Tissue-based metabolomics profiling reveals metabolic signatures and major metabolic pathways of gastric cancer

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Abstract

Purpose

This study was aimed to screen differential metabolites between gastric cancer (GC) and paracancerous (PC) tissues and find new biomarkers of GC.

Methods

GC (n = 28) and matched PC (n = 28) tissues were collected and LC-MS/MS analyses were performed to detect metabolites of GC and PC tissues in positive and negative models. Principal component analysis (PCA) and orthogonal projections to latent structures-discriminate analysis (OPLS-DA) were conducted to describe distribution of origin data and general separation and estimate the robustness and the predictive ability of our mode. Differential metabolites were screened based on criterion of variables with p value < 0.05 and VIP (variable importance in the projection) > 1.0. Receiver operating characteristic (ROC) analysis was performed to evaluate the diagnostic power of differential metabolites. Kyoto Encyclopedia of Genes and Genomes (KEGG) was performed to search for metabolite pathways and MetaboAnalyst was used for pathway enrichment analysis.

Results

Several metabolites were significantly changed in GC group compared with PC group. Thirteen metabolites with high VIP were chose and among which 1-methylnicotinamide, dodecanoic acid and sphinganine possessed high AUC values (AUC > 0.8) indicating an excellent discriminatory ability on GC. Pathways such as pentose phosphate pathway and histidine metabolism were focused based on differential metabolites demonstrating their effects on progress of GC.

Conclusions

In conclusion, we investigated the tissue-based metabolomics profile of GC and several differential metabolites and signaling pathways were focused. Further study is needed to verify those results.

Background

Gastric cancer (GC) is one of the most common malignant tumors, and ranked fifth as the cause of death among 36 cancers in the world (Bray et al. 2018). About 50% of cancer patients in China are gastrointestinal tumors, mainly for GC, and five-year survival rate is not more than 35% (Feng et al. 2019). GC shows a multistep progression (Wang et al. 2016). The 5-year survival rate of patients with advanced GC is less than 20%, while it may reach more than 90% that just invades mucosal or submucosal layer (Jung et al. 2014). However, most patients have reached middle and advanced stage by the time GC is found, which leads to a poor prognosis since GC has no specific clinical symptoms in the early stage. Therefore, it’s important significantly to explore mechanism of GC and identify biomarkers for early diagnosis.

Metabolomics is a novel technique that explores biological states of metabolites among tissue extracts and body fluids such as plasma, serum and urine (Holmes et al. 2008; Nicholson et al. 2008) and has been used to characterize the metabolic disorders and identify potential biomarkers of various cancers (Cala et al. 2018; Chen et al. 2019; Denkert et al. 2008b). Studies on GC metabolomics were originate from plasma (Choi et al. 2016; Kuligowski et al. 2016; Lario et
al. 2017), serum (Corona et al. 2018; Liu et al. 2018), urine (Chan et al. 2016) samples mostly, but there are fewer studies related to GC tissues. Metabolic profiling of GC patients based on tissue samples with and without lymph node metastasis (LNM) was performed and some differential metabolites were identified as potential factors of diagnose and prognosis of GC patients with or without LNM (Zhang et al. 2018). This study was aimed to screen differential metabolites between GC and paracancerous (PC) tissues and identify potential biomarkers of GC.

**Materials And Methods**

**Patient selection**

Patients recruited in the study were diagnosed as GC according to results of gastroscope and biopsied. The inclusion and exclusion criteria were made. Inclusion criteria included: (i) aged between 20 and 80 years, both male or female; (ii) primary tumor; (iii) no treatment about cancer such as radiation, operation and chemoradiotherapy. Exclusion criteria were: (i) with metabolic disease including hyperlipoidemia, diabetes mellitus and gout; (ii) with congenital diseases; (iii) severe gastric cancer (survival < 2 months); (iv) with distant metastases.

The study was approved by Ethics Committee of Henan Provincial People's Hospital and the registration number was ChiCTR2100041912.

