Analysis of synovial fluid in knee joint of osteoarthritis: 5 proteome patterns of joint inflammation based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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Abstract

Purpose The purpose of this study was to use matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) in osteoarthritis research. Our aim was to find differentially expressed disease-related and condition-specific peptide in synovial fluid in the knee joint of patients suffering from osteoarthritis (OA), and to develop and validate the peptide classification model for OA diagnosis.

Methods Based on the American College of Rheumatology criteria, 30 OA cases and ten healthy donors were enrolled and underwent analysis. Magnetic beads-based weak cation exchange chromatography (MB-WCX) was performed for sample processing, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was conducted for peptide profile. ClinProt software 2.2 was used for data analysis and a genetic algorithm was created for class prediction.

Results Two peptide peaks were found which may be characterised as the potential diagnostic markers for OA. Two other significantly different peptide peaks were found in OA patients at a medium stage compared to the early and late stages. A genetic algorithm (GA) was used to establish differential diagnosis models of OA. As a result, the algorithm models marked 100% of OA, and of 97.92% of medium-stage OA.

Conclusion This study demonstrated that use of proteomics methods to identify potential biomarkers of OA is possible, and the identified potential biomarkers may be potential markers for diagnosis and monitoring the progression of OA.

Introduction

Osteoarthritis (OA) is a degenerative joint disease of the whole joint as a result of biological, chemical and viscoelastic changes to the cartilage, synovium, subchondral bone, and synovial fluid (SF) [1, 2] affecting nearly 27 million Americans or 12.1% of the adult population of the United States [3]. Several small community surveys in China report that the current knee OA prevalence ratio was present in 9–11% of males and in 20–21% of females (aged 59–84 years) [4]. Despite the increasing molecular knowledge about OA, the pathology of this disease remains poorly understood and specific biomarkers for OA are lacking [5]. In order to improve the diagnosis and treatment of OA, efforts have been made to discover biomarkers to predict OA progression and assess therapeutic response, with limited success. Nowadays, researchers believe that SF may have significant diagnostic value for OA, because SF

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in arthritic joints may undergo biochemical and chemical changes, such as alterations in protein composition and proteomic profile [6, 7, 11, 12].

Proteome analysis has been recognised as one of the most effective tools to explore biomarkers as it can assist in diagnosis and prognosis of diseases [8–10]. As a core technology of proteome analysis, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has been widely used to analyse proteomic characteristics in biological laboratories due to its sensitivity and efficacy [11–14]. The aim of this study was to analyse peptide profiles of human synovial fluid in OA patients using the MALDI-TOF-MS technique.

Patients and methods

Clinical samples

This study was purely observational and no medical interventions were carried out as part of it. Thirty patients meeting the American College of Rheumatology (ACR) criteria were recruited in this research [15]. All samples were obtained according to the guidelines approved by the Ethics Committee of Shenzhen People's Hospital, China. The Ethics Committee of Shenzhen People's Hospital specifically approved this study and an informed consent form was signed by all patients and volunteers. In detail, 13 cases were male and 17 cases were female. The mean age was 54.8 years, range 43–79 years. Ten age-matched healthy volunteers served as normal control (four males, six females, mean age 52.4 years, range 38–68 years).

According to the Kellgren and Lawrence roentgenographic diagnostic criteria [16], patients with OA were divided into three stages as follows: A (early stage): 0–1 degree; B (medium stage): 2–3 degrees; C (late stage): 4 degrees. Typical knee joint X-ray films on patients with OA in the early stage showed a symmetrical joint space without osteophyte formation; in the medium stage of OA they showed that an asymmetric joint space occurred, with medial joint space narrowing and marginal intercondylar notch osteophyte formation; in the late stage of OA, they showed both medial and lateral joint spaces were remarkably narrowed, with osteophyte formation at the edge of joint, and subchondral bone sclerosis and ligament calcification.

Exclusion criteria

Studies were excluded in the presence of systemic inflammatory conditions or infection, such as inflammatory arthritis, rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis, reactive arthritis, septic arthritis, acute pseudogout, or any other connective tissue disease; or the

presence of peripheral vascular disease; pregnant or lactating women; patients with cognitive impairment (defined as a score of <7 on the Short Portable Mental Status Questionnaire) [17].

