Screening and Verification of Key Proteins in Patients with Rheumatoid Arthritis and Intervention of Traditional Chinese Medicine

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Abstract

Background: The aim of this study was to simultaneously and quantitatively assess the expression levels of rheumatoid arthritis (RA) proteins in serum, and explore the intervention mechanism of traditional Chinese medicine.

Methods: The differences between patients and controls were investigated by Raybiotech cytokine antibody microarray. Differentially expressed proteins between two groups were then used to build receiver operator characteristic (ROC) curves. The subsequent validation with enzyme-linked immunosorbent assay (ELISA) was widened to a larger number of RA (n=40) and healthy controls (n=10). Changes of erythrocyte sedimentation rate (ESR), high-sensitivity C-reactive protein (hs-CRP), rheumatoid factors (RF) and anti-citrullinated peptide autoantibodies (anti-CCP) in patients were statistically analyzed.

Results: Six proteins interleukin-2 (IL-2), interleukin-5 (IL-5), interleukin-11 (IL-11), interleukin-17 (IL-17), tumor necrosis factor- β (TNF- β), cytotoxic T lymphocyte associated antigen (CTLA)-4 were significantly up-regulated in RA compared with controls, while interleukin-8 (IL-8), programmed death-ligand 2 (PD-L2) and B7-2 were significantly down-regulated. ELISA was performed to verify the three proteins with the most significant differences, and the results consistent with the antibody chip results. The highest diagnostic accuracy using a ROC curve was observed for IL-11 with an area under the curve of 1.00. Further, after treatment, IL-11, IL-17 and PD-L2

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levels regulated, and compared with the herbs group (TC), xinfeng capsule (XFC) combination with herbs (UC) more regulated. The association rule results showed that the combination of XFC with herbs significantly improved IL-11, IL-17, PD-L2 and hs-CRP.

Conclusions: This study highlights the potential of antibody arrays to diagnose rheumatoid arthritis. IL-11, IL-17 and PD-L2 regulated by XFC combined with herbs.

Keywords: Rheumatoid Arthritis; Proteomics; Immune; Inflammation

Background

Rheumatoid arthritis (RA) is a chronic autoimmune disease with symmetrical polyarthritis as the main clinical manifestation, with a global prevalence of about 1% [1]. Its basic pathological changes are synovitis, pannus formation and the resulting destruction of cartilage and bone, resulting in joint deformity and loss of function [2]. The emerging area of proteomics holds promise for biomarker discovery in complex diseases. Thus far proteomic methodologies have been applied to the study the biology of RA to only a very limited extent [3].

RF has a prevalence of up to 80 % in RA patients and 15 % in healthy individuals [4, 5], thus there is a tremendous need for novel sensitive assays that can assess and eliminate potential RF interference while having a broad detection range. Antibody arrays from Raybiotech have been widely used in biological researches to identify secret molecules in culture medium or body fluid, which can elucidate novel biomarkers of the disease [6]. Antibody array is a technique of profiling multiple protein analytes simultaneously in samples or tissues [7]. Different from quantitative methods, antibody arrays generate signal intensities that allow assessment of relative concentrations of analytes among samples. Findings of significant relative differences can be assessed for quantitative validation by other methods.

Methotrexate and TNF blockers are two of the most commonly used agents [8]. Western medicine has many side effects, which many patients cannot tolerate. Chinese medicine has many advantages in improving RA. Xinfeng capsule (XFC) significantly the curative effect of treatment of patients with RA, effectively improve

the patient's symptoms, reduce inflammation, and improved function of the lungs. Therefore, XFC could improve the whole body function and enhance the quality of life of RA patients [9].

In this study, antibody arrays were performed to screen candidate biomarkers by comparing the expression variation of 50 cytokine in serum from 10 RA patients and 10 controls. Larger samples validation by ELISA in 40 patients. To identify potential markers of RA and explore the role of XFC on the screened cytokines, will allow for new directions of possible RA diagnosis, treatment and/or prevention.

