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Original Article

Proteomic analysis of synovial fluid as an analytical tool to detect candidate biomarkers for knee osteoarthritis

Weixiong Liao, Zhongli Li, Hao Zhang, Ji Li, Ketao Wang, Yimeng Yang

Department of Orthopedics, General Hospital of PLA, Beijing, People's Republic of China

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Abstract: We conducted research to detect the proteomic profiles in synovial fluid (SF) from knee osteoarthritis (OA) patients to better understand the pathogenesis and aetiology of OA. Our long-term goal is to identify reliable candidate biomarkers for OA in SF. The SF proteins obtained from 10 knee OA patients and 10 non-OA patients (9 of whom were patients with a meniscus injury in the knee; 1 had a discoid meniscus in the knee, and all exhibited intact articular cartilage) were separated by two-dimensional electrophoresis (2-DE). The repeatability of the obtained protein spots regarding their intensity was tested via triplicate 2-DE of selected samples. The observed protein expression patterns were subjected to statistical analysis, and differentially expressed protein spots were identified via matrix-assisted laser desorption/ionisation-time of flight/time of flight mass spectrometry (MALDI-TOF/TOF MS). Our analyses showed low intrasample variability and clear intersample variation. Among the protein spots observed on the gels, there were 29 significant differences, of which 22 corresponded to upregulation and 7 to downregulation in the OA group. One of the upregulated protein spots was confirmed to be haptoglobin by mass spectrometry, and the levels of haptoglobin in SF are positively correlated with the severity of OA ($r = 0.89$, $P < 0.001$). This study showed that 2-DE could be used under standard conditions to screen SF samples and identify a small subset of proteins in SF that are potential markers associated with OA. Spots of interest identified by mass spectrometry, such as haptoglobin, may be associated with OA severity.

Keywords: Osteoarthritis, proteomics, synovial fluid, biomarker, haptoglobin

Introduction

Osteoarthritis (OA) is a degenerative joint disease most commonly observed in adults around the world, which is characterised by metabolic, biochemical, and morphological changes, mainly in the articular cartilage [1]. In most individuals, there are no symptoms or abnormal imaging findings in the early stage of OA, and it is difficult to diagnose OA in its early phase through traditional methods. Proteomic analysis of SF can aid in finding candidate biomarkers that can be used as follows: (1) to reveal abnormal metabolic processes to elucidate the pathophysiological mechanisms underlying OA; (2) to diagnose preclinical OA or high-risk groups; (3) to monitor changes in joint tissue metabolism during the progression of OA; (4) to monitor the response of joint tissues to therapy and observe curative effects; and (5) to estimate prognosis [2].

In recent years, a number of studies were performed to reveal proteomic changes in SF during OA-related cartilage degeneration. Most of these studies investigated proteins of interest using enzyme-linked immunosorbent assays (ELISAs). For example, Honsawek et al. used this method to examine several proteins that could be related to the severity of OA and might serve as biomarkers for the determination of disease severity. These include bone morphogenetic protein-7 (BMP-7) [3], visfatin [4], and endoglin [5], among which BMP-7 has already been demonstrated to play a potential role in cartilage protection and the repair of OA. However, these studies were confined to investigating one or a small number of known proteins and failed to seek unknown or unproven proteins from the complex protein profile in SF. Despite these studies, the existing knowledge about the molecular mechanisms underlying cartilage degeneration in OA remains limited.

Recent progress in two-dimensional electrophoresis (2-DE) has led this method to be the most widely applied technique to separate a mass of proteins and screen for differentially

Table 1. Outerbridge classification

Grade	Pathology
I	Softening and swelling of articular cartilage
II	Fragmentation and fissuring of articular cartilage affecting an area of less than 0.5 inches
III	Fragmentation and fissuring of articular cartilage affecting an area greater than 0.5 inches
IV	Cartilage erosion to bone

expressed proteins [6]. In 2-DE, proteins are separated based on their isoelectric point (pI) in the first dimension and on their molecular weight (MW) in the second dimension [7]. Spot intensities can be compared after consecutive silver staining and visualisation, after which spots of interest can be identified by mass spectrometry (MS) [8]. There have been a few reports addressing the application of 2-DE for proteomic analysis of SF. Fritz et al. showed that the size of γ-globulin (mainly IgG) spots in SF from RA patients was increased compared to that from either non-RA or control patients [9]. In another report, Smith et al. showed that changes in acute-phase proteins in SF from RA patients tending towards normal levels could be correlated with clinical improvement and conventional clinical chemistry measurements [10]. Nevertheless, few comparisons have been made of SF from healthy versus OA-affected individuals by means of 2-DE [9]. In recent years, the development of immobilised pH gradients (IPG) used for isoelectric focusing (IEF) has greatly improved the stability, reproducibility and sample loading in 2-DE [11]. The subsequent combination of this methodology with micro-identification and mass spectrometry technologies has resulted in 2-DE being used as a prior separation method in proteomic analyses. It is for this reason that we choose 2-DE as analytic tool for proteomics analysis of SF from OA patients [11].

In most experiments, the progression of OA was classified using the Kellgren-Lawrence grading scale. However, radiological grading may not be in accordance with disease severity, so we adopted outerbridge classification to distinguish the degree of cartilage defects observed under arthroscopy to optimise the design of this experiment. In addition, two SF samples were selected and analysed in triplicate to determine intrasample variability. Based on these procedures, we developed standard conditions under which the results of 2-DE would be more persuasive. The combination of 2-DE with MS and ELISA analyses revealed that the concentration of haptoglobin was markedly increased in SF from OA patients.

Materials and methods

Patients

SF samples were obtained from 10 knee OA patients (three males, seven females; age: 52-70 years; mean age: 60 years) and 10 non-OA patients (six males, four females; age: 13-49 years; mean age: 27 years) with other articular diseases (meniscus injury or discoid meniscus of the knee without cartilage loss). All of the subjects were chosen from the group of patients who received arthroscopy or arthroplasty for treatment of knee joint disease during April to December 2011 in the orthopaedic department of PLA general hospital. The diagnosis of knee OA was based on the criteria of the American College of Rheumatology (ACR). All of the patients have signed informed consent documents. The study was approved by the Institutional Review Board (IRB). The severity of OA in the patients was evaluated according to the Outerbridge classification of cartilage defects (**Table 1**) [8]. The knee articular surface was divided into 6 areas (patella, femoral trochlea, medial and lateral femoral condyle, medial and lateral tibial plateau). Each area was assessed based on the Outerbridge classification. The total score of the knee articular cartilage lesions was the sum of that of each area, ranging from 0 to 24. The OA patients could be categorised into 6 grades of different severity (I, IIA, IIB, IIIA, IIIB, IV). According to the classification, 6 of the subjects recruited in the OA group were classified as grade IIIB, and the other 4 OA patients in this experiment were categorised as grade IIB. **Figure 1** shows images of cartilage defects under arthroscopy.

