


Monitoring tyrosine kinase inhibitor therapeutic responses with a panel of metabolic biomarkers in chronic myeloid leukemia patients

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The aim of this study is to investigate the potential biomarkers associated with chronic myeloid leukemia (CML), reveal the metabolite changes related to the continuous phases of tyrosine kinase inhibitors (TKIs), and find the potential biomarkers associated with treatment effects. Fifty-two patients with CML and 26 matched healthy people were enrolled as the discovery set. Another 194 randomly selected CML patients treated with TKI were chosen as the external validation set. Plasma samples from the patients and controls were profiled using the gas chromatography-mass spectrometry-based metabolomic approach. Multivariate and univariate statistical analyses were combined to select the differential metabolic features. The gas chromatography-mass spectrometry-based metabolomics showed a clear clustering and separation of metabolic patterns from healthy controls and pre- and post-TKI treatment CML patients in the discovery set. We identified 9 metabolites that differentiated CML patients from healthy controls, including lactic acid, isoleucine, glycerol, glycine, myristic acid, D-sorbitol, D-galactose, D-glucose, and myo-inositol. Among the 9 markers, glycerol and myristic acid had the most significant association with TKI treatment effects in both discovery and external validation sets. In the receiver operating characteristic analysis, the combination of glycerol and myristic acid showed a better discrimination performance compared to a single biomarker. The results indicated that metabolic profiling has the potential for diagnosis of CML and the panel of biomarkers including myristic acid and glycerol could be useful in monitoring TKI therapeutic responses.

KEYWORDS

chronic phase, gas chromatography-mass spectrometry, leukemia, metabolomics, myeloid

Bingyu Yang and Chang Wang contributed equally to this work.

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1 | INTRODUCTION

Chronic myeloid leukemia (CML) is a clonal hemopoietic stem cell disorder characterized by a reciprocal translocation between the long arms of chromosomes 9 and 22 (Ph-chromosome),¹ which results in the expression of the BCR-ABL fusion protein, with deregulated tyrosine kinase activity. The introduction of BCR-ABL tyrosine kinase inhibitors (TKIs) has set the current paradigm for the treatment of CML.² Meanwhile, the response to TKI therapy is determined by the measurement of hematologic, cytogenetic, and molecular responses.³ However, some patients show resistance to TKI therapy, resulting in therapeutic failure. In recent years, new TKIs with stronger efficacy have been gradually applied in clinical practice (such as second-generation nilotinib and third-generation ponatinib).⁴ Unfortunately, new TKIs are still hindered by drug resistance. Hence, biomarkers indicating CML pathogenic processes or responses to therapeutic interventions are required.

Metabonomics aims to measure the global dynamic metabolic response of living systems to biological stimuli or genetic manipulation and focuses on understanding systemic change over time in complex multicellular systems.⁵ Carcinoma development and treatment inevitably affect metabolic processes. As a result, the alterations in metabolic levels in biofluids indicate the important information in a disease-specific manner for use as diagnostic markers or therapeutic targets.⁶ The above fact has made metabolomics a useful tool in the identification of disease biomarkers,⁷ revelation of pathogenesis,⁸ and the treatment of disease.⁹ So far, little is known about the pathogenesis of CML and the impact of TKI treatment on the metabolome of CML patients.

In this study, we investigated the metabolic profiles of healthy controls (HC), newly diagnosed CML patients (ND), and the patients treated with TKIs using the untargeted gas chromatography-mass spectrometry (GC-MS) technique. Nine metabolites that differentiated CML patients from healthy controls were identified. Two of 9 biomarkers had the most significant association with TKI treatment effects in both discovery and external validation sets.