Materials and methods

Subjects

Ten (10) RA patients were selected from the Department of Rheumatology and Immunology, the First Affiliated Hospital of Anhui University of Traditional Chinese Medicine between August 2019 and December 2019 (RA group), while 10 normal controls were identified as healthy by the physical examination center of our hospital (Control group). All RA patients fulfilled the 2010 ACR/EULAR (American College of Rheumatology/European League Against Rheumatism) criteria for the classification of RA[10]. Excluded the patients who combination with other rheumatic diseases, and with serious organic diseases, pregnant and lactating women. This study was approved by the ethics committee of our hospital, and all patients provided written informed consent. There was no significant difference in age and sex between groups.

Collection of serum sample

The whole blood of patients and normal people were collected from 5ml, placed at room temperature for 30-45 min, centrifuged at 3000-5000 RPM/min for 10 min, and serum was collected and stored at -80°C until use.

Screening of differential proteins

A total of 20 serum samples from 10 RA patients and 10 controls, were subjected to Raybiotech antibody array with 50 cytokines proteins for the differential proteins screening.

Validation with large samples by ELISA

According to the above criteria, 40 RA patients were selected for ELISA verification. All serum samples and kit components were equilibrated to room temperature before the assay. The detection procedure was in accordance with the manufacturer's instructions.

Drugs

This was a randomized controlled clinical trial of 40 RA patients. Participants were assigned to the UC group (n=24) and the TC group (n=16) by block randomization. Both groups were treated with herbs. And the UC group was treated with XFC (batch number: 20150625, Drug-making Center, First Affiliated Hospital of Anhui University of Chinese Medicine, China), XFC consists of Radix Astragali (Huangqi), Caulis Tripterygi Wilfordii (Leigongteng), Semen Coicis (Yiyiren), and Scolopendra (Wugong). Each capsule contained 0.4g.

Bioinformatics analysis

Principal component analysis (PCA) and gene ontology (GO) analyses identified differentially expressed proteins and their functions, respectively. Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis then identified significantly enriched functions and pathways with p-values<0.05. Hierarchical clustering analysis grouped samples and proteins based on the differential protein expression patterns. PCA (R package “ggbiplot”), Hierarchical clustering analysis (R package “gplots”), GO analysis (R package “clusterProfiler”) and KEGG enrichment analysis (R package “clusterProfiler”) were performed using the open source program R (version 3.5.1).

Apriori algorithm

All the data were processed by R-Studio Version 3.5.1. we used the apriori algorithm to analyze the association rules of the herbs, differential proteins, laboratory indexes and XFC. The apriori algorithm is a frequent itemset algorithm for mining association rules. We used it to illustrate the specific rules of TCM and XFC in RA treatment. In our data, each herb and XFC was treated as a variable. The formulae were as follows:

$$\text{support}(X \rightarrow Y) = \sigma \frac{(XUY)}{N},$$

$$\text{confidence}(X \rightarrow Y) = \frac{\sigma(X \cup Y)}{\sigma(X)},$$

$$\text{lift}(X \rightarrow Y) = \frac{\text{confidence}(X \rightarrow Y)}{\sigma(Y)}$$

where $X \rightarrow Y$ is an association rule, X (Consequent) and Y (Antecedent) represent the set of XFC and herb items, $\sigma(X)$ is the frequency of itemset X , $X \cup Y$ is the union of itemset X and Y , $\sigma(X \cup Y)$ is the frequency with which itemset X and itemset Y appear together, $\text{support}(X \rightarrow Y)$ is the frequency with which X and Y appear together, and $\text{confidence}(X \rightarrow Y)$ is the probability that itemset Y appears in the presence of X . The lift is the ratio of the probability of itemset Y appearing in the presence of X to the frequency of Y . Support and confidence are often used to eliminate meaningless combinations; lift is the validity of the rules [11].

