



# Traditional Chinese medicine classification of knee osteoarthritis with proteomics analysis

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**Background:** Osteoarthritis (OA) is a heterogeneous disease caused by the pathology of the synovial joint. About 10–12% of adults have symptomatic OA. In this study, the proteomics method was used to find differentially expressed proteins and to explore the material basis of traditional Chinese medicine (TCM) classification in knee OA patients.

**Methods:** Fifteen patients of the treatment group with knee OA of three different classifications were divided into three groups according to the dialectical classification of TCM: kidney deficiency type (5 cases), Yang deficiency type (5 cases), blood stasis type (5 cases). Also, five patients with traumatic synovitis were enrolled as the control group. The joint fluids were drawn separately. The joint fluids before treatment were Aa, Ba, and Ca groups. After one week of medication, the three joint fluid types were drawn again, and they were Ab group, Bb, and Cb groups. Liquid from the control group was named group D. Quantitative analysis of protein expression was conducted to find out the differently expressed proteins. The treatment group was treated with prescriptions for syndromes according to the TCM classification.

**Results:** The proteomics analysis identified 251 differentially expressed protein groups, and the number of groups with quantitative information for all seven channels was 246. The three treatment groups and the control group had 32 differential proteins ( $P < 0.01$ ), of which nine might be differential proteins between OA and traumatic synovitis. Histone H4, histone H2A, S100 calbindin A8, fibrinogen  $\gamma$ , fiber protein  $\alpha$ , cDNA (FLJ92148), C4b binding protein, and partial transketolase variants were down-regulation, and basement membrane glycan was up-regulation in the treatment group compared with the control group ( $P < 0.01$ ). HIST1H2BC and myeloperoxidase levels in the Aa group were less than those in the D and Ab groups. The serum amyloid P and apolipoprotein CI variants in the Ba group were less than those in the D and Bb groups. Histone H2A and C-reactive protein, Fibrinogen  $\alpha$  levels in the Ca group were less than those in the D and Cb groups.

**Conclusions:** Nine proteins are closely related to OA of different TCM classification.

**Keywords:** Osteoarthritis (OA); proteomics; kidney deficiency; Yang deficiency; blood stasis; iTRAQ

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## Introduction

Osteoarthritis (OA) is a heterogeneous disease caused by the pathological changes of the synovial joint. About 10–12% of adults suffer from symptomatic OA (1,2). Therefore, it affects many individuals and throws a heavy burden on the medical system. OA is included in the category of “Bone Bi” in traditional Chinese medicine (TCM). The pathogenesis is complicated, the disease evolves rapidly, and multiple pathologies are interchangeable. Due to the inconsistent understanding of Bone Bi syndrome’s pathogenesis, there is a significant difference in the differentiation of syndromes among different medicines. In recent years, scholars have begun to study the essence of “syndrome” from the perspective of genes and proteomics (3). Proteomics theory has gradually been used to diagnose, treat, and research various diseases and syndromes in Chinese medicine (3–8). The substantial material basis research of “syndrome” with proteomics as the starting point has highlighted its advantages in modernizing TCM (9).

Synovial fluid (SF) is the capillary exudate present in the joint chamber. Proteins in SF also comes from surrounding tissues, articular cartilage, synovium, and bone (10). SF directly contacts the affected tissues, cartilage, synovial tissue, and bones, which will become a source of high-quality biomarkers (11). In earlier studies, many substances have been found in synovial fluid, including cytokines, chemokines (12), molecules (13), and proteins (14). SF analysis is a crucial way to understand OA (15). The quantitative proteomics method of isobaric tags for relative and absolute quantitation iTRAQ was initially reported by Ross *et al.* (16). This method can identify and quantify proteins from many samples simultaneously while retaining the critical posttranslational modification information. This study used iTRAQ technology to compare the protein in the synovial fluid between patients with knee OA and knee traumatic synovitis to obtain the relative and absolute content of histones in different joint synovial fluids, hoping to find the difference of protein expression. We present the following article in accordance with the MDAR reporting checklist (available at <http://dx.doi.org/10.21037/apm-20-2117>).

