

## Urinary Metabolomic Analysis of Human Gastric Cancer Mouse Models and Patients Using Gas Chromatography/Mass Spectrometry

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### Abstract

Gastric cancer is the second cause of cancer deaths in China. To identify potential markers for screening or diagnosis of gastric cancer, we coupled xenotransplantation mouse models with a urine metabolomic approach. SGC-7901 gastric cancer cells were subcutaneously or orthotopically implanted into nude mice to establish metastasis and non-metastasis mouse models. Urine samples from mice bearing tumors or gastric cancer patients and their healthy controls were collected and subjected to gas chromatography and mass spectrometry (GC/MS) analysis. Metabolic data were analyzed using Mann-Whitney test to find urinary biomarkers for gastric cancer. Diagnostic models for gastric cancer mice and patients were constructed using principal components analysis (PCA) and validated with the area under the curve (AUC) of receiver operating characteristic (ROC) curves. The results indicated these metabolites mainly include lactic acid, serine, proline, malic acid, and fatty acids. The PCA models discriminated all gastric cancer mice or most gastric cancer patients including six of seven early stage patients, from their healthy controls with AUC value of 1.0 or 0.996, respectively. In addition, they were able to differentiate between metastatic and non-metastatic mice with AUC value of 1.0, as well as between invasive/metastatic and non-invasive cancers with AUC value of 0.982. Our data suggest that there are significant metabolic alterations during progression of gastric cancer and the potential metabolic biomarkers could be useful for screening and early diagnosis of gastric cancer progression.

**Keywords:** Gastric cancer; Metastasis; Urine metabolite; Metabolomics; Gas chromatography and mass spectrometry

### Introduction

Gastric cancer is one of the most frequent malignancies and the second cause of cancer deaths in China. Up to now, surgical resection remains the only curative treatment option. However, early dissemination of the disease through the lymphatic system, blood and peritoneum has limited the ability of optimal surgery to cure, except in patients with relatively early tumors [1]. To improve the prognosis and the survival is to forecast the progression and recurrence of gastric cancer at an early stage. To date, endoscopy is the most effective screening tool for accurate diagnosis of gastric cancer [2]. However, the result of endoscopy is easily affected by artificial factors (e.g. the experience of the endoscopist). Over the past decades, certain tumor biomarkers including carbohydrate antigen 19-9 (CA19-9) and carcinoembryonic antigen (CEA) have been used for screening gastric cancer and its recurrence, but they have limitations clinically because of poor sensitivity and specificity. In addition, proteomics was also applied to detect gastric cancer [3,4]. However, proteomics does not provide evidence of endpoint markers for disease diagnosis. Metabolomics, an omic science in systems biology, is the comprehensive and simultaneous profiling of metabolic changes which occur in living systems in response to genetic, environmental, or lifestyle factors. It can establish the missing link between genotype and phenotype, providing information that is complementary to genomics and proteomics analysis and that improves our understanding of pathogenic mechanisms and metabolic phenotype [5,6]. It is shown that metabolomics can analyze changes of metabolite levels in biological samples and reveal significantly perturbed expression of amino acids, fatty acids, lactate, carboxylic acids and urea cycle between tumor tissue and normal mucosa [7-10]. However, compared with tissues and serum, markers acquired from urine are noninvasive and convenient, especially in the progression or recurrence of gastric cancer. Currently,

metabolomic study on cancer metastatic progression remains scarce. Recently, metabolomic analysis showed that increased sarcosine synthesis is an important metabolic change during prostate cancer progression [11]. However, to our knowledge, there has been no report on urine metabolomic investigation of gastric cancer progression until now [11].

In this study, metastasis and non-metastasis mouse models were established using human gastric cancer cell line SGC-7901. Due to the same genetic backgrounds, this pair of models provides a suitable system for comparative studies of molecular changes involved in gastric cancer progression. Gas chromatography/mass spectrometry (GC/MS) has been widely applied in metabolomic investigations for its high sensitivity, peak resolution and reproducibility [9]. Therefore, we deployed a gas chromatography/mass spectrometry (GC/MS) on urinary metabolite profiling approach to discriminate between metastasis and non-metastasis models of gastric cancer. Additionally, we also analyzed urine samples from gastric cancer patients and healthy control subjects. On the basis of pattern recognition results, we expected that the potential metabolic biomarkers could be found for early diagnosis and screening of gastric cancer progression.

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## Materials and Methods

### Materials

Tetrahydrofuran (THF), bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) plus 1% trimethylchlorosilane (TMCS) and chromatographic pure 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 severe combined immune deficiency (SCID) mice were acquired from Shanghai Experimental Animal Center of Chinese Academy of Sciences. Animals used were six-week old and weighed 20-25 g. Animal 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 three groups which included metastasis group (n=8), non-metastasis group (n=8), and normal group (n=7). Metastatic model was made using orthotopic implantation of histologically intact tissue of human gastric cancer [12]. Mice were anesthetized with 4.3% trichloroaldehyde hydrate. An incision of metastatic or normal mice 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 100mg was fixed on each injured site of the serosal surface of the metastatic group, while normal control mice received no tumor implantation. Then the stomach was 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 oter. Then a tumor piece of 100mg was fixed under the skin. Observing anesthesia circumstances, animals were sent to the breeding room until consciousness. All animals tolerated the surgical procedure well with no anesthesia-related death.

