Serum Metabolomic Profiles of Rheumatoid Arthritis Patients With Acute-Onset Diffuse Interstitial Lung Disease

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ABSTRACT

OBJECTIVE: Acute-onset diffuse interstitial lung disease (AoDILD) includes acute exacerbation of interstitial lung disease (ILD), drug-induced ILD, and Pneumocystis pneumonia, and frequently occurs in patients with rheumatoid arthritis (RA). Since AoDILD causes a poor prognosis in RA, biomarkers for AoDILD were eagerly desired. Metabolomic analyses were extensively performed in cancer patients and successfully generated better diagnostic biomarkers. In the present study, serum metabolomic profiles of AoDILD in RA were investigated to generate better potential metabolic biomarkers.

METHODS: Serum samples of 10 RA patients with AoDILD were collected on admission and in a stable state, more than 3 months after the admission. Serum metabolomic analyses were conducted on the samples from these RA patients with AoDILD.

RESULTS: Apparently distinct serum metabolic profiles in AoDILD were not observed in univariate or hierarchical cluster analyses. Partial least-squares-discriminant analysis (PLS-DA) was performed to select candidate metabolites based on variable importance in projection (VIP) scores. The PLS-DA model generated from the four metabolites with VIP scores more than 2.25 (mannosamine, alliin, kynurenine, and 2-hydroxybutyric acid) could successfully discriminate AoDILD from the stable condition (area under the curve: 0.962, 95% confidence interval: 0.778–1.000).

CONCLUSION: It was demonstrated that metabolomic profiling was useful to generate better biomarkers in AoDILD.

KEYWORDS: Rheumatoid arthritis, AoDILD, metabolomics

Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory disease characterized by the synovial joint destruction and the extra-articular manifestations. Interstitial lung disease (ILD) is one of the extra-articular lesions associated with RA and is interstitial inflammation of the lung. ILD associated with RA (RA-ILD) confers a dismal prognosis. Acute exacerbation of RA-ILD is occasionally observed, the complication of RA-ILD is reported to be a risk factor for drug-induced ILD in RA, and Pneumocystis pneumonia is associated with RA with an increased frequency. Acute exacerbation of ILD, drug-induced ILD, and Pneumocystis pneumonia were included.
in acute-onset diffuse ILD (AoDILD). These three conditions sometimes overlap. It is also difficult to distinguish them. The immune reconstitution inflammatory syndrome by the infection of *Pneumocystis jirovecii* or other unidentified organisms would explain the common pathogenesis of AoDILD. The immune reconstitution inflammatory syndrome also occurred in collagen disease patients under the treatment of immunosuppressive drugs. AoDILD frequently occurs in RA patients and encompasses a quite poor prognosis. Thus, biomarkers for AoDILD were eagerly desired for the prediction.

Krebs von den lungen-6 (KL-6) and surfactant protein-D (SP-D) were originally generated for biomarkers of idiopathic pulmonary fibrosis and have been also used for the diagnosis of chronic RA-ILD. These markers were validated for RA patients with AoDILD in a few studies. Metabolomic analyses quantify the low molecular weight metabolites, characterize the altered cellular metabolisms, and reflect the cellular responses to pathological conditions. Metabolomic analyses were extensively performed in cancer patients and successfully generated new diagnostic biomarkers using multivariate analyses. Metabolomic analyses were also conducted in patients with autoimmune or pulmonary diseases. However, few metabolomic studies on AoDILD in RA were reported, so far. Here, we investigated serum metabolomic profiles of AoDILD in RA in order to generate new potential metabolomic biomarkers with multivariate analyses.

**Materials and Methods**

**Patients and sera**

A total of 10 RA patients (patient number 1 to 10) were admitted to National Hospital Organization Sagamihara National Hospital from 2001 to 2010 for the treatment of AoDILD with corticosteroid pulse therapy (mean age of admission ± SD, 66.1 ± 8.0 years, 4 male patients). All the RA patients fulfilled the criteria for RA of American College of Rheumatology. These 10 patients with RA and AoDILD include 4 acute exacerbation of RA-ILD and 6 drug-induced ILD. Drug-induced ILD, *Pneumocystis pneumonia*, and acute exacerbation of ILD were defined, as previously described. Amino acid profiles of the RA patients with AoDILD were reported in the previous study. AoDILD progresses within a month, accompanied with clinical symptoms (fever, dry cough, or dyspnea), hypoxia, and ILD specific findings in computed tomography and patients with bacterial infection or heart disease were excluded. Serum samples of the RA patients with AoDILD were collected on admission (sample numbers 1.1, 2.1, 3.1, 4.1, 5.1, 6.1, 7.1, 8.1, 9.1, and 10.1). Sera were also collected in the stable state, more than three months before the admission (sample numbers 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0). This study was reviewed and approved by Sagamihara National Hospital Research Ethics Committee and University of Tsukuba Research Ethics Committee. Written informed consent was obtained from all study participants except patients deceased before the start of this study. The serum samples collected before this study were anonymized to prevent any link with the identification of the participants, and the analyses were approved on that condition by Sagamihara National Hospital Research Ethics Committee. This study was conducted in accordance with the principles expressed in the Declaration of Helsinki.

