Identification of Lipid Biomarkers for Skeletal Tuberculosis Using an Untargeted Metabolomics Approach

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Research

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Abstract

Background

The identification of novel biomarkers in the human body is urgently needed to improve diagnosis and the clinical management of skeletal tuberculosis (STB). This study aimed to identify potential lipid markers to differentiate *Mycobacterium tuberculosis* infection from other infections, and establish a metabolite biomarker panel suitable for STB diagnosis from abscess samples.

Methods

Participants were recruited from Beijing Chest Hospital between January 2018 and December 2019. Abscess specimens were collected from STB patients and patients diagnosed with other skeletal infections. Then we comparatively explored the lipid metabolomes of abscess specimens from STB and non-STB patients using untargeted lipid metabolomics approach.

Results

A total of 957 features in ESI+ mode and 584 in ESI- mode were extracted from the lipidomic data. Among 1541 lipid metabolites, 55 lipid metabolites were significantly changed in STB patients compared to the control group. Significant up-regulation was clearly visualized in phosphatidylethanolamine, phosphatidylinositol, lysophatidylinositols, lysophosphatidylcholines and lysophosphatidylethanolamine. Concomitantly, triacylglycerols and phosphatidylcholines were significantly down-regulated in patients affected by STB. It is worth noting that the two down-regulated monoacyl chain phosphatidylcholine, triacylglycerols and one up-regulated LPE showed excellent diagnostic potential, the AUC values of which were higher than 0.9 and the highest value was 1.0.

Conclusions

To conclude, our data firstly characterize the lipid signatures of abscess specimens from STB patients. The LPEs are significantly upregulated in the STB cases than those in non-STB control, whereas phosphocholines and triacylglycerol are markedly downregulated in the STB cases. The panel of five lipid biomarkers exhibits great capacity for differential diagnosis of STB and non-TB cases.

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, remains the major public health concern worldwide, with an estimated 10.0 million incident cases in 2019 [1]. Although it most commonly affects the lungs, it can virtually affect any other anatomic site, termed as extrapulmonary tuberculosis (EPTB) [2, 3]. Nearly 10% of global TB cases are EPTB cases, posing challenges for TB control efforts [1]. Skeletal tuberculosis (STB) is one of the most common sites of EPTB, both in terms of relative frequency and the substantial potential for permanent disability [2, 4]. Timely diagnosis and treatment are critical in achieving successful outcomes and preventing disability associated with STB [5]. Unfortunately, similar

to other forms of EPTB, STB is harder to diagnose due to its extremely paucibacillary nature [4]. Therefore, the identification of novel biomarkers in the human body is urgently needed to improve diagnosis and the clinical management of this EPTB form.

The wide application of omics technologies has extended our knowledge of new diagnostic and treatment strategies for infectious diseases, including TB [6, 7]. Metabolomics is one of the newcomers to the omics revolution that identify and quantify of the complete metabolome of a specific biological system in the nonbiased condition [8]. To date, several metabolomics studies have been conducted with the aim of explore specific metabolite biomarkers in assisting TB diagnosis [9-11]. Some of the compounds identified as potential biomarkers were verified in subsequent reports, indicating their promising approach for clinical application [8]. However, the majority of previous studies were performed in blood samples to determine biomarkers for pulmonary TB; little is known about metabolites originating directly from tubercle bacilli, as well as metabolites altered in the host due to the infection in the specimens from EPTB patients.

Lipids play an important role in the pathology of tuberculosis, which is often associated with malnutrition and wasting syndrome [12]. Previous studies confirmed that the blood lipid level in host individuals is associated with bactericidal effect of first-line antibiotics and phagosome maturation, suggesting the important role of lipids in bacteria-host interaction [13]. Given that, we comparatively explored the lipid metabolomes of abscess specimens from STB and non-STB patients using untargeted lipid metabolomics approach, to identify potential lipid markers to differentiate *Mycobacterium tuberculosis* infection from other infections. We aimed to establish a metabolite biomarker panel suitable for STB diagnosis from abscess samples.

