Final Report

Proteomic profiling of canine osteosarcoma cell lines and patients

By Miss Tassanee Jaroensong

December 2015

Contract No.MRG5480055

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Proteomic profiling of canine osteosarcoma cell lines and patients

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This project granted by the Thailand Research Fund

Content

Abstract

Project Code : MRG 5480055

Project Title : การวิเคราะห์โปรตีนโอมิกส์ของเซลล์มะเร็งของกระดูกสุนัขและในสุนัขที่ป่วยเป็นมะเร็งกระดูก

Proteomic profiling of canine osteosarcoma cell lines and patients

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Project Period : 2 years

บทคัดย่อ:

มะเร็งกระดูกในสุนัข (Canine osteosarcoma,OSA) เป็นมะเร็งชนิดที่รุนแรงในสุนัข มีการแพร่กระจายหลักไปยังปอดทางเส้นเลือด การค้นหาโปรตีนโดยใช้เทคโนโลยีโปรตีโอมิกส์ที่เกี่ยวข้องกับการแพร่กระจายไม่เพียงสามารถทำให้ค้นพบโปรตีนเป้าหมายและเส้นทางการส่งสัญญาณที่เกี่ยวข้องกันแต่ยังเป็นเป้าหมายของการรักษาใหม่ได้ด้วย จุดประสงค์ของการศึกษาคือดำเนินการวิเคราะห์โปรตีนโอมิกส์ของเซลล์มะเร็งของกระดูกสุนัขและในสุนัขที่ป่วยเป็นโรคมะเร็งในกระดูกที่มีความแตกต่างกันในด้านความรุนแรงของการแพร่กระจาย เราพบโปรตีน 5 ชนิดจากเนื้อเยื่อมะเร็งกระดูก ได้แก่ myosin (MYO7a) GTPase IMAP family member 5 isoform 1 (GIMAP5) PR domain 15 (PRDM15) A disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS4) และ interleukin receptor type 1 (IL1R1) รวมทั้งโปรตีน rCG38920 จากซีรั่ม นอกจากนี้ยังพบโปรตีน 2 ชนิดจากเซลล์ HMPOS ได้แก่ activating signal co-integrator 1 (ASC-1) complex and N-terminal kinase-like protein (NTKL) และ eukaryotic initiation factor 2 gamma (eIF-2γ) จากเซลล์ POS

คำสำคัญ: โปรตีโอมิกส์ แมสสเปกโทรเมตรี เจลอิเล็กโตรโฟรีซิส สุนัข มะเร็งกระดูก

Abstract:

Canine osteosarcoma (OSA) is an aggressive primary bone tumor in dogs. Metastasis of canine OSA occurs mainly to the lung via the hematogenous route. Identification of the proteins with proteomic technologies that are associated with metastasis is inevitable not only for the discovery of OSA targets and signaling pathways associated with metastatic OSA involved, but also to provide novel therapeutic targets. The objective of this study is to conduct a proteomic profiling analysis of canine OSA cell lines and patients with differential metastatic potential. We use mass spectrometry to examine the protein profiles. We found the overexpression of 5 proteins from OSA tissues included 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 rCG38920 protein from serum. There were 2 being detected only in HMPOS cells included activating signal co-integrator 1 (ASC-1) complex and N-terminal kinase-like protein (NTKL). There was 1 being detected only in POS cells included eukaryotic initiation factor 2 gamma (eIF-2γ).

Keywords: Proteomic, Mass spectrometry, Gel electrophoresis, Canine, Osteosarcoma

Final Report

Abstract

Canine osteosarcoma (OSA) is an aggressive primary bone tumor in dogs. Metastasis of canine OSA occurs mainly to the lung via the hematogenous route. Identification of the proteins with proteomic technologies that are associated with metastasis is inevitable not only for the discovery of OSA targets and signaling pathways associated with metastatic OSA involved, but also to provide novel therapeutic targets. The objective of this study is to conduct a proteomic profiling analysis of canine OSA cell lines and patients with differential metastatic potential. We use mass spectrometry to examine the protein profiles. We found the overexpression of 5 proteins from OSA tissues included 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 rCG38920 protein from serum. There were 2 being detected only in HMPOS cells included activating signal co-integrator 1 (ASC-1) complex and N-terminal kinase-like protein (NTKL). There was 1 being detected only in POS cells included eukaryotic initiation factor 2 gamma (eIF-2γ).

Executive summary

The objective of this study is to conduct a proteomic profiling analysis of canine OSA cell lines and patients with differential metastatic potential.

We use mass spectrometry to examine the protein profiles of HMPOS, POS, OOS, CHOS cell lines. Moreover, we also use serum and OSA lesions from patients.

In this study, we found the overexpression of 5 proteins from OSA tissues included 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 1 protein from serum included rCG38920. In cell lines, one high-metastatic (HMPOS), 2 low-metastatic (OOS and POS) and one non-metastatic (CHOS) canine OSA cell lines were used in this study. In canine OSA cell lines, 67 proteins were present in metastatic cell lines. There were 2 being detected only in HMPOS cells included activating signal co-integrator 1 (ASC-1) complex and N-terminal kinase-like protein (NTKL). There was 1 being detected only in POS cells included eukaryotic initiation factor 2 gamma (eIF-2γ).

In conclusion, we identified proteins in canine OSA tissue, serum, and cell line that can potentially discriminate patients with malignant OSA from benign or non-OSA through proteomic approach using mass spectrometry. These proteins may be useful to diagnosis, prognosis and prevent misdiagnosis among patients as they have similar clinical symptoms in the early stage. In the future, further independent validation of these biomarkers will be required using the greater numbers of patient samples and the more technique to obtain the greatest diagnostic power for differentiating malignant OSA patients from controls.

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Objective

1. To conduct a proteomic profiling analysis of canine OSA cell lines with differential metastatic potential.

2. To conduct a proteomic profiling analysis of canine osteosarcoma patients with differential malignant behaviors.

Research Methodology

Cell lines and culture conditions

Four canine OS cell lines, HMPOS [1], POS, OOS, and CHOS [2], established from spontaneous canine OS patients were kindly derived from Assist.Prof.Dr. Nakagawa Takauki from The University of Tokyo. The HMPOS cell line was derived from a cloned POS cell line from spontaneous femoral OSA. The OOS and CHOS cell lines were derived from mandibular and scapular OSA, respectively. The cells were maintained in RPMI-1640 (Wako Pure Chemical Industries Ltd., Osaka, Japan) supplemented with 10% heat inactivated fetal bovine serum (Gibco BRL, Grand Island, NY, USA) and 50 mg/L gentamicin (Sigma-Aldrich Inc.) at 37°C in a humidified atmosphere with 5% CO_2.

Plasma and tumor specimens from patients

Canine OSA plasma and tissue specimens are obtained from dogs underwent surgical resection at Veterinary Teaching Hospital, Kasetsart University, between 2013 and 2014. The clinical information of these patients, including age, gender, breed, body weight, serum ALP, lung metastasis and survival time was obtained from the medical records and telephone interviews to the owners or referred veterinarians.

Histological classification according to World Health Organization (WHO) was made by Veterinary Pathologist of Veterinary Teaching Hospital, Kasetsart University. Pulmonary metastasis was confirmed on thoracic radiography or histopathological examination after autopsy. The normal donor plasma samples were pooled and used as a normal control. All blood samples were collected in EDTA-containing tubes at room temperature and immediately centrifuged at 1,000 rpm for 10 min. The plasma supernatant was collected and divided into aliquots and stored at -80 ^{o}C until use.

Total proteins extraction

Cells were extracted with 200 µl of 0.5% SDS, incubated at 37ºC 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 -20ºC. The mixture was centrifuged at 10,000g for 15 min and the supernatant was discarded. The pellet was dried in a Speedvac, resuspended in 200 µl of 0.5% SDS and stored at -80^{o}C prior to use.

Determination of protein concentration by Lowry method

The pellets were resuspended in 0.15% Sodium Deoxycholic acid (DOC) and determined protein concentration by Lowry method (Lowry et al., 1951). The absorbance at 750 nm (OD_{750}) was measured and the protein concentration was calculated using the standard curve, plotted between OD_{750} on Y-axis and BSA concentration (µg/ml) on X-axis.