Statistical analysis

ROC curves were constructed to evaluate the diagnostic value of proteins for RA patients using the R package “pROC”. The differences in levels of proteins between RA patients and normal controls were analyzed using the Wilcoxon Rank Sum test. The clinical characteristics of the two groups were analyzed using the unpaired Student’s t-test. All statistical data were analyzed using R software (version 3.5.1) and GraphPad Prism software (version 8.0). In all cases, p -values < 0.05 were considered as statistically significant.

Results

Sample general

There were seven males and seventeen females in the UC group, with an average age of 65.88 ± 11.77 years, and four males and twelve females in the TC group, with an average age of 60.63 ± 11.70 years. There was no significant difference in age and sex between groups.

Identification of differential proteins of RA

The Raybiotech antibody array measured the concentration of 50 proteins in the serum from 10 RA patients and 10 controls. Twenty-four proteins were up-regulated, nine were down-regulated, and the rest were indistinguishable. A volcano plot depicting the proteins’ expression levels shows that six proteins were significantly

up-regulated in the RA patient group, including IL-2、IL-5、IL-11、IL-17、TNF- β and CTLA-4, while IL-8 、PD-L2 、B7-2 was significantly down-regulated ($\log_{2}FC > \log_{2}(1.2)$, $p\text{-value} < 0.05$) (Figures 1A and 1B, and Figure 2). A hierarchical clustering algorithm was used to cluster samples and differentially expressed proteins based on protein expression patterns (Figure 1C). The samples were divided into three main clusters. More specifically, the below cluster in Figure 1C contained seven patients, while the above cluster was comprised of both three patients and two controls. Although preliminary, these data suggest that the RA and controls groups can be distinguished from each other using antibody arrays and unsupervised clustering. The middle cluster was comprised of only controls. Principal component analysis (PCA) identified proteins that were differentially expressed across the two groups. A plot of the first two principal components (PC1 and PC2) for each sample shows a total variability of 52.81% and 22.65%, respectively (Figure 1D), thus implying that the protein expression patterns of SP patients and NOR are indeed different.

Figure 1. Protein expression profiles in RA and Control were detected by chip.(A) Volcano map of differentially expressed protein: the vertical line corresponds to two times above and below, and the horizontal line indicates that the p value is 0.05, while TRUSE indicates that the difference is significant.(B) Scatter plots of differentially expressed proteins: red: expression up-regulated; blue: expression down.(C) Hierarchical cluster analysis of significantly different protein expression in serum. Each row represents a sample and each column represents a protein. (D) PCA mapping of the 10 RA patients and 10 Control samples.

Figure 2. Nine differentially expressed proteins in serum. Control: normal controls, RA: RA patients.

GO and KEGG enrichment analyses were performed to investigate the enriched functions and pathways of differentially expressed proteins associated with RA patients. GO enrichment analysis revealed that 292 significant functional terms were involved in the category of “biological process” (BP) function, 20 in the category of “molecular function” (MF) and 2 in the category of “cellular component” (CC) (Figures 3A, 3B and 3C). KEGG enrichment analysis revealed that differentially expressed proteins were significantly enriched in 22 pathways (Figure 3D). These enriched functional terms and signaling pathways may reflect rheumatoid arthritis disease pathogenesis.

Figure 3. Bioinformatics analysis of different protein expression levels in RA patients and Control detected by microarray. (A-C) GO enrichment analysis of significantly different protein expression regarding (A) biological process, (B) molecular function, and (C) cellular component. (D) KEGG enrichment analysis of significantly enriched pathways in differentially expressed proteins.

ROC curves were constructed for the 9 differentially expressed proteins in serum to determine their sensitivity and specificity for diagnosing RA. ROC curve analyses revealed that, the proteins distinguished RA patients from Control with an area under the curve (AUC) > 0.70 (Figure 4). IL-11 and IL-17 and PD-L2 had the highest AUC at 1.000 and 0.830, respectively. Therefore, these proteins could be used as potential biomarkers for RA patients.

Figure 4. ROC curve analysis of differentially expressed proteins between RA patients and normal controls.