**Sample collection**

GC and PC tissues were obtained from dissected specimens of patients receiving radical gastrectomy. Samples were washed off immediately with phosphate buffered saline and frozen in liquid nitrogen and stored at -80 °C.

**Preparation of tissue extraction**

Prior to analysis, 50 mg of sample was weighted and 1000 μL extract solution (acetonitrile: methanol: water = 2: 2: 1, with isotopically-labelled internal standard mixture) was added. After 30 s vortex, samples were grind at 35 Hz for 4 min and homogenized by sonicating for 5 min in ice-water bath, and then those steps were repeated for 3 times. Samples were incubated for 1 h at -40 °C and centrifuged at 12000 rpm for 15 min at 4 °C. The supernatant was transferred to a fresh glass vial for analysis.

**LC-MS/MS analysis**

LC-MS/MS analyses were performed by an UHPLC system (Vanquish, Thermo Fisher Scientific) with an UPLC BEH amide column (2.1 mm * 100 mm, 1.7 μm) coupled to Q Exactive HFX mass spectrometer (Orbitrap MS, Thermo). Mobile phase was composed by water phase [25 mmol/L ammonium acetate and 25 ammonia hydroxide in water (pH = 9.75)] and acetonitrile (Shi et al. 2020). The injection volume was 3ml and the auto-sampler temperature was 4 °C. The Q Exactive HFX mass spectrometer was used to acquire MS/MS spectra on information-dependent acquisition (IDA) mode in the control of the acquisition software (Xcalibur, Thermo). In this mode, the acquisition software continuously evaluates the full scan MS spectrum. The ESI source conditions were set as following: sheath gas flow rate as 25 Arb, Aux gas flow rate as 20 Arb, capillary temperature 350 °C, full MS resolution as 60000, MS/MS resolution as 7500, collision energy as 10/30/60 in NCE mode, spray Voltage as 3.6 kV (positive) or -3.2 kV (negative), respectively.

**Data preprocessing and annotation**

The original data were converted to the mzXML format by ProteoWizard and R package XCMS (version 3.2) (Kuhl et al. 2012), an in-house program, was used for peak detection, extraction, alignment, and integration. Then an in-house MS2
database (BiotreeDB, V2.1) was applied in metabolite annotation (Chen et al. 2020). The cutoff for annotation was set at 0.3.

**Statistical analysis**

The data were processed by SIMCA 16.0.2 software package (Sartorius Stedim Data Analytics AB, Umea, Sweden) (Chen et al. 2020; Triba et al. 2015) for principal component analysis (PCA) and orthogonal projections to latent structures-discriminate analysis (OPLS-DA). As an unsupervised pattern recognition method, PCA shows distribution of origin data and general separation and OPLS-DA was used to obtain maximal covariance between variables and sample category in both positive and negative models. 7-Fold cross-validation and 200 permutation tests were used to estimate the robustness and the predictive ability of our mode (Deng et al. 2019).

Variables with p value < 0.05 and VIP (variable importance in the projection) > 1.0 were defined as differential metabolites between GC and PC groups and results were presented by volcano plots. Box plots were made by ggplot2 function of R package. To further evaluate the diagnostic power of the potential biomarkers, receiver operating characteristic (ROC) analysis was carried out by plotROC function of R package and 95% confidence interval (CI) was calculated by pROC function.

Kyoto Encyclopedia of Genes and Genomes (KEGG) was performed to search for metabolite pathways and MetaboAnalyst was used for pathway enrichment analysis based on differential metabolites.

**Results**

**Baseline clinical characteristics of patients**

A total of 28 GC tissues and 28 matched tissues from the same patient were collected. There were 19 males and 9 females and their average age and BMI were 60±11 years and 21.6±2.7 kg/m$^2$. The rates of patients with smoking or drinking hobbies respectively were 53.6% and 35.7%.

