Proteins related to the functions of fibroblast-like synoviocytes identified by proteomic analysis

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Abstract

Objectives

It is well known that the fibroblast-like synoviocytes (FLS) play a key role in pathogenesis of rheumatoid arthritis (RA). This study was performed to separate the differentially expressed proteins of FLS from the patients with RA or osteoarthritis (OA) by two-dimensional electrophoresis (2-DE), and found proteins associated with the functions of FLS by mass spectrometry (MS).

Methods

Total proteins were extracted and quantified from the primary cultured FLS from patients of RA (n=8) or OA (n=6). Proteins were separated by high-resolution 2-DE, and identified the differentially expressed proteins by MS. Western blot analyses was used to validated the expression of candidate proteins. The mRNA of these proteins was detected by semi-quantitative fluorescent PCR.

Results

There are 1147 protein spots from RA and 1324 protein spots from OA showed on 2-DE graphs, respectively. We have selected 84 protein spots for MS analysis, and 27 protein spots were successfully identified. We have found that protein isoaspartyl methyltransferase (PIMT) and pirin (iron-binding nuclear protein, PIR) with lower expression in RA, and thioredoxin 1(Trx-1) only expressed in RA may be associated with functions of FLS. Western Blot confirmed the expression of PIMT and pirin lower in RA, and Trx-1 expressed only in RA. The results of semi-quantitative fluorescent PCR are also consistent with 2-DE graphs.

Conclusion

PIMT, pirin and Trx-1 affect the functions of FLS in some style and can be the drug targets of RA.

Key words

rheumatoid arthritis, fibroblast-like synoviocytes, protein isoaspartyl methyltransferase, pirin, thioredoxin

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Received on July 5, 2011; accepted in revised form on September 20, 2011.

Funding: This research was supported by grants from the National Natural Science Foundation of China (NSFC) (no. 81072467), the Shanghai Municipal Science and Technology Commission for International Cooperation (no. 09410705000) and the Bureau of Shanghai Pudong New Area (no. PW2009D-1).

Competing interests: none declared.

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterised by non-suppurative inflammation of synovium (1). It is well known that fibroblast-like synoviocytes (FLS) play a key role in pathogenesis of RA. It contributes to inflammation of synovium by producing cytokines and degradation of the extracellular matrix (ECM) by expressing proteolytic enzymes (2, 3). All the behaviours of FLS in RA are based on the hyperplasia of synovium. However, hyperplasia is caused by an imbalance between proliferation and apoptosis (4, 5), so the molecules which break the imbalance may explain the hyperplasia mechanism of FLS.

Proteomic is a powerful technology used to indentify protein and biomarkers involved in the pathogenesis of specific diseases. It contains two basic methods: two-dimensional electrophoresis (2-DE) and mass-spectrometric analysis. In 2-DE, proteins are separated in a first dimension according to their isoelectric points and then in a second dimension on the basis of their molecular masses. Proteins are separated in their native state. The mass-spectrometric analysis is based on the principle that large protein molecular ions can be produced by laser desorption without much fragmentation when these bio-molecules are mixed with small organic compounds that serve as matrix for strong absorption of a laser beam. It can measure up to 100,000 Da proteins with high detection sensitivity (6). In recent years, proteomic approaches have employed mass spectrometry and bioinformatics to identify proteins in the target cell, tissue and serum of RA. Protein S100A8, S100A9 and S100A12 in synovial fluid were identified as the specific biomarkers discriminate RA from other inflammatory joint diseases by proteomic analysis (7). Protein c19orf10 (chromosome 19 open reading frame 10) with increased expression is involved in cell proliferation and differentiation in FLS, synovial fluid, and the synovium of RA which is also identified by proteomic analysis (8). Uridine diphosphoglucose dehydrogenase (UDPGD), which is very important to synovial fluid and ECM for its ability to synthesise hyaluronan, is identified by proteomic analysis (9). However, most of these studies focus on the inflammation-related proteins or as on autoantigens or biomarkers with increased expression in RA, and only a few studies have attempted to identify the proteins related to the proliferation and apoptosis of FLS, or the proteins with decreased expression in RA.

In this study, we separated the proteins of FLS from patients with RA and osteoarthritis (OA) by high-resolution two-dimensional electrophoresis and identified the differentially expressed proteins by mass spectrometry (MS). By searching the document, we found 3 candidate proteins which may be associated with functions of FLS, which provided new experimental evidence for investigating the precise role of FLS in RA.

Materials and methods

Sample collection, primary culture of FLS and total protein extraction

Eight RA patients (2 male, 6 female, mean age 58 years, range 48–71 years) and six OA patients (3 male, 3 female, mean age 60 years, range 43–77 years) undergoing knee replacement surgery in Shanghai East Hospital were enrolled in the experiments. The RA patients have suffered from the disease for 4–8 years with joint destruction. They also had high levels of anti-cyclic citrullinated peptide antibody (23–76 RU/ml), rheumatoid factor (42–115 IU/ml) and C-reactive protein (19–85 mg/L). The RA patients took disease-modifying anti-rheumatic drugs (DMARDs) for delay joint destruction before knee replacement surgery. The RA patients and OA patients were also treated with non-steroidal anti-inflammatory drugs (NSAIDs) to control inflammation. All patients signed informed consent, and the RA patients were in line with the diagnostic criteria of American College of Rheumatology (ACR). The study protocol was approved by the Research Ethics Committee of Shanghai East Hospital. The synovial tissue samples were cut into pieces immediately after removal in surgery under sterile conditions, then were placed in 25 cm^2 culture flasks (Corning, USA) with 0.25%