Prefractionation protein by SDS-PAGE

Proteins were fractionated on 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 (1970). The separating gel used for the fractionation of soluble proteins from mammalian cells contained 12.5% acrylamide. The equal volume of protein samples were mixed with 5 µl 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^{o}C for 10 min before loading onto the 12.5% SDS-PAGE. To estimate size of polypeptides, 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 according to Blum et al (1987).

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 (IAA) 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^{o}C for a few hours or overnight. To extract peptide digestion products, 30 µl of 50% ACN in 0.1% formic acid (FA) was added into the gels, and then the gels were incubated at room temperature for 10 min in a shaker. Peptides extracted were collected and pooled together in the new tube. The pool extracted peptides were dried by vacuum centrifuge and kept at -80^{o}C for further mass spectrometric analysis.

HCTUltra LC-MS analysis

Peptide solutions were analyzed using an HCTultra PTM Discovery System (Bruker Daltonics Ltd., U.K.) coupled to an UltiMate 3000 LC System (Dionex Ltd., U.K.). Peptides were separated on a nanocolumn (PepSwift monolithic column 100 µm i.d. x 50 mm). Eluent A was 0.1% formic acid and eluent B was 80% acetonitrile in water containing 0.1% formic acid. Peptide separation was achieved with a linear gradient from 10% to 70% B for 13 min at a flow rate of 300 nl/min, including a regeneration step at 90% B and an equilibration step at 10% B, one run took 20 min. Peptide fragment mass spectra were acquired in data-dependent AutoMS (2) 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 (Bruker). 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 nonredundant (NCBI nr) database (www.matrixscience.com). Default search parameters used were the following: Enzyme = trypsin, max. missed cleavages =1; fixed modifications = carbamidomethyl (C); variable modifications = oxidation (M); peptide tolerance ±1.2 Da; MS/MS tolerance ±0.6 Da; peptide charge = 1+, 2+ and 3+; instrument = ESI-TRAP.

Proteins quantitation and identification

For proteins quantitation, DeCyder MS Differential Analysis software (DeCyderMS, GE Healthcare (Johansson et al., 2006; Thorsell et al., 2007) 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 ions signal intensities in MS mode. The analyzed MS/MS data from DeCyderMS were submitted to database search using the Mascot software (Matrix

Science, London, UK, (Perkins et al., 1999)). The data was searched against the NCBI database for protein identification. Database interrogation was; taxonomy (Human or Eucaryote); enzyme (trypsin); variable modifications (carbamidomethyl, oxidation of methionine residues); mass values (monoisotopic); protein mass (unrestricted); peptide mass tolerance (1 Da); fragment mass tolerance (±0.4 Da), peptide charge state (1+, 2+ and 3+) and max missed cleavages (1). Proteins considered as identified proteins had at least two peptides with an individual mascot score corresponding to $p<0.05$ and $p<0.1$, respectively.

Result

Canine patient characteristics

A total of 25 canine patient samples were included in this serum and bone tissue proteome study. Ten canine OSA patients (serum and tumor tissue samples) compared with the control group of 15 non-OSA orthopedic patients (serum and bone samples) underwent surgical resection at Veterinary Teaching Hospital, Kasetsart University, between 2013 and 2014. No statistically significant difference was found among the data of the canine OSA patients and those with control patients regarding age, sex, and ALP. The clinical characteristics of the patients in this study are shown in Table 1.

Canine OSA cell culture

This study focused on the conducting of a proteomic profiling analysis of one high-metastatic (HMPOS), 2 low-metastatic (OOS and POS) and one non-metastatic (CHOS) canine OSA cell lines and those of the cell medium. Figure 1 shows morphological appearance of canine OSA cell lines. HMPOS cell line was medium-sized and polygonal in shape. OOS cell line consisted of spherical

cells, fibroblast-like cells, large or small polygonal cells, and multinucleated giant cells. CHOS cell line consisted of elongated fibroblastic cells. POS cells show spherical cells, fibroblast-like cells, large or small polygonal cells, and multinucleated giant cells.

Characteristic	Control	OSA patients
Age (mean ± SD years)	9 ± 3.5	9 ± 2
Sex (male:female)	11 : 4	8 : 2
ALP (median μ/L)	103.5	63
Golden Retriever	4	4
Rottweiler	0	2
Mixed	5	4
Poodle	1	0
Pomeranian	2	0
Shih Tzu	2	0
French Bulldog	1	0

Table 1. Clinicopathological characteristics of canine OS patients.

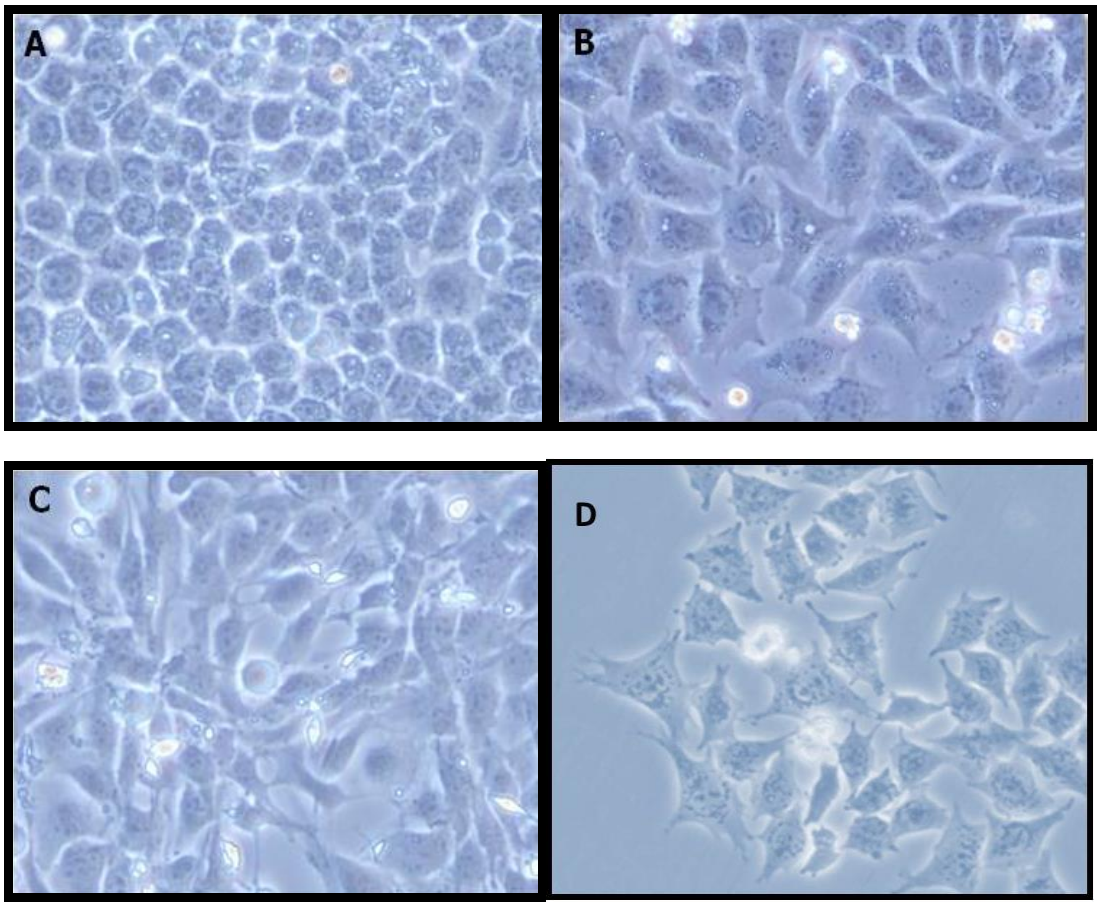

Figure 1. Morphological appearances of canine OS cell lines. (A) HMPOS cells show a medium-sized polygonal cell type; (B) OOS cells show a mixed cell type consisted of spherical cells, fibroblast-like cells, large or small polygonal cells, and multinucleated giant cells; (C) CHOS cells show a fibroblastic cell type; (D) POS cells show spherical cells, fibroblast-like cells, large or small polygonal cells, and multinucleated giant cells; Original magnification, 400×.

