Quantitative Proteomic Analysis of Peripheral Blood Mononuclear Cells in Ankylosing Spondylitis by iTRAQ

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Abstract

This study was designed to identify and quantify the different proteins expression levels in ankylosing spondylitis (AS) and to explore the pathogenesis of AS. We performed isobaric tags for relative and absolute quantitation (iTRAQ) coupled with multiple chromatographic fractionation and tandem mass spectrometry to detect the proteins profiling in peripheral blood mononuclear cells (PBMCs) from AS patients and healthy controls. Mascot software and the International Protein Index and the Gene Ontology (GO) database were used to conduct the bioinformatics analysis. The differentially expressed proteins were validated by enzyme-linked immunosorbent assay (ELISA). A total of 1,232 proteins were identified by iTRAQ, of which 183 showed differential expression and 18 differentially expressed proteins were acute phase reactants. Upon mapping of the differentially expressed proteins to GO database, we found four differentially expressed proteins involved in the biological process of cell killing, including up-regulated cathepsin G (CTSG), neutrophil defensin3 (DEFA3), protein tyrosine phosphatase receptor type C (PTPRC), and down-regulated peroxiredoxin-1 (PRDX1), which were consistent with the verified results of ELISA. Our proteomic analyses suggested that the proteins involved in the biological process of cell killing might play an important role in the pathogenesis of AS. Clin Trans Sci 2015; Volume 8: 579–583

Keywords: ankylosing spondylitis, iTRAQ, proteomic, cell killing

Introduction

Ankylosing Spondylitis (AS) is an autoimmune disease, which is characterized by involving the axial joint and ligament and tendon adhesion of inflammatory changes.^{1,2} Since it is subject to genetic and environment factors, the pathogenesis of AS is very complex.^{3,4} Recent studies have demonstrated that NK cells are generally considered to play important roles in the process of cell killing and are associated with the pathogenesis of AS.^{5,6} Isobaric tags for relative and absolute quantitation (iTRAQ) is widely used for generating quantitative proteomic expression profiles and is regarded as a powerful new tool for proteomic research in many autoimmune diseases.^{7,8} Although two-dimensional electrophoresis analysis or other proteomic approaches have recently generated proteomic profiles from serum and peripheral blood mononuclear cells (PBMCs) in AS patients,^{9,10} these approaches cannot completely quantify and screen protein expression due to their high background and low sensitivities. Until now, iTRAQ-based systematic proteomic analysis in AS has not yet been carried out.

With the rapid development of iTRAQ technology, high-quality data can be obtained. Though these data are being extensively used to explore the mechanisms of biological systems, it is very rough to analyze these huge raw data. Bioinformatics analysis plays an important part of processing the information embedded in large-scale protein expression profiling studies. For example, Gene Ontology (GO) analysis is an international standardization of gene function classification system, which can describe biological process, molecular function, and cellular component. GO functional annotation analysis is suitable for all identified proteins.

In this work, we performed an iTRAQ-based quantitative proteomic analysis to provide some useful information for AS. In addition, bioinformatics analysis was used to identify the differentially expressed proteins and analyze the biological process of cell killing involved in proteins, which may provide important clues to study the pathogenesis of AS.

Material and Methods

This study was performed at Shenzhen Sixth People's Hospital (Nanshan Hospital) China. All samples were obtained from the patients with informed consent and with approval of the institutional ethics committee of Shenzhen Sixth People's Hospital (Nanshan Hospital), China.

Patients

This study included 9 healthy controls and 12 patients with AS who fulfilled the 1984 modified New York criteria; all AS patients were HLA-B27-positive. All participants provided informed consent. The mean age of the patients with AS was 37 years old (range, 19–58 years old), and none had been diagnosed with other immune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, etc. The patients had not received treatment with any drugs prior to blood sample collection.

PBMC isolation and protein extraction

Venous blood (6 mL) was collected from all patients and healthy controls into EDTA anticoagulant tubes. PBMCs were isolated using Ficoll-Paque PLUS solution (GE HealthCare Bioscience, Marlborough, MA, USA). A total of 300 μ L lysis buffer was added to the cell sample. The suspension was sonicated (200 W) for 15 minutes, then centrifuged at 4°C, 30,000 $\times$ g for 15 minutes. The supernatant was transferred to another tube, and a fivefold volume of chilled 10% TCA in acetone was added; the samples were then incubated for at least 2 hours or overnight at –20°C. After incubation, the samples were centrifuged at 4°C, 30,000 $\times$ g for 15 minutes, and the supernatant was discarded. The precipitate was washed for at least 30 minutes with chilled acetone, and the supernatant was discarded after centrifugation at 4°C, 30,000 $\times$ g for 15 minutes. The pellet was then air-dried and resuspended in 1 mL of Lysis buffer. The suspension was then sonicated (200 W) for 15 minutes and centrifuged at 4°C, 30,000 $\times$ g for 15 minutes.

