Serum disease-associated proteins of ankylosing spondylitis: results of a preliminary study by comparative proteomics

T. Li[1], Z. Huang[1], B. Zheng[1], Z. Liao[1], L. Zhao[1,2], J. Gu[1]

[1]Department of Rheumatology, Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou, China; [2]Department of Rheumatology, Beijing Hospital, Beijing, China.

Abstract

Objective

Ankylosing spondylitis (AS) is difficult to diagnose at the early stage since it lacks simple and specific diagnostic indicators. Proteomics is a new enabling technology to screen disease-associated proteins which can be used in diagnostics or therapeutics. This study was performed by using two-dimensional electrophoresis (2-DE) in combination with mass spectrometry to search for disease-associated proteins in the sera of AS patients.

Methods

Sera from 6 AS patients and 6 healthy volunteers (HVs) were mixed respectively and high-abundance proteins were depleted by Plasma 7 Multiple Affinity Removal System. The protein expression profiles of the sera between the two groups were compared by 2-DE and proteins over/under-expressed in the AS group were identified by mass spectrometry. Two of the identified proteins were then chosen to be verified by using ELISA in the individual sera of 32 AS patients and 32 HVs.

Results

Serum amyloid A, apolipoprotein A (ApoA)-IV, ApoA-IV precursor, haptoglobin 2, ceruloplasmin (Cp) and immunoglobulin superfamily 22 were over-expressed by more than 3-fold in the sera of AS patients compared with HVs. Plasma glutathione peroxidase, similar to complement component 3 and chain A of transthyretin (TTR) were under-expressed in the sera of the AS patients. ELISA experiments showed the same trends in the expression levels of Cp and TTR with 2-DE.

Conclusion

There were 9 differentially expressed proteins in the sera of the AS group compared with the HV group, which might be candidate AS-associated proteins and might be promising diagnostic indicators or therapeutic targets for AS.

Key words

Ankylosing spondylitis, serum, proteomics, two-dimensional electrophoresis, disease-associated protein.

Tianwang Li, MD, PhD
Zhixiang Huang, MD
Benrong Zheng, MD
Zetao Liao, MD
Like Zhao, MD, PhD
Jieruo Gu, MD

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Please address correspondence and reprints requests to:
Jieruo Gu, MD,
Department of Rheumatology,
Third Affiliated Hospital of
Sun Yat-Sen University,
600 Tianhe Road,
Guangzhou, 510630, China.
E-mail: gujieruo@163.com

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Introduction

Ankylosing spondylitis (AS) is a chronic inflammatory rheumatic disease which mainly affects the spine and sacroiliac joint, and sometimes it also involves the eyes, heart, lung and skin. It is a severe disabling disease which will eventually cause fusion of the spine, a condition known as "bamboo spine". Up to now, the most widely used criterion to diagnose AS is the modified New York criteria developed in 1984. Radiographic sacroiliitis is essential to establish the diagnosis of AS according to this criteria. But definite sacroiliitis showed by plain radiography always appears at relatively late stages of AS and there may not be any abnormality in radiographic examination of the sacroiliac joint when AS is at the very early stage. This is one of the important reasons for the long diagnostic delay of 5 to 10 years in AS (1). Recently MRI has been used to detect early sacroiliitis in AS patients (2), but it might be worthless when the sacroiliac joint has not been involved. Furthermore, the high cost of MRI examination often prevents its widespread application. In addition, although HLA-B27 has been confirmed to be related with AS for more than 30 years (3, 4), it still has not been a diagnostic indicator so far because its positive rate in AS patients is just around 90% and most of the HLA-B27 positive individuals do not suffer from AS. Therefore there is an urgency in our daily clinical work to find some simple and specific serum biomarkers for early diagnosis of AS.

