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RESEARCH ARTICLE

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iTRAQ-based proteomic analysis of differentially expressed proteins in sera of seronegative and seropositive rheumatoid arthritis patients

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Abstract

Objective: The diagnosis of seronegative rheumatoid arthritis (SNRA) is often difficult due to the unavailability of reliable laboratory markers. The aim of this study was to identify differentially expressed proteins in sera of SNRA, seropositive RA (SPRA), and healthy donors (HD).

Methods: A total of 32 seropositive RA patients, 32 SNRA patients, and 35 HD were enrolled in our study. Differentially expressed proteins between 3 groups were identified via isobaric tags for relative and absolute quantitation (iTRAQ)-based proteomic analysis, and an ELISA test was used for the validation test. Correlation analysis was conducted by GraphPad Prism.

Results: Using iTRAQ quantitative proteomics, we identified 14 proteins were significantly different between SPRA and SNRA, including 4 upregulated proteins and 10 downregulated proteins. Four differentially expressed proteins were validated by ELISA test, and the results showed that SAA1 protein was significantly higher in SPRA and SNRA patients compared with HD, and PSME1 was elevated in SPRA patients. What's more, SAA1 was increased in the anti-CCP or RF high-level group in RA patients, and PSME1 was increased in the RF high-level group. Alternatively, SAA1 was positively correlated with inflammation indicators in RA patients, while PSME1 showed no correlation with inflammation indicators.

Conclusions: iTRAQ proteomic approaches revealed variations in serum protein composition among SPRA patients, SNRA patients, and HD and provided new idea for advanced diagnostic methods and precision treatment of RA.

KEYWORDS
iTRAQ, seronegative rheumatoid arthritis, seropositive rheumatoid arthritis

Yujue He and Junyu Lin have contributed equally to this work and are considered co-first authors.

1 | INTRODUCTION

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease. Rheumatoid factor (RF) and anti-cyclic citrullinated peptide antibodies (ACPAs) are the mainstay of serological markers for RA.

In the 20th century, the laboratory indicators of rheumatoid arthritis mainly depended on RF, which was the first serological marker of rheumatoid arthritis. Although the sensitivity of RF detection was high, the specificity for RA was limited, especially in the first year of the disease.[1] ACPA was measured by the anti-cyclic citrullinated peptide (anti-CCP) antibody test, which has been included in the RA criteria.[2] It has been found to have 94% specificity and 75% sensitivity. For the early phases of RA, its sensitivity was 61%.[3] Because of the lack of sufficient specificity and sensitivity in these indicators to some extent, RA patients can be defined according to the presence or the absence of autoantibodies as follows: seropositive rheumatoid arthritis (SPRA) and seronegative rheumatoid arthritis (SNRA).[4,5] However, the differences between SNRA patients and SPRA patients in protein levels remain unclear.

According to the guidelines issued by the American College of Rheumatology (ACR)/European Union Against Rheumatology (EULAR) in 2010,[2] serological indicators were included in the scoring system along with clinical manifestations and imaging biomarkers. If the score is greater than or equal to 6 points, the patient can be diagnosed with definite RA. Compared with the previous version of the guideline, RF and ACPA accounted for 3 points in the 2010 ACR/EULAR, which shows that serological indicators are valued for the diagnosis of RA. Due to the negative serological results, the SNRA patients have higher disease activity in clinical manifestations and ultrasound examinations when they were considered as RA.[6]

About one-third of RA patients are seronegative RA patients in the clinic.[7] The diagnosis of seronegative RA is usually full of uncertainty, leading to the progress of this devastating disease. The differential diagnosis of seronegative RA may be difficult because of the absence of markers for early diagnosis. So, how do we achieve early and accurate diagnosis of seronegative RA? The purpose of this study was to identify differentially expressed proteins in sera of SNRA, SPRA, and healthy donors.

In the present study, we performed comparative proteomic analysis using isobaric tags for relative and absolute quantification (iTRAQ) approach in order to identify differentially expressed proteins in sera of SNRA, SPRA, and healthy donors. As a result, a total of 63 proteins were observed between SNRA and HD, and 16 proteins were significantly different between SPRA and HD. Compared with SPRA, 14 differentially expressed proteins were identified in the SNRA. Four of the differentially expressed proteins identified were further confirmed by ELISA analysis.