## Methods

### Study population

From January 1, 2011, to January 31, 2013, according to

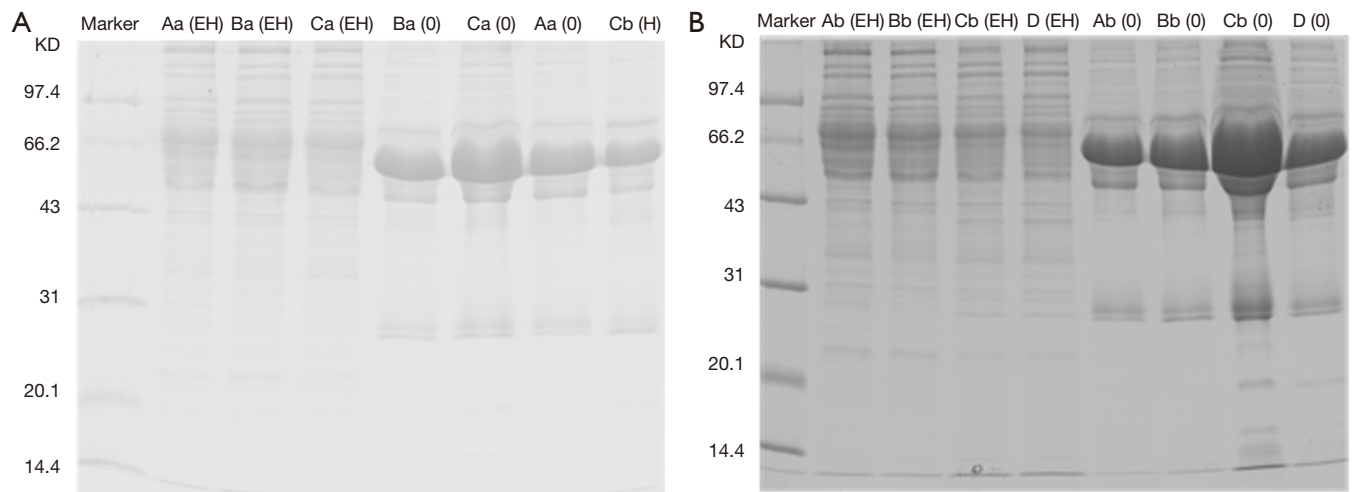
the OA diagnostic criteria established by the American Academy of Rheumatology and the TCM syndrome criteria of Zhonggu Bi in the Chinese Medicine Industry of the People’s Republic of China, “Diagnosis and Curative Effect Standards of TCM Diseases and Syndromes” (94 edition), patients aged 40–65 years old with knee OA stage I–II (according to Kellgren-Lawrence Staging) were enrolled. Knee OA with different TCM Classification of each patient was confirmed by a specialist team (consisting of 2 professors and 2 chief doctors) according to criteria mentioned previously. At the final analysis, 15 patients have enrolled: 5 patients with kidney deficiency, 5 patients with Yang deficiency, 5 patients with blood stasis. Another 5 patients with synovitis were enrolled as the control group. All subjects were excluded from diseases, including severe liver and kidney function, hematopoietic system disorders, hypertension, cardiopulmonary dysfunction, tumors, tuberculosis, infectious diseases, and acute severe injuries. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by ethics committee of The First Affiliated Hospital of Guizhou University of Traditional Chinese Medicine. All subjects were informed and signed an informed consent form before oral treatment with Chinese medicine and joint fluid acquisition.

### Classification and medication

Synovial fluid samples were divided into three groups according to the patient diagnosis of TCM: kidney deficiency group, Yang deficiency group, and blood stasis group. Patients of the kidney deficiency group were given the treatment of nourishing yin and nourishing the kidney, and choosing Liuwei Dihuang Decoction (prepared Rehmannia 15 g, dogwood 12 g, yam 12 g, dan bark 10 g, Alisma 10 g, Poria cocos 10 g); patients of Yang deficiency type were given Wenyang therapy to dispel cold and nourish the kidney and replenish Qi (the prescription uses Jinkuishenqi Decoction: 10 g Guizhi, 6 g Aconite, Rehmannia 15 g, Cornus 15 g, Chinese Yam 15 g, Poria 15 g, Dan Bark 10 g, Alisma 10 g); patients of blood stasis type were given blood circulation promotion therapy (the prescription is Taohong Siwu Decoction: Rehmannia root 15 g, angelica 15 g, white peony 10 g, chuanxiong 8 g, peach kernel 9 g, safflower 6 g). The above three prescriptions were administered to patients twice a day for two weeks.

