Proteomic profiling of canine osteosarcoma patients

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Abstract

Background Canine osteosarcoma (OSA) is an aggressive primary bone tumor in dogs. Metastasis of canine OSA occurs mainly in the lungs through the hematogenous route. Identification of the proteins that are associated with metastasis using proteomic technologies is important not only for the discovery of OSA targets and signaling pathways associated with metastatic OSA but also to provide novel therapeutic targets. The objective of this study was to conduct a proteomic profiling analysis of canine OSA patients. We used mass spectrometry to examine the protein profiles. **Results** We found the overexpression of five proteins from OSA tissues—myosin (MYO7a), GTPase IMAP family member 5 isoform 1 (GIMAP5), PR domain 15 (PRDM15), A disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS4), and interleukin receptor type 1 (IL1R1)—and one protein from the serum, rCG38920. **Conclusions** In this study, we identified numerous proteins in the tissue and serum of patients with malignant and benign OSA or non-OSA through a proteomic approach using mass spectrometry. These proteins may be useful for diagnosis and prognosis, including the prevention of misdiagnosis between malignant OSA and non-OSA as these have similar clinical symptoms in the early stages. In the future, further independent validation of these biomarkers using a greater number of patient samples and different techniques, including Western blot, is necessary to improve diagnostic power for differentiating malignant OSA patients from non-OSA patients.

Background

Canine osteosarcoma (OSA) is an aggressive primary bone tumor in dogs, accounting for up to 85% of malignancies originating from the skeleton.[1] Metastasis of canine OSA occurs mainly in the lungs through the hematogenous route and is the most common cause of death.[1] Lymph nodes are involved less often (4.4–9%).[2, 3] Despite advances in the methods of managing canine OSA, including limb amputation/sparing, chemotherapy, and palliative radiotherapy, prognosis remains poor because almost 90% of dogs will develop predominantly pulmonary metastasis. In these dogs, median survival times range from 3 months to 1 year, and less than 20% of dogs survive for more than 2 years following diagnosis.[4, 5]

Due to similarities in the biology and treatment of OSA in dogs and humans, canine OSA represents a valid and important tumor model. Increasingly, studies of OSA in pet dogs will become a standard component in the development process of novel therapies for human OSA. In addition, these studies will streamline the selection process to determine compounds that have a higher likelihood of success for the treatment of human patients. The studies of specific proteins in canine OSA are limited when compared to those in other neoplasms. Examples of such proteins include TNF,[6] MIF,[6] ezrin,[7] survivin,[8] cathepsin K,[9] MET,[10] p53,[11] erbB-2,[12] IGF-1,[13] MMP-2,[14] and MMP-9.[14] Identification of the proteins that are associated with metastasis using proteomic technologies is necessary not only for the discovery of OSA targets and signaling pathways associated with metastatic OSA but also to provide novel therapeutic targets. The objective of this study was to conduct a proteomic profiling analysis of

canine OSA patients. We used mass spectrometry to examine the protein profiles of serum and OSA tissues from patients.

Results

Canine patient characteristics

A total of 25 canine patient samples were included in this serum and bone tissue proteome study. There were 10 canine OSA patients and a control group of 15 non-OSA orthopedic patients. The median age at the time of diagnosis of the OSA group and non-OSA group was 9 years. Male OSA patients were affected four times as often as females. The males in the control group were affected three times as often as females. The breed distribution of the OSA group was as follows: 4 golden retrievers, 2 rottweilers, and 4 mixed breeds. The breed distribution of the control group was as follows: 4 golden retrievers, 5 mixed breeds, 1 poodle, 2 pomeranians, 2 shih tzus, and 1 French bulldog. The median value of serum ALP of the OSA group and non-OSA group was 63 and 103.5 U/L, respectively. The proteins were fractionated on a one-dimensional 12.5% SDS-PAGE mini slab gel and visualized using silver staining (Figure 1). The band of interest was excised and digested. The peptides were analyzed using an HCTultra LC-MS, and the data were analyzed with DeCyder MS Differential Analysis Software and submitted to a database using the Mascot program. A Mascot search of the mammalian database resulted in a total number of 1,006 differentially expressed proteins among the control and canine OSA serum and tissues.

