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Proteome Comparisons between Pre-Chemotherapy and Post-Chemotherapy Serum of Metastatic Osteosarcoma Patients Reveals Potential Novel Biomarker

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Abstract

This study is the first to show on differential protein profiles between serum isolated from healthy individual and metastatic osteosarcoma patients at different stages (Pre- and post-chemotherapy). The analyses have identified significant number of proteins that involved in the progression of the osteosarcoma metastasis. This data could provide a new insight in the osteosarcoma biological processes and use as a potential biomarker for better OS prognosis.

Osteosarcoma (OS), a malignant bone tumour, is commonly occurs in children and young adults between the ages of 10 to 30. Although the standard treatment for OS is advancing and significantly improved the survival rate in recent years, its poor prognosis continues to remain the major problem in managing the disease. In this study, we have conducted a series of systematic analysis to identify novel proteins associated with the metastatic progression of human OS using a 4-plex iTRAQ analysis. Pooled serum samples were collected from patients who were diagnosed with metastatic osteosarcoma. The serum was collected at two stages; pre-chemotherapy and post-chemotherapy. iTRAQ analysis identified 217 proteins with 104214 spectra from the patients' plasma. The proteins identified were analysed using bioinformatics software and categorized according to their role in biological processes. Most of the proteins fall under cellular component organization or biogenesis (39.4%), cellular process (35.4%), biological regulation (20.0%) and immune system process (29.3%). In addition, these proteins have also shown to be significantly altered in their expression when compare between pre- and post-chemotherapy patients samples such as C-reactive proteins, vascular adhesion molecule-1 and gelsolin. To date, this is the first differential protein expression study to use metastatic osteosarcoma patients' serum at different stages for the protein profiling. We have successfully generated a comprehensive data on the differentially expressed protein and the comparative study has revealed a significant amount of proteins expression has been altered. This data could provide a new insight in the OS biological processes and use as a potential biomarker for better OS prognosis.

Keywords: Osteoarcoma; Metastasis; Biomarker; Chemotherapy; Proteomics

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Introduction

Osteosarcoma (OS), also known as osteogenic sarcoma, is the most common type of cancer that develops in bone. Most osteosarcoma occurs in children and young adults between the ages of 10 to 30 [1]. It accounts about 10% of childhood cancer. This cancerous (malignant) bone tumor usually develops during the period of rapid growth of an adolescence maturing into an adult. The malignant bone tumour tends to develop in the bones of the tibia (shin), femur (thigh) and humerus (upper arm).

Diagnosis of OS was made by imaging test followed by histological grading of the tissue biopsied from the patients. The present standard treatment for high-grade OS includes neo-adjuvant chemotherapy followed by surgical resection and post-operative chemotherapy [2]. Although the cure rate of non-metastatic cases has improved tremendously, there were recurrences in approximately 30% of the cases and more than 80% of the relapses involve the lungs [3]. These undetectable micrometastases are commonly present at time they first seek medical attention and indicate presence of more aggressive diseases. Previous data has shown that approximately 80% of patients are believed to have metastatic diseases, yet only 8-15% is detectable with the current diagnostic tools [4]. Despite decades of trial using intensified dosing, different timing and variations in combinations of chemical agents poor prognosis has continues to be the major problem in managing osteosarcoma. Although advances of the standard treatment for OS have significantly improved the survival rate in recent years, its prognosis continues to remain less optimistic.

Identification of cancer biomarkers using blood and its products, such as plasma and serum are of paramount importance since they contain proteins secreted from the cancer cells. However, identifying clinically relevant biomarkers impose a major challenge to researcher due to presence of abundant protein such as albumin, immunoglobulin and transferrin which contribute 99% of the mass of the total plasma proteins [5]. The remaining 1% is thought to be composed of the medium/low abundance proteins and include the biomarker pool [6]. In addition, the progression of osteosarcoma is usually presents at early haematogenous metastasis which is also attributed to the poor prognosis of OS. This tumour cells are likely to invade the surrounding tissues or migrate to distant sites during tumour progression.

Recent advances in genomics and proteomics has enabled researchers to learn more about the molecular aspects of a tumour. Although there are many different protein fractionation methodologies based on differences in molecular weight, shape, charge, pI, hydrophobicity and affinity through specific biomolecular interactions, it has been reported that high abundance protein separation using the antibody based IgY-12 immunodepletion system is highly reproducible [7]. Amongst the proteomic technologies used, isobaric Tags for Relative and Absolute Quantitation (iTRAQ) has the advantages of being relatively high throughput and simultaneously provide information on peptide quantitation and identification, as previously reported [8-10].

In this study we have performed a 4-plex iTRAQ analysis in order to identify the novel proteins that play a key role in the osteosarcoma metastatic progression. In a typical workflow, samples are reduced, alkylated and proteolytically digested to generate peptides. The peptides are labelled with a set of iTRAQ reagents (in a 4 or 8-plex format), pooled and fractionated by strong cation exchange (SCX). The fractions are then analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS), with the resultant mass spectra providing sequence information (from the peptide fragments), and relative quantification (from the reporter group ions).

Materials and Method

Blood collection and serum isolation

Peripheral blood was drawn from patients attending the Orthopaedic Clinic at University of Malaya Medical Centre (UMMC) following written informed consent from the patients and upon approval by the UMMC Ethics Committee (BK-MIS-1117-E01). The blood was drawn twice; during the patients' initial visit to the clinic (pre-chemotherapy) and the second time, during the surgical removal of the tumour (post-chemotherapy). Serum was isolated by allowing the blood to coagulate for 30 minutes, centrifuged at 1,200 × g for 10 min at 4°C and then stored at −80°C in 100 µl aliquots.

Serum grouping and pooling

Twelve serum samples were carefully selected and pooled (n=6 patients/group), to form 2 patient groups. The patients were actively monitored for at least 5 years from the time of their initial blood taking. These patients were with evidence of bone metastasis from the prior imaging and histological analysis. The 2 patient groups were: Group 1: Pre-chemotherapy samples, which the blood was drawn prior to chemotherapy and any treatment to the patients. Group 2: Post-chemotherapy samples which the blood was drawn during surgical removal of the tumour (post-chemotherapy patients).

Control group was collected from voulunteer healthy individuals prior to their consent.