Recently AS has been generally accepted as a multi-gene genetic disease (5), and the disease-causing genes should code for specific proteins so as to regulate various pathophysiological processes of AS. This led us to hypothesise that there may be differences in the types of proteins or protein expression levels in the sera of AS patients compared with healthy controls. Proteomics is a new enabling technology to separate and identify disease-specific proteins which can be used as targets in disease diagnostics (6, 7), and to search for therapeutic targets of new drugs so as to understand the mechanisms underlying the therapeutic effect (8). Recent studies have demonstrated that haptoglobin precursor (9) and proteasome activator (PA28) (10) express differentially in AS patients by proteomics. Haptoglobin precursor was the first disease-associated protein reported in the sera of AS patients by means of proteomics, but it had also been found over-expressed in many other diseases such as rheumatoid arthritis (RA), tumours, trauma and infection, etc., so it was regarded as one of the acute phase reactants (11). In addition, the authors used only raw sera to run two-dimensional electrophoresis (2-DE) (9) and sera without being removed of high-abundance proteins might result in serious background interference. Since it has been shown to increase in acute hepatitis C virus infection (12), PA28 which was separated from peripheral blood mononuclear cells of AS patients might also be the result of inflammation, but not a specific protein of AS. We performed this study using 2-DE, one of the core proteomic techniques to compare the serum protein expression profiles between AS patients and healthy volunteers (HVs), aiming to search for disease-associated proteins in AS patients. To confirm the reliability of the results from 2-DE, ELISA experiments were carried out to verify the differentially expressed levels of two AS-associated proteins found by 2-DE.

Methods

Participants and serum samples

Of the 64 subjects enrolled, 32 were AS patients from the outpatient or inpatient department of the Third Affiliated Hospital of Sun Yat-Sen University and the other 32 subjects were HVs who worked or studied in the same hospital. All participants aged from 20 to 45 had gived their signed written informed consent before their enrollment. All the patients with AS met the 1984 modified New York criteria for AS. Patients who suffered from other chronic diseases or any acute infections within the last 3 months were excluded from our study. None of the HVs had previous history of back pain or arthralgia, nor had any other chronic diseases or acute infections in the 3 months before enrollment. Six individuals from the AS

group and 6 from the HV group were randomly selected to join in the 2-DE experiment, and all participants were enrolled into the following ELISA process.

Before the 2-DE experiment, the sera from the 6 AS patients were mixed together, and then high-abundance proteins of the mixed sera were depleted by Plasma 7 Multiple Affinity Removal System (Agilent, USA) according to the manufacturer's protocol. Then the same treatment was carried out on the sera from the 6 HVs.

Two-dimensional electrophoresis

The prepared sera diluted by rehydration solution (8 M urea, 2% CHAPS, 18 mM DTT, 0.5% carrier ampholyte) were focused on IPG strips (13 cm, pH 3-10 NL, GE Health Care, USA), using the following conditions: 12 hrs at 30 V, 1 hr at 500 V, 1 hr at 1000 V, 6 hrs at 8000 V, 4 hrs maintaining at 500 V in an Ettan IPGphor Isoelectric Focusing System (GE Health Care, USA). Next, after incubation for 15 mins in equilibration buffer A (6 M urea, 0.05 M Tris-HCl (pH 8.8), 2% SDS, 30% glycerol, 1% DTT, a trace of bromophenol blue) and 15 mins in equilibration buffer B (6 M urea, 0.05 M Tris-HCl (pH 8.8), 2% SDS, 30% glycerol, 2.5% iodoacetamide), the strips were placed on top of 12.5% SDS PAGE gels and electrophoresed at 15 mA per gel for 30 mins, then 30 mA per gel until the bromophenol blue reached the end of the gel in a SE-600 apparatus (GE Health Care, USA). The third step was staining the gels by the modified fast silver staining protocol (fixed with 10% ice acetic acid in 40% ethanol for 30 mins; sensitised with sensitising solution (6.8% sodium acetate, 0.2% sodium thiosulphate, 30% ethanol) for 20 mins and then rinsed using three changes of distilled water, 5 mins each; impregnated with 0.25% silver nitrate for 20 mins, then discarded the silver nitrate and the gels were rinsed twice with distilled water for 1 min each; developed with 0.15% formaldehyde in 2.5% sodium carbonate for 10 mins and then terminated with 1.46% EDTA for 10 mins). Finally, the silver-stained gels were scanned by Image Master Scanner (GE Health Care, USA) with a 300 dpi resolution and analysed by PDQuest 8.01 software (Bio-Rad, USA). Four satisfactory gels for each serum sample were gained from the repeated 2-DE experiments.