2 | MATERIALS AND METHODS

2.1 | Selection of patients and sample preservation

Fifty-two patients with CML and 26 matched healthy people as controls were enrolled in this study from March 2013 to April 2015. In

total, the data from 104 plasma samples (52 plasma samples from 26 patients at diagnosis and post-TKI treatment, 26 plasma samples from another 26 patients post-TKI treatment, and 26 plasma samples from healthy controls) were used for subsequent data treatment and statistical evaluation. Information about the patients involved in the study is summarized Table S1. The clinical diagnoses of CML in chronic phase (absolute neutrophil count $<1.0 \times 10^9/L$, and/or platelets $<50 \times 10^9/L$) and bone marrow reports from all patients were obtained from the First Affiliated Hospital of Soochow University (Suzhou, China). A total 52 post-TKI CML patients were separated into 2 groups according to the therapeutic effect: those sensitive to TKI treatment (SCML) ($n = 26$, with optimal curative effect); and those resistant to TKI treatment (RCML) ($n = 26$, with therapeutic failure). Another 194 post-TKI subjects (112 SCML and 82 RCML) were selected randomly from the First Affiliated Hospital of Soochow University to independently validate defined plasma therapeutic biomarkers. The response to the treatment was assessed according to the recent recommendations of European LeukemiaNet experts.¹⁰ Diet, weight, and levels of physical activity remained unchanged during TKI therapy. Patients with 1 or more confounding factors, such as pre-existing physical or mental disorders, medication, and/or illicit drug use, were also excluded from the study. To minimize the disturbance factors in metabonomics, each group was basically matched in age, sex, and body mass index. Moreover, the SCML and RCML groups from the discovery set were also matched in the drugs they received, to minimize differences in the drugs' metabolism. Imatinib was the initial medication for all patients. Considering some RCML patients included in this study were using second-line TKI (nilotinib) to improve better efficacy, we only selected cases of nilotinib application for more than 12 months to obtain stable plasma drug concentrations of nilotinib and eliminate the interference of imatinib in metabolism.

The study protocol was approved by the Faculty Hospital Ethics Committee at the First Affiliated Hospital of Soochow University in accordance with the guidelines in the Declaration of Helsinki. All subjects gave their written informed consent before the start of this study.

All clinical venous blood samples were collected with EDTA anti-coagulation tubes in the morning and centrifuged at 1500 g for 15 minutes. Each plasma sample was divided into equal aliquots and stored at -80°C until analysis.

2.2 | Chemicals

Acetonitrile (HPLC grade) and n-heptane were purchased from Tedia (Fairfield, OH, USA). 2,4-Dichlorobenzoic acid (internal standard), methoxamine, N-methyl-N-trimethyl-silyl trifluoroacetamide with 1% trimethylchlorosilane, and pyridine were obtained from Sigma-Aldrich (St. Louis, MO, USA). Standards for metabolite identification were from Sigma-Aldrich and JK Chemical.

2.3 | Plasma sample preparations

The plasma samples were prepared as previously reported,¹¹ with a few modifications. The plasma samples were thawed and 100 μ L aliquots were mixed with 400 μ L cold acetonitrile. Subsequently, 20 mg/mL 2,4-dichlorobenzoic acid (internal standard) was added. After vortexing for 2 minutes, the sample was centrifuged for 15 minutes to precipitate the protein (25152 g, 4°C). The supernatant was transferred into a new tube and dried with a vacuum (Martin Christ, Osterode, Germany). The dried samples were then redissolved with methoxyamine pyridine solution (15 mg/mL, 50 μ L) and ultrasound-treated for 15 minutes at room temperature. Afterwards, the sample was oximated in a water bath at 70°C for 1 h, followed by silylation reaction with 50 μ L N-methyl-N-trimethyl-silyl trifluoroacetamide in a water bath at 40°C for 60 minutes. Finally, the derivatized sample was centrifuged (25152 g, 15 minutes) and the supernatant was analyzed by GC-MS.

The quality control (QC) sample was prepared by equally mixing aliquots of plasma samples from all patients and controls to evaluate the stability of the GC-MS analytical system.