Sample collection and preparation

An SF sample 1-2 ml was aspirated from the affected knee with a sterile puncture needle prior to operating on the patients, and 50 μl of proteinase inhibitor (cocktail, ROCH) was imme-

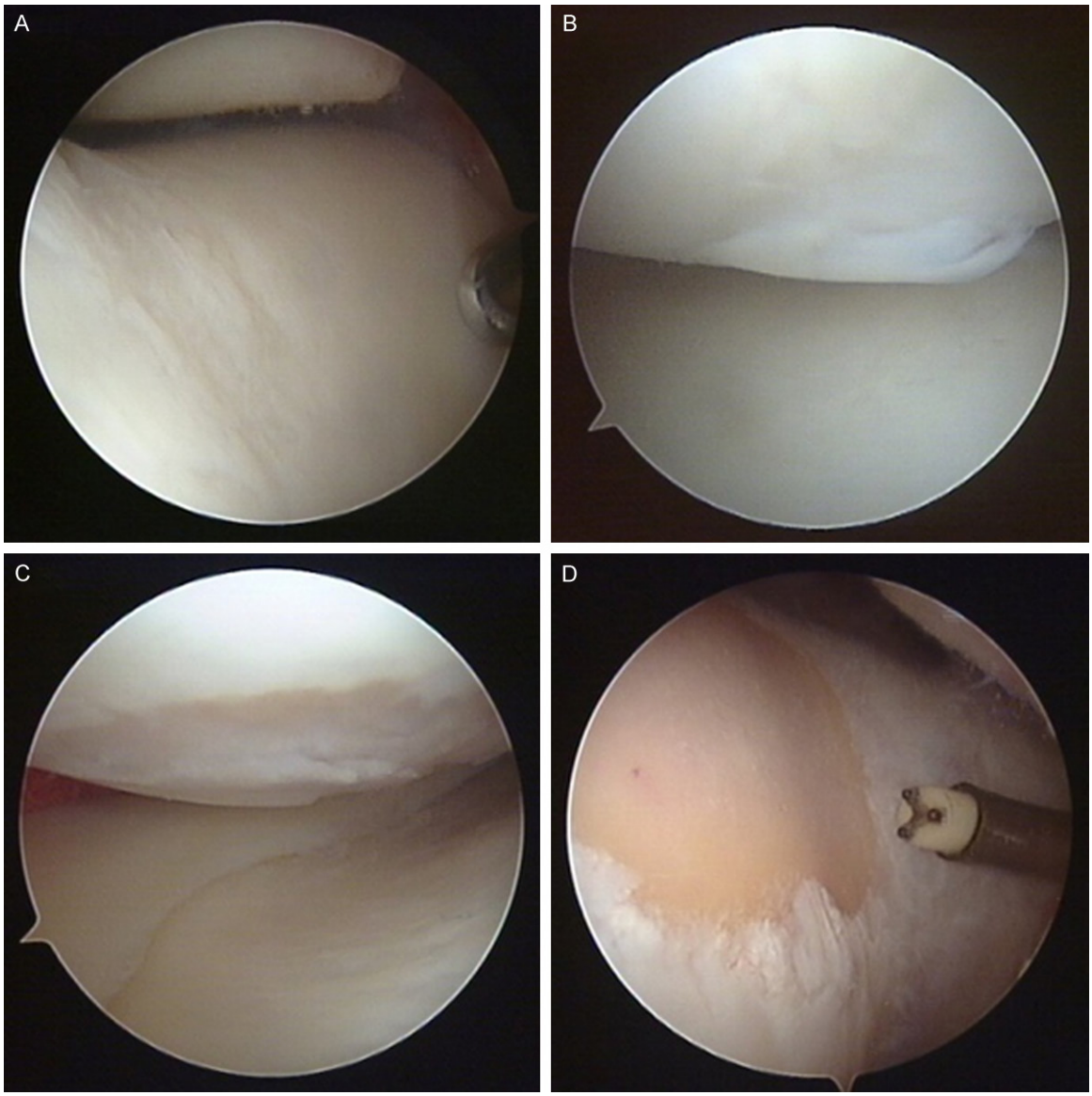


Figure 1. The Outerbridge classification of cartilage defects viewed under arthroscopy. Femoral trochlea cartilage lesion of grade I (A), Grade II cartilage defect in the femoral condylar (B), Grade III cartilage defect of the femoral condyle (C), Grade IV cartilage lesion of the femoral trochlea (D).

diately added to the sample after collection. The mixture was then applied to an Eppendorf tube (Millipore) and centrifuged at 8000 rpm for 40 min at 4°C (Hettich MIKRO 22R, Germany) to obtain a volume of 100 μl to remove cells and debris and concentrate the proteins. After centrifugation, the SF sample was prepared for protein quantification to determine the volume of sample to be loaded in the subsequent procedure, and the total protein concentration was measured via the Bradford Assay with a commercial Bradford reagent (Bio-Rad Laboratories, Hercules, CA). Then, the processed samples were stored at -80°C until use.

2-Dimensional electrophoresis (2-DE)

The 2-DE analyses were performed according to Gorg et al. [12], with some modifications. The SF samples were centrifuged for 2 min before loading, and 160 μg of protein was then diluted to a volume of 350 μl with rehydration solution containing 8 M urea, 0.02% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) (Sigma), 0.02 M dithiothreitol (DTT), and 0.05% IPG buffer (Amersham Pharmacia Biotech). The volume of the sample to be loaded could be calculated based on the total mass and concentration of the proteins. The samples

