

Original Article

Potential urinary metabolite markers for diagnosing gastric cancer

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Abstract: Background: Gastric cancer (GC) is one of the most common malignant tumors. Metabolomics has shown promise to be an important novel tool in cancer detection. This research was conducted to identify whether GC had a divergent urinary metabolic phenotype compared with healthy controls (HCs). Methods: Urines from 77 GC patients and 67 matched HCs were analyzed using gas chromatography-mass spectroscopy (GC-MS). Univariate and multivariate statistical analysis were employed. Differential metabolites were identified using orthogonal partial least-squares-discriminant analysis (OPLS-DA). Potential GC vs. HCs biomarker model was identified using logistic regression analysis. Diagnostic performance was evaluated using receiver-operating characteristic (ROC) curve analysis. Results: The 68 metabolites were identified using GC-MS. GC patients had a distinct urinary metabolic phenotype. There were 26 differential metabolites identified by OPLS-DA model, and a potential biomarker model consisting of five metabolites-lactic acid, 1-methylnicotinamide, glutamine, myo-inositol and 3-indoxylsulfate-were generated by logistic regression analysis. ROC curve analysis showed the good diagnostic performance with an area under the receiver operating characteristic curve (AUC) of 0.952 (95% CI=0.913-0.995) and 0.961 (95% CI=0.924-1.000) in the training and testing set, respectively. Conclusion: These results demonstrated that the clinical applicability of metabolic profiling for early GC diagnosis showed great promise and should be explored further.

Keywords: Gas chromatography-mass spectroscopy, GC-MS, biomarker, metabolomics, gastric cancer, GC

Introduction

Gastric cancer (GC) is the 3th leading cause of cancer-related death in the whole world [1, 2]. There are about 800,000 new cases each year in Eastern Europe, Asia and South America, and the mortality rate is up to 70% during the diagnosed patients [3]. GC causes huge economic burden for individual and society, but there is still no effective treatment methods for GC. The five-year survival rates are disappointed even among patients receiving gastrectomy [4]. An accurate early diagnosis of GC is the effective way to improve the prognosis. Chan et al. reported that the five-year survival rates of Stage IA and IB is different (71% vs. 57%) [3]. But in clinical practice, GC is often diagnosed late, because the early GC is often small and misdiagnosed. Currently, the endoscopy and biopsy are the two mainly techniques using to early diagnose GC. The detection rate of endoscopy is 2-7 times higher than photofluorography, but it depends on the experience of pathol-

ogist and endoscopist [5]. Therefore, it is urgently needed to develop an objective and convenient method to diagnose GC.

Metabolomics is the study of low-molecular-weight chemicals in a biological sample, which has been widely used to analyze the metabolic changes in various diseases [6]. Researchers found that it was an effective tool to identify potential biomarkers [7, 8] and study biological pathways [9, 10]. Nowadays, mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR) are the two most commonly used techniques for measuring the metabolome. Each technique has its advocates and possesses its unique features. Chan et al. used NMR to identify three potential urinary biomarkers (2-hydroxyisobutyrate, 3-indoxylsulfate, and alanine) for diagnosing GC [3]. Wu et al. used gas chromatography-mass spectroscopy (GC-MS) to find the different metabolomic profile of malignant GC tissue compared to normal tissue [11]. Due to its peak resolution, high reproduc-