Immunodepletion and iTRAQ labelling

Pooled serum samples were depleted of the most common plasma proteins according to the iTRAQ protocol (Applied Biosystem). Previous studies have shown that serum pooling followed by depletion of the most highly abundant proteins is an effective strategy to reduce the dynamic range of proteins, and thus enhance the identification of serum biomarkers, as demonstrated using the quantitative proteomic method of iTRAQ [11]. Serum samples from 6 patients representing each of the 2 patient groups were pooled in equal volumes to give a total volume of 200 µl for each group (40 µl of each sample). The pooled serum samples were shipped on dry ice to Proteomics International (Australia).

The samples were then being acetone precipitated, reduced, alkylated and trypsin digested according to the protocol (Applied Biosystem). Samples (Control, Pre-chemotherapy and post-chemotherapy) were labelled with iTRAQ reagents, 114 (control), 115 (control), 116 (pre-chemotherapy) and 117 (post-chemotherapy) accordingly. Peptide were desalted on Strata-X 33 µm reversed phase column (Phenomenex) and dissolved in a buffer containing 10 mM KH_2PO_4 (pH3) in 10% acetonitrile prior to separation by strong cation exchange liquid chromatography (SCX) using polysulfoethyl column (4.6 × 100 mm, 5 µm, 300 A) 1100 HPLC system (Agilent, USA). Peptides were eluted with a linear gradient of 0-400 mM KCl and eight fractions of the peptides were collected and loaded onto an Agilent Zorbax 300SB-C18, 3.5 µm (Agilent, USA) which running on nano HPLC system (Shimadzu, Japan). Peptides were resolved with a

gradient of 10-40% acetonitrile (0.1%) trifluoroacetic acid) over 160 minutes. The resultant spots were analysed on a 5600 Triple TOF mass spectrometer (AB Sciex, USA).

Data analysis

Spectral data was analysed against the SwissProt database with the taxonomy set to Homo sapiens using ProteinPilot™ 4.5 software (AB Sciex, USA). The database was downloaded on February 2014 and contained 542,503 sequences.

Immunohistochemistry

Tissue biopsied from the metastatic OS patients were fixed prior to paraffin embedding. The paraffin embedded tissue blocks will be sectioned at 4 to 5 micron thickness, applied to poly-L-lysine coated slides and dried in a hot oven at 60°C for a minimum of 2 hours. The sections was then deparaffinised in a few changes of xylene followed by graded concentration of alcohol until fully hydrated. Immunohistochemistry was performed using a Dakoimmunostaining kit (DakoCytomation, USA) to verify the presence of VCAM-1 in the tissue biopsied. Slides were washed with Tris-buffered saline (TBS) and treated with 0.03% hydrogen peroxide containing sodium azide. Slides were then incubated with primary antibody to VCAM-1 or PBS alone (negative control) for 1 hour. Slides were then further exposed to horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody/ IgG for 30 minutes. Samples were stained with diaminobenzidine (DAB kit, DakoCytomation, USA) according to manufacturer protocol and observed under the light microscope for the presence of VCAM-1.

Results

Gene ontology annotation

Gene ontology (GO) annotations for biological processes were assigned using Protein Analysis through Evolutionary Relationships (PANTHER) software. This software link the protein accession codes to the corresponding entries in the gene ontology database hence identifying the range of proteins assessed earlier [12]. Approximately 217 proteins with 104,214 spectra were analysed. The PANTHER analysis revealed the presence of many common plasma proteins such as cellular component organization (39.2%), cellular process (66.7%), biological regulation (24.2%) and localization (18.2%) (**Figure 1**). In total of 51 proteins and 69 proteins were differentially expressed in pre-chemotherapy and post-chemotherapy in relative to control serum.

Identification of proteins for biomarker leads

Differences in protein levels are reported following a t-test analysis and the p-values were calculated based on the number of peptides used for the quantification and the variance of the iTRAQ reporter ratios derived from these peptides. A p-value ≤ 0.01 was used to assign significant changes in protein levels between sample sets. These protein changes were reported as significant differential expression, were selected based upon statistical significance rather than fold change [13].

In our analysis, we were interested in proteins showing both increased and decreased expression levels, as previous studies have reported that both significantly up- and down-regulated proteins may be of clinical relevance9. Identification of proteins differentially expressed in pre-chemotherapy and post-chemotherapy were of interest as these could provide leads for potentially useful diagnostic and prognostic biomarkers. Thus, a comparison between the pre-chemotherapy groups versus the control group showed a significant differential expression of 51 proteins; 28 of which were up-regulated and 23 down-regulated (**Table 1a**). Similarly, a comparison between the post-chemotherapy groups versus the control group identified the differential expression of 69 proteins; 29 of which showed significant over-expression and 40 showed down-regulations (**Table 1b**). Comparisons of the pre-chemotherapy versus the post-chemotherapy group identified the differential expression of 57 proteins, with 41 proteins showing significant over-expression and 16 showing significant down-regulation (**Table 1c**).

Differential protein level associated with OS metastasis progression

In addition to the analysis above, protein differences were mapped according to progression of the disease to the metastatic cancer (**Figure 2**). The lists of differences are based on comparisons between the controls versus pre-chemotherapy group; control versus post-chemotherapy group and pre-chemotherapy versus post-chemotherapy groups. From the list, a number of proteins were seen to be differentially expressed at certain stages. For instance, individual proteins such as C-reactive protein, alpha-2-macroglobulin, vascular adhesion molecule-1 (VCAM-1) and gelsolin were evidently showed a significant differential expression at different stages.

Protein-protein interaction

Protein-protein interaction network was analysed by String software (version 9). In this work we have analysed the interaction of protein in response to chemotherapy. Therefore, we have used the list of post-chemotherapy proteins differential expression in relative to control that we have identified earlier as reference. From the analysis we have shown 12 proteins were involved in the protein-protein interaction out of 69 proteins identified earlier (**Figure 3**).

Immunohistochemistry

According to the above bioinformatics analysis, we have selected VCAM-1 to further verify the result obtained using immunohistochemistry assessment to the OS tissue biopsy of the patients. The relative positive signal at post-chemotherapy is much more that in the pre-chemotherapy samples (**Figure 4**). This assessment reveals good correlation between VCAM-1 staining and the proteome expression profiles.