2.4 | Gas chromatography-mass spectrometry

Metabolic profiling of plasma samples was acquired using an Agilent 7890/5975C GC-MS (Agilent Technologies, USA). Each derivatized sample (1 μ L) was injected into a DB-5 fused silica capillary column (30 m \times 0.25 mm \times 0.25 μ m; J&W Scientific, Folsom, CA, USA) with a split ratio of 10:1. The temperature program was as follows: the initial column temperature of 80°C was maintained for 5 minutes, then increased to 170°C at 5°C/min, followed by an increase to 300°C at 10°C/min, and maintained for 5 minutes. The injection temperature was 300°C. The temperatures of the inlet and ion source were set at 280 and 230°C, respectively. High-purity helium (99.9996%) was used as the carrier gas, with a constant flow rate of 1.1 mL/min. The data were acquired in a full scan with 30-600 *m/z*. Plasma samples were running at random and the QC sample was injected every 10 samples for evaluation.

Identification of metabolites in plasma was carried out by library search: NIST (<http://www.nist.gov/srd>), Wiley (<http://onlinelibrary.wiley.com/>) and Fiehn (<http://fiehnlab.ucdavis.edu/>), retention index, and confirmation of authentic standard.

2.5 | Data processing and analysis

The metabolic peak extraction, detection, and alignment referred to 1 previous report.¹² The response areas of the metabolites were finally normalized to 2,4-dichlorobenzoic acid (internal standard).

Partial least squares discriminant analysis (PLS-DA) filtered by orthogonal signal correction using SIMCA version P13.0 (Umetrics, Umea, Sweden) was chosen to establish a multivariable data model for classification of different groups. Metabolites responsible for the classification were picked out according to the variable importance for project values (VIP > 1). In addition, the Mann-Whitney *U*-test (SPSS 23; IBM, Armonk, NY, USA) was carried out to investigate the statistical significance in different groups. The discriminatory capability of potential biomarkers was evaluated by the receiver operating characteristic (ROC) analysis. Finally, false discovery rate (FDR) correction was carried out using the Benjamini-Hochberg method ($q < 0.10$) was applied to all data analyses performing multiple comparisons to reduce the false positive rate.

3 | RESULTS

3.1 | Global metabolomic profiling of patients and controls

To check the data quality, 7 QC samples were used to trace the variability during the analytical sequence. In the GC-MS analysis, the distributions of relative standard deviation in QC samples indicated that 89% and 97.25% of the peaks were lower than 15% and 30%, respectively (Figure S1). Accordingly, most of the metabolic features showed better stability and reproducibility in the GC-MS profiles analysis. Typical total ion chromatograms of samples from patients with CML and healthy individuals are shown in Figure S2. Through comparison with available library databases or standard samples, 45 compounds were identified, including amino acids, organic acids, carbohydrates, and fatty acids.

To better visualize the similarities and differences among these complex datasets, pattern recognition analysis was used to classify the metabolic phenotypes and identify the differentiating metabolites. Initially, the principal component analysis (PCA) model was used to overview the metabolic differences among HC, ND, and post-TKI (including SCML and RCML) groups. In the score plot, each point represents a sample, and the distance between different data-points reflects the degree of metabolic distinction. The PCA score plot showed a partial differentiation with overlap in different groups (Figure S3). To eliminate the unrelated information for the grouping discrimination, a further supervised analysis using PLS-DA with a preprocessing technique of orthogonal signal correction was carried out. In accordance with the score plot of plasma in the PLS-DA model, distinct separation was observed among 3 groups (HC, ND, and post-TKI groups) (Figure 1A). Figure 1B shows a clear separation in the 4 groups, which suggested a markedly different plasma