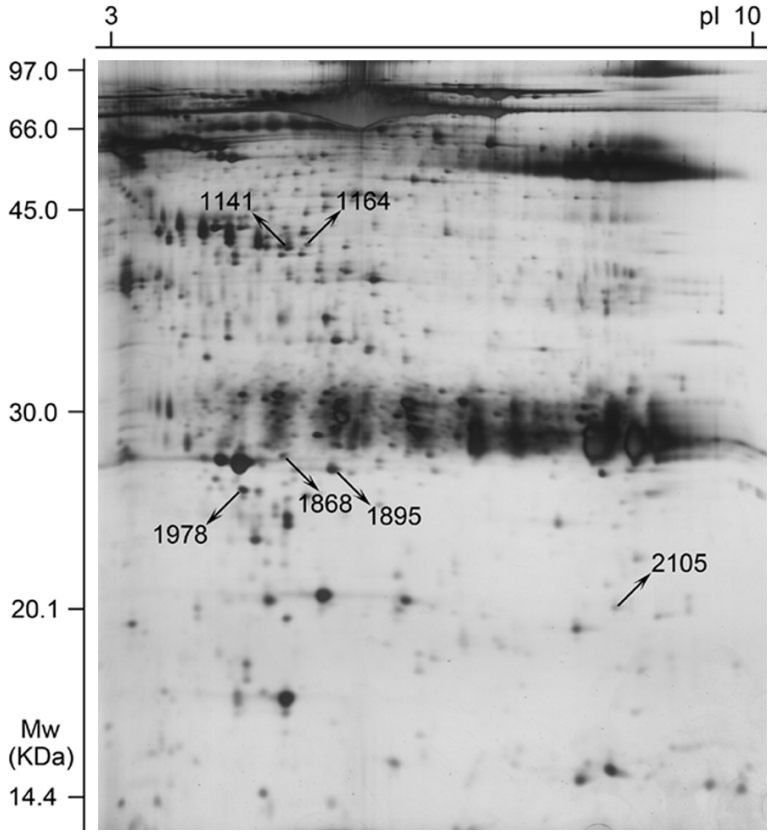


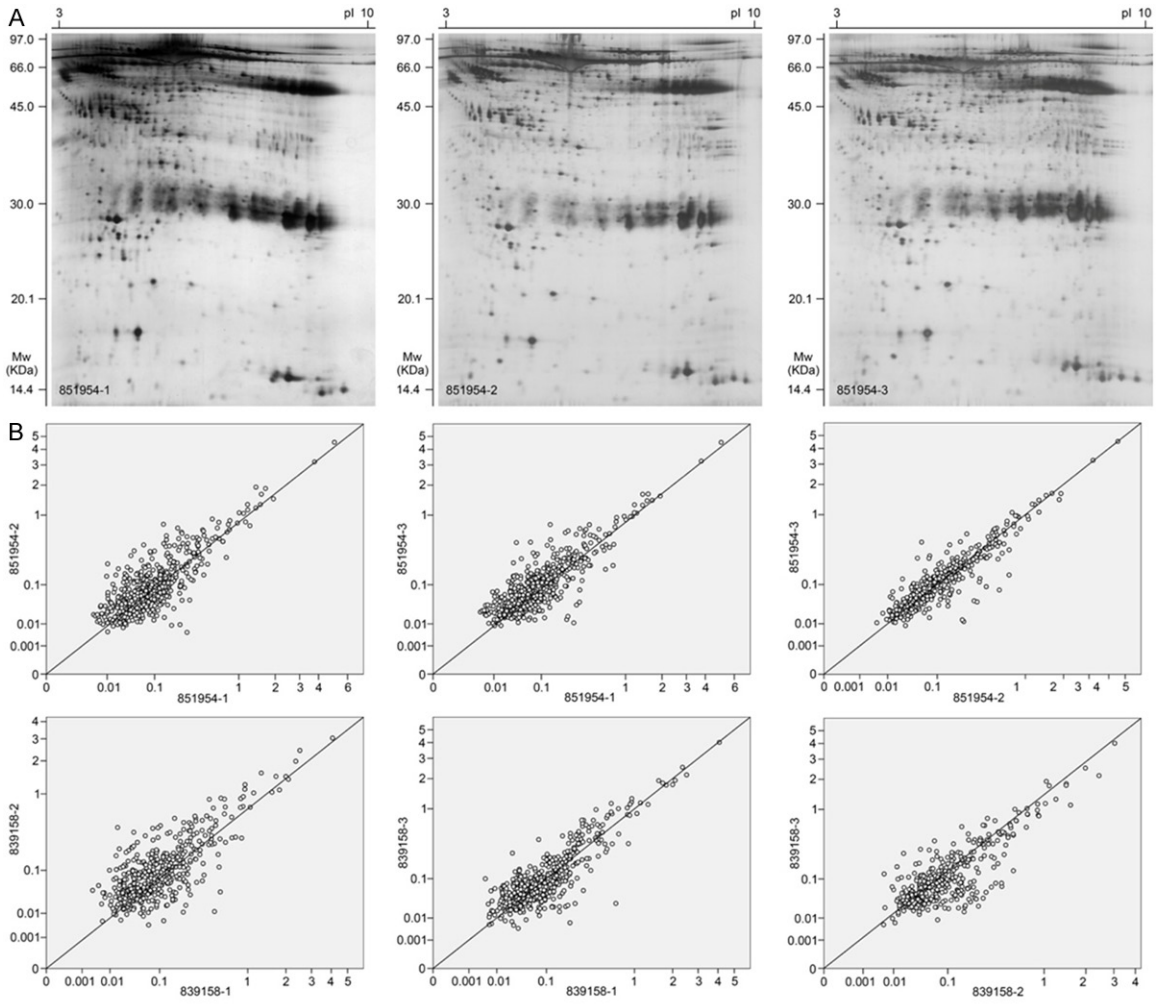
Figure 2. This electropherogram derived from a grade IIIB OA sample shows protein spots separated in 2 dimensions based on their isoelectric point (pI) and molecular weight (MW), respectively. The indicated spots show differences of more than 5 fold.

were loaded onto 18 cm (pH 3-10) non-linear immobilised pH gradient Drystrips (Amersham Pharmacia Biotech). Then, 800 µl of cover fluid was added to cover the drystrips. Isoelectric focusing (IEF) was carried out using the IPGphor system (Amersham Pharmacia Biotech) under a constant voltage of 30 V/gel for the initial 10 h, followed by 500 V/gel for 1 h, 1000 V/gel for 1 h and 8000 V/gel for the last 10 h. After IEF, the strips were equilibrated twice in a solution containing 50 mM Tris (hydroxymethyl) amino methane (Tris-Cl). pH 8.8, 30% glycerol, 6 M urea, 2% sodium dodecyl sulfonate (SDS) and bromophenol blue tracer for 15 minutes. Then, the IPG strips were transferred to vertical self-cast 13% SDS-polyacrylamide gels [13% acrylamide, 375 mM Tris-Cl, 0.1% SDS, 0.1% ammonium persulfate (APS), tetramethylethylenediamine (TEMED)] and embedded with agarose sealing solution (0.5% agarose, SDS electrode buffer, bromophenol blue tracer). Finally, SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the PROTEAN II Xi Cell vertical electrophoresis system (Bio-Rad company) under a constant current of 20 mA/gel for the initial 40 min and 30 mA/gel thereafter until the bromophenol blue tracer reached the bottom of the gel. Two SF samples from each OA and control group were selected and analysed in triplicate, respectively, to determine intra-sample variability.

Silver nitrate staining was performed in the analytical gels according to the protocol of Pasquai et al. [13]. An ImageScanner (Amersham Pharmacia Biotech) was utilised to capture the 2-DE images. ImageMaster 2D Platinum (Amersham Pharmacia Biotech) was used to quantify and revise the intensity of the protein spots. The spot intensity was normalised for every gel [(spot quantity)/$\sum$(spot quantities)] to correct for subtle variations in sample loading and gel staining between the gels. Statistically significant differences in spot intensities among different samples were identified using Student's t-test. The significance of the differences in the spot intensities among different subgroups of the OA and non-OA group was determined by analysis of variance (ANOVA). *P*-values < 0.05 were considered statistically significant.