**Serum metabolomic analysis**

Serum metabolomic analysis was conducted with capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) at Human Metabolome Technologies (HMT, Tsuruoka, Yamagata, Japan) as previously described.

**Statistical analysis**

MetaboAnalyst 4.0 (http://www.metaboanalyst.ca/MetaboAnalyst/faces/home.xhtml) was used for statistical analyses. All the zero values were replaced with the half of the minimum positive values in the original data. Data scaling was performed as mean-centered and divided by the standard deviation of each variable. Hierarchical cluster analysis was performed by BellCurve for Excel software (Social Survey Research Information Co., Ltd., Tokyo, Japan). The univariate analyses were conducted for the discovery of metabolite biomarker. Wilcoxon signed-rank test was performed in the comparison of metabolite levels and multiple testing was corrected by calculating false discovery rate (FDR). Receiver operator characteristic (receiver operating characteristic curve [ROC]) curves were generated, and area under the curve (AUC) values were calculated. Multivariate analyses were used for the development of complex metabolite biomarker models based on selected metabolites. Partial least squares-discriminant analysis (PLS-DA) was performed to select candidate metabolites based on variable importance in projection (VIP) scores. PLS-DA algorithm was used to create biomarker models with selected candidate metabolites for monitoring AoDILD, and Monte Carlo cross validation was conducted to validate them. Two third of the samples were used to create models, and the remaining samples were used for validation in Monte Carlo cross validation. The permutation test for the created biomarker model was performed to show the predictive accuracy of the model as a measure of performance. The impact of AoDILD on metabolic pathway was evaluated with *Homo sapiens* pathway library, global test, and relative-betweeness centrality.

**Results**

**KL-6 and SP-D as biomarkers of AoDILD in RA**

KL-6 and SP-D levels were compared between the stable and the AoDILD states. KL-6 levels were not significantly higher
in the AoDILD than the stable state \((P = 0.1730, \text{mean} \pm SD, \text{stable}: 540.7 \pm 272.0 \text{U/ml, AoDILD: 844.9} \pm 591.9 \text{U/ml})\). SP-D levels were not significantly higher in the AoDILD than the stable state \((P = 0.1159, \text{mean} \pm SD, \text{stable}: 79.5 \pm 34.9 \text{ng/ml, AoDILD: 127.2} \pm 78.7 \text{ng/ml})\). ROC curves of these two surrogate markers were generated and AUC values were calculated (Supplementary Figure S1, KL-6, AUC: 0.625, 95% confidence interval [CI]: 0.334–0.916, SP-D, AUC: 0.658, 95% CI: 0.377–0.939). Thus, KL-6 and SP-D were insufficient biomarkers of AoDILD in RA.

**Serum metabolomic profiles of AoDILD in RA**

In metabolomic analysis, 216 metabolites, including 140 cationic and 76 anionic compounds, were detected (Supplementary Table S1). After the normalization with data scaling, 202 metabolites were compared between the stable and the AoDILD conditions (Supplementary Table S2). No significant difference was detected in the comparison after the correction of multiple testing by FDR, though some compounds show suggestive association. These metabolites were clustered with Ward method and visualized in a clustered heat map (Supplementary Table S2, Figure 1). Clustering of the metabolites did not show any apparent discrimination of AoDILD from the stable state. Thus, apparently distinct serum metabolomic profiles in AoDILD were not observed in univariate or hierarchical cluster analyses.