Discussion

This study is conducted with the aims to identify potentially useful serum biomarkers for metastatic osteosarcoma. We have successfully profiled pooled serum samples from a carefully selected group of patients representing the stages of osteosarcoma (OS) cancer progression using a 4-plex iTRAQ approach. Following the GO annotations of the 217proteins identified and quantified,

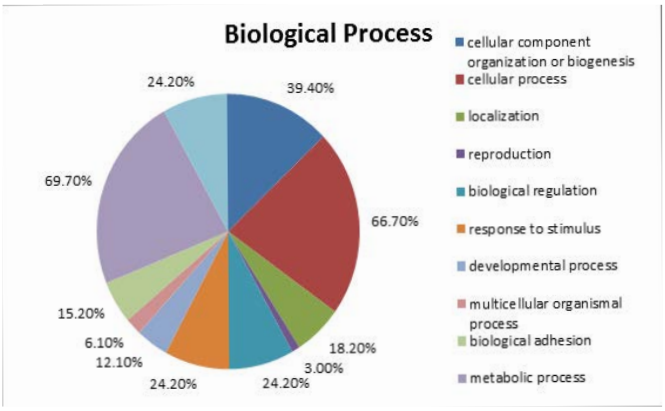

Figure 1 Bioinformatics analysis of differentially expressed proteins. The pie-chart showing the distribution of the 217 proteins identified from the samples in human biological processes. GO database was used for the analysis.

majority of these proteins were found to be classified in diverse biological processes such as cellular component organization or biogenesis (39.2%), cellular process (66.7%), biological regulation (24.2%) and localization (18.2%). Regarding the differentially expressed proteins, some of these have previously been reported as candidate for biomarkers in many other cancers such as alpha-2-macroglobulin (prostate cancer) [14], ceruloplasmin (ovarian cancer) [15] and C-reactive protein (bone cancer) [16] which provides confidence to our dataset and provides an independent confirmation of these candidates.

In comparison between normal sera and pre-chemotherapy of the metastatic OS samples, total of 52 proteins have been shown to be altered. One of the proteins that have shown to be significantly elevated is C-reactive protein (CRP). CRP is an acute phase reactant (APR) protein produced by the liver in response to inflammation. Elevation of CRP has been reported previously in patients with bone metastatic prostate cancer, and has been associated with an adverse outcome for men with castration resistant prostate

Table 1a. Proteins differentially expressed between the control (114) and pre-chemotherapy (116) groups.

Accession No.	Name	% Cov	Peptides (95%)	116:114	P Val 116:114
P04114|APOB_HUMAN	Apolipoprotein B-100	47.8	361	0.9638	0.00E+00
P0C0L5|CO4B_HUMAN	Complement C4-B	76.6	566	4.2073	4.60E-03
P01023|A2MG_HUMAN	Alpha-2-macroglobulin	68.1	153	1.0864	0.00E+00
P00450|CERU_HUMAN	Ceruloplasmin	72.4	353	1.0666	0.00E+00
P00751|CFAB_HUMAN	Complement factor B	63.6	207	1.028	0.00E+00
P02751|FINC_HUMAN	Fibronectin	47.7	104	0.2051	0.00E+00
Q14624|ITIH4_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H4	70.2	209	1.0093	7.30E-03
P01031|CO5_HUMAN	Complement C5	49.7	102	3.767	0.00E+00
P43652|AFAM_HUMAN	Afamin	64.1	84	0.0787	0.00E+00
P06727|APOA4_HUMAN	Apolipoprotein A-IV	76	73	0.912	0.00E+00
P13671|CO6_HUMAN	Complement component C6	65.9	72	1.8365	8.00E-04
P06396|GELS_HUMAN	Gelsolin	58.4	101	0.8551	0.00E+00
P02749|APOH_HUMAN	Beta-2-glycoprotein 1	79.1	114	0.4207	3.00E-04
P01008|ANT3_HUMAN	Antithrombin-III	72.2	66	1.977	2.00E-04
P01042|KNG1_HUMAN	Kininogen-1	61.8	121	0.3048	1.90E-03
P02765|FETUA_HUMAN	Alpha-2-HS-glycoprotein	66.8	122	0.955	3.00E-03
P01011|AACT_HUMAN	Alpha-1-antichymotrypsin	63.8	77	1.028	1.50E-03
P07996|TSP1_HUMAN	Thrombospondin-1	41.4	41	0.1486	0.00E+00
P02748|CO9_HUMAN	Complement component C9	61.9	41	4.7863	0.00E+00
P35858|ALS_HUMAN	Insulin-like growth factor-binding protein complex acid labile subunit	51.2	50	0.5649	3.51E-02
P00738|HPT_HUMAN	Haptoglobin	74.6	36	1.9055	0.00E+00
P02649|APOE_HUMAN	Apolipoprotein E	75.4	33	0.3281	1.00E-04
P04196|HRG_HUMAN	Histidine-rich glycoprotein	64.8	41	0.929	0.00E+00
P07357|CO8A_HUMAN	Complement component C8 alpha chain	57.7	35	1.7219	1.24E-02
Q06033|ITIH3_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H3	48.7	41	2.729	1.71E-02
P10909|CLUS_HUMAN	Clusterin	52.1	38	0.3565	1.70E-02
P36955|PEDF_HUMAN	Pigment epithelium-derived factor	76.1	44	0.1406	0.00E+00
P29622|KAIN_HUMAN	Kallistatin	60.4	32	0.1706	0.00E+00
P02750|A2GL_HUMAN	Leucine-rich alpha-2-glycoprotein	60.8	54	1.1695	0.00E+00
P00748|FA12_HUMAN	Coagulation factor XII	49.3	34	3.5318	1.18E-02
Q96PD5|PGRP2_HUMAN	N-acetylmuramoyl-L-alanine amidase	58.9	47	0.3162	3.10E-03