In-gel trypsin digestion and peptides extraction

In-gel digestion was performed using a protocol previously described by Shevchenko et al. [14], with minor variations. Protein spots that showed significant differences were chosen for gel excision. The gel spots were excised and destained with 50 mM ammonium bicarbonate in 50% acetonitrile (gradient grade; Merck, Darmstadt, Germany). The spots were then dehydrated with 100% acetonitrile and dried under flowing nitrogen. Proteins were digested with a mixture containing 10 ng/ml of sequencing grade trypsin (Promega, Madison, WI, USA) and 25 mM ammonium bicarbonate overnight at 37°C. Finally, peptides were extracted with a solution containing 50% acetonitrile and 0.5% trifluoroacetic acid (Sigma).



	851954-1	851954-2	851954-3
851954-1	1	0.947	0.955
851954-2		1	0.970
851954-3			1

	839158-1	839158-2	839158-3
839158-1	1	0.916	0.951
839158-2		1	0.936
839158-3			1

Figure 3. Analysis of intrasample variability in two samples from the OA (851954) and non-OA (839158) groups. Triplicate electropherograms from a grade IIIB OA sample (A), the non-OA sample electrophoretograms are not shown (B), Scatter plots of each pair of gels derived from the same SF sample. The X and Y axes of the plots represent the intensities of the spots (C), Correlation coefficients from each pair of gels ($P < 0.001$).

Mass spectrometry (MS) and database searches

MALDI-TOF/TOF MS was performed in a Bruker UltraflexTM III MALDI-TOF/TOF MS (Bruker Daltonics) operating in reflection mode under an accelerating voltage of 20 kV and a reflecting voltage of 23 kV. A saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.1% trifluoroacetic acid was used as the matrix to aid the ionisation of the sample, and 1 μl of the matrix solution and peptide sample solution at a ratio of 1:1 were applied to the Score384 target well. External calibration of the mass spectrometer was realised routinely by analysing a standard peptide calibration mixture. Then, the loaded samples were ionised with a pulsed nitrogen laser (337 nm) with a frequency of 4 Hz. The most abundant ions fulfilling certain preset criteria (a signal-to-noise ratio higher than 25 and quality factor higher than 50) were subjected to high-energy CID analysis. The collision energy was set to 1 keV, and nitrogen was chosen as the collision

gas to cleave the ions. Protein spots from different gels were identified separately to conform that the corresponding spots that were differentially expressed between the gels represented the same protein.

PMFs were subjected to search against the S. flexneri 2a 2457T database using the program Mascot 2.1 (Matrix Science Ltd.) to eliminate redundancy resulting from multiple members of the same protein family, and the results were checked against the NCBInr database (version October 21,2006,4,072,503 sequences).

Enzyme-linked immunosorbent assay (ELISA)

An additional 24 SF samples were acquired from 18 knee OA patients (four males, fourteen females; age: 48-74 years; mean age: 61 years) and 6 non-OA patients (five males, one female; age: 19-40 years; mean age: 29 years) using the method described above. Among the 18 OA patients, 6 were classified as grade IIB according to the Outerbridge classification, while 7 were grade IIIB and 5 were grade IV. The haptoglobin concentration in SF was determined using an ELISA (MyBioSource, R&D Systems, San Diego, California, USA) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed with SPSS 13.0 for Windows. Data were expressed as a mean ± SD. Pearson's correlation coefficient was utilised to analyse the correlation between the haptoglobin concentration in SF and the OA disease severity. *P*-values < 0.05 were considered statistically significant.

Results

Comparative proteome analysis of OA and control group

The 2-DE method described above was applied to 20 SF samples obtained from patients at PLA general hospital (10 non-OA samples, 6 grade IIIB OA samples and 4 grade IIB OA samples).The average total protein concentration in the SF was 23.58 ± 1.90 μg/μl. A total of 726 ± 30 protein spots could be detected on each gel. Spots were regarded as representing differentially expressed proteins when the intensity of the spots between the OA and control samples was proven to be significantly different ($P < 0.05$). Based on this criteria, 29 spots were identified as proteins showing significant differences, with molecular weights (MWs) ranging from 14 kDa to 97 kDa. Among these 29 protein spots, 22 were upregulated and 7 were downregulated in the OA group compared with the control group. There were 6 spots for which the difference of intensity was greater than 5 fold. The locations of these 6 spots in the gels are indicated in **Figure 2**. The results demonstrated that there were a large number of proteins showing statistically significant differences in spot intensity between these two groups.

Analysis of intrasample variability

Because of the influence of gel-to-gel variability and manual operational error, two SF samples from both the OA and control groups were selected and analysed in triplicate, respectively, to examine the reproducibility and stability of the experiment. The correlations between each pair of gels were investigated to analyse the relationships between and alterations in the intensity of the protein spots. The results revealed that gels run with aliquots from the same sample showed high similarity, which was reflected in the correlation coefficients. The analysis of intrasample variability is shown in **Figure 3**.

Protein identification by MS

As noted above, These 6 spots showing a difference of more than 5 fold were subjected to MALDI-TOF/TOF MS after excision and digestion with trypsin for the purpose of identification. The corresponding protein spots from 2 different gels were identified to ensure that spots at the corresponding locations in different electrophoretograms represented the same proteins. Subsequent MS/MS analysis was performed to obtain more information about the fragments of peptides and improve the reliability of the identification. The MS results are shown in **Table 2**. Among these 6 spots, one was confirmed to be haptoglobin, suggesting that there might be an increase in the haptoglobin concentration in SF from OA patients.

Statistical analysis of haptoglobin between different gels

The results showed that there was an increase in the intensity of haptoglobin in the OA group compared with the control group, which was statistically significant ($P < 0.001$). To determine the relationship between the spot intensity and OA severity, we compared the relative

Table 2. The identified protein spots showing statistically significant differences (P < 0.05) between samples from the OA and control groups

SSP	Protein name	NCBInr IDno.	Mr	pI	Peptides		Sequence coverage	Score
					Match	Total		
1141	haptoglobin	gi|3337390	38209	5.88	4	46	16%	137
1164	Chain B, crystal structure of fibrinogen fragment D	gi|2781208	37625	5.84	19	42	60%	168
1868	proapolipoprotein	gi|178775	28944	5.45	11	31	46%	173
1895	hypothetical cytoskeletal protein	gi|4588000	45372	5.73	22	106	60%	84
1978	Chain A, crystal structure of the trigonal form of human plasma retinol-binding protein at a 2.5-Angstrom resolution	gi|157830446	20945	5.27	9	34	74%	301
2105	Chain A, solution structure of domain 3 from human serum albumin complexed to an anti-apoptotic ligand directed against Bcl-Xl and Bcl-2	gi|71042087	22637	8.22	7	15	49%	90

SSP is the abbreviation for the standard spot number, representing the number used in 2-DE to indicate the locations of the proteins. The protein score is -10 × Log (P), where P is the probability that the observed match is a random event. Protein scores greater than 84 is significant ($P < 0.05$).

