Prediction of gastric cancer metastasis through urinary metabolomic investigation using GC/MS

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INTRODUCTION

Gastric cancer is the second leading cause of cancer death worldwide, and in many Asian countries, such as China[1,2]. Until now, there has been no effective treatment for gastric cancer. Even among patients undergoing gastrectomy,
because of locoregional relapse and distant metastases, the 5-year survival rates remain disappointing. Early dissemination of the disease through the lymphatic system, blood and peritoneum has limited the therapeutic effects of optimal surgery, except in patients with relatively early-stage tumors. Therefore, it is significant to establish an accurate early diagnosis of gastric cancer. Currently, the diagnosis or screening of gastric cancer or tumor recurrence mainly depends on endoscopy and pathological examinations. The ratio for identifying early gastric cancer with endoscopy is higher than that with X-ray, and the diagnosis of gastric cancer using endoscopy is more accurate. Nevertheless, the results of endoscopy are easily affected by artificial factors (e.g. the experience of the endoscopist). Over the past years, epidemiological data have shown that Helicobacter pylori (H. pylori) infection is strongly associated with the development of gastric cancer, and H. pylori eradication may be considered as a strategy to prevent gastric cancer. In addition, investigation of gastric cancer tissues and some biomarkers have been used for screening gastric cancer. However, compared with tissues and serum, the markers acquired from urine are noninvasive and convenient, especially in the patients with recurrent gastric cancer. The urinary metabolic profiling could be used to get urinary metabolites as gastric cancer or tumor recurrence biomarkers.

Metabolomics is a post-genomic research field for analysis of low molecular weight compounds in biological systems, and offers an analysis of metabolite level changes in biological samples. In recent years, studies of metabolomics used in various diseases have been conducted, such as stomach cancer, lung cancer, renal cancer, brain tumors, and colorectal cancer. Nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) are the most commonly employed techniques for measuring the metabolome, MS-based techniques, including gas chromatography/mass spectrometry (GC/MS), GC-MS/MS, liquid chromatography/mass spectrometry (LC-MS) and LC-MS/MS, are among the most efficient and versatile for quantitative analysis of endogenous and exogenous substances in biological samples. Because of its peak resolution, high sensitivity and reproducibility, GC/MS has been well established and widely utilized in metabolomics.

In this study, we have established a human gastric cancer non-metastasis model and a metastasis model using severe combined immune deficiency (SCID) mice, and deployed GC/MS following chemical derivatization to profile the mouse model urinary specimens and their matched urine. The metabolic differences among the three groups were characterized by principal components analysis (PCA). On the basis of its results, we expected that the potential metabolic biomarkers could be found in mice for early diagnosis and screening the metastasis or the recurrence of gastric cancer.

MATERIALS AND METHODS

Chemicals and materials

Tetrahydrofuran (THF) and bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Vacuum dryer was purchased from Shanghai NOTED Technologies. All other reagents were obtained from Sinopharm Chemical Reagent Co. Ltd.

Animal models

Male SCID mice were acquired from Shanghai Experimental Animal Center of Chinese Academy of Sciences. Animals used were 6-wk old and weighed 20-25 g. Animal and experimental procedures were performed according to the relative ethical regulations for the care and use of laboratory animals of our university. Human gastric cancer SGC-7901 (Shanghai Cancer Institute), a poorly-differentiated adenocarcinoma line, was originally derived from a primary tumor and maintained by passage in the subcutis of nude mice. Tumors were cut out aseptically. Necrotic tissues were cut and the reserved healthy tumor tissues were scissor minced into pieces (about 3 mm × 4 mm in diameter) in Hank’s balanced salt solution. Each tumor piece was weighed and adjusted to be approximately 100 mg. All animals were randomly divided into metastasis group (n = 8), non-metastasis group (n = 8), and normal group (n = 8). Animal models were made using orthotopic implantation of histologically intact tissue of human gastric cancer. Mice were anesthetized with 4.3% trichloraldehyde hydrate. An incision of the metastatic group and the normal group was made through the left upper abdominal pararectal line. Then peritoneal cavity was carefully exposed and a part of serosal membrane in the middle of the greater curvature of stomach was mechanically injured by scissors. A tumor piece of 100 mg was fixed on each injured site of the serosal surface of the metastatic group, while normal control mice received no tumor implantation. The stomach was then returned to the peritoneal cavity, and the abdominal wall and skin were closed. An incision of the non-metastatic group was made at the left oyster. A tumor piece of 100 mg was fixed under the skin. All animals were sent to the breeding room after becoming conscious.