**Potential biomarkers for AoDILD**

Furthermore, PLS-DA was performed (Figure 2), and the component 1 successfully discriminated the stable state from the AoDILD conditions. The VIP scores of the component 1 from PLS-DA were calculated (Supplementary Figure S2). The metabolites with VIP scores more than 2.00 were listed in Table 1. The metabolites with higher VIP scores than 2.25 (mannosamine, alliin, kynurenine, and 2-hydroxybutyric acid) were selected to generate new biomarkers for AoDILD. Serum mannosamine and alliin levels were decreased and serum kynurenine and 2-hydroxybutyric acid levels were increased in AoDILD. ROC curves of these four metabolites were generated and AUC values were calculated (Supplementary Figure S2, mannosamine, AUC: 0.825, 95% CI: 0.595–0.980, alliin, AUC: 0.750, 95% CI: 0.600–0.900, kynurenine, AUC: 0.780, 95% CI: 0.490–0.943, 2-hydroxybutyric acid, AUC: 0.752, 95% CI: 0.490–0.948). A PLS-DA model was generated with these four metabolites, and the performance of the model was evaluated. An ROC curve of the PLS-DA model with these four metabolites was generated based on its average performance across all runs of Monte Carlo cross validation (Figure 3) and an AUC value was calculated (AUC: 0.962, 95% CI: 0.778–1.000). To evaluate the overfitting of the model, permutation test was performed (Permutation \(P = 0.0072\). The impact of AoDILD on metabolic pathway was also analyzed.
As shown in Figure 4, it was suggested that AoDILD influenced ketone body metabolism, amino acid turnover, and niacin metabolism, though the differences failed to reach significance. Thus, this PLS-DA model with the four metabolites demonstrated better performance than KL-6 or SP-D, though distinct serum metabolic profiles in AoDILD were not detected in univariate or cluster analyses.

### Discussion

Although many studies on metabolomic biomarkers were conducted, it is difficult to develop a single biomarker for the diagnosis of the complex diseases. In this study, PLS-DA was applied for the establishment of the diagnostic model of AoDILD with the four metabolites, mannosamine, alliin, kynurenine, and 2-hydroxybutyric acid, and the created PLS-DA model showed better performance than KL-6 or SP-D, pre-existing biomarkers. Although these pre-existing biomarkers can successfully indicate the condition of chronic ILD, they were not able to indicate the condition of AoDILD, acute or subacute ILD.

The plasma mannosamine levels decreased in response to the surgical procedure of Roux-en-Y gastric bypass performed in diabetic patients; plasma mannosamine was also associated with fasting plasma glucose, suggesting the biomarker for the altered carbohydrate metabolisms. Alliin (S-allyl-cysteine sulfoxide) is a garlic derivative known as a hydroxyl radical scavenger and has an immunomodulatory effect on phagocytosis of the peripheral blood mononuclear cells. Alliin can protect lipopolysaccharide-induced acute lung injury and increased insulin sensitivity. Serum mannosamine and alliin levels were decreased in the AoDILD, suggesting the reduced glucose levels and increased oxidative stress in the AoDILD condition.

Kynurenine is an important compound in the metabolism of tryptophan and is also used for the production of niacin. Serum kynurenine levels were increased in colorectal cancer patients, suggesting the upregulation of tryptophan metabolism in colorectal cancer. Serum levels of 2-hydroxybutyric acid were also increased in colorectal cancer patients, and it was revealed to be an early insulin resistance biomarker. Since kynurenine and 2-hydroxybutyric acid levels were increased in the AoDILD state and colorectal cancer, common metabolic changes would occur between these two conditions. Regulatory T cells accelerated tryptophan catabolism in dendritic cells and kynurenine generated by dendritic cells suppressed immunological function of T cells, suggesting the immunosuppression status in the AoDILD state and colorectal cancer.

In the previous study on plasma amino acid levels in AoDILD, the plasma levels of methionine and phenylalanine were increased. Similar tendencies on the results of these two amino acids were obtained in this study. The small sample size of the patients with AoDILD is the limitation of this study, though it is quite difficult to obtain the sera from RA patients before the onset of AoDILD. Independent large-scale studies are necessary for the validation of the results of the present study. In the present study, the metabolomic profiles in other controls, RA patients never suffering from AoDILD, idiopathic pulmonary fibrosis patients with acute exacerbation of ILD, or healthy controls were not compared with AoDILD. These comparisons should be performed in future large-scale studies.

To the best of our knowledge, this is the first study of metabolomic analysis to distinguish AoDILD from the stable condition.
condition. Our results did not apparently show distinct serum metabolomic profiles in AoDILD in univariate or cluster analyses. However, the PLS-DA model generated from the four metabolites with higher VIP scores (mannosamine, alliin, kynurenine, and 2-hydroxybutyric acid) could successfully monitor the AoDILD state. It was demonstrated that metabolomic profiling could be useful to generate better biomarkers for AoDILD.

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Author Contributions
HF and ST conceived and designed the experiments. HF and SO performed the experiments. HF analyzed the data. HF, KS, AH, AK, TM, NF, and ST contributed reagents/materials/analysis tools. HF and ST contributed to the writing of the manuscript. All authors read and approved the final manuscript.

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Supplemental Material
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Figure 4. Metabolomic pathway analysis based on metabolites in sera from RA patients with the stable and the AoDILD conditions. A P value from pathway enrichment analysis with global test and a pathway impact value from pathway topology analysis with relative-betweeness centrality of each metabolomic pathway was plotted. AoDILD: acute-onset diffuse interstitial lung disease; RA: rheumatoid arthritis.
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