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Identification of osteoporosis markers through bioinformatic functional analysis of serum proteome

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Abstract

Osteoporosis is a severe chronic skeletal disorder that increases the risks of disability and mortality; however, the mechanism of this disease and the protein markers for prognosis of osteoporosis have not been well characterized. This study aims to characterize the imbalanced serum proteostasis, the disturbed pathways, and potential serum markers in osteoporosis by using a set of bioinformatic analyses. In the present study, the large-scale proteomics datasets (PXD006464) were adopted from the Proteome Xchange database and processed with MaxQuant. The differentially expressed serum proteins were identified. The biological process and molecular function were analyzed. The protein–protein interactions and subnetwork modules were constructed. The signaling pathways were enriched. We identified 209 upregulated and 230 downregulated serum proteins. The bioinformatic analyses revealed a highly overlapped functional protein classification and the gene ontology terms between the upregulated and downregulated protein groups. Protein–protein interactions and pathway analyses showed a high enrichment in protein synthesis, inflammation, and immune response in the upregulated proteins, and cell adhesion and cytoskeleton regulation in the downregulated proteins. Our findings greatly expand the current view of the roles of serum proteins in osteoporosis and shed light on the understanding of its underlying mechanisms and the discovery of serum proteins as potential markers for the prognosis of osteoporosis.

Abbreviations: BMD = bone mineral density, BMPs = bone morphogenetic proteins, GO = gene ontology, PPIs = protein–protein interactions, TGF-β = transforming growth factor beta, TMT = tandem mass tag.

Keywords: bioinformatics, differentially expressed proteins, osteoporosis, protein markers, serum proteome

1. Introduction

Osteoporosis is one of the most severe chronic skeletal disorders in the world and featured by the loss of bone mineral density (BMD).[1] It increases the risk of osteoporotic fracture, leading to a high frequency of disability and mortality of osteoporotic patients.[2]

The dynamic regulation of bone metabolisms ensures the bone homeostasis, including bone resorption, osteoblast differentiation, skeletal development, and bone formation.[3] Dysregulation of this delicate process increases bone resorption and decreases bone formation, therefore raising the occurrence of osteoporosis. Many studies have investigated the processes of bone metabolisms and the molecular features of osteoporosis. Zhu et al[4] performed a profiling of cytosolic proteome with multiomics techniques in Caucasian male osteoporotic patients and identified BMD-associated genes for the monocyte-mediated osteoporosis. Despite that osteoporosis has been increasingly studied, the mechanisms underlying low BMD and osteoporosis have not been fully understood. Recently, circulating proteins have been widely investigated and their biological significance is indicated in modulation of bone metabolisms. Transforming growth factor beta (TGF-β) is a multifunctional extracellular protein that regulates bone remodeling, osteoblast proliferation and differentiation, and bone formation.[5] Recent studies show a dose-dependent manner of TGF-β in the regulation of bone metabolisms. Low-dose TGF-β promotes differentiation of osteoclast for bone formation, whereas high-dose TGF-β inhibits the differentiation.[6,7] Another example is the bone morphogenetic proteins (BMPs), an important group of growth factors

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involved in bone metabolisms. BMP1, BMP2, and BMP4 induce bone formation; however, BMP3 prevents osteoblast differentiation and lowers bone density.[5] Some studies report that BMP2 is required in postnatal bone development and formation and the knockout of BMP2 in a mouse model led to frequent fractures and difficulty in healing.[8] Other circulating proteins have also been reported to significantly affect the bone metabolism. ANXA2 facilitates the migration of monocyte from endothelium to bone microenvironment.[9] TAGLN2 is relevant to osteoclast differentiation for the regulation of bone formation and resorption.[10,11] Several studies using omics technologies identified differential expression of extracellular proteins in various levels, from mRNA, protein expression, to posttranslational modifications, indicating a vital role of circulating proteins in the regulation of BMD and osteoporosis.[4,12,13]