Specimen collection and pathological examination

Six weeks after implantation, all mice were housed in metabolic cages and maintained in an air conditioned room (24 ± 2°C). They were only allowed free access to water during urine sample collection (8:00 pm that day to 8:00 am the next day). All animal urine was collected in frozen tubes at the sixth week after implantation, and immediately stored at -80°C until processing. The specimens were collected at the same time. Then all mice were killed, tumors growing on the stomach wall were resected and fixed in 4% formalin, and processed for routine paraffin embedding after careful macroscopic examination. In order to evaluate histologically for liver metastasis or lymph node metastasis or other organ metastasis under microscope, four-micron-thick sections were stained with hematoxylin and eosin, then observed by a blinded pathologist.

Sample pretreatment and derivatization

Each urinary specimen was transferred to a glass cen-
trifuge tube, subsequently centrifuged at 18000 × g for 3 min and 50 μL of the supernatant was collected from each sample into a 1-mL EP tube, respectively. The collected supernatant was evaporated to dryness at 60°C for 24 h, using a vacuum dryer. Then 100 μL THF was added to each of the dried urine extracts and vortex-mixed for 2 min, and 50 μL BSTFA was added to the mixture and vortex-mixed for 2 min. The mixture was incubated at 60°C and derivatized for 30 min. After returning to the ambient temperature, samples were prepared for GC/MS analysis.

GC/MS analysis
Each derivatized sample of 1 μL was injected splitless into an Agilent 6980 GC system equipped with an HP5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm), electron impact ionization at 70 eV, and a quadrupole mass spectrometric detector (Agilent Technologies, Palo Alto, CA, USA). The column temperature was initially held at 100°C for 3 min, 10°C/min up to 220°C, then 10°C/min to 280°C, and remained there for 5 min. The injector temperature was 280°C. Carrier gas flow was helium at a constant flow rate of 1.0 mL/min. The interface temperature and the ion source temperature were set at 200°C. Masses were obtained from 100-600 m/z, GC total ion chromatograms (TICs) and fragmentation patterns were acquired using GC/MSD ChemStation Software (Agilent Technologies, Palo Alto, CA, USA). Compound identification was performed by comparing the mass spectrum with a standard mass spectrum in the national institute of standards and technology (NIST) mass spectra library. Peaks with similarity index more than 70% were assigned compound names, while those having less than 70% similarity were assigned to unknown metabolites. The chromatograms were subjected to noise reduction prior to peak area integration. Any known artificial peaks, such as peaks due to noise, column bleed and BSTFA derivatization procedure, were excluded from the data set. Integrated peak areas of multiple derivative peaks belonging to the same compound were summed and considered as a single compound. The resulting three dimensional matrix included sample information, peak intensities and peak retention time, and was applied to correlation analysis and pattern recognition.

Data processing and pattern recognition
The relative peak area of each compound would be calculated as the response after the peak areas of compounds were integrated. Each sample was represented by a GC/MS TIC. t test was employed for statistical analysis. Data were expressed as mean ± SD. The differentially expressed compounds with P < 0.05 were considered statistically significant. PCA was used to differentiate the samples and performed using the SPSS 16.0 for Windows.

