Quantitative proteomic analysis of peripheral blood mononuclear cells in rheumatoid arthritis

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Abstract

In order to identify proteins differentially expressed in rheumatoid arthritis patients and healthy controls, we used isobaric tagging for relative and absolute protein quantification (iTRAQ) to generate quantitative protein profiles from peripheral blood mononuclear cells (PBMCs) from 10 patients with rheumatoid arthritis and 10 healthy donors. Proteins were extracted, concentrated, pooled, labeled with iTRAQ reagents and subjected to multi-dimensional chromatographic fractionation followed by tandem mass spectrometry. A total of 3,208 proteins were identified based on ProteinPilot[TM] 3.0 software and the International Protein Index (human 3.62) database. Of these, 83 proteins differed at least 2-fold in expression levels between patients and controls. These results indicate that iTRAQ-based technology can serve as a useful aid for identification and quantification of disease-linked proteins from PBMCs. Several proteins, such as serotransferrin, clusterin, annexin A2, and heterogeneous nuclear ribonucleoprotein A2/B1, may be served as biomarker candidates.

Introduction

Rheumatoid arthritis is an autoimmune inflammatory rheumatic disease that affects many tissues and organs and causes chronic synovial inflammation, eventually leading to joint destruction and disability [1-4]. The pathogenesis of rheumatoid arthritis is quite complex and involves genetic and environmental factors. One of the main goals of research on this disease is to identify biomarkers that can shed light on disease onset and progression as well as improve diagnosis, treatment and prognosis.

Several studies implicate lymphocytes in the pathology of rheumatoid arthritis [5-8]. Therefore, we wanted to focus on peripheral blood mononuclear cells (PBMCs) and compare their proteomic profiles from patients and healthy controls. To avoid the problems of high background and low sensitivity associated with two-dimensional electrophoresis and other proteomic approaches used previously [9-11] to examine PBMCs in patients with rheumatoid arthritis, we chose the method of isobaric tagging for relative and absolute quantitation (iTRAQ). This has proven to be a powerful new tool for proteomic research in many autoimmune diseases [10,12-16]. Up to eight tags can be used in multiplex iTRAQ [17,18], and the large amounts of data emerging from tandem mass spectrometry analysis can be analyzed using bioinformatics. Despite its efficiency as a protein discovery tool, iTRAQ has yet to be applied to rheumatoid arthritis based on our review of the literature.

Here we used iTRAQ-based quantitative proteomic analysis and bioinformatics-based protein identification in order to identify proteins linked to rheumatoid arthritis. We identified these proteins by comparing proteomic profiles of PBMCs from patients and healthy controls.

Methods

Patients

PBMCs were isolated from 10 patients with rheumatoid arthritis (five men; mean age, 45 yr; age range, 36 to 56 yr. five women; mean age, 47 yr; age range, 39 to 55 yr) who fulfilled the American College of Rheumatology Revised Criteria for Rheumatoid Arthritis, as well as from 10 healthy controls who were age- and sex-matched with the patients. Patients had no history of other immune diseases, such as ankylosing spondylitis, systemic lupus erythematosus, or multiple sclerosis. Patients had not received any drug treatment prior to blood sample collection.

All participants provided written informed consent for this study, which was performed at Xinyang Normal University and Xinyang First People's Hospital (Railway Hospital) in China. The study protocol was approved by the institutional research ethics committees at both study sites.

PBMC isolation and protein extraction

Venous blood (4 mL) was collected from all patients and controls into EDTA anticoagulant tubes. PBMC were isolated using lymphocyte separation medium following the manufacturer's instructions

(Cedarlane Laboratories, Hornby, Ontario, Canada). Total protein was extracted from PBMCs, and concentration was measured using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Pools of 100 μg of total protein from patients and controls [19] were prepared and analyzed using iTRAQ, respectively.