In this study, we compared our text-mining findings with a public proteomics data in Proteome Xchange database (PXD006464) and identified the differentially expressed serum proteins in osteoporosis. Bioinformatic analyses were performed to categorize the protein functional classifications, investigate the gene ontology (GO) terms and the signaling pathways, and analyze the protein–protein interactions (PPIs) networks and the subnetwork modules. The regulation of cytoskeleton, extracellular matrix, biomineral tissue development, and skeletal development was found closely related to osteoporosis. Interestingly, some other pathways in immune system and cancers were also found in the pathway enrichment. The bioinformatic analyses in the current study unravel the disturbed serum proteostasis and provide a systematic view of pathway alterations in osteoporosis, providing a basis for the validation of functional regulators in osteoporosis and for the construction of osteoporotic models. In addition, this study conveys a molecular view for the understanding of the mechanism and a comprehensive framework for discovering novel drug targets and biomarkers to improve the diagnosis, prognosis, and treatment of osteoporosis.

2. Materials and methods

2.1. Text mining of key genes in osteoporosis

The key word for text mining was osteoporosis. All the related protein names were extracted using an in-house R package against the MEDLINE database (www.ncbi.nlm.nih.gov) of literatures on life sciences and biomedical sciences. The osteoporosis-related protein names were verified with the GeneCards (www.genecards.org) and the subcellular localization of these proteins was searched against the Universal Protein Resource (Uniprot) database to ensure the hits are extracellular or secreted proteins.[14]

2.2. Identification of differentially expressed serum proteins

A large-scale 6-plex tandem mass tag (TMT)-labeled proteomics raw data (Proteome Xchange database: PXD006463) from osteoporotic patients was downloaded. The ethics committee or institutional review board was not formed for this study because we only acquired and analyzed the data from the public online database, and we did not perform the experiments. The basic characteristics of patients were summarized in the Supplemental Table 1, http://links.lww.com/MD/E883. Data processing was performed using MaxQuant 1.5.4.1 with an integrated Andromeda search engine.[15] The MS/MS spectra were searched against a SwissProt human database (downloaded from Uniprot on January 2015) with the following parameters: 10 ppm for both precursor and fragment mass tolerance, full tryptic peptides with maximal 2 missed cleavage, carbamidomethylation (C) as static modification, and oxidation (M) as dynamic modification, 6-plex TMT was chosen as isobaric labels with 0.01 Da reporter ion tolerance, and false discovery rate $<1\%$. Corrected reporter intensity provided by MaxQuant was used for quantification and the ratio of protein expressions was calculated as the mean of the osteoporotic samples over the mean of the controls. Two-tailed Student t test was performed and P value less than .05 was considered as statistically significant. Only the proteins with $P<.05$ and fold changes greater than 50% (the ratio >1.5 or <0.67) were considered as the differentially expressed serum proteins and used for following analyses.

2.3. Gene ontology analysis, pathway enrichment, and protein–protein interactions

Heatmap and hierarchical clustering of the differentially expressed proteins were performed with R programing language. PANTHER classification system was used to perform protein functional classifications and GO term enrichment .[16] PPIs network was analyzed using the online tool STRING with the highest confidence (interaction score >0.9).[17] The modules of PPI subnetworks were also analyzed and displayed in the Cytoscape.[18] The signaling pathways were enriched against KEGG pathway database using DAVID and only the pathways with more than 2 hits and $P<.05$ (Benjamini-Hochberg Procedure) were displayed.[19,20]

3. Results

3.1. Identification of differentially expressed serum proteins

To better characterize and evaluate the serum molecular features of osteoporosis, a total of 3236 proteins associated with osteoporosis were initially identified using a text-mining approach using our in-house R package. Subsequently, 2459 proteins were filtered out by searching against GeneCards for osteoporotic correlation and Unitprot for subcellular localization. The MaxQuant analysis of the publicly available proteomics data identified 1360 proteins. When comparing the search results, 651 proteins were shared by both protein lists (Fig. 1A), in which 209 proteins were significantly upregulated and 230 downregulated in osteoporosis (Fig. 1B and Supplemental Table 2, http://links.lww.com/MD/E884). This profiling result indicates the homeostasis of serum proteome has been significantly disturbed in osteoporotic patients when compared with the healthy individuals. To validate these differentially expressed proteins, we performed a quantitative analysis using 2 publicly available mRNA microarray datasets (GSE56815 and GSE80614). In total, 80 of the differentially expressed proteins were validated ($P<.05$ and ratio >1.5 or <0.67) (Supplemental Table 3, http://links.lww.com/MD/E885).