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Disulfide bonds in the supernatant were reduced by incubation with 10 mM dithiothreitol at 56°C for 1 hour. The cysteines were blocked with 55 mM iodoacetamide in the dark for 45 minutes. A fivefold volume of chilled acetone was added to the supernatant for 2 hours at –20°C. After centrifugation at 4°C, 30,000 × g for 20 minutes, the supernatant was discarded, and the pellet was air-dried for 5 minutes. The pellet was then dissolved in 500 µL of 0.5 M triethyl ammonium bicarbonate (TEAB) and sonicated (200 W) for 15 minutes. The sample was then centrifuged at 4°C, 30,000 × g for 15 minutes, and the supernatant was quantified.

Reduction, digestion, and iTRAQ reagent labeling

One hundred micrograms of protein was accurately removed from each sample solution. The protein was digested with Trypsin Gold (protein : trypsin = 20 : 1) at 37 °C for 4 hours. After trypsin digestion, the peptide was dried by vacuum centrifugation. The peptide was then reconstituted in 0.5 M TEAB and processed for 8-plex iTRAQ (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Briefly, one unit of iTRAQ reagent (defined as the amount of reagent required to label 100 µg of protein) was thawed and reconstituted in 70 µL of isopropanol. Peptides from digestion were labeled with different iTRAQ tags in same group by incubation at room temperature for 2 hours. The peptide mixtures were then pooled and dried by vacuum centrifugation. The pooled mixtures of iTRAQ-labeled peptides were fractionated by SCX chromatography.

Peptide fractionation with strong cation exchange (SCX)

For SCX chromatography using the Shimadzu LC-20AB HPLC Pump system (Shimadzu Corp, Kyoto, Japan), the peptide from digestion was reconstituted with 4 mL of buffer A (25 mM NaH₂PO₄ in 25% ACN, pH 2.7) and loaded onto a 4.6 × 250 mm Ultramex SCX column containing 5-µm particles (Phenomenex, Inc., Torrance, CA, USA). The peptides were eluted at a flow rate of 1 mL/minute with a gradient of buffer A for 10 minutes, 5–35% buffer B (25 mM NaH₂PO₄, 1 M KCl in 25% ACN, pH 2.7) for 11 minutes, and 35–80% buffer B for 1 minute. The system was then maintained in 80% buffer B for 3 minutes before equilibration with buffer A for 10 minutes prior to the next injection. Elution was monitored by measuring absorbance at 214 nm, and fractions were collected every 1 minute. The eluted peptides were pooled as 12 fractions, desalted using Strata X C18 columns (Phenomenex, Inc.) and vacuum-dried. Each fraction was resuspended in a certain volume of mobile phase A (2% ACN, 0.1% FA) and centrifuged at 20,000 g for 10 minutes. Within each fraction, the average final concentration of peptide was about 0.25 µg/µL.

HPLC and MS/MS analysis

A splitless nanoACQUITY system (Waters, Milford, MA, USA) was coupled to the Triple TOF 5600 (AB SCIEX Inc, Boston, MA, USA) for analytical separation. The system uses microfluidic traps and nanofluidic columns packed with Symmetry C18 (5 µm, 0.18 mm × 20 mm, Waters) for online trapping and desalting and nanofluidic columns packed with BEH130 C18 (1.7 µm, 0.1 mm × 100 mm, Waters) for analytical separations. Solvents were composed of water/acetonitrile/formic acid (A: 98/2/0.1%; B: 2/98/0.1%). A portion of the sample (9 µL) was loaded and trapping and desalting were performed at 2 µL/minute for 15 minutes with 99% mobile phase A. Using a flow rate of 300 nL/minute, analytical separation was established by maintaining 5%

B for 1 minute. Over the following 64 minutes, a linear gradient to 35% B occurred in 40 minutes. Following the peptide elution window, the gradient was increased to 80% B over 5 minutes and maintained for 5 minutes. Initial chromatographic conditions were restored in 2 minutes.

The collected fractions were subsequently pooled according to the chromatogram profile based on the peak intensity, and the products were dried in a vacuum. The peptides in these fractions were further separated on an ESI spray system, and mass spectrometry data acquisition was conducted using Q-TOF.