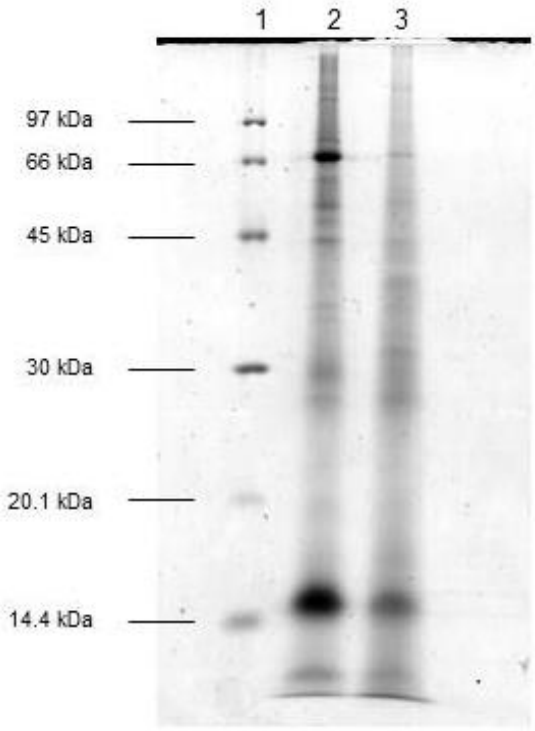

Figure 2. The proteins of OSA tissue and control were fractionated on 12.5% SDS-PAGE mini slab gel and visualized by silver staining. Lane 1, LMW-SDS Marker Kit (Product code: 17-0446-01, GE Healthcare, UK Limited) 97 kDa = Phosphorylase b (source: rabbit muscle), 66kDa = Albumin (source: bovine serum), 45 kDa = Ovalbumin (source: chicken egg white), 30 kDa = Carbonic anhydrase (source bovine erythrocyte), 20.1 kDa = Trypsin inhibitor (source: soybean), 14.4 kDa = α-Lactalbumin (source: bovine milk) ; Lane 2, Control; Lane 3, OSA tissue.

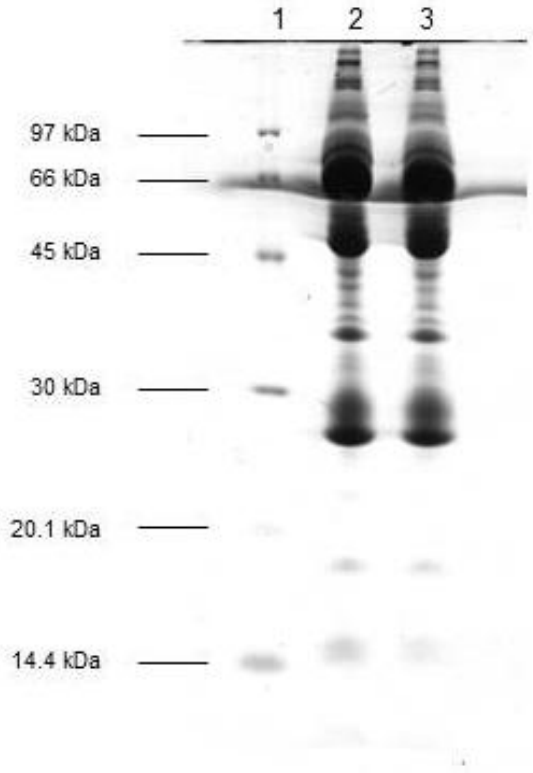

Figure 3. The proteins of OSA serum and control were fractionated on 12.5% SDS-PAGE mini slab gel and visualized by silver staining. Lane 1, LMW-SDS Marker Kit (Product code: 17-0446-01, GE Healthcare, UK Limited) 97 kDa = Phosphorylase b (source: rabbit muscle), 66kDa = Albumin (source: bovine serum), 45 kDa = Ovalbumin (source: chicken egg white), 30 kDa = Carbonic anhydrase (source bovine erythrocyte), 20.1 kDa = Trypsin inhibitor (source: soybean), 14.4 kDa = α-Lactalbumin (source: bovine milk) ; Lane 2, Control; Lane 3, OSA serum.

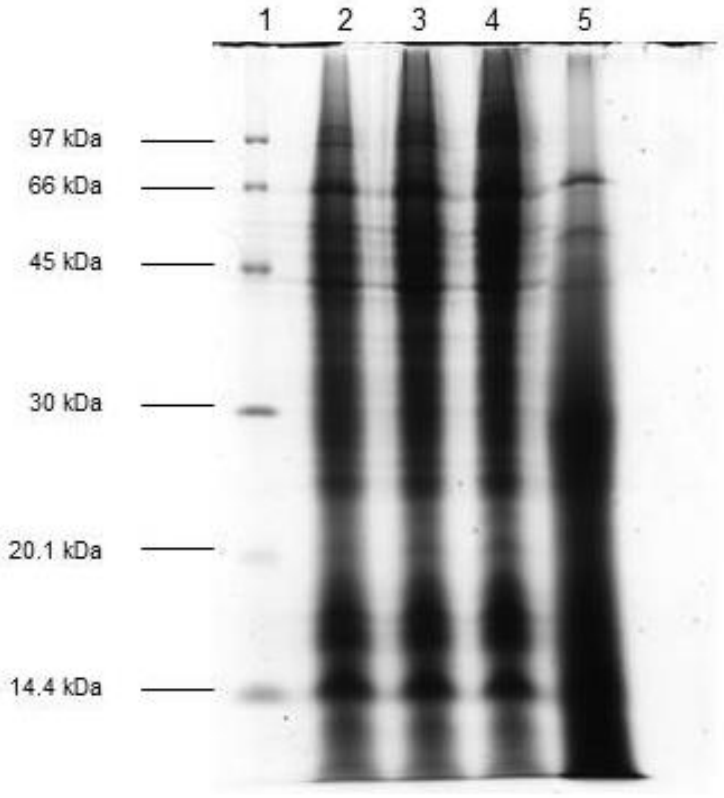

Figure 4. The proteins of OSA cell lines were fractionated on 12.5% SDS-PAGE mini slab gel and visualized by silver staining. Lane 1, LMW-SDS Marker Kit (Product code: 17-0446-01, GE Healthcare, UK Limited) 97 kDa = Phosphorylase b (source: rabbit muscle), 66kDa = Albumin (source: bovine serum), 45 kDa = Ovalbumin (source: chicken egg white), 30 kDa = Carbonic anhydrase (source bovine erythrocyte), 20.1 kDa = Trypsin inhibitor (source: soybean), 14.4 kDa = α-Lactalbumin (source: bovine milk) ; Lane 2, CHOS cell; Lane 3, HMPOS cell; Lane 4, POS cell; Lane 5, OOS cell.

