

Over-expression of talin 1 and integrin-linked kinase in PBMCs of patients with ankylosing spondylitis: a proteomic study

T. Li¹, B. Zheng¹, Z. Huang¹, H. Lu², Q. Lin¹, Z. Liao¹, Z. Lin¹, L. Zhao³,
X. Wang¹, J. Gu¹

¹Department of Rheumatology, and ²Central Laboratory, Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou, China; ³Department of Rheumatology, Beijing Hospital, Beijing, China.

Abstract

Objectives

Ankylosing spondylitis (AS) is difficult to diagnose in its early stage due to the lack of simple and specific diagnostic indicators. This study was performed to screen candidate AS-associated proteins from peripheral blood mononuclear cells (PBMCs) of AS patients by combining a two-dimensional electrophoresis (2-DE) technique with mass spectrometry (MS) analysis.

Methods

Twelve subjects consisting of 6 AS patients and 6 healthy volunteers (HVs) were enrolled in the 2-DE experiments. The protein expression patterns of PBMCs from different groups were analysed by PDQuest software, and the protein spots over/under-expressed by more than 2-fold between the two groups were identified by MS analysis. Western blot analyses were used to verify the differentially expressed proteins in 32 AS patients and 32 HVs.

Results

Six proteins including pyruvate kinase (PK), profilin 1 (PFN1), talin 1 (TLN1), Chain A of cyclophilin A (CyPA), unknown protein (gil16306948) and integrin-linked kinase (ILK) were identified from 10 over-expressed protein spots found by 2-DE in the AS group. Western blot experiments confirmed a higher expression of both TLN1 and ILK in AS group compared to the HV group ($p < 0.05$).

Conclusion

TLN1 and ILK expressed higher in PBMCs of AS patients compared to healthy controls, which were involved in the integrin signalling pathway. The two proteins are likely novel disease-associated proteins and potential disease markers of AS.

Key words

ankylosing spondylitis, two-dimensional electrophoresis, mass spectrometry, talin1, integrin-linked kinase

Tianwang Li, MD, PhD
 Benrong Zheng, MD
 Zhixiang Huang, MD
 Huiqiong Lu, Technician
 Qu Lin, MD, PhD
 Zetao Liao, MD
 Zhiming Lin, MD, PhD
 Like Zhao, MD, PhD
 Xinwei Wang, MD, PhD
 Jieruo Gu, MD

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Please address correspondence and reprint 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 that belongs to the group of diseases known as spondyloarthropathies. It mainly affects the spine and sacroiliac joint, and sometimes involves the eyes, heart, lung and skin (1). Rheumatoid arthritis (RA) is another inflammatory rheumatic disease whose clinical manifestations are sometimes similar to AS to a certain degree, but they differ in many ways including etiology, pathogenesis, pathology, clinical manifestations as well as structural outcomes (2, 3). Up to now, the modified 1984 New York criteria, which depend on the radiographic sacroiliitis, are the basic diagnosing criteria for AS. However, definite sacroiliitis shown by plain radiography is usually absent in the early stage of the disease (4). MRI has been widely used to detect early sacroiliitis in recent years (5), but it might be worthless if the sacroiliac joint has not been involved. Although genetic epidemiological studies (6) have demonstrated the association between inheritance and AS, and confirmed HLA-B27 as an important disease-associated gene in AS (7, 8), how HLA-B27 participates in the pathogenesis of AS is unclear so far. Recent genomic studies have found some other suspicious loci related with AS, but since there are many inconsistencies among different studies, most of the candidate genes remain to be identified for their exact roles of pathogenicity (9-12).