3.2. Functional classification and gene ontology analysis

To investigate the functional categories of these differentially expressed proteins in osteoporosis, a protein classification and

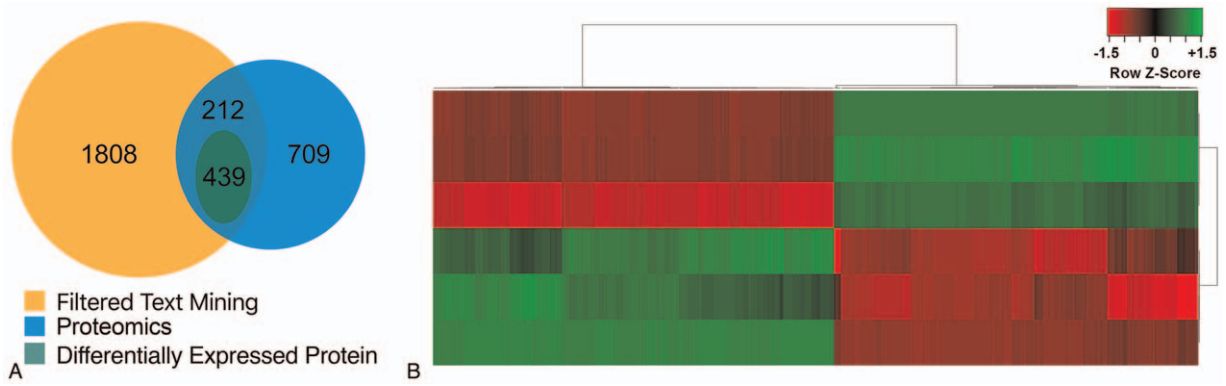

Figure 1. Identification of the differentially expressed serum proteins in osteoporosis (A). A Venn diagram shows that 651 osteoporosis-related serum proteins were overlapped between the filtered text-mining result and proteomics result, in which 439 proteins were differentially expressed (B).

GO term analysis was performed using the PANTHER classification system.[16] The serum proteins were first categorized based on their functions. The results showed that both the upregulated and downregulated serum proteins have more than a dozen of functional categories. The top 3 enriched categories in the upregulated proteins are nucleic acid binding, enzyme modulator, and hydrolase, while the top 3 enriched categories in the downregulated proteins are hydrolase, enzyme modulator, and cytoskeletal protein (Fig. 2 A and B). This is functionally controversial as the enzyme modulator and hydrolase groups were upregulated and downregulated at the same time. One possible explanation for this observation is the functional specificities of these proteases cannot be unambiguously differentiated and classified by using the general terms of protein functions. To further explore the differences between the shared categories in both upregulated and downregulated proteins, subgroup analyses of the top 3 categories were applied. Nucleic acid binding protein group was significantly upregulated in the osteoporotic patient samples and was not enriched in the downregulated proteins. Among all the nucleic acid binding proteins, RNA-binding proteins are the most enriched subgroup (see Supplemental Fig. 1A, http://links.lww.com/MD/E886). The cytoskeletal protein group in the downregulated serum proteins is composed of the actin family cytoskeletal protein and microtubule family cytoskeletal protein (see Supplemental Fig. 1F, http://links.lww.com/MD/E886). The top 2 shared protein groups between the upregulated and downregulated serum proteins are enzyme modulator and hydrolase (Fig. 2). In the enzyme modulator, the protease inhibitor is the most enriched subgroup in the upregulated protein, while the G-protein is most enriched in the downregulated proteins (see Supplemental Fig.1 B and E, http://links.lww.com/MD/E886). The protease is the most enriched subgroup of protein functional classifications in both the upregulated and downregulated serum proteins (see Supplemental Fig.1 C and D, http://links.lww.com/MD/E886).