**Metabolic profiles of gastric cancer**

An overview of study profile is showed in Fig 1. A total of 4893 peaks (negative ion mode: 2163, positive ion mode: 2730) were obtained and 485 metabolites (negative ion mode: 145, positive ion mode: 340) were identified. The UHPLC-QE-MS spectra examples of the same person were shown in Fig 2. There were some different mass spectrum peaks between GC and PC groups.

Examination of the PCA and OPLS-DA score plots (Fig 3a-3d) showed that most of samples were within 95% CI, but was failed to provide satisfactory separation of data. Permutation test of OPLS-DA models for the two groups were carried out to prevent overfit of models (Fig 3e and f). The abscissa represents correlation between random group and original group, and ordinate represents scores of R2Y and Q2. When permutation retention is 1, the values of Q2 and R2Y are close to 0.5 and 1 respectively, and Q2 is with positive slope showing that models have good predictability and do not overfit.

**Potential biomarker analysis for discrimination**

According to the criteria that p-value < 0.05 and VIP > 1, significantly differential metabolites between two groups were screened from all identified metabolites (Fig 4). There are 35 significantly different peaks (27 up-regulated and 8 down-regulated) in negative ion model, and 76 significantly different peaks (29 up-regulated and 47 down-regulated) in positive ion model existing in GC group compared with PC group.
Thirteen interestingly differential metabolites with high VIP values were chose and the differences in relative expression between two groups were shown in Fig 5 and table 1. Compared with PC group, seven high-expressed metabolites, including 1-methylnicotinamide, sphinganine, N-acetylputrescine, m-coumaric acid, imidazoleacetic acid, formiminoglutamic acid and gluconolactone were found in GC group. Meanwhile, six metabolites of dodecanoic acid, myo-inositol, D-glutamine, palmitoleic acid, camosine and prostaglandin D2 were significantly lower expressed.

ROC analysis was conducted to estimate the diagnostic value of differential metabolites. All of selective metabolites showed nice ROC curves with high area under the curve (AUC) values (showed in table 1), among which 1-methylnicotinamide, dodecanoic acid and sphinganine possessed high AUC values (AUC>0.8) indicating an excellent discriminatory ability (Fig 6).

Pathway analysis of differential metabolites

Metabolic pathways on differential metabolites were analyzed by KEGG and MetaboAnalyst. As shown in Fig 7 and table 2, mainly five significantly altered pathways, namely histidine metabolism, alanine, aspartate and glutamate metabolism, sphingolipid metabolism, pentose phosphate pathway and cysteine and methionine metabolism, were revealed.

Discussion

In this study, we analyzed metabolic profiling of GC based on GC and PC tissues and found that some metabolites were significantly changed in GC group compared with PC group. Further analysis demonstrated that 1-methylnicotinamide, dodecanoic acid and sphinganine represented excellent clinical diagnostic capability for GC patients and some pathways were focused based on differential metabolites.

1-Methylnicotinamide (1-MNA), a metabolite of nicotinamide metabolic pathway, is transformed from N-methylation of nicotinamide conducted by nicotinamide N-methyltransferase (NNMT) and plays a key role in intracellular concentration of nicotinamide. Some researches described its effects about anti-inflammatory (Bryniarski et al. 2008), anti-thrombotic (Chlopicki et al.2007) and fibrinolytic (Mogielnicki et al. 2007). Tumor microenvironment derived 1-MNA contributes to immune modulation of T cells and represents a potential immunotherapy target to treat human cancer (Kilgour et al. 2021). However expression change of 1-MNA in cancer is still unknown, but the relationship between NNMT and cancers has been reported. Expression of NNMT was increased in multiple cancers, such as gastric cancer (Lim et al. 2006), lung cancer (Tomida et al. 2009) and colorectal cancer (Roessler et al. 2005). But Negative correlation was found between NNMT expression and size and progression of tumor suggesting that NNMT may have potential effect in an initial step of malignant conversion (Lu et al. 2018). 1-MNA was deemed to play a key role in mediating cellular actions of NNMT. So 1-MNA was likely to participate in the process of cancer. Our study illustrated the expression level of 1-MNA was significantly higher in GC tissues compared with PC tissues and owned excellent diagnostic capability for GC patients though mechanism is unclear.