Database analysis

Mascot software version 2.3.02 (Matrix Science Ltd., London, United Kingdom) was used to simultaneously identify and quantify proteins. Searches were performed against the NCBI nr green plants database (20110312, 932,602 sequences after redundant processing; sequences download from <ftp://ftp.ncbi.nih.gov/blast/db/FASTA/nr.gz>). GO is an internationally standardized classification system for gene functions that provides a dynamic and controlled vocabulary to describe genes and gene product attributes in organisms. GO analysis was used to describe biological processes. Detailed information is provided on the Website <http://www.geneontology.org>.

ELISA validation analysis

For measurement of cathepsin G (CTSG), DFEA3, protein tyrosine phosphatase receptor type C (PTPRC) and peroxiredoxin-1 (PRDX1), the same PBMCs protein samples as in iTRAQ were used in this enzyme-linked immunosorbent assay (ELISA) experiment. Sandwich ELISA assays were performed according to the manufacturer's instructions. All experiments were performed in triplicate. Commercially available kits (R&D Systems, Minneapolis, MN, USA) were used, and the OD was measured using a Microplate Autoreader (DYNEX Technologies, Chantilly, VA, USA).

Statistical analysis

All statistical analyses were performed using SPSS 13.0 (IBM, Armonk, NY, USA) for Windows software. Experimental data for ELISA analysis were presented as mean ± SEM and analyzed by a paired *t*-test. The *p* < 0.05 was considered to indicate a statistically significant difference.

Results

Identification of protein expression changes by iTRAQ proteomic analysis

A total of 1,232 proteins were identified, and 183 proteins were significantly changed compared with the healthy controls. Of these altered proteins, 108 proteins were up-regulated and 75 were down-regulated. Among them, 18 differential proteins were classified as acute phase reactants (Table 1).

Upon mapping of the differentially expressed proteins to GO database

To further investigate the plausible biological processes in which these proteins might be involved, we used GO Analysis of proteins and discovered alterations in the biological process of cell killing in AS. Four differential proteins were involved in this process, including three up-regulated proteins and one down-regulated protein. These proteins are listed in (Table 2).

ID	Accession	Description	Mass	Coverage	Unique peptide	Controls vs. AS	Number
1	IPI00021727	C4BPA C4b-binding protein alpha chain	79689	6.5	3	10.534	4
2	IPI00032179	SERPINC1 antithrombin-III	64585	8.6	3	10.464	4
3	IPI00478809	F5 coagulation factor V	291624	4.3	8	1.725	8
4	IPI00553177	SERPINA1 isoform 1 of alpha-1-antitrypsin	57525	16.7	6	19.808	7
5	IPI00418163	C4B complement C4-B preproprotein	217898	7.1	9	4.208	14
6	IPI00017601	CP ceruloplasmin	143365	10.2	8	12.085	11
7	IPI00783987	C3 complement C3 (fragment)	223249	23.2	27	7.967	68
8	IPI00022418	FN1 isoform 1 of fibronectin	290085	5	8	3.037	11
9	IPI00019568	F2 prothrombin (fragment)	80601	7.6	3	4.605	4
10	IPI00019580	PLG plasminogen	108457	7.9	5	5.926	8
11	IPI00218192	ITI4H isoform 2 of interalpha trypsin inhibitor heavy chain H4	113384	1.6	1	5.486	2
12	IPI00022463	TF serotransferrin	97243	31.2	18	12.343	48
13	IPI00478003	A2M alpha-2-macroglobulin	191992	14.2	14	12.948	23
14	IPI00020091	ORM2 alpha-1-acid glycoprotein 2	28132	22.9	2	17.524	2
15	IPI00641737	HP; HPR haptoglobin	56812	21.9	8	23.645	29
16	IPI00021885	FGA isoform 1 of fibrinogen alpha chain	109041	23.3	15	3.078	60
17	IPI00296165	C1R complement C1r subcomponent	92862	7	3	3.246	2
18	IPI00021891	FGG isoform gamma-B of fibrinogen gamma chain	62753	26	9	3.151	19

Table 1. The information of acute phase reactants identified by iTRAQ in AS.

Accession	Description	Mass	Coverage	Unique peptide	Controls vs. AS	Number
Cell killing: up-regulated						
IPI00028064	CTSG cathepsin G	30682	15.7	3	7.062	5
IPI00021827	DEFA3 neutrophil defensin 3	11493	33	4	19.508	26
IPI00155168	PTPRC protein tyrosine phosphatase, receptor type, C	158996	3.1	3	2.091	6
Cell killing: down-regulated						
IPI00000874	PRDX1 peroxiredoxin-1	28408	37.2	5	0.606	7

Table 2. The information of proteins involved in the biological process of cell killing identified by iTRAQ in AS.

