Quantitative Proteomic Analyses To Reveal the Key Features of Proteins in New Onset Ankylosing Spondylitis Patients

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ABSTRACT: Ankylosing spondylitis (AS) is a chronic immune-mediated disease. Various immune cells play an essential role in the AS pathogenesis. However, the specific pathogenesis of AS has not been well understood. Proteomic profiles of peripheral blood mononuclear cells (PBMCs) were applied to reveal the specific pathogenesis of AS. Quantitative proteomic analyses were performed using liquid chromatography–tandem mass spectrometry (LC–MS/MS)-based methods to investigate the protein profiling of PBMCs from new-onset AS patients (n = 9) and healthy controls (n = 9). We identified 782 differentially expressed proteins (DEPs) and 527 differentially phosphorylated proteins (DPPs) between AS patients and healthy controls. The subcellular location of DEPs and DPPs showed that most of the DEPs were from the cytoplasm (n = 296, 38%), were extracellular (n = 141, 18%), and from the nucleus (n = 114, 15%); most of the DPPs were from the cytoplasm (n = 37, 34%), nucleus (n = 35, 32%), and plasma membrane (n = 10, 9%). We further identified 89 proteins with both expression and phosphorylation differences. The functional annotation of the 89 differentially expressed and phosphorylated proteins enriched in the antigen processing and presentation pathway. Four DEPs with six phosphorylated positions were found in the antigen processing and presentation pathway. The differentially expressed and phosphorylated proteins may be helpful to uncover the pathogenesis of AS. The six AS-specific proteins may serve as candidate markers for AS diagnosis and new treatment targets.

INTRODUCTION
Ankylosing spondylitis (AS) is a chronic inflammatory and progressive disease, usually originates in the damage of sacroiliac joints and gradually damages the spine, causing disability and reduced quality of life. The disease is prevalent in the young population, and males are more susceptibility to disease. Although AS is considered an immune-mediated disease, its pathogenesis is not yet well understood.

Increasing evidence has shown that AS is strongly related to major histocompatibility complex (MHC) class I because nearly 95% AS patients are born to human leucocyte antigen (HLA)-B27 gene positive. However, healthy people also express HLA-B27, and only a small part of the B27-positive healthy population finally develop AS. Increasing studies showed that various immune cells and their secreted-mediators play an essential role in AS pathogenesis: the interaction of killer cell immunoglobulin-like receptors (KIRs) with HLA-B27 regulates activity of natural killer (NK) cells in AS patients; the inflammatory cytokines such as TNF-α, IFN-γ, and IL-17 produced by effector CD8+ T cells that mediate the pathogenesis of AS; B cells and antibodies are involved in the pathobiology of AS, and the antibodies provide new insights for the diagnosis of AS; and dendritic cells (DCs) also play an important role in autoimmune disease and may contribute to the Th17 immune responses and therefore be associated with the onset of AS. Because of the essential role of immune cells in the pathogenesis and development of AS, proteomic profiles of peripheral blood mononuclear cells (PBMCs) may help to reveal the specific pathogenesis of AS. Quantitative profiling of proteins and post-translational modification (PTM) are crucial to understand the complex physiology and function of proteins. The liquid chromatography–tandem mass spectrometry (LC–MS/MS)-based proteomic analysis method provides a powerful capacity and high accuracy to study signal transduction. In this study, we...
performed quantitative proteomics using LC–MS/MS-based methods to investigate the key features of proteins in PBMCs of AS patients.

**Results**

The clinical and laboratory results of between AS patients and healthy controls are given in **Table 1**. Key biometric data such as gender, age, white blood cell count, lymphocyte count, monocyte count, and neutrophil count were analyzed. There was no difference in the gender and age between AS patients and healthy controls ($P > 0.05$). AS patients had higher monocyte count than healthy controls ($P = 0.012$). For more details see **Table 1**.

**Protein Identification.** For protein expression in LC–MS/MS, we identified 2240 proteins. We found 919 quantitative proteins from the 2240 proteins. Compared with the healthy controls, there were 782 DEPs, including 646 proteins upregulated ($\geq 1.5$-fold) and 136 downregulated ($\leq 0.67$-fold) in AS patients (**Figure 1a**). For protein phosphorylation, we identified 2417 phosphorylation sites in 1281 proteins. A total of 527 phosphorylation sites in 361 proteins were quantified, and 210 phosphorylation sites were identified in 122 DPPs between the AS patients and the healthy controls. Fifty phosphorylation sites were upregulated ($\geq 1.5$-fold) in 37 proteins, and 125 phosphorylation sites were downregulated ($\leq 0.67$-fold) in 85 proteins (**Figure 1b**). Hierarchical clustering analysis of DEPs and DPPs is shown in **Figure 1c,d**, respectively.

