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Perturbations in amino acids and metabolic pathways in osteoarthritis patients determined by targeted metabolomics analysis

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ABSTRACT

Osteoarthritis (OA) is a degenerative synovial joint disease affecting people worldwide. However, the exact pathogenesis of OA remains unclear. Metabolomics analysis was performed to obtain insight into possible pathogenic mechanisms and diagnostic biomarkers of OA. Ultra-high performance liquid chromatography-triple quadrupole mass spectrometry (UPLC-TQ-MS), followed by multivariate statistical analysis, was used to determine the serum amino acid profiles of 32 OA patients and 35 healthy controls. Variable importance for project values and Student's *t*-test were used to determine the metabolic abnormalities in OA. Another 30 OA patients were used as independent samples to validate the alterations in amino acids. MetaboAnalyst was used to identify the key amino acid pathways and construct metabolic networks describing their relationships. A total of 25 amino acids and four biogenic amines were detected by UPLC-TQ-MS. Differences in amino acid profiles were found between the healthy controls and OA patients. Alanine, γ-aminobutyric acid and 4-hydroxy-L-proline were important biomarkers distinguishing OA patients from healthy controls. The metabolic pathways with the most significant effects were involved in metabolism of alanine, aspartate, glutamate, arginine and proline. The results of this study improve understanding of the amino acid metabolic abnormalities and pathogenic mechanisms of OA at the molecular level. The metabolic perturbations may be important for the diagnosis and prevention of OA.

1. Introduction

Osteoarthritis (OA), the most common joint disease, is characterized by articular cartilage destruction, joint space narrowing, osteophyte formation, subchondral sclerosis and inflammation of the synovial membrane and subchondral bone [1]. This proliferative or degenerative joint disease leads to stiffness, pain and immobility in OA patients [2]. OA can affect multiple joints, most commonly the knee [3]. There are no effective approaches to halt, reverse or delay the progression of OA. The innate immune system and genetic and environmental factors, such as occupational exposure, obesity, ethnicity and physical activity, play a major role in the pathogenesis of OA [4–6]. However, the exact pathogenesis of OA remains unclear. Notably, alterations in small-molecule metabolites, the downstream endpoints of the gene and protein, have been poorly delineated. Thus, in-depth understanding of the metabolic alteration in OA should aid in early clinical diagnosis, treatment and prevention.

Amino acids (AAs), small-molecule metabolites, not only participant the construction of peptides and proteins, but also have important physiological functions as precursors of low-molecular-weight molecules, such as serotonin, nitric oxide, polyamines, dopamine and creatine [7]. AAs also play important roles in immune function, and cytokine secretion and response [8]. The detection of AAs may contribute to the screening, diagnosis and treatment of some inflammatory diseases. Alterations in plasma AAs have been found in inflammatory disease and rheumatoid arthritis [9]. Recently, several studies have reported that alterations in the metabolism of some AAs are involved in pathogenesis and inflammation in OA. For example, glutamate-mediated events and arachidonic acid-derived inflammatory mediators have been found to contribute to the pathogenesis and the ongoing processes of peripheral nociceptive transduction and inflammation in OA [10,11]. However, the changes in AA and nicotinamide metabolite profiles in early OA have not been reported. Thus, it is necessary to investigate the metabolic changes in AA profiles and the functions of these AAs in the

Abbreviations: OA, osteoarthritis; AA, amino acid(s); UPLC-TQ-MS, ultra high performance liquid chromatography-triple quadrupole mass spectrometry; ESI, electrospray ionization; ROC, receiver operating characteristic; AUC, areas under the curve; PCA, principal component analysis; PLS-DA, partial least squares discriminates analysis; VIP, variable importance for project; CRP, C reaction protein; BCAAs, branched-chain amino acids; Hyp, 4-hydroxy-L-proline; GABA, γ-aminobutyric acid; SF, synovial fluid; QC, quality control

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initiation, establishment and development of OA.

Because metabolomics could quantitatively detect many small-molecule metabolites in body fluids or tissues, it has been used to investigate the metabolic changes in OA [12]. Furthermore, alterations in metabolites, the end products of cellular processes, may also serve as a favorable method for the diagnosis of OA [13]. Metabolic and molecular abnormalities result in anatomical and/or physiological changes in the joints of OA [14]. Metabolomics methods can be used to provide more in-depth analysis of AA metabolism profiles and may be useful in gaining a more comprehensive biomedical picture of OA.

Thus, to investigate the metabolic changes in the physiological responses in early OA, we analyzed serum AAs and nicotinamide profiles in OA patients through Ultra-high performance liquid chromatography-triple quadrupole mass spectrometry (UPLC-TQ-MS). Additional OA patients, as independent samples, were recruited to validate the observed alterations in AAs. The AA metabolic pathway changes were also further studied. Our results will improve understanding of the catabolism of proteins and the pathophysiology of OA diseases.