trypsin (Sigma, USA) and digested at 37°C for 2 h. After centrifugation, the FLS were collected by adding RPMI 1640 medium (containing 10% fetal bovine serum, glutamine, gentamicin, penicillin and streptomycin) and were cultured overnight at 37°C in 5% CO_2 environment for adherent growth. The FLS was sub-cultured until it grew to 80%. After these experiments, the third passage FLS was used. Before the total protein extraction, the FLS from the 8 RA patients and 6 OA patients were mixed together. The FLS in the logarithmic phase was washed twice with phosphate buffered saline (PBS), and then centrifuged at 1000g for 10 mins. Having removed the supernatant, 1ml lysate was added (7M urea, 2M thiourea, 4% CHAPS, 100 mM DTT, 0.5 mM PMSF, 0.5% IPG buffer) and mixed for 15 mins. After centrifugation at 12000g for 15 mins at 4°C, the protein samples were obtained form the supernatant. The protein concentration was quantified by the modified Bradford method, and the protein samples were stored in -80°C until use.

2-DE analysis

300μg protein was used to run 17cm pH 3–10 linear IPG strips (Bio-Rad, USA) for 2-DE. The first isoelectric focusing was carried on the Protean IEF Cell (Bio-Rad, USA), voltage settings: 500V for 30 mins, 1000V for 1 h, 2000 V for 1 hr, 4000 V for 1 h, 8000V for 1 h and 64000V for 1 h. After IEF, the strips were placed in a balanced solution (6M urea, 30% glycerol, 2% SDS, 1% DTT) for 15 mins, then in another balanced solution (6M urea, 30% glycerol, 2% SDS, 2.5% iodoacetamide) for 15 mins. 12% non-gradient gel and 0.5% agarose gel for sealing were used in the second SDS-PAGE. Electrophoresis was carried on the Protean II electrophresis apparatus (Bio-Rad, USA) with constant current mode (20mA/gel), and then switched to separation gel (40mA/gel) until the bromophenol blue (Amresco, USA) reached the bottom line. The silver-staining was performed rapidly after the total protein separation: fixed with 45% ethanol and 5% acetic acid for 60 mins; washed twice for 45 mins; sensitised with 0.02% $Na_2S_2O_3$ for 2 mins; washed with water for 1m×3 times; immersed in 0.1% $AgNO_3$ for 30 mins at 4°C; washed with water for 1min×3 times; developer was added (containing 2% Na_2CO_3, 0.04% formaldehyde), until the protein spots were visible; then stored in 5% acetic acid. Each experiment was carried out in parallel 3 times. Gel stained was transmission scanned with the Labscan scanner (Amersham Pharmacia Biotech, Sweden), and digital image files were analysed by Image Master software (Amersham Pharmacia Biotech, Sweden). The protein spots in the RA group and OA group were selected for identification.

In-gel digestion

Excised gel pieces were placed in a 96-well plate and destained with adding 200μl per well distaining solution (15 mM $K_3Fe(CN)_6$, 50 mM $Na_2S_2O_3$ [v/v 1:1]) to destained proteins for 30 mins. The gel pieces were washed with water for 30 mins until colourless. After removal of solution, 200μl per well of 100% acetonitrile (ACN) was added and stranded for 10 mins until gel completely dehydrated to white. Then, ACN was discarded and the gel pieces were incubated at 37°C for 10 mins in order to remove the excess ACN. Trypsin solution (12.5ng/ml, sequence grade, freshly diluted in 25mM NH_4HCO_3, Promega) was added to each of the dry gel pieces and incubated at 4°C for 30 mins to completely rehydrate the gel pieces. Then the excess trypsin solution was vacuumed out to prevent the degradation of the peptides in MS. About 5μl per well of 25mM ammonium bicarbonate solution was added to prevent drying the solution in the hydrolysis process. It was then incubated at 37°C for 12–16hrs. Peptides were extracted three times by adding 5μl per well matrix (50% ACN, 0.1%TFA) and was allowed to stand for 30 mins. The extracts were combined and the solution was dried by using high purity nitrogen. The completely dried peptides were dissolved again in CHCA solution (0.5g/L, 0.1%TFA, 50% ACN), then they were spotted onto a stainless steel MALDI target plate and naturally dried at room temperature. The dried peptides were analysed by using 4700 Proteomics Analyzer (Applied Biosystems, USA).

Mass spectrometry

PMF mass scan range is 800–3200Da, and the ionisation source is a laser (337 nm, 200 Hz) with an acceleration voltage of 20 kV. The instrument was used in reflector-positive mode and automatic data acquisition mode for data collection. The 6 strongest precursor ions were selected automatically from one MS scan for MS/MS analysis. Trypsin digested peptides of horse myoglobin were used as the internal mass standard to calibrate the instrument. The results were carried out with the GPS software (Applied Biosystems, USA) for database search. The search parameters were the following: the database is NCBI; search species is Homo Sapian (human); data retrieval method is combined search. The maximum allowable leakage cutting bit is 1, and the enzyme is trypsin. The mass tolerance was set as 0.3 Da, and MS/MS tolerance was 0.4 Da for automatic data analysis. Also, the signal-to-noise ratio (S/N) of fragment ion peaks in tandem mass spectra was 10. In the database retrieval, the peak of degradation by trypsin and the peaks of pollutants were removed by hand.