Proteomics is a new enabling technology that could identify protein networks in a high-throughput discovery approach with high sensitivity and accuracy. It has been applied successfully to screen potential biomarkers in many diseases such as tumours, hepatic diseases, neural and rheumatic diseases, *etc.* (13-18). These potential biomarkers may be used as targets for molecular diagnosis and therapeutics of corresponding diseases. Liu *et al.* (19) first studied AS patients' sera by using proteomic technology and found extremely high expression of haptoglobin precursor in sera of AS patients compared to healthy controls. However, haptoglobin precursor was regarded as

one kind of acute phase reactants and it had been found highly expressed in many other diseases such as RA (20) and tumours (21, 22). Similarly, a differential expression of proteasome activators (PA28) in monocytes of AS was likely a result of inflammation (23). In addition, we recently performed a serum proteomic study in AS patients and found 9 proteins such as serum amyloid A, apolipoprotein A-IV, haptoglobin 2, ceruloplasmin, transthyretin and plasma glutathione peroxidase differentially expressed in sera of AS patients (24). However, most of these proteins were related to inflammation and had been found to over-express in other inflammatory diseases. Therefore, it is still a challenge to search for some specific and sensitive biomarkers to help with a better understanding of the pathogenesis and early diagnosis of AS.

In this present study, proteomic technology has been performed to screen new disease-associated proteins in peripheral blood mononuclear cells (PBMCs) of AS patients. Western blot analyses were used for validation.

Methods

Participants

Thirty-two AS patients consisting of 18 males and 14 females who met the 1984 modified New York criteria were enrolled in the current study. All patients were newly diagnosed as AS, and they either had received none of the anti-rheumatic treatments, or only received non-steroid anti-inflammatory drugs (NSAIDs) therapies but not any disease modifying anti-rheumatic drugs (DMARDs), steroid or anti-tumour necrosis factor (TNF)-alpha therapies. The age range was from 20 to 45 years and the mean age was 27.3 years. Patients who suffered from other chronic diseases or any acute infections within 3 months were excluded from this study. Baseline erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) of AS patients were 25.6 ± 18.1 mm/h and 15.5 ± 16.7 mg/l, respectively. Thirty-two gender and age matched healthy volunteers (HVs) containing 16 males and 16 females were enrolled as healthy controls. The age of HVs had a range from 20 to 45 years, and the mean age

Competing interests: none declared.

was 27.2 years. None of the HVs had any previous history of back pain or arthralgia, nor did they have any other chronic diseases or acute infections during 3 months before enrolment. Six individuals consisting of 3 males and 3 females were randomly selected from each group to be enrolled in the two-dimensional electrophoresis (2-DE) experiment, but all individuals were subsequently enrolled in the Western blot validation tests. The age did not differ significantly between the AS patients and HVs individuals enrolled in the 2-DE experiments. All participants had given their written informed consents in accordance with the Declaration of Helsinki before their enrolment.

Sample collection and protein extraction

Twelve ml peripheral venous blood was collected from each individual in ethylenediamine tetraacetic acid (EDTA) anticoagulant tubes and diluted with phosphate-buffered saline (PBS) to 24ml of total volume. The diluted blood was gently overlaid on top of 12ml Ficoll-Paque Plus solution (GE Healthcare, USA) and centrifuged at 2000rpm for 20mins. Then PBMCs were collected from interphase of PBS/Ficoll and washed twice in PBS. Detergent soluble proteins were extracted from PBMCs by incubation in 65mM dithiothreitol (DTT), 20 mM Tris-HCl, 0.2% Bio-Lyte 3/10 Ampholyte, 1/50 (v/v) Protease Inhibitor Cocktail Set III (Merck KGaA, Germany), 1mM PMSF, 7M urea, 2M Thiourea for 1hr at room temperature, followed by ultrasound disruption on ice and centrifugation at 20,000g for 40mins at 4°C. The protein concentration of this soluble fraction was determined using the Bradford method as described by the manufacturer (Bio-Rad, USA).

Two-dimensional electrophoresis

The protein samples (200µg per gel) were focused on 17cm immobilised pH 3-10 gradient IPG strips (Bio-Rad, USA) in a Protean IEF Cell (Bio-Rad, USA) apparatus at 20°C using the following conditions: 12hrs rehydration, 1hr at 150V, 1hr at 250V, 1hr at 500V, 1hr at 1000V, 3hrs at 5000V,