### Animal specimen collection and pathological examination

All animal urine was collected in frozen tubes at the fourth week after implantation, and stored at -80°C

until processing. 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.

### Human gastric cancer and control samples

Human urine samples were collected from a total of 23 gastric cancer patients and 10 healthy volunteers from Shanghai Sixth People's Hospital, Shanghai Jiaotong University (Shanghai, China). All gastric cancer patients were diagnosed with different histopathological features and stages according to TNM classification: non-invasive (T1 and T2) gastric cancers, 7 patients; invasive (T3 and T4)/metastatic gastric cancers, 16 patients. All subjects signed an informed consent under local research ethics committee approval. The clinicopathological characteristics of the gastric cancer patients are summarized in Table 1. None of the patients received chemotherapy or radiotherapy prior to sampling. All urine samples were collected in the morning before breakfast and stored at -80°C until analysis.

### Sample pretreatment and derivatization

Each urinary specimen was transferred to a glass centrifuge tube, subsequently centrifuged at 18,000×g for 3 min and 50μL of the supernatant was gathered from each sample into a 1-mL EP tube, respectively. The collected supernatant was evaporated to dryness at 60°C for 24 hours, using vacuum dryer. Then 100μL THF, was added to each of the dried urine extracts, vortex-mixed for 2min, then we added 50μL TMCS to the mixture, vortex-mixed for 2min, and left the mixture to be incubated at 60°C, derivatized for 30min, so most compound have been completely derivatized. After return to ambient temperature, samples were prepared for GC/MS analysis.

### GC/MS analysis

1μL of each derivatized sample was injected splitless into

	number	Age (median, range)	Male/female	Stage IA/IB	Stage II	Stage IIIA/IIIB	Stage IV
non-invasive cancers	7	63, 53-72	6/1	2/5	/	/	/
invasive/metastatic cancers	16	67.6, 43-84	10/6	/	1	3/3	9
healthy controls	10	45.4, 37-57	6/4	/	/	/	/

Table 1: Clinical characteristics of patients and controls.

metabolites	retention time	P value <sup>a</sup>	A(normal)	B(cancer <sup>b</sup> )	R <sup>c</sup>
lactic acid	7.787	0.001	0.0278±0.0038	0.0369±0.0059	0.33
glycerol	13.253	0.001	0.0864±0.0251	0.0358±0.0158	-0.59
butanedioic acid	14.701	0.005	0.0191±0.0035	0.0138±0.0028	-0.28
L-serine	16.014	0.002	0.0009±0.0001	0.0006±0.0002	-0.33
L-threonine	16.832	0.008	0.0016±0.0002	0.0010±0.0008	-0.38
malic acid	20.084	0.033	0.0069±0.0019	0.0046±0.0020	-0.33
citric acid	29.589	0.003	0.1833±0.0343	0.1166±0.0478	-0.36
hexadecanoic acid	35.467	0.003	0.1402±0.0202	0.1877±0.0376	0.34

<sup>a</sup>P values were calculated based on Mann-Whitney test (significance at P<0.05). <sup>b</sup>Cancer group includes the non-metastasis group and the metastasis group. <sup>c</sup>R 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 present in cancer group while a negative value means a relatively lower concentration as compared to the normal.

Table 2a: List of different metabolites identified in normal control group and cancer group (metastasis and non-metastasis mice).

metabolites	retention time	P value <sup>a</sup>	A (non-metastasis)	B (metastasis)	R <sup>b</sup>
glycerol	13.253	0.046	0.0437±0.0157	0.0278±0.0119	-0.36
butanedioic acid	14.701	0.046	0.0153±0.0025	0.0124±0.0024	-0.19
L-serine	16.014	0.006	0.0007±0.0001	0.0005±0.0002	-0.29
L-threonine	16.832	0.027	0.0014±0.0008	0.0005±0.0005	-0.64
malic acid	20.084	0.036	0.0055±0.0018	0.0036±0.0019	-0.35
L-proline	21.329	0.046	0.0101±0.0040	0.0064±0.0024	-0.37
myo-inositol	36.316	0.003	0.0062±0.0013	0.0103±0.0027	0.66

<sup>a</sup>P values were calculated based on Mann-Whitney test (significance at  $P<0.05$ ). <sup>b</sup>R 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 present in metastasis group while a negative value means a relatively lower concentration as compared to the non-metastasis.