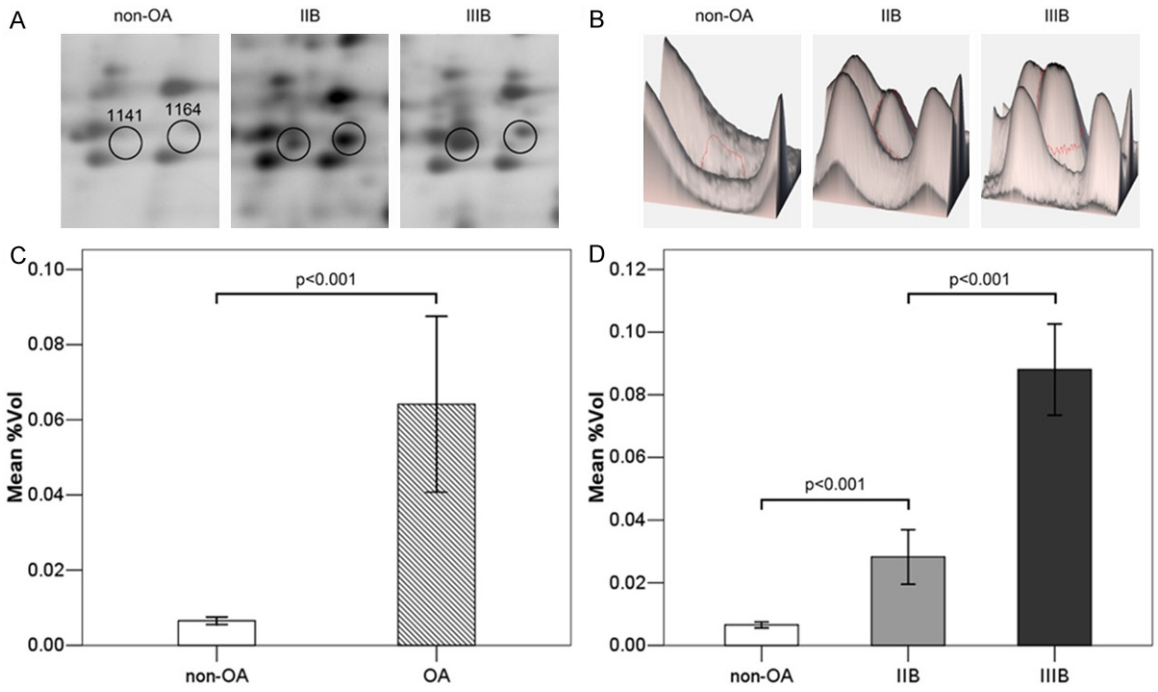


Figure 4. Comparison of the haptoglobin protein intensity between non-OA samples, grade IIB OA samples and grade IIIB OA samples. Close-up sections of electrophoretograms showed upregulation of haptoglobin in samples from the OA group (A). A 3D view of haptoglobin levels produced using ImageMaster 2D Platinum (B). Error bars indicate the haptoglobin levels in SF from different groups (C). Bars refer to the mean % vol (95% CI) of spots per group. White: non-OA; shaded: OA. Error bars indicating the haptoglobin intensity in SF from the non-OA group and different OA subgroups according to the Outerbridge classification (D). Bars represent the mean % vol (95% CI) of spots per group. White: non-OA; light gray: grade IIB; dark gray: grade IIIB.

intensities of the spots between non-OA samples, grade IIB OA samples and grade IIIB OA samples by analysis of variance (ANOVA). It was found that difference in the haptoglobin intensity between the non-OA samples and grade IIB OA samples was statistically significant ($P < 0.001$), as was that between non-OA samples and grade IIIB OA samples ($P < 0.001$). The difference between samples of grade IIB and IIIB also reached statistical significance ($P < 0.001$). The statistical results revealed that the haptoglobin level might be correlated with disease progress and could be used as potential biomarker to determine OA disease severity. The differences are presented in **Figure 4**.