ELISA was used to verify the results

For further validation of antibody screening result of chip, selecting the most significant difference expression of three proteins IL-11, IL-17, PD-L2 verified

ELISA, tested the 10 cases of normal people and 40 cases of RA patients before and after treatment the serum samples of IL-11, IL-17, PD-L2, found its expression in line with the results of antibody microarrays, compared with normal group, IL -11 in patients with RA, IL - 17 high expression, PD-L2 lower expression ($P<0.05$), and the expression of the three proteins was significantly improved after treatment, in addition, after treatment, IL-11 and IL-17 significantly decreased, while PD-L2 significantly increased as shown in Figure 5.

Figure 5. The expression levels of differentially expressed proteins (A, B) in serum (IL-11, IL-17) were detected by ELISA with the same increasing trend as those detected by microarray. (C) PD-L2 and chip detection results showed the same downward trend. NC: normal controls; before: the level before treatment; after: the level after treatment.

*Compared to normal people, $p<0.05$; #Compared to before treatment, $p<0.05$.

Changes of proteins and laboratory indexes

The results showed that IL-11, IL-17, PD-L2, hs-CRP and ESR of the two groups

were significantly improved ($p<0.05$). By comparing the difference of the improvement of indicators between the two groups (positive PD-L2), it was found that UC group was significantly better than TC group in improving IL-11, IL-17, PD-L2, hs-CRP and ESR, and the difference was statistically significant ($p<0.05$).

Figure 6. Changes of different proteins and laboratory indexes in RA patients before and after treatment.* Compared with the difference before and after treatment between the two groups, $p<0.05$; # $P<0.05$, TC group compared with the difference before and after treatment. ^ $P<0.05$, UC group compared with the difference before and after treatment.

Analysis of association rules between the improvement of patient differential

proteins and laboratory index and traditional Chinese medicine

We used the apriori algorithm to analyze the association rules of the herbs and XFC in the treatment of RA. Set the antecedent item as 1, namely XFC, and the consequent item as the proteins and laboratory index. The use of XFC or a herbs is T, otherwise is F. After the treatment, the difference between different proteins and laboratory indexes was normalized. The difference greater than 0.5 was set as T, while the difference was set as F. We focused on two parameters: support and confidence level. Support was set as $\geq 50\%$ and confidence level as $\geq 90\%$ [12] and a total of three results and suitable association rules were obtained. The detailed association rules are shown in Table 1, the support degree of the three results was the same at 58.54%, but the confidence degree of XFC improved IL-11 was the highest at 100%, and the lift degree was the highest at 1.23.

Set the antecedent item as 2, XFC combined with herbs. Support was set as $\geq 30\%$ and confidence level as $\geq 75\%$ and a total of 23 results and suitable association rules were obtained. The detailed association rules are shown in Table 2, XFC and Safflower (Honghua) improved PD-L2 had the highest degree of support at 48.78%, most results have the highest confidence at 100%, and three results showed the lift degree was the highest at 1.28.

Table 1. Association rules of XFC for proteins and laboratory indexes improvement

Antecedent	Consequent	Support (%)	Confidence (%)	Lift
XFC	IL-11 = T	58.54	100.00	1.23
XFC	IL-17=T	58.54	95.83	1.11
XFC	PD-L2 = T	58.54	91.67	1.07

Table 2. Association rules of XFC and herbs for proteins and laboratory indexes improvement