From a total of 1,006 peptides, there were 970 unique proteins (Figure 2). Of these 970 proteins, 101 proteins were not present in all of the control group's bone samples, with 5 being detected only in the OSA group's tissues, 1 only in the control group's serum, and 1 only in the OSA group's serum. The 5 proteins found only in OSA tissues included myosin (MYO6), GTPase IMAP family member 5 (GIMA5), PR domain zinc finger protein 15 (PRDM15), disintegrin and metalloproteinase with thrombospondin motifs 4 precursor, and interleukin-1 receptor type 1. Dynein was the only protein detected in the control serum, and rCG38920 was the only protein found in the OSA serum (Table 1).

Distribution of the proteins in each functional class

Categorization of the most abundant proteins in each functional category using the Software Tool for Rapid Annotation of Proteins (STRAP) bioinformatics suite[15] revealed differences between the OSA proteome and the control proteome in canine patient samples. By biological process, the analysis showed that the majority of proteins were classified under cellular process (26.2%), regulation (26.1%), and "other" (14.7%); however, other proteins were mapped to the developmental process (8.8%), interaction with cells and organisms (6.8%), localization (6.6%), response to stimulus (4.4%), metabolic process (4.3%), immune system process (2.0%), and growth (0.1%) (Figure 3). By cellular component, the analysis showed that while the majority of proteins were classified under "other" (19.7%), nucleus (15.8%), and

cytoplasm (13.7%), other proteins were mapped to the plasma membrane (10.0%), extracellular proteins (9.0%), cytoskeleton (7.0%), other intracellular organelles (7.0%), the macromolecular complex (5.9%), mitochondria (3.3%), endoplasmic reticulum (ER) (3.0%), chromosome (2.0%), endosome (1.2%), cell surface (0.7%), ribosome (0.7%), and peroxisome/microbody (0.6%) (Figure 4). Categorization of the most abundant proteins in canine patient samples by molecular function showed that while the majority of proteins fell under binding (48.5%), catalytic activity (26.5%), and “other” (14.4%), other proteins were mapped to molecular transducer activity (7.0%), structural molecule activity (3.2%), and antioxidant activity (0.3%) (Figure 5).

Protein network analysis

To better understand the biological interaction of the identified proteins, we also constructed a protein-protein functional network using the online software Stitch 4.0(<http://stitch.embl.de/>)[16]. The network of differentially expressed protein-protein and protein-chemotherapeutic drugs, carboplatin and doxorubicin - the effective agents for adjuvant chemotherapy in dogs with OSA, were related to the apoptosis caspase signaling pathway, ezrin, and the proteins ADAMTS4 and IL1R1 in the canine OSA tissues and serum is shown in Figure 6. The protein network analysis provided a clearer view of a complex framework of proteins that may be a result of the differences in canine malignant OSA and non-OSA patients.

Discussion

The findings of this study provided useful insight into OSA tumorigenesis and identified multiple signaling pathways to enrich our understanding of this cancer. Using an extraction method followed by SDS-PAGE, digestion, and mass spectrometry, we detected several proteins that are related to this tumor. We also evaluated the differential proteome in tissue and serum from the non-OSA bone controls and OSA patients.

We found the overexpression of five proteins in OSA tissues—myosin (MYO7a), GTPase IMAP family member 5 isoform 1 (GIMAP5), PR domain 15 (PRDM15), A disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS4), and interleukin receptor type 1 (IL1R1) —and one protein in the serum: rCG38920. We found that ADAMTS4 and IL1R1 were involved in the ezrin and apoptosis caspase signaling pathways. High ezrin expression in canine tumors was associated with the early development of metastases and poor outcomes in pediatric OSA patients.[7, 17]

The complex protein networks indicated that carboplatin and doxorubicin (the effective agents for adjuvant chemotherapy in dogs with OSA) were related to the apoptosis caspase signaling pathway, ezrin, and the proteins ADAMTS4 and IL1R1. Therefore, these two proteins should be further evaluated as potential novel therapeutic target proteins for canine OSA.