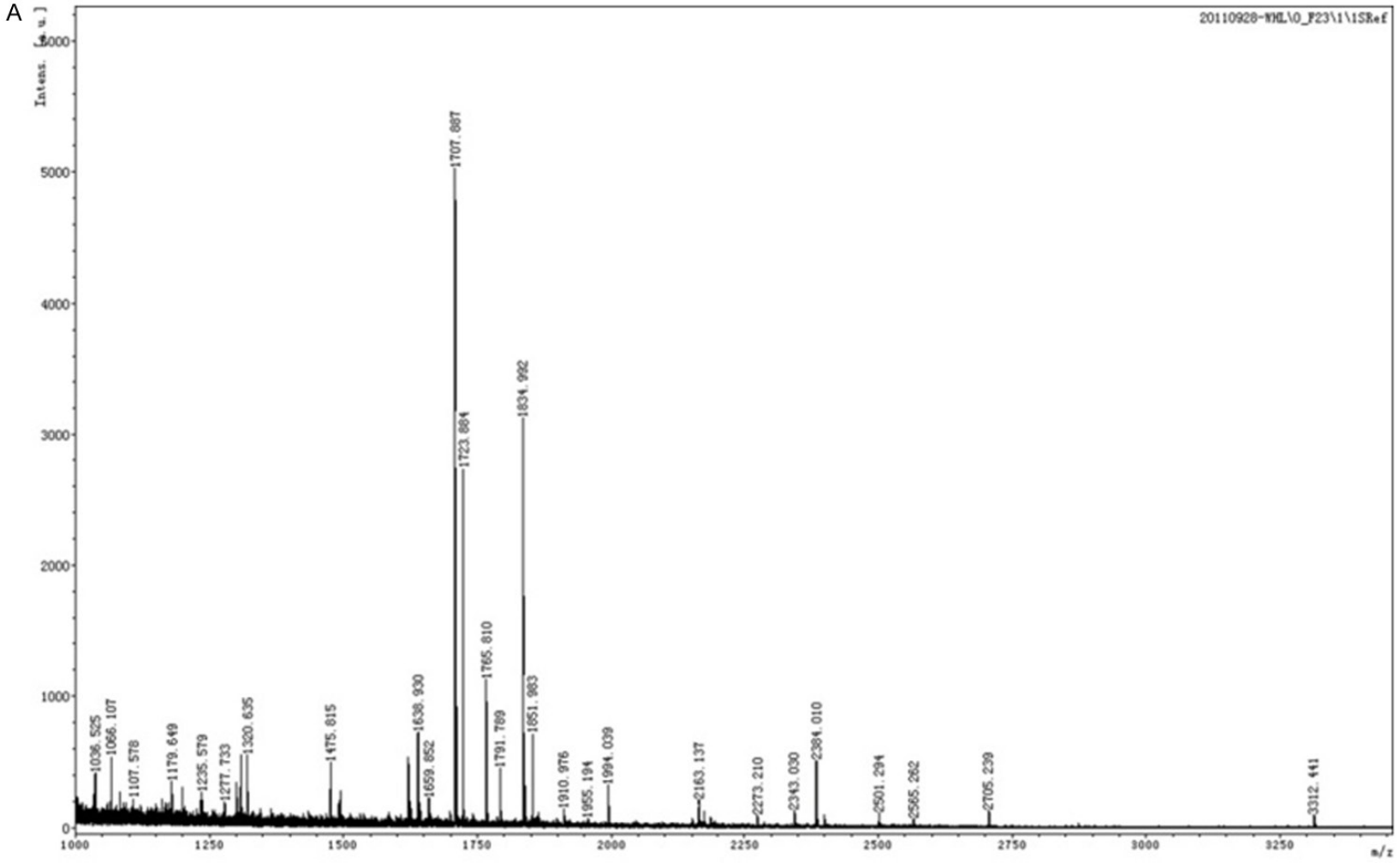
MS identification of haptoglobin

Figure 5 shows the PMFs produced through MALDI-TOF-MS and subsequent MS/MS of protein spot 1141. Peptides exhibiting mass-to-electric charge ratios (m/z) of 1707.887 and 1834.992 were chosen for subsequent high energy CID analysis because of their high ionic strengths. The PMF of protein spot 1141 was subjected to searches against the S. flexneri 2a 2457T and NCBInr databases to match it with proteins in these databases. The data returned from the databases showed that protein spot 1141 is likely to represent haptoglobin.

ELISA of haptoglobin

As is shown in **Figure 6**, the mean level of haptoglobin in SF from OA group patients (22.62 ± 11.48 μg/ml) is higher than that of non-OA group (2.54 ± 0.79 μg/ml), and this difference is statistically significant ($P = 0.001$). According to the Outerbridge classification, the mean level of haptoglobin in SF from grade IIB patients is 10.85 ± 2.74 μg/ml; that from grade IIIB patients is 25.20 ± 8.50 μg/ml; and that from grade IV patients is 33.15 ± 9.17 μg/ml. It was found that the mean level of haptoglobin in SF from grade IV patients is significantly elevated compared with those from non-OA group ($P < 0.001$), grade IIB group ($P < 0.001$) and grade IIIB group ($P = 0.046$). Moreover, the mean level of haptoglobin in SF from grade IIIB patients is markedly higher than those from non-OA group ($P < 0.001$) and grade IIB group ($P = 0.001$). The difference between the mean level of haptoglobin in SF from non-OA patients and grade IIB patients is also statistically significant ($P =$

