Combined Application of NMR- and GC-MS-Based Metabonomics Yields a Superior Urinary Biomarker Panel for Bipolar Disorder

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Bipolar disorder (BD) is a debilitating mental disorder that cannot be diagnosed by objective laboratory-based modalities. Our previous studies have independently used nuclear magnetic resonance (NMR)-based and gas chromatography-mass spectrometry (GC-MS)-based metabonomic methods to characterize the urinary metabolic profiles of BD subjects and healthy controls (HC). However, the combined application of NMR spectroscopy and GC-MS may identify a more comprehensive metabolite panel than any single metabonomic platform alone. Therefore, here we applied a dual platform (NMR spectroscopy and GC-MS) that generated a panel of five metabolite biomarkers for BD-four GC-MS-derived metabolites and one NMR-derived metabolite. This composite biomarker panel could effectively discriminate BD subjects from HC, achieving an area under receiver operating characteristic curve (AUC) values of 0.974 in a training set and 0.964 in a test set. Moreover, the diagnostic performance of this panel was significantly superior to the previous single platform-derived metabolite panels. Thus, the urinary biomarker panel identified here shows promise as an effective diagnostic tool for BD. These findings also demonstrate the complementary nature of NMR spectroscopy and GC-MS for metabonomic analysis, suggesting that the combination of NMR spectroscopy and GC-MS can identify a more comprehensive metabolite panel than applying each platform in isolation.

Bipolar disorder (BD) is one of the top ten most disabling disorders in working age adults and affects up to 1% of the general population1,2. Due to the lack of objective diagnostic modalities, the diagnosis of BD still relies on the subjective identification of symptomatic clusters3. However, the clinical symptoms of BD are considerably complex and diverse, resulting in a high rate of underdiagnosis and misdiagnosis that contributes to increased suicide risk and poorer prognosis4. Given these facts, there is an urgent need to identify objective laboratory-based diagnostic biomarkers for BD.

Metabonomics – the comprehensive analysis of low-molecular-weight endogenous metabolites in a biological sample – has been widely applied to capture the metabolic changes in various disease states5. Currently, there are three major analytical techniques that are suited for non-targeted metabonomic mapping: nuclear magnetic resonance (NMR) spectroscopy, gas chromatography-mass spectrometry (GC-MS), and liquid chromatography-mass spectrometry (LC-MS)6–8. Each of these analytical techniques has its advocates and possess their own unique features. An increasing number of researchers have applied these techniques to identify diagnostic biomarkers for neuropsychiatric disorders, including stroke, multiple sclerosis, schizophrenia, and autism9–11. Using NMR and GC-MS, our group has successfully identified several potential metabolite biomarkers in the plasma and urine of major depressive disorder (MDD) patients, which could effectively distinguish depressed subjects from healthy controls12–14.

With regards to BD, previous metabonomic studies have used a NMR metabonomic platform to identify differential metabolites in post-mortem brain tissue and plasma17–18. Meanwhile, in our group, Zheng et al. employed NMR to identify four potential urinary metabolite biomarkers, and Xu et al. employed GC-MS to identify 2,4-dihydroxyxypyrimidine as a potential urinary metabolite biomarker for diagnosing BD19–20. These
previous metabonomic studies have been helpful in developing objective laboratory-based testing for BD while providing valuable data on the physiopathologic mechanism(s) of BD. However, one limitation shared by all these studies was that the researchers only used one metabolomic platform. Irrespective of the unique advantages of any particular methodology, no single metabolomic platform can provide adequate coverage of the entire human metabolome in any given biological sample. Previous studies have demonstrated that the use of multiple metabolomics platforms and technologies allowed us to identify several previously unknown urine metabolites and to substantially enhance the level of metabolome coverage. Therefore, the combined application of NMR spectroscopy and GC-MS may identify a more comprehensive metabolite panel than any single metabolomic platform alone.

Here, in order to investigate the complementary nature of NMR spectroscopy and GC-MS for metabonomic analysis, a novel urinary metabolite panel for diagnosing BD was constructed using a dual platform approach (NMR spectroscopy and GC-MS). The diagnostic performance of the current composite biomarker panel was then comparatively assessed against the previous single platform-derived metabolite panels.

Results

Univariate analysis. Prior to analysis, data was scaled to unit variance. We did univariate analysis using all subjects to find metabolites that would be worthy of further analysis ($p < 0.10$), which identified 67 different metabolites (18 NMR-derived and 49 GC-MS-derived metabolites) from 94 metabolites. These 94 differential metabolites including the four metabolites biomarkers (choline, N-methylnicotinamide, $\alpha$-hydroxybutyrate, isobutyrate) identified by NMR and one metabolite (2,4-dihydroxypyrimidine) identified by GC/MS were included in this study. The 94 metabolites were described in supplementary Table S1. And a typical NMR and GC-MS spectrum was described in supplementary figure S1.

