors of the skeletal system. In: *Withrow and MacEwen's Small Animal Oncology*. 3rd edn., SJ Withrow, DM Vail and RL Page Eds., St. Louis, Elsevier Inc, 2013: 463–503.
2. Paoloni M, Davis S, Lana S, Withrow S, Sangiorgi L, Picci P, *et al.* Canine tumor cross-species genomics uncovers targets linked to osteosarcoma progression. *BMC Genomics* 2009; **10**: 625.
3. Ehrhart N, Dernell WS, Hoffmann WE, Weigel RM, Powers BE and Withrow SJ. Prognostic importance of alkaline phosphatase activity in serum from dogs with appendicular osteosarcoma: 75 cases (1990–1996). *Journal of the American Veterinary Medical Association* 1998; **213**: 1002–1006.
4. Garzotto CK, Berg J, Hoffmann WE and Rand WM. Prognostic significance of serum alkaline phosphatase activity in canine appendicular osteosarcoma. *Journal of Veterinary Internal Medicine* 2000; **14**: 587–592.
5. Phillips B, Powers BE, Dernell WS, Straw RC, Khanna C, Hogge GS, *et al.* Use of single-agent carboplatin as adjuvant or neoadjuvant therapy in conjunction with amputation for appendicular osteosarcoma in dogs. *Journal of the American Animal Hospital Association* 2009; **45**: 33–38.
6. Moore AS, Dernell WS, Ogilvie GK, Kristal O, Elmslie R, Kitchell B, *et al.* Doxorubicin and BAY 12-9566 for the treatment of osteosarcoma in dogs: a randomized, double-blind, placebo-controlled study. *Journal of Veterinary Internal Medicine* 2007; **21**: 783–790.
7. Boerman I, Selvarajah GT, Nielen M and Kirpensteijn J. Prognostic factors in canine appendicular osteosarcoma – a meta-analysis. *BMC Veterinary Research* 2012; **8**: 56.
8. Schmidt AF, Nielen M, Klungel OH, Hoes AW, de Boer A, Groenwold RH, *et al.* Prognostic factors of early metastasis and mortality in dogs with appendicular osteosarcoma after receiving surgery: an individual patient data meta-analysis. *Preventative Veterinary Medicine* 2013; **112**: 414–422.
9. Selvarajah GT, Kirpensteijn J, van Wolferen ME, Rao NA, Fieten H and Mol JA. Gene expression profiling of canine osteosarcoma reveals genes associated with short and long survival times. *Molecular Cancer* 2009; **8**: 72.
10. Thamm DH, O'Brien MG and Vail DM. Serum vascular endothelial growth factor concentrations and postsurgical outcome in dogs with osteosarcoma. *Veterinary and Comparative Oncology* 2008; **6**: 126–132.
11. Kow K, Thamm DH, Terry J, Grunerud K, Bailey SM, Withrow SJ, *et al.* Impact of telomerase status on canine osteosarcoma patients. *Journal of Veterinary Internal Medicine* 2008; **22**: 1366–1372.
12. Sottnik JL, Rao S, Lafferty MH, Thamm DH, Morley PS, Withrow SJ, *et al.* Association of blood monocyte and lymphocyte count and disease-free interval in dog with osteosarcoma. *Journal of Veterinary Internal Medicine* 2010; **24**: 1439–1444.
13. Bacci G, Dallarin D, Battistini A, Orlandi M, Ferrari S, Avella M, *et al.* The prognostic value of serum alkaline phosphatase in osteosarcoma of the limbs. *La Chirurgia degli Organi di Movimento* 1992; **77**: 171–180.
14. Bacci G, Picci P, Ferrari S, Orlandi M, Ruggieri P, Casadei R, *et al.* Prognostic significance of serum alkaline phosphatase measurements in patients with osteosarcoma treated with adjuvant or neoadjuvant chemotherapy. *Cancer* 1993; **71**: 1224–1230.
15. Bielack SS, Kempf-Bielack B, Delling G, Exner GU, Flege S, Helmke K, *et al.* Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. *Journal of Clinical Oncology* 2002; **20**: 776–790.
16. Bramer JA, Abudu AA, Tillman RM, Carter SR, Sumathi VP and Grimer RJ. Pre- and post-chemotherapy alkaline phosphatase levels as prognostic indicators in adults with localized osteosarcoma. *European Journal of Cancer* 2005; **41**: 2846–2852.
17. Min D, Lin F, Zhen Z, Zheng S, Tam L, Yu W, *et al.* Analysis of prognostic factors in 333 Chinese patients with high-grade osteosarcoma treated by multidisciplinary combined therapy. *Asian Pacific Journal of Cancer Prevention* 2013; **9**: 71–79.
18. Ford S, Saithna A, Grimer RJ and Picci P. Comparison of the outcome of conventional osteosarcoma at two specialist international orthopaedic oncology centres. *Sarcoma* 2004; **8**: 13–18.
19. Pochanugool L, Subhadharaphandou T, Dhanachai M, Hathirat P, Sangthawan D, Pirabul R, *et al.* Prognostic factors among 130 patients with osteosarcoma. *Clinical Orthopaedics and Related Research* 1997; **345**: 206–214.
20. Saam DE, Liptak JM, Stalker MJ and Chun R. Predictors of outcome in dogs treated with adjuvant carboplatin for appendicular osteosarcoma: 65 cases

(1996–2006). *Journal of the American Veterinary Medical Association* 2011; **238**: 195–206.
21. Harris HH. The human alkaline phosphatases: what we know and what we don't know. *Clinica Chimica Acta* 1989; **186**: 133–150.