Materials And Methods

Participants

Participants were recruited from Beijing Chest Hospital, a tuberculosis-specialized hospital, between January 2018 and December 2019. Abscess specimens were collected from 10 STB patients with confirmed etiology by mycobacteria culture and/or GeneXpert MTB/RIF. For control group, abscess specimens were collected from 4 patients diagnosed with other skeletal infections as listed in Table 1. Five milliliters of abscess samples were obtained from each patient, and then transported to the BioBank of Beijing Chest Hospital for storage. All samples were centrifuged at 12,000 g for 15 min, filtered with a disposable 0.22 µm cellulose acetate and stored at -80°C for further analysis. All procedures are performed in studies involving human participants were approved by the Ethics Committee of Beijing Chest Hospital. Signed informed consent was obtained from each participant prior to enrolment.

Lipid Extraction

One milliliter of each sample was pipetted in a 2 ml centrifuge tube and lyophilized in a freeze-dryer. Then samples were firstly bath sonicated for 15 min with 400 µl ice-cold 75% methanol to lyse cells. After

mixing samples with 1 ml methyl-tert-butyl ether (MTBE, Merck, Darmstadt, Germany) for 1 h at room temperature, 0.25 ml of deionized water was added into mixture, and incubated at room temperature for 10 min. Then the samples were centrifuged for 15 min at 4 °C. The upper organic phase was collected and freeze-dried.

Additionally, to ensure data quality for metabolism profiles, quality control (QC) samples were prepared by pooling aliquots of all samples that were representative of the all samples under analysis, and used for data normalization. Dried extracts were then dissolved in 50% acetonitrile. Each sample was filtered with a disposable 0.22 µm cellulose acetate and transferred into 2 ml HPLC vials and stored at -80°C prior to analysis.

UHPLC-MS/MS analysis

Metabolomics LC–MS analysis was conducted on a Thermo (Waltham MA, USA) UltiMate 3000 UHPLC system coupled to a Thermo Q Exactive Orbitrap mass spectrometer. A Hypersil GOLD C18 (100×2.1mm, 1.9 µm) (Thermo Scientific) was used for lipid separation. The mobile phase A was prepared by dissolving 0.77g of ammonium acetate to 400ml of HPLC-grade water, followed by adding 600ml of HPLC-grade acetonitrile; the mobile phase B was the flow rate was set as 0.3 mL/min. The gradient was 30% B for 0.5 min and was linearly increased to 100% in 10.5 min, and then maintained to 100% in 2 min, and then reduced to 30% in 0.1 min, with 4.5 min re-equilibration period employed. Both electrospray ionization (ESI) positive-mode and negative mode were applied for MS data acquisition. The positive mode of spray voltage was 3.0 kV and the negative mode 2.5 kV. The ESI source conditions were set as follows: Heater Temp 300 °C, Sheath Gas Flow rate, 45arb, Aux Gas Flow Rate, 15 arb, Sweep Gas Flow Rate, 1arb, Capillary Temp, 350 °C, S-Lens RF Level, 50%. The full MS scans were acquired at a resolution of 70,000 at m/z 200, and 17,500 at m/z 200 for MS/MS scan. The maximum injection time was set to for 50 ms for MS and 50 ms for MS/MS. MS data was acquired using a data-dependent Top10 method dynamically choosing the most abundant precursor ions from the survey scan (200–1500 m/z) for HCD fragmentation. Stepped normalized collision energy was set as 15, 25, 35 and the isolation window was set to 1.6 Th. Blank samples (75% ACN in water) and QC samples were tested after every six samples for quality control purpose.

Data preprocessing and filtering

Lipids were identified and quantified using Lipid Search 4.1.30 (Thermo). Mass tolerance of 5 ppm and 10 ppm were applied for precursor and product ions. Retention time shift of 0.25 min was performed in “alignment”. M-score and chromatographic areas were used to reduce false positives. The lipids with less than 30% RSD of MS peak area in QC samples were kept for further data analysis.

