Metabolite Profiling of Serum from Patients with Tuberculosis

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Tuberculosis (TB) is a major infectious disease that threatens the life and health of people globally. Here, we performed a metabolomic analysis of serum samples from patients with intractable TB to identify biomarkers that might shorten the TB treatment period. Serum samples collected at the commencement of patients' treatment and healthy controls were analyzed using the capillary electrophoresis and time-of-flight mass spectrometry metabolome analysis method. The analysis identified the metabolites cystine, kynurenine, glyceric acid, and cystathionine, which might be useful markers for monitoring the TB treatment course. Furthermore, our research may provide experimental data to develop potential biomarkers in the TB treatment course.

Keywords: Biomarker, CE-TOFMS, *Mycobacterium tuberculosis*, metabolites

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (MTB) and is a major health problem globally [1]. MTB has infected 10 million people and caused 1.8 million deaths per year worldwide [2]. However, a rapid and accurate TB diagnosis remains a major challenge for global control efforts [3]. Recently, the incidence of pulmonary tuberculosis is high in immunocompromised individuals, and the emergence of multidrug-resistant bacteria has made diagnosis and treatment more complicated [4, 5].

A standard diagnosis method for TB is a smear and culture for acid-fast bacilli in clinical specimens. Although these techniques are important steps for TB diagnosis, they have several drawbacks [6–8]. Smear microscopy is only effective for TB diagnosis when the bacterial load is >10,000 organisms/ml of sample [9].

Moreover, the positivity rate of a TB sputum smear is only 20–30%. Sputum cultures have been widely used in the diagnosis of MTB, but it can take 4–8 weeks to obtain results. This can lead to delayed treatment in patients with potential TB [10, 11]. Therefore, it is necessary to develop biomarkers that can be used for an accurate diagnosis that might lead to early treatment.

Moreover, metabolomics has recently emerged as a potential tool to make striking advances in new biomarker research by enabling multiple profiling and comparing multiple metabolites in a biological sample [12]. Therefore, metabolomics has been widely used to understand biological processes and metabolic behavior pathways of various diseases and to figure out host responses, allowing the researcher to develop advanced diagnostic tools and treatment strategies [13, 14].

In this study, we used capillary electrophoresis and time-of-flight mass spectrometry (CE-TOFMS) to profile and compare metabolites found in the serum of patients with active TB versus healthy controls.

Study participants were recruited from an intractable

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TB cohort from 2013 to 2014. Peripheral blood samples were obtained by venipuncture from 7 patients with active TB disease and 7 healthy controls. Blood was collected in serum separating tubes and centrifuged at 1500 rpm for 15 min at 4°C to obtain serum. The serum was immediately frozen and stored at -80°C until analysis. The Institutional Review Board (IRB) of the Korea Disease Control and Prevention Agency (KDCA) approved this study (2019-04-05-2C-A).

Metabolite extraction from samples was carried out according to the protocol provided by Human Metabolome Technologies (HMT, Japan). Briefly, 50 µl of thawed serum was treated with methanol (450 µl) containing internal standards. Chloroform (450 µl) and ultrapure water (200 µl) were sequentially added to the serum-methanol sample, and the mixture was centrifuged at 2,300 ×g at 4°C for 5 min. The upper aqueous phase (800 µl) was filtered by centrifugation, and the filtrate was concentrated and resuspended in ultrapure water (25 µl) for analysis. Cationic compounds and anionic

compounds were measured in the CE-TOFMS system (Fused silica capillary, i.d. 50 µm × 80 cm, Agilent Technologies, Santa Clara, California). Peaks detected by CE-TOFMS were extracted using automatic integration software (MasterHands ver. 2.17.1.11 developed at Keio University) to provide the requisite information, including *m/z*, migration time (MT), and peak area. Putative metabolites were then assigned from the standard library at HMT and the Known-Unknown peak library based on *m/z* and MT. The tolerance range for the peak annotation was configured at ± 0.5 min for MT and ± 10 ppm for *m/z*. In addition, all of the target metabolite concentrations were quantified by normalizing the peak area of each metabolite to the area of the internal standard and by using standard curves, which were obtained by single-point (100 µM or 50 µM) calibrations.

