Increased levels of plasma nucleotides in patients with rheumatoid arthritis

SHORT COMMUNICATION

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Abstract

Novel biomarkers of rheumatoid arthritis (RA), in addition to antibodies against cyclic citrullinated peptides, are required. Metabolome analysis is a promising approach to identify metabolite biomarkers for clinical diagnosis. We adopted a comprehensive non-targeted metabolomics approach combining capillary electrophoresis time-of-flight mass spectrometry (TOFMS) and liquid chromatography TOFMS. We constructed metabolomics profiling of 286 plasma samples of a Japanese population [92 RA patients, 13 systemic lupus erythematosus (SLE) patients and 181 healthy controls). RA case–control association tests showed that seven metabolites exhibited significantly increased levels in RA samples compared with controls ($P < 1.0 \times 10^{-4}$; UTP, ethanolamine phosphate, ATP, GDP, ADP, 6-aminohexanoic acid and taurine), whereas one exhibited a decreased level (xanthine). The plasma levels of these eight metabolites were not significantly different between seropositive and seronegative RA patients ($P > 0.05$; $n = 68$ and 24, respectively). The four nucleotide levels (UTP, ATP, GDP and ADP) were significantly higher in the non-treatment patients in comparison between patients with and without treatment ($P < 0.014$; $n = 57$ and 35, respectively). Furthermore, we found that none of the four nucleotide levels showed significant differences in SLE case–control association tests ($P > 0.2$; 13 patients with SLE and the 181 shared controls) and psoriatic arthritis (PsA) case–control association tests ($P > 0.11$; 42 patients with

PsA and 38 healthy controls), indicating disease specificity in RA. In conclusion, our large-scale metabolome analysis demonstrated the increased plasma nucleotide levels in RA patients, which could be used as potential clinical biomarkers of RA, especially for seronegative RA.

Keywords: biomarker, metabolome, psoriatic arthritis, systemic lupus erythematosus

Introduction

Early diagnosis and treatment of rheumatoid arthritis (RA) are important for preventing joint destruction. This requires identification of novel biomarkers in addition to antibodies against cyclic citrullinated peptides and/or proteins (ACPA). Metabolome analysis can comprehensively evaluate metabolic profiles in the context of biological systems [1]. It is a promising approach to identify metabolite biomarkers for clinical diagnosis, such as type 2 diabetes and Parkinson's disease [2, 3]. While there exist several previous metabolome analyses on RA [1, 4], their sample size and the number of metabolites were relatively small, providing less consistent findings.

In this study, we adopted a comprehensive non-targeted metabolomics approach combining capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) and liquid chromatography TOFMS (LC-TOFMS). This dual analysis works well in a complementary manner to detect a large number of metabolites with high resolution and high sensitivity [5]. We constructed metabolomic profiling of a total of 273 plasma samples in an RA case–control cohort of the Japanese population [6], which, to our knowledge, is one of the largest RA metabolome analyses ever reported (92 RA patients and 181 healthy controls). We also performed metabolome case–control association tests in 13 patients with systemic lupus erythematosus (SLE) and the 181 shared controls. Furthermore, we assessed findings of the association tests in an additional cohort of 42 patients with psoriatic arthritis (PsA) and 38 healthy controls.

Methods

Patient participation

We examined 92 RA patients and 14 SLE patients at Osaka University Hospital, the National Hospital Organization Osaka Minami Medical Center, Sumitomo Hospital, and Daini Osaka Police Hospital. RA patients were diagnosed according to the American College of Rheumatology/European League Against Rheumatism 2010 criteria for RA [7]. Exclusion criteria for both sequencing groups were as follows: (i) extreme diets (e.g. strict vegetarians) or (ii) known history of malignancy or serious diseases of the heart, liver or kidney. We also examined 182 healthy controls at the National Hospital Organization Osaka Minami Medical Center and Osaka University Graduate School of Medicine. All subjects provided written informed consent before participation.

As an additional cohort, we examined 43 PsA patients and 38 healthy controls at Osaka University and the National Hospital Organization Osaka Minami Medical Center (15 healthy controls were shared with the original cohort). PsA patients were diagnosed by dermatologists and rheumatologists considering the Classification Criteria for Psoriatic Arthritis [8] comprehensively.