GC-MS for diagnosing GC

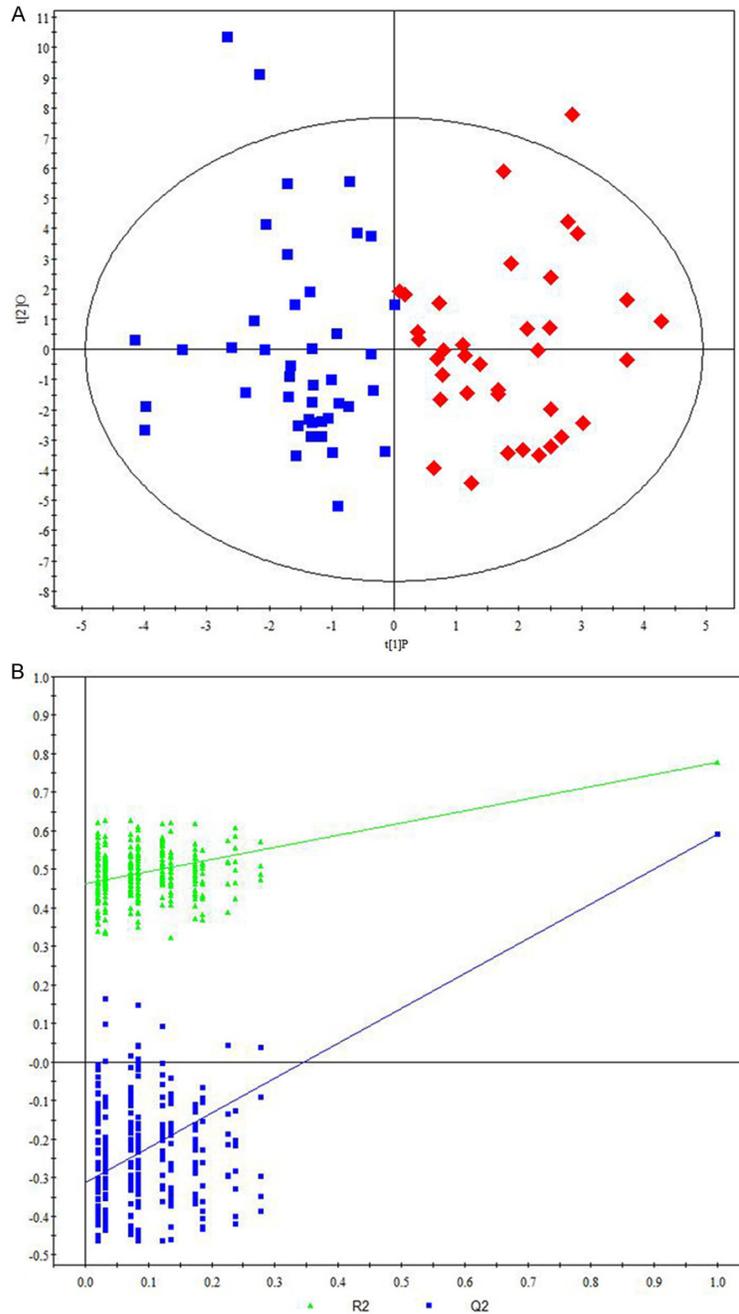


Figure 1. Metabolomic analysis of urine samples (A) and 299-iteration permutation test (B).

ability and sensitivity, GC-MS has been well established and widely used to identify and quantify the metabolites [12, 13].

Using metabolomics to identify the distinct urinary metabolomic profile for GC could provide a non-invasive, efficient, objective and cost effective method towards accurate diagnoses. Therefore, in this study, a GC-MS based

metabolomic platform was applied to profile the metabolites in urine samples from 77 GC patients and 67 healthy controls (HCs). The first purpose was to find the differential urinary metabolites in GC patients compared to HCs using training set (42 HCs patients and 36 GC). The second purpose was to identify the potential biomarkers for diagnosing GC and independently validate its diagnostic performance using testing set (35 HCs patients and 31 GC).

Methods and materials

Patient selection

GC patients and HCs were recruited from January 2011 to September 2016 from the Department of Gastrointestinal Colorectal and Anal Surgery, China-Japan Union Hospital, Jilin University, Jilin, China. The GC patients were biopsy-confirmed, >18 years of age and had no metastases on their staging computed tomography scans. Meanwhile, the HCs were recruited from the medical examination center of our hospital. The HCs had no gastrointestinal symptoms and no history of any cancers. Additionally, subjects met the following criteria were excluded: pregnancy, breastfeeding, significant cardiac disease, prior cancer, systemic infection and glomerular filtration rate <30 ml/min. Finally, 77 GC patients and 67 HCs were recruited. All

the included subjects provided the written informed consents. This study were reviewed and approved by the Ethical Committee of Jilin University.

