Metabolomics of gastric cancer metastasis detected by gas chromatography and mass spectrometry

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Supported by Grants from Shanghai Key Program of Science and Technology Committee (09JC1411600) and Shanghai Natural Science Foundation (08ZR1411300)

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Received: September 5, 2010 Revised: October 19, 2010 Accepted: October 26, 2010 Published online: December 14, 2010

Abstract

AIM: To elucidate the underlying mechanisms of metastasis and to identify the metabolomic markers of gastric cancer metastasis.

METHODS: Gastric tumors from metastatic and non-metastatic groups were used in this study. Metabolites and different metabolic patterns were analyzed by gas chromatography, mass spectrometry and principal components analysis (PCA), respectively. Differentiation performance was validated by the area under the curve (AUC) of receiver operating characteristic curves.

RESULTS: Twenty-nine metabolites were differentially expressed in animal models of human gastric cancer. Of the 29 metabolites, 20 were up-regulated and 9 were down-regulated in metastasis group compared to non-metastasis group. PCA models from the metabolite profiles could differentiate the metastatic from the non-metastatic specimens with an AUC value of 1.0. These metabolites were mainly involved in several metabolic pathways, including glycolysis (lactic acid, alanine), serine metabolism (serine, phosphoserine), proline metabolism (proline), glutamic acid metabolism, tricarboxylic acid cycle (succinate, malic acid), nucleotide metabolism (pyrimidine), fatty acid metabolism (docosanoic acid, and octadecanoic acid), and methylation (glycine). The serine and proline metabolisms were highlighted during the progression of metastasis.

CONCLUSION: Proline and serine metabolisms play an important role in metastasis. The metabolic profiling of tumor tissue can provide new biomarkers for the treatment of gastric cancer metastasis.

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Key words: Gastric cancer; Metastasis; Metabolite; Metabolomics; Gas chromatography and mass spectrometry

Peer reviewer: Ki-Baik Hahm, MD, PhD, Professor, Gachon Graduate School of Medicine, Department of Gastroenterology, Lee Gil Ya Cancer and Diabetes Institute, Lab of Translational Medicine, 7-45 Songdo-dong, Yeonsu-gu, Incheon 406-840, South Korea

Chen JL, Tang HQ, Hu JD, Fan J, Hong J, Gu JZ. Metabolomics of gastric cancer metastasis detected by gas chromatography and mass spectrometry. World J Gastroenterol 2010; 16(46): 5874-5880 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i46/5874.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i46.5874

INTRODUCTION

Gastric cancer is one of the most common malignancies and the second cause of cancer-related death worldwide and in most Asian countries, such as China1[2]. So far, surgical resection remains the only curative treatment option[3]. However, because of its asymptomatic properties,
most patients are frequently misdiagnosed until local and distant metastases occur, leading to a poor prognosis of gastric cancer patients with a 5-year survival rate of less than 30%. Most gastric cancer-related deaths occur as a result of metastasis. Metastatic recurrence is the main obstacle to the improvement of therapy for gastric cancer. No effective treatment modality is available for this deadly disease at present. Similarly, almost no prophylactic therapies can block dissemination of gastric cancer cells and prevent its metastasis. Currently, histological staging of gastric cancer is mainly based on the depth of its invasion and metastasis, both of which are considered the most important indicators of recurrence and prognosis of gastric cancer after curative resection. It has been shown that some gene candidates, such as cell adhesion molecules, are involved in the process of gastric cancer metastasis. However, no routine molecule markers for predicting gastric cancer metastasis and prognosis are available because of their high variability in expression levels.