Western Blot analysis

30μg protein was extracted from the FLS and resolved by 12%SDS-PAGE, then it was electro-transferred to polyvinylidene fluoride (PVDF) membranes. After blocking in confining solution (containing 10% dry milk) for 2 h at room temperature, membranes were incubated with specific primary antibodies overnight at 4°C. The specific primary antibodies used in this study were the anti-PIMT monoclonal mouse antibody (Abcam, 1:2000), the anti-pirin monoclonal mouse antibody (Abcam, 1:200), and the anti-thioredoxin polyclonal rabbit antibody (Abcam, 1:2000). The membrane was washed 3 times for 10 mins, then goat anti-mouse or goat anti-rabbit HRP-conjugated (Abcam, 1:5000) were added as secondary antibodies, and

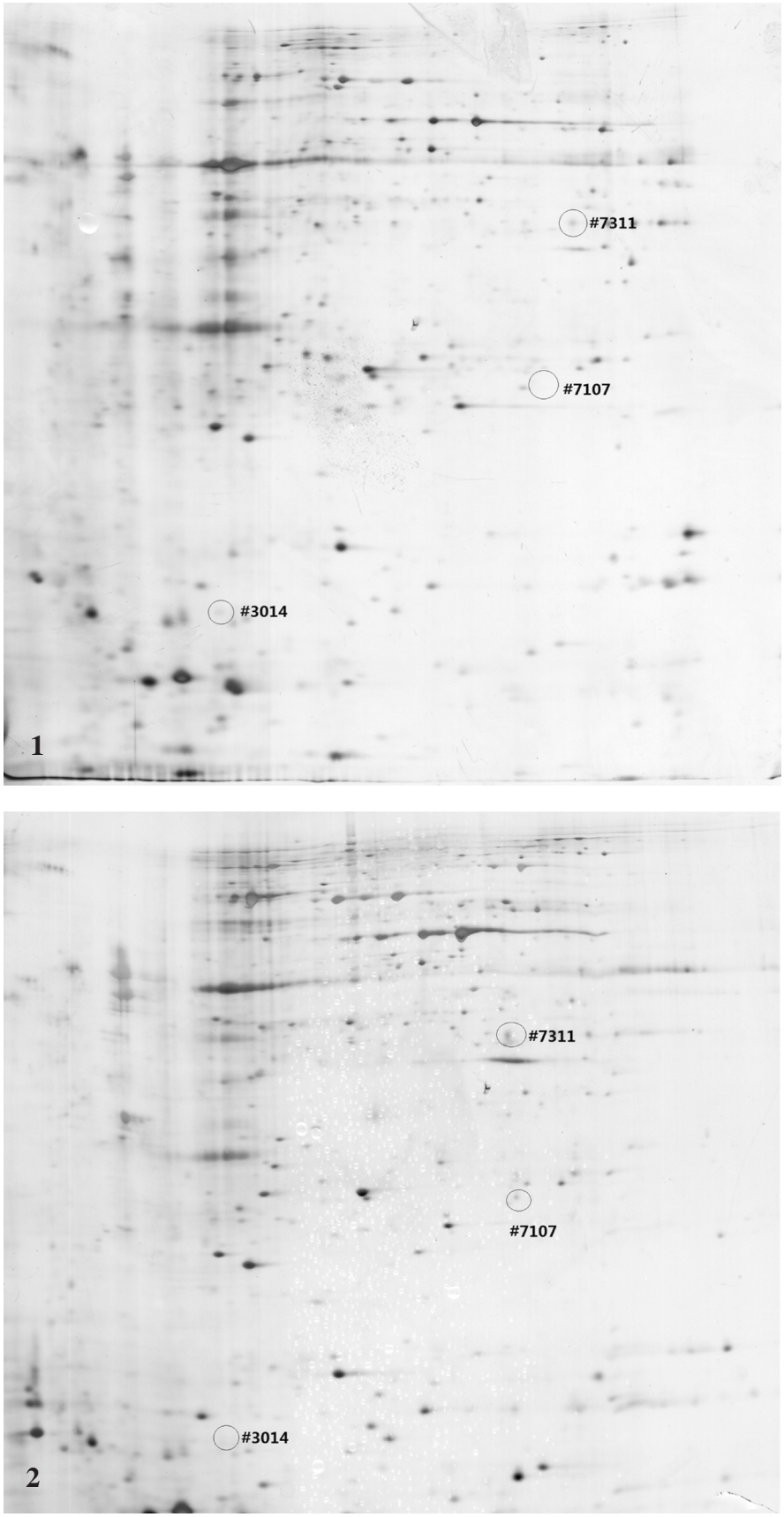


Fig. 1 and **Fig. 2.** The 2-DE graph of RA (upper panel) and OA (lower panel). There are 1147 protein spots on the graph of RA and 1324 protein spots of OA. PIMT (n. 7107), PIR (n. 7311), and Trx-1 (n. 3014) protein spots were evidenced by the circles.

finally they were incubated for 2 h at room temperature. The membrane was washed 3 times for 10 mins. The image was scanned, saved and analysed by gel imager (Tiangen, China).