Sample collection

The samples were collected between 9:00 am and 10:00 am. GC patients were told to collect

GC-MS for diagnosing GC

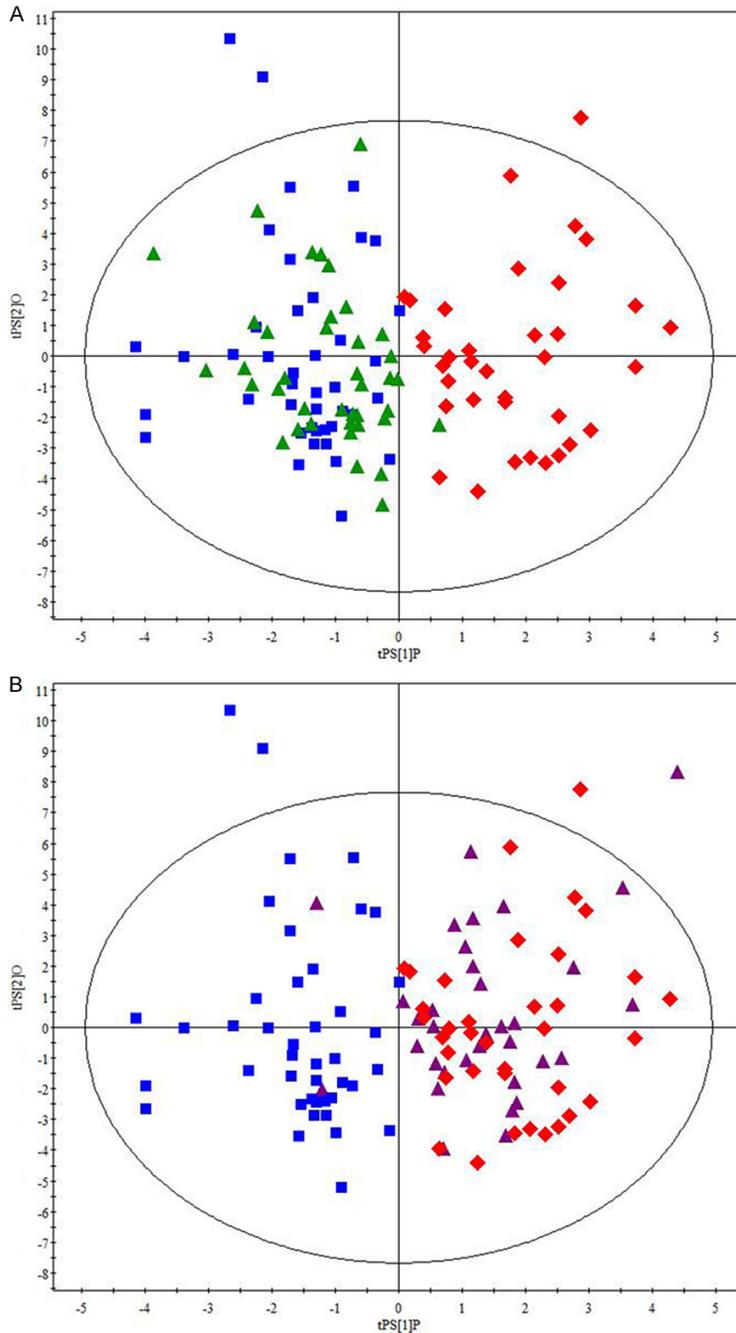


Figure 2. T-predicted scatter plots: A. HCs; B. GC patients in the testing set.

the midstream urine samples prior to chemoradiotherapy or surgery. The collected urine samples were quickly transferred into a sterile tube, and sent to the Lab under low temperature. After centrifuge (1500 g × 10 minutes) in the Lab, we obtained the supernatant and immediately divided it into equal aliquots. Finally, the processed urine samples were stored at -80°C for later use.

the carrier gas was helium (1.0 ml/minutes flow rate); full scan was performed at 50-600 m/z.

Data analysis

The relative peak areas were used to express the concentrations of the metabolites. Firstly, orthogonal partial least-squares discriminant

GC-MS acquisition

After thawing, 10 µl L-leucine-13C6 (0.02 mg/ml) as internal standard solution was added into 15 µl urine. After vortex, 15 µl urease was added into the mixture to degrade the urea (37°C, 60 minutes). The mixture was extracted in sequence with 240 µl and 80 µl of ice-cold methanol. Then, the obtained mixture was centrifuged (4°C, 14000 rpm, 5 minutes), and 224 µl obtained supernatant was moved into a glass vial for vacuum-dried at room temperature. The 30 µl of methoxyamine (20 mg/ml) was used to derivatize the dried metabolic extract (37°C, 1.5 hours). Subsequently, we added 30 µl of BSTFA with 1% TCMS into the mixture, and heated it (70°C, 1 hour) to form trimethylsilyl derivatives. After derivatization and cooling to room temperature, 1.0 µl derivative was placed in injection tube for GC-MS analysis. The GC-MS analysis conditions were as following: inject 1.0 µl samples under a temperature of 270°C; solvent delay continued for 5 minutes; the temperature programming required an initial temperature of 85°C and remained for 5 minutes, then increased the temperature to 300°C at a speed of 10°C per minutes and remained for 5 minutes; 280°C interface temperature; 230°C ion source temperature; -70eV ionization voltage; 150°C quadrupole temperature;

