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Original article

Metabolite profiles of synovial fluid change with the radiographic severity of knee osteoarthritis

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ABSTRACT

Objectives: To investigate potential pathogenic pathways in the synovial fluid of osteoarthritis (OA) patients at different disease stages [early vs. late, determined based on the Kellgren–Lawrence (KL) grading scale], through metabolite profiles that were performed by using gas-chromatography/time-of-flight mass spectrometry (GC/TOF MS).
Methods: Synovial fluid samples were obtained from 15 patients with knee OA, divided into early- (KL grade: 1 and 2) and late-stage OA (KL grade: 3 and 4). Metabolite profiles of OA based on KL grading scale were performed using GC/TOF MS, with multivariate statistical analyses conducted by orthogonal partial least squares discriminant analysis (OPLS-DA) and hierarchical clustering analysis (HCA).
Results: A total of 114 metabolites were identified and classified into various classes, such as amino acids, sugars and sugar alcohols, fatty acids, and organic acids. Significant discrimination of metabolite profiles between the early- and late-stage OA groups was shown by OPLS-DA and HCA. Twenty-eight metabolites, including malate, ethanolamine, squalene, glycerol, myristic acid, oleic acid, lanosterol, heptadecanoic acid, and capric acid, were identified as critical metabolites for discriminating between the early- and late-OA groups by using Student's *t*-test, as they showed significant differences in abundance between the two OA groups. These metabolites were related to fatty acid metabolism, glycerolipid metabolism, and the tricarboxylic acid cycle.
Conclusions: These results revealed that metabolite profiles are robustly altered along the radiographic stage of knee OA. Metabolomic approaches based on GC/TOF MS could provide valuable information on the underlying pathogenic mechanisms of OA progression.

1. Introduction

Osteoarthritis (OA) is one of the most common forms of arthritis and a leading cause of pain and disability in elderly people. It is a chronic progressive condition affecting the whole joint structure, including subchondral bone, ligaments, capsule, synovial membrane, and periarticular muscles. Therefore, it is referred to as a degenerative joint disease [1]. The knee is the most frequently involved joint in OA. Radiography is the first step to assess whether a patient has knee OA or not, and the Kellgren–Lawrence (KL) scale is the most widely used scale to assess the severity of knee OA on a plain radiogrph [2]. The radiographic definition of knee OA deals with pathophysiological joint signs shown on radiographic images, although there is a high discordance between knee pain and radiographic images [3].

The synovial fluid (SF) provides nutrition and lubrication to the articular cartilage in minute amounts. It accumulates in a pathologic joint, and reflects the ongoing process of the joint disorder [4]. Considering that OA is a disease not only of the synovium but of the whole joint structure, the investigation of metabolic pathways using SF might be more reasonable than using cultured synovial cells.

Metabolomics, the study of global changes of the entire set of metabolites from living organisms, can be a powerful tool in metabolic and physiological study [5]. In rheumatic diseases,

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metabolomics would find out the altered metabolic process, identifying specific metabolite profiles to a given condition as pertinent biomarkers or potential therapeutic targets [6]. Biological fluids, such as SF can be good sources of metabolites [7,8]. Previously, metabolomic analysis of SF has been applied to understand the metabolism of OA and to identify the biomarkers of OA [9,10]. However, there are no studies on how the metabolism of OA changes with the structural severity according to the KL grading scale through SF metabolomics.

In this study, we have performed metabolite profiles of SF from OA patients in the early and late-stages of the KL grading scale by gas-chromatography/time-of-flight mass spectrometry (GC/TOF MS) and investigated the underlying pathogenesis from the viewpoint of metabolic pathways of OA.

2. Methods

2.1. Study patients and synovial fluid collection

Among patients visiting the rheumatology clinic at the Samsung Medical Center in Seoul, 15 patients with OA who underwent knee arthrocentesis were retrospectively recruited. They were diagnosed by experienced rheumatologists based on clinical data pertinent to the classification criteria of the American College of Rheumatology (ACR) for the classification of knee OA [11]. Medical records were reviewed for age, sex, duration of disease, radiographic findings, and laboratory data, such as rheumatoid factor (RF), erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP). The severity of knee OA of each patient was assessed by KL grading scales based on radiography, looking at osteophyte formation, joint space narrowing, sclerosis, and joint deformity:

- KL grade 1, minute osteophytes with doubtful joint space narrowing;
- KL grade 2, definite osteophytes with possible joint space narrowing;
- KL grade 3, definite osteophytes with moderate joint space narrowing;
- KL grade 4, definite osteophytes with severe joint space narrowing and subchondral sclerosis [2].