RESULTS
General state of mice and pathological results
The mean weight of mice was 23.81 ± 0.16 g, 23.87 ± 0.19 g and 23.98 ± 0.19 g for normal group, non-metastasis group and metastasis group, respectively (P > 0.05). All animals from the three groups were alive at the sixth week. The normal group mice had no tumor and metastasis. The non-metastasis group and metastasis group developed localized tumors at the implanted site, which were poorly-differentiated adenocarcinomas under microscope (Figure 1A). The non-metastasis group tumor tissues (4.28 ± 0.20 g) were located at the left oxter, and have no metastasis in regional lymph nodes, liver and other organs. The metastasis group mice had cancer tissues (4.3 ± 0.3 g in non-metastasis group, P > 0.05) in the stomach, while metastatic tumors were also found in liver (Figure 1B), regional lymph nodes, and other organs. Six mice developed metastatic tumors in regional lymph nodes, four in liver, and two in other organs.

Metabolomic profiling of samples
GC/MS TIC chromatograms of urine samples derived from the normal group, the non-metastatic group and the metastatic group are presented in Figure 2. In the GC/MS TICs of urinary samples from the three groups, some peaks were identified based on NIST mass spectra library, and several examples of peaks had statistical significance (Figure 2).

With GC/MS, around 120 signals were detected per sample using mass spectral deconvolution software for peak detection. However, many of them were not consistently found in other samples or were of too low abundance or too poor spectral quality to be obviously assigned to unique metabolites. Several choline, amino acids, and fatty acids could not be found, which may be
associated with the efficiency of chemical derivatization. Table 1 shows that 46 signals could be auto-identified by the NIST library through comparing with a standard mass chromatogram. The remaining peaks which could not be identified were not listed. In addition, the retention time of metabolites and the match percentage to the NIST library are also listed in Table 1.

Three TIC profiles of consecutively injected samples of the same aliquot are presented in Figure 3, which showed stable retention time with no drift in all of the peaks. The stable TICs reflected the stability of GC/MS analysis and reliability of the metabolomic data.

Urine GC/MS data from the three groups were analyzed. Metabolites selected by t-test are listed in Tables 2 and 3 after normalization of data. Lactic acid, butanoic acid, propanoic acid, glycerol, pyrimidine, butanedioic acid, malic acid, citric acid, hexadecanoic acid and uric acid were found at higher levels in the urine of cancer group (non-metastasis group and metastasis group) than in normal control group (Table 2). Furthermore, the decreased levels of alanine, butanoic acid, glycerol, L-proline and L-threonic acid were found in the metastasis group as compared with the non-metastasis group. However, the levels of butanedioic acid and myo-inositol were significantly higher in the metastasis group than in the non-metastasis group (Table 3).

Pattern recognition and function analysis
A PCA model for gastric cancer was constructed using

Table 1 Urine metabolites of mice in the three groups (normal, non-metastasis and metastasis)