Table 1. Metabolites responsible for separating the different subjects

No	Metabolite	VIP ^a	P-value ^b	P-value ^c	FC ^d (GC/HC)
1	Lactic acid	1.43	4.74E-06	8.06E-05	0.52523
2	Butanoic acid	1.25	4.21E-04	4.63E-03	0.513362
3	Alanine	1.23	8.32E-05	1.08E-03	0.43703
4	Serine	1.25	6.93E-06	1.11E-04	0.528872
5	1-methylnicotinamide	1.50	5.05E-07	1.06E-05	-0.89182
6	Myo-inositol	2.00	4.36E-07	9.59E-06	1.234229
7	Uric acid	1.47	4.15E-06	7.46E-05	0.743345
8	Butanedioic acid	1.28	3.68E-07	8.47E-06	0.666588
9	Hexadecanoic acid	1.75	1.41E-06	2.67E-05	0.697564
10	Glutamine	2.21	1.21E-10	3.02E-09	1.130167
11	Sucrose	1.30	1.22E-06	2.44E-05	0.57478
12	Phosphate	1.06	1.27E-01	1.27E-01	0.339045
13	Pyrimidine	1.09	2.74E-04	3.29E-03	0.394708
14	2-hydroxyisobutyric acid	1.05	3.26E-02	9.78E-02	-1.059
15	Methionine	1.85	1.83E-11	4.77E-10	2.01019
16	Proline	1.39	1.23E-05	1.85E-04	1.050868
17	Propanoic acid	1.04	3.71E-03	2.97E-02	0.682118
18	Leucine	1.52	6.74E-03	3.37E-02	1.157517
19	Mannofuranose	1.64	1.41E-09	3.39E-08	-1.68715
20	Benzoic acid	1.40	2.83E-03	2.83E-02	0.839306
21	Cysteine	1.33	4.54E-03	3.18E-02	0.905551
22	Citric acid	1.32	7.78E-03	3.11E-02	-1.05789
23	3-indoxylsulfate	1.66	5.09E-05	7.13E-04	2.872616
24	Glucose	1.03	5.79E-02	1.16E-01	0.548311
25	Pantothenate	1.29	3.26E-03	2.94E-02	-1.29521
26	Malic acid	1.01	5.15E-03	3.09E-02	0.557709

^aVIP was from the OPLS-DA with a threshold of 1.0. ^bP-values were from the nonparametric Mann-Whitney U test. ^cP-values were from the Bonferroni Step-down (Holm) correction. ^dPositive and negative values indicated the higher and lower levels, respectively, in GC patients.

analysis (OPLS-DA) was performed to visualize the discrimination between GC patients and HCs and obtain the differential metabolites. The values of R²Y and Q² were applied to assess the quality of the built OPLS-DA model [14]. The 299-iteration permutation test was applied to check whether there was over-fitting or not. Variable Importance in Projection (VIP) score indicates the relative importance of each metabolite in discriminating different groups. Metabolites with VIP score >1.0 (equivalent to a p-value of less than 0.05) were viewed as differential metabolites [15]. Secondly, logistic-regression analysis with Akaike's information criterion (AIC) [16] was used to derive an optimal and simplified GC vs. HCs biomarker model. Thirdly, receiver-operating characteristic (ROC)

curve analysis was conducted to assess the diagnostic performance of the biomarker model. Statistical analyses were done using STATA Version 13 (StataCorp LP, College Station, TX, USA) and SIMCA version 12 (Umetrics, Umea, Sweden).