The prognosis of patients with advanced gastric cancer remains very poor because the molecular mechanism underlying its metastasis is not fully understood. Gastric cancer metastasis, which is a complex and multistep process, involves release, migration and penetration of its cells through the vessel walls, arrest of its cells in microcirculation of distant organs and their subsequent migration and growth at the metastatic sites. To get a better insight into the mechanism of such a process, metastasis and non-metastasis animal models of gastric cancer were established using the human gastric cancer cell line SGC-7901. Because of the same genetic backgrounds, the animal is a suitable comparative system for studying the molecular changes in gastric cancer metastasis. At present, most investigations are focused on the identification of altered genes and proteins that play an important role in gastric cancer progression. However, only a few reports are available on the identification of key metabolites characterizing gastric cancer metastasis. Metabolomics, an omic science in systems biology, is the comprehensive and simultaneous profiling of metabolic changes occurring in living systems in response to genetic, environmental, or lifestyle factors. The missing link between genotype and phenotype can be established, which may provide information about gastric cancer that is complementary to genomics and proteomics analysis, thus improving our understanding of the pathogenic mechanisms and metabolic phenotype of gastric cancer. It has been recently shown that metabolomic method has great potentials in identifying the new diagnostic markers and therapeutic targets for different cancers, such as breast, prostate, pancreatic, liver, colon and gastric cancer, suggesting that metabolic alterations play a role in the biology of cancer. A more recent metabolomic analysis showed that increased sarcosine synthesis is an important metabolic change during prostate cancer progression. However, to our knowledge, there is no metabolomic study on metastasis of gastric cancer.

In this study, metabolomic difference in metastasis and non-metastasis models of gastric cancer was detected by gas chromatography (GC) and mass spectrometry (MS), respectively, in accordance with our hypothesis that there were metabolite clusters associated with metastasis of gastric cancer.

**MATERIALS AND METHODS**

**Chemicals**

Tetrahydrofuran (THF), N-methyl-N-t-butyldimethylsilyl trifluoro-acetamide (MBDSTFA) and chromatographic pure were purchased from Sigma Chemical Co. (St Louis, MO, USA). Pyridine, sodium hydroxide, chloroform, anhydrous ethanol, and anhydrous sodium sulfate, purchased from China National Pharmaceutical Group Corporation (Shanghai, China), were of analytical grade. Vacuum dryer was the product of Shanghai NOTED Technologies (China). Distilled water was obtained from the Milli-Q System (Millipore, MA, USA).

**Animals**

Male mice with severe combined immune deficiency (SCID) at the age of 6 wk, weighing 20-25 g, were obtained from Shanghai Experimental Animal Center, Chinese Academy of Sciences (China). The mice were housed under specific pathogen-free conditions with free access to food and drinking water. Experiment was performed in accordance with the Chinese National Guidelines for the Care and Use of Laboratory Animals and the relative ethical regulations of our university.

**Animal treatment**

Twenty-two SCID mice were randomly divided into metastasis group (n = 8), non-metastasis group (n = 8), and control group (n = 6). An animal model of metastasis was induced by orthotopic implantation of histologically intact tissue of human gastric carcinoma as previously described with some minor modifications. 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 subcutis of nude mice. Tumors were removed aseptically with necrotic tissues cleared away. The adjacent healthy tissues were cut into pieces (about 3 mm x 4 mm) in Hank’s balanced salt solution, weighed and adjusted to 100 mg. After the SCID mice were anesthetized with 4.3% trichloroacetaldehyde hydrate, an incision was made through the left upper abdominal pararectal line of mice in metastasis group with the peritoneal cavity carefully exposed. A part of the serosal membrane in middle of the greater curvature of stomach was mechanically injured using scissors. One hundred mg of tumor pieces was fixed at each injured site on the serosal membrane surface. After the stomach was returned to the peritoneal cavity, the abdominal wall and skin were closed. After the SCID mice were anesthetized with 4.3% trichloroacetaldehyde hydrate, an incision was made in the left inner of mice in non-metastasis group. Then, 100 mg of tumor tissue pieces was implanted into the subcutis (ectopic implantation) of mice. Meanwhile, the mice in non-metastasis group underwent the same ortho-
topic operation as those in metastasis group but with no tumor implantation into the gastric wall. The mice that underwent the same procedure with no tumor implantation served as a control group. All animals tolerated the surgical procedure well with no anesthesia-related death.

**Sample collection and pathological examination**

After anesthesia, all mice were sacrificed and subjected to autopsy. Tumors growing at the orthotopic or subcutaneous sites were harvested. Half of each tumor was snap-frozen at -80°C, while the other half was fixed in 4% formalin and embedded in paraffin. Tissues were collected from lymph nodes and all organs, fixed in 4% formalin, and processed for routine paraffin embedding after careful macroscopic examination. The tissue was cut into 4 μm-thick sections which were stained with hematoxylin and eosin, and evaluated histologically for metastasis of lymph nodes, liver or other organs by microscopy.