2 | METHODS

2.1 | Study population

This study included 32 SPRA patients and 32 SNRA patients who visited the First Affiliated Hospital of Fujian Medical University from February to April 2018. The control group was composed of 35 healthy donors (HD) from the physical examination center of the First Affiliated Hospital of Fujian Medical University during the same period. The diagnostic of all RA patients fulfilled the 2010 American College of Rheumatology/European League against Rheumatism criteria for RA.[2] All patients received therapy with disease-modifying anti-rheumatic drugs (DMARDs), nonsteroidal anti-inflammatory drugs (NSAIDs), and glucocorticoids. Patients were excluded from the study for any of the following reasons: anemia, age less than 18 years, pregnancy, postpartum, mellitus, hypertension, atherosclerosis, diabetes, Alzheimer's disease, and concomitant other autoimmune diseases. This study was approved by the Institutional Medical Ethics Review Board of the First Affiliated Hospital of Fujian Medical University (IEC-FOM-013-1.0).

2.2 | iTRAQ-based proteomic analysis

The experiments were divided into two parts. The pre-experiment procedure consisted of the following steps: protein extraction, protein quantification, proteolysis, mass spectrometry, and database comparison. The formal experiment was performed on the basis of the pre-experiment, including the iTRAQ peptide labeling, classification, mass spectrometry analysis, and database comparison. For the sera of SPRA patients and SNRA patients, every 10 cases were mixed into one protein sample while every 15 cases of healthy donor sera were mixed into one protein sample. iTRAQ-based proteomic analysis was accomplished by Genechem.

2.3 | ELISA

Serum samples from SPRA patients, SNRA patients, and healthy donors were collected for the measurement of SAA1 by ELISA according to the manufacturer's recommendations (R&D Systems). In addition, ryanodine receptor 3 (RYR3), thymosin beta 4 (Tβ4), and proteasome activator subunit 1 (PSME1) were also measured according to the manufacturer's recommendations (Inselisa).

2.4 | Laboratory analyses and assessment

Erythrocyte sedimentation rate (ESR) was measured by Westergren's method. Anti-CCP and antinuclear antibody (ANA) were measured

by enzyme-linked immunosorbent assays (EUROIMMUNAG). Serum C-reactive protein (CRP) and RF were quantified by immunoturbidimetric assay (Dade Behring). Anti-extractable nuclear antigen (ENA) antibodies, including anti-Sm, anti-SSA, anti-SSB, anti-RNP, anti-Ro-52, anti-Scl-70, anti-Jo-1, anti-CENP-B, anti-nucleosome, anti-histone, and anti-Rib-P antibodies, were detected using immunoblotting assay (EUROIMMUN). The disease activity score assessing 28 joints (DAS28) was evaluated by a rheumatologist using the formula: DAS28 = 0.56 × √(TJC28) + 0.28 × √(SJC28) + 0.70 × ln (ESR) + 0.014 × VAS (range 0–100 mm, 0 = inactive disease), TJC28 = number of tender joints, SJC28 = number of swollen joints, VAS = visual analog scale.

2.5 | Statistical analysis

Data were presented as median (interquartile range) or *n* (%). Multiple-group comparisons were performed by one-way analysis of variance (ANOVA) followed by Bonferroni-corrected *t* tests. Comparisons of categorical variables were conducted using χ^2 testing. Correlation analyses were performed using Spearman's correlation test. All statistical analyses were performed using the Statistical Package for the Social Sciences, version 22.0 (SPSS Inc.) or GraphPad Prism 7.0 (GraphPad Software).

3 | RESULTS

3.1 | Demographic characteristic of SPRA patients, SNRA patients, and healthy donors

Thirty cases of SPRA, SNRA patients, and healthy donors were collected for iTRAQ. For RA patient sera, every 10 cases were mixed into one protein sample. In addition, every 15 cases of healthy donor sera were mixed into one protein sample. The mixed protein samples were subjected to quality inspection by SDS-PAGE electrophoresis. Samples from a total of 32 SPRA patients, 32 SNRA patients, and 35 healthy controls were collected for ELISA analysis, which was employed to validate the quality of iTRAQ results. The basic demographic and laboratory data of 32 SPRA patients, 32 SNRA patients, and 35 healthy controls are shown in Table 1.

3.2 | Protein quantification and differential analysis

iTRAQ-based quantitative proteomic approach was performed to determine the proteomic changes in serum of SNRA patients and SPRA patients. Proteins with fold change higher than 1.2 and with a *p*-value lower than 0.05 were considered as differentially expressed. A total of 16 differentially expressed proteins were observed

TABLE 1 Demographic characteristic of SPRA patients, SNRA patients, and healthy donors