For all DEPs and DPPs, we found 89 proteins with expression difference and phosphorylation difference (**Figure 1e**).

**Functional Characterization of Differentially Quantified Proteins.** We investigated the subcellular location of the DEPs and DPPs. The results showed that most of the DEPs were from the cytoplasm ($n = 296, 38\%$), were extracellular ($n = 141, 18\%$), and from the nucleus ($n = 114, 15\%$) (**Figure 2a**); most of the DPPs were from the cytoplasm ($n = 37, 34\%$), nucleus ($n = 35, 32\%$), and plasma membrane ($n = 10, 9\%$) (**Figure 2b**).

We further investigated the functional category distribution of the DEPs and DPPs based on the GO analysis tool. Thirty different groups of affected biological functions were established for the DEPs and further divided into three classifications: cellular component (43.3\%), molecular function (30.0\%), and biological process (26.7\%) (**Figure 2c**). Thirty different groups of affected biological functions were established for the DPPs and further divided into three classifications: cellular component (46.9\%), molecular function (31.2\%), and biological process (21.9\%) (**Figure 2d**). Within the biological process category, the majority of DEPs and DPPs was related to the cellular process (706 and 97, respectively) and the single-organism process (675 and 92, respectively). The cellular component showed that DEPs and DPPs were mainly involved in the cell (749 and 106, respectively) and organelle (725 and 98, respectively). For the molecular function category, 734 and 357 of the DEPs and 104 and 31 of the DPPs were associated with the binding and catalytic activity, respectively.

**Functional Enrichment of Differentially Quantified Proteins.** To better understand the biological function of these DEPs and DPPs, we performed GO annotation analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis.

In GO terms, 30 different groups of pathways were established for the DEPs and further divided into three classifications: cellular component ($n = 8$), molecular function ($n = 8$), and biological process ($n = 14$) (**Figure 3a**). Thirty-two different groups of pathways were established for the DPPs, and further divided into three classifications: cellular component ($n = 8$), molecular function ($n = 8$), and biological process ($n = 14$) (**Figure 3b**).

KEGG pathway-based enrichment analysis revealed DEPs were significantly enriched in tyrosine metabolism, arachidonic acid metabolism, malaria, metabolism of xenobiotics by cytochrome P450, and so forth (**Figure 4a**). Regarding phosphorylation, the most significantly enriched signal pathways included galactose metabolism, cell signaling in helicobacter pylori infection, vibrio cholerae infection, antigen processing and presentation, and so forth (**Figure 4b**). Furthermore, the DEPs and DPPs were enriched in six same pathways: antigen processing and presentation, pentose phosphate pathway, platelet activation, pathogenic Escherichia coli infection, proteoglycans in cancer, and tight junction.

**Verification of the Antigen Processing and Presentation Signal Pathway.** Antigen processing and presentation plays an essential role in AS pathogenesis.

Functional Enrichment of the Antigen Processing and Presentation Signal Pathway. Antigen processing and presentation pathway was related to cellular component (31.2\%), and biological process (26.7\%) (**Figure 2c**). Thirty different groups of affected biological functions were established for the DPPs and further divided into three classifications: cellular component (46.9\%), molecular function (31.2\%), and biological process (21.9\%) (**Figure 2d**). Within the biological process category, the majority of DEPs and DPPs was related to the cellular process (706 and 97, respectively) and the single-organism process (675 and 92, respectively). The cellular component showed that DEPs and DPPs were mainly involved in the cell (749 and 106, respectively) and organelle (725 and 98, respectively). For the molecular function category, 734 and 357 of the DEPs and 104 and 31 of the DPPs were associated with the binding and catalytic activity, respectively.

**Discussion**

In the present study, we performed LC–MS/MS-based proteomic analysis and identified 782 DEPs and 527 DPPs.
between AS patients and healthy controls and 89 proteins with both expression and phosphorylation difference. The functional annotation of the 89 proteins enriched in the antigen processing and presentation pathway. We further explored that the antigen processing and presentation pathway revealed four differentially expressed proteins (DEPs) (HSP90AA1, HSP90AB1, HLA-E, and CANX) with six phosphorylated positions (HSP90AA1_S263, HSP90AB1_S255, HLA-E_357, CANX_S554, CANX_S564, and CANX_S583) between AS patients and healthy controls.