iTRAQ proteomics

Protein (100 μg) from two patient pools and two control pools was blocked, digested, and labeled according to the iTRAQ protocol (Applied Biosystems, Foster City, CA, USA). Four iTRAQ tagging reactions were prepared (Patient Pool 1-iTRAQ 117, Patient Pool 2-iTRAQ 118, Control Pool 1-iTRAQ 119 and Control Pool 2-iTRAQ 121), then all reactions were pooled into a single mixture, which was fractionated by multi-dimensional liquid chromatography involving a Zorbax Bio-SCX column (35×0.3 mm, 300 Å, 3.5 μm particles; Agilent Technologies, Santa Clara, CA, USA) and a gradient of potassium formate in 25% acetonitrile, which generated 10 strong cation exchange fractions; these fractions were further separated on a Tempo™ LC nanoflow and MALDI spotting system (Applied Biosystems) equipped with a reverse-phase Magic C18AQ column (150×0.2 mm, 200 Å, 3 μm particles; Michrom Bioresources, CA, USA). Each multidimensional chromatography run yielded approximately 380 MALDI spots on a stainless steel MALDI target plate.

Mass spectrometry

Mass spectrometry was performed on an Applied Biosystems 4800 Plus MALDI TOF/TOF system. Only results associated with a signal-to-noise ratio of at least 40 were selected for tandem mass spectrometry. Mass spectra from 500 laser shots were acquired for each spot. The tandem mass spectrometry data from all 10 fractions were input into a Paragon algorithm search conducted using Protein Pilot™ 3.0 software (Applied Biosystems/MDS-Sciex) against a database of the International Protein Index (human, version 3.62) [20], which was downloaded from the EBI website (www.ebi.ac.Uk/IPI/IPIhelp.html). Only proteins identified with at least 95% confidence, or a ProtScore of 1.3, were analyzed in detail. Relative quantification of proteins was based on the ratio of peak areas of m/z 117, 118, 119, and 121 from tandem mass spectrometry spectra.

We used the following procedure to define the minimal difference in relative levels of a protein between patients and controls that would allow us to define a protein as linked to rheumatoid arthritis. Two equal amounts of 10-fraction mixtures (Applied Biosystems) were trypsin-digested and labeled with iTRAQ reagents. The standard deviation (SD) of all the ratios of the labeled peptides was computed to be 0.15. This led to a cut-off value of 2 SD + 1 = 1.3 as the minimal fold-change in patients relative to controls necessary for defining a protein as up-regulated in disease. The reciprocal cut-off of 0.77 was used as the maximal fold-change in patients relative to controls necessary for defining a protein as down-regulated in disease. These same cut-offs were also used to filter out proteins that differed by more than 1.3-fold or less than 0.77-fold between the two patient pools and the two control pools (technical replicates).

Bioinformatics analysis

Candidate biomarkers of rheumatoid arthritis were functionally annotated based on the Gene Ontology (GO) classification system (http://wego.genomics.org.cn), and a network of interacting molecules was built using on-line Ingenuity Pathway Analysis (IPA, www.ingenuity.com).

Results

Identification of protein expression changes by iTRAQ proteomic analysis

A total of 3,208 proteins were identified from patient and control PBMC extracts, of which 42 were significantly up-regulated in rheumatoid arthritis and 41 were down-regulated (Table 1). Figure 1 shows a representative tandem mass spectrum of serotransferrin, one of the proteins up-regulated in disease. Figure 2 shows the spectrum of annexin A2, one of the proteins down-regulated in disease.

Potential functions of proteins linked to rheumatoid arthritis

GO analyses using the WEGO online tool indicated that the proteins significantly up- and down-regulated in patients relative to controls are involved in cellular and metabolic processes, biological regulation, and molecular functions of binding, catalysis and transport (Figure 3).

Pathway and networks involving proteins linked to rheumatoid arthritis

IPA was used to construct pathways and networks involving proteins significantly up- or down-regulated in rheumatoid arthritis in order to analyze their possible relationships (Figure 4). The pathways are focused on receptor signal activation and acute phase response signaling, while the six networks involve primarily organismal injury and abnormalities, immunological disease, cellular movement, connective tissue disorders, cell death and survival, post-translational modification, and cell-to-cell signaling and interaction (Figure 5).

