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Integrated semi-targeted metabolomics analysis reveals distinct metabolic dysregulation in pleural effusion caused by tuberculosis and malignancy

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ABSTRACT

Background: Tuberculous pleural effusion (TPE) and malignant pleural effusion (MPE) are the 2 most frequent causes of exudative pleural effusions (PEs). However, the clinical differentiation is challenging.

Methods: Metabolic signatures in pleural effusion from 156 patients were profiled. An integrated semi-targeted metabolomics platform was incorporated for high throughput metabolite identification and quantitation. In this platform, orbitrap based mass spectrometry with data dependent MS/MS acquisition was applied in the analysis. In-house database containing ~1000 MS/MS spectra were established and "MetaInt" was developed for metabolite alignment.

Results: Using this strategy, lower levels of amino acids, citric acid cycle intermediates and free fatty acids accompanied with elevated acyl-carnitines and bile acids were observed, demonstrating increased energy expenditure caused by TPE. Kynurenine pathway from tryptophan was significantly enhanced in TPE. The ratio of tryptophan/kynurenine exhibited decent performance in differentiating TPE from MPE with sensitivity of 92.7% and specificity of 86.1%. After two further independent validations, it turns out that the ratio of tryptophan/kynurenine can be applied confidently as a potential biomarker together with adenosine deaminase (ADA) for clinical diagnosis of TPE.

Conclusions: Conclusively, the integrated in-house platform for high throughput semi-targeted metabolomics analysis reliably identified great potential of tryptophan/kynurenine ratio as a novel diagnostic biomarker to distinguish pleural effusion caused by tuberculosis and malignancy.

1. Introduction

Tuberculous pleural effusion (TPE) and malignant pleural effusion (MPE) are the 2 most frequent causes of exudative pleural effusions (PEs) and the clinical differentiation is challenging [1–3]. Detection of *Mycobacterium tuberculosis* (*M. tuberculosis*) in the PE by culture or molecular methods shows poor sensitivities due to the paucity of organisms [4–6]. Besides, cytological test also shows high false-negative results for MPEs [7,8]. Surgical intervention is reported to be accurate for both TB and neoplasm but is invasive and not widely available [9]. Biomarkers like adenosine deaminase (ADA) and carcinoembryonic antigen (CEA) are often used but the performance seems to be variable. Other biomarkers specific for these two diseases have been investigated by immunological, proteomic and transcriptomic approaches [10–14]. However, metabolomic changes between TPE and MPE are not clear.

Nuclear magnetic resonance (NMR) spectroscopy [15–18] and mass spectrometry (MS) [19–23] are 2 major techniques for metabolic analysis. In recent years, mass spectrometry (MS) is widely used in metabolomic analysis. Mainstream of metabolomics has untargeted and targeted analysis. Untargeted screening is the major technique for biomarker discovery and molecular mechanism investigation. Coupling of chromatography techniques enables analysis of complicated samples due to the separation prior to mass spectrometry [24–26]. Untargeted metabolomic analysis using liquid chromatography-mass spectrometry (LC-MS) enables to cover wide range of metabolites with various chemical properties in a single approach; thereby allowing to acquire utmost metabolite information systematically.

Untargeted metabolomics analysis requires metabolite

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identification, which is one of the major bottlenecks and rate-limiting step in the entire analysis. Various analysis platforms with different algorisms were developed for high throughput metabolomics analysis [27–29]. However, most of them incorporate online database, which is not compatible with high throughput metabolite identification, especially with MS/MS confirmation.

2. Materials and methods

2.1. Chemicals and materials

Water, methanol and acetonitrile used in the experiment were HPLC grade were from Fisher Scientific. Ammonium acetate, formic acid and ammonium hydroxide were from Sigma Aldrich.

2.2. Study population and collection of pleural effusion samples

Inpatients aged ≥ 18 y with evidence of exudative pleural effusion demonstrated by X-ray and biochemical tests according to the criteria of Light et al. [30], and who agreed to participate in the study were enrolled from June 2013 to May 2016 at Beijing Chest Hospital, Beijing, China. The study was approved by the Ethical and Institutional Review Boards for Human Investigation of the Beijing Chest Hospital. Patients were divided into two groups: TPE group and MPE group. TPE was confirmed according to the definition of tuberculous pleurisy diagnosis previously published[2], while MPE was confirmed by pathological diagnosis. At least 2 ml of PE were collected from each patient for ADA assay and metabolomic analysis. ADA were assayed within 4 h after PE collection. PE samples for metabolomic analysis were centrifuged at 3000 × *g* for 10 min. The supernatants were stored at − 80 °C until metabolite extraction.

2.3. Adenosine deaminase assay

Adenosine deaminase (ADA) activity was determined using supernatant from 1 ml of PE by adenosine deaminase assay kit (Beijing Strong Biotechnologies, Beijing, China) according to the manufacturer's instructions. The most widely accepted cut-off value 40 U/l was used for TP diagnosis.