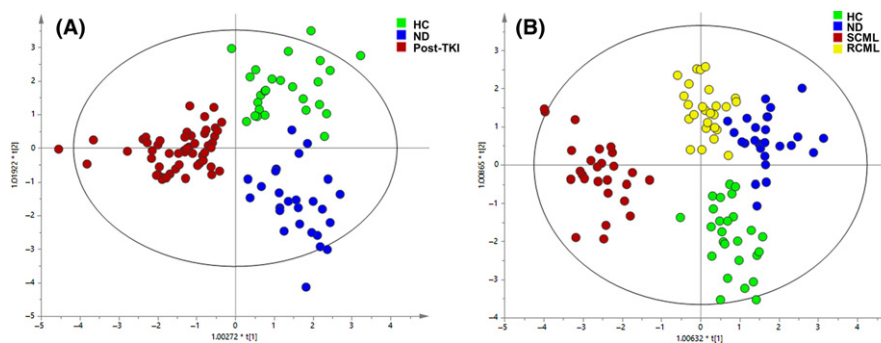


FIGURE 1 Score plot of the partial least squares discriminant analysis model for classification of responses to tyrosine kinase inhibitor (TKI) therapy in chronic myeloid leukemia (CML) patients. A, Healthy controls (HC) and newly diagnosed CML patients (ND), and CML patients after TKI therapy. B, Global metabolic profiles of HC and ND groups, and CML patients sensitive (SCML) or resistant (RCML) to TKI therapy

metabolome among different groups. The SCML and RCML groups were also classified clearly, indicating that there were some differences in the mechanisms of their therapeutic effects on CML. In addition, the SCML group was clearly deviated from the ND group, whereas minimal separation between the RCML and ND group was observed.

3.2 | Metabolic profiles of CML patients at diagnosis were different from healthy controls

To discover the potential diagnostic biomarkers, the same PLS-DA model was constructed to investigate the metabolic changes between HC and ND. Score scatter plots for the PLS-DA model showed a clear differentiation of HC and ND groups (Figure 2A), suggesting that the plasma metabolic profiles of the 2 groups were significantly different. In this model, R^2 describes how well the data could be reproduced by the training model and Q^2 provides an estimation of the predictive ability of the model, indicating that the model had good interpretative ability and predictive ability. To verify the PLS-DA model, a permutation test was carried out with 200 random permutations generated intercepts of R^2 and Q^2 , which were significantly lower than corresponding original values. The negative value of Q^2 on the vertical axis (Figure 2B) indicated that the

goodness of fit of the data and the model had good predictive ability and were reliable. Potential biomarkers were determined by referring to VIP values. The VIP value reflects the influence of every variable on the classification. Variables with a VIP value >1.0 had an above average influence on the explanation of the Y matrix and were considered primary contributors for the classification of the groups.

To evaluate the significance of the difference in concentration between variables identified by these methods in tested groups, metabolites with $VIP > 1$ were tested using the Mann-Whitney U -test and FDR correction ($P < .05$ and $FDR < 0.10$). A total of 9 metabolites were tentatively identified as potential biomarkers for the diagnosis of CML, as shown in Table 1 (all $VIP > 1$, $P < .05$, $FDR < 0.10$).

3.3 | Metabolic perturbations of CML after TKI treatment

To evaluate the metabolic perturbation related to the response to TKI treatment, the plasma profiles of the pre-TKI group (ND) and post-TKI group, including both optimal response (SCML) and treatment failure (RCML), were compared. Two separate PLS-DA models were used to study the changes of these 9 diagnostic biomarkers pre- and post-TKI treatment in SCML and RCML groups.

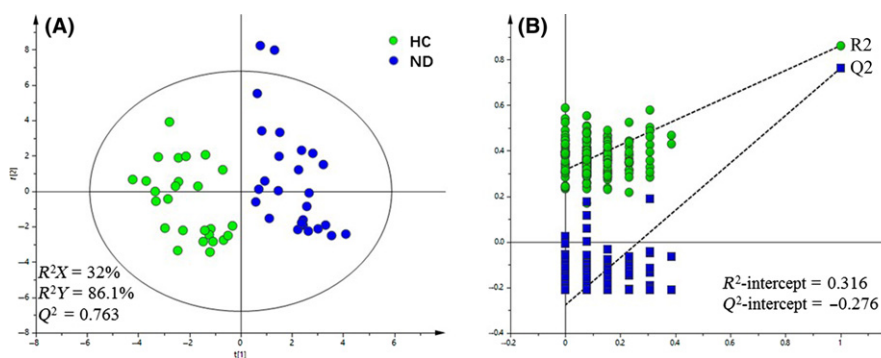


FIGURE 2 Pattern recognition in the partial least squares discriminant analysis model for classification of responses to tyrosine kinase inhibitor therapy in chronic myeloid leukemia patients. A, Score plot. HC, healthy controls; ND, newly diagnosed chronic myeloid leukemia patients. B, Results of 200 permutation tests