Discussion

Little is known about proteins associated with the pathogenesis of rheumatoid arthritis, and several are likely to play key roles, given that

Table 1. Proteins showing the greatest fold-changes in patients with rheumatoid arthritis relative to healthy controls.

| No.	Accession code	Name	Average folds (arthritis/control)
Top 10 proteins up-regulated in rheumatoid arthritis			
1	sp|P01859	Ig gamma-2 chain C region	4.823439138
2	sp|P02768	Serum albumin	4.773611854
3	sp|P01834	Ig kappa chain C region	4.750356564
4	sp|P02787	Serotransferrin	4.667309404
5	sp|P00738	Haptoglobin	4.089293455
6	sp|P01009	Alpha-1-antitrypsin	3.903263238
7	sp|P01857	Ig gamma-1 chain C region	3.803608909
8	sp|P01615	Immunoglobulin kappa variable 2D-28	3.693985617
9	sp|P01876	Ig alpha-1 chain C region	3.424910075
10	sp|P02671	Fibrinogen alpha chain	3.371755769
Top 10 proteins down-regulated in rheumatoid arthritis			
1	sp|Q16777	Histone H2A type 2-C	4.24889502
2	sp|P16402	Histone H1.3	4.101023024
3	sp|P41218	Myeloid cell nuclear differentiation antigen	3.913870339
4	sp|Q96L21	60S ribosomal protein L10-like	3.653741806
5	sp|P18859	ATP synthase-coupling factor 6, mitochondrial	3.34532721
6	sp|P62266	40S ribosomal protein S23	3.209125754
7	sp|P06454	Prothymosin alpha	3.192761477
8	sp|P61769	Beta-2-microglobulin	3.176176391
9	sp|Q07020	60S ribosomal protein L18	3.155362286
10	sp|Q9H299	SH3 domain-binding glutamic acid-rich-like protein 3	3.122316334
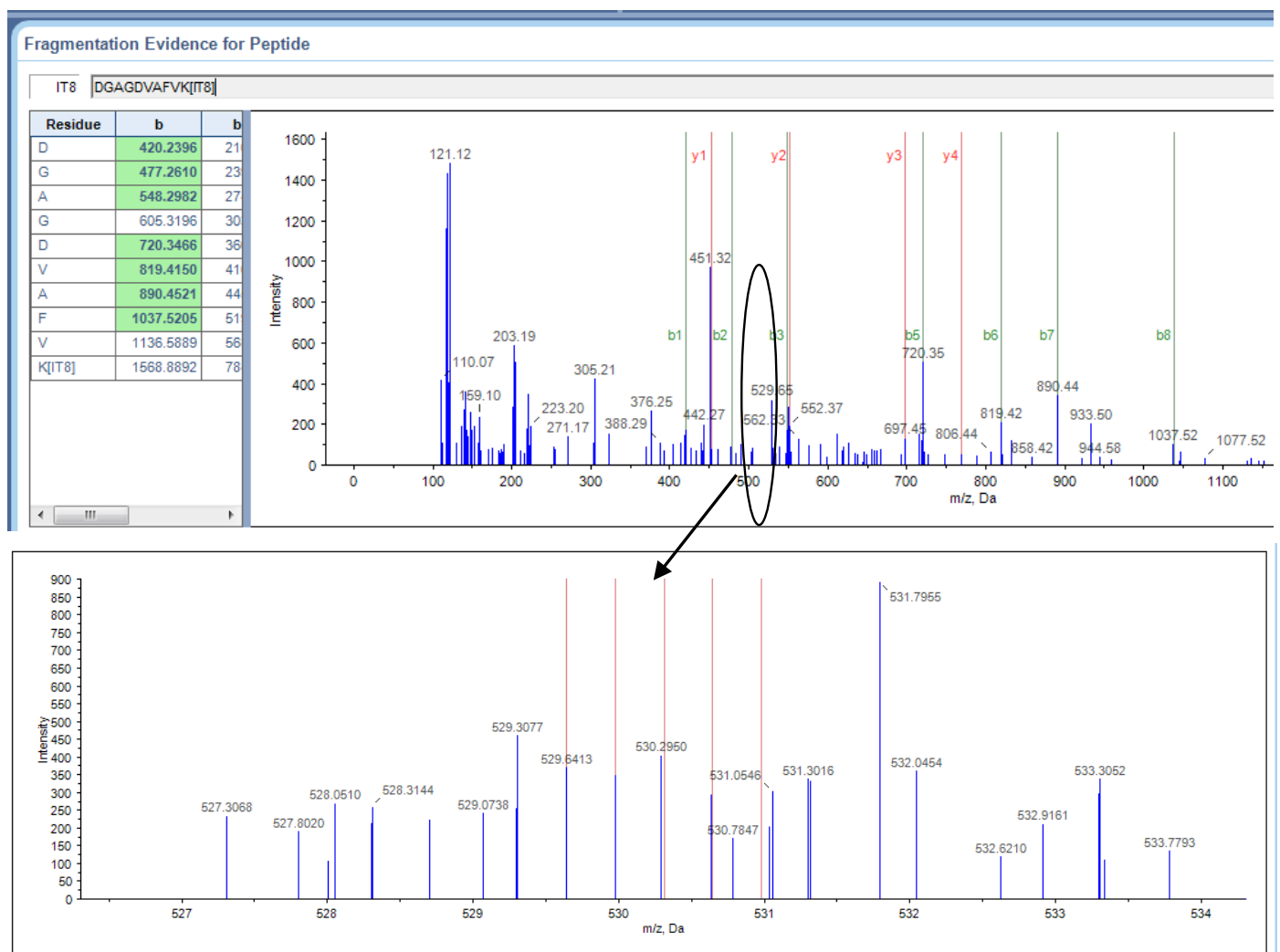
Figure 1. Serotransferrin sequencing and quantification using iTRAQ. A representative MALDI TOF/TOF MS/MS spectrum of the serotransferrin-derived peptide DGAGDVAFVK is shown with fragmented b-ions and y-ions.