Clinical data	SPRA	SNRA	HD	p^a	p^b	p^c
Number	32	32	35	/	/	/
Age, median(quartile), years	55 (16)	59 (18)	44 (12)	0.7	0.27	<0.05
Sex, no. (%) female	24 (75)	21 (65.63)	25 (71.43)	0.585	0.744	0.793
Sex, no. (%) male	8 (25)	11 (34.38)	10 (28.57)			
Anti-CCP, median(quartile), (RU/mL)	99.35 (114)	1.1 (1.3)	/	<0.05	/	/
CRP, median(quartile), mg/L	9.74 (10.48)	3.88 (8.16)	/	<0.05	/	/
RF, median(quartile), (IU/mL)	186.5 (329.58)	<20	/	<0.05	/	/
ESR, median(quartile), mm/h	29 (28.25)	18 (26.75)	/	0.025	/	/
ANA, median(quartile), S/CO	0.5 (0.475)	0.2 (0.25)	/	0.948	/	/
ENA, no. of negative	31	31	/	1	/	/
ENA, no. of positive	1	1				
Duration of disease, median (quartile), months	9 (28)	9 (19)	/	0.945	/	/
DAS28, median(quartile)	3.52 (1.68)	3.46 (1.36)	/	0.782	/	/
Treatment						
DMARDs + NSAIDs + Glucocorticoid	32	32	/	1	/	/

Abbreviations: ANA, antinuclear antibody; anti-CCP, anti-cyclic citrullinated peptide antibodies; CRP, C-reactive protein; DAS28, disease activity score assessing 28 joints; DMARDs, disease-modifying antirheumatic drugs; ENA, extractable nuclear antigen; ESR, erythrocyte sedimentation rate; NSAIDs, nonsteroidal anti-inflammatory drugs; RF, rheumatoid factor; SNRA, seronegative rheumatoid arthritis; SPRA, seropositive rheumatoid arthritis.

[a]The difference between SPRA and SNRA group.

[b]The difference between SPRA and HD group.

[c]The difference between SNRA and HD group.