OPLS-DA model. OPLS-DA analysis was carried out to explore the metabolic differences between BD subjects and healthy controls. The 67 differential metabolites were used to perform OPLS-DA analysis. In the training set, the score plots of the OPLS-DA model showed that the BD subjects were obviously separated from healthy controls with little overlap ($R^2_X$ cum = 0.36, $R^2_Y$ cum = 0.66, $Q^2$ = 0.57; Figure 1a). The values of those parameters quantifying OPLS-DA model were positive, demonstrating a robust metabolic difference between BD subjects and healthy controls. Furthermore, the permutation test showed the constructed OPLS-DA model was positive and valid (Figure 1b).

In the test set, 28 BD subjects and 48 healthy controls were used to independently validate the diagnostic performance of the OPLS-DA model. Consequentially, samples were correctly predicted by the OPLS-DA model, yielding a predictive accuracy of 96.1% (Figure 1c, d).

Differential metabolites. The coefficient loading plots of the OPLS-DA model identified 33 differential metabolites ($|r| > 0.301$).

Figure 1 | Metabonomic analysis of urine samples. (a) Orthogonal partial least-squares discriminant analysis (OPLS-DA) score plots showing a clear discrimination between BD subjects (red box) and healthy controls (black box) in the training set. (b) Permutation test showing the original $R^2$ and $Q^2$ values (top right) as significantly higher than the corresponding permuted values (bottom left), demonstrating the OPLS-DA model’s robustness. (c) OPLS-DA model was used to predict the healthy controls (green box) from the test set. (d) OPLS-DA model was used to predict the BD subjects (blue box) from the test set.
Figure 2 | Identification and validation of urinary metabolite panel. Akaike information criterion (AIC) of each model was presented. The current model constructed with five urinary metabolites (2,4-dihydroxyxypirimidine, azelaic acid, β-alanine, pseudouridine, and α-hydroxybutyrate) showed the highest predictive ability. Receiver operating characteristic (ROC) curve analysis was performed to evaluate the diagnostic performance of this five-biomarker panel. Area under the curve (AUC) values of the training set, test set, and whole set were 0.974, 0.964, and 0.960, respectively.
Combined platform panel versus single platform panel. All BD subjects were used to compare the diagnostic efficacy of the panel found by the combined analytical platforms with the panels found by each single analytical platform. The two cut-off values were from our previous studies. Finally, we obtained the following results: (i) 22 and 19 samples were wrongly predicted by NMR-derived panel and GC-MS-derived panel, respectively; (ii) 12 samples were wrongly predicted by the combined panel; (iii) 10/13 and 9/10 samples were wrongly predicted by the NMR-derived panel and the GC-MS-derived panel, respectively, but were correctly predicted by the combined panel; (iv) 6/9 samples were wrongly predicted both by the NMR-derived panel and the GC-MS-derived panel were also wrongly predicted by the combined panel; and (v) 37/39 samples were correctly predicted by the NMR-derived panel and the GC-MS-derived panel were also correctly predicted by the combined panel.

We also compared the panel derived in this work (β-alanine, 2,4-dihydroxyxypyrimidine, azelaic acid, pseudouridine, and α-hydroxybutyrate) with a composite panel directly generated by the five biomarkers identified in our previous studies. The specificity of the two panels were comparable (current panel 91.3% versus previous panel 90.5%), but the sensitivity of the current panel was higher (current panel 83.1% versus previous panel 71.8%).

Discussion

BD is a common and debilitating mental disorder. However, there is still no empirical laboratory-based test for BD to facilitate its diagnosis. Over the past few years, our research group has focused on addressing this issue. Zheng et al. has found four potential metabolite biomarkers by a NMR-based analytical platform, and Xu et al. has found one metabolite biomarker by a GC-MS-based analytical platform. However, due to the diverse physicochemical properties and biomarkers by a NMR-based analytical platform, and Xu et al. targeting the more complex chemical profile of BD.

Xu et al. reported that increased oxidative stress is associated with the pathophysiology of BD, the significantly decreased level of 2,4-dihydroxyxypyrimidine implies that disturbance of glutamine was involved in the onset of BD. In agreement with this speculation, many previous metabonomic analyses of post-mortem brain tissue and plasma obtained from BD patients have also observed significant alterations in glutamine levels.