Camosine possessed inhibitory effects on growth of tumor cells and tumor cell glycolysis (Horii et al. 2012) that was associated with cellular mitochondrial bioenergetics (Cheng et al. 2019; Shen et al. 2014). Our study found that the level of camosine was decreased in GC tissues compared with PC tissues that was corresponded to studies mentioned above. Histidine metabolism was enriched due to metabolites including camosine, formiminoglutamic acid and imidazoleacetic acid. Histidine was identified as a potentially discriminant metabolite (Jing et al. 2018), and histidine metabolism was found in enrichment of functions and signaling pathways in patients with GC (Lario et al. 2017) illustrating that histidine metabolism was involved in progression of GC.
Prostaglandin D2 (PGD2), as an important metabolite of arachidonic acid (AA) metabolism, was showed to have antitumor effects (Iwanaga et al. 2014; Kim et al. 2005; Murata et al. 2011). A signaling pathway involved in PGD2 and PTGDR2 (prostaglandin D2 receptor 2) restricted the self-renewal of GC cells and suppressed tumor growth and metastasis indicating their effects on GC (Zhang et al. 2018a). Our study showed that the level of PGD2 was decreased in GC tissues compared with PC tissues verifying its anticancer effect (Zhang et al. 2018a) in terms of clinical samples. AA metabolism was enriched in our study and reported to play a key role in inflammation and tumorigenesis (Cuendet et al. 2000; Wang et al. 2010; Yarla et al. 2016). It has been evaluated as chemopreventive agent for tumor treatment by some inhibitory synthetic molecules (Brandao et al. 2013; Smalley et al. 1997).

Fatty acid biosynthesis pathway was focused due to dodecanoic acid and palmitoleic acid. Both of two metabolites were at relatively low level in GC tissues and dodecanoic acid showed nice diagnostic function on GC. Polyunsaturated fatty acid biosynthesis pathway has an effect on GC by determining ferroptosis sensitivity (Lee et al. 2020) that is associated with several pathological conditions including cancer (Li et al. 2020). Signal transduction and transcriptional activator 5A (STAT5A) promotes tumorigenesis of GC cells though reprograming fatty acid metabolism stating indirectly the relationship between fatty acid metabolism and tumorigenesis of GC (Dong et al. 2019). Fatty acid metabolism was enriched in a study about urinary metabolic profiling of GC (Liang et al. 2015) that is consistent with our results.

Pentose phosphate pathway (PPP) was deemed to participate in the progression and/or poor prognosis of GC (Wang et al. 2018; Yin et al. 2013). Rev-erba, a nuclear receptor, inhibited proliferation of human gastric cancer cells by reducing glycolytic flux and PPP (Tao et al. 2019). As a controller of PPP, silencing of transketolase-like protein1 (TKTL1) inhibited proliferation of human GC cells in vitro and in vivo (Yuan et al. 2010). In this study, PPP was enriched and one of its metabolite, gluconolactone, was increased significantly in GC.

There are some limitations in the study. Firstly, the sample size was relatively small, although the GC and PC tissues were matched. Secondly, all patients were recruited from single center that might limit their generalization. Our study may be regarded as a preliminary study to explore metabolic characteristic of GC and PC tissues and further research with large sample, multiple research centers and verification in other omics technology was expected to confirm our results.

In conclusion, we investigated the tissue-based metabolomics profile of GC and several differential metabolites and signaling pathways were focused. Further study is needed to verify those results.

**Declarations**

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**Availability of data and material:** Not applicable.

**Code availability:** Not applicable.

**Authors' contributions:** Not applicable.

**Ethics approval:** The study was approved by Ethics Committee of Henan Provincial People's Hospital and the registration number was ChiCTR2100041912.
Consent to participate: This study was exemption of informed consent because samples involved in the study were the remaining samples of other trials.