Peptide extraction by magnetic beads

Briefly, 1–2-ml synovial fluid samples were drawn from the supra-patellar bursa of the knee joint cavity, collected in vacuum glass tubes, and coagulated at room temperature for five minutes, then centrifuged at 3,000 rpm for five minutes at room temperature and 0.1-ml aliquots of the supernatant were stored immediately at -80°C . Then the following process was performed according to the manufacturer's recommendation [18]. First, magnetic bead pellets in solution were resuspended thoroughly on a vortex device for one minute. Then 200 μl of microfuge tube containing 10 μl of magnetic bead binding buffer (BS) and 10 μl of magnetic bead-based weak cation exchange chromatography (MB-WCX) (Bruker Daltonics, Ettlingen, Germany) were mixed by pipetting up and down. Five microlitres of each synovial fluid sample was added to the solution and mixed intensively and rested at room temperature for five minutes. After that the tubes were placed in a magnetic bead separator (MBS) and agitated back and forth three times. One minute later the beads were collected on the wall of the tubes in the MBS, and the supernatant was removed carefully, using a pipette. Approximately 100- μl MB-WCX wash suspension solution was added to the tubes, beads were pulled and moved back and forth in the MBS ten times, and supernatant was removed carefully, repeated twice. Five microliters of elution suspension (ES) was added to disperse beads in tubes by pipetting up and down ten times. Two minutes later, the beads were collected on the walls of the tubes, and the clear supernatant was transferred into fresh 0.5-ml tubes. Five microlitres of stability suspension (SS) was added to the eluate, mixing intensively by pipetting up and down. Lastly, MALDI-TOF-MS targets and measurement was analysed.

MALDI time-of-flight peptide profiles

Mass spectrometry was performed on ClinProt platform. Synovial fluid peptide profiles were analysed with an Autoflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonik, Bremen, Germany). Preparation was described as follows. First, the sample application painter (Bruker Daltonik, Germany) was cleaned; second, the sample application was taken, each sample point was 1 μl and was repeated four times; third, samples were dried, followed by the matrix application, for each point 1 μl (solution composition was 3 mg/ml CHCA, 50% CAN and 2% TFA); fourth, the calibration machine was used with the

calibrator composition of 1 pmol/μl peptide mixture and 4pmol/μl protein mixture by 1:5 mixing; and fifth, sample testing was done (separate spectra were obtained for two restricted m/z ranges, corresponding to polypeptides with molecular mass of 0.7–4 kDa and 4–15 kDa [assuming $z=1$], under specifically optimised instrument settings) and acquisition signal was taken (400 laser shots delivered in four sets of 100 shots at 50-Hz frequency). Spectra were collected automatically using the flexAnalysis software (Bruker, Bremen, Germany) for fuzzy controlled adjustment of critical instrument settings to generate raw data of optimised quality.

Statistical analysis

The ClinPro Tools software 2.2 (Bruker, Daltonik) was used for analysis and data processing. The preprocessing of data included normalisation of a set of spectra, internal signal alignment by prominent internal signal peaks, and a peak picking procedure. In order to further visualise and statistically analyse the intensities of peptide peaks, t test was employed for the p -values. The calculated p -values, less than 0.05, were considered to be significant. Then a class prediction model was set up by genetic algorithm (GA), and a cross-validation was implemented to determine the accuracy of the class prediction. 80% of stochastically selected samples were taken as a training set in the class predictor algorithm and the remaining 20% of the samples was applied as a test set.

Results

Identification of differential OA related peptide peaks

In this study, 61 peptide peaks were detected, showing distinct differences in multiple peptide clusters in this cohort, demonstrating the diagnostic function of peak amount and peak area of peptide panels using ClinProTools software. Among the 61 peptide peaks, 32 of those indicated lower abundance in patient's synovial fluid than those from healthy controls, while the other 29 expressed higher peak intensities (Table 1). A significant difference of two peptide peaks (m/z 1864.28 and 5335.63) were detected between the OA group and the controls ($p<0.01$), which increased nearly two-fold in OA than in controls (Fig. 1). Therefore, these two peptide peaks may represent specific pathological changes of OA.