**Table 1** Protein quantification results

Sample	Aa (n=5)	Ba (n=5)	Ca (n=5)	Ab (n=5)	Bb (n=5)	Cb (n=5)	D (n=5)
Density ( $\mu\text{g}/\mu\text{L}$ )	1.48 $\pm$ 0.32	1.87 $\pm$ 0.37	2.68 $\pm$ 0.43	1.09 $\pm$ 0.28	2.53 $\pm$ 0.41	4.28 $\pm$ 0.76	3.73 $\pm$ 0.55



**Figure 1** (A) Before treatment; (B) after 2 weeks of treatment. SDS-PAGE electrophoresis of the sample (loading amount 10  $\mu\text{g}$ ). Remarks: a means before treatment, “EH” means the sample after removing the peak protein, “0” means the original sample, and “H” means the peak protein. SDS-PAGE electrophoresis separated the seven samples after the peak was removed, and the protein bands were relatively uniform.

### Sample collection and processing

The synovial fluids of different types of OA of kidney deficiency, yang deficiency, and blood stasis before treatment were drawn into the Aa group, Ba group, and Ca group. The synovial fluids were acquired as described before (12). After two weeks of medication, the three joint fluid types were drawn again as the Ab group, Bb group, and Cb group, the untreated control group’s joint fluid, drawn as the D group. The amount of synovial fluid in 35 samples was about 2 to 4 mL. The samples were frozen and stored in a refrigerator at  $-80\text{ }^{\circ}\text{C}$ . Use iTRAQ analysis to analyze the samples (Shanghai Shanghai Zhongke New Life Biotechnology Co. coordinates iTRAQ analysis, Ltd., China).

### Statistical analysis

Using SPSS 20.0 statistical software for statistical processing. Quantitative data were expressed as mean  $\pm$  standard deviation, and the F test performed a comparison between groups.  $P < 0.05$  indicates that the difference is

statistically significant.

## Results

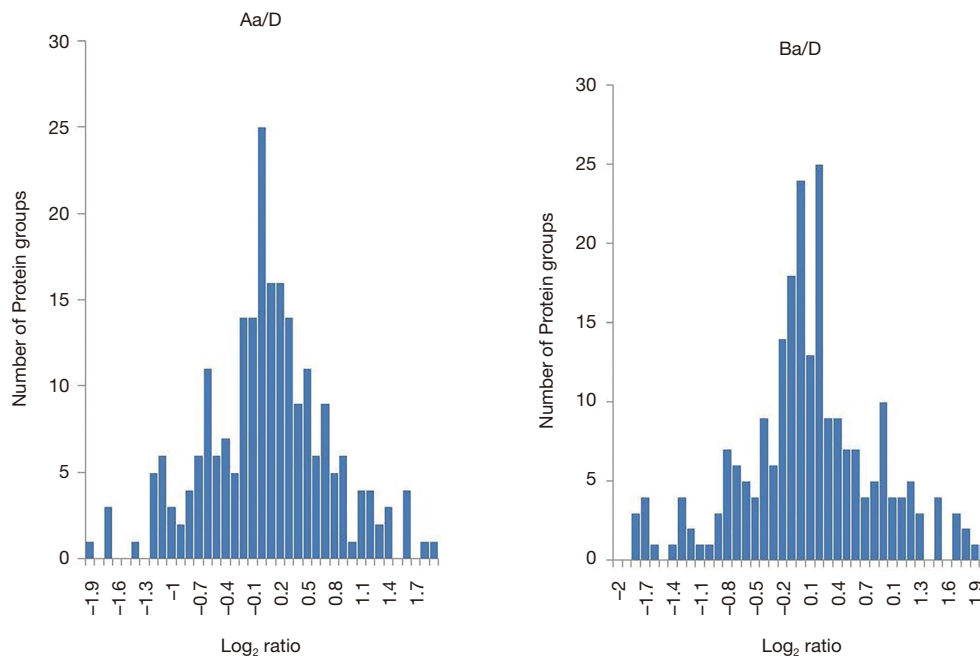
After 2 weeks of medication, all patients reported no significant suspected side effects. Repeated blood tests revealed no significant liver injury or renal injury.