In gel tryptic digestion and protein identification

Protein spots were excised from the preparative gels and transferred into 1.5 ml eppendorf tubes. Then the gel pieces were destained with 100 mM NH_4HCO_3 in 30% acetonitrile (ACN). After removing the destaining buffer, the gel pieces were lyophilised and rehydrated in 30 μl of 50 mM NH_4HCO_3 containing 50 ng trypsin (Promega, USA). After overnight digestion at 37°C, the peptides were extracted three times with 100 μL 0.1% trifluoroacetic acid (TFA) in 60% ACN. Extracts were pooled together and lyophilised. The resulting lyophilised tryptic peptides were kept at -80°C until mass spectrometric analysis. A protein-free gel piece was treated as above and used as a control to identify autoproteolysis products derived from trypsin.

The tryptic digests were analysed using a Bruker-Daltonics AutoFlex TOF-TOF Mass Spectrometer (MS) with LIFT technology (Bruker Daltonics, Germany). Samples were prepared on an AnchorChip sample plate (Bruker Daltonics, Germany) according to the manufacturer's protocol. Both the MS and MS/MS data were acquired with a N_2 laser at 25-Hz sampling rate and were submitted to MASCOT for protein identification. National Center for Biotechnology non-redundant (NCBInr) database with other metazoa as taxonomy was searched against.

ELISA validation

The ELISA tests were carried out on all the enrolled individuals. Two of the identified proteins, ceruloplasmin (Cp) and transthyretin (TTR) were confirmed by Human Ceruloplasmin ELISA kit and Human Transthyretin ELISA kit (USCNLIFE, China). The process began with adding 100 μl sample or standard to each well, then covered the plate with a plate sealer at 37°C for 120 mins. Secondly, 100 μL detection reagent A working solution was added to each well after emptying the plate and incubated at 37°C for 60 mins and followed by washing the wells 3 times, then repeated the similar procedure again while the detection reagent A working solution was changed to detection reagent B working solution. The third step was to add 90 μl substrate solution to each well, then covered the plate with a new plate sealer and incubated for 30 mins. Lastly, the OD values of each well were read by a BIO-TEK ELx800 microplate reader at 450 nm after stopping the reaction by adding 50 μl stop solution to each well. Primary concentrations of each sample were gained by calculating the linear regression equation of the OD values.

Statistical analysis

PDQuest 8.01 software was performed to analyse the 2-DE images, and the other data were processed with SPSS 11.0 software based on the independent-samples *t*-test (2-sided) or Fisher's exact test. A *p*-value of less than 0.05 was considered statistically significant.

Results

Clinical status of the participants

All participants were enrolled into the ELISA test and only 6 individuals in each group were involved in the 2-DE experiment. For all the participants, there were 16 males and 16 females in HV group and their mean age was 27.2±6.1 years, while there were 18 males and 14 females in AS group and their mean age was 27.3±6.4 years. The gender factor and age between these two groups had no significant difference. The average disease duration, baseline ESR and CRP of AS patients were 3.7±2.6 years, 25.6±18.1 mm/hr and 15.5±16.7 mg/L in ELISA test, while 3.5±1.4 years, 24.0±14.0 mm/hr and 15.8±21.5 mg/L in 2-DE experiment respectively. All these clinical parameters showed no significant difference between the patients participating in ELISA test and those being enrolled into 2-DE experiment (Table I).

Reproducibility and precision of 2-DE analysis

In this study, both the mixed sera from the two groups were used to run 2-DE

Table I. The clinical data and general characteristics of each group.

Group	M/F(n)	Age(yr)	Disease duration (yr)	ESR(mm/hr)	CRP(mg/L)
HV (ELISA)	16/16	27.2 ±6.1			
HV (2-DE)	3/3	29.5 ±3.8			
AS (ELISA)	18/14*	27.3 ±6.4*	3.7±2.6	25.6±18.1	15.5±16.7
AS (2-DE)	3/3*	26.3 ±6.2*	3.5±1.4**	24.0±14.0**	15.8±21.5**

M: male; F: female; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; HV: healthy volunteer; AS: ankylosing spondylitis; 2-DE: two-dimensional electrophoresis; ELISA: enzyme-linked immunosorbent assay

*p>0.05 compared with HV group and with AS patients enrolled in ELISA or 2-DE experiment; **p>0.05 compared with AS patients enrolled in ELISA experiment.