Receiver operating characteristic curve (ROC) analysis of differential peptide peaks and diagnostic tests

Two peptide peaks (1864.28 Da and 5335.63 Da) were analysed for ROC respectively using SPSS 13.0 software

Table 1 Sixty-one differential peptide peaks in synovial fluid from patient groups

m/z values	Normal	OA	PTTA
Up-regulated protein peaks in patient groups			
1864.28	9.94	21.35	0.00529
5335.63	1.65	2.94	0.00529
5350.99	1.52	2.56	0.00529
1777.15	10.52	22.18	0.0109
1895.58	5.67	6.91	0.0374
4208.7	3.32	4.42	0.126
5309.78	1.13	1.47	0.126
4052.39	2.04	2.62	0.0839
1689.85	6.57	10.4	0.164
2020.62	12.99	16.78	0.251
1136.8	8.08	10.76	0.251
1346.55	5.95	7.87	0.251
1341.3	5.31	6.7	0.251
1154.26	2.42	2.95	0.251
959.6	5.26	6.81	0.321
4417.7	5.4	7.87	0.428
6947.58	0.69	0.81	0.431
4432.98	3.72	5.55	0.541
2552.65	3.64	4.25	0.431
6931.92	0.71	0.83	0.431
1448.13	4.84	5.73	0.541
8926.79	1.19	1.39	0.541
3240.63	3.41	4.02	0.556
4403.46	1.77	2.07	0.556
8806.44	0.79	0.85	0.57
2931.32	3.64	3.95	0.697
2659.45	11.16	12.52	0.732
8682.27	0.72	0.75	0.804
1516.23	9.83	10.06	0.869
Down-regulated protein peaks in patient groups			
4962.26	9.93	3.87	0.00605
4935.94	3.05	1.43	0.0109
5580.83	1.65	0.95	0.126
2228.01	6.09	4.26	0.231
6172.68	1.21	0.91	0.231
6430.47	3.43	2.28	0.251
8593.83	1.42	1.14	0.251
6383.61	1.05	0.85	0.251
807.05	2.75	2.06	0.431
3442.03	4.06	2.67	0.541
2080.25	5.84	4.82	0.541
1057.77	2.8	2.38	0.541
5861.13	1.18	1.02	0.541
8562.67	0.99	0.87	0.541
7442.34	0.92	0.76	0.556
5871.72	1.18	1.06	0.556
1541.41	8.69	7.89	0.6

Table 1 (continued)

<i>m/z</i> values	Normal	OA	PTTA
1566.14	7.34	6.55	0.6
6628.45	3.16	2.7	0.6
9128.47	0.89	0.78	0.6
9065.96	0.85	0.8	0.6
7478.03	0.75	0.69	0.673
2861.32	5.2	4.91	0.778
5902.56	1.66	1.52	0.778
6800.64	1.34	1.2	0.778
5535.11	1.21	1.15	0.778
9282.67	0.76	0.72	0.778
2208.89	5.42	5.03	0.804
4089.77	2.34	2.24	0.804
7761.53	0.68	0.66	0.852
8119.82	0.67	0.66	0.909
6546.78	0.89	0.88	0.985

Normal mean intensity in normal controls, OA mean intensity in osteoarthritis (OA) patients, PTTA *P* value of *T* test or ANOVA (analysis of variance)

(see Fig. 2). The area under the ROC curve (A_Z) of 1864.28 Da was 0.926, and the cut-off point was 18.92. The area under the ROC curve of 5335.63 Da was 0.908, and the cut-off point was 2.71. Since it has been commonly believed that $A_Z > 0.9$ indicated high diagnostic value, according to the former result, these two peptide peaks may be treated as potential biomarkers of OA. We then used the above cut-off points 18.92 and 2.71 as threshold value for diagnostic test, and applied the threshold value to determine the sensitivity, specificity and accuracy of the peptide peaks 1864.28 Da and 5335.63 Da (see Table 2). It was shown that the peptide peak of 1864.28 Da reached

90% sensitivity, 80% specificity and 87.5% accuracy. The peptide peak of 5335.63 Da obtained 83.3% sensitivity, 80% specificity and 82.5% accuracy. These identified peptide peaks possessed diagnostic sensitivity and specificity for OA patients.

Identification of differential peptide peaks in different pathology stages of OA

The average intensity of peptide peaks in three stages of OA subgroups were compared, and different expressions of 62 peptide peaks in the OA subgroups were examined, two peptide peaks (m/z 1864.33 and 1448.15) were identified manifesting significantly different levels in the medium stage group. The expression of two peptide peaks was significantly higher in the medium stage group than the early stage and late stage groups ($p < 0.05$, Fig. 3), while no significant difference between the early stage group and late stage group was seen ($p > 0.05$, Fig. 3).