Results

OPLS-DA model

Totally, 68 metabolites were identified using GC-MS platform. The training samples were used to build the OPLS-DA model. The score plot of the model showed that the GC patients could be effectively separated from the HCs with little overlap (**Figure 1A**). The values of R²Y (0.73) and Q² (0.60) demonstrated that the goodness-of-fit and predictability of this model was good. Meanwhile, the 299-iteration permutation test indicated that there was no over-fitting in this model (**Figure 2B**). The testing samples were used to independently validate the discrimination power of the OPLS-DA model. The T-predicted scatter plot showed that 33 of the 35 HCs and 28 of the 31 GC patients were correctly predicted by the OPLS-DA model. The predictive accuracy was 92.4%. These

results indicated that the OPLS-DA model generated by the 68 urinary metabolites could be a potential empirical diagnostic tool for GC.

Differential metabolites

We obtained 26 differential metabolites (VIP >1.0) responsible for the discrimination between HCs and GC (**Table 1**). As compared to HCs, the levels of 1-methylnicotinamide, 2-hydroxyisobutyric acid, mannofuranose, citric acid and pantothenate were significantly decreased in the urine of GC patients. The other metabolites were significantly increased in the urine of GC patients. The univariate statistical analysis (nonparametric Mann-Whitney U test) was then performed to validate the metabolic

GC-MS for diagnosing GC

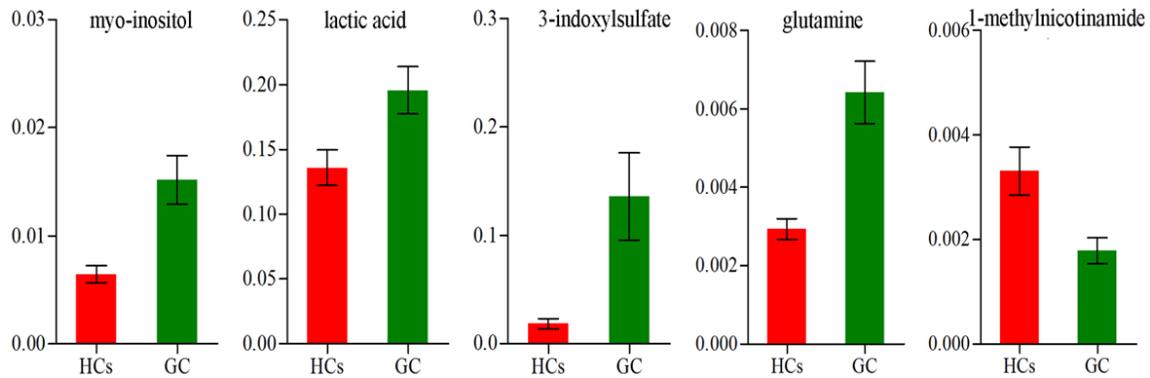


Figure 3. Five differential metabolites in the potential GC vs. HCs biomarker model.

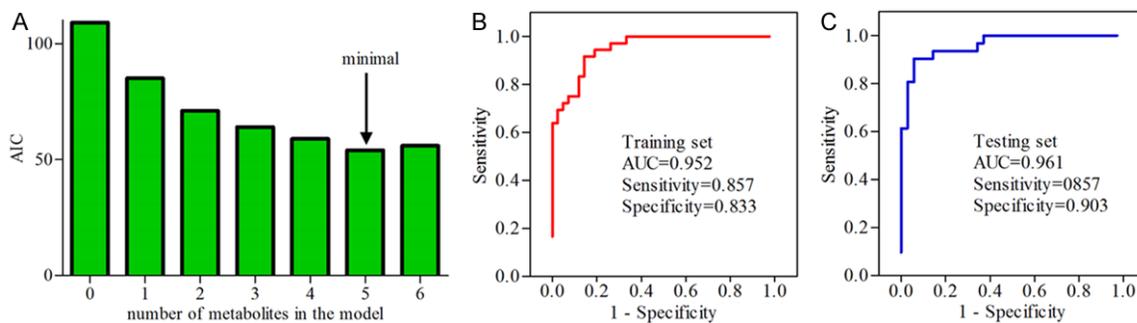


Figure 4. AIC rule (A) and the values of AUC in the training set (B) and testing set (C).

changes found by the multivariate statistical analysis. Bonferroni Step-down (Holm) method was applied to do the correction for multiple comparisons. The results showed that 23 of the 26 differential metabolites remained significantly changed.

Potential biomarker model

Using 26 metabolites to clinically diagnose GC is not a convenient and cost effective method. Then, the logistic-regression analysis with AIC was conducted. The 26 differential metabolites were used as independent variable, and the group was used as dependent variable. The results showed that the five metabolites-lactic acid, 1-methylnicotinamide, glutamine, myo-inositol and 3-indoxylsulfate-could describe the most significant deviations between GC patients and HCs (Figure 3). The optimal GC vs. HCs biomarker model consisting of these five differential metabolites could effectively predict the GC patients in both training set and testing set.