7hrs at 10000V and 24hrs maintaining at 500V. The strips were immediately equilibrated by incubating in equaliser buffer (6M urea, 20% glycerol, 2% SDS, 0.375M Tris-HCl pH 8.8) containing 2% DDT for 15mins at room temperature, followed by incubation for 15mins at room temperature in equaliser buffer containing 2.5% iodoacetamide and a trace of bromophenol blue. Strips were placed on top of 12% SDS PAGE gels and sealed by low melting point agarose containing a trace of bromophenol blue. Then the gels were electrophoresed at 25mA per gel until the bromophenol blue reached the end of the gel. The gels were silver stained using a modified silver-staining protocol as described (25). At least 3 satisfactory gels for each protein sample were gained from repeated 2-DE experiments. The gels were scanned in a GS-800 optical density scanner (Bio-Rad, USA) and then analysed by PDQuest 8.01 software (Bio-Rad, USA). The protein spot with differential expression level was defined as exhibiting the same expression tendency within at least 2/3 of the gels from the same group and expressing at least 2-fold higher/lower than the other group.

In gel tryptic digestion

Protein spots were excised from the gels and transferred into 1.5ml eppendorf tubes with serial numbers by the 2-DE performer for further identification. The performers in charge of tryptic digestion and mass spectrometry (MS) analysis didn't know anything about the experimental design and any message of the 2-DE gel except for the numbers of each protein spots to be identified. The gel pieces were destained with 100mM NH_4HCO_3 in 30% acetonitrile (ACN). After removing the destaining buffer, the gel pieces were lyophilised and rehydrated in 30µl of 50mM NH_4HCO_3 containing 50ng trypsin (Promega, USA). After overnight digestion at 37°C, the peptides were extracted three times with 100µl 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 MS analysis. A protein-

free gel piece was treated as above and used as an internal standardisation to identify autoproteolysis products derived from trypsin.

MALDI-TOF/TOF-MS analysis and database searching

The tryptic digests were analysed using a Bruker-Daltonics AutoFlex TOF-TOF Mass Spectrometer with LIFT technology (Bruker Daltonics, Germany). Samples were prepared on an Anchor-Chip 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. The National Center for Biotechnology Information (NCBI) database with Other Metazoa as taxonomy was searched against. The other parameters for searching were enzyme of trypsin; one missed cleavage; fixed modifications of carbamidomethyl (C); variable modifications of oxidation (Met); peptide tolerance of 100 ppm; fragment mass tolerance of $\pm 0.5\text{Da}$; peptide charge of 1+ were selected. Only significant hits, as defined by the MASCOT probability analysis ($p < 0.05$), were accepted.

Western blot validation

The two selected proteins, talin1 (TLN1) and integrin-linked kinase (ILK), which were found differentially expressed in AS group by 2-DE experiment, were validated by using Western blot. The same PBMCs protein samples as in 2-DE were used in this Western blot experiment. Protein samples (15µg per lane) were separated on SDS-PAGE gels (10% for TLN1 and 9% for ILK), followed by transferring to polyvinylidene fluoride (PVDF) membranes. After being blocked in TBST plus 5% fat-free milk for 1 hr at room temperature, the membranes were then incubated with mouse anti-human TLN1 monoclonal antibody diluted 1:6000 (Chemicon, USA), or mouse monoclonal antibody to ILK diluted 1:6000 (Abcam, UK). After being washed 3 times for 10mins each, the membranes were incubated with HRP-labelled secondary antibodies diluted 1:8000 (Santa-Cruz, USA) at room temperature for 2hrs, and the

immunoreactive bands were visualised by enhanced chemiluminescence (ECL; GE Healthcare, USA) and exposed to Hyperfilm ECL (Kodak, USA). Then, equal protein loading was routinely confirmed by stripping the antibody off the membrane and reprobing with anti- β -actin. Quantity One software was used to quantitate individual bands of Western blot, and the values of TLN1 or ILK were corrected by β -actin.

Statistic analysis

PDQuest 8.01 and Quantity One software (Bio-Rad, USA) were used for the analysis of the 2-DE and Western blot images, respectively. The enumeration data was calculated by Pearson's chi-square test or Fisher's exact test. Independent-samples *t*-tests were utilised for statistical analysis of the measurement data; $p < 0.05$ was considered statistically significant. The statistical process was performed through SPSS 11.0 software.