The study was performed in accordance with the 1964 Helsinki Declaration and was approved by the Institutional Review Board of Samsung Medical Center.

SF samples were obtained from arthrocentesis of knee joints for the therapeutic relief of symptoms. After sampling, each specimen was immediately aliquoted into small amount, such as 1 μg and stored at −80 °C. A single frozen aliquoted sample was thawed and used only once for one experiment. The samples were divided into two groups: the early- (KL grades 1 and 2) group vs. the late-stage OA group (KL grades 3 and 4), to investigate the alterations of metabolism with the structural severity of knee OA.

2.2. Metabolite extraction from synovial fluid

Metabolite extraction was accomplished using a slight modification of the method used in a previous study [12]. To eliminate cells and debris, the synovial fluid samples were thawed on ice for 3 min and then centrifuged at 500 × *g*. The supernatant was extracted with 80% (v/v) methanol at −20 °C and vortexed for 3 min, after which it was centrifuged at 16,100 × *g* for 5 min at 4 °C. Next, the supernatant was collected and completely dried in a vacuum concentrator (Labconco, Kansas City, MO, USA). For derivatization, the dried samples were mixed with 5 μL of 40 mg/mL of methoxyamine hydrochloride in pyridine (Sigma-Aldrich, St. Louis, MO, USA) for 90 min at 30 °C, and 45 μL of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (Fluka, Buchs, Switzerland) for 30 min at 37 °C. A mixture of fatty acid methyl esters was added to the derivatized metabolite extract as retention index markers.

2.3. Metabolite analysis

An Agilent 7890B GC (Agilent Technologies, Wilmington, DE, USA) coupled with a Pegasus HT TOF MS (LECO, St. Joseph, MI, USA) was used for the analysis of derivatized metabolite samples. A 1 μL aliquot of the metabolite sample was injected in splitless mode and separated on an Rtx-5Sil MS column (30 m × 0.25 mm, 0.25 μm of film thickness; Restek, Bellefonte, PA, USA) and an additional 10-m long integrated guard column. The GC oven was initially set to 50 °C for 1 min and was then increased to 330 °C at a rate of 20 °C/min, where it was finally held for 5 min. The temperatures of the transfer line and the ion source were 280 °C and 250 °C, respectively. The ionization mode was subjected to an electron impact of 70 eV. Mass spectra of the metabolites were recorded in the mass range of 85–500 m/z at an acquisition rate of 17 spectra/s. The GC/TOF MS data were pre-processed using Chroma TOF software (ver. 4.50; LECO) with peak detection and deconvolution of the mass spectra. As previously described, BinBase, an in-house programmed database, was used for metabolite identification and alignment [13,14]. The spectra data were normalized by dividing the median of sums of peak intensity for each sample.

2.4. Statistical analyses

To maximize the covariance between the measured data and the response variables, orthogonal partial least squares discriminant analysis (OPLS-DA) was performed using SIMCA-P+ (ver. 12.0; Umetric AB, Umea, Sweden). Hierarchical clustering analysis (HCA) based on the Euclidean distance coefficient and average linkage methods was performed using a MultiExperiment Viewer to cluster groups and identified metabolites. Univariate analysis and pathway analysis were performed using Statistica (ver. 7.1; StatSoft, Tulsa, OK, USA) and MetaboAnalyst [15], a web-based data processing tool, respectively.

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3. Results

3.1. Patient characteristics

The baseline characteristics of patients with knee OA are summarized in Table 1. The mean age of the enrolled patients was 61.5 ± 10.9 years, and the median symptom duration of OA was 2.5 years ranging from 6 months to 15 years. Eight patients (5 women and 3 men) with KL grades of 1 and 2 were allocated to the early-stage group while 7 patients (all women) with KL grades of 3 and 4 were allocated to the late-stage group. The mean age, the proportion of women, and the symptom duration between the groups were not statistically different. The prevalence of diabetes and dyslipidemia, and the proportion of overweight and obesity were comparable between the two groups. RF was checked in 10 patients, and 4 patients had positive RF values with low titers. There were no differences of ESR based on age and sex between the