Biological process and molecular function of the differentially expressed serum proteins were also analyzed to characterize protein functionalities. Both the biological process and molecular function are shared by the upregulated and downregulated serum proteins in osteoporosis (Fig. 3). Subsequently, subgroups of the top 3 biological process and molecular function in either the upregulated or downregulated serum proteins were analyzed. The subgroups of cellular process and metabolic process under biological process are shared by both the upregulated and downregulated proteins (see Supplemental Fig. 2 A, B, D, and E, http://links.lww.com/MD/E886). Cellular component organization or biogenesis is one of the top 3 biological processes in the upregulated proteins and it contains the cellular component organization and the cellular component biogenesis (see Supplemental Fig. 2C, http://links.lww.com/MD/E886). In the downregulated proteins, the biological regulation is one of the top 3 biological processes and its subgroups are composed of the

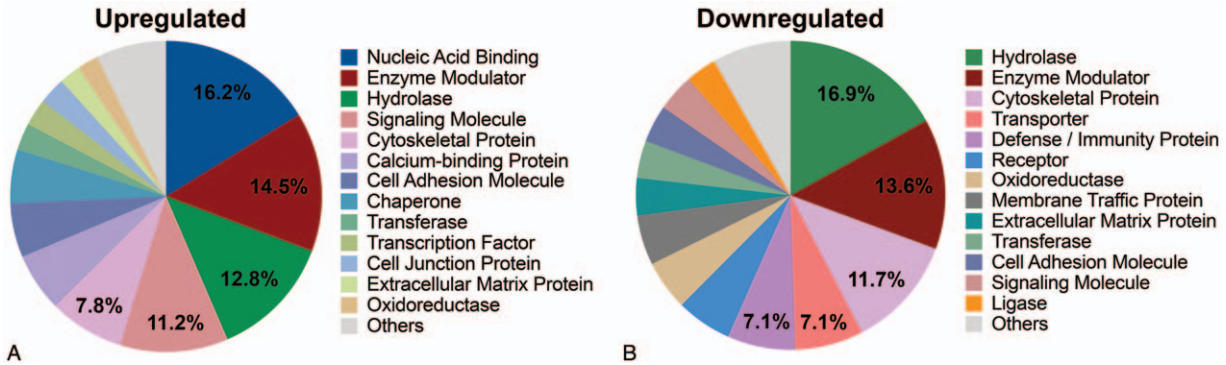

Figure 2. Functional classification of the differentially expressed serum proteins. Functional classifications and gene ontology analysis were performed with the PANTHER. Both the upregulated (A) and downregulated (B) serum proteins were classified based on protein functions.