Results

2-DE patterns

Every sample was tested at least 3 times till 3 satisfactory gels were gained from repeated 2-DE experiments in this study. Then the best 3 gels of each sample were selected to be included in the final analysis. There were 821.50 ± 14.77 and 807.92 ± 13.86 protein spots visualised in HV group and AS group, respectively. The average repetition rates of protein spots reached over 90%, and the average spot positional deviation in IEF direction and SDS-PAGE direction were both less than 1.2 mm in all the 2 groups. Five hundred and eight common protein spots were found on every gel of all the subjects, and 10 distinct protein spots which expressed differentially by at least 2-fold between the 2 groups were found. All these 10 spots were over-expressed in AS group compared with HV groups (Fig. 1).

MALDI-TOF/TOF MS analysis

Ten distinct protein spots were analysed by the MALDI-TOF/TOF MS analysis, and reliable results were obtained from 6 of them. The 6 identified proteins included pyruvate kinase (PK), profilin 1 (PFN1), TLN1, Chain A of cy-

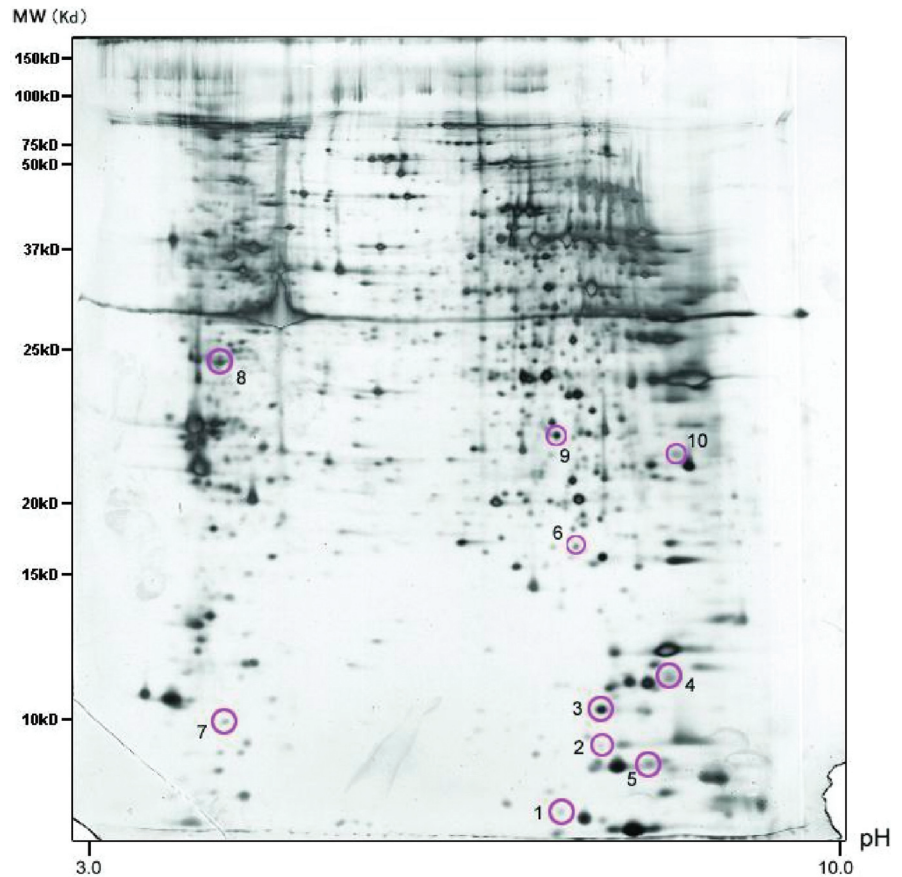


Fig. 1. The 2-DE pattern and the distribution of the 10 over-expressed protein spots in AS patient. The corresponding protein spots were showed in cycles. Cycles No. 1-6 show the 6 spots identified successfully by MALDI-TOF/TOF MS analysis and cycles No. 7-10 refer to the 4 spots failed to be identified.