TABLE 1 Identification of plasma differential metabolites in chronic myeloid leukemia (CML) patients (n = 26)

Metabolite ^a	Retention time (min)	VIP value ^b	P-value*	Adj. P-value**	FC ^c (ND/HC)	Tendency
Lactic acid	7.28	2.91	.001	.003	1.81	↑
Isoleucine	9.63	1.06	.017	.026	1.25	↑
Glycerol	11.46	1.26	<.001	<.001	-0.67	↓
Glycine	11.93	1.18	.012	.021	1.35	↑
Myristic acid	19.80	1.31	.005	.012	-0.86	↓
D-sorbitol	20.24	1.18	.044	.059	1.24	↓
D-galactose	21.22	2.81	.005	.010	1.67	↑
D-glucose	21.52	1.39	<.001	<.001	2.14	↑
Myo-inositol	24.46	1.68	<.001	<.001	2.35	↑

*P-values were calculated from the Mann-Whitney U-test ($P < .05$).

**Adjusted (Adj.) P-value obtained from the false discovery rate correction using the Benjamini-Hochberg method.

^aMetabolites were identified using available library databases and standard samples.

^bVariable importance in the projection (VIP) value was obtained from partial least squares discriminant analysis with a threshold of 1.0.

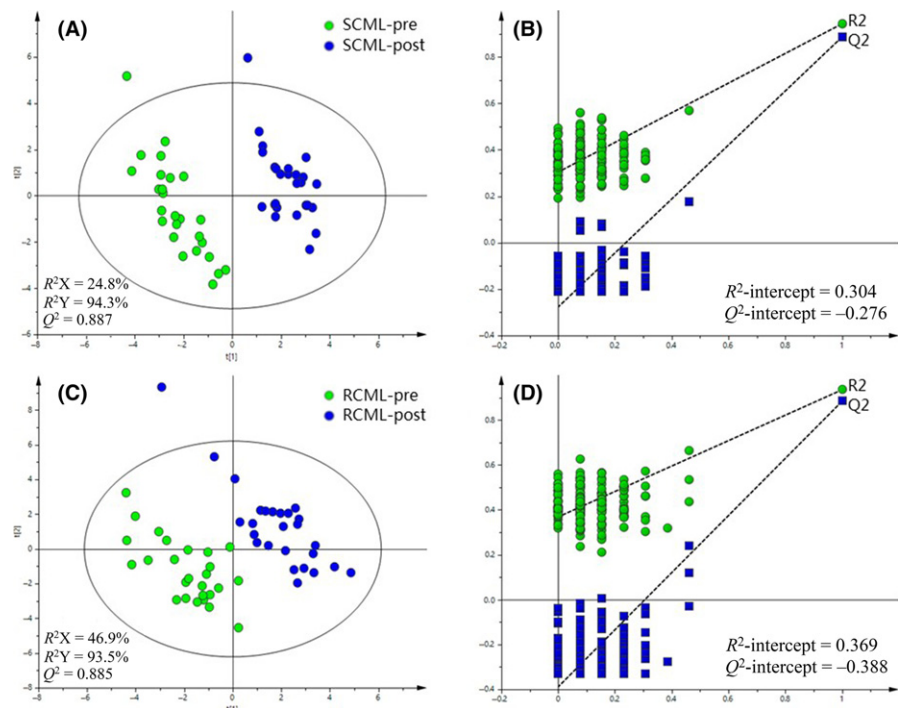
^cFold change (FC) was calculated from the arithmetic mean values of each group. Positive values indicate a relatively higher concentration present in CML patients (ND); negative values indicate a relatively lower concentration as compared to the healthy controls (HC).