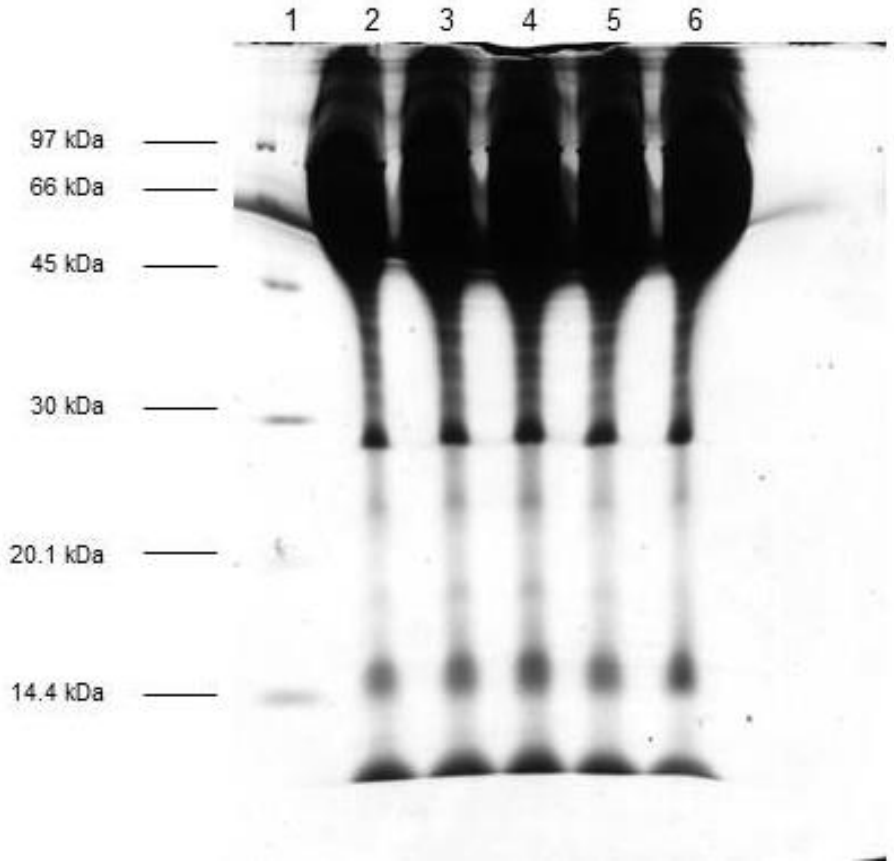

Figure 5. The proteins of OSA cell media were fractionated on 12.5% SDS-PAGE mini slab gel and visualized by silver staining. Lane 1, LMW-SDS Marker Kit (Product code: 17-0446-01, GE Healthcare, UK Limited) 97 kDa = Phosphorylase b (source: rabbit muscle), 66kDa = Albumin (source: bovine serum), 45 kDa = Ovalbumin (source: chicken egg white), 30 kDa = Carbonic anhydrase (source bovine erythrocyte), 20.1 kDa = Trypsin inhibitor (source: soybean), 14.4 kDa = α-Lactalbumin (source: bovine milk) ; Lane 2, CHOS cell media; Lane 3, HMPOS cell media; Lane 4, POS cell media; Lane 5, OOS cell media; Lane 6, Control media.

Identification of differentially expressed proteins

The proteins were fractionated on one-dimension 12.5% SDS-PAGE mini slab gel and visualized by silver staining (Figure 2,3,4 and 5). The interested band were excised and digested.

The peptides were analyzed by HCT Ultra LC-MS and the data was analyzed with DeCyder MS Differential Analysis software and submitted to database search using the Mascot program. A Mascot search of the canine database resulted in a total number of differentially expressed, 1,006 proteins among control and canine OSA serum and tissues, 781 proteins among 4 cell lines, and 1,151 proteins among cell culture medium (Figure 6).

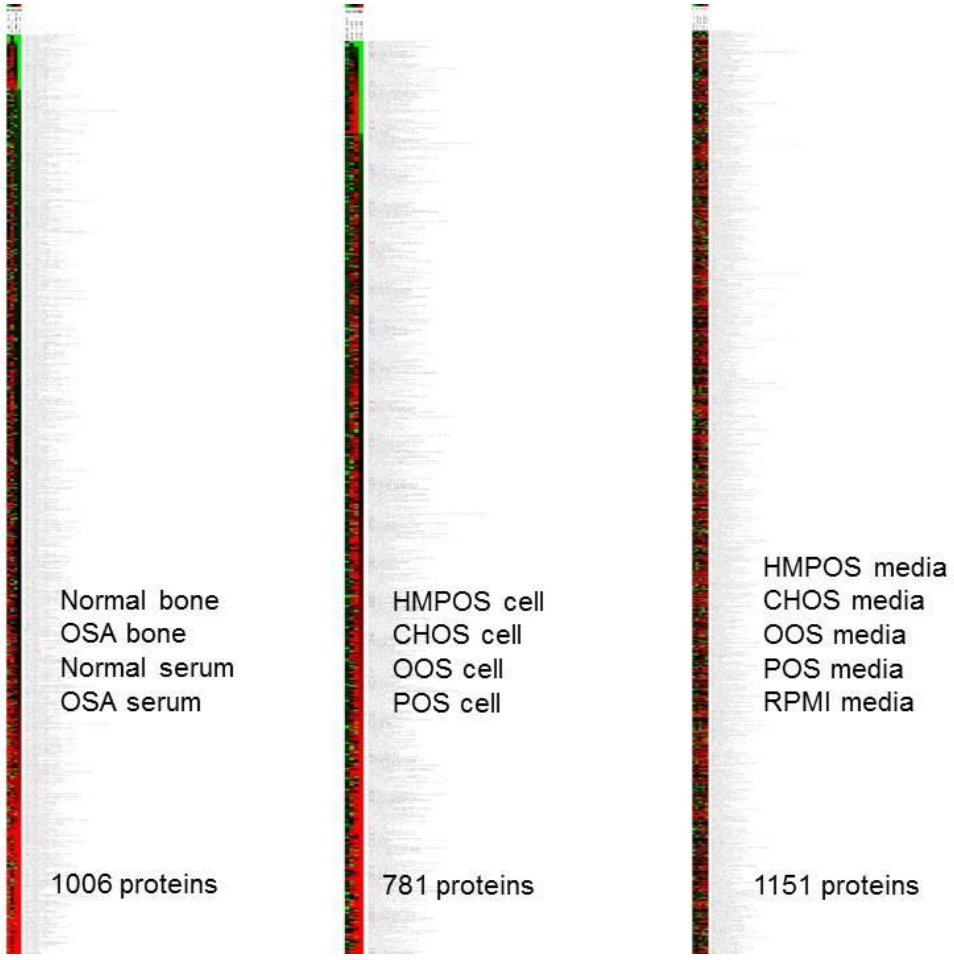

Figure 6. A hierarchical clustering analysis was carried out on the basis of the protein expression pattern. The protein expression intensities were indicated by a color code. Red indicated that the level of the protein expression was high and green indicated that the level was low.

From a total of 1,006 peptides, there are 970 unique proteins which mapped to (Figure 7). Of these 970 proteins, 101 proteins are not present in all control bone samples, with 5 being detected only in OSA tissues, 1 only in control serum and 1 only in OSA 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, only 1 protein detected in control serum. The rCG38920, only 1 protein in OSA serum (Table 2 and Appendix in DC: Bone_serum-1_final).

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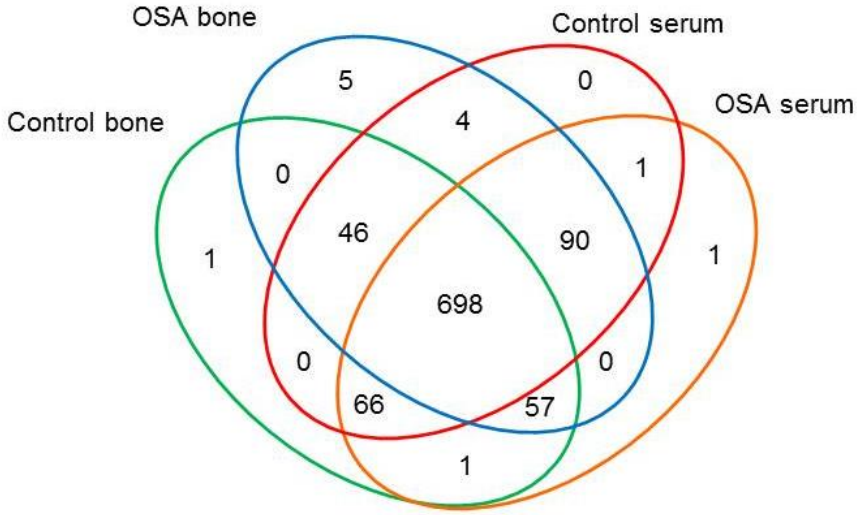


Figure 7. Summary of significant proteins of canine OSA serum and bone samples. Peptides identified after LC-MS/MS were searched against the Mascot mammalian database. A total of 1,006 proteins were identified, with 1, 5, 0, and 1 proteins detected only in control bone, OSA bone, control serum and OSA serum, respectively. A total of 6 proteins were identified in OSA serum and bone samples.

From a total of 781 peptides, there were 754 unique proteins which mapped to (Figure 8). Of these 754 proteins, 67 proteins were not present in CHOS cell lines which had benign behavior, with 3 being detected only in CHOS included MLX-interacting protein isoformX2 (MLXIP; transcription regulation), Cadps2 protein (cell differentiation and survival regulation) and Chain A, Crystal Structure of The Run Domain of Mouse Rap2 Interacting Protein X. There were 2 being detected only in HMPOS cells included activating signal co-integrator 1 complex subunit 2 isoform X1 (enhancing NF-kappa-B, SRF and AP1 transactivation) and N-terminal kinase-like protein (protein tyrosine kinase activity and transcription regulation). There was 1 being detected only in POS cells included eIF-2 gamma (Table 3 and Appendix in DC: Cell-1_final).

