Identification of potential metabolite markers for colon cancer and rectal cancer using serum metabolomics

Jianping Wu | Minyi Wu | Qianxia Wu

Department of Clinical Laboratory, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, China

Correspondence
Qianxia Wu, Department of Clinical Laboratory, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310003, Zhejiang, China. Email: syjinfei100@sina.com; 1715318@zju.edu.cn

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Abstract

Background: To determine the metabolic characteristics of patients with colon cancer (CC) and rectal cancer (RC) using gas chromatography-mass spectrometry (GC-MS)-based metabolomics.

Methods: In this study, serum samples were collected from 22 CC patients and 23 RC patients preoperatively and postoperatively and 45 healthy volunteers (HVs), and subjected to metabolomics analysis by GC-MS. Differential metabolites in the preoperative RC and CC samples and HVs were identified as potential biomarkers and evaluated for their utilities by receiver operating characteristic analyses.

Results: The different metabolic markers between CC and RC patients were identified, which may assist in distinguishing the two types of cancers. The area under the curve (AUC) was 0.805 for combination of d-glucose and d-mannose for CC diagnosis, and 0.889 for combination of 2-aminobutanoic acid, 3-hydroxypyridine, d-glucose, d-mannose, isoleucine, l-tryptophan, urea, and uric acid for RC diagnosis. The combinations of metabolite markers showed a better predictability than CEA and CA199 two commonly used protein markers for CRC diagnosis in clinical practice. Combining the metabolite markers with these two protein markers effectively improved the diagnostic accuracy with the AUC reaching 0.936 and 0.937 for CC and RC diagnosis, respectively.

Conclusions: Metabolic profiles are different in the blood samples between CC and RC patients. The study has established a panel of metabolic markers as a predictive and multiplexing signature for CC and RC diagnosis.

KEYWORDS
biomarker, colon cancer, diagnosis, metabolomics, rectal cancer

Abbreviations: AUC, area under the curve; CC, colon cancer; CRC, colorectal cancer; GC-MS, gas chromatography-mass spectrometry; HVs, healthy volunteers; QC, quality control; RC, rectal cancer; ROC, receiver operating characteristic.

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1 | INTRODUCTION

Colorectal cancer (CRC) is currently the third most common cancer worldwide. Occurrence of CRC is related to lifestyle, health management, and other genetic factors.\(^1\)\(^2\) Its incidence and mortality have continued to increase in recent years.\(^3\) The 5-year survival rate of CRC is still dismal (only 8%-9%) when cancer had metastasized.\(^4\)

In the United States, it was estimated that the colon cancer (CC) ratio was almost 1:1 in men and women, while the rectal cancer (RC) ratio was 1.5:1 in 2015.\(^5\) Because 40% of CC cases tend to be mistakenly classified as RC, it is difficult to obtain definitive numbers of deaths from CC and RC.\(^6\) The overall 5-year survival rate of CRC patients in China was also only 66.3%.\(^7\)

With effective diagnosis methods detecting the CRC at its early stage, the incidence and mortality of CRC could be significantly reduced and a better prognosis could also be obtained because it is still limited to the mucosa and submucosa.\(^8\) The 5-year survival rate could be significantly increased to 90% when the CRC is diagnosed at its early stage and its treatment is performed at the in situ stage of cancer development.\(^9\) In addition, the patterns of spread of lesions are different between RC and CC. When the CRC tumor identity is confirmed by both endoscopic and histopathologic examinations, different strategies shall be employed for RC and CC treatments. Thus, it is important to distinguish between these two types of tumors.