One-way analysis

The discriminating metabolites were obtained using a statistically significant threshold of variable influence on projection (VIP) values obtained from MetaboAnalyst 5.0 (<http://www.metaboanalyst.ca>)

and two-tailed Student's *t* test (*p* value) on the normalized raw data at univariate analysis level. The *p* value was calculated by one-way analysis of variance (ANOVA) for multiple groups' analysis. Metabolites with VIP values greater than 1.0 and *p* value less than 0.05 were considered to be statistically significant metabolites. Fold change was calculated as the logarithm of the average mass response (area) ratio between two arbitrary classes. On the other side, the identified differential metabolites were used to perform cluster analyses with R package.

Results

Lipid identification

In this study, untargeted lipid metabolomics were conducted to investigate the metabolic dysregulation in paraspinal abscesses of STB. A total of 957 features in ESI+ mode and 584 in ESI- mode were extracted from the lipidomic data. These 1541 lipids were classified into 39 lipid subclasses. Pooled QC samples were used to monitor the stability of the LC-MS system (Fig.1). When conducting differential lipid analysis between STB and non-STB, we used univariate analysis methods include Fold Change Analysis (FC Analysis), *t* test, and Volcano Plot that integrates the first two Analysis methods. We used $|FC| > 1.5$ and *p* value < 0.05 as the screening criteria to declare significant differences in the comparison group (Fig.2A).

Differential metabolites between STB and non-STB

Among 1541 lipid metabolites, 55 lipid metabolites were significantly changed in STB patients compared to the control group. The VIP, FC, *p*-values and area under a ROC curve (AUC) values of differential metabolites were listed in Table S1. Significant up-regulation was clearly visualized in phosphatidylethanolamine (PE), phosphatidylinositol (PI), lysophatidylinositols (LPI), lysophosphatidylcholines (LPC) and lysophosphatidylethanolamine (LPE). Concomitantly, triacylglycerols (TG) and phosphatidylcholines (PC) were significantly down-regulated in patients affected by STB. In order to evaluate the rationality of different lipids, and to display the relationship between samples and the differences in lipid expression patterns in different samples more comprehensively and intuitively, we used qualitatively significant differences in lipid expression to perform hierarchical analysis on the two sets of samples. Fig.2B shows the results of significant difference in lipid hierarchical clustering analysis.

Potential diagnostic biomarkers of STB

As demonstrated in volcano plot (Fig. 2A) and heatmap (Fig. 2B), the biomarkers had a clearly differential distribution between two groups. The AUC value was used to evaluate the diagnostic ability of biomarkers for STB. We presented diagnostic performances of 10 representative lipid metabolites consisting of 4 phospholipids, 4 lysophospholipids and 2 triacylglycerols (as the order of least *q*-values, Fig. 3A-D and Fig. S1). It is worth noting that the two down-regulated monoacyl chain

phosphatidylcholine, triacylglycerols and one up-regulated LPE showed excellent diagnostic potential, the AUC values of which were higher than 0.9 and the highest value was 1.0. Due to the small sample size of this study, to further optimize the diagnostic performance of the biomarkers, we combined these 5 ether lipids as a panel. In multivariate ROC analysis, this panel yielded a better AUC value of 0.991 for diagnosis of STB patients (Fig. 3E).

Pathway analysis of STB

Based on the list of significantly regulated lipids, MetaboAnalyst (<http://www.metaboanalyst.ca>) was applied to investigate which pathway might be markedly perturbed. The result of the pathway analysis was graphically presented in Fig.4. From the enrichment analysis results, the Glycerophospholipid metabolism pathway had a statistically significant raw p-value (raw $p < 0.05$, as shown in the Y-axis). Pathway impact results indicated that the Glycerophospholipid metabolism pathway presented higher impact than the other pathways, as indicated in the X-axis value. Combining the above two analysis results, we postulated that the Glycerophospholipid metabolism pathway to be a markedly perturbed pathway that correlated with the lipid rearrangement process induced by MTB infection in STB.