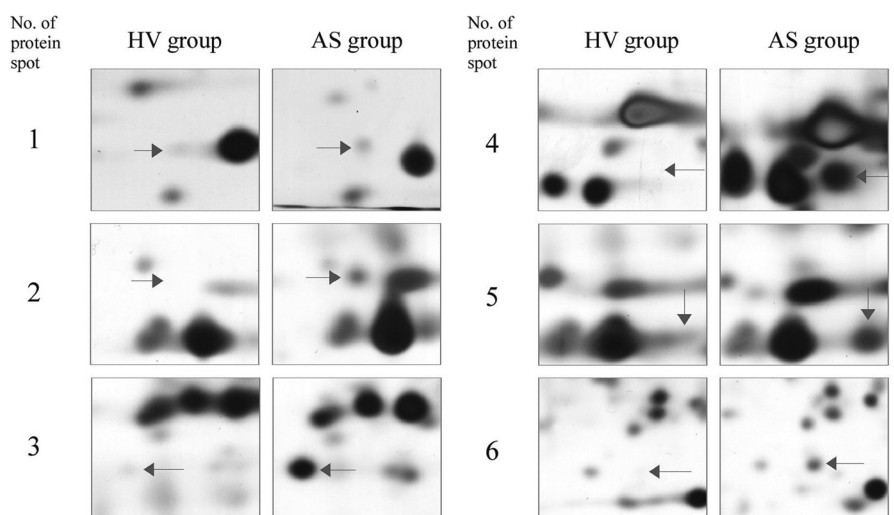


Fig. 2. The enlarged images of the 6 differentially expressed protein spots successfully identified in different groups. The corresponding protein spots were shown by arrows.

clophilin A (CyPA), unknown protein (gil16306948) and ILK. The enlarged images of these 6 protein spots from different groups were showed in Figure 2. Of all the identified proteins, 5 were

identified by MS analysis; the other one (Spot n.1) that failed to be identified by initial MS analysis finally acquired its reliable result by subsequent MS-MS analysis. All the 5 proteins identified by

Table I. MALDI-TOF/TOF MS analysis results of the 6 differentially expressed proteins between AS patients and healthy volunteers.

Spot No.	Method	Protein code	Protein name	Matched peptide	AA sequence coverage (%)	Accepted threshold value	Mascot score	Molecular weight(d)	Isoelectric point
1	MS-MS	gil35505	Pyruvate kinase	2/12	4	37	83	58411	7.58
2	MS	gil4826898	Profilin 1	6/19	47	65	86	15216	8.44
3	MS	gil55859708	Talin 1	11/21	31	65	98	30437	6.03
4	MS	gil1633054	Chain A, Cyclophilin A Complexed with Dipeptide Gly-Pro	9/30	51	65	116	18098	7.82
5	MS	gil16306948	Unknown (protein for IMAGE:3897065)	8/24	37	65	117	17998	5.20
6	MS	gil4758606	Integrin-linked kinase	11/21	23	65	108	51899	8.30

Matched peptide: This column showed the number of mass value matched/number of mass value searched; AA: amino acid; Accepted threshold value: It is the cut point for a probability equal to 5% of null hypothesis; Mascot score: It is actual score in the Mascot system. If the Mascot score is higher than the accepted threshold value, the result is reliable.

MS analysis had ≥ 6 matching peptide, $\geq 20\%$ amino acid sequence coverage rate and >80 Mascot Score. Although the matched peptides of spot n.1 were only 2/12, and the amino acid sequence coverage was just 4%, the results were obtained from MS-MS analysis which is more powerful in identifying low levels of target proteins. Additionally, the Mascot Score of this protein was 83, more than twice of accepted threshold value (Table I). Therefore, the identified result of spot n.1 was considered to be reliable.

Among 5 of the 6 identified proteins, PK, CyPA and ILK are enzymes, TLN1 is a cytoskeletal protein and PFN1 can regulate the function of cytoskeletal proteins. Moreover, TLN1 and ILK belong to the regulatory proteins of integrin signaling pathway, which is important to the initiation and development of inflammation (26-28). Thus, TLN1 and ILK were selected to be verified by Western blot experiments.