P04004|VTNC_HUMAN	Vitronectin	49.6	54	0.3981	2.70E-03
P02768|ALBU_HUMAN	Serum albumin	55.7	18	5.8614	0.00E+00
P68871|HBB_HUMAN	Hemoglobin subunit beta	89.8	23	18.197	1.00E-04
Q16610|ECM1_HUMAN	Extracellular matrix protein 1	49.3	13	0.2858	1.40E-02
P05452|TETN_HUMAN	Tetranectin	74.3	20	0.2965	6.50E-03
P04003|C4BPA_HUMAN	C4b-binding protein alpha chain	45.4	14	4.5709	9.00E-04
P02753|RET4_HUMAN	Retinol-binding protein 4	65.2	34	0.3251	7.80E-03
P02787|TRFE_HUMAN	Serotransferrin	34	10	0.1629	0.00E+00
P17936|IBP3_HUMAN	Insulin-like growth factor-binding protein 3	44	11	0.3664	1.92E-02
P15169|CBPN_HUMAN	Carboxypeptidase N catalytic chain	40.8	15	1.7378	2.46E-02
P08519|APOA_HUMAN	Apolipoprotein(a)	33.3	14	15.9956	1.66E-02
P63261|ACTG_HUMAN	Actin, cytoplasmic 2	32.5	12	2.208	2.20E-02
P02775|CXCL7_HUMAN	Platelet basic protein	64.8	14	0.15	2.30E-03
P02741|CRP_HUMAN	C-reactive protein	31.3	9	2.729	2.90E-03
P02656|APOC3_HUMAN	Apolipoprotein C-III	57.6	7	0.0824	3.00E-02
P06702|S10A9_HUMAN	Protein S100-A9	64	8	1.7219	4.81E-02
P69905|HBA_HUMAN	Hemoglobin subunit alpha	64.8	10	20.7014	1.00E-04
P05109|S10A8_HUMAN	Protein S100-A8	52.7	6	2.355	1.78E-02
P02652|APOA2_HUMAN	Apolipoprotein A-II	57	5	0.52	1.51E-02
O00187|MASP2_HUMAN	Mannan-binding lectin serine protease 2	18.7	7	1.8707	4.46E-02

Table 1b. Proteins differentially expressed between the control (114) and post-chemotherapy groups (117).

Accession No.	Name	% Cov	Peptides (95%)	117:114	P Val 117:114
P04114|APOB_HUMAN	Apolipoprotein B-100	47.8	361	1.0093	0.00E+00
P08603|CFAH_HUMAN	Complement factor H	79.2	346	0.9727	0.00E+00
P01023|A2MG_HUMAN	Alpha-2-macroglobulin	68.1	153	1.0471	0.00E+00
P02751|FINC_HUMAN	Fibronectin	47.7	104	6.1376	0.00E+00
P02790|HEMO_HUMAN	Hemopexin	89.2	416	0.955	0.00E+00
P00747|PLMN_HUMAN	Plasminogen	82.1	136	0.9462	0.00E+00
P01031|CO5_HUMAN	Complement C5	49.7	102	1.7378	1.90E-03
P00734|THRB_HUMAN	Prothrombin	71.9	159	0.955	0.00E+00
P02774|VTDB_HUMAN	Vitamin D-binding protein	81.4	130	0.9727	7.00E-04
P19823|ITIH2_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H2	49.7	171	0.1514	1.00E-04
P06727|APOA4_HUMAN	Apolipoprotein A-IV	76	73	0.9036	0.00E+00
P10643|CO7_HUMAN	Complement component C7	60.7	85	2.9376	0.00E+00
P06396|GELS_HUMAN	Gelsolin	58.4	101	0.8318	0.00E+00
P02749|APOH_HUMAN	Beta-2-glycoprotein 1	79.1	114	0.1614	0.00E+00
P01008|ANT3_HUMAN	Antithrombin-III	72.2	66	0.2512	9.00E-04
P19827|ITIH1_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H1	59.5	114	0.0938	0.00E+00
P01042|KNG1_HUMAN	Kininogen-1	61.8	121	0.1923	1.00E-04
P00736|C1R_HUMAN	Complement C1r subcomponent	71.5	79	0.3373	2.93E-02
P02765|FETUA_HUMAN	Alpha-2-HS-glycoprotein	66.8	122	0.955	1.26E-02
P01011|AACT_HUMAN	Alpha-1-antichymotrypsin	63.8	77	1.0471	1.00E-04
P07996|TSP1_HUMAN	Thrombospondin-1	41.4	41	0.2512	0.00E+00
P03952|KLKB1_HUMAN	Plasma kallikrein	57.1	46	0.1419	0.00E+00
P05156|CFAI_HUMAN	Complement factor I	59.9	43	0.3873	9.00E-04
P06681|CO2_HUMAN	Complement C2	55.1	49	0.4325	3.63E-02
P09871|C1S_HUMAN	Complement C1s subcomponent	59.3	48	0.2489	2.20E-03
P05155|IC1_HUMAN	Plasma protease C1 inhibitor	52.2	59	1.9953	3.59E-02
P08123|CO1A2_HUMAN	Collagen alpha-2(I) chain	40.3	49	48.7528	5.00E-04
P02748|CO9_HUMAN	Complement component C9	61.9	41	2.6546	1.15E-02
P26927|HGFL_HUMAN	Hepatocyte growth factor-like protein	57.5	27	0.4055	4.40E-03
P35858|ALS_HUMAN	Insulin-like growth factor-binding protein complex acid labile subunit	51.2	50	0.0982	0.00E+00
P25311|ZA2G_HUMAN	Zinc-alpha-2-glycoprotein	70.8	68	0.118	2.10E-03
P00738|HPT_HUMAN	Haptoglobin	74.6	36	1.1912	0.00E+00

