Metabolomic Identification of Exosome-Derived Biomarkers for Schizophrenia: A Large Multicenter Study

Yang Du¹, Lei Chen², Xue-Song Li³, Xiao-Lin Li³, Xiang-Dong Xu⁴, Shao-Bin Tai⁵, Geng-Lin Yang⁴, Quan Tang², Hua Liu¹, Shu-Han Liu², Shu-Yao Zhang², and Yong Cheng^{*,1,2,6,✉}

¹Key Laboratory of Ethnomedicine of Ministry of Education, Center on Translational Neuroscience, School of Pharmacy, Minzu University of China, Beijing, China; ²College of Life and Environmental Sciences, Minzu University of China, Beijing, China; ³Department of Psychiatry, The Third People's Hospital of Foshan, Foshan, Guangdong, China; ⁴Department of Psychiatry, Urumqi Fourth People's Hospital, Urumqi, Xinjiang, China; ⁵Department of Psychiatry, Huangshan Second People's Hospital, Huangshan, Anhui, China; ⁶NHC Key Laboratory of Birth Defect Research, Prevention, and Treatment, Hunan Provincial Maternal and Child Healthcare Hospital, Changsha, Hunan, China

*To whom correspondence should be addressed; 27 South Zhongguancun Avenue, Beijing 100081, China; tel: 86-10-68931383, fax: 86-10-68936927, e-mail: yongcheng@muc.edu.cn

Exosomes have been suggested as promising targets for the diagnosis and treatment of neurological diseases, including schizophrenia (SCZ), but the potential role of exosome-derived metabolites in these diseases was rarely studied. Using ultra-performance liquid chromatography-tandem mass spectrometry, we performed the first metabolomic study of serum-derived exosomes from patients with SCZ. Our sample comprised 385 patients and 332 healthy controls recruited from 3 clinical centers and 4 independent cohorts. We identified 25 perturbed metabolites in patients that can be used to classify samples from patients and control participants with 95.7% accuracy (95% CI: 92.6%–98.9%) in the training samples (78 patients and 66 controls). These metabolites also showed good to excellent performance in differentiating between patients and controls in the 3 test sets of participants, with accuracies 91.0% (95% CI: 85.7%–96.3%; 107 patients and 62 controls), 82.7% (95% CI: 77.6%–87.9%; 104 patients and 142 controls), and 99.0% (95% CI: 97.7%–100%; 96 patients and 62 controls), respectively. Bioinformatic analysis suggested that these metabolites were enriched in pathways implicated in SCZ, such as glycerophospholipid metabolism. Taken together, our findings support a role for exosomal metabolite dysregulation in the pathophysiology of SCZ and indicate a strong potential for exosome-derived metabolites to inform the diagnosis of SCZ.

Key words: exosome/schizophrenia/metabolomics/biomarker

Introduction

Schizophrenia (SCZ) is a severe neuropsychiatric disease that affects approximately 1% of the population

worldwide¹ and is characterized by positive symptoms (delusions and hallucinations), negative symptoms (apathy and social withdrawal), and cognitive impairment.^{2,3} Currently, diagnosis relies solely on a somewhat lengthy subjective evaluation by a clinician. Because earlier identification and treatment of SCZ are known to improve clinical outcomes, the last 2 decades have seen rapidly growing interest in identifying molecules associated with SCZ, in the hope of finding biomarkers that could inform an early and objective diagnosis.⁴ Researchers have applied “omics” methods, including proteomics and metabolomics, to the search for SCZ biomarkers, leading to several candidate molecules being proposed.^{5,6} However, an objective and reliable diagnosis of SCZ based on biomarkers remains elusive, and achieving a better understanding of the etiology of SCZ and subsequently developing new biomarkers for the diagnosis and prognosis of this disease remain important goals.