Western blot

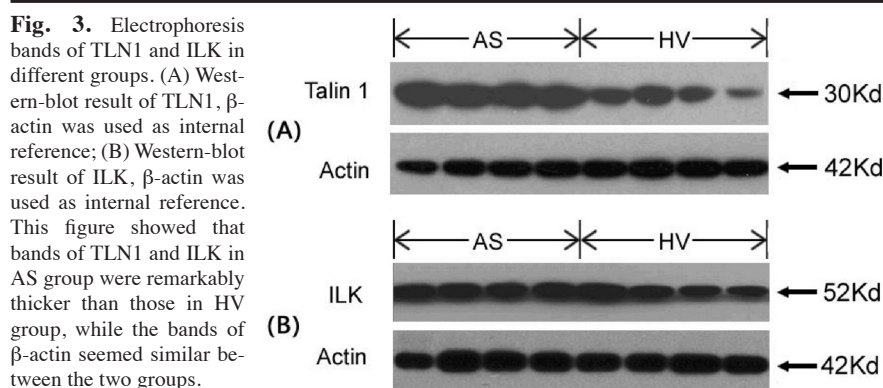
The Western blot validations were carried out in all the 32 subjects of each group. Bands of TLN1 and ILK in AS group were remarkably thicker than those in the HV group (Fig. 3). And the values of TLN1 and ILK in AS group normalised by β -actin were significantly higher than those of HVs (Table II).

Discussion

AS is an immuno-hereditary disease which involves the skeletal system and external skeletal tissue. The hallmark

of AS is acute and chronic spinal inflammation initiating in the sacroiliac joints, often coupled with enthesitis, presenting as chronic inflammation at the sites of ligamentous and tendinous insertions into bone (29). However, most of the involved lesions such as sacroiliac joint, uvea, ligament and tendon are difficult to get by biopsy. On the other hand, since AS is a systemic disease, lesions from specific organisation might not reflect the pathophysiological process of AS in complete picture. PBMCs consist of a heterogeneous population which includes monocytes, T cells, B cells, and natural killer cells. They are a group of immune cells that

participate in most of the immune reactions and provide specific information on inflammatory pathways. Recently, Schulz *et al.* (30) successfully screened some differentially expressed proteins from PBMCs of RA patients by proteomic method. And Wright *et al.* (23) found some up-regulated proteins mainly involved in inflammation and the ubiquitin proteasome pathway from monocytes of AS by 2-DE, liquid chromatography coupled to electrospray ionisation MS analysis. Additionally, genetic studies have demonstrated abnormalities in gene expression of PBMCs from AS patients (31). Therefore, in this study, PBMCs were chosen as a

**Table II.** The normalised quantitative value of TLN1 and ILK from western-blot validation in different groups.

Group	TLN1	ILK
HV(n=32)	0.4686 \pm 0.2139	0.7404 \pm 0.2310
AS(n=32)	1.1411 \pm 0.4292*	1.1201 \pm 0.2705*

TLN1: talin 1; ILK: integrin-linked kinase; HV: healthy volunteer; AS: ankylosing spondylitis

* $p < 0.05$ compared with HV group.

study sample to search for disease-associated proteins of AS.

In this 2-DE experiment, 10 protein spots that differentially over-expressed by at least 2-fold in the AS group were obtained, and 6 proteins were subsequently identified by MS analysis. As seen from the representative gel shown in Figure 1, the molecular weight (MW) of each spot with differential expression was lower than 25kDa. However, half of the 6 proteins identified by MS analysis were more than 30kDa. The MWs of protein spots that appeared in the gels were not completely consistent with the MS results, and there was discrepancy between the MW of TLN1 appeared in the 2-DE gel and bands of Western blot. We considered that all these MW discrepancies might due to protein degradation. During the complex procedure of 2-DE experiment performed in this study, the temperature should be kept constantly around 20°C for at least 26 hours for a single run in case of precipitation of the components such as urea from the sample solution. However, at such a temperature, and for such a long time, the proteins tend to degrade into proteolytic fragments by proteases. Although we had added protease inhibitors to inhibit the action of many proteases, it was impossible to inhibit all of them. Thus, degradation of some proteins was inevitable. While for the Western blot test, most of the operations were performed under relatively low temperature and the protein samples had been boiled before electrophoresis so that the proteins were not easy to degrade during the process. We thought this can partly explain why the MWs of some proteins gained from 2-DE were different from the corresponding data obtained from MS analysis and Western blot experiment.

Based on our experiments, 6 proteins were found to be over-expressed in PBMCs of AS patients and 5 of them were proteins with definite function. Among them, TLN1, ILK and PFN1 have been known to be involved in the integrin signalling pathway, which is important for cell migration, cell proliferation and pathogenesis of inflammation (26-28, 32). None of these 3 proteins has ever been reported in previous studies

on AS. In this proteomics study, we found that they expressed higher in PBMCs of AS patients than those of HVs. Western blot experiments also validated the differential expression levels of TLN1 and ILK in AS patients.

