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Original Article

Discovery and identification of serum biomarkers for postmenopausal osteoporosis based on TMT labeling and HPLC-MS/MS technology

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Abstract: *Objective*: To screen the serum protein molecular markers of postmenopausal osteoporosis by the proteomics analysis using Tandem Mass Tag (TMT) coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS). *Methods:* Serum protein samples were recruited from 10 cases of postmenopausal patients with osteoporosis and 10 cases of postmenopausal women without osteoporosis and the high abundance ratios protein was removed, differentiation protein was extracted and labeled with TMT reagent. Then, mass spectrometric detection, data analysis of differentially expressed proteins, and analysis of biological information were carried out. *Results:* 87 significantly differentially expressed proteins were screened from the differentiated protein expression profile by LC-ESI-MS/MS combined with TMT labeling, including 50 proteins up-regulated and 37 proteins down-regulated. Differentially expressed proteins were analyzed by GO annotation, these proteins are mainly involved in 15 kinds of biological processes, seven kinds of cellular component and six kinds of molecular function. RAB7A, TSP1, GAS6, SPP24 were screened as candidate proteins which were related to the mechanism of bone remodeling of osteoporosis. By STRING10.0 protein interaction network analysis tools, RAB7A, TSP1, GAS6 were located in the center of the interaction network. SPP24 was located at edge of the network, but it is directly related to the protein BMP2 of bone remodeling. *Conclusion:* These results provide that the proteomics analysis by using TMT combined with LC-ESI-MS/MS was a feasible method for screening the molecular biomarkers. It suggests that RAB7A, TSP1, GAS6 and SPP24 may be a useful biomarker which can be used in diagnosis and treatment of postmenopausal osteoporosis.

Keywords: Postmenopausal osteoporosis, biological markers, proteomics

Introduction

Osteoporosis is a kind of low bone mass, bone microstructure damage of systemic skeletal system disease, characterized by enhanced bone fragility, degradation of bone strength (reduced bone strength) and a consequent increase in fracture risk [1]. The latest research shows that in China, the average incidence rate of osteoporosis was two times higher in women than in men and that a total incidence (rate) of osteoporosis was 19.74 percent, a population of about 112 million [2]. Primary osteoporosis is also known as the "silent epidemic". At the early stage of osteoporosis, the human body is hardly any symptoms and feel, so that it is typically not detected until occur of a fragility fracture [3]. A study predicts that the total osteoporosis-related fractures will be more than 3 million, costing nearly $28.5 billion in the United States in 2025 [4]. According to the statistical analysis of hip fracture in Beijing in 2011, hip fracture incidence (rate) is gradually increasing.

Osteoporosis, a major health issue worldwide, seriously influences people's life and health with high morbidity and disability of fragility

fracture and causes huge economic burden for individual, family and society. The studies on medication of osteoporosis have achieved obvious advances lately. Among the various strategies to prevent and cure this extremely complex and challenging disease, the drugs mostly include bone absorption-inhibitor drugs and bone formation-acceleration drugs, but the safety and effectiveness data for these drugs raise concerns for patients with osteoporosis requiring long term treatment.

Bone absorption-inhibitor drugs such as bisphosphonates have become a mainstay of treatment, but concerns have emerged that long-term use of these drugs may suppress bone remodeling, leading to the senescence of bone and fragility fractures. Bone formation-acceleration drugs such as teriparatide face severe problems, such as poor compliance of patients, drug toxicity, high price, risk of inducing tumor, increased the incidence of cardiovascular disease and stroke, and so on. Thus, early detection, diagnosis and treatment of osteoporosis are particularly important.

Bone turnover markers combined with bone mineral density (BMD) are important in monitoring curative effect of osteoporosis and predicting risk of fracture. The markers such as PINP, S-CTX can be used as a clinical tool to identify postmenopausal women who have high levels of bone turnover. Bone turnover markers are significantly associated with several lifestyle factors, age, gender, diurnal rhythm, several diseases and treatments influence, and food intake and so on. Significant fluctuation had been reported between BTMs levels in both healthy individuals and subjects with osteoporosis, so that the sensitivity and specificity of bone turnover markers was low or lack in monitoring curative effect of osteoporosis and predicting risk of fracture.

Because of the limited technological conditions, using sensitive and specific molecular markers have remained a challenge to early screening and diagnosis of osteoporosis. However, the rapid progress in proteomics and mass spectrometry technology provides a novel platform, with the characteristics of high-throughput and high efficiency, which can be used to find specific disease-related proteins as biomarkers for early diagnosis and early detection of osteoporosis.

Human serum/plasma containing the huge information closely related to human physiological or pathological states has become the most common and important samples used for disease diagnosis and prognosis and can be collected without any interventional procedures [5]. Human blood serum contains thousands of proteins that are synthesized and secreted, shed, or lost from the cell, tissues, and organs throughout the body. To date, more than 10,000 proteins have been identified in human plasma (http://www.plasmaproteomedatabase.org), most of which would be present at very low relative abundances [6].

The popular approach toward mining the blood for biomarkers is the ProteinChip system or two-dimensional electrophoresis coupled with a tandem mass spectrometer. However, they are laborious and difficult to automate [7, 8]. More significantly, these experiments showed poor reproducibility and limited dynamic range, and the proteins, after electrophoretic separation, were not immediately compatible with mass spectrometry [9, 10]. Therefore, detection of low-abundance proteins by ProteinChip system or two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) approach remains challenging.

To resolve the challenging feature of low relative abundances and exceptional wide concentration range, the research strategy of serum-based comparative proteomics was used, mainly including removing the high abundance ratios protein [11], labeling and fractionation [12] and tandem mass spectrometry (MS/MS) [13].