Semi-quantitative fluorescent PCR analysis

Total RNA was extracted from FLS using Trizol (Invitrogen, USA) according to the manufacturer's instructions. The cDNA was synthesised from 5mg total RNA using a reverse transcription kit (Invitrogen, USA) as described in the manufacturer's instructions. Semi-quantitative fluorescent PCR on an ABI PRISM 7300 (Applied Biosystems, USA) was carried out using Premix Ex Taq SYBR Green PCR (TaKaRa, China) according to the manufacturer's instructions. The GAPDH sequence of primers was sense: 5'- TGACTTCAACAGCGA-3', antisense: 5'- CACCCTGTTGCTGTA GCC AAA-3'; PIMT sense: 5'-TGGGAAAACTGCTGGGCACCG-3', antisense: 5'- GGTTTCCGCCTGCAGGACCAA-3'; pirin sense: 5'-CGGGGCATTCTGCACGCTGA-3', antisense: 5'- TGTTGTGCATCATCGGGCCC-3'; Trx-1 sense: 5'- TGTCAGGGAACAGCTGCCACC-3', antisense: 5'- TGTTGCCTGGACTGGAGTACCAA-3'. PCR reactions were performed in 25µl volume, including 12.5µl 2×SYBR Premix EX Taq, 2µl cDNA samples, 0.5µl ROX, 0.5µl of each primer, and 9µl sterile water. The PCR conditions were as follows: 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 5 sec, annealing at 60°C for 31 sec, and extension at 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec. Relative to GAPDH, expression levels of mRNA were calculated according to $2^{-\Delta Ct}$, ΔCt = Ct (tested gene) - Ct (GAPDH) (10). All assays were conducted in triplicate.

Results

2-DE result

The 2-DE graphs of RA and OA are displayed in Figures 1 and 2. The differentially expressed protein spots were analysed by Image Master to draw the points and match them to each other. The isoelectric point and molecular weight distribution of these protein

Table I. Information of successfully identified protein spots.

Gel idx	Accession n	Protein name	Pro. score	PI	Protein MW (kDa)	Pep. count
113	gi 229383	cytochrome b5 fragment	131	5.22	10,026.4	5
118	gi194376310	actin	187	5.19	38,608.2	7
119	gi28876	ash protein	115	6.65	18,675.2	6
121	gi119599451	mitochondrial ribosomal protein S22	165	6.34	36,782.9	9
122	gi189053405	NCK adaptor protein 1	238	6.06	42,879.5	17
123	gi178375	aldehyde dehydrogenase	102	5.99	50,303.8	3
126	gi194376310	ACTB protein	231	5.19	38,608.2	12
127	gi 15680064	stathmin 1/oncoprotein 18	70	5.76	17,325.9	3
139	gi 49456601	pirin	142	6.27	32,137.2	10
129	gi 194375660	cytidylate kinase	199	9.06	20,545.9	8
141	gi 119582952	annexin A1	158	7.6	40,056.7	10
130	gi 197313672	carnitine deficiency-associated gene expressed in ventricle 3 isoform a	75	5.68	22,065.8	5
142	gi 189181759	electron transfer flavoprotein, alpha polypeptide isoform b	129	8.75	30,006.9	8
131	gi 194384036	phosphoglycerate kinase 1	170	8.49	35,023.1	10
132	gi 203282367	chain A of human Enolase 1	166	6.99	47,008.3	14
133	gi 20150076	protein-L-isoaspartate-O-methyl-transferase	97	6.78	24,489.5	3
135	gi 119617243	hCG2016179	74	5.87	21,810.5	4
136	gi 109074331	similar to heterogeneous nuclear ribonucleoprotein D-like isoform 5	101	8.76	27,174.6	8
149	gi 259090349	chain A of the human 70kda heat shock protein 5	214	5.88	45,114.1	12
162	gi 119583439	thioredoxin 1	69	4.62	18,922.2	4
152	gi181969	elongation factor 2	86	5.81	39,750.3	7
164	gi194375947	peroxiredoxin 2 isoform a	168	5.66	32189392	8
153	gi32455260	peroxiredoxin 5 isoform b precursor	82	9.02	17,383.1	6
165	gi4504517	heat shock protein beta-1	239	5.98	22,768.5	8
154	gi2852648	crystalline- alpha B	110	6.76	20,146.4	10
166	gi11056044	pyrophosphatase 1	88	5.54	32,639.2	4
167	gi220702506	chain A of Tapasin ERP57	404	5.61	54,199.2	26