It is well-known that antigen processing and presentation plays an essential role in AS pathogenesis. 17−19 HLAs are
proteins encoded by genes of the human MHC.\textsuperscript{20} The expression of HLA-B27 is strongly correlated with AS.\textsuperscript{21--23} HLA-B27 plays an essential role in antigen presentation cytotoxic CD8+ T-cells, thus mediated inflammation conditions in AS patients.\textsuperscript{24,25} In addition, misfolded HLA-B27-induced endoplasmic reticulum (ER) stress activates the unfolded protein response (UPR), which triggers the NF-\(\kappa\)B activation and stimulates proinflammatory cytokine expression, such as TNF-\(\alpha\), interleukin (IL)-1, IL-6, and IL-23.\textsuperscript{17} Besides the well-known HLA-B27, HLA-E also showed association with AS pathogenesis.\textsuperscript{26--28} HLA-E is a nonclassical member of MHC I, which binds and presents peptides to NK cells bearing CD94/NKG2 receptors.\textsuperscript{29} Higher expression and lower phosphorylation of HLA-E in AS patients in the present study may indicate HLA-E-regulated activity of NK cells, which involves in the development of AS.

Target antigen determination is essential to understand the pathogenesis and develop new therapy strategies in autoimmune diseases. Increasing studies indicated that heat-shock proteins (HSPs) are indispensable in innate and adaptive immunities.\textsuperscript{30--35} HSPs and their synthetic peptides help develop potential immunotherapy for autoimmune diseases.\textsuperscript{34} Compared with healthy controls, AS patients showed higher serum level HSP-65 antibody (anti-HSP65).\textsuperscript{33} In our study, we identified two HSP90 (HSP90AA1 and HSP90AB1) with both expression and phosphorylation differences in PBMCs between AS patients and healthy controls. Based on the previous studies\textsuperscript{30--33} and our findings, HSP90AA1 and HSP90AB1 may involve in the development of AS.

Calnexin (CANX) belongs to the calnexin family of molecular chaperones. Calnexin is an ER-associated calcium-binding protein that facilitates protein folding and assembly through briefly interacting with newly synthesized N-linked glycoproteins.\textsuperscript{36} Calnexin may also be essential in protein folding quality control by maintaining wrongly folded protein subunits within the ER for degradation.\textsuperscript{37} UPR triggered by accumulation of misfolded proteins in the ER decreases protein production and enhances capacity to handle excess ER protein load.\textsuperscript{38} The HLA-B27 transgenic rat model showed that the activation of UPR was strongly associated with the
expression of HLA-B27.39 Previous studies suggested that the pathogenesis of AS is correlated with UPR in the HLA-B27-related ER.40,41 Higher expression and lower phosphorylation of CANX in AS patients in the present study may indicate that CANX involves in the development of AS. However, the role of CANX in UPR activation in the ER shall be confirmed by further well-designed study.

Our study had some limitations which should be considered. First, it is a small number size cross-sectional study. Second, the results of DEPs and DPPs were average values of the pooled samples of AS patients and healthy controls. The LC−MS/MS measurement could have neglected individual information. Third, although thousands of proteins were detected, there were no more than one thousand proteins with expression and phosphorylation quantitative data. Some
The quantitative proteomic analyses revealed differentially expressed and phosphorylated proteins between AS patients and healthy controls. Key features of the DEPs and DPPs suggested that the AS onset is a complex process. The DEPs and DPPs may further help reveal the pathogenesis of AS and serve as candidate markers for AS diagnosis and new treatment targets.