**Table 2b:** List of different metabolites identified in metastasis and non-metastasis groups.

metabolites	retention time	P value <sup>a</sup>	A(healthy controls)	B(cancer)	R <sup>b</sup>
lactic acid	7.069	0.000	0.0022±0.0002	0.0031±0.0008	0.41
glycerol	12.373	0.038	0.0145±0.0069	0.0086±0.0054	-0.41
butanedioic acid	13.801	0.014	0.0005±0.0001	0.0004±0.0001	-0.20
L-serine	15.134	0.000	0.0014±0.0004	0.0006±0.0003	-0.57
L-threonine	15.938	0.002	0.0011±0.0002	0.0006±0.0004	-0.45
citric acid	28.678	0.026	0.0045±0.0013	0.0034±0.0012	-0.24
hexadecanoic acid	34.543	0.000	0.0665±0.0075	0.1213±0.0282	0.82

<sup>a</sup>P values were calculated based on Mann-Whitney test (significance at  $P<0.05$ ). <sup>b</sup>R 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 present in cancer group while a negative value means a relatively lower concentration as compared to the healthy controls.

**Table 3a:** List of different metabolites identified in healthy controls and gastric cancer patients.

metabolites	retention time	P value <sup>a</sup>	A(non-invasive cancers)	B(invasive/metastatic cancers)	R <sup>b</sup>
glycerol	12.373	0.045	0.0119±0.0052	0.0071±0.0049	-0.40
butanedioic acid	13.801	0.027	0.0005±0.0001	0.0003±0.0001	-0.40
L-serine	15.134	0.003	0.0009±0.0002	0.0005±0.0003	-0.44
L-threonine	15.938	0.005	0.0010±0.0002	0.0004±0.0004	-0.60
L-proline	20.391	0.082	0.0041±0.0013	0.0029±0.0011	-0.29
myo-inositol	35.389	0.027	0.0029±0.0006	0.0039±0.0011	0.34

<sup>a</sup>P values were calculated based on Mann-Whitney test (significance at  $P<0.05$ ). <sup>b</sup>R 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 present in invasive/metastatic cancers while a negative value means a relatively lower concentration as compared to the non-invasive cancers.

**Table 3b:** List of different metabolites identified in non-invasive and invasive/metastatic gastric cancer patients.

an Agilent 6980 GC system equipped with an HP5MS capillary column(30m×0.25mm i.d., 0.25µm), electron impact ionization at 70eV, and a quadrupole mass spectrometric detector (Agilent Technologies, Palo Alto, CA, USA). The column temperature was initially held at 100°C for 3min, 10°C min<sup>-1</sup> up to 220°C, then 10°C min<sup>-1</sup> to 280°C, and remained there for 5min; The injector temperature was 280°C; Carrier gas was helium at a constant flow rate of 1.0 mL min<sup>-1</sup>. 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 from 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 NIST (National Institute of Standards and Technology) mass spectra library. Peaks with similarity index more than 70% were assigned compound names, while those having less than 70% similarity were listed as unknown metabolites [13]. 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 TMCS 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 single compound. The resulting three dimensional matrix includes sample information, peak intensities and peak retention time. Four

TIC profiles of consecutively injected samples of the same aliquot were presented (Figure 1), showing 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.

### Data processing and analysis

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. Comparisons between two groups were performed by Mann-Whitney test and for three groups or more, one-way ANOVA Kruskal-Wallis test was employed. Data were expressed as mean±SD. The differentially expressed compounds with  $P<0.05$  were considered statistically significant. Principal component analysis (PCA) was used to differentiate the samples. The differentiation performance was validated by the area under the curve (AUC) of receiver operating characteristic (ROC) curves. Statistical analyses were carried out using SPSS 16.0 for windows.

## Results

### Metabolic profiles between model mice and healthy controls

The mean body weight of mice was 26.79±0.57g, 27.06±0.62 and 27.13±0.58g for normal group, non-metastasis group and metastasis

group, respectively ( $P>0.05$ ), suggesting that the weight of mice did not differ among three groups. All animals of these three groups were alive at the fourth week. No tumor was found in any of normal control mice. All mice from non-metastasis and metastasis groups developed localized tumors at the implanted sites, which were poorly-differentiated adenocarcinomas under microscope. The non-metastasis group tumors ( $3.58\pm 0.10\text{g}$ ) were limited at the left oexter, and had no metastasis in regional lymph nodes, liver and other organs. The metastasis group had cancer tissues ( $3.64\pm 0.75\text{g}$ , vs non-metastasis group,  $P>0.05$ ) in the stomach, while metastatic tumors were also found in liver, regional lymph nodes, and other organs. From these, four mice developed metastatic tumors in regional lymph nodes, two in liver, and three in other organs. Consistent with our previous results, the orthotopically implanted human gastric cancer mouse model

developed metastases in a manner similar to that of human gastric cancer and at an early stage [12,14].

