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Exploration of the serum metabolite signature in patients with rheumatoid arthritis using gas chromatography–mass spectrometry

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ABSTRACT

Rheumatoid arthritis (RA) is a systemic autoimmune disease with complicated pathogeny. There could be obvious alterations of metabolism in the patients with RA and the discovery of metabolic signature may be helpful for the accurate diagnosis of RA. In order to explore the distinctive metabolic patterns in RA patients, a gas chromatography–mass spectrometry (GC–MS) method was employed. Serum samples from 33 RA patients and 32 healthy controls were collected and analyzed. Acquired metabolic data were assessed by the principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA), and the data analysis results showed RA patients and healthy controls have very different metabolic profiles. Variable importance for project values (VIP) and Student's *t*-test were combined to screen the significant metabolic changes caused by RA. Serums from RA patients were featured by decreased levels of amino acids and glucose, increased levels of fatty acids and cholesterol, which were primarily associated with glycolytic pathway, fatty acid and amino acid metabolism, and other related pathways including TCA cycle and the urea cycle. These preliminary results suggest that GC–MS based metabolic profiling study appears to be a useful tool in the exploration of the metabolic signature of RA, and the revealed disease-associated metabolic perturbations could help to elucidate the pathogenesis of RA and provide a probable aid for the accurate diagnosis of RA.

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease primarily featured by joint inflammation and multitudinous inflammatory manifestations [1]. It has a global distribution and its prevalence was about 1% which is remarkably concurrent in most study populations [2]. Though there has been apparent progress in studying its etiology and pathogenesis, the mechanism of RA is still unclear [3].

To elucidate the pathogenesis of RA in different stages is crucial for clinical diagnosis and therapy. A great number of studies have been carried out on the mechanism of RA which revealed that RA results from a complex interaction of environmental exposures and susceptible genetic background [4]. It was reported that the innate immune system, various cells, gene variants and environmental risk factors played a pivotal role in the pathogenesis of RA [5–7]. Furthermore, recent molecular studies have demonstrated human factors including small molecule inflammatory mediators, autoantibodies, cytokines, chemokines and cell adhesion molecules were also implicated in RA pathogenesis [8–12]. Especially, several of these novel molecules were found to be associated with RA susceptibility and development [13,14], and their corresponding regulators might be new drug targets for the treatment of RA. Despite numerous researches on RA etiology and pathogenesis, the exact mechanism is still ambiguous.

Early diagnosis is important for the treatment of RA. Currently, serum autoantibodies, rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPAs) are widely used in clinical RA diagnosis [15]. However, rather low specificity and sensitivity of RF have been reported, especially in early arthritis cohorts [16,17]. ACPAs are highly useful for RA diagnosis with higher specificity and stability than RF. However, there were still several reports about the false positive error of ACPAs [18–21]. Taking into account of the limitation of current clinical diagnostic tools, there is a press-

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ing need to find a new way to improve the diagnosis accuracy and sensitivity of RA.

Metabolomics is a branch of omics. It aims to study a comprehensive set of small molecular weight metabolites in an organism and explore the change of endogenous metabolism induced from environmental stimuli or perturbation [22,23]. To find out the metabolic changes between unperturbed and perturbed systems can lead to insights into the potential pathological mechanism [22]. Human body fluid, such as blood, urine, cerebrospinal fluid, *etc.,* contains a significant number of metabolites which provides a wealth of valuable information in response to specific physical condition. In recent years, metabolomics has been applied more broadly to investigate disease pathogenesis, including RA. Based on the metabolic fingerprints of synovial fluid and serum by nuclear magnetic resonance spectroscopy (NMR), significant changes were detected in the patients with RA [24–26]. Metabolites disorders in urine were found in adjuvant-induced arthritis rats by using liquid chromatography-mass spectrometry (LC–MS) [27,28]. However, there were fewer reports in investigating RA by using GC–MS. GC–MS is a very effective technique for an overall analysis due to its high sensitivity and separation efficiency. In addition, ion suppression observed in LC–MS is hardly present in GC–MS. Also, identification of metabolites through searching commercial mass spectra library is easier than other techniques [29]. Metabolic profiling using GC–MS reveals the changes of sugars, organic acids, fatty acids, amines which provides complementary metabolite coverage to LC–MS. Thus, to further investigate the metabolic changes in patients, we applied GC–MS to extend our knowledge about RA other than those found by LC–MS.

In the study, serum samples from RA patients and healthy controls were collected and analyzed by GC–MS. Based on multivariate statistical analysis, metabolic alterations between two groups were identified, RA–related perturbations in metabolic pathways were further investigated.