Additionally, β-alanine, azelaic acid, and pseudouridine were identified as metabolite biomarker here. Xu et al. reported that only azelaic acid and pseudouridine, but not β-alanine, were differential metabolites between BD subjects and healthy controls. Meanwhile, Zheng et al. identified choline, isotubrurate and N-methyl-nicotinamide, none of which were included in the current work. Considering the higher accuracy of the current panel, these results show the advantage of a dual platform approach in detecting the sometimes subtle metabolic differences between experimental groups. Therefore, the combined use of these two techniques is recommended for future metabonomic studies to better enable the discovery of novel diagnostic biomarkers.

When we only used the GC/MS metabolites to identify potential metabolite biomarker panel, seven metabolites (24-dihydroxyxypyrimidine, aminoethanol, arabitol, RS23dihydrobutanoicacid, phenylalanine, azelaic acid, β-alanine) were included into the potential diagnostic model. And the discrimination power of this model was comparable to the model consisting of the four GC/MS metabolites (β-alanine, 2,4-dihydroxyxypyrimidine, azelaic acid, and pseudouridine) in the current biomarker panel. Meanwhile, the sensitivity, specificity and AUC of the four GC/MS metabolites were all lower than that of the current biomarker panel. These results suggested that the combined application of NMR- and GC-MS-Based metabolomics could identify the most typical and representative metabolite panel than applying each platform in isolation.

To analyze the biological functions of these identified potential metabolite biomarkers, the pathway analysis was performed by online software MetaboAnalyze. These metabolites were mainly involved in four metabolic pathways (Supplementary Table S2). About these metabolic pathways, previous study has found that genetically determined aberrations in pyrimidine metabolism were associated clinically with various degrees of mental retardation and/or unexpected and often devastating neurological dysfunction. Pantothenate and CoA biosynthesis was found to be unique to major depression in a study exploring thalamic transcriptome screening in three psychiatric states, but, in the current work, the results indicated that it might be affected in patients with BD. Beta-alanine appeared to act via glycine and GABA (A) receptors (both inhibitory neurotransmitters) with comparable efficacy to glycine and GABA themselves. And, expression of GABA (A) receptors was altered significantly in the lateral cerebellum of subjects with BD. In a study listing potential targets for novel therapeutics for BD, one of the suggested targets was a glutamate propionic acid receptor, which is part of the propionate metabolism pathway.

A few limitations of this study should be addressed here. First, a relatively small sample size of non-medicated BD subjects was recruited. Larger samples are needed to determine the influences of drugs upon these identified biomarkers. Second, these biomarkers were confirmed solely by comparing BD patients with healthy controls. Therefore, it was unknown whether or not these biomarkers can be effectively used to discriminate BD from other psychiatric disorders that can present in a similar fashion, such as depression and schizophrenia. Third, future studies should collect cerebrospinal fluid from BD patients to ensure that these urinary biomarkers are physiologically relevant to disease pathogenesis. Finally, although a dual platform approach was used here, these biomarkers still require verification through additional metabonomic methods such as LC-MS.
In conclusion, using a dual platform metabonomic approach, a novel composite urinary metabolite biomarker panel for diagnosing BD was identified. This panel, consisting of one metabolite detected by NMR (α-hydroxybutyrate) and four metabolites detected by GC-MS (2, 4-dihydroxypyrimidine, azelaic acid, β-alanine, and pseudouridine), could differentiate BD subjects from healthy controls with a higher accuracy than our previous single platform-derived metabolite biomarker panels. Thus, the urinary biomarker panel identified here shows promise as an effective diagnostic tool for BD. These findings also demonstrate the complementary nature of NMR spectroscopy and GC-MS for metabonomic analysis, suggesting that the combination of NMR spectroscopy and GC-MS can identify a more comprehensive metabolite panel than applying each platform in isolation.

**Methods**

**Subjects & sample collection.** Prior to sample collection, written informed consents were obtained from all recruited subjects. The protocols of this study were reviewed and approved by the Ethical Committee of Chongqing Medical University. The methods were carried out in accordance with the approved guidelines and regulations. BD subjects were recruited from the psychiatric center of the First Affiliated Hospital at Chongqing Medical University. The inclusion criteria of healthy controls were: (i) no DSM-IV Axis I/Axis II disorder; (ii) no current or previous lifetime history of neurological disease; (iii) no systemic examination center of the First Affiliated Hospital at Chongqing Medical University. The inclusion criteria of healthy controls were: (i) no DSM-IV Axis I/Axis II disorder; (ii) no current or previous lifetime history of neurological disease; (iii) no systemic medical illness; and (iv) provision of written informed consents. In total, 126 healthy controls were recruited. Demographic and clinical characteristics of BD subjects and controls were described in Table 2.