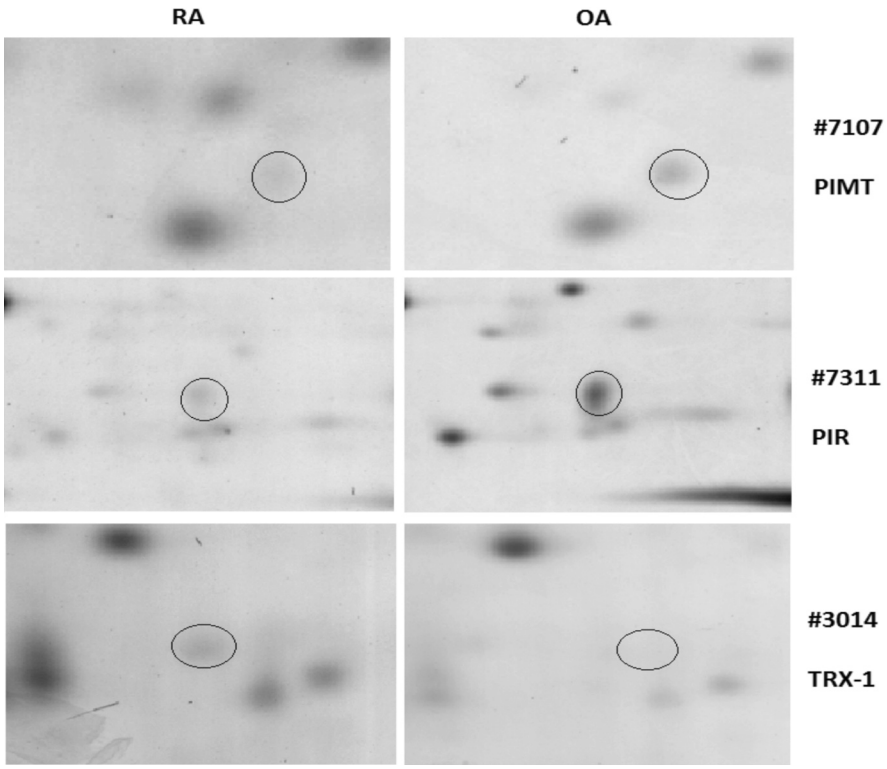


Fig. 3. The enlargement graph for differentially expressed proteins which may be related to the functions of synovium (the circles show the protein spots).

spots covered a wide range. There are 1147 protein spots of RA showed on the graphs and 1324 protein spots of OA. When the expression level of protein in one group is 3 times greater than another group, those spots can be regarded as differentially expressed spots. There are 84 protein spots differentially expressed on the graphs; among them 47 protein spots on the graphs of RA or OA have more than 3 times the expression level, 15 protein spots are only on the graphs of RA, and 12 protein spots are only present on the graphs of OA.

MS identification of differentially expressed protein spots of FLS

The 84 differentially expressed protein spots were run in-gel digestion and then were identified by 4700 Proteome Analyzer. According to the algorithm of GPS software, if the resulting scores were greater than 66, the protein identification would be successful. There are 27 protein spots identified successfully (detailed information about these proteins is shown in Table I). Among these proteins, 6 protein spots were only expressed in OA FLS; these proteins were identified as cytochrome B5 fragment, actin, ash protein, mitochondrial ribosomal protein S22, NCK adaptor protein 1 and aldehyde dehydrogenase; the expression of 12 protein spots was 3 times higher or more in OA FLS than in RA FLS, these proteins were identified as ACTB protein, oncoprotein 18, pirin(PIR), cytidylate kinase, annexin A1, carnitine deficiency-associated gene expressed in ventricle 3 isoform a, electron transfer flavour protein, alpha polypeptide isoform b, phosphoglycerate kinase 1, chain A of human enolase 1, protein-l-isoaspartate-o-methyltransferase(PIMT), hCG2016179 and heterogeneous nuclear ribonucleo protein D-like isoform 5; 6 protein spots were only expressed in RA FLS, these proteins were identified as thioredoxin 1(Trx-1), peroxiredoxin 2(PRDX2), heat shock protein beta-1, pyrophosphatase 1, chain A of Tapasin-ERP57, chain A of the human 70kDa heat shock protein 5; 3 protein spots were 3 or more times in RA FLS than in OA FLS, these proteins were iden-