| Peak No. | Retention time | Metabolites | Match percent (%) |
|----------|----------------|-------------|-------------------|
| 1        | 7.196          | Lactic acid | 91                |
| 2        | 7.508          | Acetic acid | 90                |
| 3        | 8.105          | Alanine     | 90                |
| 4        | 8.518          | Glycine     | 91                |
| 5        | 8.936          | Pentanoic acid, 4-oxo- | 94 |
| 6        | 9.412          | Butanoic acid | 83 |
| 7        | 11.940         | Urea        | 95                |
| 8        | 12.511         | Glycerol    | 91                |
| 9        | 12.590         | Silanol     | 97                |
| 10       | 13.933         | Butanedioic acid | 97 |
| 11       | 14.293         | Propanoic acid | 94 |
| 12       | 14.705         | Pyrimidine  | 93                |
| 13       | 14.774         | Triacetic   | 83                |
| 14       | 15.657         | 2-Piperidinecarboxylic acid | 90 |
| 15       | 16.070         | L-threonine | 87                |
| 16       | 16.460         | N-(3-methyl-1-oxobutyl)-Glycine | 90 |
| 17       | 17.270         | N-(2-methyl-1-oxopropyl)-Glycine | 91 |
| 18       | 17.530         | (R*,S*)-3,4-Dihydroxybutanoic acid | 94 |
| 19       | 19.301         | Malic acid  | 90                |
| 20       | 19.497         | N-(3-methyl-1-oxobutyl)-Glycine | 98 |
| 21       | 19.566         | 2,3,4-oxy-Butanal | 90 |
| 22       | 19.814         | 1,2,3,4-oxy-Butanol | 90 |
| 23       | 20.497         | L-proline   | 96                |
| 24       | 20.909         | L-threonic acid | 90 |
| 25       | 21.475         | Creatinine  | 96                |
| 26       | 25.108         | Hexadecanoic acid | 90 |
| 27       | 25.632         | Arabitol    | 91                |
| 28       | 25.843         | Nonadecane  | 83                |
| 29       | 26.018         | Xylitol     | 93                |
| 30       | 26.166         | Ribitol     | 91                |
| 31       | 26.838         | 4-Pyrimidinobutyric acid | 98 |
| 32       | 26.938         | 1-Propene-1,2,3-tricarboxylic acid | 91 |
| 33       | 27.208         | Phosphoric acid | 90 |
| 34       | 28.768         | Citric acid | 91                |
| 35       | 29.032         | Myo-inositol | 83 |
| 36       | 30.328         | Mannonic acid | 95 |
| 37       | 30.540         | Hydravone   | 96                |
| 38       | 30.730         | N-Phenylacetyl glycine | 93 |
| 39       | 31.037         | Silane      | 91                |
| 40       | 31.449         | L-Gluconic acid | 99 |
| 41       | 32.422         | D-Gluconic acid | 91 |
| 42       | 33.025         | Dehydrocholic Acid | 92 |
| 43       | 34.612         | Hexadecanoic acid | 99 |
| 44       | 35.691         | Uric acid   | 98                |
| 45       | 36.388         | Retinoic acid, methyl ester | 95 |
| 46       | 39.065         | Octadecanoic acid | 99 |

Peaks in the total ion chromatograms are numbered according to their retention time. The identification of metabolite is based on national institute of standards and technology mass spectra database according to the match of masses (m/z) between the interested peak’s fragmentation pattern and that from the standard database.

Figure 2 Representative gas chromatography/mass spectrometry total ion chromatograms of the samples from the three groups (normal group, non-metastasis group and metastasis group) after chemical derivatization.
the marker metabolite intensities as variables (lactic acid, butanoic acid, propanoic acid, glycerol, pyrimidine, butanedioic acid, malic acid, citric acid, hexadecanoic acid and uric acid). The PCA scores plot showed that the normal group and cancer group (non-metastasis group and metastasis group) samples were scattered into different regions (Figure 4A). ROC analysis, which was performed using the values determined by the first two components of the PCA model, confirmed the robustness of the PCA model. These first two components could present the majority of all significantly different metabolites among the groups (the percentage is 82.7%). Area under the curve (AUC) value of this PCA model was 1.00 (Figure 4B), which demonstrated a good diagnostic value for gastric cancer. In addition, another PCA model for gastric cancer metastasis constructed by seven marker metabolites (alanine, butanoic acid, glycerol, L-threonic acid, L-proline, butanedioic acid and myo-inositol) could differentiate between the non-metastasis group and the metastasis group (Figure 5A). This PCA model was also validated by

![Graph](image-url)

**Table 2** Marker metabolites found in normal and cancer groups

| Metabolites      | Retention time | P value | A (normal)     | B (cancer)     | R value |
|------------------|----------------|---------|----------------|----------------|---------|
| Lactic acid      | 7.196          | 2.4 × 10^-5 | 79.24 ± 6.1 | 187.04 ± 71.99 | 1.36    |
| Butanoic acid    | 9.412          | 0.000   | 16.79 ± 0.52 | 27.33 ± 4.98  | 0.63    |
| Propanoic acid   | 14.293         | 0.000   | 60.58 ± 9.79 | 147.77 ± 15.3 | 1.43    |
| Glycerol         | 12.511         | 0.000   | 147 ± 8.98   | 269.13 ± 50.31 | 0.83    |
| Pyrimidine       | 14.705         | 0.000   | 61.68 ± 8.05 | 163.11 ± 12.23 | 1.64    |
| Butanedioic acid | 13.933         | 0.1 × 10^-5 | 161.51 ± 5.85 | 267.89 ± 54.64 | 0.66    |
| Malic acid       | 19.301         | 0.000   | 10.7 ± 1.91  | 32.15 ± 1.16  | 2.00    |
| Citric acid      | 28.768         | 1.4 × 10^-4 | 1291.89 ± 364.74 | 2164.74 ± 529.58 | 0.68    |
| Hexadecanoic acid| 34.612         | 4.17 × 10^-4 | 1347.84 ± 304.67 | 2066.57 ± 437.28 | 0.53    |
| Uric acid        | 35.691         | 0.000   | 172.2 ± 17.03 | 214.52 ± 7.74  | 0.25    |