The PLS-DA model was used to elucidate the metabolic changes of pre- and post-TKI treatment CML patients in both SCML and RCML groups (Figure 3). A clear separation was observed in the score plot (Figure 3A) with a good fitness and prediction ability. The results of permutation tests with 200 permutations for plasma are shown in Figure 3B, which indicated the model was not over-fitting. The model between pre- and post-TKI treatment in RCML patients (Figure 3C) also illustrated a clear separation obtained between the 2 groups. The permutation results indicated that the model was robust and valid (Figure 3D). Overall,

the results indicated the significant impact of TKI treatment on global metabolism in CML patients.

According to the trends of metabolic alterations pre- and post-TKI treatment in SCML and RCML patients, potential biomarkers of CML could be divided into 2 categories (Table S2): (i) 7 metabolites had similar changes after TKI treatment in SCML and RCML patients, including lactic acid, isoleucine, glycine, D-sorbitol, D-galactose, D-glucose, and myo-inositol; and (ii) myristic acid and glycerol showed opposite changes after TKI treatment in SCML and RCML patients. Those metabolites with opposite trends in the 2 groups and significantly altered in SCML

FIGURE 3 Metabolic profiles of chronic myeloid leukemia (CML) patients pre- and post-treatment with tyrosine kinase inhibitors. Patients are classified as sensitive to therapy (SCML) (A, B) or resistant to therapy (RCML) (C, D) based on pattern recognition in the partial least squares discriminant analysis model



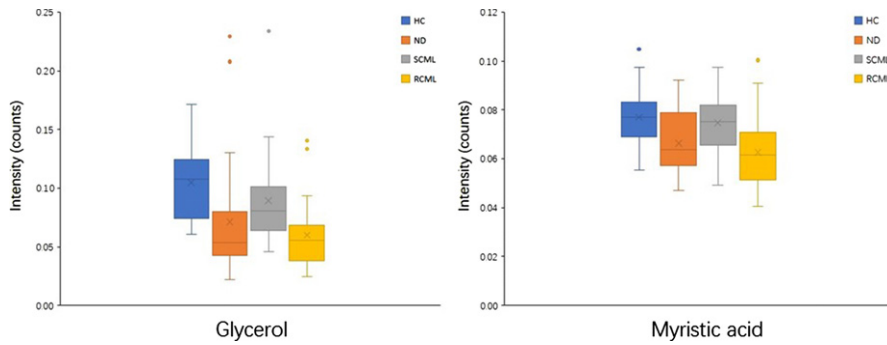


FIGURE 4 Typical variations in levels of 2 metabolites related to therapeutic effect following treatment with tyrosine kinase inhibitors. Treatment groups were healthy controls (HC), newly diagnosed chronic myeloid leukemia (CML) patients (ND), and patients sensitive (SCML) or resistant (RCML) to therapy

were more likely to be associated with therapeutic effects. As illustrated in Figure 4, typical variations in levels of the 2 metabolites related to therapeutic effect showed a tendency toward gradual recovery to the healthy state in SCML patients. In RCML patients, no obvious changes in the levels of these metabolites were observed.

3.4 | Panel of myristic acid and glycerol has potential as therapeutic-related biomarker

An independent set with 194 samples (112 SCML and 82 RCML) was collected and analyzed in the external validation phase to validate the reliability of these 2 metabolites for therapeutic evaluation. First, the statistical significance ($P < .05$) when all SCML subjects were compared with RCML subjects in the validation set was required. Both myristic acid ($P < .001$) and glycerol ($P = .001$) were validated successfully.

Subsequently, the discriminatory power of the 2 candidates was evaluated by the ROC curve analysis. The ROC curve was exploited based on the results of the area under the curve (AUC), sensitivity, and specificity at best cut-off points. In the discovery set, ROC analysis revealed AUC of 0.812 with a sensitivity of 88.5% and specificity of 76.9% for discriminating SCML patients from RCML patients (Figure 5A), showing satisfactory discrimination by the panel of 2 biomarkers. The ROC curve based on the same 2 representative metabolites repeatedly yielded satisfactory results in the external validation set; the AUC, sensitivity, and specificity were 0.758%, 75.9%, and 65.9%, respectively (Figure 5B). Compared with a single

biomarker (glycerol or myristic acid), it was clear that the rational combination of these 2 metabolites achieved better AUC values, sensitivity, and specificity in both the discovery and external validation sets.