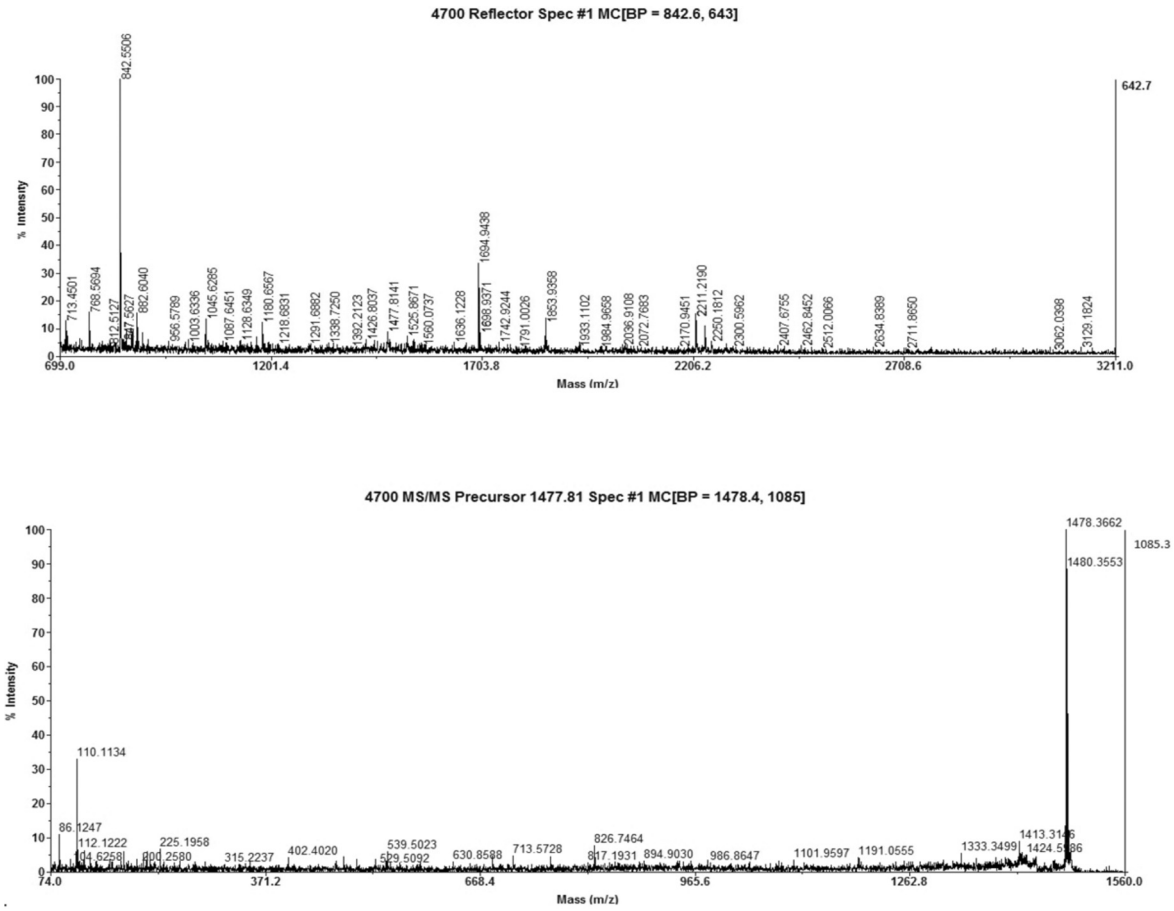


Fig. 4. Typical mass spectrum and MS/MS spectrum for protein identification of spot n. 7107.

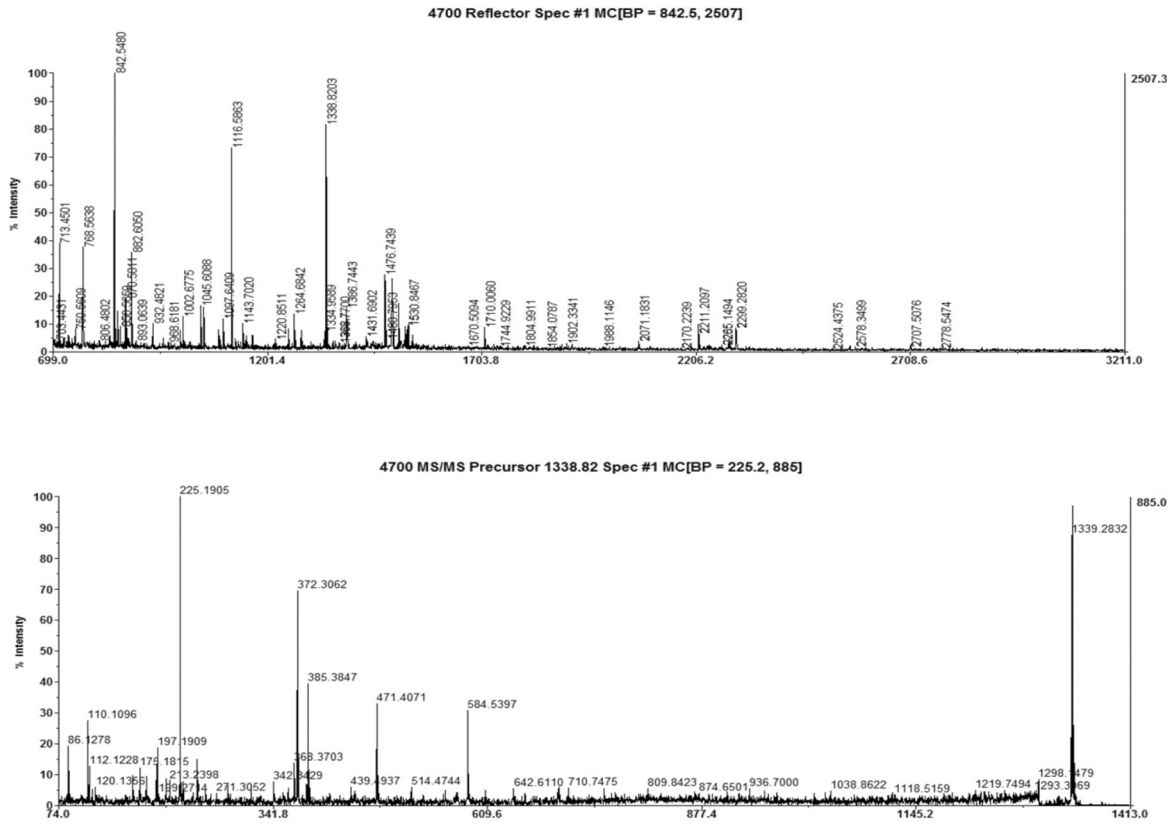
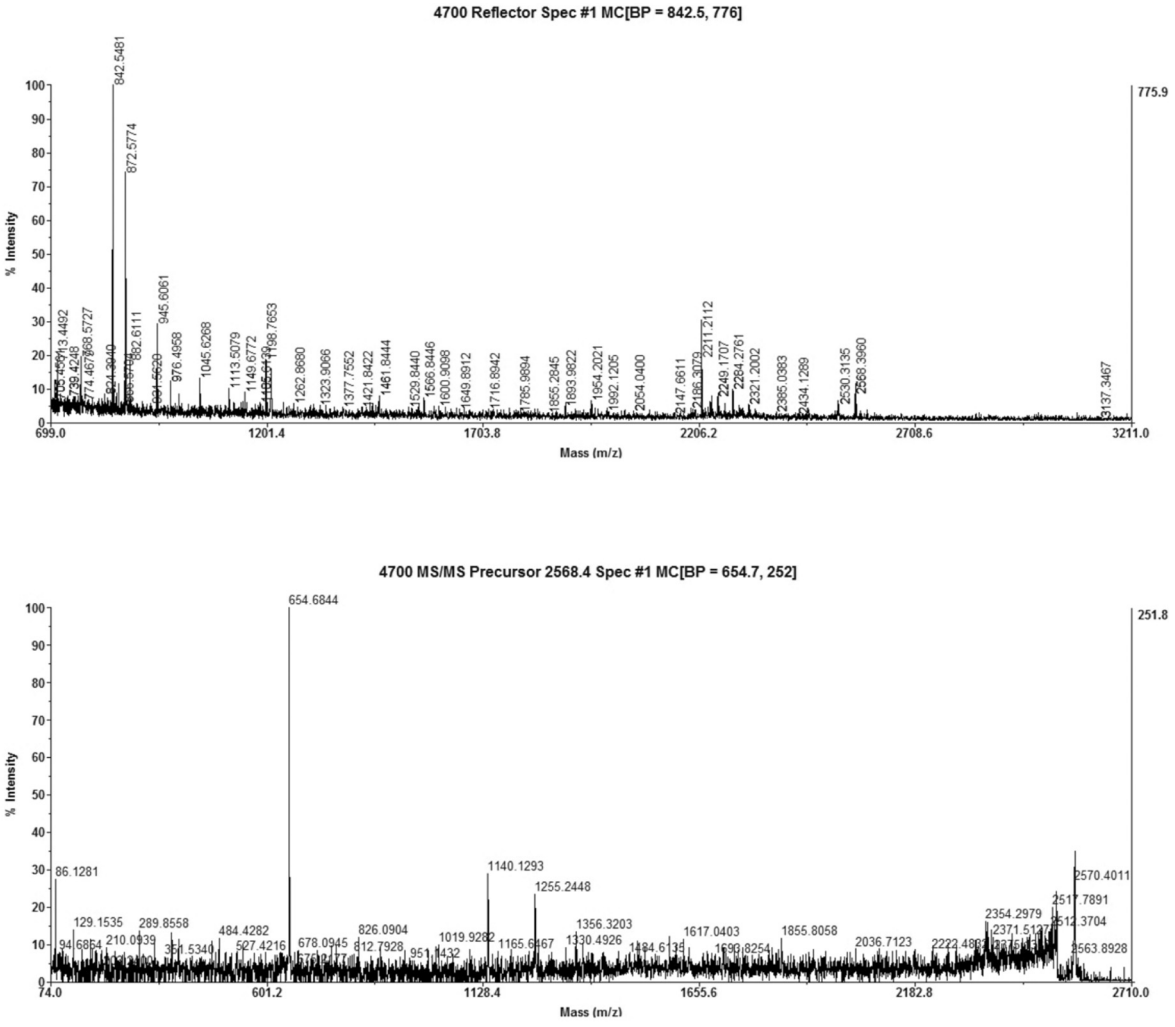