Establishment of genetic algorithm classification model

ClinPro Tools software 2.2 was used to analyse the raw data, and three independent algorithm processing stages were introduced to classify samples and establish a diagnostic model. Results to distinguish OA from normal controls by each algorithm were as follows: quick classifier algorithm (QC) with a cross-validation of 68.54% and a recognition capability of 78.75%, neural network (SNN) with a cross-validation of 74.54% and a recognition capability of 58.75%, genetic algorithm (GA) with a cross-validation of 87.23% and a recognition capability of 100%. To distinguish medium stage to early and late stages in OA subgroups, the QC with a cross-validation of 42.58% and a recognition capability of 70.17% were found, SNN

Fig. 1 Pseudo-gel maps of peptide peaks 1864.28 Da and 5335.63 Da by the comparison of the osteoarthritis (OA) group and normal controls. Peptide peaks 1864.28 Da and 5335.63 Da distinguished OA significantly from normal controls

Fig. 2 a Receiver operating characteristic curve (ROC) of 1864.28 Da. b ROC of 5335.63 Da

with a cross-validation of 58.63% and a recognition capability of 90.97% as well, and GA with a cross-validation of 85.21% and a recognition capability of 97.92%. GA was proven to have higher sensitivity and specificity than other algorithms, being the best choice for establishing a classification model.

Discussion

The new ClinProt platform which is based on magnetic bead separation and mass spectrometry analysis, performs all major steps of profiling, screening, and monitoring applications in clinical proteomics [19–22], in order to explore specific biomarkers from complex body fluids, e.g. serum, plasma, urine, saliva, cerebrospinal fluid, tissue lysate, and cell culture medium. As the core technology of ClinProt platform, the MALDI-TOF-MS features rapid analysis, high sensitivity, and low sample consumption, which has been widely applied in cancer research [23–27]. Many attempts have been reported for the development of biochemical markers of OA [28–31], including Uchida et al. who compared protein profiles of the SF of patients with rheumatoid arthritis (RA) and OA by using surface-enhanced laser desorption/ionization mass spectrometry (SELDI-TOF MS) technology and

found several reproducible and discriminatory biomarker candidates for distinction between rheumatoid arthritis and osteoarthritis. The 10,850 Da protein peak among these candidates was the clearest example of a single signal found specifically in the rheumatoid arthritis samples [32]. Kamphorst et al. analysed a synovial fluid sample from an OA patient and a healthy control, and identified a total 501 peptides were originating from 40 proteins. Peptide cleavage products from six proteins have been associated with OA in earlier studies (collagen II, proteoglycan 4, serum amyloid A, tubulin, vimentin, and Matrix Gla) [33]. To our knowledge, it is the first time that MALDI-TOF-MS has been used in the analysis of synovial fluid samples of OA for detection of characteristic markers.

In this study, synovial fluid samples of the OA group and normal controls were collected, and two significant differentially expressed peptide peaks were detected between these two groups (m/z 1864.28 and 5335.63), both of which increased nearly two-fold in the OA group, indicating that the proteomic composition of OA was significantly changed. Then, verified with ROC analysis, the two typical peptide peaks were featured for potential diagnostic sensitivity and specificity. In the OA subgroups, two significant differentially expressed peptide peaks (m/z 1864.33 and 1448.15) were detected, which were particu-

Table 2 Diagnostic tests of 1864.28 Da and 5335.63 Da peptides peaks

Diagnosis	OA group		Controls		Total	
m/z (Da)	1864.28	5335.63	1864.28	5335.63	1864.28	5335.63
Positive	27	25	2	2	29	27
Negative	3	5	8	8	11	13
Total	30	30	10	10	40	40

OA osteoarthritis

The 1864.28 Da peptide peak had sensitivity of 90%, specificity of 80% and accuracy of 87.5%, and the 5335.63 Da peptide peak had sensitivity of 83.3%, specificity of 80% and accuracy of 82.5%