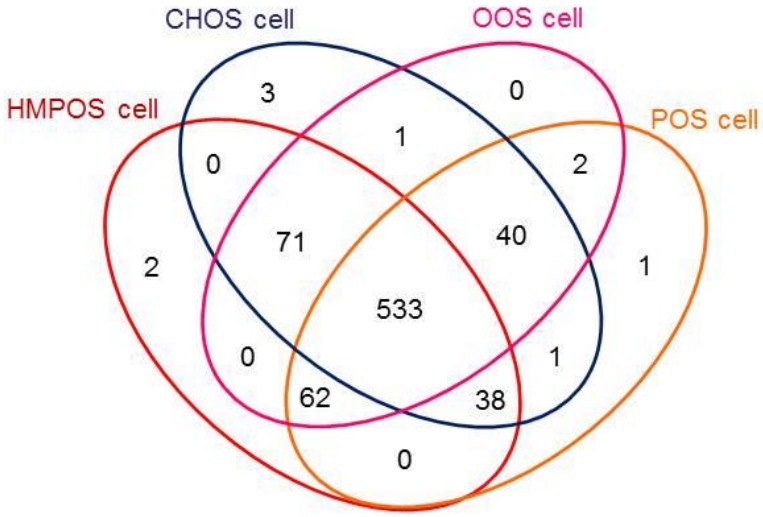


Figure 8. Summary of significant proteins of canine OSA cell lines. Peptides identified after LC-MS/MS were searched against the Mascot mammalian database. A total of 1,006 proteins were identified, with 2, 3, 0, 1 proteins detected only in HMPOS, CHOS, OOS and POS cells, respectively..

From a total of 1,151 peptides, there were 1,093 unique proteins which mapped to (Figure 9). Of these 1,093 proteins, 81 proteins were not present in CHOS cell medium, with 2 being detected only in CHOS cell medium included inhibin alpha subunit precursor and olfactory receptor 4K14-like. There was 1 being detected only in OOS cell medium included transducin-like enhancer protein 2-like. There were 6 being not detected in CHOS and RPMI control medium included hypothetical protein PANDA_006974, zona pellucida-binding protein, AWN-1=C12 fragment, melanoma-associated antigen B10-like, integrin alpha-10 isoform X2, RASA1 protein, and golgin subfamily B member 1 isoform 1(Appendix in DC: Media-1_final).

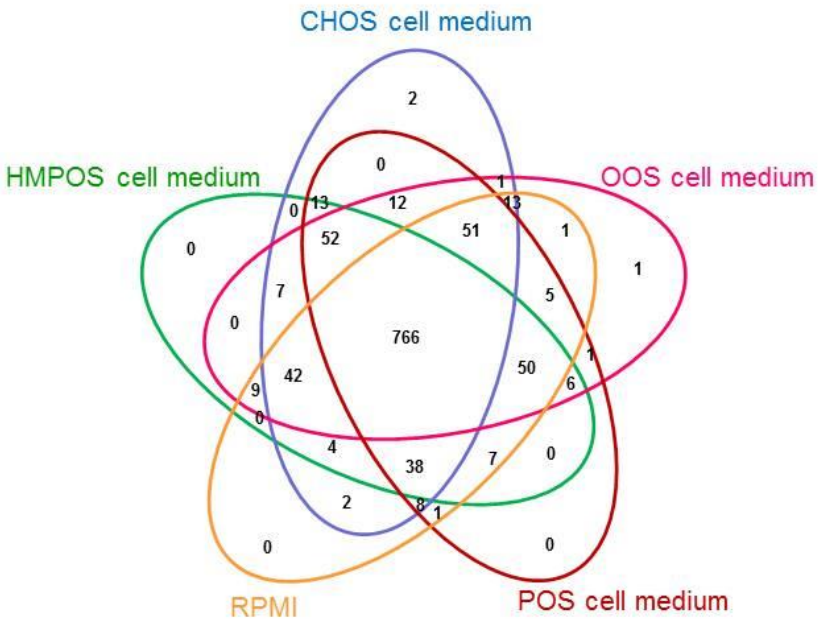


Figure 9. Summary of significant proteins of canine OSA cell lines. Peptides identified after LC-MS/MS were searched against the Mascot mammalian database. A total of 1,006 proteins were identified, with 1, 5, 0, 1, and 1 proteins detected only in non OSA bone, OSA bone, non OSA serum and OSA serum, respectively.

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)	15.1723	0	0	0	< 0.01

Table 2. The identified proteins from OSA bone and serum compared to control.

Accession No.	Protein name	HMPOS	CHOS	OOS	POS	*p* value
	activating signal cointegrator 1 complex					
gi|291409871	subunit 2 isoform X1	15.7793	0	0	0	< 0.01
	N-terminal kinase-like					
gi|301762568	protein	18.2382	0	0	0	< 0.01
	MLX-interacting protein					
gi|73994513	isoformX2	0	15.667	0	0	< 0.01
gi|28703972	Cadps2 protein	0	16.355	0	0	< 0.01
	Chain A, Crystal Structure Of The Run Domain Of Mouse Rap2 Interacting					
gi|114793770	Protein X	0	17.86	0	0	< 0.01
gi|236825	eIF-2 gamma	0	0	17.04326	0	< 0.01

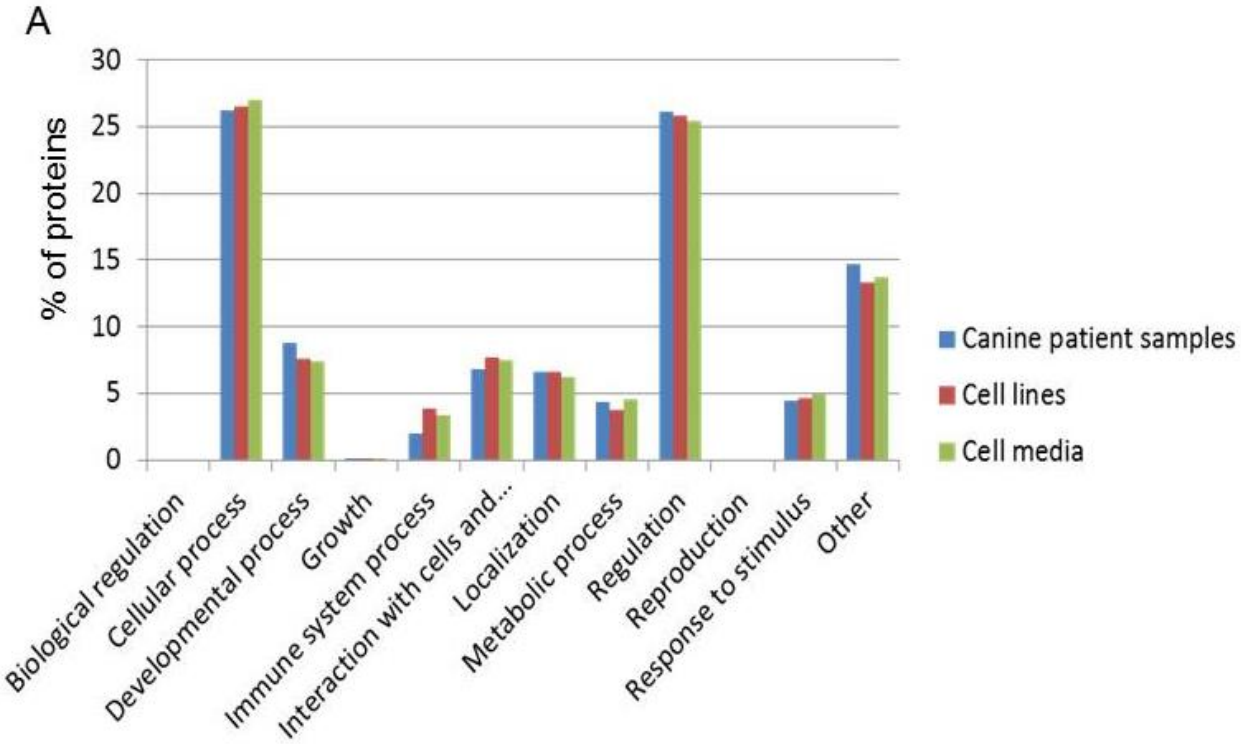
Table 3. The identified proteins from HMPOS, CHOS, OOS and POS cells.