A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1320.635
1475.615
1638.930
1659.852
1707.887
1723.864
1765.810
1791.789
1834.992
1851.983
1910.976
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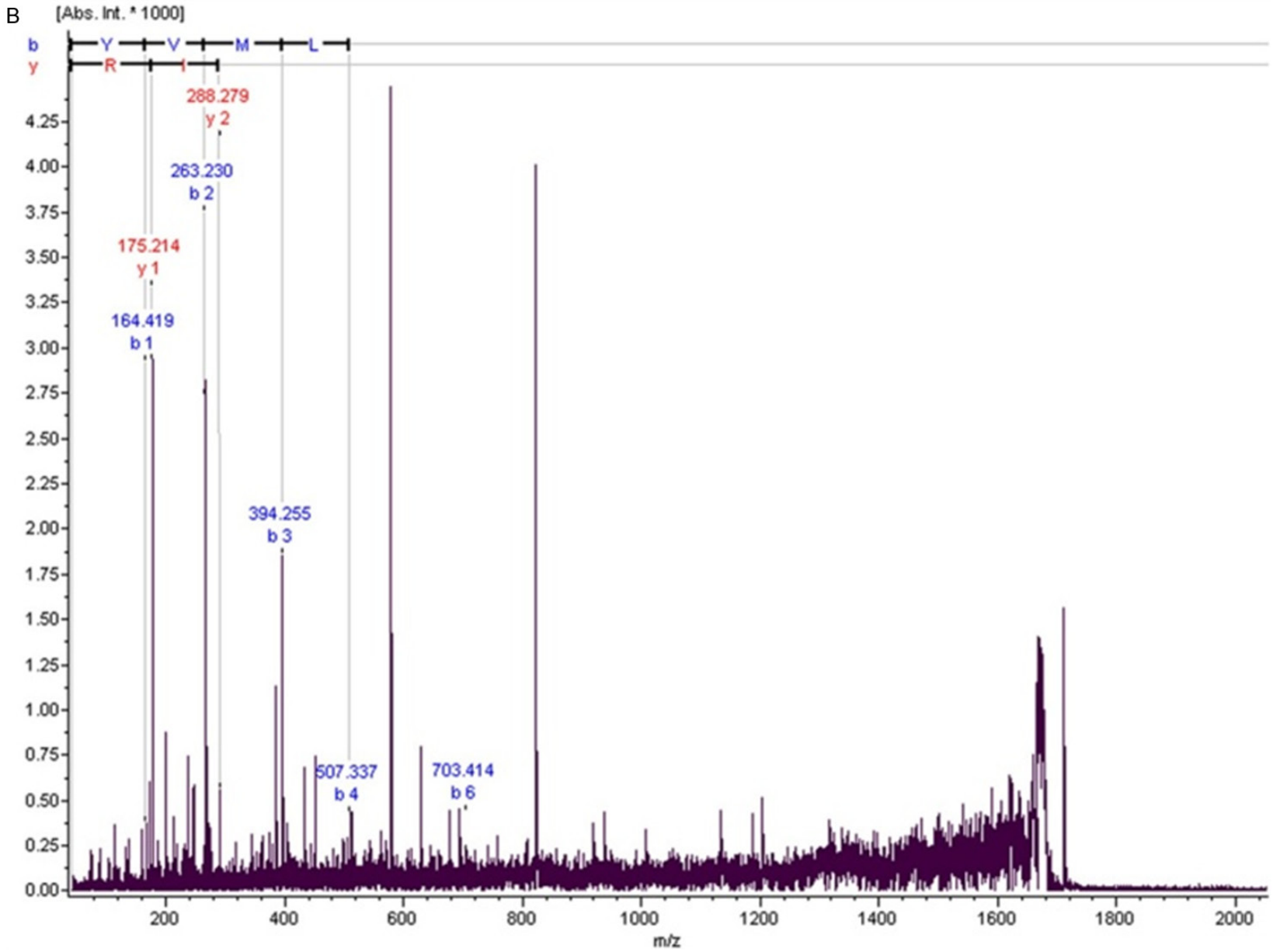
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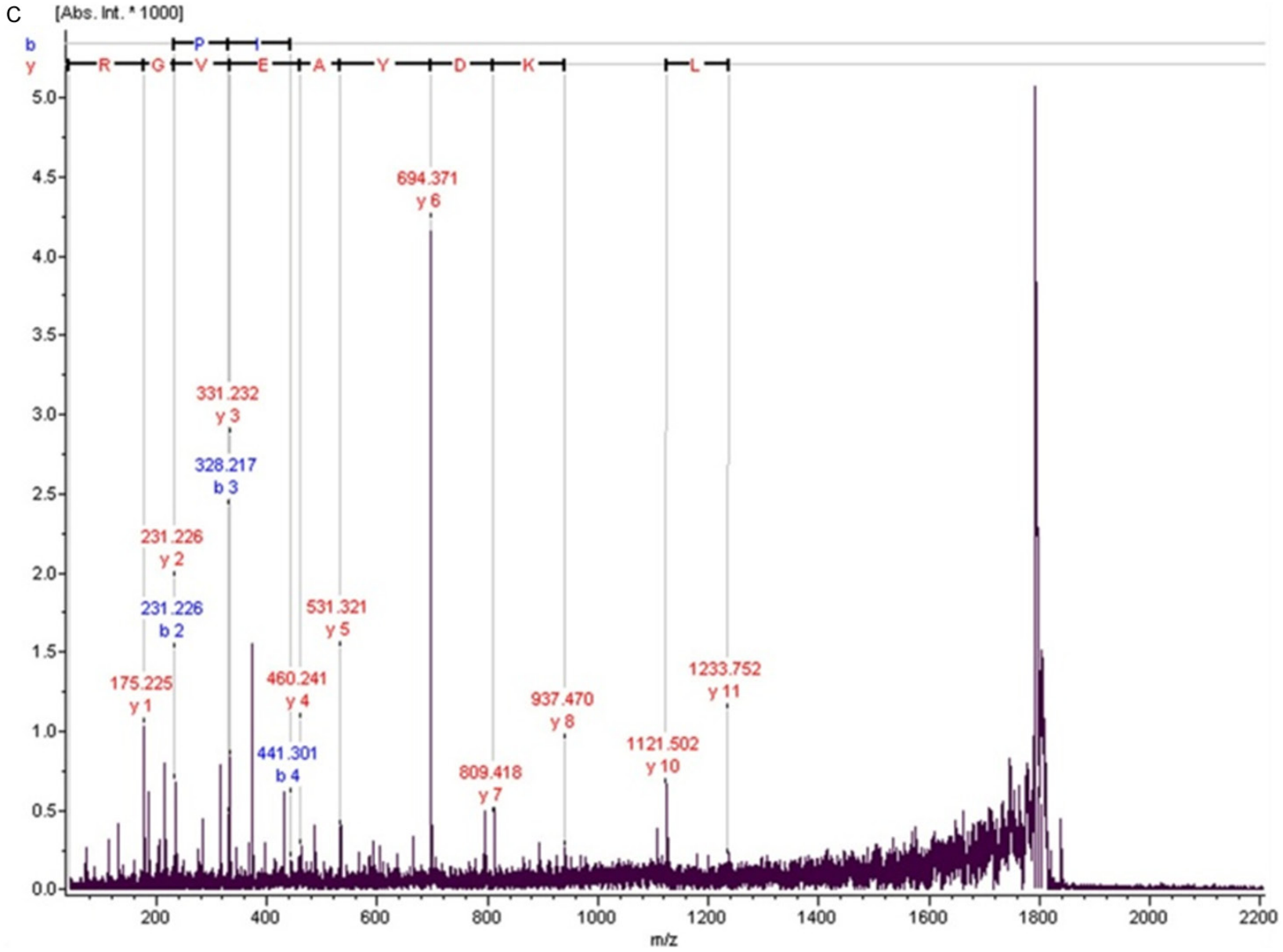
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y 3
328.217
b 3
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y 2
231.226
b 2
531.321
y 5
175.225
y 1
460.241
y 4
441.301
b 4
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y 7
937.470
y 8
1121.502
y 10
1233.752
y 11
5.0
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Figure 5. Mass spectrogram of protein spot 1141 identified by MALDI-TOF-MS. PMF of spot 1141 by MALDI-TOF-MS (A). The protein was identified as haptoglobin by subjecting its PMF to searches against the database. The protein score (137) is statistically significant (P < 0.05). Peptides of 1707.887 and 1834.992 selected from the PMF of spot 1141 were subsequently analysed using an MS/MS system (B and C).

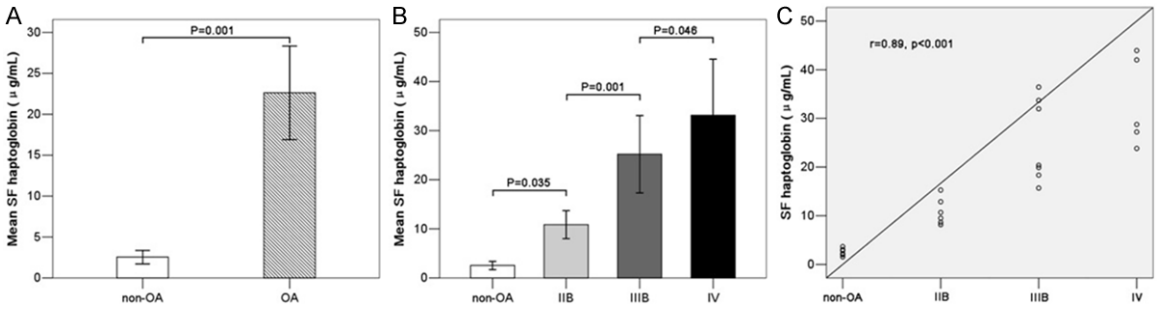

Figure 6. ELISA results. Comparison of the haptoglobin levels in SF from different groups. Bars refer to the mean concentration (95% CI) of haptoglobin in each group. White: non-OA; shaded: OA (A). Comparison of the haptoglobin intensity in SF from the non-OA group and different OA subgroups. Bars represent the mean concentration (95% CI) of haptoglobin in each group. White: non-OA; light gray: grade IIB; dark gray: grade IIIB; black: grade IV (B). The relationship between haptoglobin levels and OA severity. The horizontal axis represents the severity of OA, and the vertical axis represents the concentration of haptoglobin in SF (C). This plot shows that these two parameters are positively correlated (r = 0.89, P < 0.001).

0.035). Thus, the levels of haptoglobin in SF are positively correlated with the severity of OA (r = 0.89, *P* < 0.001).