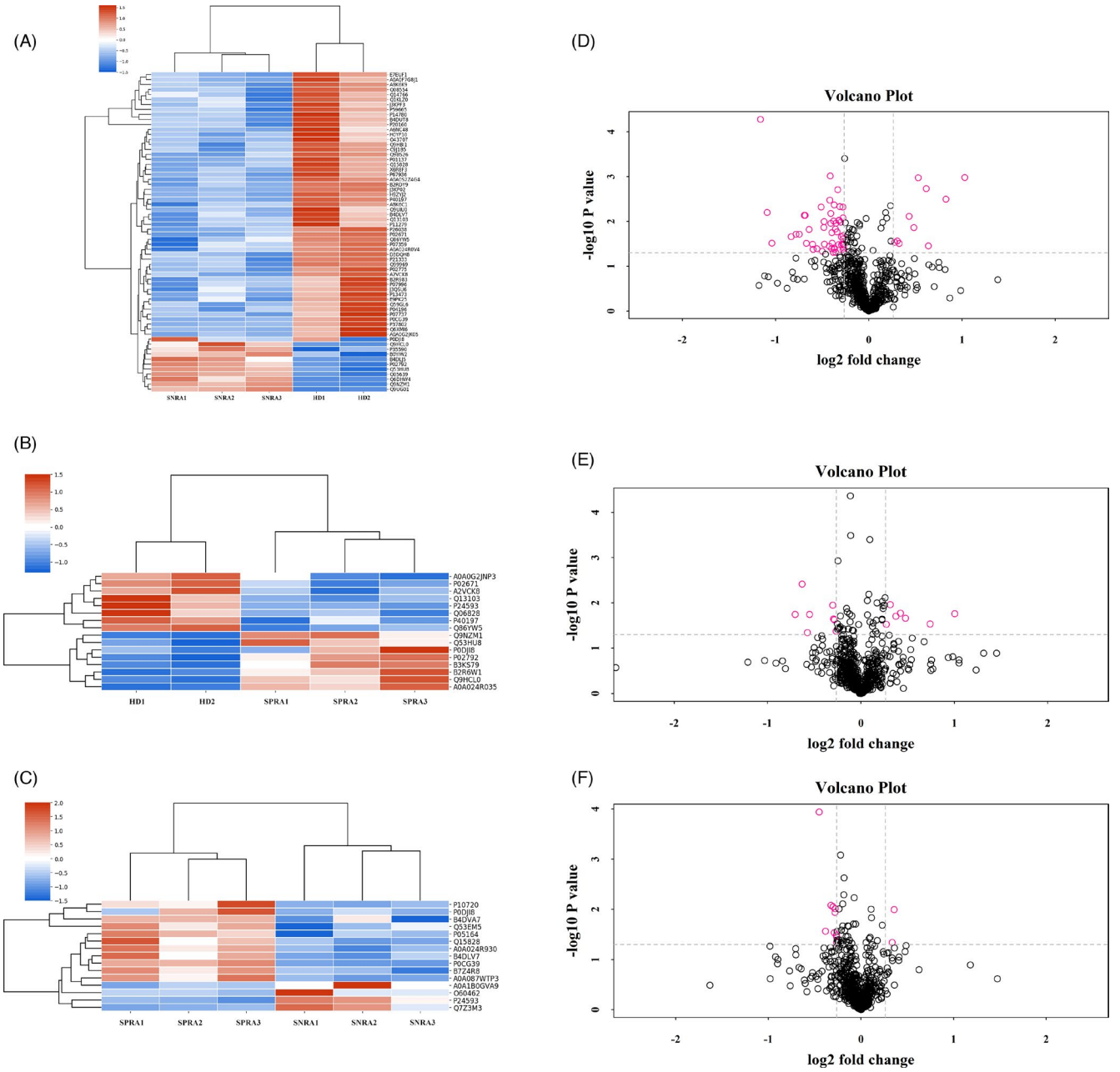


FIGURE 1 iTRAQ-based quantitative proteomic analysis. (A) Proteins cluster analysis of SNRA patients and HD. (B) Proteins cluster analysis of SPRA patients and HD. (C) Proteins cluster analysis of SPRA patients and SNRA. (D) Volcano plot analysis of differences in protein distribution between SNRA patients and HD. The x-axis is the logarithmic transformation with 2 as base of the fold change. The y-axis is the logarithmic transformation with 10 as base of the statistical significance. (E) Volcano plot analysis of differences in protein distribution between SPRA patients and HD. (F) Volcano plot analysis of differences in protein distribution between SNRA patients and SRRA

between SPRA and HD, among which 8 proteins were upregulated and 8 proteins were downregulated (Figure 1B,E). While between SNRA and HD, 63 differentially expressed proteins were determined, 10 of which were upregulated and 53 were downregulated (Figure 1A,D). In addition, compared with SPRA, a total of 14 proteins were differentially expressed, of which 4 were upregulated and 10 were downregulated in the SNRA (Figure 1C,F). Specific information about differentially expressed proteins in different groups is shown in Tables S1–S3, respectively. In addition, the functions of the differentially expressed proteins were determined by gene ontology (GO) enrichment analysis, which including biological processes, cellular components, and molecular functions (Figure 2A–C).

3.3 | Verification of four differentially expression proteins by ELISA

According to the fold change of the differentially expressed proteins, we selected 4 differentially expressed proteins for further assessment. ELISA was performed to analyze differential proteins SAA1,

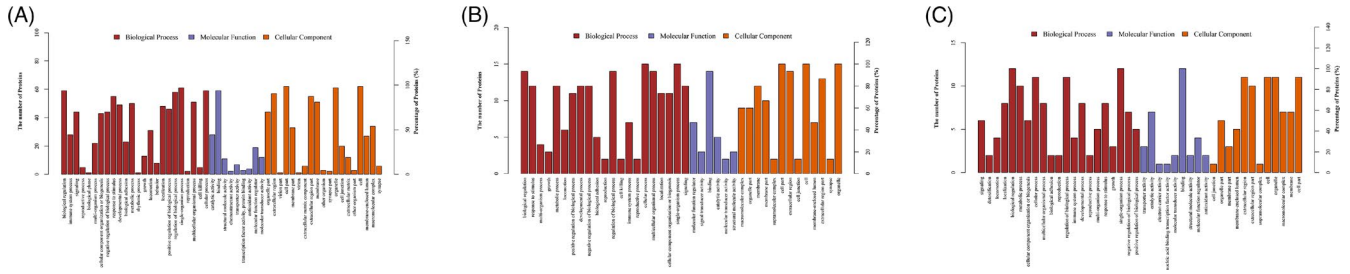


FIGURE 2 Gene ontology (GO) enrichment analysis. (A) GO analysis between SNRA and HD. (B) GO analysis between SPRA and HD. (C) GO analysis between SNRA and SPRA

FIGURE 3 Differential protein expression levels in serum of SPRA patients, SNRA patients, and healthy donors. (A–D) the level of SAA1, RYR3, Tβ4, and PSME1 in serum of SPRA patients, SNRA patients, and healthy donors. SAA1: serum amyloid A1, RYR3: ryanodine receptor 3, Tβ4: thymosin beta 4, PSME1: proteasome activator subunit 1, $^{*}p < 0.05$