All subjects provided written informed consent before participation. The study protocol was approved by the ethical committees of Osaka University and related medical institutions.

Sample collection

In the original cohort, plasma samples were collected in BD™ P100 Blood Collection Tubes (Franklin Lakes, NJ, USA), which contains spray-dried K2 EDTA anticoagulant, proprietary proteinase inhibitors and a mechanical separator to obtain plasma [9]. In the additional cohort, plasma samples were collected in vacuum blood collection tubes containing K2 EDTA or heparin anticoagulant in addition to the P100 tubes. Within 1 h of collection, the samples were centrifuged for 15 min at 2500 × *g* and stored at –80°C until analysis.

Metabolite extraction

Metabolite extraction and metabolome analysis were conducted at Human Metabolome Technologies (HMT), Japan. For CE-TOFMS measurement, 50 μl of plasma was added to 450 μl of methanol containing internal standards (H3304–1002, HMT) at 0°C to inactivate enzymes. The extract solution was thoroughly mixed with 500 μl of chloroform and 200 μl of Milli-Q water and centrifuged at 2300 × *g* and 4°C for 5 min. The 350 μl of the upper aqueous layer was centrifugally filtered through a Millipore 5-kDa cutoff filter to remove proteins. The filtrate was centrifugally concentrated and resuspended in 50 μl of Milli-Q water for CE–MS analysis. For LC-TOFMS measurement, 500 μl of plasma (or serum) was added to 1500 μl of 1% formic acid/acetonitrile containing internal standard solution (Solution ID: H3304-1002, Human Metabolome Technologies, Inc., Tsuruoka, Japan) at 0°C to inactivate enzymes. The solution was thoroughly mixed and centrifuged at 2300 × g and 4°C for 5 min. The supernatant was filtrated by using Hybrid SPE phospholipid (55261-U, Supelco, Bellefonte, PA, USA) to remove phospholipids. The filtrate was desiccated and then dissolved with 100 μl of isopropanol/Milli-Q for LC–MS analysis.

Metabolome analysis

Metabolome analysis was conducted by the Dual Scan package of HMT using CE-TOFMS and LC-TOFMS for ionic and nonionic metabolites, respectively, on the basis of the methods described previously [10, 11]. Briefly, CE-TOFMS analysis was carried out using an Agilent CE system equipped with an Agilent 6210 TOFMS, Agilent 1100 isocratic HPLC pump, Agilent G1603A CE-MS adapter

kit and Agilent G1607A CE-ESI-MS sprayer kit (Agilent Technologies, Santa Clara, CA, USA). The systems were controlled by Agilent G2201AA ChemStation software version B.03.01 for CE (Agilent Technologies) and connected by a fused silica capillary (50 μm *i.d.* × 80 cm total length) with commercial electrophoresis buffer (H3301-1001 and I3302-1023 for cation and anion analyses, respectively, HMT) as the electrolyte. The spectrometer was scanned from *m/z* 50 to 1000. LC-TOFMS analysis was carried out using an Agilent LC System (Agilent 1200 series RRLC system SL) equipped with an Agilent 6230 TOFMS (Agilent Technologies). The systems were controlled by Agilent G2201AA ChemStation software version B.03.01 (Agilent Technologies) equipped with ODS column (2 × 50 mm, 2 μm). Peaks were extracted using MasterHands, automatic integration software (Keio University, Tsuruoka, Yamagata, Japan) to obtain peak information including *m/z*, peak area and migration time for CE-TOFMS measurement (MT) or retention time for LC-TOFMS measurement (RT). Signal peaks corresponding to isotopomers, adduct ions and other product ions of known metabolites were excluded, and remaining peaks were annotated according to the HMT metabolite database based on their *m/z* values with the MTs and RTs determined by TOFMS. Areas of the annotated peaks were normalized based on internal standard levels and sample amounts to obtain relative levels of each metabolite. As a result of CE-TOFMS and LC-TOFMS, we detected 566 and 553 metabolites in the original cohort and the additional cohort, respectively. We applied quality controls to the individuals (exclusion of three individuals; one SLE patient and one healthy control because of outliers of principal component analysis, and one PsA patient because of excessively low levels of metabolites) and the metabolites (exclusion of metabolites detected in <3% of the samples). The levels of each metabolite were normalized using log transformation.