Distribution of the proteins in each functional class

Categorization of the most abundant proteins of each functional category revealed differences between OSA proteome compared to the control proteome in canine patient samples, cell lines, and cell media using the Software Tool for Rapid Annotation of Proteins (STRAP) bioinformatics suite[3]. By biological process, the analysis showed that while the majority of proteins were classed under cellular process (26.2%, 26.5%, 27.0%), regulation (26.1%, 25.8%, 25.5%), and other (14.7%, 13.3%, 13.7%); other proteins mapped to developmental process (8.8%, 7.6%, 7.4%), interaction with cells and organisms (6.8%, 7.7%, 7.5%), localization (6.6%, 6.7%, 6.2%), response to stimulus (4.4%, 4.7%, 5%), metabolic process (4.3%, 3.7%, 4.5%), immune system process (2.0%, 3.9%, 3.3%), and growth (0.1%, 0.1%, 0.1%) (Figure 10A). other (19.7%, 19.3%, 20.0%), nucleus (15.8%, 16.7%, 14.5%), and cytoplasm (13.7%, 13.4%, 14%); other proteins mapped to plasma membrane (10.0%, 10.8%, 11.0%), extracellular (9.0%, 9.8%, 10.8%), cytoskeleton(7.0%, 6.8%, 5.0%), other intracellular (7.0%, 5.7%, 6.4%), macromolecular complex (5.9%, 4.3%, 4.6%), mitochondria (3.3%, 4.1%, 5.3%), endoplasmic reticulum (ER) (3.0%, 2.3%, 2.5%), chromosome (2.0%, 3.0%, 2.7%), endosome (1.2%, 1.3%, 1.5%), cell surface (0.7%, 1.3%, 1.0%), ribosome (0.7%, 0.7%, 0.6%), and peroxisome/microbody (0.6%, 0.5%, 0.3%) (Figure 10B). Categorization of the most abundant proteins in canine patient samples, cell lines, and cell media by molecular function showed that while the majority of proteins were classed under binding (48.5%, 48.6%, 47.2%), catalytic activity(26.5%, 28.6%, 27.0%), and other (14.4%, 13.4%, 16.0%); other proteins mapped to molecular transducer activity (7.0%, 6.2%, 6.0%), structural molecule activity (3.2%, 3.0%, 3.5%), antioxidant activity (0.3%, 0.2%, 0.5%), and translational regulator activity(0%, 0%, 0.1%) (Figure 10C).

The protein network analysis

To gain an overview of the biological interaction of the identified proteins, we also constructed the protein-protein functional network using the online software Stitch 4.0 (http://stitch.embl.de/). The network of the differentially expressed proteins on canine OSA tissues and serum generated were shown in Figure 11 and those proteins on canine OSA cell lines were shown in Figure 12. The protein network analysis provided a clearer view of a complex framework of proteins that may be result in the differences in canine malignant OSA and benign/non-OSA.


A
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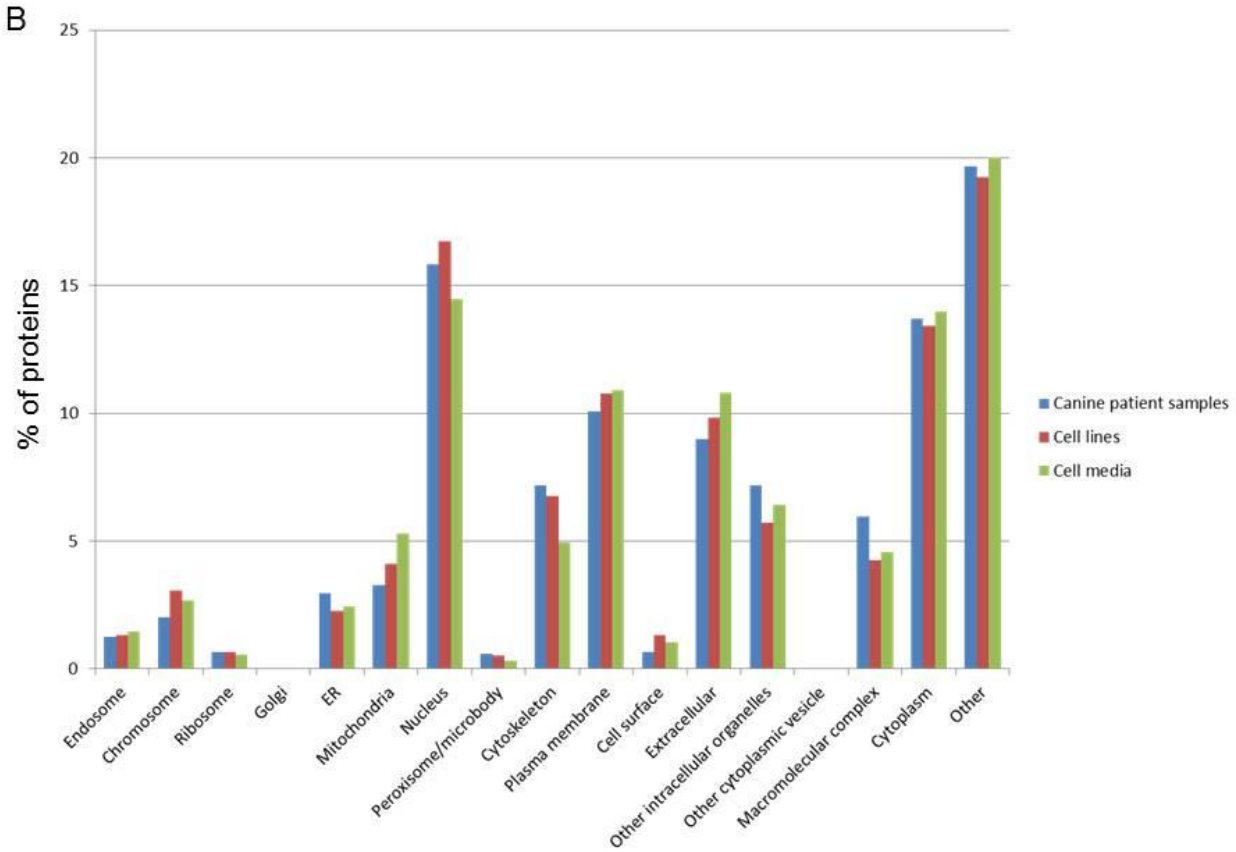
30
25
20
15
10
5
0
Biological regulation
Cellular process
Developmental process
Growth
Immune system process
Interaction with cells and...
Localization
Metabolic process
Regulation
Reproduction
Response to stimulus
Other
Canine patient samples
Cell lines
Cell media


B
% of proteins
25
20
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Endosome
Chromosome
Ribosome
Golgi
ER
Mitochondria
Nucleus
Peroxisome/microbody
Cytoskeleton
Plasma membrane
Cell surface
Extracellular
Other intracellular organelles
Other cytoplasmic vesicle
Macromolecular complex
Cytoplasm
Other
Canine patient samples
Cell lines
Cell media