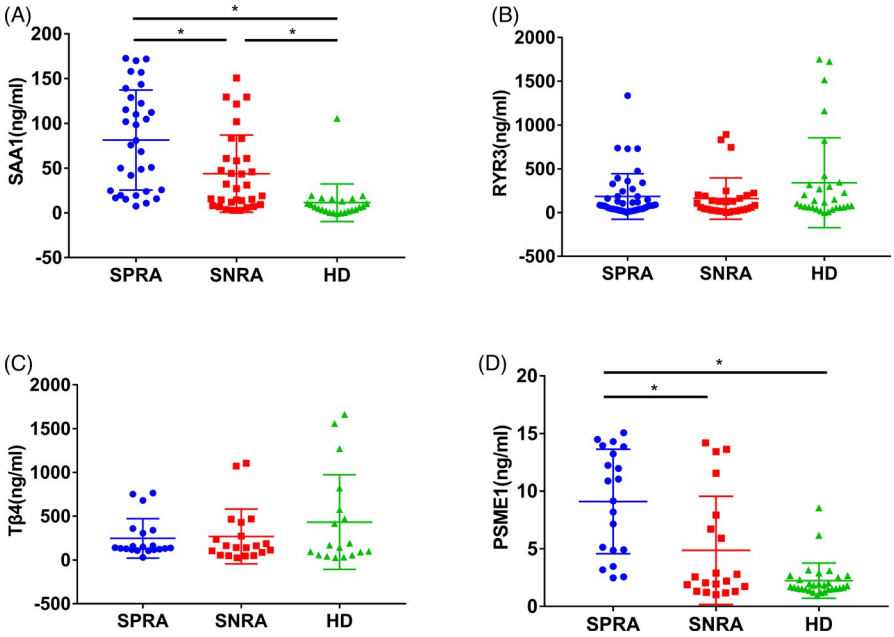
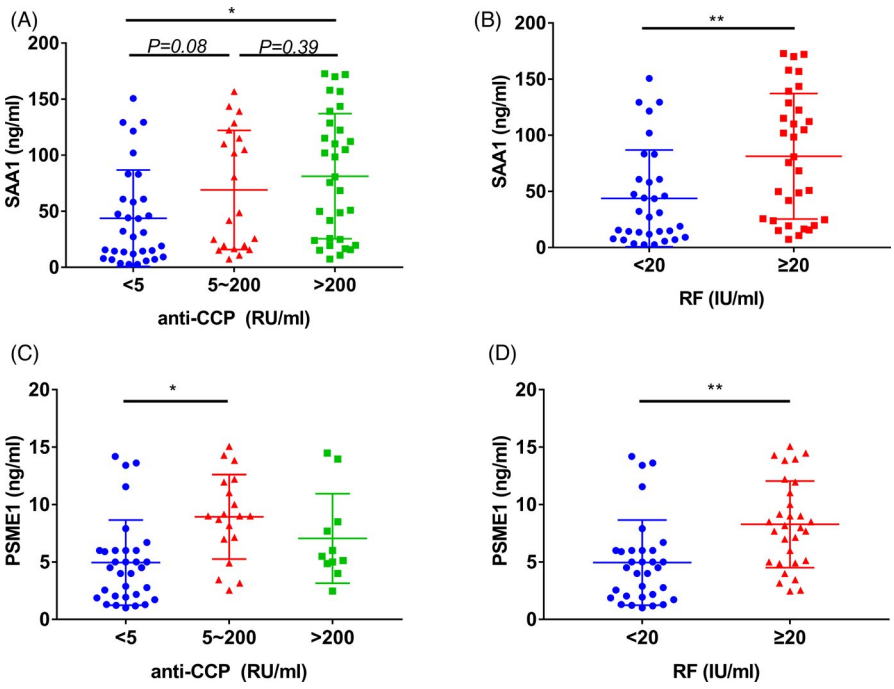


FIGURE 4 Relationship between SAA1, PSME1, and autoantibodies in RA patients. (A) SAA1 in anti-CCP antibodies low-level group (<5 RU/mL), medium-level group (5~200 RU/mL), and high-level group (>200 RU/mL). (B) SAA1 in RF negative (<20 IU/mL) and RF positive (≥20 IU/mL). (C) PSME1 in anti-CCP antibodies low-level group (<5 RU/mL), medium-level group (5~200 RU/mL) and high-level group (>200 RU/mL). (D) PSME1 in RF negative (<20 IU/mL) and RF positive (≥20 IU/mL). SAA1: serum amyloid A1, PSME1: proteasome activator subunit 1, $^{*}p < 0.05$, $^{**}p < 0.01$



RYR3, Tβ4, and PSME1 (Figure 3A–D). We found that the expression of SAA1 protein in SPRA patients was significantly higher than that of SNRA patients and healthy donors, and SAA1 in SNRA patients was significantly higher than it in healthy donors (Figure 3A). In addition, the expression of PSME1 protein in SPRA patients was significantly higher than that of SNRA patients and healthy donors, while there was no statistical difference between SNRA patients and healthy donors (Figure 3D).