Fig. 5. Typical mass spectrum and MS/MS spectrum for protein identification of spot n. 7311.



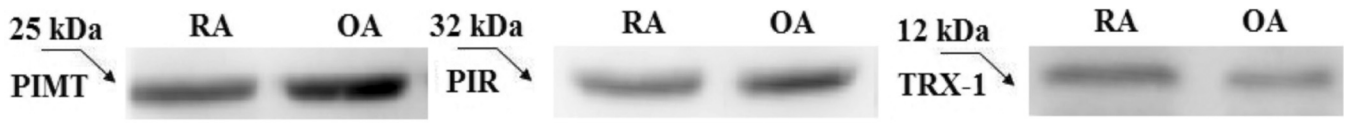


Fig. 6. Typical mass spectrum and MS/MS spectrum for protein identification of spot n. 3014.

Fig. 7. Western blot analysis for PIMT, PIR and Trx-1in FLS of RA and OA.

tified as elongation factor 2, peroxiredoxin 5 isoform b precursor(PRDX5) and crystalline-alpha B.

After searching the document, we have selected 3 candidate proteins which related to functions of FLS in RA (Fig. 3 shows the 3 candidate proteins in the enlargement graph). Figure 4 represents the MS and MS/MS graphs for protein spot n. 7107 respectively. The identified protein spot n. 7107 is PIMT. Figure 5 represents protein spot n. 7311 identified as pirin. Figure 6 represents protein spot n. 3014 identified as Trx-1.

Western blot result

The Western blot result (Fig. 7) shows that the protein expression level of PIMT and pirin in RA was significantly lower than the level in OA, the expression of Trx-1 is higher in RA than the level in OA. All the results are consistent with 2-DE graphs.

Semi-quantitative fluorescent PCR result

According to the formula, relative to GAPDH, mRNA expression levels of PIMT, pirin and Trx-1 are shown in Figure 8. As Figure 7 shows, the mRNA expression levels of PIMT and pirin of OA FLS are much higher than the levels of RA FLS; mRNA expression levels of Trx-1of RA FLS are much higher than the levels of OA FLS.