Table 2. DEPs in the Antigen Processing and Presentation Signal Pathwaya

| protein accession | gene name | protein description | AS/HC ratio | regulated type |
|-------------------|-----------|---------------------|-------------|---------------|
| P08238            | HSP90AB1  | HSP 90-β            | 12.333      | up            |
| P27824            | CANX      | calnexin            | 5.944       | up            |
| P07858            | CTsb      | cathepsin B         | 5.231       | up            |
| P07900            | HSP90AA1  | HSP 90-α            | 4.988       | up            |
| P34932            | HSPA4     | heat shock          | 4.141       | up            |
| P27797            | CALR      | calreticulin        | 3.914       | up            |
| Q06323            | PSME1     | proteasome activator complex subunit 1 | 2.656 | up |
| Q9U146            | PSME2     | proteasome activator complex subunit 2 | 2.597 | up |
| P30101            | PDIA3     | protein disulide-isomerase A3 | 2.521 | up |
| Q03518            | TAP1      | antigen peptide transporter 1 | 2.521 | up |
| P11021            | HSPA5     | ER chaperone BiP    | 2.49        | up            |
| P0IDMV8           | HSPA1A    | heat shock          | 2.236       | up            |
| P13747            | HLA-E     | HLA class I histocompatibility antigen, α chain E | 2.205 | up |
| P10319            | HLA-B     | HLA class I histocompatibility antigen, α chain E | 2.195 | up |
| P11142            | HSPA8     | heat shock cognate 71 kDa protein | 1.933 | up |
| P13746            | HLA-A     | HLA class I histocompatibility antigen, A-11 α chain | 1.695 | up |

aDEPs, differentially expressed proteins.

Table 3. DPPs in the Antigen Processing and Presentation Signal Pathwaya

| protein accession | gene name | position | protein description | AS/N1 ratio | regulated type |
|-------------------|-----------|----------|---------------------|-------------|---------------|
| P07900            | HSP90AA1  | 263      | HSP 90-α            | 1.954       | up            |
| P08238            | HSP90AB1  | 255      | HSP 90-β            | 0.538       | down          |
| P13747            | HLA-E     | 357      | HLA class I histocompatibility antigen, α chain E | 0.453 | down |
| P27824            | CANX      | 554, 564, 583 | calnexin | 0.294       | down          |

aDPPs, differentially phosphorylated proteins.

CONCLUSIONS

The quantitative proteomic analyses revealed differentially expressed and phosphorylated proteins between AS patients and healthy controls. Key features of the DEPs and DPPs suggested that the AS onset is a complex process. The DEPs and DPPs may further help reveal the pathogenesis of AS and serve as candidate markers for AS diagnosis and new treatment targets.

PATIENTS AND METHODS

Patients. This observational study was conducted at the Department of Nephrology, The Affiliated Hospital of Jinan University (Guangzhou, China). The study was approved by the local ethical committee of The Affiliated Hospital of Jinan University and was carried out in accordance with the Declaration of Helsinki. Nine new onset B27-positive AS patients in a positive status and nine age-matched healthy controls were recruited [median age interquartile range (IQR), 27 (30, 34) vs 34 (29.5, 36)]. The diagnosis of AS was following the 1984 revised New York classification criteria.13 Patients with autoimmune diseases and infectious diseases were excluded from this study. After obtaining an informed consent, cubital vein blood samples were collected from all patients and healthy controls. Blood routine examination was performed, and the white blood cell count, lymphocyte, monocyte, neutrophil differentials were determined using an automated blood cells counter (Beck-man Coulter, Ireland).

Isolation of PBMCs. PBMCs were isolated using the density gradient centrifugation method. Briefly, the heparinized whole blood was diluted 1:1 with phosphate buffered saline (PBS, Gibco), and 20 mL of diluted blood was layered onto 10 mL of Ficoll-Paque Plus (Amershams Biociences, Uppsala, Sweden). Samples were then centrifuged at 500g for 20 min at room temperature. PBMCs were collected from the interface of spun blood samples and were washed twice with PBS by centrifugation at 500g for 10 min at 4 °C. The supernatant was discarded, and the PBMCs were then resuspended at a final concentration of 1 × 10⁶ cells/mL in RPMI 1640 (Gibco) with 10% heat inactivated fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The viability of the cells was consistently greater than 95% as measured by trypan blue solution (Sigma, Germany).

Protein Extraction and Trypsin Digestion. The extraction and digestion procedures of proteins followed the published method.14 We took samples from a −80 °C cryogenic refrigerator and then sonicated them three times on ice using a high intensity ultrasonic processor (JY92-IIIN, Scientz, China) in four-fold volume lysis buffer (containing 8 M urea, HALT protease, and phosphatase inhibitor cocktail). The cell fragments were removed by centrifugation for 10 min, at 12,000g at 4 °C. Finally, the supernatant was collected and transferred to a new centrifuge tube. The protein concentration was determined with a BCA kit in accordance with the manufacturer’s instructions (Beyotime, China). We extracted 200 μg of proteins from each sample and then combined to a pool of 1.8 mg of AS patient sample and a pool of 1.8 mg of healthy control sample.