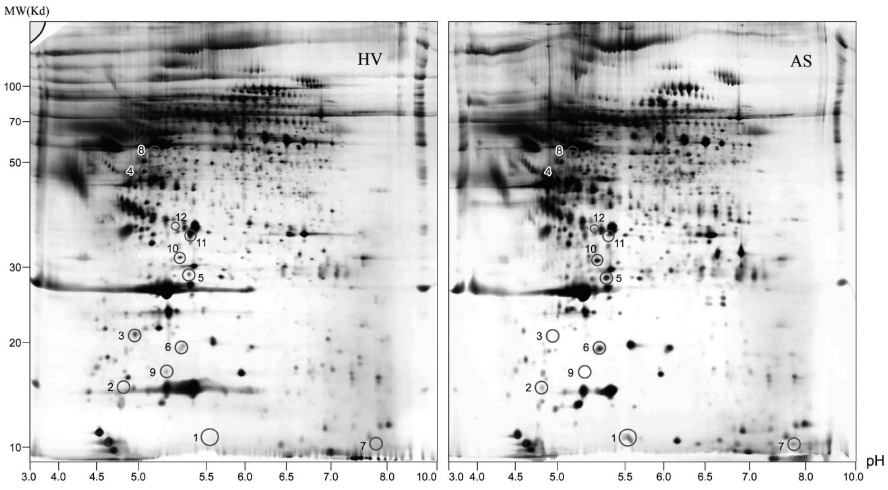


Fig. 1. The 2-DE patterns of one HV (left) and one AS patient (right). The distribution of the different protein spots between the two groups were showed by the cycles, among which spot 1 to 9 were successfully identified by MALDI-TOF/TOF MS analysis while spot 10 to 12 were failed to get reliable results. Spot 1, 2, 5, 6, 7, 8, 10 and 12 were over-expressed, and spot 3, 4, 9 and 11 were under-expressed in AS group compared with HV group.

for at least 4 times till 4 satisfactory gels were obtained from each group. Four gels from each group were selected to be included in the analysis. Using the gel analysis software, under the same parameters, there were 662.50±13.67 protein spots visualised in the 2-DE gels of AS group and 668.50±12.40 in HV group, the average repetition rate was 92.97% in AS group and 94.56% in HV group, both the average spot positional deviations in IEF direction and SDS-PAGE direction were less than 1.0 mm in each group.

Comparison protein spots between the two groups

All 8 gels were analysed by PDQuest software 8.01, and there were 450 commonly matched protein spots visualised on 2-DE gels. The protein spot with differential expression level was defined as exhibiting the same expression tendency within at least 3 of 4 gels from the same group and expressing at least 3 times differentially from the other group. By this approach, 12 protein spots with differential expression levels were found and their distribution in gels from different groups was shown in Figure 1. Among the 12 protein spots differentially expressed, eight were over-expressed and 4 were under-expressed.

Identification of the proteins with differential expression levels

Twelve protein spots with differential expression levels were analysed by MALDI-TOF/TOF MS analysis and 9 of them produced reliable results (Table II). The enlarged images of these 9 spots from different groups are shown in Fig. 2. Of all the identified proteins, eight were identified by MS analysis, while the other one (Spot no. 6), which failed to be identified by initial MS analysis, finally acquired its reliable result by subsequent MS/MS analysis. Among the 9 successfully identified proteins, serum amyloid A (SAA), apolipoprotein A (ApoA)-IV, ApoA-IV precursor, haptoglobin 2 (Hp2), Cp and immunoglobulin superfamily22 (IGSF22) were over-expressed, and plasma glutathione peroxidase (GPx3), similar to complement component 3 and chain A of TTR were under-expressed in AS patients (Table III). Found by bioinformation seaching, most of the identified proteins were related to the acute inflammatory response and regulated the inflammatory process, and some of them also involved in other physiologic or pathologic process such as lipid metabolism, lipid peroxidation and amyloidosis.