1P values were calculated based on Student t test (significance at P < 0.05); 2Cancer group included the non-metastasis group and the metastasis group; 3R value was calculated from the arithmetic mean values of each group. R = (B-A)/A. R with a positive value indicates a relatively higher concentration in cancer group while a negative value means a relatively lower concentration as compared with the normal group.

**Table 3** Metabolic differences in the two groups

| Metabolites        | Retention time | P value | A (non-metastasis) | B (metastasis) | R value |
|--------------------|----------------|---------|--------------------|----------------|---------|
| Alanine            | 8.105          | 0.000   | 173.75 ± 39.59     | 19.28 ± 10.63  | -0.89   |
| Butanoic acid      | 9.412          | 0.000   | 32.09 ± 1.00       | 22.58 ± 0.72   | -0.30   |
| Glycerol           | 12.511         | 0.003   | 303.23 ± 26.16     | 235.04 ± 45.64 | -0.22   |
| Butanedioic acid   | 13.933         | 0.1 × 10^-5 | 216.36 ± 2.63     | 319.43 ± 17.89 | 0.48    |
| L-proline          | 20.497         | 0.000   | 184.99 ± 10.26     | 117.78 ± 7.05  | -0.36   |
| L-threonic acid    | 20.909         | 2.28 × 10^-4 | 284.94 ± 46.47    | 181.48 ± 37.25 | -0.36   |
| Myo-inositol       | 29.032         | 0.000   | 33.08 ± 3.58       | 114.8 ± 2.20   | 2.47    |

1P values were calculated based on Student t test (significance at P < 0.05); 2R value was calculated from the arithmetic mean values of each group. R = (B-A)/A. R with a positive value indicates a relatively higher concentration in metastasis group while a negative value means a relatively lower concentration as compared with the non-metastasis group.
DISCUSSION

In this study, we investigated urinary metabolite profiling using GC/MS. This was assessed non-invasively by measuring two voxels (tumor and healthy controls). We have discriminated the gastric cancer model mice from their healthy controls in a PCA analysis of GC-MS urinary metabolite spectra. Moreover, we could also discriminate the gastric cancer metastasis model mice from the non-metastasis group by GC-MS and PCA of urinary metabolites. Some marker metabolites were worth investigating in the future. Compared with the normal group, the level of lactic acid was higher in the cancer group urine. It could be explained that glucose is often converted into lactic acid in cancer cells, which is known as the “Warburg effect”, and cancer cells have a higher rate of aerobic glycolysis[31]. The levels of butanedioic acid, malic acid and citric acid, intermediates of tricarboxylic acid (TCA) cycle, were also found to be higher in the gastric cancer mice. The abnormalities of these metabolite expressions demonstrated a close correlation of TCA cycle with gastric cancer morbidity along with disordered aerobic respiration and mitochondrial functions. The disorder of aerobic respiration (mainly TCA cycle) and the impairment of mitochondrial enzymes have been reported in other malignancies including colorectal cancer, pheochromocytoma and paraganglioma[22,32,33]. Uric acid, the final metabolite of purines, at enhanced level in cancer mice urine, suggests the abnormalities of purine metabolism in gastric cancer[34]. In our study, the significantly higher levels of glycerol and hexadecanoic acid in cancer than in normal groups were interpreted as increased adipocyte lipolysis in cancer and enhanced expression and function of adipocyte hormone-sensitive lipase (HSL)[35].