4 | DISCUSSION

Metabolic models can provide a framework for analyzing the information-rich omics datasets and are increasingly being used to investigate metabolic alternations in human diseases.¹³ In the current work, a GC-MS-based metabolic profiling method was used to find the metabolic signature of CML patients. Our results showed a clear clustering and separation of metabolic patterns from healthy control and pre- and post-TKI treatment CML patients, suggesting that the metabolic perturbations were evident in the patients, dependent on pathological conditions and treatment interventions. The SCML group was clearly deviated from the ND group, whereas minimal separation between the RCML and ND group was observed, suggesting that after TKI treatment, SCML patients underwent an obvious metabolic perturbation, whereas RCML patients were not significantly affected.

Compared with healthy controls, ND CML patients had higher levels of lactic acid, myo-inositol, carbohydrates (d-galactose and d-glucose), and amino acids (glycine and isoleucine), whereas the levels of fatty acids (myristic acid and glycerol) and D-sorbitol were decreased. We found that CML patients showed increased levels of

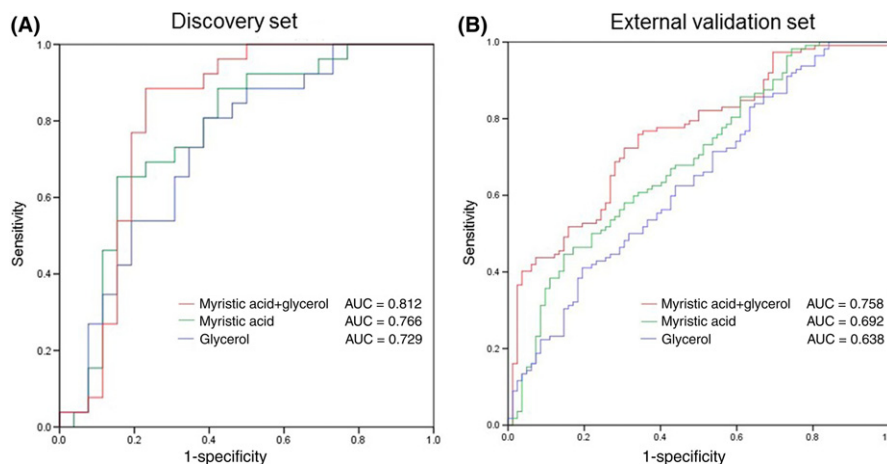


FIGURE 5 Evaluation of the discriminatory powers of individual and combined potential therapeutic biomarkers in chronic myeloid leukemia (CML) patients treated with tyrosine kinase inhibitors. A, Receiver operating characteristic curves using data of 52 patients with CML and 26 matched healthy controls (discovery set). B, Receiver operating characteristic curves using data of 194 randomly selected CML patients treated with tyrosine kinase inhibitors (validation set)

many amino acids in plasma. Specifically, the levels of glycine and isoleucine were significantly higher ($P < .05$). The similar rising tendency of glycine in CML was consistent with a previous study.¹⁴ As a branched-chain amino acid, isoleucine could promote protein synthesis and turnover, signaling pathways, and metabolism of glucose,¹⁵ particularly in the regulation of proteins. Studies have shown that expression of the BCR-ABL kinase in hematopoietic cells stimulates increase in glucose uptake, and high glycolytic activity could be observed in BCR-ABL-positive cells.^{16,17} In conclusion, we found that the intensities of amino acids, D-galactose, D-glucose, and lactic acid were upregulated in newly diagnosed CML patients, which may indicate a cellular requirement for a higher turnover of energy and structural proteins.