A tandem mass tag (TMT) is an isobaric mass tag chemical label used for protein quantification and identification from different sources in a single experiment [14]. Recent successes using TMT labeling and high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) illustrate the role of tandem mass spectrometry (MS/MS)-based platform as an indispensable tool for the emerging field of serum biomarkers [15]. The use of MS/MS-based tag detection produces high-quality data with good sensitivity, excellent signal-to-noise ratios, and abroad dynamic range [16].

This study utilized the TMT labeling coupled with online HPLC-MS/MS proteomics approach

to profile the levels of proteins in plasma from patients with postmenopausal osteoporosis versus postmenopausal normal women. The aims of the study were to find the differential expression proteins which may contribute to the early diagnosis and correlation mechanism of osteoporosis and may be directly related to bony remodeling.

Materials and methods

Subjects and samples collection

Since December 2012 to August 2013, ten patients (mean age, 55.2±2.35 years) with newly diagnosed postmenopausal osteoporosis at the Department of Orthopaedics and Trauma, the Second Affiliated Hospital of Zhejiang Chinese Medical University, China, have been prospectively enrolled with informed consent. Ten healthy postmenopausal women with normal bone mass ranges (mean age, 54.4± 2.07 years) served as controls. The demographic characteristics of subjects showed that there were no significant differences between the two groups (age, t=0.0.809, P=0.429, P> 0.05). Both patients and controls were from the same geographic region (Southeast China) and of the same ethnic origin (Han). None of the patients and controls had prior health conditions such as diabetes, cardiovascular disease, mental illness, osteomalacia, rheumatoid arthritis, multiple myeloma, bone tumor, osteoarthrosis, Paget's disease, osteogenesis imperfect, Alzheimer's disease, and so on. They also had never received treatment or undergone drug therapy with in the last six months before their inclusion in the study. World Health Organization (WHO)'s recommended criteria on the diagnosis of osteoporosis were used. The lumbar vertebra normal position bone density was surveyed using dual energy X-ray absorptiometry [17, 18], compared with a normal adult of the same gender and race, T≤-2.5 could be diagnosed as osteoporosis, where T=(the standard deviation of measured value-peak bone mass)/normal adult bone density. This study enrolls postmenopausal women aged 50 to 60 years who fulfilled the criterion on the diagnosis of osteoporosis. The same standard techniques of blood collection protocols used in this study were guided by reports on the Plasma Proteome Project. Blood paired normal and control groups. Using the Vacuette Blood Collection Tubes (Greiner bio-one, Chonburi, Thailand) without anticoagulation, venous blood samples (5 mL) were collected with equal amount to reduce the individual variability in the morning before consuming any food and allowed to clot at room temperature for 1 h after blood collection in the clinic. The samples were then centrifuged at 4°C for 5 min in 943 g. The serum was frozen and stored at -80°C for future analysis. Samples that did not satisfy the specified time frame would not be used for analysis. This study was approved by the local Ethics Committee of the Second Affiliated Hospital of Zhejiang Chinese Medical University in accordance with the Helsinki Declaration. The patients and volunteers provided written informed consent for their participation.

Protein extraction and digestion

The serum was used to measure the concentrations of total proteins with 2-D Quant kit (GE Healthcare, London, UK) according to the manufacturer's instructions. Then, the different serums were mixed and the high abundance ratios proteins of the mixed serum were removed with ProteoMiner protein enrichment kit (Bio-Rad, California, USA). The residual protein content was determined with 2-D Quant kit (GE Healthcare, London, UK) according to the manufacturer's instructions.

About 150 ug protein was reduced with 10 mM DTT for 1 h at 56°C and alkylated with 55 mM iodoacetamide for 45 min at room temperature in darkness. The protein was precipitated with 20% TCA for 2 h at 4°C and washed with cold acetone for three times. After centrifugation, the pellet was dissolved and sonicated in 0.5 M Tetraethylammonium Bromide (TEAB). The protein suspension was digested with trypsin (Promega, Wisconsin, USA) at an enzyme-to-substrate ratio of 1:50 for 12 h at 37°C. To ensure complete digestion, additional trypsin at an enzyme-to-substrate ratio of 1:50 was added, and the mixture was incubated for an additional 4 h.

TMT labeling and frationation with high-pH reverse-phase chromatography

After trypsin digestion, peptide was desalted by Strata X C18 SPE column (Phenomenex, California, USA) and vacuum-dried. Peptide was reconstituted in 1 M TEAB and processed according to the manufacturer's protocol for 6-plex