### Quantitative determination results

See *Table 1* for the quantitative results of protein analyzed by iTRAQ. The comparison showed statistical differences between each group at different time points ( $P < 0.05$ ). The protein electrophoresis has similar results (*Figure 1*).

### Quantitative comparative analysis

We used a frequency distribution histogram to analyze iTRAQ quantitative data. The ratio of each group of labels to the reference label is Log<sub>2</sub> logarithm, the group distance is set to 0.1, and the distribution range is  $-2.0$  to  $2.0$ . Excel



**Figure 2** iTRAQ quantitative data analysis. The ordinate is the protein number, and the abscissa is the  $\text{Log}_2$  logarithm of the two sets of samples and the internal reference value.

draws *Figure 2*. *Figure 2Aa/D* and *2Ba/D* are typically distributed and similar, and the ratio between the two sets of samples and the reference (*2Aa/D* and *2Ba/D*) is used for Pearson correlation analysis, as shown in the scatter diagram 3. The points in the figure are close to a straight line, suggesting that there is a correlation.

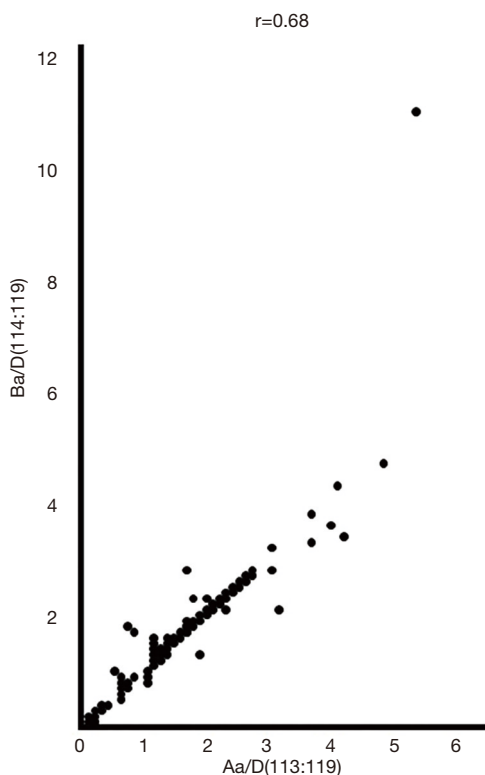
### Significantly different proteins

The total number of protein groups identified in the experiment is 251, and all seven channels have quantitative information, and the number of protein groups is 246. The seven samples were analyzed for Aa and D, Ba and D, Ca and D, Aa, and Ab, Ba, and Bb, Ca and Cb, and the significance index (P value) calculated. A total of 32 differential proteins were detected between the treatment group and the control group ( $P < 0.01$ ), as shown in [Table S1](#). Compared with the control group, there were eight significant reductions in the treatment group: histone H4, histone H2A, S100 calcium-binding protein A8, fibrinogen  $\gamma$ , fibrinogen  $\alpha$ , cDNA (FLJ92148), C4b binding protein, part of transketolase variants; one significantly increased: basement membrane glycan. Among these differentially expressed proteins, fibrinogen  $\gamma$  and C4b binding protein

were still significantly reduced compared with the control group after treatment. Two proteins were significantly reduced in the Aa group than the control group and the Ab group: HIST1H2BC and myeloperoxidase. Compared with the control group and the Bb group, the Ba group also had two significantly reduced proteins: serum amyloid P protein and apolipoprotein C-I variants. Compared with the control group and the Cb group, the Ca group had three proteins significantly reduced: histone H2A, C-reactive protein, and fibrinogen  $\alpha$ . Compared with the control group, lactoferrin and prefibrin-1 were significantly decreased in the Aa and Ba groups, and hemoglobin  $\alpha 1$  was significantly increased.

### Discussion

The iTRAQ technology uses 4 or 8 isotopic labels to label the amino groups of polypeptides specifically and then performs tandem mass spectrometry analysis to compare the relative or absolute content of proteins in 4 or 8 different samples simultaneously. Many scholars have used it to find the differentially expressed proteins of some diseases (17-21). In this experiment, we used iTRAQ technology to investigate OA patients and found 32 proteins with significant differences.



**Figure 3** Correlation analysis of Aa/D and Ba/D. The abscissa and ordinate base two logarithmic values of the ITRAQ ratio of Aa/D and Ba/D, respectively.