Prior to moving forward with the use of potential biomarkers identified in clinical studies, the use of independent samples to validate the biomarkers is essential. Therefore, the 71 BD subjects and demographically-matched 126 healthy controls were segregated into a training set (~60%) and a test set (~40%). The training set, including 43 BD subjects and 78 demographically-matched healthy controls, was used to identify potential urinary biomarkers, and the remaining samples in the test set were used to independently validate the diagnostic performance of these biomarkers.

After overnight fasting, morning urine samples were collected in a sterile cup and transferred into a sterile tube. Urine samples were then centrifuged at 1500 g for 10 min. The resulting supernatant was immediately divided into equal aliquots and stored at −80°C.

**NMR acquisition.** Prior to NMR analysis, urine samples were thawed and centrifuged at 1500 g for 10 min to remove precipitation. To ensure stabilization of urinary pH, 500 μl of urine was mixed with 100 μl of phosphate buffer (90% D2O, 1 mM 3-trimethylsilyl-1-[2, 2, 3, 3, 3-H4] propionate (TSP), and 3 mM sodium azide; pH 7.4). After centrifugation at 12000 rpm for 10 min, 500 μl samples of supernatant were transferred into 5 mm NMR tubes. The proton spectra of the urine samples were collected on a Bruker Avance 600 spectrometer operating at a 600.13 MHz 1H frequency with a standard 1-dimensional (1D) pulse sequence. Typically, 64 transients were collected into 16 K data points with a spectral width of 8000 Hz, an acquisition time of 0.945 s, and a relaxation delay of 2 s. Prior to Fourier transformation, the free induction decay (FID) was zero-filled and multiplied by an exponential function corresponding to a line-broadening factor of 0.3 Hz in the frequency domain. Urine resonance assignments were performed according to previous literature and NMR database.

**GC-MS acquisition.** The procedure for GC-MS preparation was performed according to our previous study (15). Briefly, a 15 μl aliquot of urine was vortexed after adding 10 μl internal standard solution (L-leucine-13C6, 0.02 mg/ml). Then, 15 μl urease was added into this mixture solution. The urea was degraded for 60 min at 37°C. The mixture was extracted with 240 μl of ice-cold methanol and then 80 μl of ice-cold methanol. After vortexing for 30 s, the mixture was centrifuged at 14000 rpm for 5 min at 4°C. The 224 μl supernatant was transferred to a glass vial and vacuum-dried at room temperature. The dried metabolic extract was derivatized with 30 μl of methoxyamine (20 mg/ml) for 1.5 h at 37°C. Subsequently, 30 μl of BSTFA with 1% TCMS was added into the mixture and heated for 1 h at 70°C, forming trimethylsilyl (TMS) derivatives. After derivatization and cooling to room temperature, 1.0 μl of this derivative was injected into the GC/MS for analysis. GC/MS analysis was carried out according to this group’s previously published work.

**Data analysis.** The overall workflow of identifying a simplified set of urinary metabolite biomarkers for BD is summarized in Figure 3. SIMCA-P + 12.0 and SPSS 19.0 were used for all analysis. Spectral data from NMR and GC-MS was collected and unit variance scaled. The data resulting in a p-value less than 0.10 in univariate analysis were then used to perform a multivariate analysis. Orthogonal partial least-squares discriminant analysis (OPLS-DA) was applied to the unit variance-scaled spectral data to visualize discrimination between BD subjects and healthy controls. Three parameters (R’X, R’Y and Q’Y), calculated by the default leave-one-out (LOO) validation method, were employed to evaluate the model performance. The overall accuracy of the model was determined using the receiver operating characteristic (ROC) curve analysis and the area under the curve (AUC). AUC values of 0.70 to 0.99 are considered highly accurate, while values of 0.60 to 0.69 are regarded as having acceptable diagnostic accuracy.
procedure, were used to describe the quality of OPLS-DA model. A 99-iteration permutation test was performed to rule out non-randomness of separation between groups. The metabolites with variable importance (VIP) values of no less than 1 (equivalent to a p-value of less than 0.5) and a correlation coefficient of |r| > 0.301 (equivalent to a p-value of less than 0.5) were identified as the differential metabolites responsible for sample differentiation and then entered into a multivariate logistic-regression model. A forward selection method was used to obtain a model in which all data had a p-value of less than 0.01. In order to make the clinical practice more feasible and convenient, a step-wise optimization algorithm based on Akaike’s information criterion (AIC) was employed to optimize the metabolite biomarker combination. A receiver-operating characteristic (ROC) curve analysis was used to further evaluate the diagnostic performance of this simplified set of BD biomarkers in the training and test sets. Given the biological reproducibility observed in the independent training and test sets, we repeated the ROC analysis using all subjects to increase the statistical power.

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