ELISA results

To confirm the reliability of the results from 2-DE, two proteins including Cp and TTR were chosen for ELISA analysis. The results of ELISA test were shown in Table IV. The expression level of Cp was obviously higher in the AS group than that in the HV group, and the difference was statistically significant (p<0.05). While the expression level of TTR was statistically lower in AS group than that of HV group (p<0.05). The ELISA results showed entirely consistent trends with those of 2-DE experiment.

Discussion

Although a number of evidence-based recommendations and outcome measures in AS have been developed on the basis of sound scientific principles (13), the early diagnosis of AS and evaluation of its disease activity by more simple and specific indicators are still a challenge. Over the years, AS has been classified as seronegative spondyloarthropathies owing to its shortage of serum rheumatic factors. However, AS is a hereditary disease, the pathogenic genes must translate into some kinds of active proteins before they play essential roles in disease development. On the other hand, the clinical manifesta-

Table II. The information of the 9 proteins identified successfully by MALDI-TOF/TOF MS analysis.

No.	Method	Protein code	Protein name	Molecular weight(d)	Isoelectric point
1	MS	gil225986	serum amyloid A	11675	5.89
2	MS	gil563320	apolipoprotein A-IV	28141	5.39
3	MS	gil404108	plasma glutathione peroxidase (Homo sapiens)	16760	8.93
4	MS	gil169214179	PREDICTED: similar to complement component 3 (Homo sapiens)	45642	4.94
5	MS	gil178779	apolipoprotein A-IV precursor	43358	5.22
6	MS/MS	gil223976	haptoglobin 2	42344	6.23
7	MS	gil47125416	ceruloplasmin (Homo sapiens)	24839	8.52
8	MS	gil109730305	IGSF22 protein (Homo sapiens)	97226	7.03
9	MS	gil73535906	chain A, crystal structure of human transthyretin with bound iodide	13941	5.35

MS: mass spectrometer.

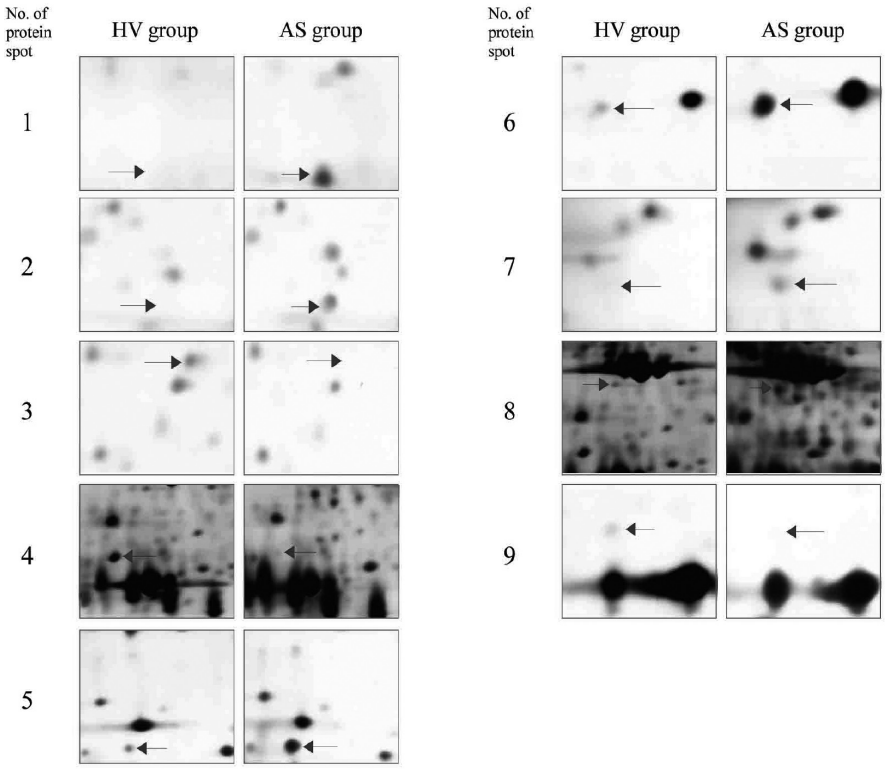


Fig. 2. The enlarged images of the 9 identified protein spots with differential expression levels between HV group and AS group (The arrows show the corresponding spots or locations). Spot 1, 2, 5, 6, 7 and 8 were over-expressed, and spot 3, 4 and 9 were under-expressed in AS group compared with HV group.