ADAMTS4 is a cartilage proteoglycan that may be involved in cartilage turnover and may play an important role in the destruction of aggrecan in arthritic diseases. IL1R1 is a receptor for interleukin-1 alpha (IL-1A), interleukin-1 beta (IL-1B), and interleukin-1 receptor antagonist protein (IL-1RA). Binding to the agonist leads to the activation of NF-kappa-B. Signaling involves formation of a ternary complex containing IL1RAP, TOLLIP, MYD88, and IRAK1 or IRAK2 (cell surface receptor signaling pathways), after binding to interleukin-1 associated with the co-receptor IL1RAP to form the high affinity interleukin-1 receptor complex, which mediates interleukin-1-dependent activation of NF-kappa-B, MAPK, and other pathways.

MYO7a, GIMAP5, and PRDM15 also were found to be differentially expressed between the OSA and control patients. MYO7a encodes for certain myosins, cytoskeletal-associated proteins that bind to actin filaments that are concentrated beneath the plasma membrane. They are important for short-range transport during endocytosis and exocytosis and generate mechanical force for muscle contraction, cell migration, and cytokinesis.[18] In human cervix cancer cells (HeLa) and OSA cells (U2OS), myosin 1G (MYO1G) and myosin heavy chain 1 (MYH1) are essential for cancer cell survival.[19] Myosin 6 (MYO6) is a reverse-direction motor protein that moves toward the minus-end of actin filaments. Myosins have functions in a variety of intracellular processes, such as vesicular membrane trafficking and cell migration.[20] Myosin[21, 22] and ezrin,[23] the actomyosin-associated proteins, have been reported as putative physiological substrates for Rho-kinase. Rho-kinase can regulate the phosphorylation of both proteins during cell migration.[24] These findings are in agreement with previous studies using primary human OSA and benign bone tumor samples with 2D gel electrophoresis in which the protein spots were identified with MALDI-TOF MS.[25] The fact that cytoskeleton and microtubule-associated proteins were identified suggests that they play a role in the tumor cell migration and metastasis that are characteristic of OSA. The GIMAP family members are associated with immune cell function and seem to be confined to the same tissues, indicating that different GIMAP family members may act in concert to mediate their functions. The up-regulation of GIMAP family member 5 has been associated with human B-cell malignancy, possibly by inhibiting apoptosis.[26] GIMAP family member 6 is the biomarker identified for human prostate cancer.[27] PRDM15 is a putative histone methyltransferase, a class of enzymes frequently deregulated in human cancer, such as breast cancer, lung cancer, hepatoma, colorectal cancer, lymphoma, and osteosarcoma.[28] The function of the only protein detected in the OSA serum, rCG38920, is still unknown. Validation of these identified proteins was necessary to determine if they can be useful as novel biomarkers for canine OSA in the future.

Conclusions

We identified the overexpression of five proteins from OSA tissues—myosin (MYO7a), GTPase IMAP family member 5 isoform 1 (GIMAP5), PR domain 15 (PRDM15), A disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS4), and interleukin receptor type 1 (IL1R1) —and one protein from the serum: rCG38920. These proteins detected in the tumor tissue and serum can potentially discriminate

between patients with malignant and benign OSA or non-OSA through a proteomic approach using mass spectrometry. These proteins may be useful for diagnosis and prognosis, including the prevention of misdiagnosis between malignant OSA and non-OSA as these have similar clinical symptoms in the early stages. In the future, further independent validation of these biomarkers using a greater number of patient samples and different techniques, including Western blot, is necessary to improve diagnostic power for differentiating malignant OSA patients from non-OSA patients.

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 Kasetsart University of Veterinary Medicine Animal Care and Use Committee (protocol: ACKU60-VET-020). Owner consent was obtained prior to the collection of serum and tumor tissue.