For digestion, we reduced the protein solution with 5 mM dithiothreitol for 30 min at 56 °C and then alkylated with 11 mM iodoacetamide for 15 min at room temperature in darkness. The urea concentration of the protein sample was diluted to less than 2 M by adding 100 mM triethyl ammonium bicarbonate (TEAB). Finally, trypsin (Promega, USA) was added at 1:50 trypsin-to-protein mass ratio for the first digestion overnight and 1:100 trypsin-to-protein mass ratio for the second 4 h-digestion.

Modification Enrichment and Affinity Enrichment. After trypsin digestion, the peptide was desalted by the Strata X C18 solid-phase extraction column (Phenomenex, USA) and vacuum-dried. Peptides were reconstituted in 0.5 M TEAB and...
processed in accordance with the manufacturer’s protocol for the TMT kit/iTRAQ kit (Thermo Fisher Scientific). The peptide mixtures were then incubated for 2 h at room temperature and pooled, desalted, and dried by vacuum centrifugation.

The tryptic peptides were fractionated into fractions by high pH reverse-phase high-performance liquid chromatography using the Thermo Betasil C18 column (5 μm particles, 10 mm ID, 250 mm length). Briefly, peptides were first separated with a gradient of 8−32% acetonitrile (pH 9.0) over 60 min into 60 fractions. Then, the peptides were combined into eight fractions and dried by vacuum centrifuging.

For the enrichment of the modified peptides, tryptic peptides were dissolved in NETN buffer (containing 100 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, and 0.5% NP-40; pH 8.0) and then incubated with prewashed antibody beads (lot number 001, PTM Bio) at 4 °C overnight with gentle shaking. Then, we washed the beads four times using NETN buffer and twice using H2O. The bound peptides were eluted from the beads with 0.1% trifluoroacetic acid. For LC−MS/MS analysis, the resulting peptides were desalted with C18 ZipTips (Millipore) according to the manufacturer’s instructions.

**LC−MS/MS Analysis.** We performed LC−MS/MS analysis on an EASY-nLC 1000 ultraperformance liquid chromatography (UPLC) system (Thermo Fisher Scientific), followed by MS/MS using Q Exactive Plus (Thermo Fisher Scientific) coupled online to the UPLC system. When the ion source voltage was set to 1.4 kV, peptides were detected in the Orbitrap one MS/MS scan of 70,000, followed by 20 MS/MS scans with 15.0 s dynamic exclusion. We have submitted the LC−MS raw spectral data to ProteomeXchange via the PRIDE database. The project name is quantitative proteomic analyses to reveal the key features of proteins in new onset AS patients, and the project accession number is PXD019667 in ProteomeXchange.

**Database Search and Bioinformatic Analysis.** The MS/MS data were retrieved by the Maxquant search engine (v1.6.6.0). A human database was searched (Swiss-Prot, downloaded 2019-05-13, 20422 protein sequences). The decoy database antilibrary was used to reduce false positive rate (FDR). The FDR was adjusted to <1%, and the minimum score for modified peptides was set >40. Proteins with a fold-change ≥1.50 or ≤0.67 between AS patients and healthy controls were considered as expression/phosphorylation significant. Based on the protein sequence alignment method, the protein domain functions were defined by InterProScan (http://www.ebi.ac.uk/interpro/). The subcellular localization was predicted by WoLF PSORT. Functional annotation enrichments of DEPs and differentially phosphorylated proteins (DPPs) were performed by Gene Ontology (GO) annotation analysis and KEGG analysis. The enrichment
significant was identified as $p < 0.05$ in Fisher’s exact test and $q < 0.05$ in Benjamini-Hochberg’s procedure. We conducted hierarchical clustering analysis for the DEPs and DPPs using the “heatmap.2” function form and “gplots” R package.

**Statistical Analysis.** The clinical and laboratory data were analyzed with SPSS version 22.0. Results of normally distributed parameters are expressed as arithmetic mean ± SD. Two-group comparisons were performed using the two-sample $t$-test; results of non-normally distributed parameters are expressed as the median (IQR)., and two-group comparisons were performed using the Wilcoxon rank-sum test. Pearson’s $\chi^2$ test was used for testing qualitative data. A $p$ value $< 0.05$ was considered significant.

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**Author Contributions**

Y.-P.L., X.-L.Z., and C.Y. contributed equally to this work. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

**Notes**

The authors declare no competing financial interest.

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