Results and discussion

We assessed a total of 490 metabolites obtained from plasma samples of the 92 RA patients and 181 controls (Supplementary Table 1). Case–control association tests were performed with sex, age, sampling hospitals and the top 30 principal components as covariates, using the generalized linear model function in the R package glm2 (version 1.2.1). Significant associations were identified for the eight metabolites that satisfied the Bonferroni's correction ($P < 1.0 \times 10^{-4}$; Fig. 1, Table 1). Of these, seven exhibited increased levels in RA samples compared with in controls [UTP, ethanolamine phosphate (EAP), ATP, GDP, ADP, 6-aminohexanoic acid and taurine], whereas one exhibited a decreased level (xanthine). The majority of RA-associated metabolites are nucleotides (UTP, ATP, GDP and ADP). Extracellular nucleotides are increased during conditions of hypoxia or inflammation (12). The purinergic system, which comprises the signaling and metabolism of purines such as adenosine, ATP and UTP, is indicated to affect the pathogenesis of many chronic inflammatory diseases (13, 14). For example, in inflammatory bowel disease, enteric-nerve death driven by ATP induced signaling pathways and barrier-protective effects of adenosine-receptor signaling during intestinal inflammation was reported (15, 16). As for RA, the release of nucleotides, especially ATP, into the synovial fluid is triggered by the hypoxic environment in the synovium and hypotonic nature of the synovial fluid typical of RA (17). Our study initially identified increased levels of nucleotides in RA plasma. The capability of CE-TOFMS to detect nucleic acids was considered to be responsible for providing this novel result, while the classical methods such as gas chromatography and LC have less ability to detect them. As an example, the previous large-scale metabolome study of RA detected almost 1000 metabolites but few nucleotides (18). Another study using
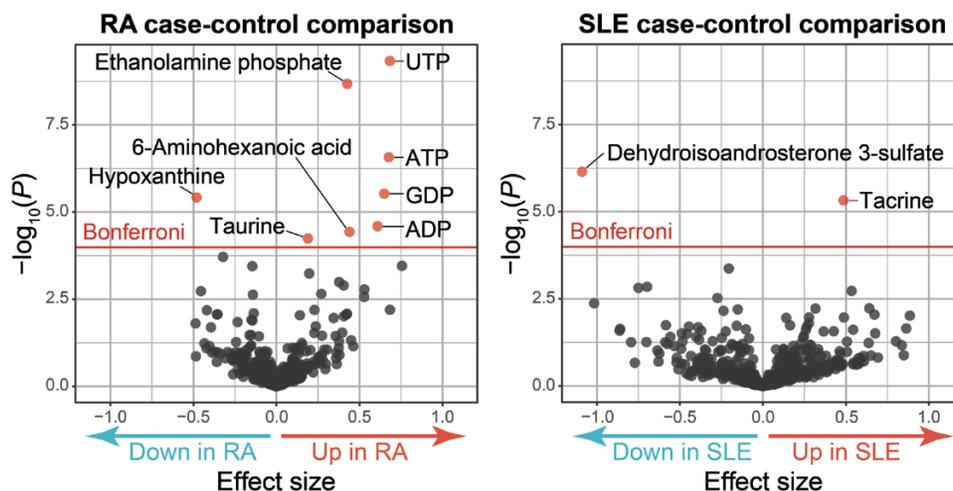
Fig. 1. Results of case–control association tests. Volcano plots of RA case–control association tests (left) and SLE case–control association tests (right). The *x*-axes indicate beta values of the generalized linear model as effect sizes. The *y*-axes indicate $-\log_{10} P$-values. The horizontal red lines indicate the Bonferroni-corrected threshold ($P = 1.0 \times 10^{-4}$). Metabolites with *P*-values less than the Bonferroni thresholds are plotted as red dots.