Consent for publication: Not applicable.

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Table 1 Major differential metabolites between GC and PC groups
| NO | Metabolites                              | Regulation | Polarity | VIP  | P value | Fold chang | AUC       | 95% CI      | Metabolism pathways                                      |
|----|-----------------------------------------|------------|----------|------|---------|------------|-----------|-------------|--------------------------------------------------------|
| 1  | 1-Methylnicotinamide                     | Up         | POS      | 2.89 | 0.0000  | 0.30       | 0.904     | 0.823-0.986  | Nicotinate and nicotinamide metabolism                  |
| 2  | Sphinganine                              | Up         | POS      | 2.45 | 0.0042  | 0.22       | 0.847     | 0.748-0.946  | Sphingolipid metabolism                                |
| 3  | N-Acetylputrescine                       | Up         | POS      | 2.44 | 0.0125  | 0.34       | 0.760     | 0.630-0.890  | Arginine and proline metabolism                        |
| 4  | Dodecanoic acid                          | Down       | NEG      | 2.31 | 0.0000  | 2.07       | 0.856     | 0.749-0.963  | Fatty acid biosynthesis                                |
| 5  | myo-Inositol                             | Down       | NEG      | 2.29 | 0.0003  | 1.70       | 0.777     | 0.653-0.900  | No                                                     |
| 6  | D-Glutamine                              | Down       | NEG      | 2.06 | 0.0069  | 1.24       | 0.724     | 0.588-0.861  | No                                                     |
| 7  | m-Coumaric acid                          | Up         | NEG      | 2.04 | 0.0011  | 0.60       | 0.728     | 0.594-0.862  | Phenylalanine metabolism                               |
| 8  | Imidazoleacetic acid                     | Up         | NEG      | 2.01 | 0.0459  | 0.44       | 0.689     | 0.539-0.839  | Histidine metabolism                                   |
| 9  | Palmitoleic acid                         | Down       | NEG      | 1.47 | 0.0019  | 2.01       | 0.767     | 0.638-0.895  | Fatty acid biosynthesis                                |
| 10 | Formiminoglutamic acid                   | Up         | POS      | 1.45 | 0.0435  | 0.57       | 0.649     | 0.501-0.797  | Histidine metabolism                                   |
| 11 | Carnosine                                | Down       | POS      | 1.45 | 0.0131  | 2.08       | 0.716     | 0.578-0.853  | Histidine metabolism                                   |
| 12 | Gluconolactone                           | Up         | NEG      | 1.36 | 0.0352  | 0.81       | 0.661     | 0.515-0.807  | Pentose phosphate pathway                              |
| 13 | Prostaglandin D2                         | Down       | NEG      | 1.15 | 0.0055  | 2.66       | 0.723     | 0.587-0.859  | Arachidonic acid metabolism                            |

AUC: area under the ROC curve; Fold change: PC group vs GC group.