the disease is likely to involve many genes. Here we identified several proteins significantly up- and down-regulated in the disease, providing the basis for further molecular studies aimed at identifying biomarkers useful for diagnosis, treatment and prognosis. While iTRAQ has proven effective at identifying disease biomarkers from tissue or serum [9-11,15,19,21-23], its usefulness for analyzing proteins from PBMCs is only beginning to be explored in the case of rheumatoid arthritis.

In this study, 83 proteins were identified as differentially expressed in rheumatoid arthritis. Most are inflammation-associated proteins and many were found to be up-regulated in the disease, including complement, immunoglobulin, and ribosomal protein. These proteins seem less useful as potential rheumatoid arthritis biomarkers, since they are also up-regulated in other auto-immune diseases. In contrast, other proteins that we identified as linked to rheumatoid arthritis merit further attention as biomarker candidates, including serotransferrin, clusterin, annexin A2, and heterogeneous nuclear ribonucleoprotein A2/B1.

GO analysis suggested that many of the proteins linked to rheumatoid arthritis in our study are involved in cellular and metabolic processes, biological regulation, and molecular functions related to binding, catalysis and transport. Interaction networks constructed from all 83 disease-linked proteins are involved primarily in organismal injury and abnormalities, immunological disease, cellular movement, connective tissue disorders, cell death and survival, post-translational modification, and cell-to-cell signaling and interaction.

Several proteins identified in our study as linked to rheumatoid arthritis have already been shown in previous work to contribute to pathogenesis of the disease. Clusterin, which was up-regulated in disease in our study, is expressed predominantly by synoviocytes and is present in synovial fluid. It promotes apoptosis and can regulate the NF-kappaB pathway, strongly affecting inflammation and immunity [24,25]. Annexin A3, which was up-regulated in disease in our study, induces migration and tube formation of human umbilical vein endothelial cells; transfecting cells with an annexin A3 expression plasmid leads to high levels of vascular endothelial growth factor (VEGF), a major angiogenic factor. Annexin A3 also enhances the transactivation activity of hypoxia-inducible factor-1 (HIF-1) [26]. The consistency between these previous studies and our own findings suggests that our iTRAQ results are reliable.