According to the National Comprehensive Cancer Network (NCCN) guideline of colorectal cancer screening, fecal occult blood tests and colonoscopy are the main screening options for CRC.\(^10\) However, these two methods contain the limitation in weak compliance, missing diagnosis, and generating false positives in large-scale CRC screening.\(^11\) At present, non-invasive screening methods involving tumor markers carcinoembryonic antigen (CEA) and sugar chain antigen (CA199) are widely used. However, these two serum biomarkers have relatively poor sensitivity and specificity, thereby producing high false-negative rates. They are inferior for diagnosing asymptomatic patients. In benign diseases such as pancreatitis, cholangitis, and other gallbladder diseases, CA199 level shows different degrees of elevation.\(^12\) Moreover, these two markers cannot be used to differentiate CC and RC. Thus, it is necessary to identify efficient, non-invasive, and specific markers for CC and RC screening.

Metabolomics is a technique used to investigate life phenomena by exploring the changes in endogenous metabolites after stimulation or disturbance by external factors. Because the metabolome is downstream of the genome, transcriptome, and proteome, changes in the metabolome can reflect the real physical status in the functional level. Since the development of metabolomics, it has become increasingly widely used in research on diseases, including studies on disease subtypes, prognostic evaluation, and discovery of diagnostic markers.\(^13\)\(^-\)\(^16\)

In this study, metabolomics was used to determine the metabolic differences among RC patients, CC patients, and healthy volunteers (HVs). The differentially expressed metabolites were screened to establish a diagnostic model for RC and CC.

2 | MATERIALS AND METHODS

2.1 | Participants

The trial was approved by the Ethics Committee of the First Affiliated Hospital of Zhejiang University. The informed consents were obtained when the patients were selected to enroll into the study. The study subjects were admitted to the hospital between December 2016 and May 2017 and comprised 22 CC patients and 23 RC patients in the middle-differentiation period. Samples were obtained from all patients preoperatively and postoperatively (3 days after surgery). In addition, 45 age- and sex-matched HVs were also enrolled as a control group and showed no significant changes in routine biochemical and tumor index test results. Fasting blood samples were collected in the morning into coagulation-promoting tubes, and serum was isolated after centrifugation. All samples were stored at -80°C for further analysis. The background characteristics of the study subjects are shown in Table 1.

| Number of subjects | Healthy volunteers | Colon cancer | Rectal cancer |
|--------------------|--------------------|--------------|--------------|
| Total | 45 | 22 | 23 |
| Male | 31 | 15 | 16 |
| Female | 14 | 7 | 7 |
| Age | | | |
| Range | 49-84 | 49-84 | 54-80 |
| Mean | 66.49 | 64.41 | 68.48 |

TABLE 1 Background characteristics of the subjects included in the study

2.2 | Reagents

Methoxyamine, pyridine, methyl-trimethyl-silyl-trifluoroacetamide (MSTFA), and deionized water were purchased from ANPEL Laboratory Technologies Inc. HPLC-grade methanol and acetonitrile were purchased from Merck. Other reagents employed were analytically pure grade and purchased from Sinopharm Chemical Reagent Co. Ltd.

2.3 | Sample preparation

After thawing in an ice-water bath, the serum samples were vortexed. A 50-μL aliquot of each sample was placed in a 1.5-mL
Eppendorf tube, and 200 µL of cold methanol containing 20 µg/mL tridecanoic acid was added. After vortexing for 30 seconds and centrifugation at 21 130 g for 15 minutes, 200 µL of supernatant was lyophilized in a centrifugal concentrator.

Before analysis, the sample was dissolved in 50 µL of methoxyamine-pyridine solution (20 mg/mL) and oximated for 90 minutes in a 37°C water bath. Silylation was then performed by addition of 40 µL of MSTFA and incubation in a 37°C water bath for 60 minutes. After centrifugation at 21 130 g for 15 minutes, 70 µL of supernatant was removed for GC-MS analysis. Quality control (QC) samples containing the same amount of each serum sample were processed in the same way as the actual samples and used to monitor the stability during the analysis.