TLNs are large adaptor proteins that link the integrin family of adhesion molecules to F-actin. In vertebrates, there are two TLN genes: TLN1 and TLN2. TLN1 is essential for integrin-mediated cell adhesion, but the role of TLN2 is unclear so far (33). TLNs were component of podosome, which was an adhesive structure and played an important role in mediating transmigration of monocytes and (or) macrophages through the endothelium (34). Meanwhile, TLNs were found redistributed and accumulated in the peripheral supramolecular activation cluster of activated cytotoxic T lymphocytes and the myotendinous junctions (35, 36). Frenette *et al.* (37) reported that TLNs were concentrated and colocalised at myotendinous junctions and increased mechanical loading could promote TLN synthesis. TLN1, the key TLN member in immune cells (38), formed a platform for the recruitment of diverse focal adhesion proteins related to integrin signalling pathway (28). Manevich *et al.* (39) discovered that TLN1 was essential for the generation of very late antigen-4 (VLA-4) and could contribute to VLA-4-mediated adhesions of T lymphocytes under both shear stress and shear-free conditions. ILK, a 59 kDa serine-threonine protein kinase, has been proved to be closely associated with the cell proliferation, migration and signal transmission (28). It has been found to highly express in human mononuclear leukocytes and be activated by the exposure to chemokine in a PI3K-dependent manner (40). Tan *et al.* (41) reported that ILK could mediate nitric oxide production by regulating inducible nitric oxide synthase expression in macrophages. Furthermore, over-expression of ILK could diminish the firm adhesion of endothelial cells, suggesting that ILK was involved in the regulation of leukocytes adhesion via integrins (40). Interestingly, ILK was reported to regulate the expression of cyclooxygenase-2 (COX-

2) in a NF- κ B dependent manner (41) and non-steroidal anti-inflammatory drugs (NSAIDs) could inhibit ILK activity *in vivo* (42). Thus, ILK might be a candidate target of anti-inflammatory therapy. Meanwhile, ILK is also important in the myotendinous junctions. On the other hand, the ILK knockout osteoblasts highly expressed differentiated markers, collagen I, bone sialoprotein and osteocalcin (43). All of the above indicated that ILK not only played important roles in the formation and stabilisation of myotendinous junctions, but also took part in the acceleration of bone formation by osteoblasts.

TLN1 and ILK have never been reported in previous AS studies, but a previous study showed a significantly different expression involving the integrin pathway in AS monocytes compared to healthy controls (23). In this proteomic study, TLN1 and ILK were found higher expressed in PBMCs of AS patients than those in HVs. The results of this study suggested that TLN1 and ILK might be novel candidate AS-associated proteins, or potential biomarkers for the diagnosis of AS. Moreover, because the integrin signalling pathway plays an important role in pathogenesis of inflammation, and for the close association of the integrin signalling pathway with TLN1 and ILK, we supposed that the integrin signalling pathway might be involved in the pathogenesis of AS. As to the other 3 proteins found to be over-expressed in PBMCs of AS patients, PFN1 is known to be relevant to inflammatory adhesion (44), CyPA may contribute to the inflammatory processes in arthritis (45), and PK was reported to be an autoimmune target in Tourette syndrome and associated disorders (46). Though they were found in our study by 2-DE to be over-expressed in PBMCs of patients with AS, the detailed roles they play in inflammation or autoimmunity are still to be determined.

In summary, six proteins were found highly expressed in PBMCs of AS patients compared with healthy controls. Among them the differential expression levels of TLN1 and ILK were verified by Western blot. TLN1 and ILK, which were known to be involved in

the integrin signalling pathway, might be novel candidate disease-associated proteins and potential disease markers of AS. However, there were some potential limits of this study. For example, the small number of patients enrolled in the 2-DE experiment, and that there was not any disease-controlled group to underline the real utility of the identified proteins in AS. Therefore, further investigations must be conducted to verify whether the 6 proteins identified in this study were specific for AS. In future studies, 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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