Cancer metastasis could be considered as an essential prognostic factor[36]. Figure 5A shows the new constructed tumor metastatic model by seven marker metabolites for the non-metastasis group and the metastasis group. This PCA model was also validated by ROC analysis (AUC = 1.00, Figure 5B). Seven metabolites in this model are capable of predicting the gastric cancer metastasis. Compared with the non-metastasis group, levels of alanine receiver operating characteristic (ROC) analysis (AUC = 1.00, Figure 5B).
and glycerol were found to be lower in the metastasis group. Alanine and glycerol could get into the glycolytic pathway through gluconeogenesis, which produced more energy for the tumor progression and metastasis. The decreased level of L-proline in the metastasis group may be interpreted as increased demand for structural proteins synthesis. These proteins, including receptors, membrane channels and enzymes, play an important role in tumor progression and metastasis[17–19]. Moreover, the higher level of myo-inositol in metastasis group urine, was consistent with the reduction of myo-inositol in lung cancer tissues[40]. The amount of myo-inositol may be a potential indicator for gastric cancer metastasis, as it has been reported that the Gly:Myo-inositol ratio may be a useful index for brain tumor classification[41].

What the difference of metabolite changes of butanoic acid and pyrimidine between the normal and the cancer groups, and the decreased levels of butanoic acid and L-threonine in the metastasis group indicates remains unclear.

In conclusion, GC/MS revealed detailed information on the metabolic profile of normal and cancer urine and was found to be suitable, in tandem with the PCA model, for the identification of metabolic variations characteristic of the gastric cancer. Furthermore, seven metabolites have been selected, which constructed a diagnostic model for distinguishing the non-metastatic and the metastatic gastric cancer. To our knowledge, this is the first report on urinary metabolomic investigation of gastric cancer metastasis by GC/MS. On the basis of this research, we believe that urinary metabolomic information obtained by GC/MS might play a significant role in the early diagnosis and screening metastasis or recurrence of gastric cancer.

**COMMENTS**

**Background**

Gastric cancer is the second leading cause of cancer death worldwide, and in many Asian countries. Tumor metastasis is one of the leading causes of cancer death. Metabolic alterations play a role in the biology of cancer. The urinary metabolites as gastric cancer or tumor recurrence biomarkers can be obtained by investigating the urinary metabolic profiling.

**Research frontiers**

Metabolomics is a post-genomic research field for analysis of low molecular weight compounds in biological systems, and its approaches offer an analysis of metabolite level changes in biological samples. Recently, metabolomic method has shown great potentials in identifying the new diagnostic markers and therapeutic targets for cancers. However, metabolomic studies on cancer metastasis remain scarce.

**Innovations and breakthroughs**

Recently, metabolomic studies on gastric cancer and colon cancer tissues have been conducted. Compared with tissues and serum, markers acquired from urine are noninvasive and convenient, especially in the patients with recurrent gastric cancer. This is the first report on urinary metabolomic investigation in gastric cancer using gas chromatography/mass spectrometry (GC/MS).

**Applications**

Potential metabolomic biomarkers in urine could be used for early diagnosis and screening the metastasis or the recurrence of gastric cancer.

**Terminology**

Metabolomics is a post-genomic research field for analysis of low molecular weight compounds in biological systems, and its approaches offer an analysis of metabolite level changes in biological samples. Because of its peak resolution, high sensitivity and reproducibility, GC/MS has been widely utilized in metabolomics.

**Peer review**

This manuscript evaluates tumor metabolism with a goal to identify possible biomarkers with potential diagnostic value and the potential for prediction of tumor metastasis. The authors concluded that the urinary metabolomic profiling of each group is different, and the selected metabolites might be instructive to clinical diagnosis or screening metastasis for gastric cancer. This is a relevant randomized controlled trial using an animal model to evaluate a non-invasive method for surveillance of gastric cancer.

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