Serum and tumor specimens from patients

Canine OSA plasma and tissue specimens were obtained from 10 dogs, according to the rules of the ethical committee at the Faculty of Veterinary Medicine, Kasetsart University that underwent surgical resection at the university's Veterinary Teaching Hospital. The clinical information of these patients, including age, gender, breed, body weight, serum alkaline phosphatase (ALP), and lung metastasis, was obtained from medical records. Histological classification was made by the veterinary pathologist of the Veterinary Teaching Hospital, Kasetsart University. Pulmonary metastasis was confirmed on thoracic radiography or through histopathological examination after autopsy. The control group consisted of 15 non-OSA orthopedic patients, from which serum and bone tissue samples were taken. Serum was collected and stored at -80 °C until use. Proteins from bone tissues were extracted with 200 µl of 0.5% SDS, incubated at 37 °C for a few hours, and centrifuged at 10,000g for 15 min. The supernatant was transferred to a new tube, mixed well with 2 volumes of cold acetone, and incubated overnight at -20 °C. The mixture was centrifuged at 10,000g for 15 min and the supernatant was discarded. The pellet was dried in a Speedvac, re-suspended in 200 µl of 0.5% SDS, and stored at -80 °C prior to use. The pellets were re-suspended in 0.15% sodium deoxycholic acid, and protein concentration was determined using the Lowry method [29].

Prefractionation of proteins using SDS-PAGE

Proteins were fractionated on an SDS-PAGE mini slab gel (8 x 9 x 0.1 cm, Hoefer miniVE, Amersham Biosciences, UK). The polyacrylamide gel was prepared according to the standard method described by Laemmli.[30] The separating gel used for the fractionation of soluble proteins from mammalian cells contained 12.5% acrylamide. An equal volume of protein samples was mixed with 5 ml of 5X sample buffer (0.125M Tris-HCl, pH 6.8, 20% glycerol, 5% SDS, 0.2M DTT, 0.02% bromophenol blue), boiled at 95

°C for 10 min before being loaded onto the 12.5% SDS-PAGE. To estimate the size of polypeptides, a low molecular weight protein standard marker (Amersham Biosciences, UK) was used. Electrophoresis was performed in SDS electrophoresis buffer (25mM Tris-HCl, pH 8.3, 192mM glycine, 0.1% SDS) until the tracking dye reached the bottom of the gel. After the electrophoresis finished, gels were silver stained.

In-gel digestion

After protein bands were excised, the gel plugs were dehydrated with 100% acetonitrile (ACN), reduced with 10mM DTT in 10mM ammonium bicarbonate at room temperature for 1 h, and alkylated at room temperature for 1 h in the dark in the presence of 100mM iodoacetamide in 10mM ammonium bicarbonate. After alkylation, the gel pieces were dehydrated twice with 100% ACN for 5 min. To perform in-gel digestion of proteins, 10 µl of trypsin solution (10 ng/µl trypsin in 50% ACN/10mM ammonium bicarbonate) was added to the gels, followed by incubation at room temperature for 20 min, and then 20 µl of 30% ACN was added to keep the gels immersed throughout digestion. The gels were incubated at 37 °C for a few hours or overnight. To extract peptide digestion products, 30 µl of 50% ACN in 0.1% formic acid was added to the gels, and then the gels were incubated at room temperature for 10 minutes in a shaker. Extracted peptides were collected and pooled together in the new tube. The pooled extracted peptides were dried by vacuum centrifuge and kept at -80 °C for further mass spectrometric analysis.