GC/MS TICs of urine samples derived from the normal, non-metastatic and metastatic groups are presented in Figure 2. With GC/MS, around 120 signals were detected per sample using mass spectral deconvolution software for peak detection. Urine GC/MS data from these three groups were analyzed. Metabolites that were selected by Mann-Whitney test were listed in Table 2, after normalization of data. Eight metabolites were different between the normal group and the cancer group (non-metastasis and metastasis models, Table 2a). Lactic acid and hexadecanoic acid were found at higher levels, while glycerol, butanedioic acid, serine, L-threonine, malic acid and citric acid were lower in cancer urine samples (non-metastasis and metastasis mice,

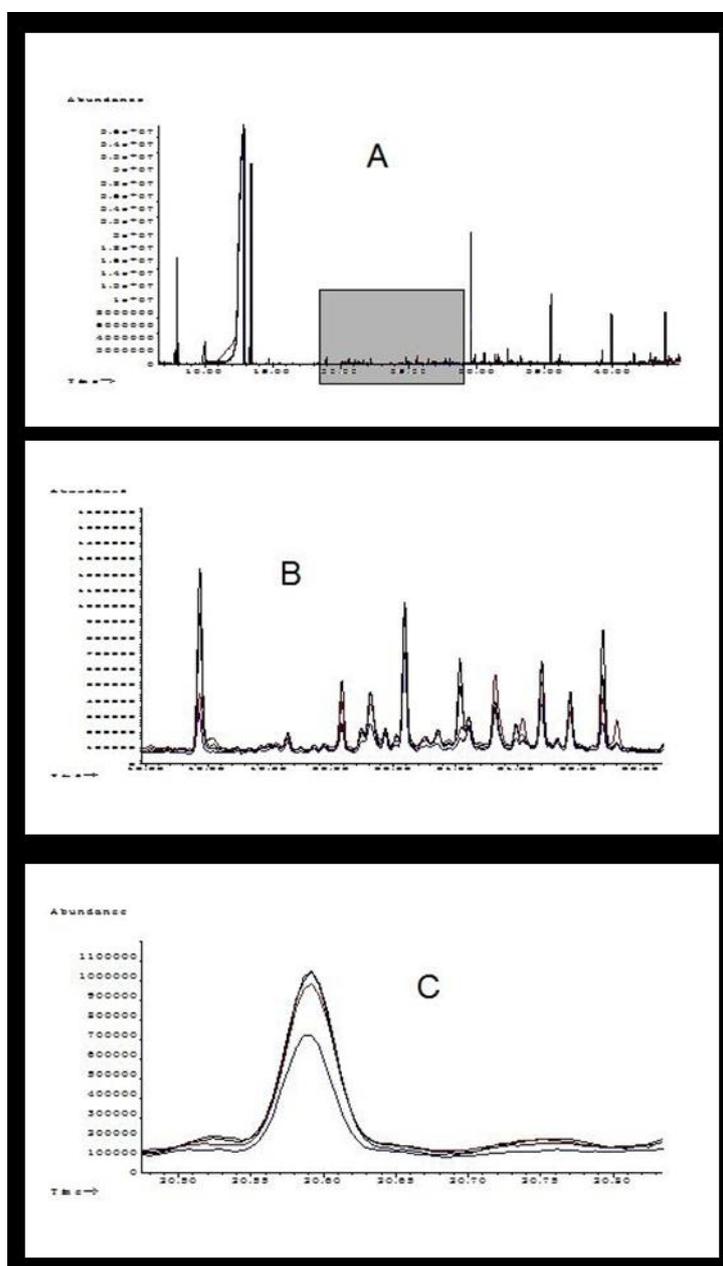
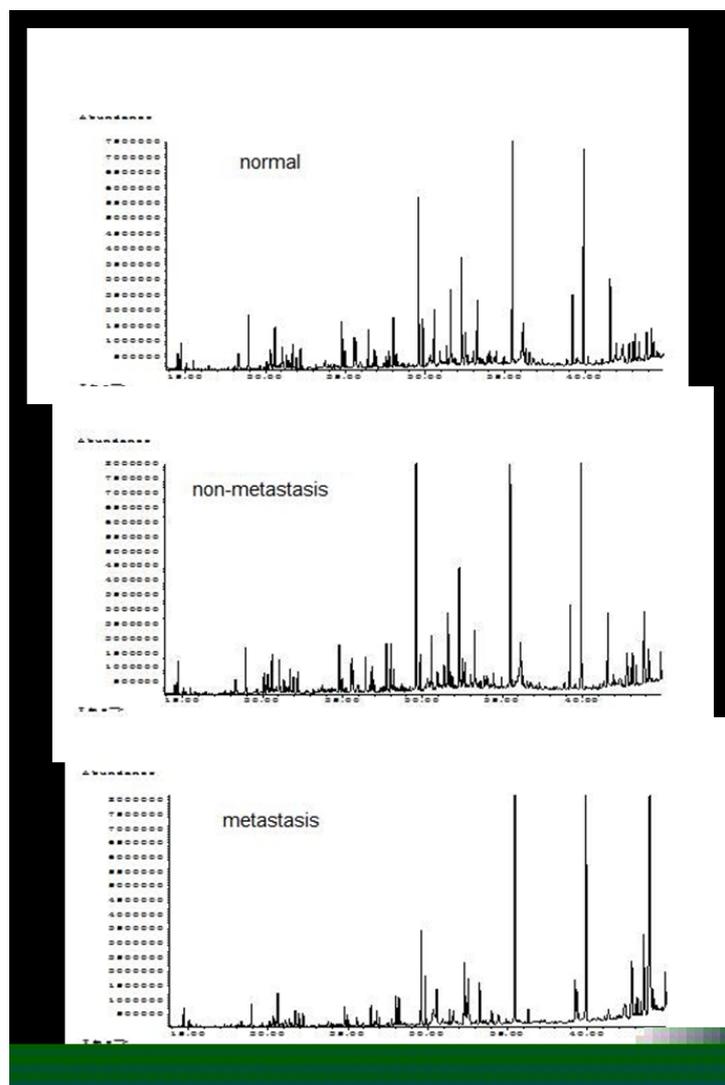