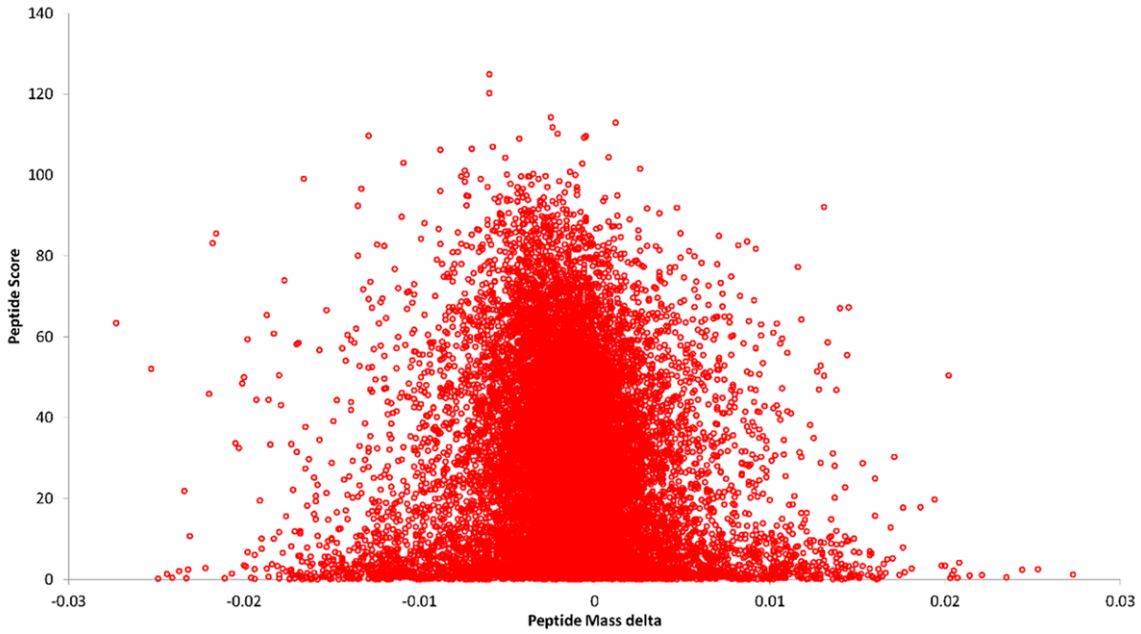

Figure 1. Mass Error Distribution. Note: X-axis: Peptide Mass Delta, mass tolerance between identified peptide mass and theoretical peptide mass, unit: Dalton (Da). Y-axis: Peptide Score to evaluate the reliability of the peptides. Each red dot in the figure represents a peptide.

TMT kit (Thermo Scientific Pierce, USA). Briefly, six units of TMT reagent (defined as the amount of reagent required to label 100 µg of protein) were thawed and reconstituted in 80 µL acetonitrile. Peptide from each sample was labeled with TMT-130 and TMT-131 respectively by incubation at room temperature for 2 h. The peptide mixtures were then pooled and dried by vacuum centrifugation.

The labeled peptides were further separated with reverse-phase HPLC. The reverse-phase column (Agilent, ZORBAX Extended-C18 4.6 mm × 250 mm, 5 µm particle, 80 Å pore size) was equilibrated with 2% buffer B (10 mM ammonium formate in 90% acetonitrile, pH 10.0). The peptide mixture in buffer A (10 mM ammonium formate in 2% acetonitrile, pH 10.0) was loaded onto the column and eluted with linear gradient of 5% to 8% buffer B in 5 min, 8-18% B in 35 min, 18-32% B in 22 min, 32-95% B in 2 min, at a constant flow rate of 1 mL/min using analytical HPLC (Rigol). 56 fractions were collected and combined equally into 14 final fractions.

LC-ESI-MS/MS analysis by Q exactive

The peptide from each fraction was vacuum-dried and re-suspended in buffer A (2% ACN, 0.1% FA) and centrifuged at 20000 g for 2 min. The supernatant was transferred into sample tube and loaded onto an Acclaim PepMap 100 C18 trap column (Dionex, 75 um×2 cm) by EASY nLC1000 nanoUPLC (Thermo Scientific) and the peptide was eluted onto an Acclaim PepMap RSLC C18 analytical column (Dionex, 50 um×15 cm). A 34 min gradient was run at 300 nl/min, starting from 5 to 30% B (80% ACN, 0.1% FA), followed by 2 min linear gradient to 40% B, then 2 min to 80% B, and maintenance at 80% B for 4 min.

The peptides were subjected to NSI source followed by tandem mass spectrometry (MS/MS) in Q Exactive (Thermo) coupled online to the UPLC. Intact peptides were detected in the Orbitrap at a resolution of 70000. Peptides were selected for MS/MS using 27% NCE with 12% stepped NCE; ion fragments were detected in the Orbitrap at a resolution of 17500. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans was applied for the top20 precursor ions above a threshold ion count of 3E4 in the MS survey scan with 5.0 s dynamic exclusion. The electrospray voltage applied was 1.8 kV. Automatic gain control (AGC) was used to prevent overfilling of the ion trap; 1E5 ions were