Exosomes are small vesicles with a typical diameter of 50–150 nm, which are widely present in the blood, urine, and cerebrospinal fluid.⁷ They are released into circulation from multiple cell types and reach both distant and neighboring cells, where their contents of nucleic acids, proteins, and lipids regulate the phenotypes of the recipients.⁸ The roles of exosome-derived microRNAs (miRNAs) in intercellular communications have attracted great attention in recent years, and they have been found to regulate physiological functions and the pathological processes of many diseases, including angiogenesis, inflammation, aging, and major depression.^{9–11} Moreover, blood exosomal miRNAs are reportedly promising biomarkers for various diseases, such as breast cancer,¹² atherosclerosis,¹³ and SCZ.¹⁴ More recently, metabolomic

data have suggested that exosome-associated metabolites can potentially serve as biomarkers for the diagnosis of pancreatic cancer¹⁵ and premalignant liver disease.¹⁶ However, until now, this “omics” method has not been applied in the investigation of exosomal metabolite aberrations in neuropsychiatric diseases.

Here, we used ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) to study the exosome metabolomic profile of patients with SCZ and healthy control (HC) participants. We recruited 4 sets of participants from 3 clinical centers to provide 1 training set for predictor discovery and 3 test sets for class prediction. We found a cluster of 25 exosome-derived metabolites with good to excellent performance in differentiating patients with SCZ from HCs in all 4 participant sets.

Materials and Methods

The training participant set included 78 patients with SCZ and 66 HCs, recruited from Huangshan Second People's Hospital. The first test set included 107 patients with SCZ and 62 HCs, recruited from Urumqi Fourth People's Hospital. The second test set included 104 patients with SCZ and 142 HCs, from the Third People's Hospital of Foshan. The third test set included 96 patients with SCZ and 62 HCs, also from the Third People's Hospital of Foshan. All patients with SCZ had been diagnosed by experienced psychiatrists according to the International Classification of Diseases-10 and the Structured Clinical Interview of DSM-IV. The patients' psychiatric symptoms were evaluated according to the Positive and Negative Syndrome Scale (PANSS). Exclusions were any medical illness, including cancer, hypertension, diabetes, mental retardation, autoimmune diseases, substance abuse, and infectious diseases. Furthermore, the HC subjects were recruited from similar communities from the patients with SCZ. The demographic and clinical characteristics of the study participants are presented in [supplementary table 1](#).

All participants or their relatives signed an informed consent form. The research plan was reviewed and approved by the Ethics Committee of Huangshan Second People's Hospital, the Ethics Committee of the Third People's Hospital of Foshan, and the Ethics Committee of Urumqi Fourth People's Hospital. The experiments were conducted in accordance with the Declaration of Helsinki.

Exosome Isolation and Validation

We collected blood samples from patients and HCs after overnight fasting. The samples were allowed to clot at room temperature for 1 h and the serum obtained by centrifugation at 3000g for 10 min. Serum exosomes were isolated on a qEV column according to the manufacturer's

protocol (Izon). Then, the exosomes were concentrated at 10 000g for 60 min using a protein concentrator based on a polyethersulfone membrane having a 30-kDa molecular-weight cutoff (Vivaspin). The concentrated exosomes were resuspended in 200 μ l of phosphate-buffered saline for further analysis. The exosome validation methods such as negative-staining electron microscopy, nanoparticle tracking analysis, and Western blotting were applied, as described in our recent article.¹⁴

Metabolite Measurements

Widely targeted metabolomics of blood exosome samples from patients and HCs were acquired using an UPLC (Shim-pack UFLC SHIMADZU CBM30A system) and tandem mass spectrometer (MS/MS) (4500 QTRAP; Applied Biosystems) equipment. Qualitative analysis of the first- and second-order mass spectra employed a public database of metabolite information and metabolomics data curation environment (MetWare). Multiple-reaction monitoring in triple quadrupole mass spectrometry was used for the quantitation of metabolites. The detailed assay procedure, including quality control, is described in a recent article.¹⁷ The metabolomics data are presented in [supplementary tables 2–5](#).

Bioinformatics Analysis

To understand the biological functions of the differentially expressed metabolites, they were annotated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (the major public database on metabolic pathways), then mapped to the KEGG pathway database by the MetaboAnalyst software.¹⁸ Significant enrichments were defined as pathways with $P < .05$.