Table 1. Metabolites with significant case–control discrepancy in RA or SLE

Metabolite	HMDB ref	RA versus control		SLE versus control		PsA versus control	
		P-value	Fold-change	*P*-value	Fold-change	*P*-value	Fold-change
Metabolites with significant RA case–control discrepancy							
UTP	HMDB00285	**4.6×10^{-10}**	**19.3**	0.68	6.20	—	—
Ethanolamine phosphate	HMDB00224	**2.1×10^{-9}**	**2.77**	0.12	1.70	0.27	1.15
ATP	HMDB00538	**2.7×10^{-7}**	**4.62**	0.22	2.56	0.11	1.44
GDP	HMDB01201	**3.0×10^{-6}**	**6.85**	0.79	2.99	0.68	5.10
Hypoxanthine	HMDB00157	**3.8×10^{-6}**	**0.63**	0.0015	0.30	0.27	6.45
ADP	HMDB01341	**2.5×10^{-5}**	**3.87**	0.89	1.85	0.29	1.50
6-Aminohexanoic acid	HMDB01901	**3.7×10^{-5}**	**2.57**	0.15	1.43	—	—
Taurine	HMDB00251	**5.7×10^{-5}**	**1.75**	0.15	1.16	0.93	1.40
Metabolites with significant SLE case–control discrepancy							
Dehydroisoandrosterone 3-sulfate	HMDB01032	**0.0086**	**0.37**	**7.2×10^{-7}**	**0.43**	0.37	0.44
Tacrine	—	0.29	33.1	**4.7×10^{-6}**	**44.8**	—	—

The numbers were in bold when the Bonferroni's correction was satisfied ($P < 1.0 \times 10^{-4}$). HMDB, Human Metabolome Database.
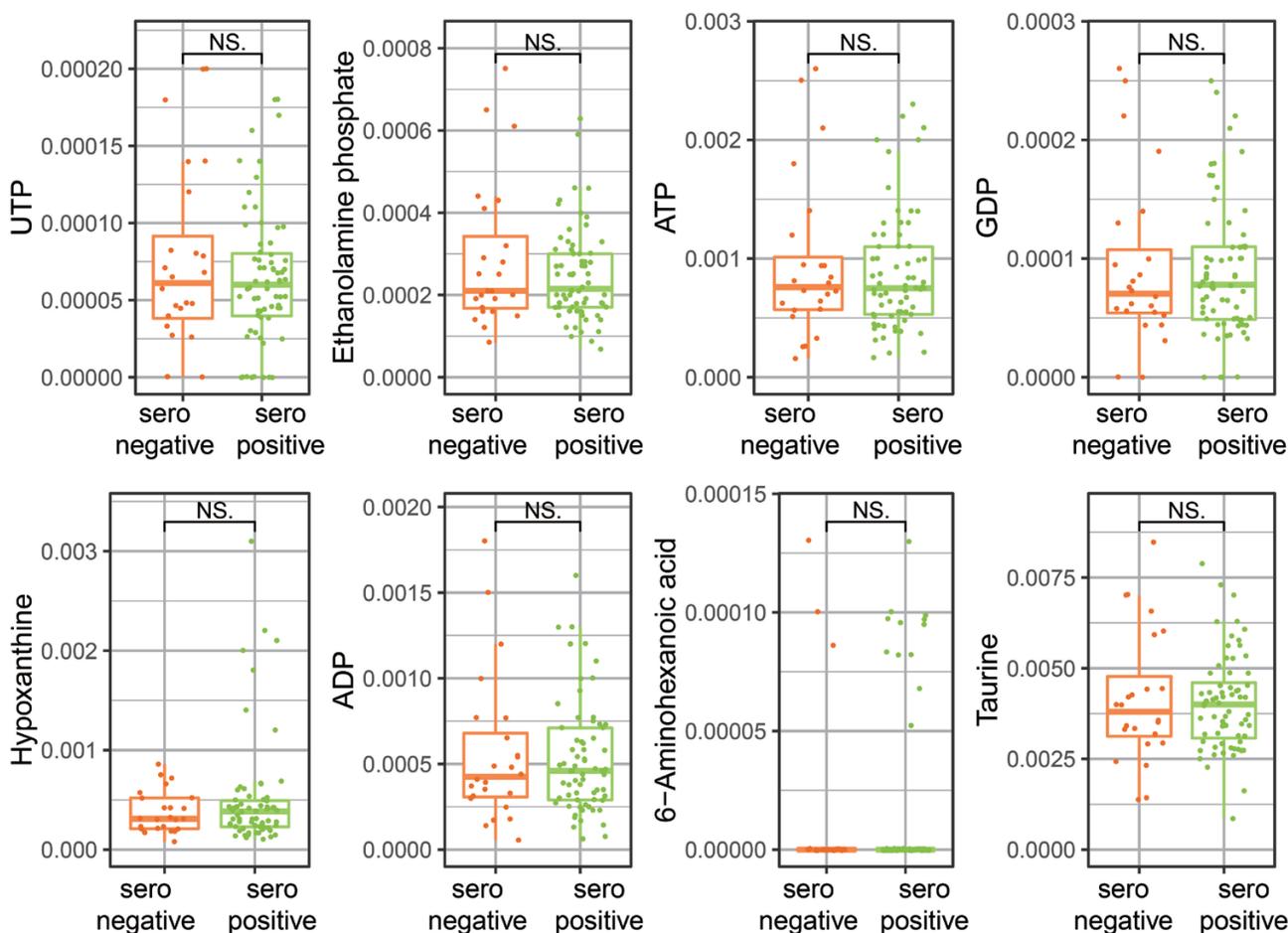
Fig. 2. Differences in metabolites with significant RA-control discrepancy between seropositive and seronegative RA patients. The *y*-axes indicate plasma levels of metabolites. The lower and upper hinges of the boxes indicate the first and third quartiles. The horizontal lines within the boxes indicate median levels. The Mann–Whitney *U* test was used to compare seropositive and seronegative RA patients: not significant (NS), $P \geq 0.05$.