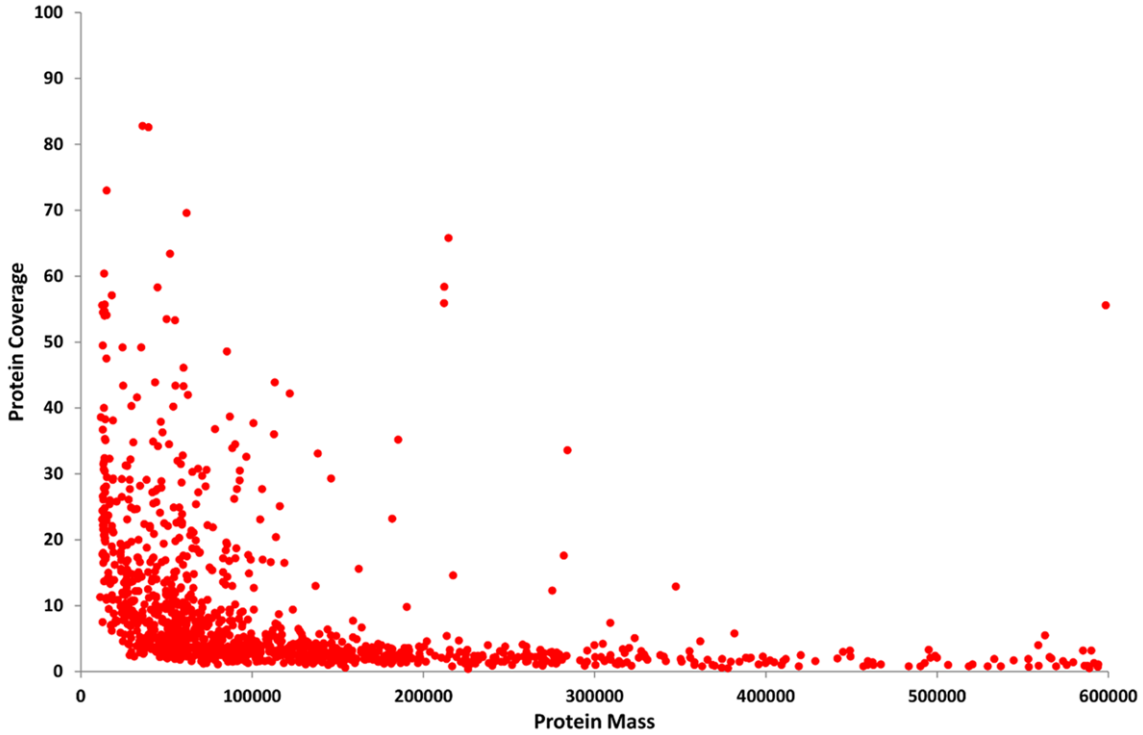

Figure 2. Identified Protein Mass Distribution. Note: X-axis: Molecular Weight (Da), Y-axis: Coverage of protein identified region.

accumulated for generation of MS/MS spectra. For MS scans, the m/z scan range was 350 to 1600 Da.

Database search

The instrument data files (.raw) were merged and converted into a .mgf file by Proteome Discoverer (ver. 1.3.0.339, Thermo). Peptide and protein identifications were performed using the Mascot search engine (ver. 2.3.0, Matrix Science) against protein database SwissProt_Human (20268 sequences). Database searching was restricted to tryptic peptides. Carbamidomethyl (C), TMT6plex (N-term), TMT6plex (K) were selected as fixed, oxidation (M) as variable modifications, 2 missed cleavage allowed and precursor error tolerance at 10 ppm, fragment deviation at 0.02 Da. T High confidence peptides were used for protein identifications by setting a target false discovery rate (FDR) threshold of 1% at the peptide level. Only unique peptides with high confidence were used for protein identifications. The quantitative protein ratios were weighted and normalized by the median ratio of peptide ratios.

Bioinformatic analysis

Gene Ontology (GO) annotation proteome was derived from the UniProt-GOA Database (www.http://www.ebi.ac.uk/GOA/). Proteins were classified by Gene Ontology annotation based on three categories: biological process, cellular compartment and molecular function. Protein-protein interactions playing a critical role in biological processes are the basis and characteristics of life structure and life activities. With the rapid development of high-throughput experimental technology, the protein-protein interaction network becomes an effective method used to study the interaction of proteins. The protein-protein interaction network was analyzed by Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) online tool software (ver. 10.0, http://string-db.org).

Statistical analysis

Statistical analyses were performed with SPSS software, version 20.0 (SPSS, Chicago, IL, USA). Clinical data values were presented as mean ± SD and $P<0.05$ was considered as statistically significant. T-test was used to com-