Discussion

In this study, using a lipid metabolomics approach, we comparatively explored the altered metabolomes that would differentiate MTB infection from other infections. Our data demonstrated that the lipid metabolic profiles of STB and non-STB patients were significantly different from that of abscess specimens, providing potential novel biomarkers for STB diagnosis. The varying metabolites were mainly related to the pathways of lysophospholipid, glycerophospholipid and phosphatidylcholine metabolism. In particular, the levels of two LPEs were significantly higher in the STB cases than those in non-STB control. LPE is a constituent of cell membranes in the human host, which is derived from the hydrolysis of PE[14]. We speculate that the elevated abscess levels of LPEs originates from the destruction of cell membranes within necrotic lesions. The accumulation of LPEs may reflect more severe bone and tissue destruction by tubercle bacilli in STB patients than other patients. Our findings may also be in line with previous observations of higher plasma levels of LPEs in rats with severe induced-liver injuries [15, 16]. Therefore, it is also possible that the high LPE levels may be an indicator of disease severity in STB patients. In addition, previous experimental studies have shown that LPEs can serve as immune modulators that can stimulate the activation of multiple immune cells, including macrophage and natural killer T cell [17, 18]. Similar results were found in a recent study by Lau and colleagues that the higher plasma levels of LPE in sepsis patients significantly correlated with proinflammatory cytokines [19]. Thus an interesting question yet to be answered is whether LPE may be involved in the immunity against tubercle bacilli. Further studies are urgently needed to verify this hypothesis.

Phosphocholines are reservoirs and transporters of fatty acids, phosphate, glycerol, and choline, and are also essential nutrients that maintaining health in adults [20]. In a recent metabolism study, a decrease in PC was noted in plasma samples of pulmonary TB patients [21]. In consistent with previous

observations, we also observed lower abscess levels of PC in STB cases than non-STB cases. A host-pathogen metabolic flux model revealed that tubercle bacilli are able to consume PC on mycobacterial growth, thereby resulting in its decrease during infection [22]. Therefore this altered metabolism in PC may be the results of consuming nutrients by intracellular bacteria. In addition to energy source, PC participate in the innate immunity to fight intracellular bacteria [23]. Exogenous application of PC inhibits pro-inflammatory signaling in macrophages, thereby facilitating the survival of mycobacteria [23]. In view of these findings, it is exciting to speculate whether their decrease in abscess specimens is actively regulated by host immune cells, thereby resulting in induction of MTB killing. The change in expression pattern of genes involving in PC metabolism in MTB-infected lesions is of importance to elucidate the molecular mechanism of PC against intracellular pathogen.

In addition, we also found that triacylglycerols, another major source of carbon and energy for MTB, were substantially decreased in abscess specimens from STB patients. This decrease may reflect the energy wasting in patients with STB, as noted in PC. A recent study on MTB by Daniel et al demonstrated that the bacteria could use host triacylglycerol to acquire a dormancy-like phenotype in macrophage [24]. According to this model, the decreased level of triacylglycerol served as an effective indicator for formulating dormant, non-replicating tubercle bacilli in lesions. In this aspect, the decreased triacylglycerol may be repurposed for energy storage in tubercle bacilli, thus improving their survival under hypoxia stress (Fig. 5). Further studies are also needed to identify these fatty acids that function in the lipid metabolism to MTB survival within host niches.

We also acknowledged several limitations to the present study. First, Due to the low recovery rate of pathogen from abscess specimens, only four patients with confirmed etiology were included in the control group. The small sample size may weaken the overall significance of our study. Second, although our method offered great sensitivity and specificity to differentiate STB from non-STB cases, the biomarkers require further validation using a separate larger sample cohort. Finally, we observed the varying regulation of lipid profiles between two different disease groups; however, its underlying molecular mechanism remains unclear. Despite these limitations, our results firstly provide important foundation for facilitating the diagnosis of STB patients using a panel of differential lipids.

Conclusions

To conclude, our data firstly characterize the lipid signatures of abscess specimens from STB patients. The LPEs are significantly upregulated in the STB cases than those in non-STB control, whereas phosphocholines and triacylglycerol are markedly downregulated in the STB cases. The panel of five lipid biomarkers exhibits great capacity for differential diagnosis of STB and non-TB cases. Further studies are required for validating the performance of this novel diagnostic panel in a separate larger sample cohort.