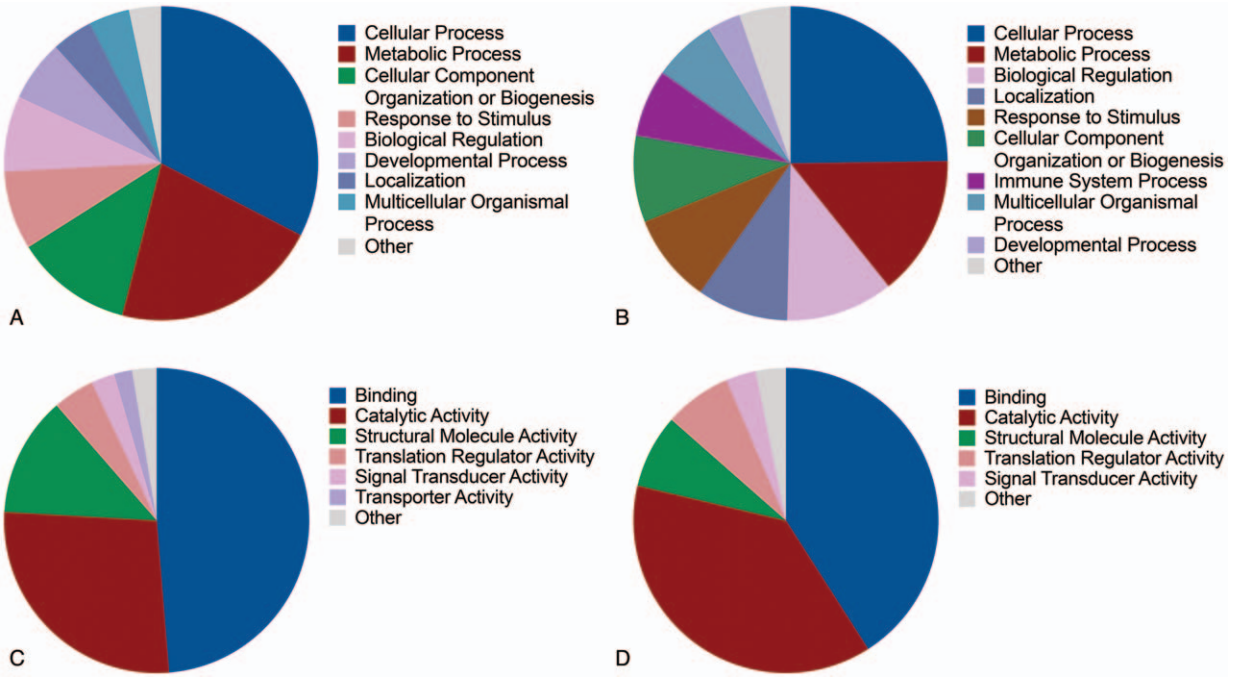

Figure 3. Gene ontology analysis of the differentially expressed serum proteins. Biological process was analyzed for the upregulated (A) and downregulated (B) serum proteins. Molecular function was analyzed for the upregulated (C) and downregulated (D) serum proteins.

regulation of biological process, homeostatic process, and regulation of molecular function (see Supplemental Fig. 2F, http://links.lww.com/MD/E886). Considering the subgroups of the molecular function, the binding and the catalytic activity are shared by both the upregulated and downregulated proteins (see Supplemental Fig. 3A, B, D and E, http://links.lww.com/MD/E886). However, the structural molecular activity subgroup shows the difference, in which the upregulated proteins possess the functional terms structural constituent of ribosome and structural constituent of cytoskeleton, while the downregulated proteins have structural constituent of cytoskeleton and extracellular matrix structural constituent (see Supplemental Fig. 3 C and E, http://links.lww.com/MD/E886). The GO analysis indicates that even though the protein expressions are significantly differentiated between the healthy controls and osteoporotic patients, the general functions of these proteins may be similarly categorized and cannot be unambiguously distinguished by the general GO terms, which is in agreement with our protein classification analysis.

3.3. Protein–protein interaction network analysis

To investigate the relationships between the differentially expressed serum proteins in osteoporosis and to discover the difference behind the highly similar functional classifications, a PPI network analysis was performed by STRING with a more detailed functional analysis of the highly interacted subnetwork modules.[17] The top 3 modules of PPI subnetworks were analyzed in both the upregulated and downregulated proteins. As shown in the analysis, each PPI module is unique and different from others with the specific biological process terms (Fig. 4). In the PPI modules derived from the upregulated serum proteins, the biological process is enriched in mRNA processing and protein synthesis (Fig. 4, A–C). Of note, the negative regulation of biomineral tissue development was highly enriched, suggesting an inhibition or dysregulation of bone metabolism in osteoporosis. On the other hand, the skeletal system development was also highly enriched, indicating a potential recovery event for bone formation in osteoporosis. Among all the PPI modules from the downregulated serum proteins, the biological process is highly enriched in immune system and cytoskeletal development, indicating that most of the immune activities, including innate immune response and phagocytosis, are downregulated and the ability of actin cytoskeleton organization is inhibited in osteoporosis (Fig. 4, D–F).