Peptide identification by HCTultra LC-MS analysis

Peptide solutions were analyzed using an HCTultra PTM Discovery System (Bruker Daltonics Ltd., UK) coupled to an UltiMate 3000 LC System (Dionex Ltd., UK). Peptides were separated on a nanocolumn (PepSwift monolithic column, 100 µm diameter x 50 mm length). Eluent A and eluent B solutions were prepared from 0.1% formic acid, and from 80% ACN in water containing 0.1% formic acid, respectively. Peptide separation was achieved with a linear gradient from 10% to 70% of eluent B for 13 minutes at a flow rate of 300 nl/minute. A regeneration step and an equilibration step were at 90% of eluent B and at 10% of eluent B, respectively, for 20 minutes/run. Peptide fragment mass spectra were acquired in the data-dependent AutoMS mode with a scan range of 300–1500 *m/z*, 3 averages, and up to 5 precursor ions selected from the MS scan 50–3000 *m/z*. Peptide peaks were detected and deconvoluted automatically using DataAnalysis version 4.0. Mass lists in the form of Mascot generic files were created automatically and used as the input for Mascot MS/MS ions searches of the National Center for Biotechnology Information (NCBI) mammal database (www.matrixscience.com) for protein identification. For protein quantitation, DeCyder MS Differential Analysis Software (DeCyderMS, GE Healthcare) was used. Acquired LC-MS raw data were converted, and the PepDetect module was used for automated peptide detection, charge state assignments, and quantitation based on the peptide ion signal intensities in MS mode. The analyzed MS/MS data from DeCyderMS were submitted to the database using the Mascot software (Matrix Science, London, UK). The data were searched against the NCBI mammal database for protein identification. Proteins considered as identified proteins had at least two peptides with an individual mascot score corresponding to $p < 0.05$. The relationship of candidate proteins and chemotherapeutic drugs were performed by the Stitch program, version 5.0[16].

Abbreviations

OSA: Osteosarcoma; GIMAP5: GTPase IMAP family member 5 isoform 1; PRDM15: PR domain 15; ADAMTS4: A disintegrin and metalloproteinase with thrombospondin motifs 4; IL1R1: interleukin receptor type 1; ALP: Alkaline phosphatase; ACN: acetonitrile

Declarations

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Author's contributions

TJ designed the study, recruited the patients, and wrote the manuscript. CT collected the samples and made the statistical analysis. SR did the laboratory work and revised the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study is available from the corresponding authors on reasonable request.

Ethic approval and consent to participate

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 Kasetsart University of Veterinary Medicine Animal Care and Use Committee (protocol: ACKU60-VET-020). Owner consent was obtained prior to the collection of serum and tumor tissue.

Consent for publication

Not applicable.

Competing interest

The authors declare that they have no competing interest.

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References

1. Withrow SJ, Vail DM (eds.): **Small Animal Clinical Oncology**, 4th edn. St. Louis: SAUNDERS; 2007.
2. Spodnick GJ, Berg J, Rand WM, Schelling SH, Couto G, Harvey HJ, Henderson RA, MacEwen G, Mauldin N, McCaw DL *et al*: **Prognosis for dogs with appendicular osteosarcoma treated by amputation alone: 162 cases (1978-1988)**. *J Am Vet Med Assoc* 1992, **200**(7):995-999.
3. Hillers KR, Dernell WS, Lafferty MH, Withrow SJ, Lana SE: Incidence and prognostic importance of lymph node metastases in dogs with appendicular osteosarcoma: 228 cases (1986-2003). *J Am Vet Med Assoc* 2005, **226**(8):1364-1367.
4. Straw RC, Withrow SJ: **Limb-sparing surgery versus amputation for dogs with bone tumors**. *Vet Clin North Am Small Anim Pract* 1996, **26**(1):135-143.
5. Boston SE, Duerr F, Bacon N, Larue S, Ehrhart EJ, Withrow S: Intraoperative radiation for limb sparing of the distal aspect of the radius without transcarpal plating in five dogs. *Vet Surg* 2007, **36**(4):314-323.
6. Cates JM, Friedman DB, Seeley EH, Dupont WD, Schwartz HS, Holt GE, Caprioli RM, Young PP: **Proteomic analysis of osteogenic sarcoma: association of tumour necrosis factor with poor prognosis**. *Int J Exp Pathol* 2010, **91**(4):335-349.
7. Khanna C, Wan X, Bose S, Cassaday R, Olomu O, Mendoza A, Yeung C, Gorlick R, Hewitt SM, Helman LJ: **The membrane-cytoskeleton linker ezrin is necessary for osteosarcoma metastasis**. *Nat Med* 2004, **10**(2):182-186.