Table 2 Detailed results of pathway analyses based on metabolomics data.
| No. | Pathway                                      | Total | Hits | Raw $p$ | $-\ln(p)$ | Hits names                                                  | Impact |
|-----|---------------------------------------------|-------|------|---------|-----------|------------------------------------------------------------|--------|
| 1   | Sphingolipid metabolism                     | 25    | 2    | 0.05    | 2.94      | Sphinganine                                                | 0.23   |
|     |                                             |       |      |         |           | Sphingosine                                                |        |
| 2   | Histidine metabolism                        | 44    | 3    | 0.03    | 3.62      | Formiminoglutamic acid; Carnosine; Imidazoleacetic acid   | 0.07   |
| 3   | Alanine, aspartate and glutamate metabolism | 24    | 2    | 0.05    | 3.02      | N-Acetyl-L-aspartic acid; Succinic acid semialdehyde       | 0.06   |
| 4   | Cysteine and methionine metabolism          | 56    | 1    | 0.57    | 0.55      | 5'-Methylthioadenosine                                     | 0.05   |
| 5   | Pentose phosphate pathway                    | 32    | 1    | 0.38    | 0.96      | Gluconolactone                                            | 0.04   |
| 6   | Arachidonic acid metabolism                 | 62    | 2    | 0.24    | 1.44      | Prostaglandin D2; Prostaglandin B2                        | 0.03   |
| 7   | Butanoate metabolism                        | 40    | 1    | 0.46    | 0.79      | Succinic acid semialdehyde                                 | 0.03   |
| 8   | Pentose and glucuronate interconversions   | 53    | 1    | 0.55    | 0.59      | D-Xylitol                                                  | 0.03   |
| 9   | Phenylalanine metabolism                    | 45    | 1    | 0.50    | 0.70      | m-Coumaric acid                                            | 0.03   |
| 10  | Pyrimidine metabolism                       | 60    | 1    | 0.60    | 0.51      | Cytidine                                                   | 0.03   |
| 11  | beta-Alanine metabolism                     | 28    | 1    | 0.35    | 1.06      | Carnosine                                                  | 0.02   |
| 12  | Nicotinate and nicotinamide metabolism      | 44    | 2    | 0.14    | 1.97      | NAD; 1-Methylnicotinamide                                  | 0.02   |
| 13  | Glycerophospholipid metabolism              | 39    | 2    | 0.11    | 2.17      | Triethanolamine; Glycerophosphocholine                     | 0.01   |
| 14  | Tyrosine metabolism                         | 76    | 1    | 0.69    | 0.37      | Succinic acid semialdehyde                                 | 0.00   |
| 15  | Arginine and proline metabolism             | 77    | 1    | 0.69    | 0.37      | N-Acetylputrescine                                         | 0.00   |
| 16  | Purine metabolism                           | 92    | 1    | 0.76    | 0.28      | Deoxyinosine                                               | 0.00   |
| 17  | Fatty acid biosynthesis                     | 49    | 2    | 0.17    | 1.80      | Dodecanoic acid; Palmitoleic acid                          | 0.00   |
| 18  | Linoleic acid metabolism                    | 15    | 1    | 0.20    | 1.60      | Bovinic acid                                               | 0.00   |
| 19  | Ether lipid metabolism                      | 23    | 1    | 0.29    | 1.22      | Glycerophosphocholine                                      | 0.00   |
| 20  | Vitamin B6 metabolism                       | 32    | 1    | 0.38    | 0.96      | Succinic acid semialdehyde                                 | 0.00   |
| 21  | Steroid hormone biosynthesis                | 99    | 1    | 0.78    | 0.25      | Cholesterol sulfate                                        | 0.00   |

Hits: the matched number of metabolites in one pathway. Raw P: the original P value calculated from the enrichment analysis. Hits names: names about the matched differential metabolites in one pathway.

**Figures**
Figure 1

An overview of study profile for identifying metabolites of GC
Figure 2

Representative UHPLC-QE-MS total ion chromatograms of GC and PC tissue. Negative ion mode of GC tissue (A) and PC tissue (B), and positive ion mode of GC tissue (C) and PC tissue (D) of the same patient.

Figure 3

Identification of discriminating biomarkers by metabolome analysis. The PCA (A) and OPLS-DA (C) score plot and OPLS-DA (E) model are in negative ion mode. PCA (B) and OPLS-DA (D) score plot and OPLS-DA (F) model are in positive ion mode.
Figure 4

Volcano plots for the GC vs. PC groups. A was derived from negative ion mode of GC patients. B was derived from positive ion mode of GC patients.
Figure 5

Relative intensity of the significantly altered metabolites
Figure 6

ROC graphs of metabolites with the high AUC values. A: 1-methylnicotinamide; B: dodecanoic acid; C: sphinganine
Figure 7

Bubble analysis of metabolic pathways between GC and PC groups