Discussion

In this study, we screened proteins with different expression in FLS of RA and OA using 2-DE and MS. 27 proteins were identified successfully. Among the 27 proteins, the expression of 18 proteins in OA is higher than in RA, and the expression of 9 proteins in RA is higher than in OA. By searching the document, we have identified 3 candidate proteins related to the functions of RA-FLS. The 3 candidate proteins are: protein isoaspartyl-methyltransferase (PIMT), pirin (iron-binding nuclear protein, PIR) and thioredoxin 1(Trx-1). Western blot and semi-quantitative fluorescent PCR confirmed a

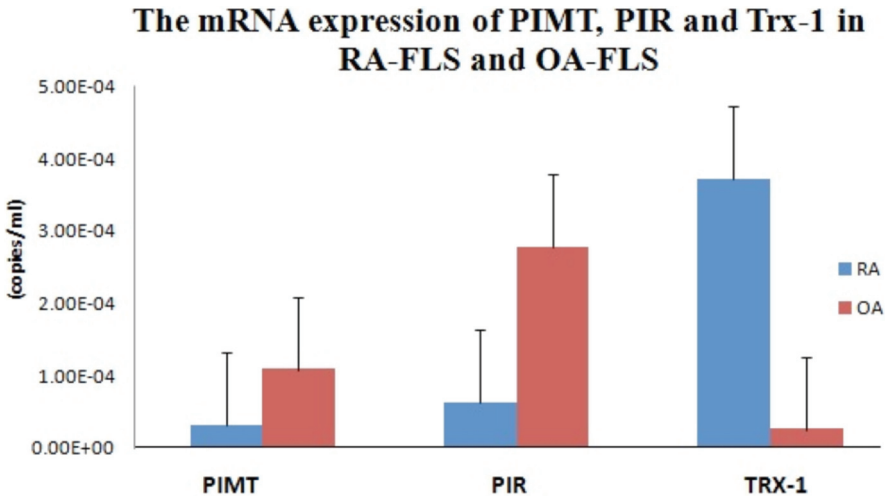


Fig. 8. Semi-quantitative fluorescent PCR analysis for the mRNA expression levels of PIMT, PIR and Trx-1 in RA and OA.

decreased expression of PIMT and pirin in the RA-FLS and an increased expression of Trx-1 in OA-FLS.

PIMT is encoded by gene PCMT1 (protein carboxyl-O-methyltransferase-1), and it can identify and repair the abnormal l-aspartyl residues in proteins. The abnormal l-aspartyl residues would affect the protein activity and destroy the cell function (11). This repair function of PIMT is very important for the mitogen-activated protein kinase (MAPK) signal system. In PIMT knockdown cells, Raf-1, MEK1/2 and extracellular regulating kinase (ERK)1/2, members of the MAPKs cascade, were phosphorylated to significantly higher levels than in control cells, and the hyperphosphorylation of Raf-1, MEK1/2 and ERK1/2 lead to proliferation of cells (12). In our study, it was found that PIMT expression level in RA is much lower than in OA. Thus, it is possible that the decreased expression of PIMT leads to the dysfunction of MAPKs, resulting in the proliferation of FLS.

Pirin is a 32-kDa nuclear proteins consisting of 290 amino acids, it is highly conserved and belongs to the cupin super-family (13). Pirin has interactions with some nucleoprotein, such as the oncoprotein B-cell lymphoma 3-encoded (Bcl-3) and nuclear factor I (NFI) *in vivo,* suggesting that PIR is a transcription cofactor involved in transcriptional regulation, apoptosis, stress response and other activities (14-16). Although its precise role in these functions has not yet been defined, pirin expression is known to be deregulated in several human malignancies, such as human salivary glands cystic adenocarcinomas, ascolorectal cancer and glioma. It was found that knockdown of pirin inhibited melanoma cell migration and overexpression of pirin in human bronchial epithelial cells were associated with an increase in the number of apoptotic cells (17, 18). We have found that pirin in RA was significantly lower than in OA, so we hypothesised that decreased expression of pirin may be affect the apoptosis and migration of FLS in the pathogenesis of RA.

Thioredoxin is a multi-functional protein and belongs to conserved redox protein family. Human thioredoxin 1 (Trx-1) is a 12 kDa polypeptide, and has the disulfide/thiol bond in the regulation of redox activity (19). It has anti-oxidative, anti-apoptotic and anti-inflammatory effects (20, 21). Trx-1 is mainly in the cytoplasm, and could migrate to the nucleus under the ionic rays or ultraviolet irradiation. Most of the mutant cells secreted Trx-1, but the specific pathway is unknown (22). Many studies have shown that Trx-1 plays a different role in the different stages of tumourigenesis. In the early stages of tumour, Trx-1 can inhibit the oxidative stress caused by a variety of carcinogens, so it inhibits the development of a tumour; but in the progression stage, Trx-1 of high concentration promotes tumour growth and resists to apoptosis. In the advanced stage, Trx-1 is also involved in angiogenesis and tumour metastasis (23-26). It was found that human regulatory *t*-cells (Tregs) express and secrete higher levels of Trx-1, and the levels of Tregs are increased in different cancer types as well as in inflammatory diseases, such as RA (27). Lemarechal *et al.* have found Trx-1 in the serum of RA patients has significantly higher than in controls, and the activity of Trx-1 is high (28). In this study, we have found Trx-1 expressed in RA is much higher than in OA, and we hypothesised that the anti-apoptotic, anti-oxidative and oncogenic effects of Trx-1 make it a key protein in the pathogenesis of RA, and also the drug targets of RA.

We have detected 3 candidate proteins in RA-FLS and OA-FLS by applying proteomic. Western blot and semi-quantitative fluorescent PCR confirmed the decreased expression of PIMT and pirin in the RA-FLS and increased expression of Trx-1 in RA-FLS. Functional classification indicated that these identified genes were related to cell proliferation, apoptotic and migration of FLS. These 3 candidate proteins affect the functions of FLS in some style and can be the drug targets of RA.

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