tions of AS are similar to those of RA to some extent and that RA has been well known for its autoimmunity in pathogenesis. So it is reasonable to deduce that there might be some special differentially expressed proteins including autoantibodies in sera of AS patients. Such proteins might be used as new diagnostic indicators, evaluation index of disease activity as well as therapeutic targets of AS, and might provide new clues to studies on pathogenesis of AS. For these reasons, we performed this comparative study to screen some differentially expressed proteins from sera of AS patients by using proteomics technology.

High-abundance proteins of serum, which consist of 22 proteins such as albumin, immunoglobulin, transferrin, haptoglobin, antitrypsin, fibrinogen, α2-macroglobulin, α1-acidglycoprotein, ApoA-I, ApoA-II and complement component 3, add up to around 99% of the mass of serum proteins. These high-abundance proteins, especially albumin and immunoglobulin, always limit the sample volume of 2-DE, and often obstruct the identification of low-abundance protein spots from the electrophoresis patterns. A serum 2-DE experiment without depleting of multiple high-abundance proteins usually results in terrible background interference in the gels. As a result, effectively removing of the high-abundance proteins and enrichment of low-abundance proteins in serum at the same time are the key issue of a successful 2-DE experiment (14, 15). In this present study, Plasma 7 Multiple Affinity Removal System, which is currently regarded as an effective way to deplete the most important seven high-abundance proteins and to enrich low-abundance proteins of serum was used to ensure the gels with clear protein spots and distinct background.

Another key issue of 2-DE technology is the repeatability and stability of the gels. Because of the complex procedures, the gels often present poor repeatability and instability (16). To solve this problem, some researchers advised mixing the experimental samples. According to the literatures, sample pooling can effectively reduce the individual differences, improve the repeatability of 2-DE gels, and simplify the statistical analysis in 2-DE (17). So, in this study, we randomly selected 6 AS patients and 6 HVs, mixed their sera respectively and repeated the 2-DE experiments till 4 satisfactory gels were gained from each group. The results were satisfactory that the number of protein spots in each gels of the same group was stable and the average spot positional deviations were slight. The

Table III. The data of MS analysis and standardised quantization of the 9 identified serum proteins.

No.	Matched peptide	Sequence coverage (%)	Reliability threshold	Actual score	Standardised quantisation	
					HV (n=4)	AS (n=4)
1	4/12	61	65	69	0.41 ± 0.11	534.15 ± 169.22
2	5/10	20	65	66	0.41 ± 0.11	263.93 ± 38.56
3	7/11	35	65	88	529.59 ± 43.99	0.44 ± 0.07
4	8/36	26	66	68	275.09 ± 19.55	51.84 ± 16.56
5	10/19	24	65	113	190.43 ± 25.32	952.30 ± 85.06
6	2/31	8	36	73	295.44 ± 53.85	1126.24 ± 70.95
7	5/14	20	65	72	0.42 ± 0.12	67.23 ± 4.56
8	7/11	9	65	70	5.12 ± 1.79	184.11 ± 34.76
9	6/19	69	65	94	150.62 ± 33.53	15.53 ± 574.56

Table IV. The ELISA results of Cp and TTR between HV group and AS group.

Group	Cp(mg/ml)	TTR(mg/ml)
HV(n=32)	0.2035±0.0374	0.1544±0.0431
AS(n=32)	0.2963±0.0650*	0.0993±0.0197*

Cp: Ceruloplasmin; TTR: Transthyretin
*$p<0.05$.

good repeatability promised the possibility of comparing the differences between the two groups. Furthermore, the ELISA results showed entirely consistent trends with those of the 2-DE experiments. The results of this study indicated that sample pooling was a feasible method to reduce the individual differences and our results were reliable.