Some of the proteins down-regulated in rheumatoid arthritis in our study have previously been shown to be down-regulated in other
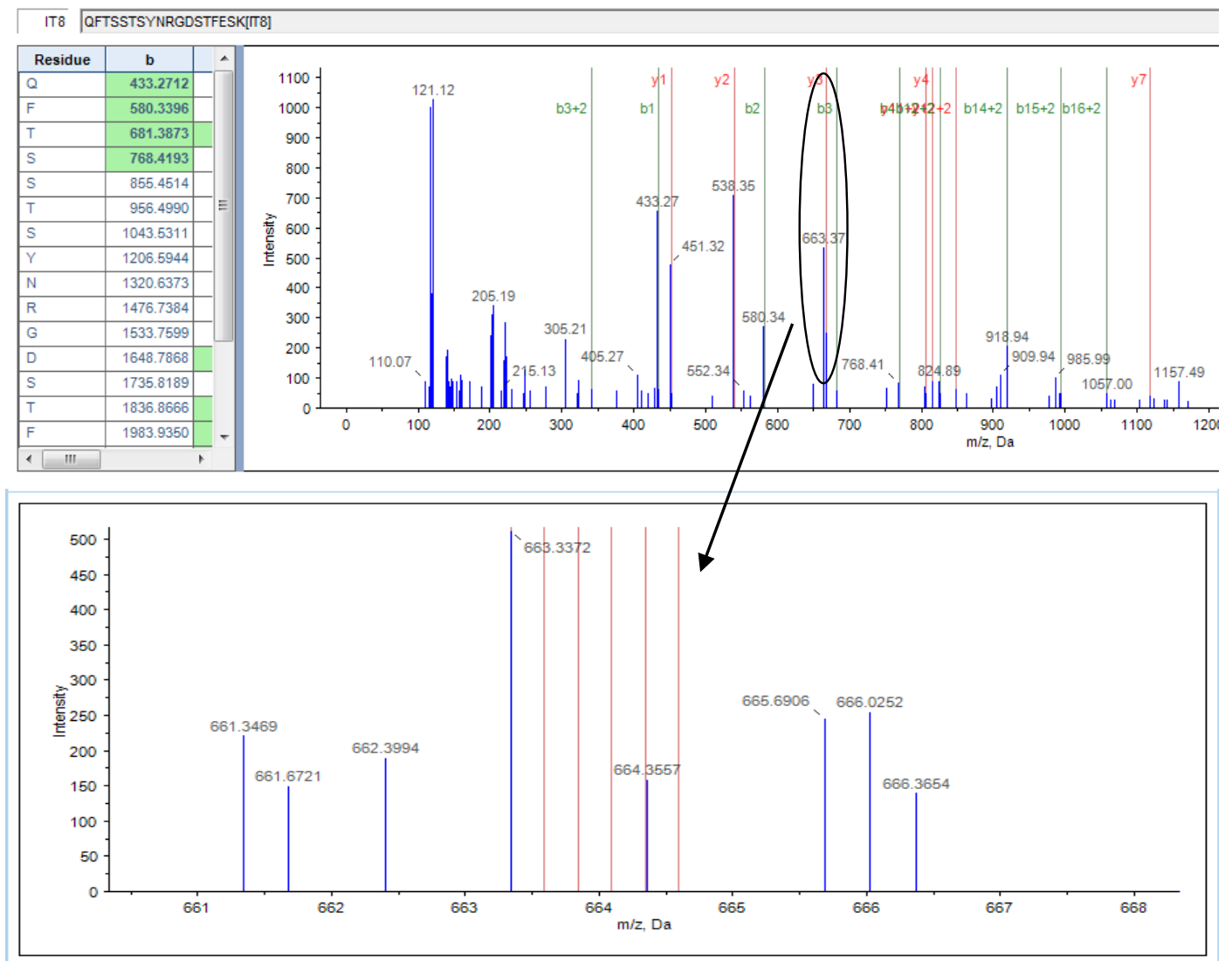
Figure 2. Annexin A2 sequencing and quantification using iTRAQ. A representative MALDI TOF/TOF MS/MS spectrum of the Annexin A2-derived peptide QFTSSTSYNRGDSTFESK is shown with fragmented b-ions and y-ions.

disease contexts. Prothymosin α exerts in vitro immunomodulatory effects on autoimmune diseases [27-29]. Cytochrome c oxidase (CytcO) is a multi-subunit enzyme that acts as both an O_2 sensor and a regulatory enzyme that determines mitochondrial respiration capacity. Hypoxia inhibits CytcO expression in vitro [30], which is linked with joint inflammation and decreased vascularity [31,32]. Positively charged cytochrome c and negatively charged truncated prothymosin α, when meeting in cytosol, can interact with each other. Truncated prothymosin α inhibits cytochrome c oxidation by H_2O_2, catalyzed by peroxidase [33]. Down-regulation of CytcO has been reported in juvenile idiopathic arthritis [34] and inflammatory arthritis.