P02649|APOE_HUMAN	Apolipoprotein E	75.4	33	2.9107	1.00E-04
P04196|HRG_HUMAN	Histidine-rich glycoprotein	64.8	41	0.9376	0.00E+00
Q06033|ITIH3_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H3	48.7	41	4.1687	1.00E-04
P02760|AMBP_HUMAN	Protein AMBP	78.4	65	0.4699	1.03E-02
P10909|CLUS_HUMAN	Clusterin	52.1	38	0.1803	2.10E-03
P05546|HEP2_HUMAN	Heparin cofactor 2	52.9	47	0.1644	1.00E-04
P36955|PEDF_HUMAN	Pigment epithelium-derived factor	76.1	44	0.2512	2.10E-03
P29622|KAIN_HUMAN	Kallistatin	60.4	32	0.1675	0.00E+00
P08697|A2AP_HUMAN	Alpha-2-antiplasmin	67.6	47	0.1318	6.00E-03
P07358|CO8B_HUMAN	Complement component C8 beta chain	55.2	27	0.631	1.27E-02
P02750|A2GL_HUMAN	Leucine-rich alpha-2-glycoprotein	60.8	54	1.1482	0.00E+00
P04275|VWF_HUMAN	von Willebrand factor	19.6	21	1.7378	2.70E-03
P07225|PROS_HUMAN	Vitamin K-dependent protein S	42.3	24	0.5445	5.50E-03
P01024|CO3_HUMAN	Complement C3	28.1	27	1.0965	4.86E-02
Q96PD5|PGRP2_HUMAN	N-acetylmuramoyl-L-alanine amidase	58.9	47	0.2032	1.80E-03
P04004|VTNC_HUMAN	Vitronectin	49.6	54	0.1419	1.00E-04
P02768|ALBU_HUMAN	Serum albumin	55.7	18	5.7016	0.00E+00
Q96KN2|CNDP1_HUMAN	Beta-Ala-His dipeptidase	43.4	17	0.1096	0.00E+00
P68871|HBB_HUMAN	Hemoglobin subunit beta	89.8	23	5.2481	2.90E-03
P05452|TETN_HUMAN	Tetranectin	74.3	20	0.2032	1.10E-03
P05543|THBG_HUMAN	Thyroxine-binding globulin	42.7	21	3.8371	3.77E-02
P02753|RET4_HUMAN	Retinol-binding protein 4	65.2	34	0.1472	1.50E-03
P02787|TRFE_HUMAN	Serotransferrin	34	10	0.0492	0.00E+00
P17936|IBP3_HUMAN	Insulin-like growth factor-binding protein 3	44	11	0.1585	2.90E-03
P15169|CBPN_HUMAN	Carboxypeptidase N catalytic chain	40.8	15	1.4454	3.75E-02
P08519|APOA_HUMAN	Apolipoprotein(a)	33.3	14	21.2814	1.45E-02
P43251|BTD_HUMAN	Biotinidase	23.4	11	0.2679	2.43E-02
P02741|CRP_HUMAN	C-reactive protein	31.3	9	12.1339	3.70E-03
Q9Y6R7|FCGBP_HUMAN	IgGFc-binding protein	5.7	8	3.4995	2.62E-02
P06702|S10A9_HUMAN	Protein S100-A9	64	8	2.6303	2.06E-02
P69905|HBA_HUMAN	Hemoglobin subunit alpha	64.8	10	5.5976	2.30E-03
P18428|LBP_HUMAN	Lipopolysaccharide-binding protein	20.8	6	5.1523	1.39E-02
P05109|S10A8_HUMAN	Protein S100-A8	52.7	6	3.9446	9.80E-03
P01009|A1AT_HUMAN	Alpha-1-antitrypsin	33.3	4	3.1915	4.20E-03
P02647|APOA1_HUMAN	Apolipoprotein A-I	39.3	3	0.2355	3.58E-02
P18065|IBP2_HUMAN	Insulin-like growth factor-binding protein 2	16	3	13.6773	3.44E-02
P14151|LYAM1_HUMAN	Vascular cell adhesion protein 1	17.2	2	2.3988	3.27E-02

cancer [17]. This suggested that our finding is in consistent to the previous findings on the relation of CRP in metastatic progression of a cancer. In addition to CRP, many other APR protein family proteins have been seen to be altered in this data set including alpha-2-macroglobulin, ceruloplasmin, haptoglobin, and alpha-1-antichymotrypsin. Although alteration of these proteins is not surprising as it is well documented that the presence of a tumour activates an inflammatory response, but the possibility of these APR proteins could have been secreted 'ectopically' by the tumour cells is plausible. For examples, previous studies have reported that common plasma proteins; albumin, prealbumin, alpha-1-antitrypsin, ceruloplasmin, alpha-2-macroglobulin, haptoglobin, transferrin and alpha-1-antichymotrypsin have been secreted in renal cell carcinoma, squamous cell carcinoma and breast cancer cell lines [18,19]. The potential of assessing APR proteins as cancer biomarkers could aid in diagnosis and staging and further identify the metastatic OS in the patients. Elevated serum CRP has been shown in patients with poor OS disease survival16. Another study has also supported that elevated serum CRP has higher correlation with patients with high grade OS survival [20].

Comparison between post-chemotherapy groups with the control group showed further significant elevation of the CRP. This further supported our suggestion on the possibility of assaying the APR protein secreted by the tumour cells to aid at least in the prognosis of the OS patients in determining the stages or identifying the metastatic disease itself. Another protein that was shown to have been remarkably altered is gelsolin. Gelsolin is an actin-binding protein which is key regulator of actin filament assembly and disassembly [21]. Previous studies have shown that gelsolin is being down-regulated in OS patients [22,23]. This is in consistent with our findings in which, gelsolin has been shown to be down-regulated in patients with the OS patients before chemotherapy and further reduction of the protein expression after chemotherapy. However, the comparison between pre-chemotherapy and post-chemotherapy group shows an increase

Table 1c. Proteins differentially expressed between the pre-chemotherapy (116) and post-chemotherapy (117) groups.