Figure 1: The overlay chromatograms of four parallel samples. A: the total TICs of GC/MS analysis; B: enlarged part of TIC from 18 to 28 min; C: one peak enlarged.



**Figure 2:** Representative GC/MS TICs of the samples from three groups (normal, non-metastasis, and metastasis) after chemical derivatization.

Table 2a) compared to normal mice. A PCA model was constructed using the marker metabolite intensities as variables. PCA analysis demonstrated good separation when comparing urine samples from tumor-bearing mice to controls. We correctly separated all 8 non-metastatic urine samples and all 8 metastatic urine samples from controls as shown in Figure 3A. ROC analysis, which was carried out using the values determined by the first two components of the PCA model, confirmed the robustness of the PCA model (Figure 3B). AUC value of this PCA model was 1.00.

As can be seen from Table 2b, there were seven metabolites responsible for the separation of urine samples from metastatic mice from non-metastatic. Glycerol, butanedioic acid, L-serine, L-threonine, malic acid and L-proline were remarkably lower in metastasis group compared to non-metastasis group. However, the level of myo-inositol was significantly higher in metastatic group as compared to non-metastatic (Table 2b). Figure 4A showed another PCA model by seven marker metabolites between the non-metastasis and metastasis groups. The model was subjected subsequently to ROC analysis with AUC value of 1.00 as shown in Figure 4B. These results showed that we successfully

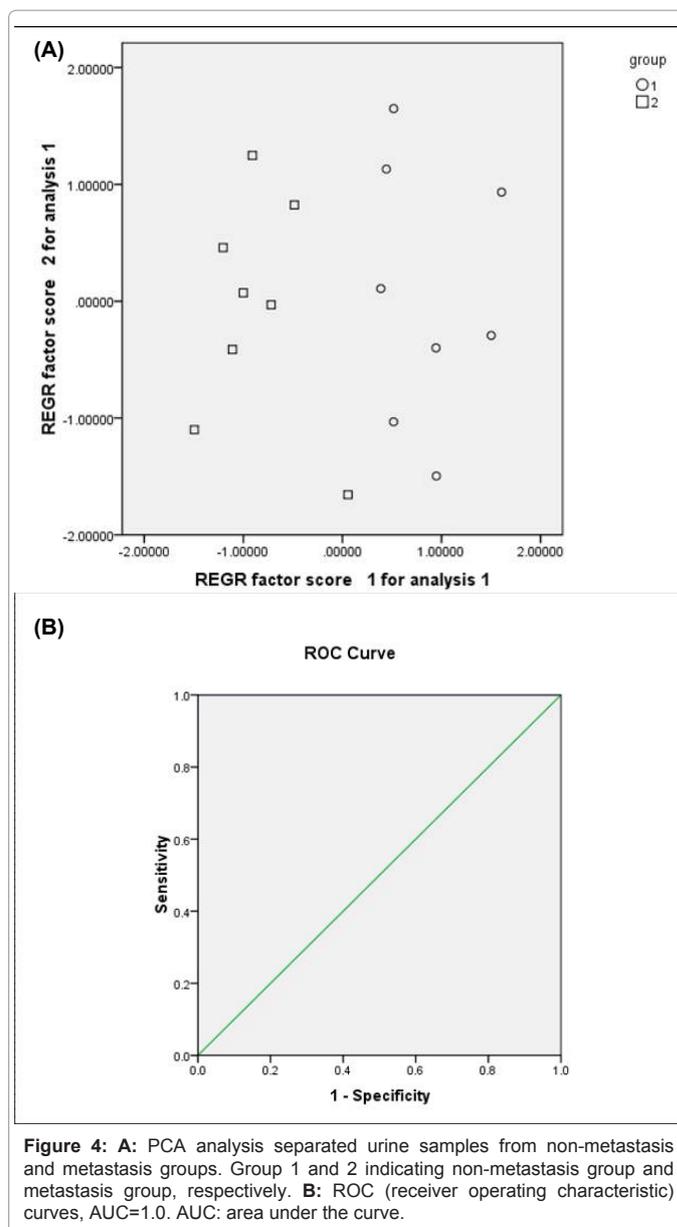
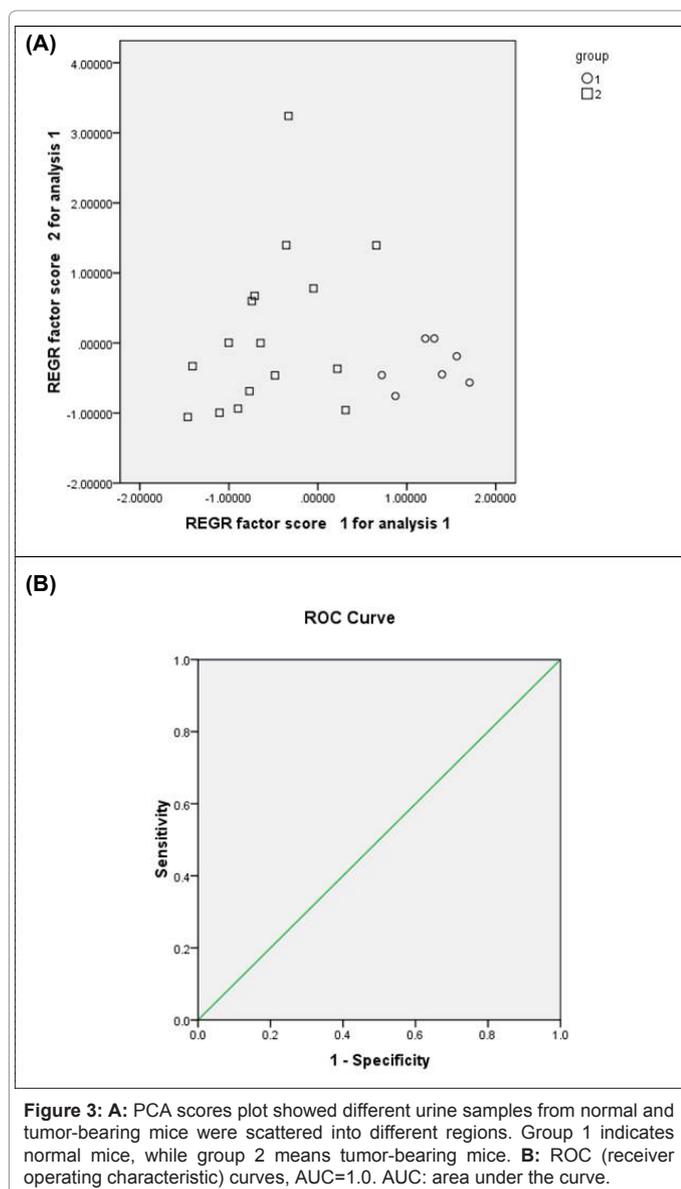
built human gastric cancer non-metastasis and metastasis models by implantation of histologically intact tissue of SGC-7901 human gastric carcinoma into SCID mice, and that we could differentiate between urinary samples from mice of different metastatic potentials as well as between tumor-bearing mice and normal controls using GC/MS based metabolomic methods.