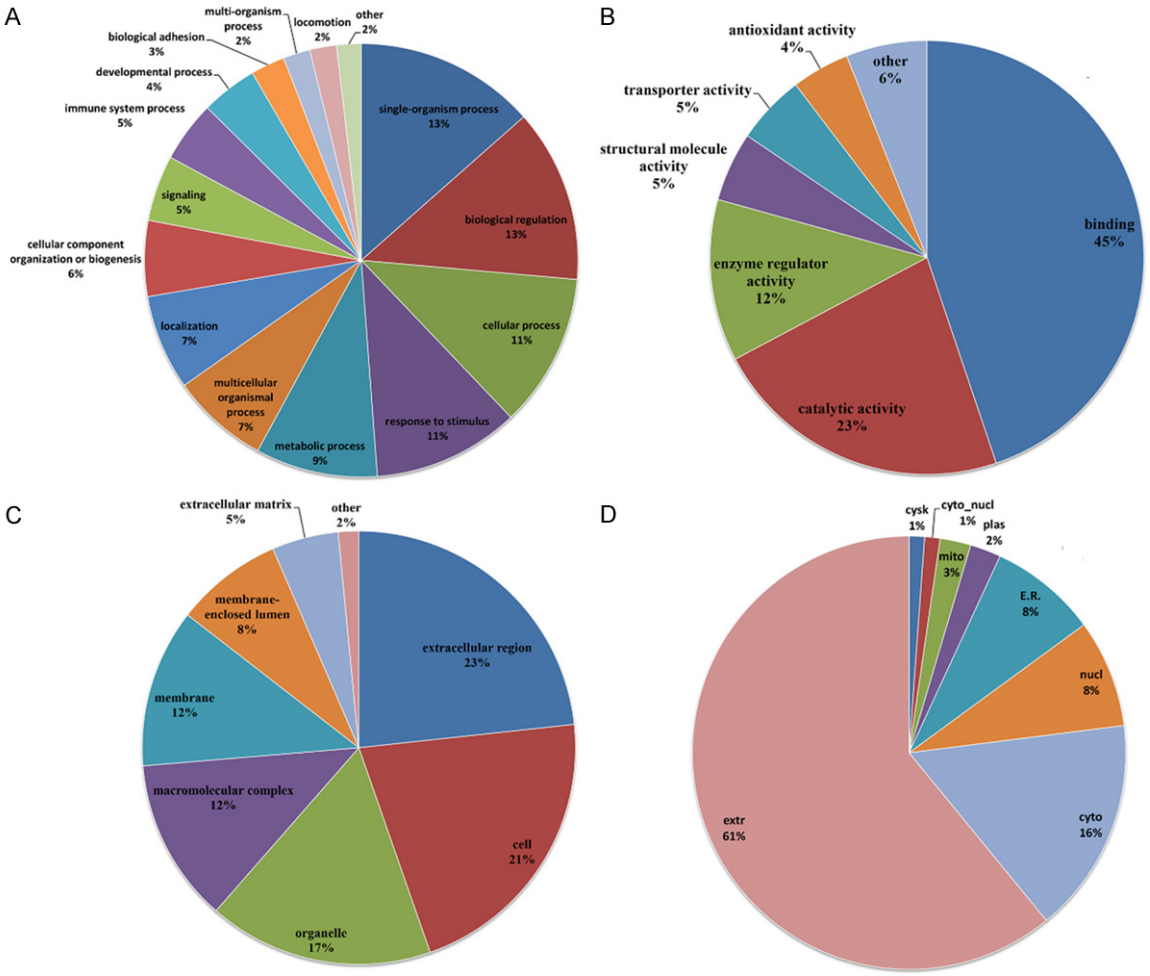

Figure 3. Gene Ontology analysis of differentially expressed proteins. A: Biological process. B: Molecular function. C: Cellular component. D: Subcellular location.

pare the difference between the two groups for continuous variables.

Results

Protein Identification and differentially expressed proteins

In this study, we employed a TMT-based quantitative proteomic approach to identify differentially expressed proteins in serum samples from ten patients with postmenopausal osteoporosis and ten age- and gender-matched healthy controls. Serum samples from patients with postmenopausal osteoporosis and control cases were depleted of abundant proteins, labeled with TMT reagents and fractionated by High-pH Reverse-Phase Chromatography. Analysis using high resolution hybrid quadrupole-orbitrap mass spectrometry led to the acquisition of 125,042 MS/MS spectra. MS/MS search against Swiss-prot Human database using Mascot search algorithms led to the identification of 6498 peptides corresponding to 1137 proteins. Peptides mass error distribution for quality control of this experiment is shown in **Figure 1**. Protein mass distribution for quality control of this experiment is shown in **Figure 2**. Based on the abundance of the protein, When the fold change is above 1.5 and T test *p*-value <0.01, we defined this protein as the differentially expressed protein. According to the criteria for protein quantification, a total of 87 were identified, within a 99% confidence interval, to be differentially regulated, including 50 upregulated (TMT ratios of ≥1.5) and 37 downregulated (TMT ratios of ≤0.67).

Table 1. The detailed information of GAS6, SPP24, RAB7A and TSP1 associated with the bone remodeling

Protein accession	Protein description	130/131 Ratio	Regulated Type	Subcellular Location
Q14393	Growth arrest-specific protein 6 (GAS6)	1.714	Up	extr
Q13103	Secreted phosphoprotein 24 (SPP24)	1.639	Up	extr
P51149	Ras-related protein Rab-7a (RAB7A)	2.534	Up	cyto
P07996	Thrombospondin-1 (TSP1)	1.69	Up	extr

Gene ontology (GO) analysis of differentially expressed proteins

To gain insights into the biological changes in the patients with OP versus controls, the differentially expressed proteins were categorized according to the Gene Ontology (GO) classes "Biological Process", "Molecular Function", "Cellular Component", and "Subcellular Location". In the cellular component by GO analysis, differentially expressed proteins were located in the extracellular region (23%), cell (21%), organelle (17%), macromolecular complex (12%), membrane (12%), membrane-enclosed lumen (8%), extracellular matrix (2%), other (2%), implying that most of the differential proteins were secretary proteins (**Figure 3A**).

According to the molecular functions, most of the differentially expressed proteins were involved in binding (45%), catalytic activity (23%), enzyme regulator activity (12%), structural molecule activity (5%), transporter activity (5%), antioxidant activity (4%) and others (6%) (**Figure 3B**). The most common functional annotation was the binding activity (**Figure 3B**).

In the biological process of GO analysis, most of the differentially expressed proteins were associated with single-organism process (13%), biological regulation (13%), cellular process (11%), response to stimulus (11%), metabolic process (9%), multicellular organismal process (7%), localization (7%), cellular component organization or biogenesis (6%), signaling (5%), immune system process (5%), developmental process (4%), biological adhesion (3%), multi-organism process (2%), locomotion (2%) and others (2%) (**Figure 3C**).

According to the subcellular location, most of the differentially expressed proteins were located in cysk (1%), cyto_nucl (1%), mito (3%), plas (2%), E.R. (8%), nucl (8%), cyto (16%), extr (61%) (**Figure 3D**). According to the Gene Ontology (GO) classification, most of these proteins were found to be located in extracellular space in our plasma proteome map.

GO-based candidate biomarkers analysis associated with bone remodeling

Bone remodeling imbalance is the pathological basis of osteoporosis. candidate biomarkers of postmenopausal osteoporosis, we took "bone remodeling" as subject and retrieved the GO annotation information of differentially expressed proteins using Excel Retrieval Tool [19]. Finally, the retrieval results showed that the annotation information of Growth arrest-specific 6 (GAS6), Secreted phosphoprotein 24 (SPP24), Ras-related protein Rab-7a (RAB7A), and Thrombospondin-1 (TSP1) were associated with the bone remodeling. The detailed information of GAS6, SPP24, RAB7A and TSP1 was shown in **Table 1**.

The HCD MS/MS spectra of peptides fragment sequence for GAS6, SPP24, RAB7A, and TSP1 were shown in **Figure 4A-D**, respectively. The analysis of GAS6 by GO annotation revealed that it was mainly involved in biological function such as positive regulation of fibroblast proliferation, negative regulation of interleukin-6 secretion, positive regulation of lymphocyte differentiation, negative regulation of interleukin-1 production, regulation of MAPK cascade, and positive regulation of ERK1 and ERK2 cascade of biological process; calcium ion transmembrane transporter activity, calcium ion binding, and calcium channel activity of molecular function. SPP24 main biological function seems to be bone remodeling, skeletal system development of biological process.