2.4. Metabolite extraction

Pre-chilled HPLC grade methanol was added to PE samples upto 80% (v/v). Samples were centrifuged at 14000 g for 30 min and supernatants were collected after 2 h storage in − 80 °C freezer. Later on, supernatants were dried and re-dissolved using 60uL of 80% methanol. After centrifugation (14,000 × *g*, 20 min), supernatants were transferred to sample vials for LC-MS/MS analysis.

2.5. Semi-targeted metabolomics profiling

Q Exactive mass spectrometer (Thermo, CA) was used for semi-targeted metabolite screening. Data dependent acquisition was applied in positive and negative ion mode separately. 95% and 50% of acetonitrile with 5 mM ammonium formate and 0.1% formic acid was used as mobile phases A and B for Atlantis HILIC Silica column (2.1 mm × 100 mm) in positive mode. In negative ion mode, 95% and 50% of acetonitrile with 5 mmol/l ammonium acetate at pH 9.0 was prepared as mobile phases for BEH Amide column (2.1 mm × 100 mm). A total of 25 min gradient was used for efficient separation. DDMS2 mode was performed with MS scan at 70,000 resolution and subsequent 10 MS/MS scans at 17,500 resolution. ESI voltages of 3.5 and 2.5 kV were used for positive and negative ion mode respectively. Mass range of *m/z* 70–1050 was applied for positive ion mode data collection, while *m/z* 80–1200 for negative mode. Stepped NCE with 30 + 50% was utilized for fragmentation.

2.6. Metabolite identification

Tracefinder 3.2 (Thermo Fisher) was applied for metabolite identification with in-house database. MS/MS raw spectra from either standards or biological samples were stored as reference in Library Manager in DB format. The confirmation of MS/MS spectra from non-standards relied on METLIN (www.metlin.scripps.edu) and HMDB (www.HMDB.ca). The in-house library contained ~1000 MS/MS spectra. A database including compound name, chemical formula, and precursor mass in comma separated values file was required for database search using Tracefinder. The software was able to implement metabolite assignment by comparing the acquired MS/MS spectra with references in Library on the basis of precursor match from database. Using this approach, 2 levels of identification were displayed in the results: one was confirmed by MS/MS spectra and the other was assigned as tentative candidates based on accurate mass. For metabolites having MS/MS confirmation, only the ones with score over 30 were considered as confident identification. Isotopic distribution and chromatographic peak shape were applied as filters to eliminate unreliable candidates. After filtering out false positives, both confirmed metabolites and potential candidates were combined for statistical analysis.

2.7. MetaInt data processing

MetaInt was developed in Python programming environment, which is compatible with output results of Tracefinder 3.2. Tracefinder 3.2 exported identified metabolites in excel format for each sample but not integrated. MetaInt was able to align and integrated the same metabolite in all samples based on compound names. The highest library score would be displayed as the score for metabolite identification. If any misidentified metabolite having obvious shift of elution time (> 0.2 min) in any sample, it would be highlighted for further confirmation. Output results in excel format can be used for further statistical analysis.

2.8. Pathway and statistical analysis

Metabolites identified from positive and negative mode were merged after normalization for analysis. Principle component analysis (PCA) and orthogonal partial least squares discriminate analysis (OPLS-DA) were performed using SIMCA 14 (Umetrics). Spearman rank correlation results, heatmaps and student's *t*-test results were acquired from Metaboanalyst 3.0 (www.metaboanalyst.ca). Missing value was replaced by half of the minimum value in data. Data was normalized and log transformed before analysis. Unequal group variance was selected for t-test in Metaboanalyst. Receiver operating characteristic curve (ROC) was achieved using SPSS software. The Human Metabolome Database (www.HMDB.ca), Kyoto Encyclopedia of Genes and Genomes (www.KEGG.jp) and Metaboanalyst (www.metaboanalyst.ca) were used for pathway analysis.

3. Results

3.1. Characteristics of pleural effusion samples

Pleural effusion samples were collected from 156 patients with tuberculous pleurisy or malignant pleurisy. The clinical features of these patients were shown in the Table S1. All patients were HIV negative. There were 113 (72.4%) males, and the average age was 46.2 ± 20.6 y. Pleural effusion samples were collected at three time points from 2013 to 2016. The second batch (2014-batch) of samples (51 TPEs and 20 MPEs) collected in 2014 was analyzed for semi-targeted metabolic profiling. Biomarker validation was carried out using the other two batches: 2013-batch with 13 TPEs/13 MPEs and 2016-batch having 51 TPEs/8 MPEs, collected retrospectively in 2013 and prospectively 2016, respectively. Meanwhile, the effect of sample