**Sample pretreatment and derivation**

One mL still water and one mL anhydrous ethanol were added to each 100 mg tissue sample. Gastric carcinoma tissue samples were ground with still water and anhydrous ethanol (1:1, v/v). The mixture was ultrasonicated at 4°C for 30 min and vortexed for 2 min. The tissue sample (0.1 g/mL) was centrifuged at 18,000 × g (10,000 rpm/min) for 10 min. The aqueous layer was adjusted to pH 7.8 with 0.1 mol/L potassium phosphate buffer solution, and 1.5 mL supernatant was obtained from each sample. The collected supernatant (500 μL) was evaporated to dryness at 50°C for 24 h in a vacuum dryer. Then 150 μL tetrahydrofuran was added to each of the dried tissue extracts, vortex-mixed for 10 min and evaporated to complete dryness in a nitrogen evaporator. MBDSTFA (100 μL) was added to each sample and derived at 60°C for 30 min. The samples were vortexed for 30 s after derivation, for GC and MS analysis.

**GC and MS analysis**

Each of the samples was injected into an Agilent 6980 GC system equipped with a HP5MS capillary column (30 mm × 0.25 mm, i.d., 0.25 μm), and a quadrupole mass spectrometric detector (Agilent Technologies, Palo Alto, CA, USA). Helium was used as the carrier gas with a constant flow rate of 1.0 mL/min. One μL sample was injected into the Agilent 6980 GC system at 280°C. The column temperature was initially kept at 100°C for 3 min, and then elevated to 220°C at an increasing rate of 10°C per min, followed by 10°C per min to 280°C for 5 min. Both of the interface and ion source temperature were 200°C. MS was conducted in an electron impact ionization mode at 70eV. Mass data were obtained in a full scan mode from m/z 100 to 600. Total ion chromatograms (TIC) and fragmentation patterns of GC were acquired using the GC/MS ChemStation Software (Agilent Technologies, Palo Alto, CA, USA). Compounds were identified by comparing the mass spectrum with a standard mass spectrum in the National Institute of Standards and Technology (NIST) Mass Spectra Library. Peaks with a similarity index more than 70% were the assigned compound names, while those with a similarity index less than 70% were considered unknown metabolites [16]. Chromatograms were subjected to noise reduction prior to peak area integration. Any known artificial peaks due to derivatization of column bleed and BSTFA should be excluded from the data set. The resulting three-dimensional matrix included sample information, peak retention time, and peak area.

**Statistical analysis**

Data, normalized by dividing the sum of all peak areas in the sample (1 mg) before multivariate analysis, were expressed as mean ± SD and introduced into SPSS 16.0 for Windows. Metabolite levels were compared by independent t test for the detection of significant differences in metastasis and non-metastasis groups. P < 0.05 was considered statistically significant. Principal components analysis (PCA) was performed to differentiate the metabolic patterns in metastasis and non-metastasis groups. The differentiation performance was validated by the area under the curve (AUC) of receiver operating characteristic (ROC) curves. Similarly, metabolomic differences in metastasis group, non-metastasis group and control group were analyzed by t test and PCA.

**RESULTS**

The mean weight of mice in three groups was 23.81 ± 0.16, 23.87 ± 0.19 and 23.98 ± 0.19 g, respectively. Microscopy showed localized poorly-differentiated adenocarcinoma in all mice of non-metastasis and metastasis groups at the implanted sites. The average tumor weight was 4.28 ± 0.20 g in non-metastasis group and 4.30 ± 0.3 g in metastasis group, indicating that the tumor grows at a similar rate in the subcutis or stomach. However, the metastatic rate was significantly different when the tumor was implanted into the ectopic or orthotopic sites. Two thirds of the mice bearing an orthotopic tumor developed metastatic tumors in the region of lymph nodes, half in liver and one fourth in other organs. In contrast, no tumor metastasis was observed in the non-metastasis group after ectopic implantation. Macroscopic and histological examination showed no gastric cancer in the mice without tumor implantation. These results indicate that GC and MS are of a high reproducibility in the retention time of metabolites. Of the 152 signals detected in the tissue samples, some were not consistently found in other samples or could not be assigned to the unique metabolites because the abundance was too low. Fifty-eight signals could be identified by comparing a standard mass chromatogram with that in the NIST Library (Table 1). According to the NIST Mass Spectra Database, most of the chromatograms were identified as endogenous metabolites, such as amino acids, organic acids, inorganic acids, fatty acids, and pyrimidines, which were involved in several metabolic pathways, including glycolysis (lactic acid, alaline), serine metabolism (serine, phosphoserine), proline metabolism (proline), glutamic acid metabolism, glutamine metabolism, tricarboxylic acid (TCA) cycle (succinate), nucleotide metabolism (pyrimidine), fatty acid
metabolism (docosanoic acid, and octadecanoic acid), and methylation(glycine).