8. Shoeneman JK, Ehrhart EJ, 3rd, Eickhoff JC, Charles JB, Powers BE, Thamm DH: **Expression and function of survivin in canine osteosarcoma.** *Cancer Res* 2012, **72**(1):249-259.
9. Schmit JM, Pondenis HC, Barger AM, Borst LB, Garrett LD, Wypij JM, Neumann ZL, Fan TM: **Cathepsin K expression and activity in canine osteosarcoma.** *J Vet Intern Med* 2012, **26**(1):126-134.
10. Ferracini R, Angelini P, Cagliero E, Linari A, Martano M, Wunder J, Buracco P: **MET oncogene aberrant expression in canine osteosarcoma.** *J Orthop Res* 2000, **18**(2):253-256.
11. Loukopoulos P, Thornton JR, Robinson WF: **Clinical and pathologic relevance of p53 index in canine osseous tumors.** *Vet Pathol* 2003, **40**(3):237-248.
12. Flint AE, U'ren L, Legare ME, Withrow SJ, Dernell W, Hanneman WH: **Overexpression of the erbB-2 proto-oncogene in canine osteosarcoma cell lines and tumors.** *Veterinary Pathology* 2004, **41**(3):291-296.
13. MacEwen EG, Pastor J, Kutzke J, Tsan R, Kurzman ID, Thamm DH, Wilson M, Radinsky R: **IGF-1 receptor contributes to the malignant phenotype in human and canine osteosarcoma.** *J Cell Biochem* 2004, **92**(1):77-91.
14. Loukopoulos P, Mungall BA, Straw RC, Thornton JR, Robinson WF: **Matrix metalloproteinase-2 and -9 involvement in canine tumors.** *Vet Pathol* 2003, **40**(4):382-394.
15. Bhatia VN, Perlman DH, Costello CE, McComb ME: Software tool for researching annotations of proteins: open-source protein annotation software with data visualization. *Anal Chem* 2009, **81**(23):9819-9823.
16. Szklarczyk D, Santos A, von Mering C, Jensen LJ, Bork P, Kuhn M: **STITCH 5: augmenting protein-chemical interaction networks with tissue and affinity data.** *Nucleic Acids Res* 2016, **44**(D1):D380-384.
17. Jaroensong T, Endo Y, Lee SJ, Kamida A, Mochizuki M, Nishimura R, Sasaki N, Nakagawa T: Effects of transplantation sites on tumour growth, pulmonary metastasis and ezrin expression of canine osteosarcoma cell lines in nude mice. *Vet Comp Oncol* 2012, **10**(4):274-282.
18. Krendel M, Mooseker MS: **Myosins: tails (and heads) of functional diversity.** *Physiology (Bethesda)* 2005, **20**:239-251.
19. Groth-Pedersen L, Aits S, Corcelle-Termeau E, Petersen NH, Nylandsted J, Jaattela M: Identification of cytoskeleton-associated proteins essential for lysosomal stability and survival of human cancer cells. *PLoS One* 2012, **7**(10):e45381.
20. Casaletto JB, Saotome I, Curto M, McClatchey AI: **Ezrin-mediated apical integrity is required for intestinal homeostasis.** *Proc Natl Acad Sci U S A* 2011, **108**(29):11924-11929.
21. Amano M, Ito M, Kimura K, Fukata Y, Chihara K, Nakano T, Matsuura Y, Kaibuchi K: **Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase).** *J Biol Chem* 1996, **271**(34):20246-20249.
22. Kimura K, Ito M, Amano M, Chihara K, Fukata Y, Nakafuku M, Yamamori B, Feng J, Nakano T, Okawa K *et al.*: **Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase).** *Science* 1996, **273**(5272):245-248.