3.4. Signaling pathway enrichment analysis

A signaling pathway analysis was performed using DAVID against KEGG pathway database.[19,20] The enriched signaling pathways from the upregulated serum proteins are involved in protein synthesis, infections, immunological reactions, and some cancer-related pathways (Fig. 5A). However, among the downregulated serum proteins, the signaling pathways are highly enriched in the regulation of cell adhesion and immune response (Fig. 5B).

4. Discussion

Using the text-mining strategy and the publicly available large-scale proteomics data, we identified the differentially expressed serum proteins in osteoporosis, in which 209 were upregulated and 230 downregulated. We further validate 80 of the differentially expressed proteins by analyzing the microarray datasets. Then we classified these serum proteins by their functions and analyzed their biological process and molecular function. To explore in detail the specific roles of these proteins in osteoporosis, we constructed the PPI networks and analyzed the biological process of each PPI subnetwork module. Finally, we

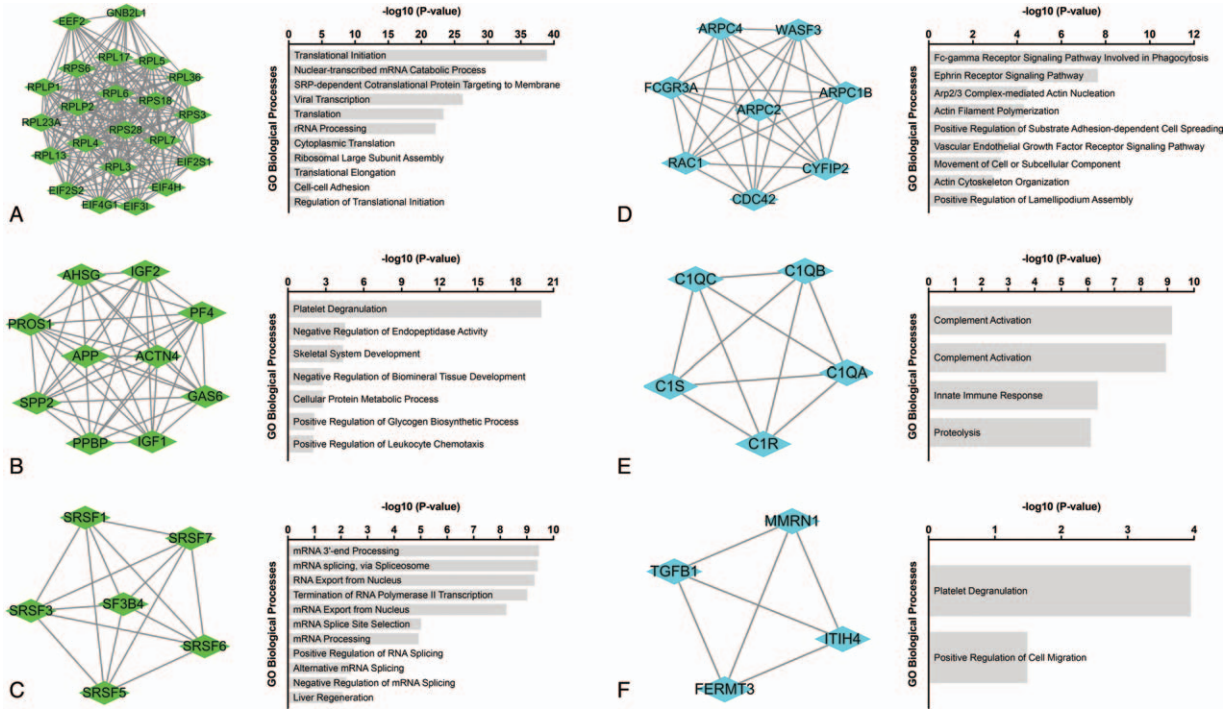

Figure 4. Subnetwork modules and the biological process of the differentially expressed serum proteins. PPI subnetwork modules and their biological process were analyzed and displayed. The top 3 modules of the upregulated (A–C) and downregulated (D–F) serum proteins and the biological processes were analyzed, respectively.