### Metabolic profiles between gastric cancer patients and healthy controls

Similarly, urine samples derived from the gastric cancer patients and healthy controls were analyzed with above GC/MS methods. Around 180 signals were detected per sample using mass spectral deconvolution software for peak detection. Seven differential metabolites were identified between gastric cancer patients and healthy controls as shown in Table 3a. Lactic acid and hexadecanoic acid were found to be up-regulated in gastric cancer, while glycerol, butanedioic acid, serine, threonine and citric acid were down-regulated compared to controls. From Figure 5A, it is clear that a PCA scores plot based on seven marker metabolites showed good separation between urine

samples from gastric cancer patients and healthy controls. AUC value of this PCA model was 0.996, which demonstrated a good diagnostic value for gastric cancer (Figure 5B).

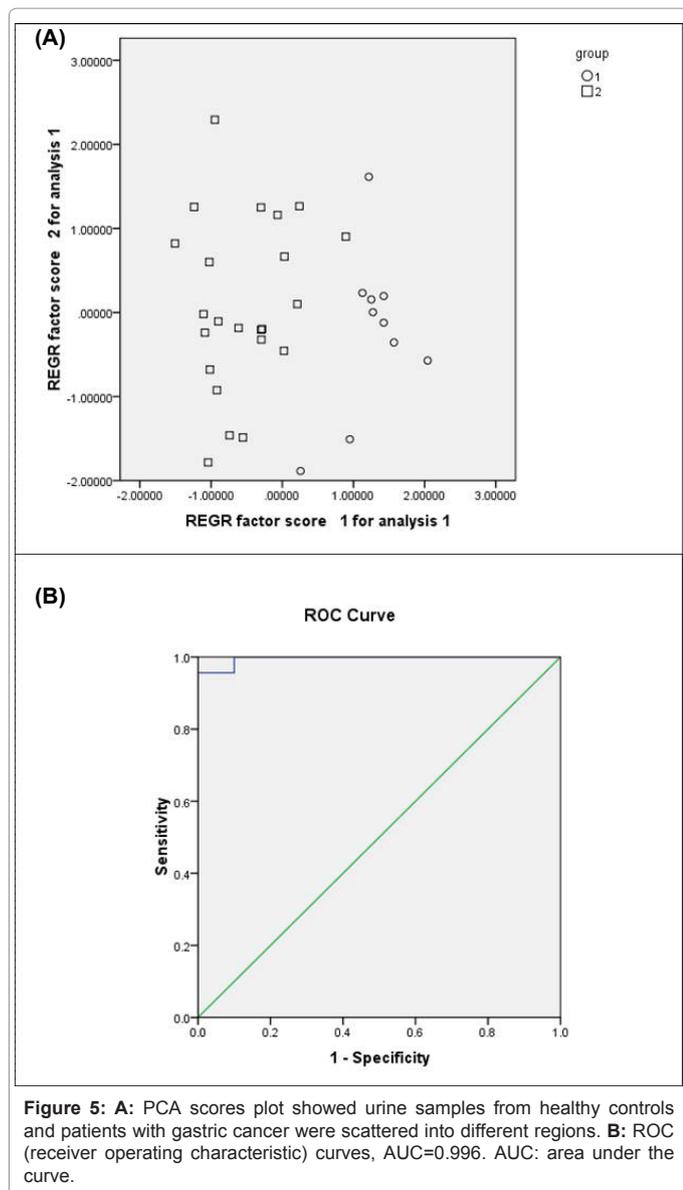
Furthermore, five metabolites with characteristic expression levels were identified between non-invasive and invasive/metastatic cancers in Table 3b. Glycerol, butanedioic acid, L-serine, and L-threonine were down-regulated in invasive/metastatic cancers when compared with non-invasive cancers. L-proline was down-regulated in invasive/metastatic cancers, although the difference was not statistically significant when compared to that of non-invasive cancers (invasive/metastatic versus non-invasive cancers,  $P=0.082$ ). However, compared with non-invasive cancers, myo-inositol was significantly up-regulated in invasive/metastatic cancers. The