22. Henthorn PS, Millán JL and Leboy P. Acid and alkaline phosphatases. In: *Dynamics of Bone and Cartilage Metabolism*. MJ Seibel, SP Robins and JP Bilezikian Eds., San Diego, Academic Press, 1999: 130–134.
23. Rodan GA. Introduction to bone biology. *Bone* 1992; **13**: S3–S6.
24. Barger A, Graca R, Bailey K, Messick J, de Lorimer LP, Fan T, *et al.* Use of alkaline phosphatase to differentiate canine osteosarcoma from other vimentin-positive tumors. *Veterinary Pathology* 2005; **42**: 161–165.
25. Lacoste H, Fan TM, de Lorimier LP and Charney SC. Urine N-telopeptide excretion in dogs with appendicular osteosarcoma. *Journal of Veterinary Internal Medicine* 2006; **20**: 335–341.
26. Limmahakhun S, Pothacharoen P, Theera-Umpon N, Arpornchayanon O, Leerapun T, Luevitoonvechkij S, *et al.* Relationships between serum biomarker levels and clinical presentation of human osteosarcoma. *Asian Pacific Journal of Cancer Prevention* 2011; **12**: 1717–1722.
27. Sternberg RA, Pondenis HC, Yang X, Mitchell MA, O'Brien RT, Garrett LD, *et al.* Association between absolute tumor burden and serum bone-specific alkaline phosphatase in canine appendicular osteosarcoma. *Journal of Veterinary Internal Medicine* 2013; **27**: 955–963.
28. Holmes KE, Thompson V, Piskun CM, Kohnken RA, Huelsmeyer MK, Fan TM, *et al.* Canine osteosarcoma cell lines from patients with differing serum alkaline phosphatase concentrations display no behavioural differences in vitro. *Veterinary and Comparative Oncology* 2013. doi:10.1111/vco.12031.
29. Kendziorski CM, Chen M, Yuan M, Lan H and Attie AD. Statistical methods for expression quantitative trait loci (eQTL) mapping. *Biometrics* 2006; **62**: 19–27.
30. Ambroszkiewicz J, Gajewska J, Klepacka T, Chelchowska M, Laskowska-Klita T and Wozniak W. Clinical utility of biochemical bone turnover markers in children and adolescents with osteosarcoma. *Advances in Medical Sciences* 2010; **55**: 266–272.
31. Singh B, Gogineni SK, Sacks PG, Shaha AR, Shah JP, Stoffel A, *et al.* Molecular cytogenetic characterization of head and neck squamous cell carcinoma and refinement of 3q amplification. *Cancer Research* 2001; **61**: 4506–4513.
32. Guardavaccaro D and Pagano M. Oncogenic aberrations of cullin-dependent ubiquitin ligases. *Oncogene* 2004; **23**: 2037–2049.
33. Sufan RI and Ohh M. Role of the NEDD8 modification of Cul2 in the sequential activation of ECV complex. *Neoplasia* 2006; **8**: 956–963.
34. Broderick SR, Golas BJ, Pham D, Towe CW, Talbot SG, Kaufman A, *et al.* SCCRO promotes glioma formation and malignant progression in mice. *Neoplasia* 2010; **12**: 476–484.
35. Yoo J, Lee SH, Lym KI, Park SY, Yang SH, Yoo CY, *et al.* Immunohistochemical expression of DCUN1D1 in non-small cell lung carcinoma: its relation to brain metastasis. *Cancer Research and Treatment* 2012; **44**: 57–62.
36. Zucchini C, Rocchi A, Manara MC, De Sanctis P, Capanni C, Bianchini M, *et al.* Apoptotic genes as potential markers of metastatic phenotype in human osteosarcoma cell lines. *International Journal of Oncology* 2008; **32**: 17–31.
37. Nakano T, Tani M, Ishibashi Y, Kimura K, Park YB, Imaizumi N, *et al.* Biological properties and gene expression associated with metastatic potential of human osteosarcoma. *Clinical and Experimental Metastasis* 2003; **20**: 665–674.
38. Namløs HM, Kresse SH, Müller CR, Henriksen J, Holdhus R, Sæter G, *et al.* Global gene expression profiling of human osteosarcomas revelas metastasis-associated chemokine pattern. *Sarcoma* 2012; **639038**: 1–12. doi:10.1155/2012/639038.
39. Cleton-Jansen AM, Anninga JK, Briaire-de Bruijn IH, Romeo S, Oosting J, Egeler RM, *et al.* Profiling of high-grade central osteosarcoma and its putative progenitor cells identifies tumourigenic pathways. *British Journal of Cancer* 2009; **101**: 1909–1918.
40. Sottnik JL, Campbell B, Mehra R, Behbahani-Nejad O, Hall CL and Keller ET. Osteocytes serve as a progenitor cell of osteosarcoma. *Journal of Cellular Biochemistry* 2014; **115**: 1420–1429.
41. Lisle JW, Choi JY, Horton JA, Allen MJ and Damron TA. Metastatic osteosarcoma gene expression differs in vitro and in vivo. *Clinical Orthopedics and Related Research* 2008; **466**: 2071–2080.
42. O'Donoghue LE, Ptitsyn AA, Kamstock DA, Siebert J, Thomas RS and Duval DL. Expression profiling in canine osteosarcoma: identification of biomarkers and pathways associated with outcome. *BMC Cancer* 2010; **10**: 506.
43. Kubista B, Klinglmueller F, Bilban M, Pfeiffer M, Lass R, Giurea A, *et al.* Microarray analysis identifies distinct gene expression profiles associated with histological subtype in human osteosarcoma. *International Orthopaedics* 2011; **35**: 401–411.