2. Material and methods

2.1. Ethics

The study protocol was approved by the Ethics Committee of Harbin Medical University (Harbin, China) and conducted in accordance with the tenets of the Declaration of Helsinki. Written informed consent was collected from each participant.

2.2. Study population

Based on review of patient symptoms (joint pain and stiffness) and radiographic evidence of structural damage (joint space narrowing with or without the presence of osteophytes) of the affected joint [15], the OA patients were diagnosed by two medical doctors at the Harbin Medical University. The exclusion criteria were: (1) a history of other known joint disorders (e.g., trauma, autoimmunity, and bone tumors); (2) a history of other known chronic disorders that could possibly influence metabolic profiles, including cancer, metabolic syndrome, diabetes, infectious disease and autoimmune disease; (3) the patients with previous therapeutic intra-articular injection or prior aspiration, and the patients had sports-related knee injuries.

The Kellgren and Lawrence grade (K-L grade) was used for assessment of severity of the disease by a radiologist and an orthopedist as the following: grade 0, no radiological changes; grade I, doubtful narrowing of joint space and possible osteophytic lipping; grade II, definite osteophytes and possible narrowing of joint space; grade III, moderate multiple osteophytes, definite narrowing of joint space, some sclerosis and possible deformity of bone contour; grade IV, large osteophytes, marked narrowing of joint space, severe sclerosis, and definite deformity of bone contour. OA patients were divided into a mild group (K-L grade II or III) and a severe group (K-L grade IV). A total of 32 OA patients were recruited from our department. In addition, they were strictly required not to have corticosteroid or nonsteroidal anti-inflammatory drugs for at least a month before the sample collection.

Thirty-five healthy individuals with none of the exclusion criteria who underwent routine annual medical examinations in our hospital were recruited as a control group. After enrollment, the healthy controls underwent physical and radiographic exams to confirm the absence of OA.

In order to eliminate any differences between two groups with their dietary, the whole diet information and dietary habits were recorded from all participants. In addition, the participants who had different diet habits with alcohol consumption or complete vegetable diet were excluded. Body mass index (BMI) and C Reactive Protein (CRP) levels for each participant were also assessed.

2.3. Serum sample preparation

All blood samples were collected in the morning before breakfast. Fasting blood samples were immediately centrifuged at 3000*g* for 10 min at room temperature. Then, the serum was moved into a clean Eppendorf tube and stored at −80 °C until analysis. Serum AAs were prepared as described elsewhere [15]. Briefly, 50 μL serum was extracted for UPLC-TQ-MS and added 250 μL of acetonitrile/methanol/formic acid (74.9:24.9:0.2 by vol.) including two additional stable isotope-labeled internal standards for phenylalanine-d8 and valine-d8. The mixture was vortexed for 1 min, then held at room temperature for 10 min, and centrifuged for 10 min (14,000*g*, 4 °C). Finally, the solution was filtered by a syringe filter (0.22 μm pore size) and used for UPLC-TQ-MS analysis [16].

2.4. Quality control sample

We used quality control (QC) samples to assess reproducibility and reliability of the UPLC-MS system. The QC samples were prepared by mixing equal volumes of different individual serum samples (5 healthy controls and 5 patients with OA). One QC sample was injected at the start of the analytical batch, followed by analysis of 1 QC sample at every 10 sample injection throughout the analytical workflow. The reproducibility and reliability of the method was assessed by coefficients of variation (CV%) of 29 metabolites from QC samples.

2.5. UPLC-TQ-MS analysis

A Waters ACQUITY UPLC system (Waters Corporation, Milford, MA, USA) combined with a Waters Xevo TQD mass spectrometer (Waters Corporation, Manchester, UK) were used for analysis. A total of 2-μL the sample solution was added into an ACQUITY UPLC™ HILIC column (100 mm × 2.1 mm *i.d.*, 1.7 μm film thickness). The flow rate of the mobile phase was kept on 300 μL/min. Analytes were recovered from the column by gradient elution with solution A (10 mM aqueous ammonium formate, 0.1% (v/v) formic acid) and solution B (0.1% (v/v) formic acid in acetonitrile). The optimized factors for the UPLC isolation and electrospray ionization (ESI)-TQ-MS test were performed in according to elsewhere [16,17].

MS analyses were conducted using ESI and multiple reaction monitoring scans in the positive ion mode. Every transition was kept on 30 ms for the cone voltage and collision energies. The ion spray voltage and the source temperature were maintained at 3.2 kV and 150 °C, respectively. Internal standard peak areas were used for QC sample. The samples with peak areas differing from the group mean were statistic. MarkerLynx Application Manager software (version 4.1; Waters Corporation, Milford, MA, USA) was utilized for automated peak integration. Finally, in order to confirm identity, the metabolite peaks were checked out and compared to a known standard.

2.6. Statistical analysis

The results are shown as mean ± SD. SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. A probability (p) value < 0.05 was considered significant. Continuous variables were analyzed with Student's t-test. To detect the predictive ability of