Fig. 3 Two pictures in the panel were pseudo-gel maps of peptide peaks 1864.33 Da and 1448.15 Da by the comparison of osteoarthritis (OA) subgroups. It was shown that peptide peaks 1864.33 Da and 1448.15 Da distinguished the medium stage of OA significantly from early and late stages

larly highly expressed in the medium stage group compared with the early stage and late stage groups. As indicated by Xiang et al. [34], a peptide (m/z 1865) was detected predominantly in sera from systemic sclerosis (SSc) patients compared to sera from patients of systemic lupus erythematosus (SLE), rheumatic arthritis (RA), and OA. They further identified the peptide as DRC3f, a degraded derivative of C3f produced by removal of the C-terminal arginine with carboxypeptidase N. The level of DRC3f (m/z 1,865) was related to vascular involvement in SSc. In recent studies, some researchers also identified peptide 1865 as the unique peptide that down-regulated in active-SLE whereas it up-regulated in RA [35]. Thus the two detected peptides (m/z 1864.28 and 1864.33) were more critical to explore their function and the role they play in OA.

In a previous study, differentially expressed proteins of cellular metabolism, structure and protein targeting were identified in OA [36]. The differences of the mitochondrial protein profiles between OA and normal chondrocytes were also described, a mitochondrial dysregulation was found in cartilage cells of OA and redox imbalance highlighting a key factor of OA pathogenesis [37]. Bo et al. compared the differential protein expression patterns of the synovial fibroblasts (SFs) between the patients suffering RA or OA with the healthy controls, and identified 25 proteins, which included enzymatic and structural proteins, signal transduction proteins, and calcium binding protein [38]. Chang et al. compared the expression profiles of synovial membranes of the RA, OA, and ankylosing spondylitis (AS) patients, showing that Ig-kappa light-chain C region, PRDX4, SOD2, TPI, and TXNDC5 in synovial tissues of RA patients were up-regulated, indicating that the abnormality of cell differentiation, immunoactivation, and endogenous antioxidant reaction may link with RA processes. Especially TPI, which is an isomerising enzyme catalysing the reversible intercon-

version of d-glyceraldehyde 3-phosphate to dihydroxyacetone phosphate, plays an important role in glycolysis and gluconeogenesis [39]. Compared with similar research, potential biomarkers were also found in sera detection. De Seny et al. identified a total of 103 serum samples from 34 RA patients, 20 psoriatic arthritis (PsA) patients, nine asthma patients, ten Crohn's disease patients, 14 OA patients and 16 healthy control subjects and found the most discriminative mass/charge (m/z) values serving as potential biomarkers for RA, suggesting that the 10,832 peak could represent myeloid-related protein 8, which was related to disease activity in juvenile rheumatoid arthritis [40].

In conclusion, as a pilot and preliminary study on the inducement of joint synovial fluid component changes for articular cartilage lesion of OA, it not only provided us a potential approach for diagnosis, target therapy and prevention of OA on a molecular level, but also paved a new way to explore pathogenesis of OA. Validation of these promising peptide peaks should be continued with further studies on higher number of patient groups, combined with comprehensive proteomics analysis of serum and urine samples. The amount of the peptide peaks represented by the MS peaks present in the circulating blood should be evaluated; if these peptide peaks are contained abundantly in the blood of healthy donors, they are less promising to be biomarkers for OA. Conversely, if the peptide peaks are found only in the blood from OA patients but not in healthy donors, they have a higher chance to be biomarkers.

Conclusion

A preliminary diagnostic standard distinguishing OA from healthy people as well as targeting OA of different stages

was summarised as the following: four peptide peak signatures (m/z 1864.28, 5335.63, 4935.94, 4962.26) maintain the potential to be treated as a cross-validation set to discriminate OA from the healthy, achieving a recognition capacity of 100% and a cross validation of 87.23%. Similarly, the peptide peaks with mass/charge (m/z) values of 1448.15, 1864.33, 2553 and 3240.79 were promising to be treated as a cross-validated classification pattern to screening OA from medium stage to early or late stage. After repeat training the pattern could be implemented to distinguish medium stage OA from early and late stage with a recognition capacity of 92.92% and a cross validation of 85.21%.

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Author contributions All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Dai had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study conception and design: Xiaohua Pan and Yong Dai. Acquisition of data: Xiaohua Pan, Jiakai Chen, and Xiaofen Chen. Analysis and interpretation of data: Xiaohua Pan and Liling Huang.

Competing interests The authors have declared that no competing interests exist.

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