To further explore the biological function, metabolite-gene interaction analysis was performed on 264 SCZ risk genes identified by genome-wide association studies and the differentially expressed metabolites identified here, using MetScape,¹⁹ a Cytoscape plug-in.

Statistical Analysis

Unsupervised principal components analysis (PCA) was performed by the statistical function *prcomp* in R. Based on the detected metabolites, an orthogonal partial least-squares-discriminant analysis (OPLS-DA) model was constructed to assess differentially expressed metabolites, and variable importance in projection (VIP) was extracted from this model.¹⁷ Differentially expressed metabolites were defined as $VIP > 1.5^{20}$ and $P < .05$ by a Mann–Whitney *U* test. The potential of blood exosomal metabolites to discriminate between patients with SCZ and HCs was evaluated in terms of a receiver operating characteristic (ROC) curve generated by the SPSS 22.0 statistical analysis software.²¹

Results

Differential Expression of Serum Exosomal Metabolites in the Training Set

We isolated serum exosomes from patients with SCZ and controls (supplementary figure 1), and the nanoparticle tracking analysis showed that the size and number of serum exosomes from patients with SCZ and controls were not significantly different (supplementary figure 2). We then used UPLC-MS/MS to analyze differentially expressed metabolites in serum exosomes between patients with SCZ and controls. Our widely targeted UPLC-MS/MS analysis detected hundreds of metabolites, including phospholipids, amino acids, organic acids, and lipid oxidation products. Plots of PCA scores showed a separation of metabolite profiles between patients and controls (figure 1A), suggesting the presence of an SCZ signature in the blood exosomal metabolite concentrations. To identify exosomal metabolites differentially expressed between SCZ patients and controls, an OPLS-DA model was constructed (figure 1B), which identified 25

metabolites by the criteria: VIP score > 1.5 and $P < .05$ (figures 1C and 1D). Of these, 10 were upregulated and 15 were downregulated (figures 1C and 1D). Significant KEGG pathway enrichment was found for the differentially expressed metabolites in pathways related to glycerophospholipid metabolism and the biosynthesis of phenylalanine, tyrosine, and tryptophan (figure 1E).

Exosomal Metabolites as Biomarkers for SCZ

To investigate whether the 25 differentially expressed exosomal metabolites that we had identified could serve as biomarkers for differentiating between patients with SCZ and controls, we initially used the training set as the test set. An ROC curve (figure 2A) yielded a sensitivity of 85.9 %, a specificity of 95.5%, and an area under the curve (AUC) of 95.7 % (95% CI = 92.6%–98.9%), suggesting that blood exosomal metabolites had potential as biomarkers for SCZ.

To validate these results, we applied the 25 metabolites to 3 additional test sets for class prediction. The first test set included 107 patients with SCZ and 62 HCs and

Fig. 1. Bioinformatic screening for blood exosomal metabolites differentially expressed in schizophrenia (SCZ). (A) Principal components analysis (PCA) and (B) an orthogonal partial least squares-discriminant analysis (OPLS-DA) model diagram based on the metabolites detected in the training participant set. (C) Volcano plot displaying metabolite differences between patients with SCZ and healthy controls (HCs) for the training participant set. (D) Illustration of the 25 differentially expressed blood exosomal metabolites in 78 patients with SCZ compared with 66 HCs. (E) KEGG enrichment pathways for the 25 differentially expressed metabolites.

Fig. 2. Biomarker performance for the set of 25 metabolites differentially expressed in schizophrenia (SCZ). Receiver operating characteristic (ROC) curves showing the accuracy of the metabolites in discriminating between patients with SCZ and HCs in (A) the training participant set, (B) the first test participant set, (C) the second test participant set, and (D) the third test participant set.

yielded a sensitivity of 86.9%, a specificity of 87.1%, and an AUC of 91.0% (95% CI = 85.7%–96.3%) (figure 2B). The second test set included 104 patients with SCZ and 142 HCs and yielded a sensitivity of 73.1%, a specificity of 80.3%, and an AUC of 82.7% (95% CI = 77.6%–87.9%) (figure 2C). The third test set included 96 patients with SCZ and 62 HCs and yielded a sensitivity of 97.9%, a specificity of 95.2%, and an AUC of 99.0% (95% CI = 97.7%–100%) (figure 2D). These results demonstrated that blood

exosomal metabolites had good to excellent performance in differentiating between patients with SCZ and HCs.