Our iTRAQ study identified some proteins, such as beta-2-microglobulin, thymosin beta4 and beta10, have not previously been linked to rheumatoid arthritis, but have been linked to other diseases. Examples are beta-2-microglobulin and thymosin β10 [35-38]. These candidate rheumatoid arthritis biomarkers deserve further attention.

The goal of the present study was to identify a set of potential candidate biomarkers of rheumatoid arthritis that could be validated and further analyzed in future work involving other patient populations. Such work should verify and extend the present results by comparing protein levels in PBMC and serum between patients and controls.

Conflicts of interest

The authors declare no conflicts of interest.
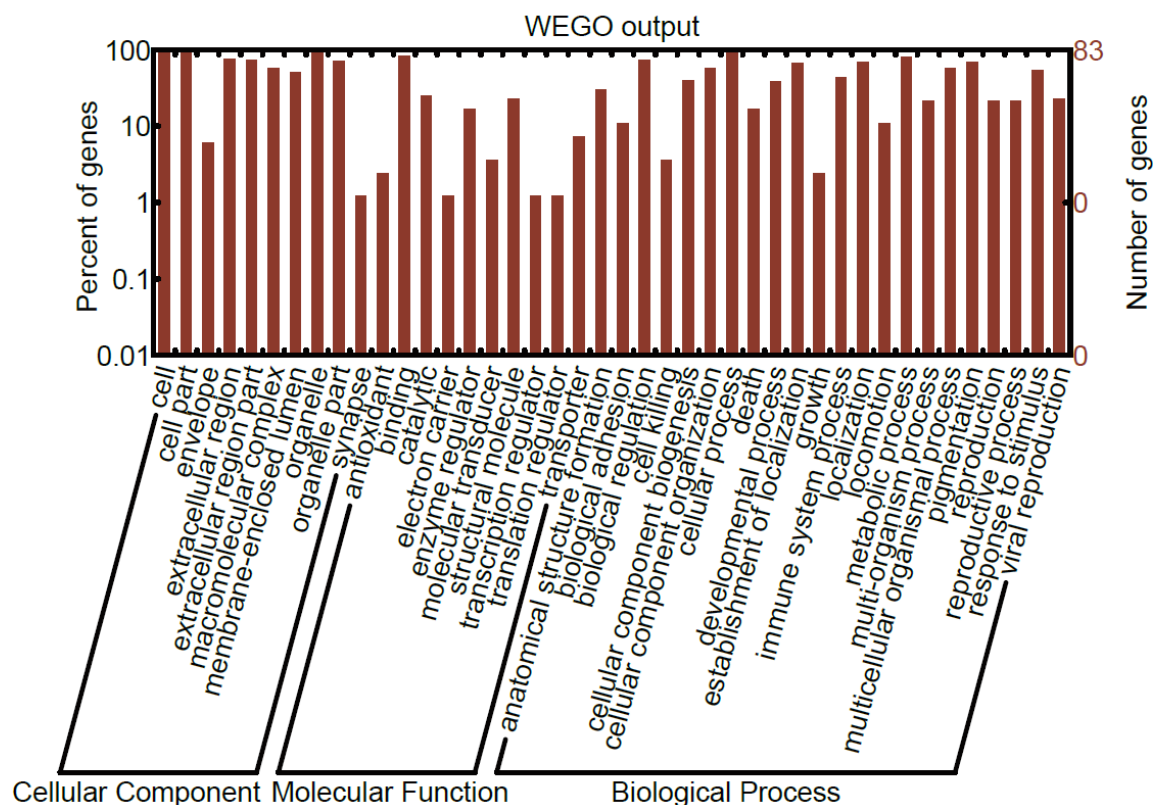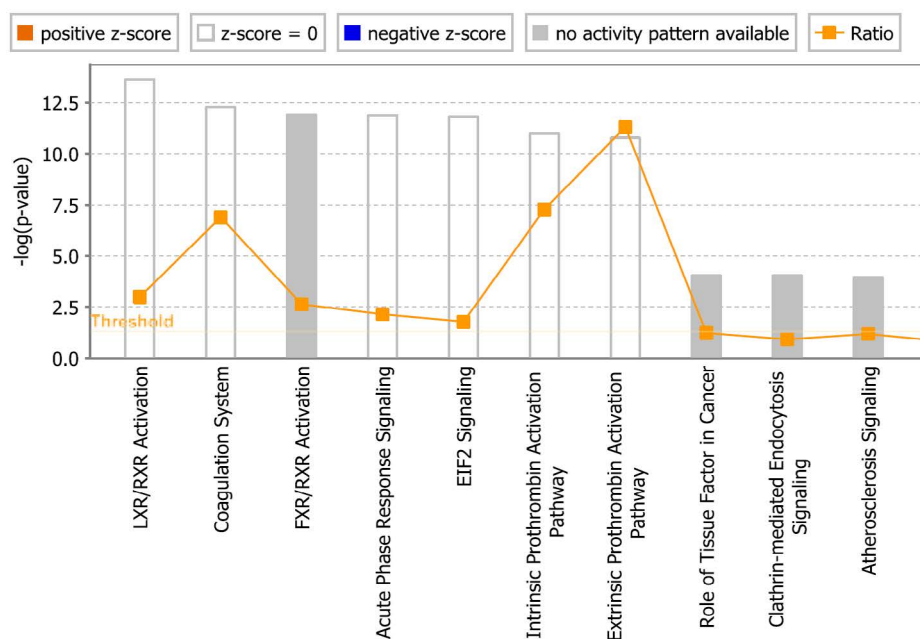
Acknowledgments