Accession No.	Name	% Cov	Peptides (95%)	116:117	PVal 116:117
P04114|APOB_HUMAN	Apolipoprotein B-100 OS	47.8	361	0.9462	0.00E+00
P0C0L5|CO4B_HUMAN	Complement C4-B	76.6	566	1.977	1.83E-02
P08603|CFAH_HUMAN	Complement factor H	79.2	346	1.028	0.00E+00
P01023|A2MG_HUMAN	Alpha-2-macroglobulin	68.1	153	1.028	0.00E+00
P00450|CERU_HUMAN	Ceruloplasmin	72.4	353	1.0471	0.00E+00
P00751|CFAB_HUMAN	Complement factor B	63.6	207	1.0186	0.00E+00
P02751|FINC_HUMAN	Fibronectin	47.7	104	0.0466	0.00E+00
Q14624|ITIH4_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H4	70.2	209	1.0093	3.80E-02
P02790|HEMO_HUMAN	Hemopexin	89.2	416	1.0186	2.90E-03
P00747|PLMN_HUMAN	Plasminogen	82.1	136	1.028	0.00E+00
P01031|CO5_HUMAN	Complement C5	49.7	102	2.1878	0.00E+00
P00734|THRB_HUMAN	Prothrombin	71.9	159	1.0375	0.00E+00
P02774|VTDB_HUMAN	Vitamin D-binding protein	81.4	130	1.028	0.00E+00
P19823|ITIH2_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H2	49.7	171	4.9659	2.20E-03
P43652|AFAM_HUMAN	Afamin	64.1	84	0.0809	0.00E+00
P10643|CO7_HUMAN	Complement component C7	60.7	85	0.3499	0.00E+00
P13671|CO6_HUMAN	Complement component C6	65.9	72	4.529	0.00E+00
P06396|GELS_HUMAN	Gelsolin	58.4	101	1.0186	1.12E-02
P02749|APOH_HUMAN	Beta-2-glycoprotein 1	79.1	114	2.6792	1.39E-01
P01008|ANT3_HUMAN	Antithrombin-III	72.2	66	7.379	0.00E+00
P19827|ITIH1_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H1	59.5	114	8.5507	0.00E+00
P01042|KNG1_HUMAN	Kininogen-1	61.8	121	1.6144	2.20E-01
P00736|C1R_HUMAN	Complement C1r subcomponent	71.5	79	3.0761	1.83E-02
P07996|TSP1_HUMAN	Thrombospondin-1	41.4	41	0.5702	4.23E-02
P03952|KLKB1_HUMAN	Plasma kallikrein	57.1	46	4.7863	0.00E+00
P05156|CFAI_HUMAN	Complement factor I	59.9	43	3.5975	0.00E+00
P06681|CO2_HUMAN	Complement C2	55.1	49	2.355	9.02E-02
P09871|C1S_HUMAN	Complement C1s subcomponent	59.3	48	3.5975	2.00E-03
P08123|CO1A2_HUMAN	Collagen alpha-2(I) chain	40.3	49	0.0119	1.60E-03
P02748|CO9_HUMAN	Complement component C9	61.9	41	1.8365	6.00E-03
P26927|HGFL_HUMAN	Hepatocyte growth factor-like protein	57.5	27	1.8707	1.69E-01
P35858|ALS_HUMAN	Insulin-like growth factor-binding protein complex acid labile subunit	51.2	50	6.1944	7.60E-03
P25311|ZA2G_HUMAN	Zinc-alpha-2-glycoprotein	70.8	68	6.8549	1.12E-02
P00738|HPT_HUMAN	Haptoglobin	74.6	36	1.5849	0.00E+00
P02649|APOE_HUMAN	Apolipoprotein E	75.4	33	0.1191	0.00E+00
P07357|CO8A_HUMAN	Complement component C8 alpha chain	57.7	35	2.0324	6.50E-03
Q06033|ITIH3_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H3	48.7	41	0.6427	1.88E-02
P02760|AMBP_HUMAN	Protein AMBP	78.4	65	2.5119	1.20E-03
P05546|HEP2_HUMAN	Heparin cofactor 2	52.9	47	6.368	1.00E-04
P08697|A2AP_HUMAN	Alpha-2-antiplasmin	67.6	47	5.2481	1.73E-02
P07358|CO8B_HUMAN	Complement component C8 beta chain	55.2	27	2.421	5.30E-03
P04275|VWF_HUMAN	von Willebrand factor	19.6	21	0.2911	3.00E-04
P01024|CO3_HUMAN	Complement C3	28.1	27	0.3532	8.00E-04
P04278|SHBG_HUMAN	Sex hormone-binding globulin	48.3	16	0.207	0.00E+00
Q96KN2|CNDP1_HUMAN	Beta-Ala-His dipeptidase	43.4	17	5.6494	1.60E-03
P68871|HBB_HUMAN	Hemoglobin subunit beta	89.8	23	3.8371	1.80E-03
Q16610|ECM1_HUMAN	Extracellular matrix protein 1	49.3	13	0.2512	1.00E-04
P51884|LUM_HUMAN	Lumican	53	23	0.4529	4.42E-02
P04003|C4BPA_HUMAN	C4b-binding protein alpha chain	45.4	14	1.6444	3.90E-03
P02787|TRFE_HUMAN	Serotransferrin	34	10	3.6644	2.57E-02
P63261|ACTG_HUMAN	Actin, cytoplasmic 2	32.5	12	2.0512	3.19E-02
P02775|CXCL7_HUMAN	Platelet basic protein	64.8	14	0.2312	2.63E-02
P0C0L4|CO4A_HUMAN	Complement C4-A	76.6	543	3.4041	4.32E-02
P02741|CRP_HUMAN	C-reactive protein	31.3	9	0.2188	4.29E-02
P69905|HBA_HUMAN	Hemoglobin subunit alpha	64.8	10	4.0179	1.60E-03
P01009|A1AT_HUMAN	Alpha-1-antitrypsin	33.3	4	0.5395	3.68E-02
P02652|APOA2_HUMAN	Apolipoprotein A-II	57	5	0.5754	2.57E-02