We then used the multi-correlation coefficient analysis to assess the relationships of the 25 metabolites to potential confounders, including age and disease severity. The results suggested that age was not a confounding factor for the levels of the 25 metabolites (supplementary figure 3). Additionally, the 25 metabolites were not significantly associated with the PANSS total

score, PANSS positive score, and PANSS negative score in the training set and the first test set ([supplementary figure 3](#)). However, the 25 metabolites showed a significant association with the PANSS negative score in both the second and third test sets ([supplementary figure 3](#)).

Biomarker Performance Stratified by Medication Status and Sex

We next investigated whether these biomarker results were influenced by the atypical antipsychotics prescribed to patients with SCZ. Because the second and third test sets included a large proportion of first-episode, drug-free patients, we tested the performance of the 25 metabolites in differentiating between first-episode, drug-free patients with SCZ and HCs. In subgroups drawn from the second test set, which included 40 first-episode, drug-free patients with SCZ and 142 HCs, these metabolites yielded a sensitivity of 77.5%, a specificity of 85.9%, and an AUC of 86.4% (95% CI = 80.3%–92.6%) ([figure 3A](#)). In subgroups drawn from the third test set, which included 49 first-episode, drug-free patients with SCZ and 62 HCs, the 25 metabolites yielded a sensitivity of 100%, a specificity of 100%, and an AUC of 100% (95% CI = 100%–100%) ([figure 3B](#)). These results demonstrated that the biomarker performance of the 25 metabolites was not reduced in unmedicated patients with SCZ.

We then tested whether sex affected the differentiation accuracy of the 25 metabolites, drawing ROC curves separately for female and male participants. Analyses of all 4 sets of participants revealed that these metabolites had good to excellent performance in discriminating between female or male patients with SCZ and their respective controls ([supplementary table 6](#)).

The participants were all Han Chinese except in the first test set, which included 36 Uyghur Chinese patients. The blood-exosome biomarker panel also showed excellent performance in discriminating between these patients and HCs, yielding a sensitivity of 92.9%, a specificity of 88.7%, and an AUC of 93.7% (95% CI = 88.9%–98.6%) ([figure 3C](#)).

We further analyzed whether there were metabolites from serum exosomes to differentiate between first-episode, drug-free, and chronically treated patients with SCZ. We identified 6 metabolites ([supplementary figure 4](#)) which had VIP score > 1.5 in both the second and third test sets. The 6 metabolites distinguished these subsets of SCZ patients, with 73.8% accuracy for the second test set and 100% for the third test set ([supplementary figure 4](#)).

Metabolite-Gene Molecular Network in SCZ

To further explore the biological roles of the differentially expressed exosomal metabolites in the pathophysiology of SCZ, we constructed a metabolite-gene molecular network specific to SCZ. From among the 264 SCZ risk genes reported by genome-wide association studies and the differentially expressed blood exosomal metabolites reported by the present study, 38 genes and 8 metabolites were found in the database. Analysis of these suggested that 4 of the risk genes (nitric oxide synthase 1 [*NOS1*], dipeptidase 2 [*DPEP2*], 5-aminolevulinic acid synthase 1 [*ALAS1*], and glutamic acid decarboxylase 1 [*GADI1*]) and 2 of the differentially expressed metabolites (L-arginine and taurine) were functionally connected ([figure 4](#)).