After TKI treatment, most of the potential diagnostic metabolites had the tendency towards healthy levels, indicating that TKIs could effectively alter the metabolic profile of CML patients, specifically blocking the metabolic pathways associated with the disease. Among those metabolites, 7 metabolites (including lactic acid, isoleucine, glycine, D-sorbitol, D-galactose, D-glucose, and myo-inositol) showed a similar tendency after TKI treatment in SCML and RCML patients, which were considered to be a result of TKI treatment intervention. Previous studies also reported that imatinib strongly suppresses cytosolic glycolysis and increases tricarboxylic acid cycle intermediates,^{18,19} which was consistent with our experimental results.

Two metabolites, myristic acid and glycerol, might not only be useful in diagnosis, but also in assessment of the TKI therapeutic effect. Myristic acid is a long chain saturated free fatty acid (FFA) and the glycerol backbone is found in all lipids known as triglycerides. Alterations in the fatty acid profiles of plasma lipids are quite common in cancer and have been shown in a variety of neoplastic processes.²⁰ Moreover, glycerolipid (GL)/FFA cycling could regulate several biological processes; disturbances in GL/FFA cycling are strongly associated with the pathogenesis of some cancers.²¹ The downregulated levels of these 2 metabolites indicated that lipolysis was likely to be decelerated under CML status. After TKI treatment, the 2 metabolites showed opposite tendencies in SCML and RCML patients and were only significantly affected in SCML patients, which was more likely to be associated with therapeutic effects. Levels of the 2 metabolites showed a tendency toward gradual recovery to the healthy state in SCML patients. In RCML patients, no obvious changes in the levels of these metabolites were observed. The glycerol of the SCML group increased significantly after TKI treatment, suggesting recovery of the lipid metabolism disorder and a change of energy metabolism mode. In contrast, there is growing evidence that imatinib-resistant leukemic cells have abnormal glucose metabolism and maintain highly elevated glycolysis, irrespective of treatment,²² which might explain why glycerol was decreased in the RCML group, but was not significant. A previous study reported that 13-methyl-myristic acid could effectively inhibit the growth of tumors *in vitro* and *in vivo* by inducing apoptosis of cancer cells.²³ Higher levels of myristic acid in SCML might be associated with suppression of BCR-ABL-positive tumor cell-mediated cell proliferation. The lower level of myristic acid in the RCML group could be related to protective

modification by myristoylation. The suggestion for a mechanistic model of TKI intervention is shown in Figure S4. Subsequently, the discriminatory power of the 2 candidates was evaluated in the external validation set.

With TKI therapy, CML conditions have become more similar to indolent disorders, where daily therapy is required indefinitely for long-term survival.²⁴ In addition to the high price of the drug itself, the cost of monitoring response to TKI therapy is still expensive. Metabolomics offers a non-invasive method to collect and analyze specimens. Metabolic biomarkers may be used as a less expensive monitoring tool that could reduce the cost to CML patients. Nevertheless, the use of metabolic biomarkers in the monitoring of CML also holds some disadvantages that need to be acknowledged. First, the metabolite profiles in plasma might fluctuate to similar or greater levels compared with other omics, such as genomics and transcriptomics, in response to many systemic conditions such as stress, diet, physical activity, and basic diseases. Second, the application of TKI during the long-term clinical course of CML still carries many problems, for instance, undertreatment and overtreatment, the TKI drug itself (such as side-effects, toxicity, and tolerability), individual disease, and patients' characteristics.²⁵ Therefore, complete clinical and pathological parameters and factors should be taken into account when selecting metabolic biomarkers to monitor individual CML treatment.

In summary, this study showed that GC-MS-based metabolomics could provide a promising strategy for complementary diagnosis of CML and offer a new insight to monitor the therapeutic responses of TKI in CML patients. Notably, myristic acid and glycerol could serve as biomarkers for characterizing CML patients and their therapeutic responses to TKI therapy. The panel of biomarkers could discriminate SCML from RCML with satisfactory sensitivity and specificity in both the discovery and external validation sets. Of note, this study is limited by its small sample size, so further research with a large perspective cohort study is still needed.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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