The GC and MS data about tissue metabolites in metastasis and non-metastasis groups were analyzed by Student’s t test. Marker metabolites selected by Student’s t test are presented in Table 2. Among these metabolites, proline was the most up-regulated tissue metabolite in metastasis group, which was 2.45-fold higher than that in non-metastasis group. Glutamine was the most down-regulated tissue metabolite in the metastasis group, which was 1.71-fold lower than that in the non-metastasis group. The lactic acid, L-alanine, L-valine, leucine, malic acid, L-aspartic acid, serine, phosphoserine, dimethylglycine, glycine, L-glutamic acid, L-lysine, myo-inositol, propanedioic acid, docosanoic acid, octadecanoic acid, arginine, pyroline, and pyrimidine were significantly up-regulated, while the glucose, succinate, L-isoleucine, L-methionine, propanamide, L-threonine acid, and butanedioic acid were remarkably down-regulated in the metastasis group compared to the non-metastasis group. The main metabolic pathways associated with metastasis of gastric cancer included glycolysis (lactic acid, alaline), serine metabolism (serine, phosphoserine), proline metabolism (proline), tri-carboxylic acid (TCA) cycle (succinate, malic acid), fatty acid metabolism (docosanoic acid, and octadecanoic acid), and methylation(glycine).

The levels of lactic acid, propanedioic acid, L-alanine,
The lactic acid level was higher while the glucose level was lower in metastasis group than in non-metastasis group. Lactic acid is the end product of glycolysis. Increased glucose uptake and consumption are frequently observed in many cancer cells even under normoxic conditions, known as the Warburg effect. It has been demonstrated that the lactic acid level is increased in various metastatic cancers, including renal, uterine cervix, head and neck, colorectal cancers[25-27]. Moreover, the high lactate level in tumor tissue is associated with its metastasis and poor prognosis[27]. In fact, increased lactic acid produced by tumor cells can result in acid-mediated matrix degradation, T cell inactivation, up-regulation of VEGF and HIF-1 alpha, and enhancement of cell motility, thus providing favorable conditions for metastatic spread[28]. Therefore, high lactic acid levels reflect an increased energy demand for tumor progression.

In the present study, GC and MS showed that proline was the most up-regulated tissue metabolite, indicating that increased proline in metastatic gastric cancer tissue may be correlated with the increased turnover of extracellular matrix in metastatic cancer cells. Tumor cells need increased degradation of collagen during the process of invasion and metastasis, thus producing a large amount of proline. Pyrroline-5-carboxylic (P5C) is the precursor of proline and also its degradation product. Proline oxidase, also known as proline dehydrogenase, catalyzes the first step of proline to P5C in mitochondria and the latter is converted to proline by the cytosolic P5C reductase. It has been demonstrated that proline oxidase can be induced by p53 due to genotoxic stress and initiates apoptosis by the mitochondrial and death receptor pathways[28]. Proline is a stress substrate and matrix metalloproteinases can degrade collagen in the extracellular matrix. So far little attention has been paid to the correlation between proline metabolism and tumor progression. It has been shown that proline consumption is increased in patients with metastatic renal cancer[29]. Increased proline biosynthesis has been recently observed in metastatic breast cancer cell lines[30]. Furthermore, proline metabolism is linked with arginine and glutamate metabolism, TCA cycle and pentose phosphate pathway (PPP) due to P5C[28], suggesting that the significantly up-regulated metabolism of proline is highly correlated with cancer metastasis.