LC–MS could not directly detect nucleotides either but identified the increased levels of D-ribofuranose, hypoxanthine, uridine and pseudouridine in RA patients that indicated an increased rate of nucleotide synthesis (19). Taurine was associated with oxidative stress and related to the inflammatory processes in RA (20). Furthermore, it has been reported that serum EAP level is a promising biomarker for diagnosing depression (21). Although the biological mechanism of EAP
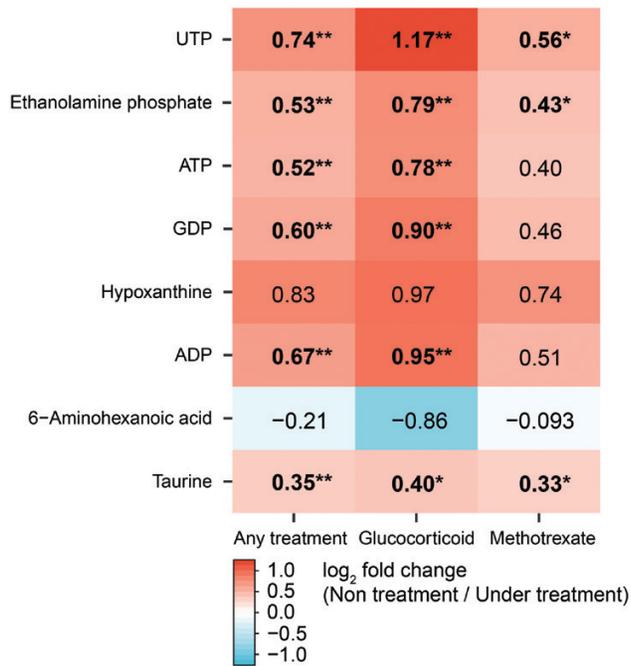
Fig. 3. Comparison of metabolites with significant RA-control discrepancy in RA patients with and without treatment. The number and color of cells represent $\log_2$ fold-change of metabolite levels in the non-treatment group to those in each under treatment group (any treatment, glucocorticoid and methotrexate). When the Mann–Whitney U test between non-treatment group and each under treatment group showed a P-value < 0.05, the number is shown in bold with asterisks: *P < 0.05; **P < 0.01.

in RA remains unclear, the increased level of EAP would reflect the pathology of RA.

While our analysis adjusted the confounding effect of sex and age by incorporating them as covariates, we additionally performed a subgroup analysis with the sex- and age-adjusted samples (Supplementary Table 2). The nucleotides exhibited high fold-changes (3.1–8.8) and nominally increased levels in RA samples compared with controls (P < 0.031; Supplementary Table 3). Thus, the plasma levels of nucleotides were increased according to the status of RA independently from age and sex.