Discussion

Our metabolomics data demonstrated a profile of differentially expressed metabolites in the blood exosomes

Fig. 3. Biomarker performance of exosomal metabolites stratified by medication status and race. Receiver operating characteristic (ROC) curves showing the accuracy of the differentially expressed metabolites in discriminating between first-episode, drug-free patients with schizophrenia (SCZ) and healthy controls (HCs) in (A) the second test participant set and (B) the third test participant set. (C) Evaluation of the accuracy of the 25 metabolites in discriminating between Uyghur Chinese patients with SCZ and HCs in the first test participant set.

of patients vs HCs, and 25 differentially expressed metabolites showed excellent performance in distinguishing patients with SCZ from HCs. The potential of the differentially expressed metabolite cluster as a biomarker for SCZ was validated in 3 additional test sets of participants from 2 other clinical centers. Further analysis suggested that the unique SCZ signatures were not due to medication effects. Sex did not affect the classification accuracy of the biomarkers. Bioinformatics analysis suggested that the 25 differentially expressed metabolites were significantly enriched in the pathways related to phenylalanine, tyrosine, and tryptophan biosynthesis and glycerophospholipid metabolism. Taken together, data from the present study revealed for the first time the dysregulation of blood exosomal metabolite contents in patients with SCZ and provided novel insights into the roles of metabolites in SCZ pathogenesis and diagnosis.

The membrane phospholipid hypothesis of SCZ, which postulates membrane phospholipid dysfunction as a biochemical basis for the neurodevelopmental concept of SCZ, was proposed by Horrobin in 1998.²² The initial rationale was the discovery of the critical roles that phospholipids play in normal brain development and in adult brain functioning, and the discovery of aberrations in essential fatty acid levels in the peripheral blood of patients with SCZ.^{22,23} Individuals at ultra-high risk of psychosis were later found to have the dysregulation of essential fatty acid metabolism.⁵ Here, we found that several fatty acids were downregulated in the blood exosomes of patients with SCZ, consistent with a recent postmortem study

showing decreased glycerophospholipid metabolism in the prefrontal cortex of patients with SCZ.²⁴ Moreover, the differentially expressed metabolites that we found here included 2 products of lipid peroxidation that were upregulated in patients. These results agree with previous reports of increased malondialdehyde levels in the blood of patients with SCZ,^{25,26} suggesting that lipid peroxidation plays a role in SCZ pathogenesis. The potential role of phospholipid metabolism in SCZ was further supported by a recent preclinical study, which showed that a deficiency of 2 phospholipids—arachidonic acid and docosahexaenoic acid—during the early neurodevelopmental period caused SCZ-like phenotypes in adult mice through the epigenetic regulation of nuclear receptor genes.²⁷ Moreover, studies by Pawelczyk et al^{28,29} suggested that dietary supplementation with *n*-3 polyunsaturated fatty acid had beneficial effects in patients with SCZ. Interestingly, Amminger et al³⁰ demonstrated that dietary supplementation with *n*-3 polyunsaturated fatty acids reduced the rate of progression to first-episode psychosis in individuals at ultra-high risk of psychotic disorder. Taken together, these results indicate that disturbances of lipid metabolism are critical for the onset and/or development of SCZ, and more investigations into lipid metabolism as a therapeutic target for SCZ are warranted.

In addition to the dysregulation of lipid metabolism, the present study found upregulation of the amino acid L-arginine in patients with SCZ, in agreement with 2 previous reports that showed elevated plasma L-arginine levels in patients with SCZ,^{31,32} but inconsistent with

Fig. 4. Molecular network associations. The network associations among 2 differentially expressed metabolites found here (L-arginine and taurine) and 4 previously known schizophrenia risk genes.

the findings of He et al.³³ Our metabolite-gene network analysis showed that L-arginine is related to 4 SCZ risk genes, *NOS1*, *DPEP2*, *ALAS1*, and *GADI*, and that taurine is related to *GADI*. The metabolite-gene connection in SCZ is supported by reports of altered NO metabolism in patients with SCZ,³⁴ it being well known that NO metabolism impacts synaptic transmission and vesicular release.³⁵ Furthermore, abundant evidence from postmortem studies shows that *GADI* is downregulated in multiple regions of the SCZ brain, including the prefrontal and medial temporal cortex, at both transcriptional and translational levels.³⁶ Since approximately 80%–90% of brain GABA is synthesized by the *GADI* enzyme, the altered expression of *GADI* was considered at least in part to contribute to the GABAergic neuron dysfunction found in SCZ.³⁶ However, the reasons for the functional involvement of *DPEP2* and *ALAS1* in SCZ are unclear; further studies are necessary to better understand the metabolite-gene molecular network in SCZ.