In this study, serine metabolism was involved in the metastatic process of gastric cancer, showing alterations in the pathway. The serine and phosphoserine increased 1.48-fold and 1.56-fold, respectively in metastatic tumors. It was reported that all the three genes involving the serine biosynthesis pathway, phosphoglycerate dehydrogenase (PHGDH), phosphoserine aminotransferase 1 (PSAT1),
and phosphoserine phosphatase (PSPH) are up-regulated in highly metastatic breast cancer cells\textsuperscript{[13]}, which is agreement with our results. Although most amino acids are increased in gastric cancer and colorectal cancer tissue compared with their adjacent normal tissue\textsuperscript{[17]}, little is known about the alterations related to metastatic behaviors. In the present study, the levels of leucine, valine glutamate and lysine were higher while the levels of methionine and threonine were lower in metastatic specimens, indicating that the demand for energy is increased in metastatic progression. Glycine can derive from serine synthesized by the glycolytic intermediate phosphoglycerate and its elevated levels may be associated with glycolysis. Sarcosine, a methylation derivative of the amino acid, glycine, is related to prostate cancer progression\textsuperscript{[10]}. Dimethylglycine is another methylation product of glycine and how it is up-regulated remains unclear.

The disturbed TCA cycle, observed in a large number of tumors including gastric cancer, is considered to be related to carcinogenesis\textsuperscript{[17]}. In this study, the TCA cycle intermediates such as succinate and malic acid were remarkably perturbed in metastatic specimens, suggesting that enhanced glycolysis contributes to metastatic progression. In this study, the expression patterns of metastatic and non-metastatic human carcinoma were compared by metabolomic analysis, and several important metabolic pathways associated with metastasis of gastric cancer were identified. Of note, proline and serine metabolites were highlighted in this study. Further functional and clinical sample analysis of the metabolic pathways is needed to demonstrate their role in gastric cancer metastasis. The metabolic pathways may be exploited as biomarkers for gastric cancer progression.

**COMMENTS**

**Background**

Gastric cancer is the second cause of cancer-related death worldwide, and its metastasis is one of the leading causes of cancer-related death. The molecular mechanisms underlying gastric cancer metastasis are still not fully understood. Recent metabolomic studies have shown that metabolic alterations play a role in the biology of gastric cancer. The metabolic profiling of tumor tissue is used to elucidate the underlying mechanisms and identify the metabolomic markers of gastric cancer metastasis for improving its diagnostic and therapeutic strategies.

**Research frontiers**

Metabolomics, an OMIC science in systems biology, is the comprehensive and simultaneous profiling of metabolic changes occurring in living systems in response to genetic, environmental and lifestyle factors. Gas chromatography (GC) and mass spectrometry (MS) have been widely applied in metabolomic investigation because of their high sensitivity, peak resolution and reproducibility.

**Innovations and breakthroughs**

Recently, most investigations have been focused on identifying the altered genes and proteins that play a role in cancer progression. In this study, the expression patterns of metastatic and non-metastatic human carcinoma were compared by metabolomic analysis, and several important metabolic pathways associated with metastasis of gastric cancer were identified. This is the first report on metabolomic investigation of gastric cancer metastasis.

**Applications**

The results of this study indicate that the metabolic pathways can be exploited as biomarkers for gastric cancer progression, which can be used in diagnosis and treatment of gastric cancer.

**Terminology**

Metabolomics, an OMIC science in systems biology, is the comprehensive and simultaneous profiling of metabolic changes occurring in living systems in response to genetic, environmental and lifestyle factors. Gas chromatography (GC) and mass spectrometry (MS) have been widely applied in metabolomic investigation because of their high sensitivity, peak resolution and reproducibility.

**Peer review**

To elucidate the underlying mechanisms of metastasis and identify metabolomic markers of gastric cancer metastasis, the authors performed GC/MS to identify the metabolomic difference in metastatic and non-metastatic lesions. The results indicate that proline and serine metabolism play an important role in gastric cancer metastasis, and that metabolic profiling of tumor tissue can provide new biomarkers for the treatment of gastric cancer metastasis.

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