Figure 3. GO analysis of proteins significantly up- or down-regulated in rheumatoid arthritis.

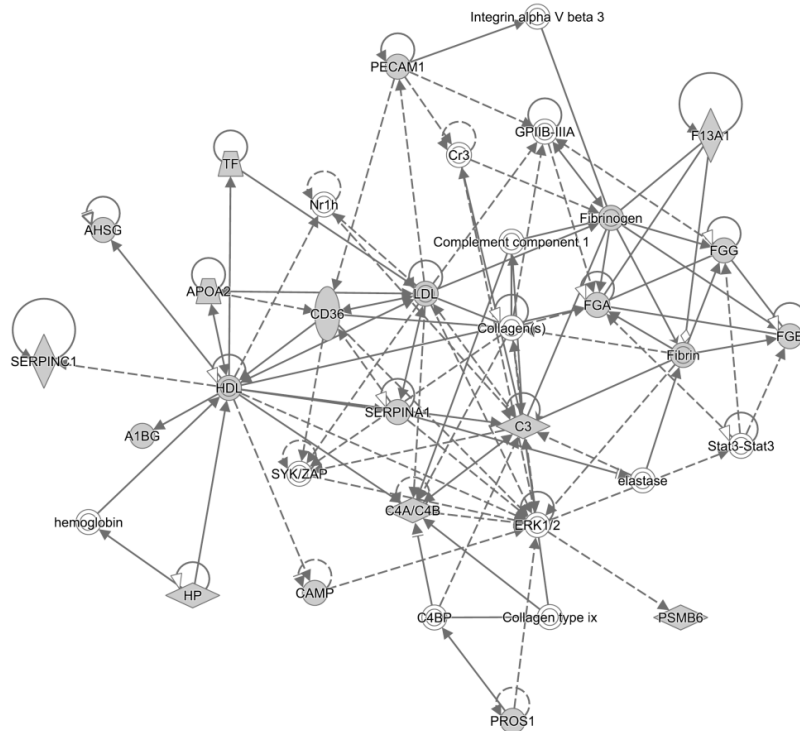Analysis: RA-different proteins-2 - 2017-02-17 04:32 PM

Figure 4. Signaling pathways involving proteins significantly up- or down-regulated in rheumatoid arthritis.

Figure 5. Two networks involving proteins significantly up- or down-regulated in rheumatoid arthritis.

(A) A network involved in organismal injury and abnormalities. (B) A network involved in organismal functions, immunological disease, and cellular movement.

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