new constructed diagnostic model by five marker metabolites between the non-invasive and invasive/metastatic cancers was showed in Figure 6A. The model was subjected subsequently to ROC analysis (AUC=0.982, Figure 6B). These metabolites were of potential values as indicators for gastric cancer progression.



## Discussion

In this study, we investigated urinary metabolite profiling using GC/MS. This was assessed noninvasively by measuring tumor and healthy controls. We have differentiated all tumor-bearing mice and most gastric cancer patients from their healthy controls in a PCA analysis of GC/MS urinary metabolite spectra. Eight differential metabolites were found between tumor-bearing mice and healthy controls, while seven differential metabolites were identified between gastric cancer patients and healthy counterparts. A majority of the identified metabolites were observed in both mice and human specimens. Moreover, we have also differentiated between metastasis and non-metastasis models, as well as invasive/metastatic and non-invasive cancers in a PCA analysis. Seven up and down-regulated metabolites were found in both mice and human subjects with the same direction.

Of 8 identified differential metabolites in gastric cancer mice, butanedioic acid, malic acid and citric acid, intermediates of TCA



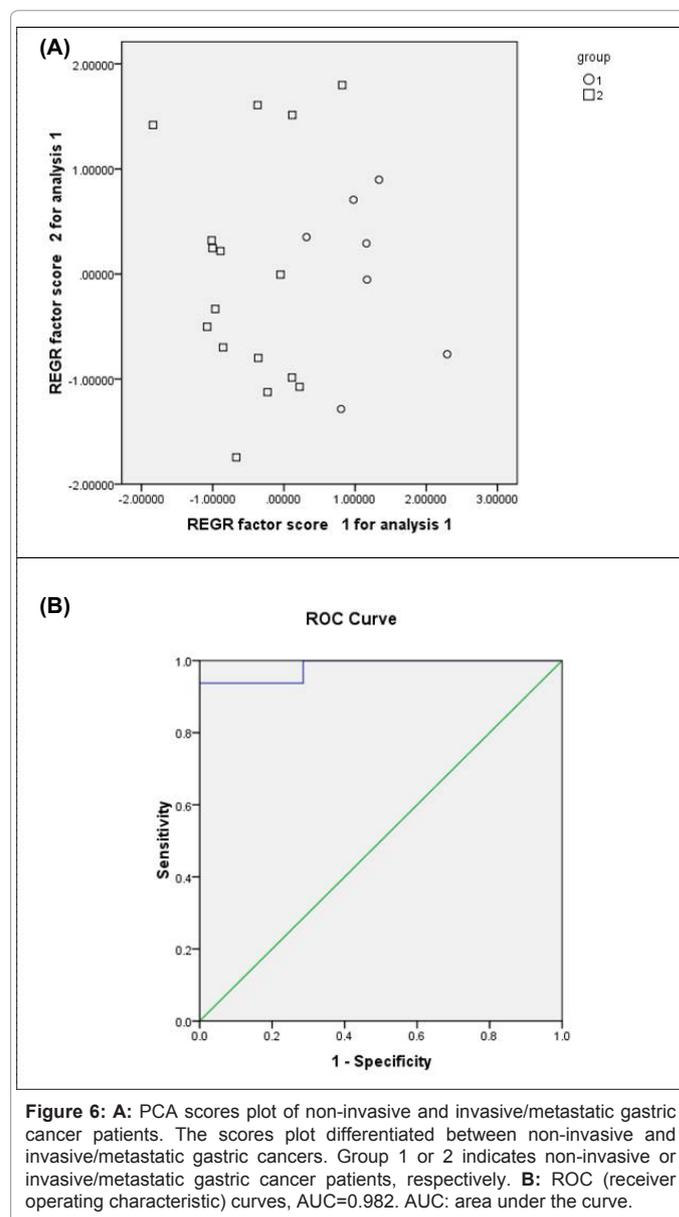
**Figure 5:** A: PCA scores plot showed urine samples from healthy controls and patients with gastric cancer were scattered into different regions. B: ROC (receiver operating characteristic) curves, AUC=0.996. AUC: area under the curve.