3.4 | The expression levels of SAA1 and PSME1 in groups of different levels of autoantibodies

According to the level of anti-CCP antibodies, RA patients were divided into three groups: low-level group (<5 RU/mL), medium-level group (5~200 RU/mL), and high-level group (>200 RU/mL). The results, as shown in Figure 4A, indicated that the level of SAA1 was significantly higher in the high-level anti-CCP group than in the low-level anti-CCP group, while no difference was found between the other groups. In addition, the level of PSME1 was significantly lower in low-level anti-CCP group than in the medium-level group (Figure 4C). Then according to the level of RF, we separated the RA patients into two groups based on the normal reference interval in the local laboratory: RF negative (<20 IU/mL) and RF positive (≥20 IU/mL). The results showed that the level of SAA1 and PSME1 was significantly higher in the high-level RF group than in the low-level RF group (Figure 4B,D), and the result of SAA1 was consistent with the anti-CCP.

3.5 | SAA1 is positively correlated with inflammation markers in RA patients

We further explored the correlation between SAA1 and inflammation markers. The results revealed that SAA1 was positively correlated with inflammation indicators CRP and ESR in SPRA patients and SNRA patients (Figure 5A–D), while SAA1 had no correlation with DAS28 in SPRA patients ($p = 0.060$) and SNRA patients ($p = 0.054$, Figure 5I,J). We also analyzed the correlation between PSME1 and inflammation markers, and no correlations were found between PSME1 and CRP or ESR (Figure 5E–H). As shown in Figure 5K,L, there was no correlation between DAS28 and PSME1 in SPRA patients ($p = 0.268$) and SNRA patients ($p = 0.474$).

3.6 | There is no correlation between differential proteins and evolution time of the disease

Finally, we explored the correlation between the differential proteins SAA1, PSME1, and the evolution time of the disease, and the analysis revealed no significant correlations (Figure 6A–D).

4 | DISCUSSIONS

Proteomics is an emerging discipline in biomedical research, which was born at the end of the 20th century. This is a powerful technique for large-scale protein analysis, identification, and quantitative of proteins. Almost all human life activities are regulated by the coordinated action of many proteins. In general, there is no one-to-one relation between gene expression level and protein expression level. Proteomic study can circumvent many invalid information at the genome and transcriptome levels. Therefore, proteomics has become an emerging research hotspot.[8,9]

According to the purpose of the experiment, we can divide proteomics technology into two categories, high-throughput proteomics and targeted proteomics. High-throughput proteomics is used for early differential protein screening, while targeted proteomics is used for subsequent target protein verification. The iTRAQ technology is a high-throughput screening technology used in proteomics. The iTRAQ is a peptide in vitro labeling technique based on stable isotope labeling, which specifically labels the amino group of the polypeptide and then performs tandem mass spectrometry analysis.[10,11] iTRAQ technology can analyze differential proteins of 8 samples in one experiment.

In order to invest the differentially expressed proteins among SPRA patients, SNRA patients, and HD, the quantitative proteomic technique iTRAQ was performed and validated by ELISA assay. The results showed that SAA1 and PSME1 were elevated in the sera of SPRA patients. PSME1 is a multi-catalytic protease complex that implicated in immunoproteasome assembly and is required for efficient antigen processing.[12] Studies have shown that PSME1 is related to the diagnosis or prognosis of many diseases, such as breast cancer, ovarian cancer, and skin cutaneous melanoma.[13] PSME1 is expressed in many different cell types, especially antigen-presenting cells. However, no difference in PSME1 between SNRA patients and HD was found, and no studies have demonstrated the role of PSME1 in the pathogenesis of RA.

Therefore, we chose SAA1 protein for subsequent experiments. SAA1 is an acute-phase protein. It is mainly produced by liver cells, but it can also be released by other cell types, including immune cells, endothelial cells, synovial cells, and epidermal keratinocytes.[14,15] Several studies have shown that SAA1 expression has been demonstrated to change in many diseases such as rheumatoid arthritis, diabetes, atherosclerosis, and Alzheimer's disease.[16–18]

Some experiments have identified that SAA1 can be used as a biomarker for disease activity of RA.[19,20] Other researches have confirmed that SAA1 can bind to RAGE on the surface of synovial cells followed by activating NF-κB signaling and promoting the progress of joint inflammation.[21] SAA1 produced in the synovial tissue of RA patients is critical in cell growth, invasion, migration, angiogenesis as well as the secretion of chemokines and metal matrix proteases.[19] We wondered whether there is any relationship between SAA1 and indicators, such as diagnostic