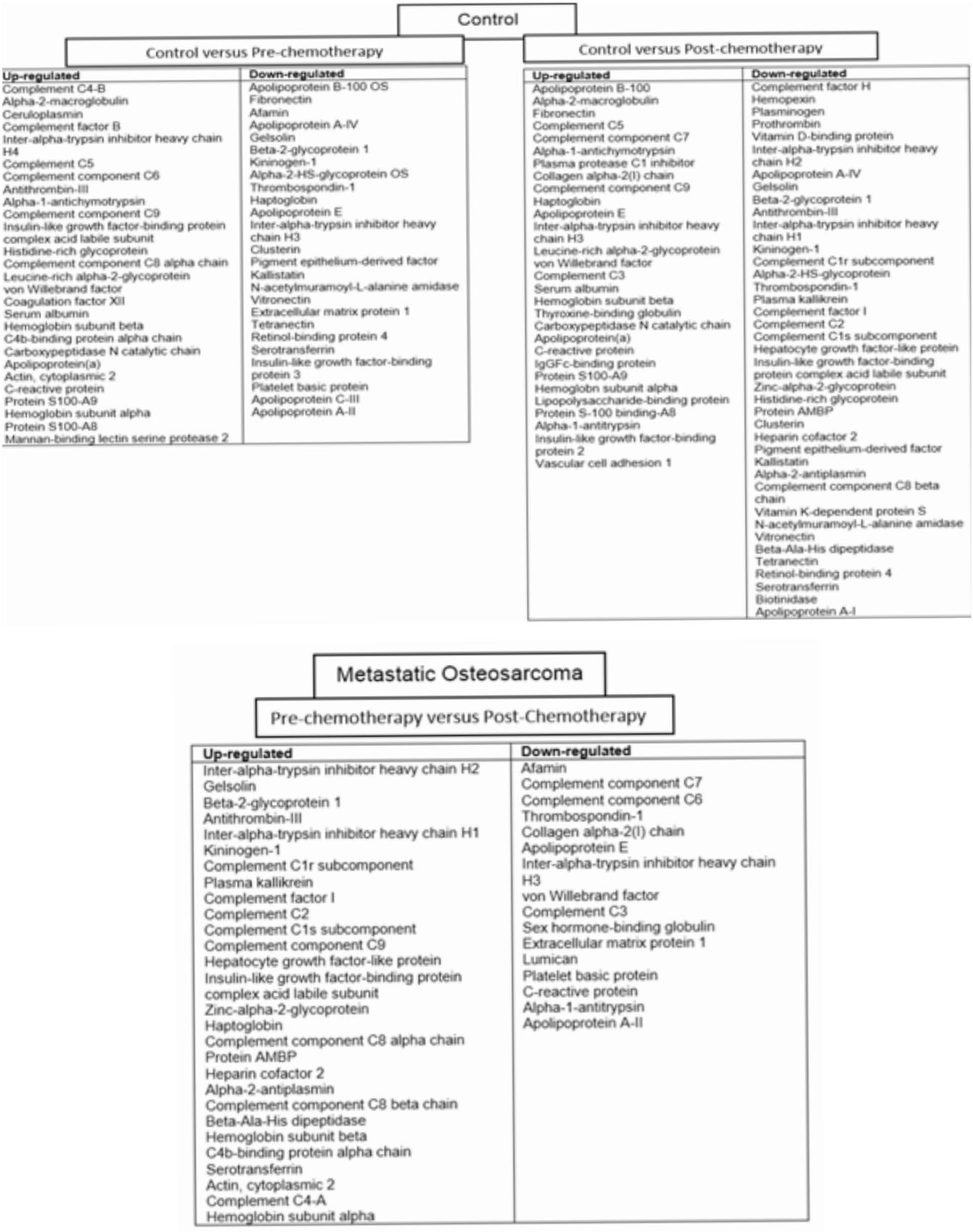

Figure 2 Proteins showing significant differential expression according to the disease different stages of metastasis (pre-chemotherapy and post-chemotherapy). The list of differentially expressed proteins shown is based on comparisons between control versus pre-chemotherapy; control versus post-chemotherapy and pre-chemotherapy versus post-chemotherapy group.

of gelsolin expression. We believed that this protein could play a role in the irresponsiveness towards the chemotherapy of the metastatic tumour cells. Vascular adhesion molecule-1 (VCAM-1) has shown a similar pattern in their expression even though the elevation in comparison between pre-chemotherapy and post-chemotherapy is not significant.

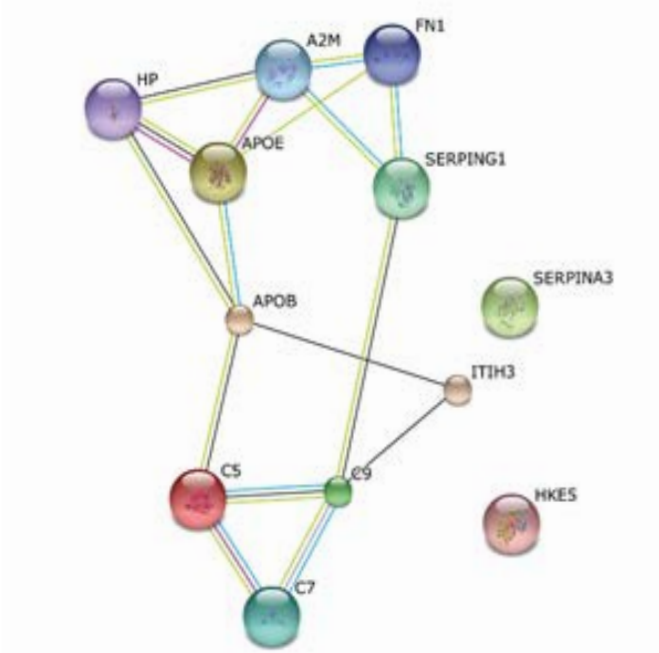

Figure 3 Protein-protein interaction networks of the differentially expressed proteins in response to chemotherapy relatives to control samples in metastatic osteosarcoma patients analyzed by String software (ver.9). The network displayed the interaction between the differential proteins (nodes) supported by the evidence (line). The network consists of up to eight lines by which each color represents an evidence for the respective interaction.

To support our hypothesis, we have carried out immunohistochemistry assessment on VCAM-1. Our data has shown increased in the expression of VCAM-1 in post-chemotherapy groups when compared with the pre-chemotherapy patients in metastatic OS patients. Our study represents one of the first steps in development of biomarkers for OS metastatic patients. To our knowledge, our study is the first to use the iTRAQ approach in identifying leads for potential biomarkers of metastatic OS using patients' serum. The panel of proteins identified in this study, together with the APR proteins from the patients' serum could be benefited future biomarker identification specific for the metastatic OS disease by further fractionation and strategies. However, these proteins warrant further validation and investigation.

In conclusion, we have successfully profile the proteins of the metastatic OS patients' serum using the iTRAQ labelling and LC-MS separation. Many common biological processes proteins have been identified with several significant differentially expressed proteins detected. Amongst the potential biomarker candidate proteins identified are CRP. Our findings could also provide a clue on understanding the tumour progression as we have also identified proteins (gelsolin and VCAM-1) that may play a role in responsiveness to the chemotherapy.

Acknowledgement

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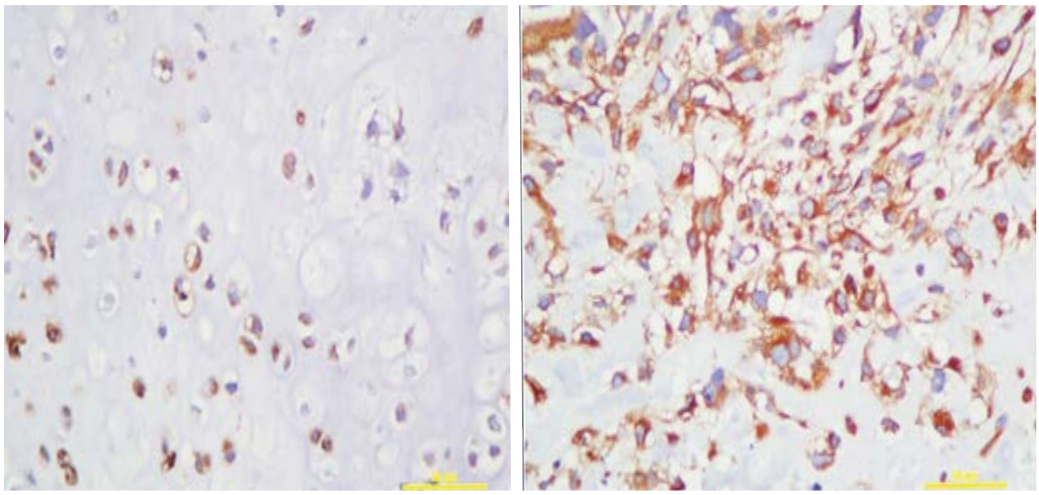

Figure 4 Immunohistochemistry photograph of pre-chemotherapy and post-chemotherapy metastatic OS tissue biopsied. The expression of VCAM-1 protein intensity was evidently higher in post-chemotherapy patients' sample (B) compare to pre-chemotherapy samples (A).