The field of blood-biomarker discovery for neurological diseases has exploded over the last 2 decades,⁴ due to the easy accessibility of blood samples and the influence of the “peripheral as a window on the brain” hypothesis.³⁷ Although these studies so far have not resulted in breakthroughs such as international acceptance of blood biomarkers capable of informing diagnosis, prognosis, or treatment response, potential blood biomarkers for SCZ have been proposed. These potential biomarkers implicate processes such as inflammatory response, neurotrophin deficit, and epigenetic regulation in the etiology of SCZ.^{14,38,39} In recent years, the field has grown to encompass metabolomics approaches to biomarker discovery in neuropsychiatric disease. Although several studies have found differentially expressed metabolites in the serum or plasma of patients with SCZ, findings have been largely inconsistent across studies, as summarized by Davison et al.⁵ in a systematic review. Most of these studies had a small sample size and did not test the accuracy of the metabolites as biomarkers for SCZ. Therefore, the usefulness of these metabolites for the diagnosis of SCZ has been uncertain. Nevertheless, a few studies have identified metabolite biomarker panels that differentiate between SCZ patients and controls with reasonable to excellent performance. However, these results were limited by sample size and/or single-center data.^{20,33,40} In the present study, we discovered a panel of biomarkers consisting of 25 blood exosomal metabolites that could discriminate between patients with SCZ and HCs. The strength of our study is that this panel showed good to excellent performance consistently across 4 independent cohorts from 3 clinical centers, located in the Eastern, Central, and Western parts of China. One possible reason for this consistency was the large sample size of our study. It is also very likely that exosomal metabolites reflect the pathophysiology of the brain, since it is

known that exosomes cross the blood-brain barrier easily. Furthermore, our analysis demonstrated that the SCZ signature of the biomarker panel was present at the early stages of the disease and prior to the initiation of antipsychotic treatment, suggesting that this set of 25 blood exosomal metabolites can potentially aid in the diagnosis of SCZ.

A limitation of this study was that some potential confounding factors were not considered in this study, such as smoking status and socioeconomic status, which may affect the levels of blood exosomal metabolites. An example is that the disease severity may have moderating effects on the 25 metabolites in blood exosomes of patients with SCZ. Another limitation of this study was the ethnic homogeneity of the participants, who were mostly Han Chinese, although we also obtained data with a relatively small sample size on Uyghur Chinese patients with SCZ, which also suggested the reliability of the biomarker panel. However, due to the multifactorial nature of SCZ etiology, there is increasing recognition that the integration of different biomarkers reflective of the various etiological pathways relevant to SCZ should lead to better diagnosis. Our previous study showed that blood exosomal miRNAs had approximately 90% (training samples) and 75% (testing samples) accuracies in differentiating between patients with SCZ and controls.¹⁴ It is likely that a combination of miRNAs and metabolites from blood exosomes could improve the diagnostic accuracy for SCZ. The limitation of this study was that only a small proportion of samples were overlapping between the 2 studies, and the usefulness of combination of miRNAs and metabolites to diagnose SCZ is unclear. Therefore, more studies are needed, hopefully with international cooperation, to translate our findings on blood exosomal metabolite biomarkers into benefits for patients with SCZ.

Supplementary Material

Supplementary material is available at *Schizophrenia Bulletin*.

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The authors have no biomedical financial interests or potential conflicts of interest to declare.

Authors Contributions

Y.C. conceived and designed the study; X.-S.L., X.-L.L., X.-D.X., S.-B.T., and G.-L.Y. diagnosed the patients and collected the samples; Y.D., L.C., Q.T., and H.L. performed the experiments; Y.D., S.-H.L., and S.-Y.Z. did statistical analyses; all the authors analyzed and interpreted the data. Y.C. drafted the manuscript with critical revisions from all the authors.

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