Antecedent	Consequent	Support (%)	Confidence (%)	Lift
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XFC & Honeysuckle stem(Rendongteng)	IL-11 = T	41.46	100.00	1.28
XFC & Poria(Fulin)	IL-11 = T	41.46	100.00	1.28
XFC & Dandelion(Pugongying)	IL-11 = T	41.46	100.00	1.28
XFC & Salvia miltiorrhiza(Danshen)	IL-11 = T	43.90	94.44	1.21
XFC & Poria(Fulin)	IL-17 = T	41.46	94.12	1.10
XFC & The dandelion(Pugongying)	IL-17 = T	41.46	100.00	1.17
XFC & Poria(Fulin)	PD-L2 = T	41.46	100.00	1.11
XFC & Dandelion(Pugongying)	PD-L2 = T	41.46	100.00	1.11
XFC & Salvia miltiorrhiza(Danshen)	PD-L2 = T	43.90	100.00	1.11
XFC & Herba Siegesbeckiae(Xixiancao)	PD-L2 = T	43.90	100.00	1.11
XFC & Honeysuckle stem(Rendongteng)	PD-L2 = T	41.46	100.00	1.11
XFC & Salvia miltiorrhiza(Danshen)	PD-L2 = T	43.90	100.00	1.11
XFC & Safflower(Honghua)	PD-L2 = T	48.78	100.00	1.11
XFC & Dandelion(Pugongying)	hs-CRP = T	41.46	76.47	1.16
XFC & Pericarpium cirri reticulate(Chenpi)	hs-CRP = T	46.34	76.68	1.12
XFC & Honeysuckle stem(Rendongteng)	hs-CRP = T	41.46	76.47	1.16
XFC & Salvia miltiorrhiza(Danshen)	anti-CCP = T	43.90	77.78	1.10
XFC & Poria(Fulin)	anti-CCP = T	41.46	82.35	1.16
XFC & Dandelion(Pugongying)	anti-CCP = T	41.46	82.35	1.16
XFC & Pericarpium cirri reticulate(Chenpi)	anti-CCP = T	46.34	78.95	1.12

XFC & Safflower(Honghua)	anti-CCP = T	48.78	75.00	1.06
XFC & Herba Siegesbeckiae(Xixiancao)	anti-CCP = T	43.90	83.33	1.18
XFC & Honeysuckle stem(Rendongteng)	anti-CCP = T	41.46	82.35	1.16

Discussion

RA is an inflammatory autoimmune disease with unclear pathogenesis. Chronic, symmetrical and extra-articular diseases are the main clinical manifestations, which are autoimmune diseases. The pathogenesis of RA is still unclear, and cellular immune inflammatory response and other factors affect the occurrence and development of RA. Evidence suggests that the activity and the course of RA strongly correlate with the cytokines. In addition, the role of helper T cells, such as helper T cell 1 (Th1), Th17 and regulatory T cell dysfunction, in the pathogenesis of RA has been widely recognized and was considered as the central link of RA [13, 14].

In the present work, we applied Raybiotech antibody chip and ELISA to observe different proteins and index changes during the course and treatment of RA. Compared with controls, the RA patients in this study showed significantly higher levels of IL-11 and IL-17, but lower PD-L2.

As a new member of the IL-6 family of cytokines, IL-11 played an important role in promoting the proliferation and differentiation of platelets, regulating immune response, regulating bone metabolism, and inducing the synthesis of acute reactive protein, and was related to the inflammatory state of various tissues. Current studies have shown that IL-11 was highly expressed in synovial tissues, joint fluid and serum of RA patients [15].

Th17 was one of the most important inflammatory factors in vivo, and the degree of infiltration was positively correlated with the severity of RA, while IL-17 was the main secretory factor of Th17 and the key protein for the function of Th17 [16]. IL-17 enhanced osteoclast differentiation and functional activity in bone destruction by up-regulating synovial osteoclast differentiation factor, and aggravated bone

destruction [17]. IL-17 can produce inflammatory cytokines, proteases and adhesion molecules, activate secondary neutrophils and macrophages, and at the same time weaken inflammatory cytokines in the process of RA inflammation, and participate in RA inflammation and cartilage destruction through a variety of mechanisms [18]. High levels of IL-17 can be detected in both peripheral blood and synovial fluid of RA patients [19].