Discussion

The long-term goal of our experiment was to analyse the proteomic pattern in SF and identify potential biomarkers for OA in SF via 2-DE and MALDI-TOF/TOF MS analyses. To this end, we compared the proteomic profiles of SF samples obtained from OA and non-OA patients. The subjects in the OA group were divided into two subgroups based on the Outerbridge classification of cartilage defects to investigate the relationship between differentially expressed proteins and disease severity.

It was found that significant differences were present for 29 protein spots between the OA group and the non-OA group, of which 22 were upregulated, while 7 were downregulated and 6 exhibited a difference of more than 5 fold in spot intensity. The identification of haptoglobin was of particular interest, as haptoglobin has previously been reported to be elevated in RA compared with OA. Furthermore, the differences in the intensity of the haptoglobin spots in 2-DE gel images observed between non-OA samples compared with grade IIB and IIIB OA samples are all statistically significant. The results confirm the hypothesis that there is marked intersample variability in the protein spot intensity between the OA and control groups. Haptoglobin may be correlated with cartilage degeneration and could be used as a potential biomarker to evaluate the severity of OA. This conclusion has been verified by ELISA in our study. Another notable protein identified by MS is cytoskeletal protein, which is important in the physiology and biomechanics of chondrocytes. The increase in the concentration of this protein in OA samples may coincide with the apoptotic processes involved in the pathogenesis of OA chondrocytes.

Smeets et al. reported that haptoglobin could be expressed in extravascular sites such as tendons, ankles and knees in rats [15]. Stevens et al. showed that haptoglobin could also be synthesised in chondrocytes in cartilage explants from bovine stifle joints. In addition, they found that this process could be stimulated and promoted by IL-1β and TNF-α [16]. Similarly, Samut et al. reported that the expression and activity of haptoglobin were primarily augmented by IL-6 [17], which is produced through the activities of IL-1 and TNF-α. Therefore, the increase in haptoglobin levels observed in the SF of OA patients may be partly due to increases in the levels of upstream cytokines.

It has been found in numerous studies that haptoglobin is elevated in the serum and SF of patients with RA [1]. Immune disorders are con-

sidered to be mainly responsible for the pathogenesis of RA, which is characterised by the infiltration of activated $CD4^+$ T lymphocytes and MHC class II-positive APCs in the synovium and other joint tissues. Haptoglobin is able to not only inhibit the proliferation and activation of T lymphocytes, but also to suppress the release of the Th1 and Th2 cytokines [18]. Therefore, local expression of haptoglobin may play an important part in relieving an excessive immune response and reducing the damage to articular tissues in RA. Additionally, leukotrienes (LTs) and prostaglandins (PGE) are crucial inflammatory factors that can promote the release of IL-1β and TNF-α in the RA inflammatory response. Due to its antioxidant properties, Haptoglobin could suppress the activities of LOX and COX to inhibit the synthesis of LTs and PGE, the attenuation of which would alleviate potential damage to the surrounding tissues [19]. A recent study demonstrated that decreases in the concentration of acute phase proteins in SF, including haptoglobin, might be correlated with clinical improvement of RA. Therefore, variations in the concentration of haptoglobin in SF may be associated with the progress and prognosis of the articular inflammation.

Haptoglobin might also have a vital function in the pathophysiological process of OA. For instance, it is well known that increased activity and amounts of MMPs contribute to the metabolic imbalance in the articular cartilage in most OA patients [20]. Haptoglobin might act as a nonspecific inhibitor of MMP-2 and MMP-9 [21], which constitute the gelatinase subgroup and are capable of catabolising the macromolecules in the extracellular matrix [22]. Therefore, it is reasonable to deduce that the increased concentration of haptoglobin in the SF of OA patients probably represents feedback from the enhanced MMP activity to protect the macromolecules in the extracellular matrix from being broken down excessively. Additionally, the concentration and molecular weight of hyaluronic acid (HA) in SF observed under inflammatory and degenerative arthropathy are lower than in normal SF [23] suggesting that there is prevalent and pervasive depolymerisation of HA in most arthropathy cases. haptoglobin might play a crucial role in protecting HA from being depolymerised by phagocytes. It was found that haptoglobin could prevent HA from the degradative effects of oxygen-derived free radicals (ODFR), mainly by combining with HA to form a complex [24] Formation of HA-Hp complexes in SF could protect HA from the deterioration due to hydrogen peroxide, which is derived from hydroxyl free radicals in activated polymorphonuclear leucocytes (PMNLs) [25]. In addition, free radicals can damage basement membranes and cartilage matrix components [26] as well as inhibit the synthesis of macromolecules [27], so increases in haptoglobin levels could be regarded as a protective mechanism to preserve tissue integrity by suppressing the activities of free radicals. Consequently, acute phase proteins, including haptoglobin, might be exuded into the osteoarthritic SF from the peripheric circulatory system during the previous inflammatory phase [24] or have been synthesised by chondrocytes in articular cartilage, as described above. Regardless of where they originate, variations in the levels of SF proteins, including haptoglobin, can provide important information about the pathophysiological process of OA. However, there is currently not sufficient evidence to determine whether an individual's susceptibility or resistance to OA is associated with their haptoglobin genotype. Thus, further studies addressing the relationship between haptoglobin and diseases are required.

Disease-related biomarkers are generally present in low levels and might be obscured by highly abundant proteins [28]. In the initial stage of the experiments described herein, we attempted to remove unwanted highly abundant proteins in SF with a 2-D Clean-Up Kit to increase the sensitivity of the 2-DE analysis. However, the results showed that some proteins of interest might also be eliminated when we remove these highly abundant proteins with the 2-D Clean-Up Kit. This phenomenon was in agreement with the situation many other researchers have reported [29]. The most frustrating problem we came across in the experiment was contamination from serum proteins. We do not have sufficient evidence to prove that the differentially expressed proteins we detected through 2-DE represent actual alterations in SF rather than contamination from serum. The best way to solve this problem is to aspirate SF under the surveillance of musculoskeletal ultrasound, which is limited in our experiment. Another solution to this problem is to increase the volume of the samples collected, which is

what we are planning to do next. As noted above, SF presents enormous potential regarding the diagnosis and prognosis of OA, though more normalised sample preparation methods and advanced equipment are needed to ensure that the results of SF analysis are accurate and repeatable.

The present study mainly focused on comparative proteomics analysis of SF, aimed at searching for differentially expressed proteins under OA. However, we did not conduct a corresponding functional analysis of these differentially expressed proteins. Therefore, we cannot infer whether the alterations in these proteins we found in SF are primary causes of or secondary changes due to the disease. Furthermore, we are not yet able to determine whether these proteins of interest play a role in abnormal metabolic process or only represent non-functional biomarkers, which will require further research.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Zhongli Li, Department of Orthopedics, General Hospital of PLA, 28 Fuxing Road Haidian District, Beijing 100853, People's Republic of China. Tel: +86-1066938106; Fax: +86-13910630653; E-mail: yangyangz82@163.com

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