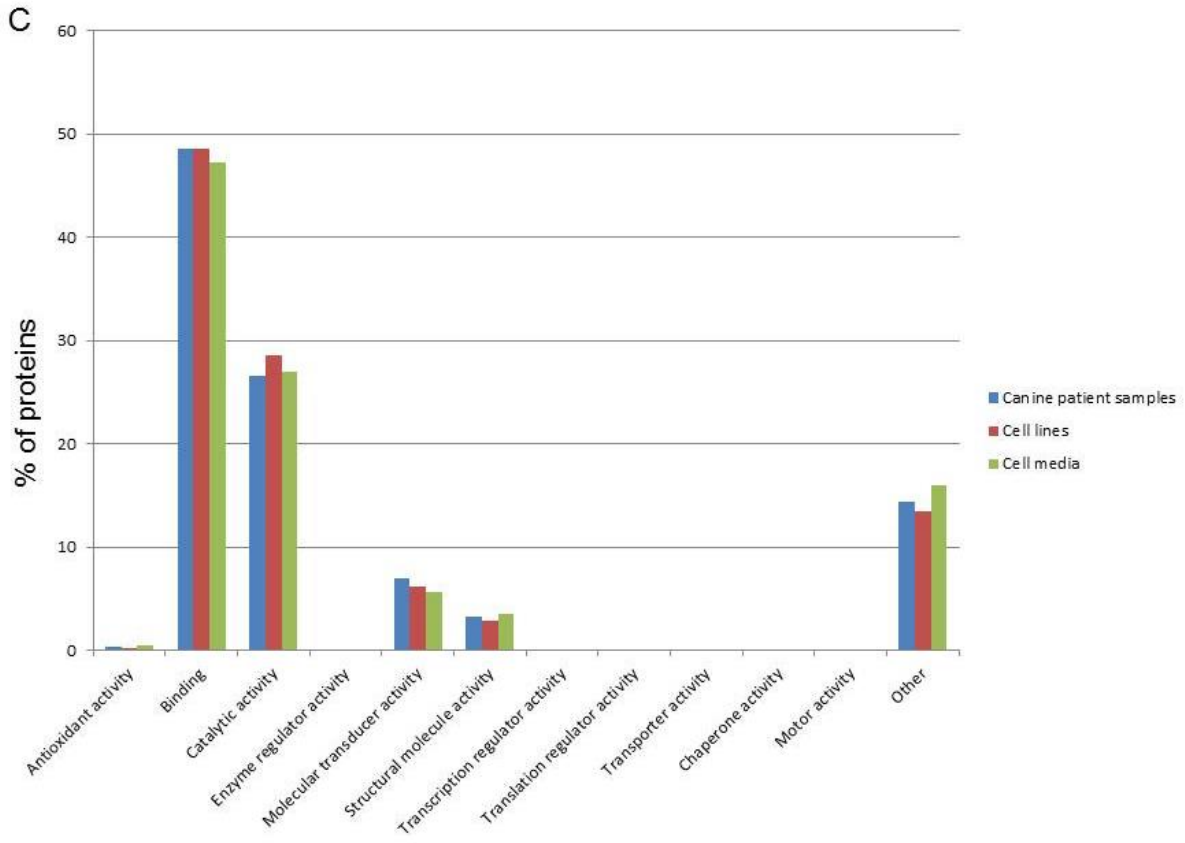

Figure 10. Distribution of the proteins among canine OSA serum and control, OSA bone and control, OSA cell lines and media. The bar chart shows the number of proteins in each functional class analyzed by STRAP 1.5 software. The biological process (A), the cellular component (B) and the molecular function (C).

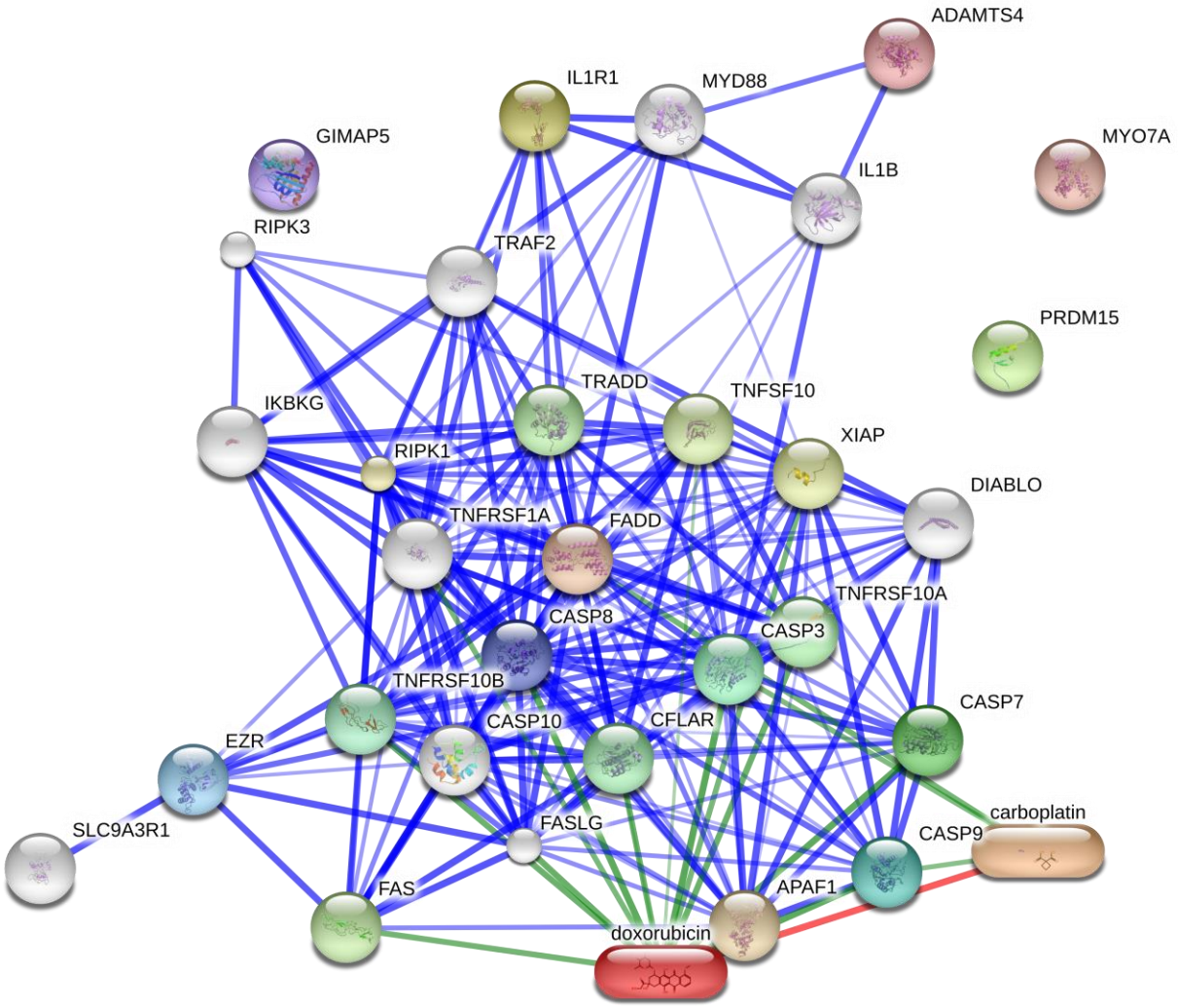

Figure 11. The protein-protein interaction networks of the differentially expressed proteins on canine OSA tissues and serum predicted by using the software Stitch 4.0 (http://stitch.embl.de/). Stronger associations are represented by thicker lines. Protein-protein interactions are shown in blue, chemical-protein interactions in green and interactions between chemicals in red.