Abbreviations

TB: Tuberculosis

EPTB: extrapulmonary tuberculosis

STB: skeletal tuberculosis

MTBE: methyl-tert-butyl ether

QC: quality control

LC–MS: Liquid Chromatography / Mass Spectrometry Instruments

HPLC:High Performance Liquid Chromatography

UHPLC: Ultra High Performance Liquid Chromatography

ESI: electrospray ionization

VIP: variable influence on projection

FC: Fold Change

PE: phosphatidylethanolamine

PI: phosphatidylinositol

PC: phosphatidylcholines

TG: triacylglycerols

LPE: lysophosphatidylethanolamine

LPI: lysophatidylinositols

LPC: lysophosphatidylcholines

Declarations

Availability of data and materials

All data underlying the findings are fully available.

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Contributions

YX, FMH and YYS performed all experiments. JF and SBQ performed the surgery and provided specimens. QL, YX, JF and YP participated in data analysis, carried out sequence alignments. QL, YX, JF and YP provided helpful discussions. QL and YP drafted the manuscript. JF and YP designed the project. YP revised the manuscript. All authors read and approved the final manuscript.

Ethics declarations

Ethics approval and consent to participate

Animal studies were carried out under protocols approved by the Capital Medical University Institutional Animal Care and Use Committee.

Consent for publication

Informed consent for publication was obtained from all participants.

Potential conflicts of interest

The authors declare no conflict of interest regarding the publication of this paper.

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Tables

Table 1. Demographic and clinical characteristics of clinical cases

Characteristics	STB	Non-STB
Total	10(100.00)	4(100.00)
Sex		
Male	9(90.00)	3(75.00)
Female	1(10.00)	1(25.00)
Age, years	40.90±5.77	49.75±12.7
HIV	0(0.00)	0(0.00)
Complication		
Osteoporosis	4(40.00)	2(50.00)
Liver injury	6(60.00)	3(75.00)
Hyperuricemia	5(50.00)	1(25.00)
Anemia	4(40.00)	2(50.00)
Malnutrition	2(20.00)	3(75.00)
Dyslipidemia	1(10.00)	0(0.00)

Figures

Figure 1

QC sample ion mode mass spectrum basepeak overlay spectrum The LC-MS/MS basepeak mass spectrum of the QC sample, the response intensity and retention time of each chromatographic peak basically overlap.

Figure 2

Metabolites volcano plot and heatmap of significantly different metabolites A. Volcano plot of differential lipids classification of the STB group and the non-STB group. The abscissa is a FC; the ordinate is the p value of one-way analysis of variance (ANOVA). Lipids with p value <0.05 obtained by t-test and FC $\geq$ 1.5 verified by Fold Change Analysis were identified as significantly differential metabolites. Colored plots indicate upward trend and downward trend of substances, and gray plots indicate that they are not statistically significant. B. Heatmap of significantly different metabolites in STB and non-STB

samples. Cells in each row represent individual samples. Red and blue color indicate increased and decreased levels, respectively.

Figure 3

Area under curve (AUC) of biomarkers Receiver operating characteristic (ROC) curves and boxplots of peak intensity distribution showing diagnostic ability of lipid biomarkers with top five AUC values. (A-E) ROC curves for individual biomarkers; (F) ROC curve for the diagnostic panel consisting of the 5-biomarker panel.

Pathway Enrichment Analysis

Figure 4

Statistics of KEGG substance enrichment The ordinate represents the enriched pathway, and the abscissa represents the rich factor. The results show significant enrichment in glycerophospholipid metabolism (red color).

Figure 5

Pathway of glycerophospholipid and triacylglycerols metabolic The infection of tubercle bacilli within bone and surrounding tissue triggers the formulation of granulomas lesions, thus resulting in a hypoxic environment devoid of nutrients. The glycerophospholipids and triacylglycerols of host cells are repurposed by MTB to biosynthesize the intracellular lipid essential for survival and dormancy.

Supplementary Files

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