#### *Analysis of the difference between OA and traumatic synovitis proteome*

Eight (histone H4, histone H2A, S100 calcium-binding protein A8, fibrinogen  $\gamma$ , fibrinogen  $\alpha$ , cDNA (FLJ92148), C4b binding protein, partial transketone) significantly decreased and a significantly increased protein (basement membrane glycan) OA patients compared with patients with traumatic synovitis. Among these proteins, fibrinogen gamma and C4b binding protein were still significantly reduced after treatment, suggesting that OA patients' systemic symptoms are negatively related to fibrinogen gamma and C4b binding protein in synovial fluid.

#### *Correlation between kidney deficiency and marrow deficiency and yang deficiency and cold coagulation*

This study found that lactoferrin and prefibrin-1 were significantly reduced in the Aa and Ba groups compared with the control group hemoglobin  $\alpha 1$  was significantly

increased. According to *Figures 2 and 3*, the two groups A and B, have a positive correlation. Both lactoferrin and prefibrin-1 have a specific effect on the structure of bone cells. TCM believes that kidney yin deficiency mainly causes kidney deficiency, while kidney yang deficiency mainly causes yang deficiency, and both are dysfunction of the kidney, and there is a view that yang damages yin and yin damages yang. The two types have common points in symptoms and genes (22). In the treatment of OA, both kidney yin and kidney yang can be cured. Therefore, although both kidney and yang deficiency can find their different proteins, there is a clear correlation between these two types.

#### *Differential proteins of three types of OA in kidney deficiency and marrow deficiency, yang deficiency, and cold coagulation, and blood stasis block*

HIST1H2BC and myeloperoxidase were significantly reduced in the Aa group compared with the control group and the Ab group, suggesting that the two are characteristic proteins of kidney deficiency type OA. Serum amyloid P protein and apolipoprotein C-I variants were significantly reduced in the Ba group compared with the control group and the Bb group, suggesting that the two may be the characteristics of the Yang deficiency type OA. At present, many scholars have found the differentially expressed proteins of kidney-yin deficiency and kidney yang deficiency, but there are no reports of differences in HIST1H2BC, myeloperoxidase, serum amyloid P protein, and apolipoprotein CI variants. Histone H2A, C-reactive protein, and fibrinogen  $\alpha$  were significantly reduced in the Ca group compared with the control group and the Cb group, suggesting that the three may be characteristic proteins of blood stasis type OA. Studies have shown that fibrinogen is a differential protein of coronary heart disease with blood stasis syndrome (23), and the relationship between histone H2A, C-reactive protein, and blood stasis syndrome has not been studied.

In general, through the analysis of this study, it is concluded that the synovial fluid in OA with kidney deficiency, Yang deficiency n has a significant protein correlation, which further confirms that kidney yang deficiency is related to kidney yin deficiency, which is supportive for further research on the theory of kidney deficiency syndrome. It is also concluded histone H4, histone H2A, S100 calcium-binding protein A8, fibrinogen  $\gamma$ , fibrinogen  $\alpha$ , cDNA (FLJ92148), C4b binding protein,

and partial transketol and basement membrane glycan may be used as markers to identify OA. HIST1H2BC and myeloperoxidase may be differential proteins that distinguish kidney-deficiency OA from the other two types. Serum amyloid P protein and apolipoprotein CI variants may be differential proteins of yang deficiency. Histone H2A, C-reactive protein, and fibrinogen  $\alpha$  may be differential proteins of blood stasis type. The protein differences between the three types of OA syndromes of different TCM types provide a material basis for TCM clinical types and provide theoretical support for the subsequent treatment stage.

However, there are still many limitations to this study. Since some patients have no synovitis changes or, there is very little synovial fluid. It is not easy to get for the test. Secondly, the sample size is small, which decreases the credibility of this study. Future studies should enroll more patients to confirm our findings.

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### Footnote

*Reporting Checklist:* The authors have completed the MDAR reporting checklist. Available at <http://dx.doi.org/10.21037/apm-20-2117>

*Data Sharing Statement:* Available at <http://dx.doi.org/10.21037/apm-20-2117>

*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/apm-20-2117>). The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by ethics committee

of The First Affiliated Hospital of Guizhou University of Traditional Chinese Medicine. All subjects were informed and signed an informed consent.

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