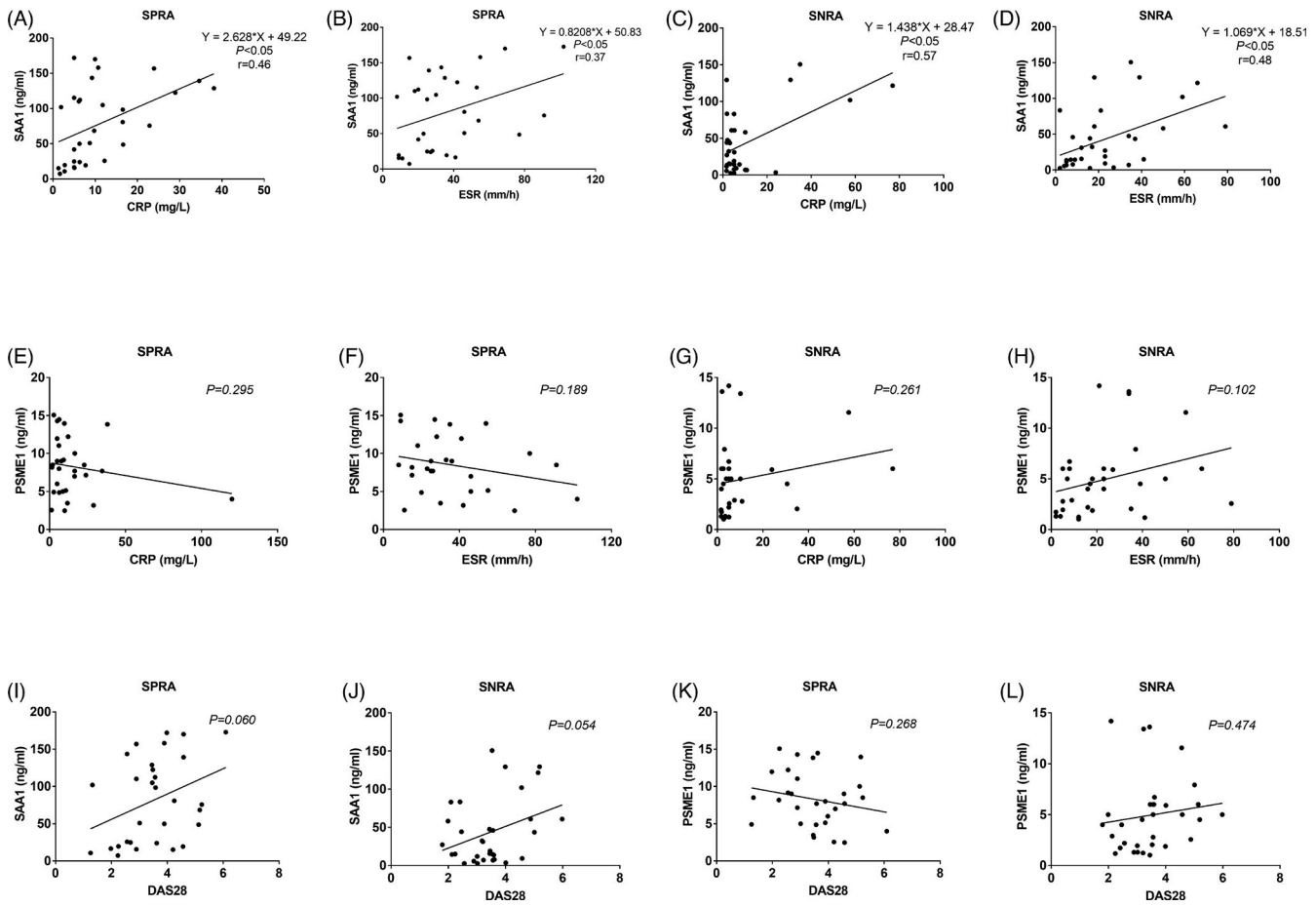


FIGURE 5 Correlation between SAA1, PSME1, and inflammation markers CRP and ESR in RA patients. (A–D) Correlation between SAA1 and inflammation markers in different RA patients. (E–H) Correlation between PSME1 and inflammation markers in different RA patients. (I and J) Correlation between SAA1 and DAS28 in different RA patients. (K and L) Correlation between PSME1 and DAS28 in different RA patients. SAA1: serum amyloid A1, PSME1: proteasome activator subunit 1, DAS28: disease activity score assessing 28 joints, SPRA: seropositive rheumatoid arthritis, SNRA: seronegative rheumatoid arthritis

indicators and inflammatory factors. Therefore, we first calculated the correlation between SAA1 and autoantibodies, such as anti-CCP and RF. The results demonstrated that the subset of RA patients with high basal levels of autoantibodies expressed significantly higher level of SAA1 protein compared with the subset with low basal level of autoantibodies. Then, we further explored the correlation between SAA1 and inflammation markers, such as CRP and ESR. The results illustrated that there were significant positive correlations between SAA1 and inflammation markers, indicating that SAA1 has the potential to become a diagnostic indicator and inflammatory marker in RA patients, especially in SNRA patients.