23. Matsui T, Maeda M, Doi Y, Yonemura S, Amano M, Kaibuchi K, Tsukita S, Tsukita S: Rho-kinase phosphorylates COOH-terminal threonines of ezrin/radixin/moesin (ERM) proteins and regulates their head-to-tail association. *J Cell Biol* 1998, 140(3):647-657.
24. Kosako H, Yoshida T, Matsumura F, Ishizaki T, Narumiya S, Inagaki M: Rho-kinase/ROCK is involved in cytokinesis through the phosphorylation of myosin light chain and not ezrin/radixin/moesin proteins at the cleavage furrow. *Oncogene* 2000, 19(52):6059-6064.
25. Zhao ZL, Li QF, Zheng YB, Chen LY, Shi SL, Jing GJ: The aberrant expressions of nuclear matrix proteins during the apoptosis of human osteosarcoma cells. *Anat Rec (Hoboken)* 2010, 293(5):813-820.
26. Zenz T, Roessner A, Thomas A, Frohling S, Dohner H, Calabretta B, Daheron L: hlan5: the human ortholog to the rat lan4/lddm1/lyp is a new member of the lan family that is overexpressed in B-cell lymphoid malignancies. *Genes Immun* 2004, 5(2):109-116.
27. Neuhaus J, Schiffer E, von Wilcke P, Bauer HW, Leung H, Siwy J, Ulrici W, Paasch U, Horn LC, Stolzenburg JU: **Seminal plasma as a source of prostate cancer peptide biomarker candidates for detection of indolent and advanced disease.** *PLoS One* 2013, 8(6):e67514.
28. Schneider R, Bannister AJ, Kouzarides T: **Unsafe SETs: histone lysine methyltransferases and cancer.** *Trends Biochem Sci* 2002, 27(8):396-402.
29. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: **Protein measurement with the Folin phenol reagent.** *J Biol Chem* 1951, 193(1):265-275.
30. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970, 227(5259):680-685.

Tables

Table1. The proteins identified in the OSA bone and serum compared to the control samples.

Accession No.	Protein name	Control bone	OSA bone	Control serum	OSA serum	p value
gi 325054036	Myosin Viia Myth4-Ferm-Sh3 In Complex With The Cen1 Of Sans (MYO7A MyTH4-FERM/CEN)	0	15.33	0	0	< 0.01
gi 77874419	GTPase IMAP family member 5 (GIMAP5)	0	15.584	0	0	< 0.01
gi 297707978	PR domain zinc finger protein 15 (PRDM15)	0	16.046	0	0	< 0.01
gi 31982399	A disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS4)	0	18.302	0	0	< 0.01
gi 332078461	Interleukin-1 receptor type 1 (IL1RL1)	0	19.167	0	0	< 0.01
gi 149031360	rCG38920	0	0	0	12.97	< 0.01
gi 41473345	Dynein, axonemal, heavy polypeptide 11 (DNAH11)	11	15.1723	0	0	< 0.01

Figures

Figure 1

Silver stain of the fractionated proteins. (A) The serum proteins from the OSA tissue and control groups. (B) The bone tissue proteins from the OSA tissue and control groups.

Figure 2

Summary of significant proteins in the canine OSA serum and bone samples. Peptides identified after LC-MS/MS were searched against the Mascot mammalian database. A total of 1,006 proteins were identified, with 1, 5, 0, and 1 proteins detected only in the control bone, OSA bone, control serum, and OSA serum, respectively. A total of 6 proteins were identified in the OSA serum and bone samples.

Figure 3

Distribution of the proteins among canine OSA and non-OSA patients. The bar charts show the number of proteins in the biological process analyzed by STRAP 1.5 software.

Figure 4

Distribution of the proteins among canine OSA and non-OSA patients. The bar charts show the number of proteins in the cellular component analyzed by STRAP 1.5 software.

Figure 5

Distribution of the proteins among canine OSA and non-OSA patients. The bar charts show the number of proteins in the molecular function analyzed by STRAP 1.5 software.

Figure 6

The protein-protein interaction networks. Interaction of the differentially expressed proteins in canine OSA tissues and serum predicted using the software Stitch 4.0 (<http://stitch.embl.de/>) indicated that carboplatin and doxorubicin were related to the apoptosis caspase signaling pathway, ezrin, and the proteins ADAMTS4 and IL1R1. Stronger associations are represented by thicker lines. Protein-protein interactions are shown in blue, chemical-protein interactions in green, and interactions between chemicals in red.