MALDI-TOF/TOF MS is the most widely used method to identify proteins in proteomics study. But MS analysis are always influenced by various factors such as characteristics of the target proteins, enzymolysis efficacy, acquirement rate of peptide fragments and MS performance, so it is impossible to get reliable result from every MS analysis. In our MALDI-TOF/TOF MS analysis, nine proteins were successfully identified from 12 protein spots with differential expression levels between the two groups, but the other 3 protein spots were failed to get reliable results. In our future study, these 3 protein spots should be identified by more advanced technology such as quadrupole time-of-flight (Q-TOF) MS.

In this study, SAA, ApoA-IV, ApoA-IV precursor, Hp2, Cp and IGSF22 were found over-expressed in AS group. SAA is a kind of apolipoprotein, involving in lipid transportation, G receptor coupling reaction, cell adhesion, and regulation of the activation of neutrophils, lymphocytes, macrophages as well as platelet. Furthermore, SAA is also related to secondary amyloidosis. Lange's study (18) showed that SAA was an indicator of inflammation in AS and Jung *et al*. (19) reported that the level of SAA was correlated with the disease activity of AS. Hp2 is one of the acute-phase proteins. It plays an important role in the course of inflammatory response which macrophages, lymphocytes, neutrophils, monocytes and Langer-han's cells are involved in. Hp2 has been found increased in AS patients, but it did not correlate well with Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) (20). Cp is a metal protein which involves in the excretion of copper and the absorption of iron. The lack of Cp will cause liver damage, diabetes and nervous system lesions. In addition, Cp exhibits a copper-dependent oxidation and antioxidation activity (21). It can help to eliminate free radicals, reduce oxidative stress, and thus prevent tissue from being damaged. A number of studies had already found that serum Cp level was elevated in kinds of arthritis (22) including AS (23), which showed entirely consistent trend with our finding. But there was no significant difference in Cp level found by the past studies between active and inactive AS patients (24), implying that Cp did not correlate well with the disease activity of AS.

Based on this study, TTR, GPx3 and similar to complement component 3 were under-expressed in AS patients. The ELISA test confirmed the lower expression trend of serum TTR level in AS group. TTR is a carrier protein of thyroid hormones and belongs to prealbumin. It exhibits anti-inflammatory as well as anti-microbial activities and prevents organisations from inflammatory damage (25, 26). Richter's study showed that the serum TTR levels of patients with active AS were lower than those of healthy controls, and TTR increased while ESR and CRP decreased in patients with active AS after methylprednisolone treatment (27). But Surrall *et al*. did not find any obvious decrease of serum TTR in AS patients (22). The contradictory results of different studies might be due to their difference in disease status of AS patients. Despite this, the change of serum TTR levels of AS patients implied that TTR might have a close relationship with disease activity of AS. GPx3 is an antioxidant enzyme found in the extracellular fluid. It belongs to the glutathione peroxidase (GPx) family, which function in the detoxification of hydrogen peroxide. GPx3 decreased in patients with cancer, diabetes and cerebral thrombosis, Barrett esophagitis and chronic renal insufficiency, but so far there have been few studies which focused on the status of GPx3 in AS patients. Túnez (28) reported that GPx level decreased in active AS patients compared with inactive AS patients, and it increased significantly after 6 weeks' treatment of infliximab. The result was similar with our study, which suggested that the level and function change of GPx might have close contact with the disease course of AS.

Unfortunately, there is little information about IGSF22 and similar to complement component 3, especially their relationship with AS. Similarly, despite

their involvement in inflammation has been reported (29), ApoA-IV and ApoA-IV precursor have never been studied in the field of AS. It is still a mystery that what roles these proteins play in the pathogenesis and development of AS, but it will become clear as more researchers focus on it.

In conclusion, this study has successfully set up serum protein profiles of AS patients and HVs, and screened 9 proteins with differential expression levels in AS patients. These proteins might be serum disease-associated proteins of AS patients. However, there were some potential limits of this study. For example, the small number of patients enrolled and the potential influence of patients' treatment on the results was not fully taken into account. Moreover, in order to refine the specificity of these candidate biomarkers, it would have been informative to compare the serum profiles of AS patients to other disease controls such as RA patients. So further investigation must be carried out to test whether the 9 proteins identified in this study can be used as useful diagnostic indicators or therapeutic targets of AS. In a future study, we should enlarge the sample size, and enrol patients with different diseases as controls to support the results with more evidence.

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