Diagnostic performance

The AIC showed that five differential metabolites were enough to build the optimal and simplified GC vs. HCs biomarker model (Figure 4A). Then, we further used the ROC analysis to obtain the value of the area under the curve (AUC), which was used to evaluate the diagnostic performance of this model. The value of AUC greater than 0.8 indicates the good diagnostic performance; and it is closer to 1.0, the diagnostic performance is better [17]. Here, we found that the AUC value of this model was 0.952 (95% confidence interval (CI)=0.913-0.995) in the training set (Figure 4B) and 0.961 (95% CI=0.924-1.00) in the testing set (Figure 4C). These results showed that the diagnostic performance between this simplified model and the OPLS-DA model was similar.

Discussion

GC is a highly morbid and fatal disease, and the delayed diagnosis of GC is common. Previous NMR metabolomics study found that there was

divergent urinary metabolic phenotype between GC patients and HCs. The present study used the GC-MS based metabolomic method to characterize a urinary metabolic profile of GC patients, which was also found to be distinct from HCs. The OPLS-DA model built with 68 metabolites showed that there was little overlap between GC patients and HCs, and 26 differential metabolites responsible for separating GC patients from HCs were identified. Meanwhile, a potential GC vs. HCs biomarker model consisting of five differential metabolites-lactic acid, glutamine, 1-methylnicotinamide, myo-inositol and 3-indoxylsulfate-were identified. This potential biomarker model could yield an AUC of 0.952 (95% CI=0.913-0.995) in the training set and 0.961 (95% CI=0.924-1.00) in the testing set. These results demonstrated that the clinical applicability of metabolic profiling for early GC diagnosis showed great promise and should be explored further.

The univariate statistical analysis found that the levels of phosphate, 2-hydroxyisobutyric acid and glucose were not significantly altered in the urine of GC patients. But, the multivariate statistical analysis still viewed these three metabolites as differential metabolites. Previous metabolomics studies also reported some similar results, and even found that the non-significantly differential metabolites in univariate statistical analysis could be identified as the potential biomarkers in multivariate statistical analysis [18-20]. The reason was that the multivariate statistical analysis thought the addition of these metabolites could obtain the highest discrimination power. These results showed that compared to univariate statistical analysis, the multivariate statistical analysis had an advantage in identifying the subtle yet significantly altered metabolites in biosamples [21].

Among the five biomarkers, 3-indoxylsulfate was also identified as potential biomarker for GC diagnosis [3]. Previous study reported that treating with 1-methylnicotinamide could inhibit the gastric acid secretion, increase the mucosal blood flow and accelerate healing [22]. The decreased 1-methylnicotinamide level in GC patients found here might indicate the loss of this mucosal protective mechanism. The myo-inositol level was found to be decreased in GC tissue [11]. Similarly, in this study, we found the significantly decreased myo-inositol level in

urine of GC patients. An animal study showed that the level of lactic acid was higher in the urine of cancer group [23]. Here, we also found the significantly higher level of lactic acid in the urine of GC patients. These results indicated that the identified five biomarkers here might be helpful for future developing a better method for clinical diagnosing GC.

Limitations of this study included: i) the recruited sample size was relatively small, which might limited the power of identifying different metabolites, and conversely, there was possible that the identified different metabolites were spurious; ii) the included patients were from the same place, which might limited the applicability of our results; iii) the two groups were only matched on three common confounders (sex, age and BMI), the potential influence of other confounders could not be ruled out; iv) only one metabolomic platform was used, previous studies reported that the use of multiple metabolomics platforms could identify a more comprehensive metabolite panel than any single metabolomic platform alone [24, 25]. Future studies should use the multiple metabolomics platforms to validate and support our findings.

In conclusion, we found that there was different urinary metabolic phenotype between GC patients and HCs. The 26 differential metabolites were identified, and the five potential biomarkers could be helpful for researchers to develop a urine-based diagnostic method for GC. This study showed that there was clinical potential to using metabolic profiling for GC diagnosis, although numerous work are still needed to complete before moving this test into reality.

Disclosure of conflict of interest

None.

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