analyzed the signaling pathways disturbed in osteoporosis. This study has demonstrated that the serum proteome has been largely altered and the signaling pathways have been disturbed in osteoporosis, and an imbalanced serum protein homeostasis and dysregulation of cellular functions were also observed. However, the causality of osteoporosis cannot be demonstrated by the current study due to the lack of intervention and biological validation. A detailed and comprehensive in vitro or in vivo study is required to thoroughly elucidate the molecular mechanisms and causal relationships between the proteins, pathways, and osteoporosis, thus providing a basis for diagnosis, prognosis, and development of potential treatments for this disease.

In the present study, we have identified the upregulated and downregulated serum proteins by combing text mining and bioinformatics analyses. We have further comprehensively analyzed the functions of the upregulated and downregulated proteins in osteoporosis, respectively. In the protein classifications, the subgroup analyses indicate that RNA-binding proteins and protease inhibitor are upregulated in osteoporosis; however, cytoskeletal proteins and G-proteins are downregulated. Interestingly, the protease subgroup of hydrolase possesses a large proportion in both the upregulated and downregulated serum proteins, which is functionally controversial. One explanation for this observation might be the functional specificities of these

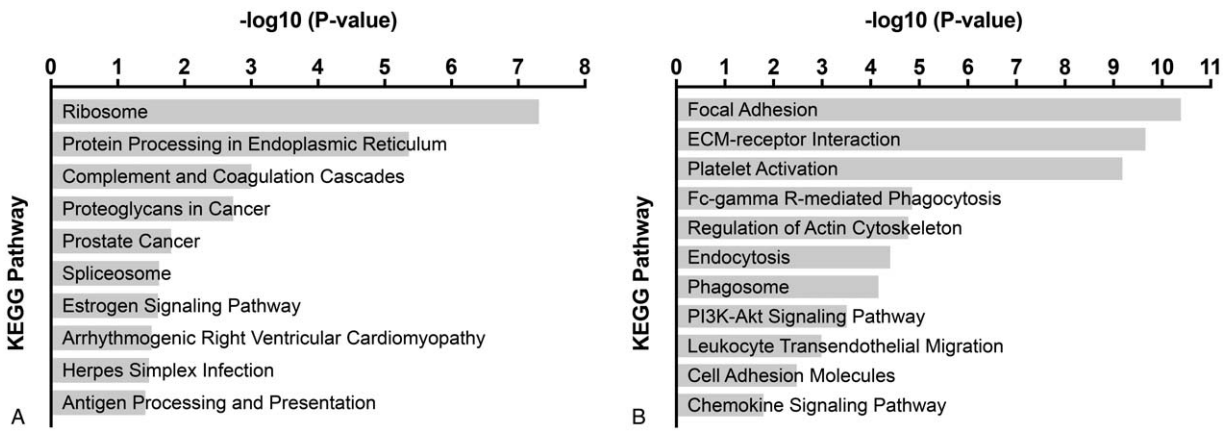

Figure 5. Signaling pathway analysis of the differentially expressed serum proteins. The signaling pathways disturbed in osteoporosis were analyzed for the upregulated (A) and downregulated (B) serum proteins, respectively.

proteases cannot be unambiguously differentiated and classified by using the general terms of protein functions. Among the upregulated serum proteins, the functional terms, in general, are protein synthesis, infection, and immune response. Complement and coagulation cascades is one of the highly enriched pathways in the upregulated proteins. Complement system, coupled with blood coagulation, is a mediator of innate immunity that regulates inflammatory processes, recruits inflammatory and immunocompetent cells, and eliminates pathogens.[21] However, recent studies show that complement activation plays a role in cell turnover, tissue growth, and regeneration.[22,23] Another enriched pathway is the antigen processing and presentation, which immunologically prepares antigens for presentation to the immune system for pathogen clearance.[24] The activation of this pathway induces $CD8^+$ T cells and natural killer cells to eliminate pathogens, as well as the induction of $CD4^+$ T cells for an assistant role.[24–26] Insulin-like growth factor 1 (IGF-1) is a convergent point of a few pathways and was previously identified as upregulated in the early-onset breast cancer.[27] In this study, the pathway analysis shows that IGF-1 is involved in prostate cancer and proteoglycans in cancer. These 2 pathways, combined with Herpes simplex infection, might suggest an easier infection process and high-level inflammation, probably due to an impaired immune system in osteoporosis.[28] In addition, the analysis of the subnetwork modules, as well as the signaling pathways, derived from the upregulated serum proteins shows a high enrichment in protein synthesis. Combined with the viral infection and transcription in the GO terms, this observation may suggest an enhancement of inflammation and dysregulation of immune system, which can be anticipated as the role of inflammation in bone degradation.[29,30]