Compared with SPRA, 14 differentially expressed proteins were identified in the SNRA. GO analysis was further used to analyze the functions of the differentially expressed proteins, which included biological processes, cellular components, and molecular functions. To explore the function of the differentially expressed proteins, we performed a GO enrichment analysis. The 30 most enriched GO terms are presented in Figure S1. For interpretation of our data, we chose GO terms with $p < 0.01$. The result showed that primary lysosome (GO:0005766), azurophil granule (GO:0042582), hydrogen ion transmembrane transporter activity (GO:0015078), and hydrogen ion transmembrane transport (GO:1902600) were found to be upregulated in SNRA, and response to stress (GO:0006950) and positive regulation of biological process (GO:0048518) were downregulated in SNRA. At present, the response to stress pathway in RA patients is still inconclusive. Studies have found that RA patients have a different immune response to stress than patients with psoriasis or healthy controls.[22] Other studies have shown that the cortisol response to stress was heightened in patients with psoriasis compared with RA patients and healthy controls.[23] Thus, the functions of response to stress pathway in initiation and progression of RA need to be elucidated in the future study.

However, our study has some limitations. Although the iTRAQ technology has powerful protein qualitative ability, its sensitivity needs to be improved in protein quantification. In addition, our research is a retrospective cross-sectional study. The results show that

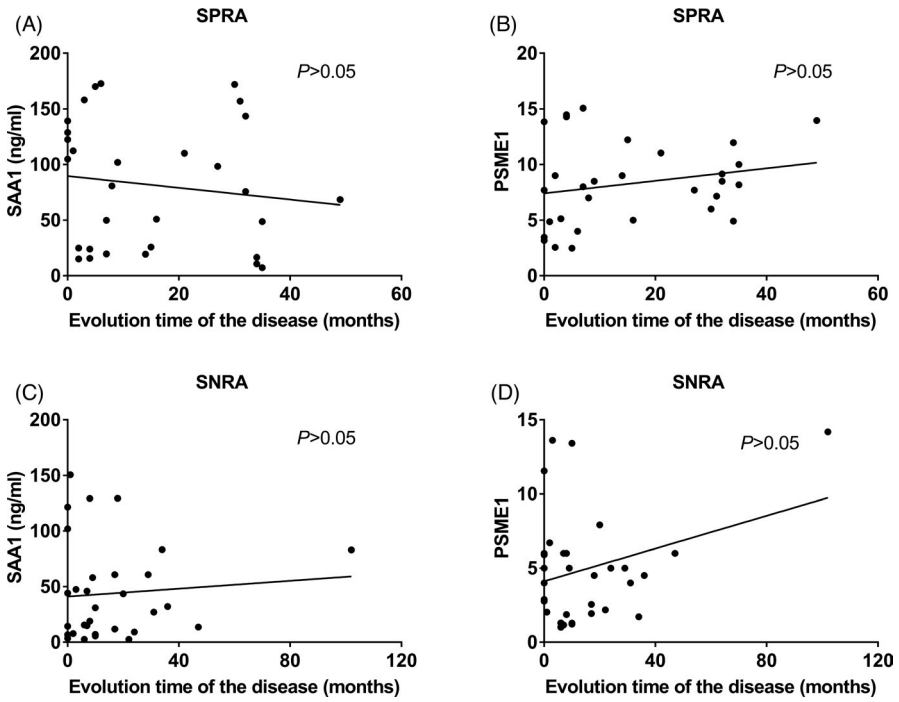


FIGURE 6 Correlation between SAA1, PSME1, and evolution time of the disease. (A and B) Correlation between SAA1, PSME1, and evolution time of SPRA. (C and D) Correlation between SAA1, PSME1, and evolution time of SNRA

SAA1 is positively correlated with the level of inflammation indicators and autoantibodies in RA patients, and future longitudinal research is needed to further corroborate whether SAA1 can predict the appearance of autoantibodies and whether it can be used as a diagnostic indicator for RA.

5 | CONCLUSIONS

Our results revealed that there was a difference among SPRA patients, SNRA patients, and HD in protein level which were found using the iTRAQ proteomic analysis. Furthermore, ELISAs confirmed that SAA1 and PSME1 were elevated in the serum of SNRA and SPRA patients. Based on our research results, the differentially expressed proteins may be useful for the development of advanced diagnostic methods and precision treatment for RA.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

Yujue He and Junyu Lin participated in the design of the study and drafted the manuscript. Ziqing Yu and Jifeng Tang performed the statistical analyses. Qishui Ou and Jinpiao Lin participated in the design of the study and revised the manuscript. All authors read and approved the final manuscript. We thank all the RA patients and healthy donors for kindly cooperation in this study. We also thank Qiang Fu and Jingyi Lu for technical assistance.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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