Significant differences in metabolites among the experimental groups were identified using a *t*-test. Statistical significance was set at *p* < 0.05. Principal

Fig. 1. Principal component analysis and hierarchical cluster analysis. (A) Principal component score plot showing the separation of groups consisting of patients with active tuberculosis (TB) or healthy controls. PC1 and PC2 show the first principal component and second principal component, respectively. The number in parentheses is the contribution rate. Healthy control group: T01, T02, T03, T04, T05, T06, and T07. Baseline group: T08, T14, T19, T22, T26, T29, and T33. The 2 weeks after treatment start group: T09, T15, T20, T23, T27, T30, and T34. The 1 month after treatment start group: T10, T16, T21, T24, T28, T31, and T35. (B) The rows display metabolites and the columns represent samples.

component analysis (PCA) and hierarchical cluster analysis (HCA) were performed using statistical analysis software developed at HMT.

The TB cohort project collected 21 serum samples from patients with active TB ($n = 7$) and 7 serum samples from healthy people ($n = 7$). Patients were identified as positive for TB based on clinical symptoms classified as a TB infection, a positive MTB smear, and culture. The healthy control group included patients with a negative MTB smear and culture, a negative interferon-gamma release assay, and no TB lesions observed on chest X-ray. Patients with active TB were classified into the following groups according to changes in bacterial load during the first month of treatment: treatment start (baseline), 2 weeks after treatment commencement (2 weeks), and 1 month after treatment commencement (1 month).

CE-TOFMS was used to detect mass spectrometry (MS) data in 28 serum samples according to the classification. CE-TOFMS analysis detected and identified 272 peaks based on the HMT standard library. We performed multivariate statistical analysis using the MS data to identify metabolites variation between active TB patients and healthy controls. PCA visualized the relative distance between the two groups. The PCA plot distribution showed a clear variation between the active TB

patient group at baseline, 2 weeks after treatment commencement, 1 month after treatment commencement, and healthy controls (Fig. 1A).

Using the profiles of 272 significant differentiating serum metabolites, we drew a heat map to classify the upregulated and downregulated metabolites for TB infection (Fig. 1B). These upregulated and downregulated metabolites may be important for early infection diagnosis and biomarker discovery [15]. The relationship between metabolites in each group is represented by color change. Metabolites with low levels appear in green, while those at high levels are represented in red. The heat map, which standardized the relative area of metabolites, confirmed that a clear cluster was formed between the active TB patient group at baseline, 2 weeks after treatment commencement, 1 month after treatment commencement, and healthy controls. The main mechanisms and substances that appeared between each group were identified by grouping the formed clusters into dendrograms. Through these metabolic changes, the characteristics of each group were confirmed.

A *t*-test was performed to evaluate the statistically significant variation in metabolites in patients across the treatment periods. Compared with healthy controls, there were significant differences in 38 metabolites in the baseline, 44 in the 2 weeks after treatment com-

Fig. 2. Dynamic changes in serum metabolite concentrations. Metabolites showing significant differences between the healthy control and tuberculosis treatment groups are shown. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$

mencement, and 37 in the 1 month after treatment commencement ($p < 0.05$). In addition, according to the patients' treatment, there were significant differences between the baseline and treatment duration groups ($p < 0.05$). Major metabolomic pathways, such as central carbon and amino acid, were identified. Metabolites with significant differences between groups such as cystine, kynurenine, cystathionine, glyceric acid, isonicotinic acid, and pyrazinamide were identified (Fig. 2). Cystine and kynurenine were upregulated in patients with active TB. The ratios of cystine and kynurenine between the groups may be potentially suitable biomarkers to detect active TB infection [16, 17]. Indoleamine 2,3-dioxygenase (IDO) has an immunomodulatory function, i.e., it catalyzes tryptophan through the kynurenine pathway. Kynurenine helps suppress the immune response by managing T cell anergy and proliferation [18]. Additionally, the suitability of sample selection and response to treatment was confirmed by expressing drug-derived metabolites in patients with active TB [19]. Cystathionine was downregulated and glyceric acid was upregulated in the 1 month after treatment commencement group. Using this significant difference in metabolite expression, we identified candidates that can determine the effects of TB treatment [20, 21]. Currently, there have been few studies that have analyzed the metabolism of patients with active TB in South Korea. In this study, through metabolite analysis of patients with active TB in South Korea, serum metabolites that affected TB infection and treatment were identified.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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