RAB7A is mainly involved in biological function such as bone remodeling of biological process. TSP1 is mainly involved in biological function such as angiogenesis, sprouting angiogenesis, blood vessel development, endochondral bone

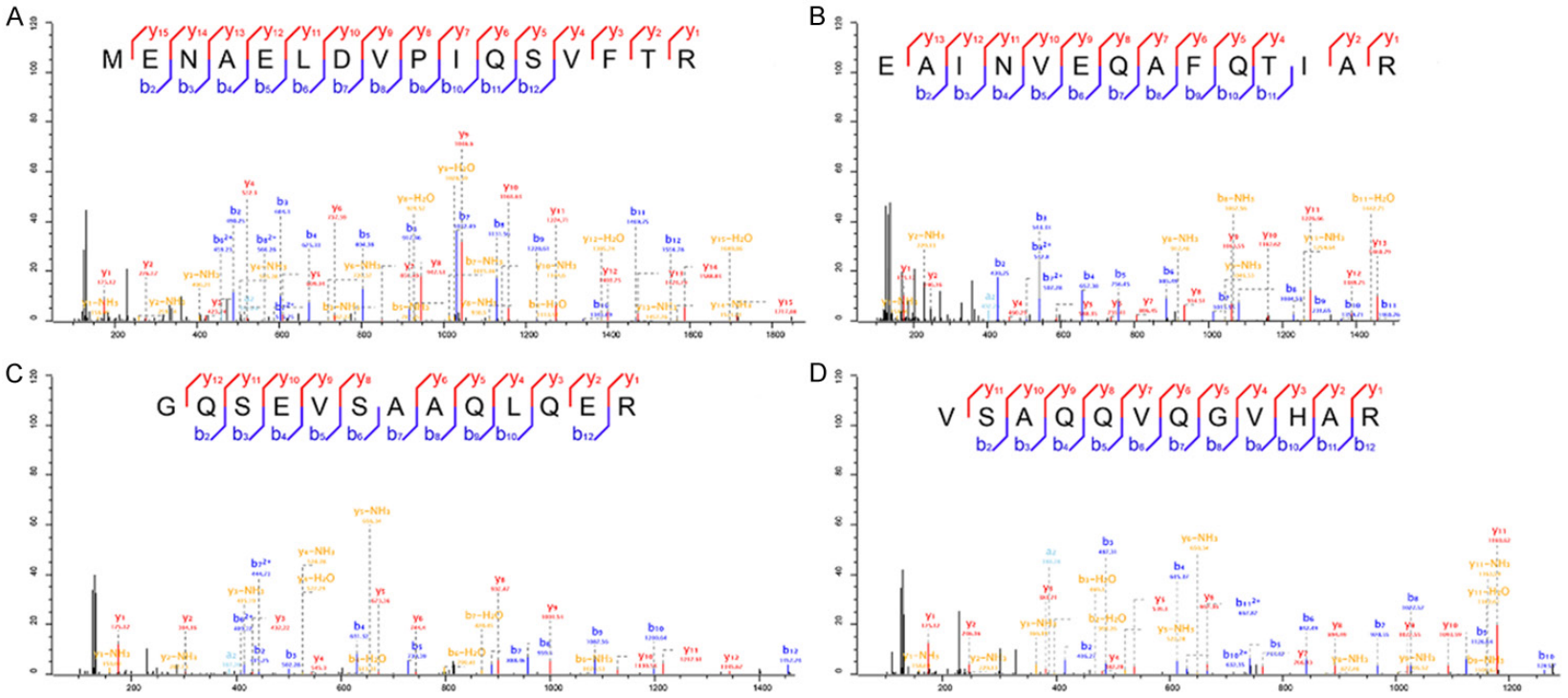

Figure 4. MS/MS spectrum of unique peptides fragment sequence for GAS6, SPP24, RAB7A, and TSP1. "GQSEVSAAQLQER" is the unique peptides fragment sequence belonged to GAS6; "VSAQQBQGVHAR" belongs to SPP24; "EAINVEQAFQTIAR" belongs to RAB7A; "MENAELDVPIQSVFTR" belongs to TSP1.

