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Metabolomics analysis of oral mucosa reveals profile perturbation in reticular oral lichen planus

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ABSTRACT

Background: Oral lichen planus (OLP) is a chronic inflammatory mucosal disorder and potentially oral premalignant lesion affecting the stratified squamous epithelia. In OLP, reticular type is the most common clinical form of the disease. However, little is known about it. Metabolomics analysis may help to investigate the disease pathogenesis and to improve clinical treatment.
Methods: Liquid chromatography (LC)-mass spectrometry (MS) system, XCMS software, SIMCA software, and OSI / SMMS software were integrated to identify differentially expressed metabolites for the pathways and pathology analysis.
Results: Totally, 21 modulated metabolites were identified, whose dysregulations affected 30 metabolic pathways. Through an impact-value screen (impact-value > 0.1), 8 pathways were selected as the significantly dysregulated pathways. Pathological network showed that these metabolites participated in 5 pathological processes, that is, inflammatory lesion, DNA damage and repair disorder, apoptosis process, oxidative stress injury, and abnormal energy expenditure.
Conclusion: The study revealed the metabolic perturbation of oral mucosa in reticular OLP, which may provide an important reference for the understanding of the pathogenesis of the disease and the discovery of therapeutic targets.

1. Introduction

Oral lichen planus (OLP) is a chronic inflammatory mucosal disorder and potentially oral premalignant lesion affecting the stratified squamous epithelia [1–3]. The disease affects 0.5%–3% of the population between 30 and 60 years of age with a female to male ratio of 1.4:1 [1,2,4]. Clinically, the lesions can be classified into 6 variants: reticular, erosive, papular, plaque-like, atrophic, and bullous. Among them, the reticular type is the most common form of OLP, followed by the erosive type [4]. In the previous studies [1,2], we have investigated the pathogenesis of erosive OLP with metabolomics analysis, and put forward some new viewpoints. However, little is known about the reticular type with the highest incidence rate. Researches on it may benefit more patients.

Metabolomics provides an opportunity to study the pathogenesis of the diseases, which is a well-established method and one of the major components in systems biology [1,2]. The analytical samples can be obtained from tissues, urine, or serum, of which tissue metabolomics can most directly analyze the pathological changes of the lesion. In this research, metabolomics was used to study the metabolic perturbation of oral mucosal tissues from reticular OLP patients, based on which the pathogenesis of this type of the disease was analyzed.

2. Material and methods

2.1. Chemicals and reagents

All chemicals and reagents were analytical or HPLC grade. Methanol, deionized water, acetonitrile, and formic acid were purchased from CNW Technologies GmbH (Düsseldorf, Germany). L-2-

Abbreviations: ESI, electrospray ionization; LC, liquid chromatography; MS, mass spectrometry; NO, nitric oxide; OLP, oral lichen planus; OPLS-DA, orthogonal partial least-squares-discriminant analysis; PCA, principle component analysis; PEs, phosphatidylethanolamines; QC, quality control; RSD, relative standard deviation; TCA, tricarboxylic acid; VIP, variable importance in the projection

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Chlorophenylalanine was from Shanghai Hengchuang Bio-technology Co., Ltd. (Shanghai, PR China).

2.2. Samples collection and preparation

All subjects signed informed consent, and the research protocol was approved by the Ethical Committees of First Affiliated Hospital of Heilongjiang University of Chinese Medicine (date of approval: December 29, 2014, approval No. HZYLLKY201400601) and was performed in accordance with the principles stated in the Declaration of Helsinki. Oral mucosa tissue samples were collected between January 1, 2016 and June 30, 2016 at the First Affiliated Hospital of Heilongjiang University of Chinese Medicine, PR China. Oral lesion mucosa samples were obtained from 10 female and 6 male reticular OLP patients and their age ranged from 20 to 54 years. Normal gingival mucosa samples were collected from 15 female and 9 male healthy individuals (age ranged from 18 to 48 years) after tooth extractions. The subjects did not have other diseases such as diabetes, hypertension, cardiovascular diseases, kidney disease, and other intraoral inflammation, etc. All subjects were maintained on a regular diet and did not obtain any treatment prior to admission, and the samples were collected at their initial visit. The diagnosis of reticular OLP was made by clinical and laboratory criteria (Supplementary Fig. S1).

20 mg oral mucosa tissue sample, 10 μl internal standard (L-2-chlorophenylalanine in methanol, 0.3 mg/ml), and 400 μl extraction solvent (methanol/water, 4/1, v/v) were added to Eppendorf tube. Samples were stored at −80 °C for 5 min, and then grinded in a ball mill (JXFSTPRP-24/32, Shanghai Jingxin Industrial Development Co., Ltd., PR China) at 60 Hz for 2 min. The homogenate was ultrasound-extracted for 10 min, and then stored at −20 °C for 30 min. The extraction was centrifuged at 13000 rpm, 4 °C for 10 min. The supernatant was transferred to the vials. The vials were stored at −80 °C until liquid chromatography (LC)-mass spectrometry (MS) analysis. Quality control (QC) samples were prepared by mixing aliquots of the all samples to be a pooled sample.