2. Materials and methods

2.1. Patients

The trial was approved by the ethics committee of Zhejiang Chinese Medical University. 33 patients were screened according to the inclusion criteria-the American College of Rheumatology (ACR) 1987 classification criteria for RA. Clinical information on gender, age, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), ACPAs and RF values of patients were collected (Table 1). 33 serum samples from RA patients and 32 samples from healthy volunteers were collected with promoting coagulating tubes and then stored at −80 °C until analysis.

2.2. Chemicals

The methanol as the extraction solvent in the study was procured from Tedia (Fairfield, OH, USA). Methoxyamine, methyltrimethyl-silyl-trifluoroacetamide (MSTFA) and pyridine were provided by Sigma-Aldrich (St. Louis, MO, USA). Standards for metabolite identification were bought from Sigma-Aldrich (St. Louis, MO, USA). A Milli-Q system (Millipore Corp, Millipore, MA, USA) was used to provide ultra-pure water.

2.3. Sample preparations

Serum samples were prepared according to our previous method [30]. After thawing and mixing, 50 μL of serum sample was placed in a 1.5 mL eppendorf vial and kept on ice, subsequently 200 μL cold methanol with 20 μg/mL tridecanoic acid (internal standard) was added. After vortexing for 30 s, the sample was centrifuged for 10 min to precipitate the protein (12,000g, 4 °C). The supernatant was concentrated and dried under a vacuum condition produced by a Labconco CentriVap System (Labconco. Kansas, MO, USA). Fifty microlitre of methoxyamine pyridine solution (20 mg/mL) was added to the dried residue to re-dissolve and oximated for 90 min in a 40 °C water bath. Afterward, 40 μL of MSTFA was added for trimethylsilylation for 60 min. After a series of above-mentioned processing, the supernatant was prepared for analysis. A quality control (QC) strategy was applied to monitor the variability within analytical batch and ensure the data quality [31]. QC sample was prepared by equally mixing the sera from all patients and controls, and QCs were processed together with samples by using the same method.

2.4. GC–MS

Metabolic profiling of serum sample was acquired by Agilent 7890/5975C GC–MS (Agilent Technologies, Santa Clara, CA, USA). 1 μL of the sample which had been processed was injected onto a DB-5 fused silica capillary column (30 m × 0.25 mm × 0.25 μm, J&W Scientific, Folsom, CA, USA) with a split ratio of 10:1. The temperature program was as follows: started with the initial GC oven temperature at 70 °C and maintained for 3 min, then increased to 300 °C at 5 °C/min, and kept for 5 min. The injection temperature was 300 °C. The temperature of the transfer line was kept at 280 °C. High-purity helium (99.9996%) was used as the carrier gas, with a constant flow rate of 1.2 mL/min. Mass spectra were acquired at a scan speed of 2 spectra per second after a solvent delay of 4.8 min, and the mass scan range was set at m/z 33–600. Serum samples were analyzed in random and QC was injected every 10 samples within the batch.

2.5. Data processing and statistical analysis

GC–MS data of QC samples were imported into the AMDIS 2.62 software (NIST, Boulder, CO, USA) for resolution of co-eluting peaks and each individual peak was marked by m/z and retention time pairs. Peak integration was finished in MSD ChemStation (Agilent Technologies, Santa Clara, CA, USA) and a compound Table composed of retention time, m/z and the corresponding peak area was generated for further statistical analysis.

The metabolites in each chromatogram were scaled to unit variance before statistical analysis. Then metabolic data were subjected to a principal component analysis (PCA) using the SIMCA-P 11.0 version (Umetrics AB, Umea, Sweden) to give an overview of the differences between the metabolic profiles of serum samples from RA patients and controls. Partial least squares discriminant analysis (PLS-DA), which is more focused on the class discrimination, was carried out to identify the metabolic variations in RA patients. Metabolites contributed strongly to the classification were picked out according to the variable importance for project values (VIP > 1). In addition, Student's t-test (SPSS 21, International Business Machines Corp., Armonk, USA) was carried out to exclude metabolites with no statistical significance between patients and controls, and the significance level was adjusted by the Bonferroni method for multiple testing.

Significantly changed metabolites were further identified by similarity searches in the NIST05 mass spectral library (National Institute of Standards and Technology, Gaithersburg, MD, USA) and partially were verified with standards. The process was performed using the NIST Mass Spectral Search Program Version 2.0 (http://www.nist.gov/srd/mslist.htm). Moreover, commercial standards were used for confirmation.