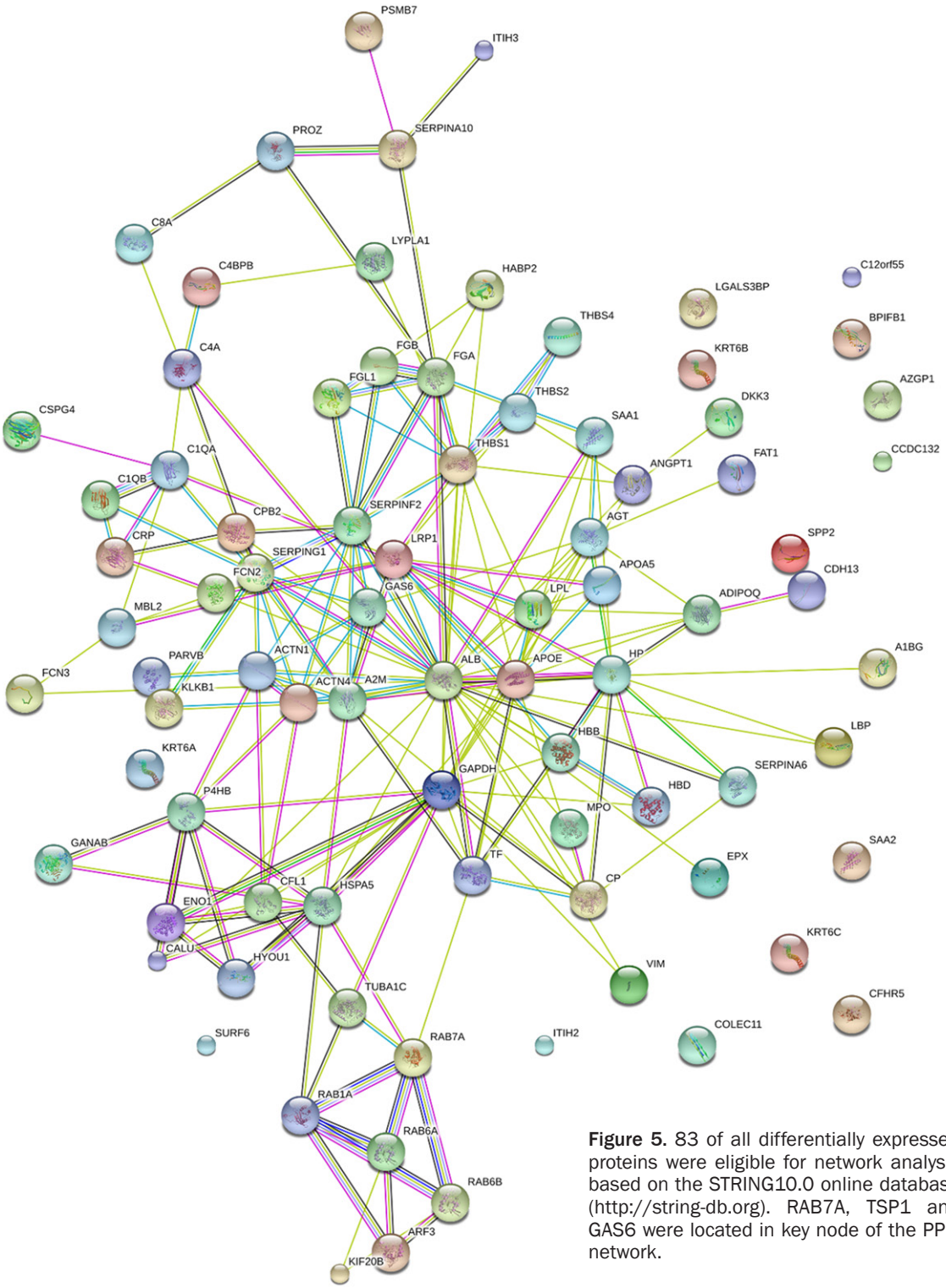

Figure 5. 83 of all differentially expressed proteins were eligible for network analysis based on the STRING10.0 online database (http://string-db.org). RAB7A, TSP1 and GAS6 were located in key node of the PPIs network.

morphogenesis; blood vessel morphogenesis, negative regulation of interleukin-12 production, regulation of leukocyte chemotaxis, cartilage development, cartilage development involved in endochondral bone morphogenesis, endochondral bone growth, bone development, skeletal system morphogenesis, bone morphogenesis, regulation of locomotion, positive reg-

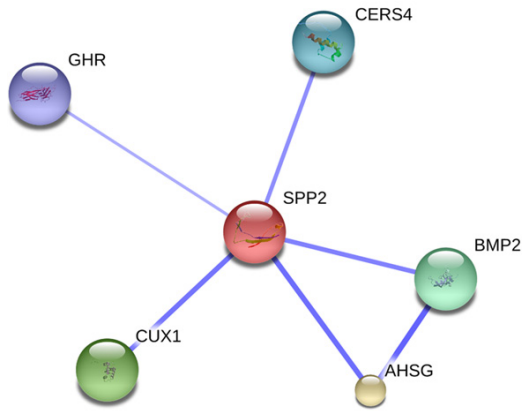

Figure 6. The PPIs network of SPP24. SPP24 was shown at edge of the protein-protein interaction maps, but it was directly related to BMP2, an important protein of bone reconstruction of osteoporosis.

ulation of transforming growth factor beta receptor signaling pathway, positive regulation of tumor necrosis factor biosynthetic process and positive regulation of MAPK cascade of biological process.

Interaction networks analysis of differentially expressed proteins

The new version 10.0 of STRING database (http://string-db.org) provides a critical assessment and integration of protein-protein interactions, including direct (physical) as well as indirect (functional) associations [20]. Protein-protein interactions (PPIs) aiding in the interpretation of MS-based proteomics data are crucial for all biological processes to understand of the functional organization of the proteome. In the protein-protein interaction network analysis, 83 of all differentially expressed proteins were eligible for network analysis based on the STRING10.0 online database (http://string-db.org) (**Figure 5**).

According to the network, RAB7A, TSP1 and GAS6 were located in key node of the PPIs network. SPP24 was shown at edge of the protein-protein interaction maps, but it was directly related to BMP2, an important protein of bone reconstruction of osteoporosis (**Figure 6**).

Discussion

In this study, our aim was to identify human serum low abundance ratios protein profiling for postmenopausal osteoporosis biomarker discovery using the TMT-based proteomic approach coupled with 2D-LC-MS/MS to compare the serum proteome between postmenopausal patients with osteoporosis and postmenopausal women without osteoporosis. Results of mass spectrometry analysis showed that a total of 87 were differentially expressed proteins, including 50 proteins up-regulated and 37 proteins down-regulated.