Regarding the metabolites with significant case–control associations, we compared seropositive and seronegative patients (n = 68 and n = 24, respectively), but no significant difference was detected (Mann–Whitney U test, P > 0.05; Fig. 2). The diagnosis of seronegative RA is still challenging due to the absence of clinical biomarkers. These eight metabolites identified in our study should be potential candidates of novel biomarkers for seronegative RA. We further compared patients with and without treatment (n = 52 and n = 40, respectively). The levels of the four nucleotides, EAP and taurine were significantly higher in the non-treatment patients (Mann–Whitney U test, P < 0.0078; Fig. 3). We added subgroup analyses of each patient group taking glucocorticoid and methotrexate, finding that the nucleotide levels of non-treatment patients were higher compared with both subgroups (P < 0.0025 and P < 0.10 for glucocorticoid and methotrexate, respectively; Fig. 3). This result indicates that

the increments in plasma nucleotide levels would reflect the RA naive pathophysiology, rather than that modified by medication. We further added the score of DAS28-CRP or the level of CRP as a covariate in the comparison between patients with and without treatment. We did not observe apparent changes in the results compared with those without the covariate of DAS28-CRP or CRP. The levels of three nucleotides (UTP, ATP and ADP) were significantly higher in the non-treatment patients as well (P < 0.039; Supplementary Table 4). This result suggests that the reduction of the nucleotide levels according to treatment would not be solely explained by the change in the systemic inflammation.

To assess the correlation between the disease duration and the eight metabolite levels, we divided the RA patients into three groups (<1 year, from 1 to 5 years and >5 years) and conducted linear regression analysis with sex and age as covariates. We found no significant correlation (P > 0.51). Especially when focusing on the non-treatment patients for excluding the effect of treatment, the differences between groups were slight (Supplementary Figure 2).

Furthermore, we performed metabolome case–control association tests in 13 patients with SLE and the 181 shared controls. We identified significant associations for the two metabolites that satisfied the Bonferroni's correction (Fig. 1, Table 1). One metabolite demonstrated an increased level in SLE samples (tacrine), whereas the other demonstrated a decreased level [dehydroisoandrosterone 3-sulfate (DHEAS)]. DHEAS levels also decreased nominally in RA samples (P = 0.0086; Table 1; Supplementary Figure 1), which is concordant with previous findings (18, 22). On the other hand, the levels of none of the four nucleotides that were increased in the RA samples showed a significant difference in the SLE case–control association tests (P > 0.2), indicating disease specificity in RA. To further evaluate the disease specificity, we conducted metabolome analysis in an additional cohort consisting of 42 PsA patients and 38 healthy controls with the same procedure as the original cohort. UTP and 6-aminohexanoic acid were not detected in the additional cohort, while they were only detected in a very small number of the healthy controls in the original cohort. As for the other six metabolites, PsA case–control association tests showed no significant differences (P > 0.11) with less fold-changes than RA case–control association tests (Table 1). This result was consistent with the previous report that the levels of metabolites indicating nucleotide synthesis were increased in RA patients compared with PsA (19). It has been suggested that RA differs from other inflammatory arthritis in the hypoxia-inducible factor-related signal and the mechanism of angiogenesis (23–25). Further functional studies are required to investigate the RA specificity of increased nucleotide levels in plasma.

In conclusion, our large-scale metabolome analysis demonstrated the increased plasma nucleotide levels in RA patients, which could be used as potential clinical biomarkers of RA, especially for seronegative RA.

Funding

Acknowledgement

We thank Ms Miho Kaneda for supporting the study.

Author contributions

T.K. and Y.O. designed the study, conducted data analysis and wrote the manuscript. T.K., Y.M., T.N., N.A., J.H., K.S., K.Y., T.M., K.O., Shig.T., M.M., H.M., M.Y., M.N., Shin.T., S.O., A.O., and Y.S. collected the samples. H.I., A.K., K.T., and Y.O. supervised the study.

Conflicts of interest statement: the authors declared no conflicts of interest.

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