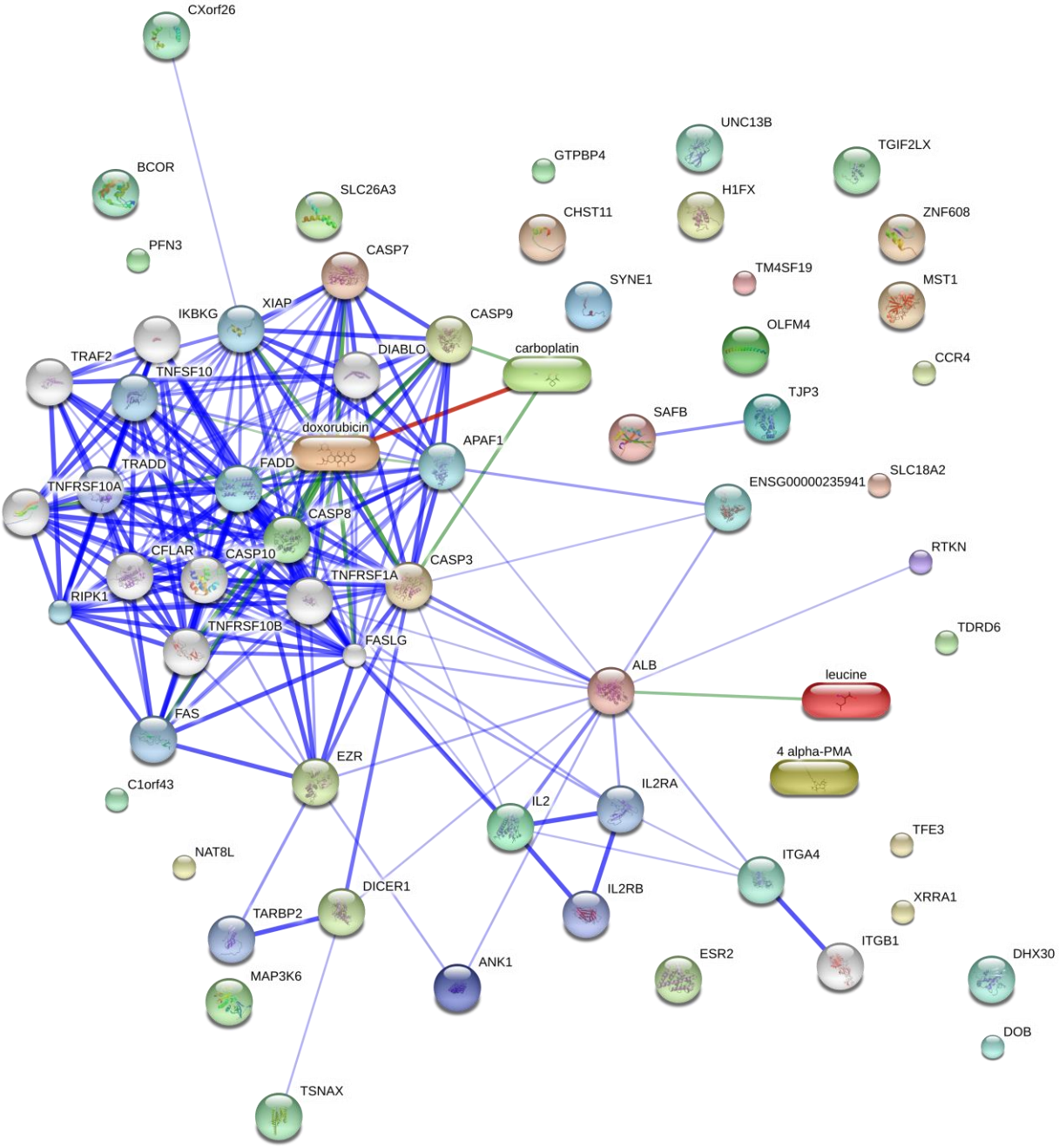

Figure 12. The protein-protein interaction networks of the differentially expressed proteins on canine OSA cell lines predicted by using the software Stitch 4.0 (http://stitch.embl.de/). Stronger associations are represented by thicker lines. Protein-protein interactions are shown in blue, chemical-protein interactions in green and interactions between chemicals in red.

Discussion and Conclusion

OSA is the most common malignant primary bone tumor in dogs and humans. Despite of advances in diagnosis and treatments for the primary bone tumor, development of the lung metastasis continues to be the most significant cause of death in both species[4,5].

The proteomic studies in OSA have been widely used for identifying early diagnostic, prognostic, predictive biomarkers and new therapeutic targets from multiple studies[6-9]. All differentially expressed proteins identified in these studies can be found online. This information provided useful insight into OSA tumorigenesis and multiple signaling pathways to enrich our understanding of this cancer. In this study, using an extraction method followed by SDS-PAGE, digestion and mass spectrometry, we can identify several proteins that were related to this tumor. We evaluated the differential proteome in tissue and serum between the none-OSA bone controls and OSA patients. Moreover, we also evaluated those in canine OSA cell lines with differential metastatic potential. In this study, we found the overexpression of 5 proteins from OSA tissues included 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 1 protein from serum included rCG38920. We found that ADAMTS4 and IL1R1 were involved in ezrin and apoptosis caspase signaling pathway. High ezrin expression in canine tumors was associated with early development of metastases and poor outcome in pediatric osteosarcoma patients[10,11]. From complex protein networks, carboplatin and doxorubicin (the effective agents for adjuvant chemotherapy in dogs with OSA) were related to apoptosis caspase signaling pathway, ezrin, and our 2 identified proteins (ADAMTS4 and IL1R1). Therefore, these 2 proteins may be more evaluated to be the novel therapeutic target proteins for canine OSA. Other 3 proteins also found differentially expressed between OSA and control patients including MYO7a, GIMAP5, and PRDM15. MYO7a

was myosins, cytoskeletal-associated protein, bind to actin filaments that are concentrated beneath the plasma membrane. They are important for short-range transport during endocytosis and exocytosis. They also generate mechanical force for muscle contraction, cell migration and cytokinesis[12]. In human cervix cancer (HeLa) and OSA cells (U2OS), myosin 1G (MYO1G) and myosin heavy chain 1 (MYH1) are essential for cancer cell survival[13]. Myosin 6 (MYO6) is a reverse-direction motor protein that moves towards the minus-end of actin filaments. Myosins have functions in a variety of intracellular processes such as vesicular membrane trafficking and cell migration[14]. Myosin[15,16] and ezrin[17] , the actomyosin-associated proteins have been reported as the putative physiological substrates for Rho-kinase. Rho-kinase can regulate the phosphorylation of both proteins during cell migration[18]. These findings are in agreement with the previous studies using primary human OS and benign bone tumor samples with 2D gel electrophoresis and the protein spots was identified with MALDI-TOF MS[19]. The cytoskeleton and microtubule-associated proteins were identified, suggesting they play a role in the tumor cell migration and metastasis that is characteristic of OSA. The GIMAP family members are associated with function in immune cells and seem to be confined to the same tissues suggesting that different GIMAP family members may act in concert to mediate their functions. The up-regulation of GIMAP family member 5 was associated with human B-cell malignancy, possibly by inhibiting apoptosis[20]. GIMAP family member 6 was the biomarker identified for human prostate cancer[21]. 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[22]. ADAMTS4 is a cartilage proteoglycan that may be involved in its turnover and may play an important role in the destruction of aggrecan in arthritic diseases. IL1R1 is receptor for interleukin-1 alpha (IL-1A), 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 pathway, after binding to interleukin-1 associates 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. The rCG38920, only 1 protein in OSA serum, still had unknown function. However, the validations of these identified proteins were needed in order to determine if they can be useful as the novel biomarkers for canine OSA in the future.

In cell lines, one high-metastatic (HMPOS), 2 low-metastatic (OOS and POS) and one non-metastatic (CHOS) canine OSA cell lines were used in this study. From 754 proteins, 67 proteins were not present in CHOS cell lines which had benign behavior. There were 2 being detected only in HMPOS cells included activating signal co-integrator 1 (ASC-1) complex and N-terminal kinase-like protein (NTKL). The ASC-1 complex is a transcriptional coactivator that plays an important role in gene transactivation by multiple transcription factors including activating protein 1 (AP-1), nuclear factor kappa-B (NF-kB) and serum response factor (SRF). The encoded protein contains an N-terminal KH-type RNA-binding motif which is required for AP-1 transactivation by the ASC-1 complex. Mutations in this gene are associated with Barrett esophagus and esophageal adenocarcinoma[23]. NTKL, protein tyrosine kinase activity and transcription regulation, was frequently upregulated in human hepatocellular carcinoma (HCC), which was significantly correlated with vascular invasion, cell cycle progression, cell motilty and poor prognosis[24]. There was 1 being detected only in POS cells included eukaryotic initiation factor 2 gamma (eIF-2γ). Eukaryotic translation initiation factor 2 alpha (eIF2α), which is a component of the eukaryotic translation initiation complex, functions in cell death and survival under various stress conditions. The eIF2α phosphorylation had the role in cell death using the breast cancer cell lines MCF-7 and MCF-7/ADR.

MCF-7/ADR cells are MCF-7-driven cells that have acquired resistance to doxorubicin[25]. However, the studies of ASC-1, NTKL, and eIF2γ on OSA have not yet been found. From complex protein networks, carboplatin and doxorubicin were related to apoptosis caspase signaling pathway, ezrin, and our several identified proteins. Contrastly, several identified proteins were not related to this signaling pathway. However, further study should be validated these identified proteins if they can be useful as the novel biomarkers for canine OSA in the future.

In conclusion, we identified proteins in tumor tissue, serum, cell line and cell medium that can potentially discriminate patients with malignant OSA from benign or non-OSA through proteomic approach using mass spectrometry. These proteins may be useful to diagnosis, prognosis and prevent misdiagnosis between malignant OSA from benign or non-OSA as they have similar clinical symptoms in the early stage. In the future, further independent validation of these biomarkers will be required using the greater numbers of patient samples and the more technique including Western blot to obtain the greatest diagnostic power for differentiating malignant OSA patients from controls.

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Appendix

Output (Acknowledge the Thailand Research Fund)

8.1 International Journal Publication

In submission (กำลังรอตีพิมพ์)

8.2 Application

8.3 Others e.g. national journal publication, proceeding, international conference, book chapter, patent

CD : file 1) Bone_serum-1_final.xls 2)Cell-1_final.xls 3) Media-1_final.xls