2.3. LC-MS analysis

A Dionex Ultimate 3000 RS UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA) was equipped with ACQUITY UPLC BEH C_{18} column (1.7 μm, 2.1 × 100 mm, Waters Corp, Milford, USA). The binary gradient elution system consisted of (A) water (containing 0.1% formic acid, *v*/v) and (B) acetonitrile (containing 0.1% formic acid, v/v), and separation was achieved using the following gradient: 5–20% B over 0–2 min, 20–60% B over 2–4 min, 60–100% B over 4–11 min, the composition was held at 100% B for 2 min, then 13–13.5 min, 100% to 5% B, and 13.5–14.5 min holding at 5% B. The flow rate was 0.4 ml/min and column temperature was 45 °C. All the samples were kept at 4 °C during the analysis. The injection volume was 5 μl.

MS analysis was performed on a Q-Exactive quadrupole-Orbitrap mass spectrometer equipped with heated electrospray ionization (ESI) source (Thermo Fisher Scientific, Waltham, MA, USA) in both positive and negative ion modes. The mass range was from *m/z* 66.7 to 1000.5. The resolution was set at 70,000 for the full MS scans and 35,000 for HCD MS/MS scans. The Collision energy was set at 10, 20, and 40 eV. The mass spectrometer operated as follows: spray voltage, 3000 V (positive) and 2500 V (negative); sheath gas flow rate, 45 arbitrary units; auxiliary gas flowrate, 15 arbitrary units; capillary temperature, 350 °C.

The QC samples were injected at regular intervals (every 10 samples) throughout the analytical run to provide a set of data from which repeatability can be analyzed.

2.4. Data analysis

Metabolomics data were acquired using the XCMS software (1.50.1 version), which produced a matrix of features with the associated retention time, accurate mass, and chromatographic. The variables presented in least 50% of either group were extracted. Internal standard was removed from the data set. Then all ions were normalized to the total peak area of each sample to achieve a minimum relative standard deviation (RSD). The metabolite ions with RSD less than 30% were used for the further data processing.

The positive and negative data were combined to get a combine data set which was imported into SIMCA software package (version 14.0, Umetrics, Umeå, Sweden). Principle component analysis (PCA) and orthogonal partial least-squares-discriminant analysis (OPLS-DA) were carried out to visualize the metabolic alterations among experimental groups, after mean centering and unit variance scaling. Variable importance in the projection (VIP) ranks the overall contribution of each variable to the OPLS-DA model, and the variables with VIP > 2 are considered relevant for group discrimination. Student's *t*-test was used for statistical analysis and performed with the R Programming Language (version 3.3.1; https://www.r-project.org/). Differentially expressed metabolites were chosen according to their variable VIP statistics (VIP > 2) and predefined *P*-value thresholds ($p < 0.05$).

In the present study, the default 7-round cross-validation was applied with 1/seventh of the samples being excluded from the mathematical model in each round, in order to guard against overfitting.

2.5. Differentially expressed metabolites identification

Differentially expressed metabolites were identified using OSI / SMMS software co-developed by Dalian Institute of Chemical Physics, Chinese Academy of Sciences and Dalian ChemData Solution Information Technology Co., Ltd., PR China. Reference material database (Dalian Institute of Chemical Physics, Chinese Academy of Sciences and Dalian ChemData Solution Information Technology Co., Ltd., PR China), HMDB, and METLIN were used as the database source.

2.6. Metabolic pathway analysis and pathological network construction

MetaboAnalyst was applied for the metabolic pathway analysis of differentially expressed metabolites based on database source including the HMDB, SMPDB, and KEGG. Pathological network was constructed by Cytoscape software (version 3.6.0).

3. Results

3.1. Metabolomics analysis and differentially expressed metabolites identification

2042 metabolite ions were acquired in positive ion mode, and 1167 metabolite ions were acquired in negative ion mode. 75.31% of ions in positive ion mode and 82.76% in negative ion mode exhibited less than 30% of RSD, which displayed good reproducibility of the metabolomics method. QC samples in PCA score plot (Supplementary Fig. S2) clustered well, indicating good reproducibility of the sample process procedure and the instrumental system. Both PCA and OPLS-DA score plots (Supplementary Figs. S2 and S3) showed obvious separation between control and reticular OLP groups, suggesting that biochemical perturbation significantly occurred in oral mucosa tissues from the patients.

With the help of OSI / SMMS software, 21 differentially expressed metabolites were identified (Table 1). Compared with the control group, the levels of 13 metabolites were up-regulated, and 8 metabolites were down-regulated in reticular OLP group.

3.2. Metabolic pathway analysis and pathological network construction

Metabolic pathway analysis (Fig. 1) indicated that these 21 metabolites in oral mucosa tissues were involved in 30 pathways. Through an impact-value screen (impact-value > 0.1), 8 pathways were selected