References

1 Luu HH, Kang Q, Park JK, Si W, Luo Q, et al. (2005) An orthotopic model of human osteosarcoma growth and spontaneous pulmonary metastasis. Clinical and Experimental Metastasis 22: 319-329.

2 Ferguson WS, Goorin AM (2001) Current treatment of osteosarcoma. Cancer Invest 19: 292-315.

3 Gorlick R, Anderson P, Andrulis I, Arndt C, Beardsley GP, et al. (2003) Biology of childhood osteogenic sarcoma and potential targets for therapeutic development: meeting summary. Clin Cancer Res 9: 5442-5453.

4 Ward WG, Mikaelian K, Dorey F, Mirra JM, Sassoon A, et al. (1994) Pulmonary metastases of stage IIB extremity osteosarcoma and subsequent pulmonary metastases. J Clin Oncol 12: 1849-1858.

5 Anderson NL, Anderson NG (2002) The human plasma proteome: history, character, and diagnostic prospects. Mol Cell Proteomics 1: 845-867.

6 Qian WJ, Kaleta DT, Petritis BO, Jiang H, Liu T, et al. (2008) Enhanced detection of low abundance human plasma proteins using a tandem IgY12-SuperMix immunoaffinity separation strategy. Mol Cell Proteomics 7: 1963-1973.

7 Liu T, Qian WJ, Mottaz HM, Gritsenko MA, Norbeck AD, et al. (2006) Evaluation of multiproteinimmunoaffinity subtraction for plasma proteomics and candidate biomarker discovery using mass spectrometry. Mol Cell Proteomics 5: 2167-2174.

8 Glen A, Evans CA, Gan CS, Cross SS, Hamdy FC, et al. (2010) Eight-plexiTRAQ analysis of variant metastatic human prostate cancer cells identifies candidate biomarkers of progression: An exploratory study. Prostate 70: 1313-1332.

9 Glen A, Gan CS, Hamdy FC, Eaton CL, Cross SS, et al. (2008) iTRAQ-facilitated proteomic analysis of human prostate cancer cells identifies proteins associated with progression. J Proteome Res 7: 897-907.

10 Keshamouni VG, Michailidis G, Grasso CS, Anthwal S, Strahler JR, et al. (2006) Differential protein expression profiling by iTRAQ-2DLC-MS/MS of lung cancer cells undergoing epithelial-mesenchymal transition reveals a migratory/invasive phenotype. J Proteome Res 5: 1143-1154.

11 Boylan KL, Andersen JD, Anderson LB, Higgins L, Skubitz AP (2010) Quantitative proteomic analysis by iTRAQ(R) for the identification of candidate biomarkers in ovarian cancer serum. Proteome Sci 8: 31.

12 Thomas PD, Kejariwal A, Campbell MJ, Mi H, Diemer K, et al. (2003) PANTHER: a browsable database of gene products organized by biological function, using curated protein family and subfamily classification. Nucleic Acids Res 31: 334-341.

13 Noirel J, Evans C, Salim M, Mukherjee J, Ow SY, et al. (2011) Methods in Quantitative Proteomics: Setting iTRAQ on the Right Track. Current Proteomics 8: 17-30.

14 Burgess EF, Haman AJL, Pietenpol JA (2008) Prostate cancer serum biomarker discovery through proteomic analysis of alpha-2 macroglobulin protein complexes. PROTEOMICS - Clinical Applications 2: 1223-1233.

15 Lee CM, Lo HW, Shao RP, Wang SC, Xia W, et al. (2004) Selective Activation of Ceruloplasmin Promoter in Ovarian Tumors Potential Use for Gene Therapy Cancer Res 64: 1788.

16 Nakamura T, Grimer RJ, Gaston CL, Watanuki M, Sudo A, et al. (2013) The prognostic value of the serum level of C-reactive protein for the survival of the patients with a primary sarcoma bone. Bone Joint J 95-B: 411-418.

17 Prins RC, Rademacher BL, Mongoue-Tchokote S, Alumkal JJ, Graff JN, et al. (2012) C-reactive protein as an adverse prognostic marker for men with castration-resistant prostate cancer (CRPC): Confirmatory results. Urol Oncol 30: 33-37.

18 Yoshimura S, Tamaoki N, Ueyama Y, Hata J (1978) Plasma protein production by human tumors xenotransplanted in nude mice. Cancer Res 38: 3474-3478.

19 Mbeunkui F, Metge BJ, Shevde LA, Pannell LK (2007) Identification of differentially secreted biomarkers using LC-MS/MS in isogenic cell line representing a progression of breast cancer. J Proteome Res 6: 2993-3002.

20 Funovics PT, Edelhauser G, Funovics MA, Laux C, Berzaczy D, et al. (2011) Pre-operative serum C-reactive protein as independent prognostic factor for survival but not infection in patients with high-grade osteosarcoma. Int Orthop 35: 1529-1536.

21 Sun HQ, Yamamoto M, Mejillano M, Yin HL (1999) Gelsolin, a Multifunctional Actin regulatory protein. J Biol Chem 274: 33179-33182.

22 Jin S, Shen JN, Peng JQ, Wang J, Huang G, et al. (2012) Decreased expression of serum gelsolin in patients with osteosarcoma. Chin Med J 125: 262-269.

23 Shen JN, Jin S, Wang J, Huang G, Yin JQ, et al. (2008) Detection of serum biomakers of osteosarcoma by proteomic profiling. Chinese Journal of Oncology 30: 519-522.