cycle, were decreased. This shows that TCA cycle is down-regulated in gastric cancer model. The similar results were also found in the human studies. The abnormalities of these metabolites expression demonstrate 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 stomach cancer and other malignancies including colorectal cancer, brain tumors [11,15-16]. The onset of gastric cancer and its several pathological stages are in energy consumption status, and cancer metabolizes the majority of the glucose through glycolysis, even in the presence of oxygen [17]. It could be explained that lactic acid, an end-product of glycolysis, was elevated in gastric cancer mice and patients as well.

Several differentially expressed metabolites were also found in amino acid metabolism. L-serine, L-threonine, glycogenic amino acid, were decreased both in tumor-bearing mice and gastric cancer patients, as compared with their controls. These two amino acids were involved in glycolysis. This is why the two amino acids are often declined in

gastric cancer urine. In addition, nucleotide synthesis is markedly up-regulated in tumor cell [18], while L-serine is a precursor of purine and pyrimidine. It also explains that L-serine was detected at decreased level in cancer urine samples. Furthermore, some fatty acids and their metabolites were expressed differentially between gastric cancer and healthy controls. In the study, glycerol was decreased and hexadecanoic acid was elevated both in mice and patients with gastric cancer, as compared with their controls. Fatty acid synthase (FAS), a key enzyme of fatty acid synthesis, is highly expressed in stomach cancer [19]. Therefore, hexadecanoic acid was at elevated level in cancer urine. The decreased level of glycerol in the urine samples could be interpreted as it got via the way of gluconeogenesis into the glycolytic pathway, which produced energy for the tumor growth.

Tumor invasion and metastasis could be considered as a significant prognostic factor [20]. Apart from these differentially expressed metabolites which were found in model mice and gastric cancer patients, compared with healthy controls. Six metabolites also have



**Figure 6:** A: PCA scores plot of non-invasive and invasive/metastatic gastric cancer patients. The scores plot differentiated between non-invasive and invasive/metastatic gastric cancers. Group 1 or 2 indicates non-invasive or invasive/metastatic gastric cancer patients, respectively. B: ROC (receiver operating characteristic) curves, AUC=0.982. AUC: area under the curve.

differential expression between non-metastasis and metastasis mice, and the similar results were also found between non-invasive and invasive/metastatic cancers in the human studies. Some metabolites, such as glycerol, butanedioic acid, L-serine and L-threonine have participated in glycolysis or TCA cycle to provide more energy for tumor progression. This is why levels of these metabolites were found to be lower in metastasis mice and in invasive/metastatic cancers, compared with non-metastasis mice and non-invasive gastric cancer patients.

Moreover, the expression of some serine/threonine protein kinases, like integrin-linked kinase (ILK), mammalian target of rapamycin (mTOR) was involved in the development and progression of gastric carcinoma in recent reports [21,22]. It could also explain that L-serine and L-threonine were at decreased level in metastasis model and invasive/metastatic gastric cancers. The decreased level of L-proline in urine samples from metastasis mice and invasive/metastatic gastric cancers may be interpreted as increased demand for structural proteins synthesis. These proteins, including receptors, membrane channels and enzymes, play a significant role in tumor progression and metastasis [23-25]. Some scholars have reported that myo-inositol trispyrophosphate (ITPP) markedly reduced tumor progression [26]. Excessive amount of myo-inositol has been lost in urine so that it weakly reduced tumor progression. Therefore, myo-inositol was at elevated level in mice and patients during progression of cancer.

In this study, we identified several important metabolic pathways associated with progression of gastric cancer. These metabolic pathways were mainly involved in glycolysis (lactic acid), serine metabolism (serine), proline metabolism (proline), TCA cycle (malic acid), and fatty acid metabolism. To the best of our knowledge, this is the first urinary metabolomic study on gastric cancer progression. Further clinical sample analysis of the metabolic pathways is needed to demonstrate their roles in gastric cancer development. The metabolic pathways may be exploited as biomarkers for gastric cancer progression, which could be applied for screening and early diagnosis of gastric cancer.

#### Conflict of Interest

The authors state no conflict of interest.

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