In this study, Thermo Scientific Q Exactive quadrupole-Orbitrap mass spectrometers were used for intact mass measurement which had the ability to generate higher-energy collisional dissociation (HCD) with high-resolution, accurate-mass (HR/AM) fragment ions. The Q Exactive mass spectrometer is the ideal qualitative/quantitative screening platform with high-confidence confirmation with MS/MS sensitivity and selectivity superior to triple quadrupole, Q-TOFs, or Q-Trap levels [21, 22]. The superior quantitation capabilities of Q Exactive MS make it possible to identify, quantify, and confirm metabolites, peptides and proteins of complex serum samples [23].

Compared with the traditional experimental techniques, TMT-based proteomic approach coupled with 2D-LC-MS/MS technology has the important advantages of sensitivity, accuracy and throughput in the identification and quantification of human serum low abundance proteins, so it is an effective method for seeking the potential therapeutic targets and biomarkers.

Using GO annotation and protein interaction networks of bioinformatics to analysis 87 differentially expressed proteins, the results showed that these differentially expressed proteins derived from a variety of cell or tissue and played a variety of molecular biological functions involved in relevant biological process. By the analysis of “Cellular Component” and “Subcellular Location”, serum proteomic protein not only came from cells, and also from the extracellular matrix. Therefore, these results indicated that serum proteins could reflect the body’s metabolism.

Normal bone remodeling is the dynamic balance between bone resorption and bone formation regulated by many hormones and bone metabolism regulators from osteoclast, osteoblast and osteocyte [24]. Combined with the

pathophysiology of osteoporosis, 4 differentially expressed proteins (TSP1, RAB7A, SPP24 and GAS6) were selected as candidate markers associated with postmenopausal osteoporosis.

GAS6, a ligand for tyrosine-protein kinase receptors AXL, TYRO3 and MER (TAM) family is a member of the vitamin K-dependent protein family. GAS6 binds with TAM receptors to regulate the cell cycle, such as survival, proliferation, migration, differentiation, adhesion, and apoptosis. GAS6 has been reported to be involved in diverse human diseases. Obesity and hormones disorder have been shown to be a potential risk factor in the progression of osteoporosis. A study reports shown that plasma GAS6 levels were significantly lowered in postmenopausal than in premenopausal overweight females [25]. Another study suggests that plasma Gas6 was associated with sex hormones in premenopausal and postmenopausal women.

Further, in recent years, a growing number of studies have shown Vitamin K is involved in the regulation of biological functions such as normal coagulation, bone mineralization, calcium homeostasis, apoptosis, cell growth and signal transduction and be used in the prevention and treatment of postmenopausal osteoporosis [26].

In this experiment, GAS6 protein was upregulated in patients with postmenopausal osteoporosis, indicating a potential role of Gas6 in early diagnosis.

TSP1, a widely expressed secreted ligand, is an adhesive glycoprotein consisting of multiple structural domains. TSP1 is released from a variety of cells in response to thrombin stimulation, developing and repairing tissues. While TSP1 plays important roles in bone metabolic process, the role of TSP1 in bone remodeling is becoming one of the hot-spots. TSP1-/- mice were reported to have increased bone mass and increased cortical bone size and thickness compared to wild type (WT) [27]. TSP1 was proved as a critical regulator of bone development and remodeling by blocking osteoblast differentiation of MSCs through its ability to stimulate transforming growth factor-β (TGF-β) activation [28]. In this experiment, TSP1 protein upregulated in patients with postmenopausal osteoporosis, indicating a potential role of TSP1 in early diagnosis, but the exact mechanism is unclear.

RAB7A, an isoform of Rab-7 locatesat the cytoplasmic face of distinct membrane compartments, was a small RAS-related GTP-binding proteins of the RAB family members. Rab7a has been shown to have critical roles in regulating autophagy, lipid metabolism, growth factor signaling, bone resorption, and phagolysosome biogenesis by controlling intracellular vesicular traffic [29]. In bone-resorbing osteoclasts, it is reported that Rab7 is involved in formation of the ruffled border, which is a late endosomal-like compartment in the plasma membrane by Rac1-Rab7 interaction. The decreased expression of Rab7 caused a significant inhibition of bone resorption in the osteoclast *in vitro*. In this experiment, RAB7A protein upregulated in patients with postmenopausal osteoporosis, indicating a potential role of RAB7A in early diagnosis [30].

SPP24 is an extracellular bone matrix phosphoprotein regulating bone remodeling and repair by binding to and affecting the activity of members of the BMP/TGF-b family of cytokines [31, 32].

In conclusion, this study showed that TSP1, RAB7A, SPP24 and GAS6 may be potentially effective molecular markers in postmenopausal osteoporosis. In the future, we will detect and validate expression level of these proteins in a large clinical sample study by Western blotting and ELISA. Apart from the biological processes of bone remodeling, they were involved in the processes of inflammation and vascular remodeling. Therefore, the results of this study provided another new perspective for the research on the diagnosis and treatment of osteoporosis. In addition, this present study only selected the proteins associated with the bone remodeling of postmenopausal osteoporosis, this did not reflect show the full view of serum proteome of the postmenopausal osteoporosis. But, we firmly believe that with the rapid development and application of proteomics technologies, the relationship between the other differentially expressed proteins and postmenopausal osteoporosis will gradually be revealed.

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Disclosure of conflict of interest

None.

Authors' contribution

Xiaolin Shi made substantial contributions to the conception and design, the acquisition of data, the analysis and interpretation of data, and the drafting of the manuscript. Chunwen Li carried out the TMT labeling and HPLC-MS/MS technology. Bocheng Liang participated in the design of the study and performed the statistical analysis. Bo Wang, Peng Wu, Jianliang Yao, Zhenyu Shi, and Lingcheng Kong participated in the design and coordination of the study, and assisted with drafting the manuscript. All authors carried out the analyses, and read and approved the final manuscript.

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