Among the downregulated serum proteins in osteoporosis, the signaling pathways are highly enriched in cell adhesion and immune response. Of note, several pathways are closed related to cytoskeletal physiology, including focal adhesion, extracellular matrix-receptor interaction, regulation of actin cytoskeleton, and cell adhesion molecules. These pathways are involved in the modulation of arrangement and disassembly of cytoskeletal structures consisting of actin filaments and the interacting partners.[31–33] Previous studies show that the status of cytoskeleton affects the morphology and functions of osteoclasts in bone metabolism.[34] Interestingly, these cytoskeletal pathways have crosstalk with other highly enriched pathways, such as PI3K-Akt pathway, chemokine pathway, and leukocyte transendothelial migration, which are related to cell survival, immune surveillance, and inflammation.[35–37] Rac1 is the intersection of these pathways and the downregulation of this protein inactivates PI3K-Akt pathway, blocks chemokine signaling, and disturbs direction sensing of leukocyte, therefore resulting in activation of apoptosis, dysregulation of cell motility and migration, and leukocyte dysfunction.[38–41] The downregulation of cytoskeletal pathways also affects the capability of immune system to eliminate pathogens, trigger and exacerbate inflammation.[42] Vesicular trafficking is playing a crucial role in osteoclastic bone resorption[43] and the vesicular trafficking-mediated endocytosis is regulated by the cyclic adenosine monophosphate.[44,45] Consistent with these findings, our study shows that endocytosis pathway is highly enriched among the downregulated serum proteins, suggesting the pathophysiological significance of endocytosis in osteoporosis. TGF-β has been found as a key regulator in bone formation and the loss of TGF-β was previously shown to induce chondrocyte hypertrophy and cartilage degeneration.[46] In this study, we found that TGF-β was downregulated in the osteoporotic patients and inactivates the endocytosis, which suggests an additional role of TGF-β in the regulation of inflammation, as well as bone formation, in osteoporosis. Although complement activation and innate immune response are enriched among the downregulated proteins, which seems controversial to the previous studies and our current data, the deficiency of the member in C1 protein complex (C1QA, C1QB, C1R, C1S, and C1QC) has been shown to associate with dysfunction of the immune system and elicit lupus erythematosus and glomerulonephritis.[47]

In the present study, we combined the text-mining strategy with the adopted proteomics data to characterize the differentially expressed serum proteins in osteoporosis. We also performed the integrative analysis of protein functions, PPI networks and module analysis, and signaling pathway enrichment. Our study combining various bioinformatics techniques shows the important roles of extracellular proteins in osteoporosis and reveals the relationship between immune system and bone metabolisms including bone resorption and formation. In addition, this bioinformatics study elucidates an interaction between cytoskeletal pathways, cell proliferation/migration pathways, and osteoporosis from a systematic point of view rather than targeted proteins in traditional molecular biology, indicating the crosstalk among differential protein complexes and pathways.

Collectively, this study identifies the molecular features for understanding the underlying mechanisms of osteoporosis and provides a basis for the screening of biomarkers, prognostic factors, or drug targets for the treatment of osteoporosis.

Author contributions

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Methodology: Mengying Lv, Ziqi Li.
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Writing – review & editing: Mengying Lv, Peng Chen, Ziqi Li.

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