Activation of T cells requires signal stimulation, CTLA-4 was a natural costimulatory signal regulatory molecule, and the B7 family was the only costimulatory molecule capable of one-way signal transmission from antigen presenting cells to T cells [20]. The important regulatory role of CTLA-4 in T cell activation, and the immune regulatory role of CTLA-4 in T-reg cells is also necessary [21]. The study showed that CTLA-4 regulated the whole course of disease, included the chronic stage of RA. CTLA-4 expressed on the surface of traditional T cells and Treg cells was very important for the body. The former controlled the initiation of naive self-reactive T cells, while the latter prevented the attack of inflammatory tissues [22]. In addition, PD-L2, a member of the B7 family, was not only distributed in dendritic cells and macrophages, but also expressed on activated T cells [23]. Interestingly, there was researched evidence that PD-L2 was low expressed in patients with rheumatoid arthritis, while our results showed opposite result, which may be related to the small sample number and serum sample of the test in this study [24].

TNF- β is a pro-inflammatory factor, and although it is still controversial whether the TNF gene is associated with RA susceptibility, its phenotype has been confirmed to play an important role in the pathogenesis and prognosis of RA [25]. TNF- β levels in the blood and synovial fluid of RA patients increased [26], consistent with the study results.

In this study, after treatment, the differential proteins IL-11, IL-17 and PD-L2, as well as the laboratory indicators ESR and hs-CRP, which were acute proteins that rise sharply in plasma when the body was infected or tissue damaged, were significantly improved in the UC group compared with the TC group [27]. Hs-CRP and ESR were commonly used in the laboratory to diagnose the activity of rheumatic diseases, and

can be used as indicators to monitor the activity of diseases and evaluate the treatment stability. Association rules show that compared with single use Chinese traditional medicine group, XFC combined traditional Chinese medicine on IL-11, IL-17, PD-L2, hs-CRP, anti-CCP have obvious correlation, showed that after the combined traditional Chinese medicine can significantly improve the indicators, and the mechanism may be related to the pharmacological effects of XFC, semen coicis extract exerts anti-RA effects via inhibiting pro-inflammatory factors and alleviating oxidative stress [28]; The active components of radix astragali can strengthened humoral and cellular immunity and improved the regulation of human immunity [29]. *Tripterygium wilfordii hook.f.* was anti-inflammatory and regulated a variety of immune factors [30].

Conclusions

Raybiotech antibody chip was used to detect the expression of immune inflammation-related proteins in the serum of RA patients to further clarify the pathogenesis of RA and find new diagnostic markers. It is true that RA patients have an imbalance in the expression of key proteins of immune inflammation. IL-11 and IL-17 are expressed up-regulated, while PD-L2 is down-regulated. The combination of XFC with traditional Chinese medicine can significantly improve the laboratory indexes of hs-CRP and anti-CCP immune inflammation in RA patients, which may be related to the regulation of the expression of IL-11, IL-17 and PD-L2, and the mechanism remains to be further explored.

Abbreviations

RA: rheumatoid arthritis; ROC: receiver operator characteristic; ELISA: enzyme-linked immunosorbent assay; ESR: erythrocyte sedimentation rate; hs-CRP: high-sensitivity C-reactive protein; RF: rheumatoid factors; anti-CCP: anti-citrullinated peptide autoantibodies; IL-2: interleukin-2; IL-5: interleukin-5; IL-11: interleukin-11; IL-17: interleukin-17; TNF- β : tumor necrosis factor- β ; CTLA-4: cytotoxic T lymphocyte associated antigen-4; IL-8: interleukin-8; PD-L2: programmed death-ligand 2 ; XFC: xinfeng capsule ; TC: herbs group; UC: XFC combination with

herbs; PCA: Principal component analysis; GO: gene ontology; KEGG: kyoto encyclopedia of genes and genomes; BP: biological process; MF: molecular function; CC: cellular component; Th1: helper T cell 1; Th17: helper T cell 17.

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Authors' contributions

All authors reviewed the final version of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All donors gave written informed consent for inclusion in the study and ethical approval was obtained from the following ethics committee: First Affiliated Hospital of Anhui University of Traditional Chinese Medicine (Study No. 2019AH-12).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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