ELISA validation

Based on the GO analysis of biological processes, the four differentially expressed proteins associated with cell killing (CTSG, PTPRC, DEFA3, and PRDX1) were validated using ELISA analysis. As shown in *Figure 1*, compared with healthy controls, the expression levels of CTSG, DEFA3, and PTPRC were obviously higher in AS ($p < 0.05$), while the expression level of PRDX1 was significantly lower in AS ($p < 0.05$), which were consistent with the results obtained from iTRAQ.

Discussion

The genetic studies show that AS is a multigene genetic disease, and the pathogenic genes must code for proteins to be involved in development of AS.¹¹ However, the proteins associated with the pathogenesis of AS know very little. So we performed iTRAQ

technology to identify the differentially expressed proteins from the PBMCs of AS patients.

In this study, 183 proteins were identified as differentially expressed in AS. Most of these proteins are classified as APRs which are a group of proteins that undergo dramatic changes in concentration during periods of inflammation.¹² APRs have also been reported to be involved in the development of AS¹³ and assess the drug therapy for AS.^{14,15}

GO analysis was applied to classify proteins in terms of their involvement in three main categories (biological process, cellular component, and molecular function). In this study, upon mapping of the differentially expressed proteins to GO database, we identified four differentially expressed proteins involved in the biological processes of cell killing, including the up-regulated CTSG, DEFA3, and PTPRC and the down-regulated

Figure 1. Validation of the four differentially expressed proteins involved in cell killing (CTSG, PTPRC, DEFA3, and PRDX1) of PBMCs from AS and health control using ELISA analysis. The significance of the differences between AS and control is indicated by * $p < 0.05$.

PRDX1, which were consistent with the results obtained from ELISA.

CTSG is a serine protease located in the azurophil granules of neutrophils. As a leukocyte proteinase, CTSG is primarily involved in the early stages of the immune response.¹⁶ CTSG is released from granulocytes and has been reported to bind to NK cells and augment NK cytotoxicity.¹⁷ Our findings indicated that CTSG was clearly increased in AS patients, the result implied that CTSG involved in the development of AS through binding NK cells and augmenting NK cytotoxicity. Additionally, Tamiya et al. have reported that CTSG may serve as a potential biomarker for the disease activity of systemic lupus erythematosus.¹⁸

DEFA3, also known as human alpha defensin 3, is thought to participate in the host defense. The up-regulated DEFA3 was involved in the development of disease by enhancing the ability of host defense.¹⁹ Recently, Jespersgaard et al. have demonstrated that a high DEFA3 copy number is important in hindering the normal inflammatory response in Crohn's disease.²⁰ Our results found that DEFA3 was enhanced in the PBMCs of AS patients, and we hypothesize that the increased DEFA3 might be linked to the development of AS owing to increasing the host defense and inhibiting hindering of the normal inflammatory response.

Earlier studies have demonstrated that PTPRC, also known as CD45, is a transmembrane receptor-like molecule specifically expressed on the cell surface of all nucleated hematopoietic cells and plays a significant role in regulating CD4⁺ and CD8⁺ autoreactive T cells and interferon-beta responsiveness.²¹ In addition, Rhee et al. have found that PTPRC plays a key role in autoimmune diseases, such as multiple sclerosis and Rheumatoid arthritis.²² In this study, PTPRC was increased and identified as cell killing involved in protein. Based on our findings, we speculated that PTPRC might affect the biological process of cell killing by regulating the T cells responses in AS.

PRDX-1 is known to be a factor that can evoke inflammatory reactions.²³ Extracellular PRDX-1 enhances NK cell activity and suppresses viral replication in cells.²⁴ Our experiments showed that PRDX-1 was down-regulated; the down-regulated PRDX-1 might have resulted from the decrease of NK cell killing activity. As a result, we assumed that PRDX-1 was involved in the development of AS by affecting the NK cell killing activity.

The iTRAQ technology is a powerful tool for quantitative proteomic expression analysis in AS. However, this study contains some potential limitations. The limited number of samples prevents reliable conclusions. Thus, increasing the number of patients and controls will be necessary. Moreover, further investigation will be required to confirm the function study of the differential proteins involved in the biological processes of cell killing.

In summary, we performed iTRAQ analysis to identify and quantify candidate proteins in AS patients. The differential expression of these proteins may provide valuable information for the understanding of the pathogenesis of AS.

Conflict of interest

The authors declare no conflicts of interest.

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