2.4 | GC-MS analysis

The chromatographic separation was performed on an Agilent 7890A GC coupled to an Agilent 5975B MSD with electron ionization (EI) source. A DB-5 MS column (30 m × 250 µm × 0.25 µm; J&W Scientific) was used for separation with high-purity helium as the carrier gas at a consistent line flow rate of 40.0 cm/s. The initial oven temperature was 70°C for 3 minutes, followed by a temperature increase to 300°C in steps of 5°C/min, and maintenance at 300°C for 10 minutes. A 1-µL aliquot of sample was injected with a split ratio of 10:1 and an inlet temperature of 300°C. The transfer line and ion source temperatures were 280°C and 230°C, respectively. Electron ionization was used for the metabolic profiling analysis with an ionization energy of 70 eV. Mass spectra were acquired in the full scan mode using a mass scan range of 33-600 m/z. The solvent delay time was adjusted to 5.5 minutes, and the scan cycle was set at 0.2 seconds.

The QC samples were used to balance the GC-MS system before analysis of the actual samples. During the batch analysis, a QC sample was inserted every 10 samples to monitor the repeatability and stability of the pretreatment and instrument.

2.5 | Statistical analysis

The peak detection was performed with R version 3.3.2 software. The original data were exported into an online website (http://www.metaboanalyst.ca/) for peak matching by using XCMS for metabolanalyst. The algorithm was used for peak picking with a resolution of 25 ppm and a signal-to-noise threshold (snthr) set to 5. Features that appeared in less than 75% of the samples which underwent the same extraction method were removed (minfrac = 0.75). The peak width range was set to c (5.15), and bw was set to 4 seconds. The generated peak table was retained for multivariate statistical analysis. A principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were conducted with Simca-P 11.0 software (Umetrics AB). The differential metabolites were identified by using NIST 17 Mass Spectral Library. Further, the retention time and elution order of compounds were also used to assist in characterization of the compounds by using a database from Prof. Guowang Xu (Institute of Chemical and Physics, Chinese Academy of Sciences). R software was used to perform the Kruskal-Wallis test and receiver operating characteristic (ROC) curve analyses. SPSS 22.0 software (SPSS Inc) was used for binary logistic regression analyses.

3 | RESULTS

3.1 | Analytical characteristics

As a GC-MS instrument can show fluctuation during a runtime, tridecanoic acid was used as an internal standard to examine the stability of the analytical conditions. The retention time and peak area of tridecanoic acid in each QC sample were recorded. No drift of retention time was observed. The QC chart revealed that the internal standard in all QC samples showed small fluctuations (within 2 SD), indicating that the sample pretreatment, derivatization, and instrument analysis protocols adopted in the experiment were stable (Figure S1A).

In the study, a total of 638 peaks were detected, and the peak area was normalized by the internal standard. QC samples were employed to measure the variability of each peak during the analytical sequence. The relative standard deviation (RSD) of each peak was calculated based on the normalized peak area. In the 18 QC samples, 521 peaks had RSD < 30%, and only 38 peaks had RSD > 60%, indicating that most of the detected metabolites were relatively stable during the process (Figure S1B). Finally, 521 peaks were used for data processing.

3.2 | Metabolic characteristics revealed by statistical analysis

After peak extraction and normalization, the PLS-DA, with UV scaling, was performed to discriminate the potential marker candidates among the RC, CC, and HV groups. Preoperative and postoperative samples were investigated separately. The preoperative CC, RC, and HV samples were well discriminated in the PLS-DA score plot (Figure 1A). The cumulative R2Y and Q2 were 0.515 and 0.382, respectively, when the first three components were calculated. It indicated that the two types of patients had different metabolic characteristics. Cross-validation results (the intercepts of R2Y and Q2 are 0.204 and −0.217, respectively) indicated the model were not over-fitted (Figure 1C).

However, the postoperative samples from the three groups appeared to partially overlap in the model (Figure 1B), with R2Y = 0.409, Q2 = 0.16. Cross-validation results (the intercepts of R2Y and Q2 are 0.2 and −0.283, respectively) were showed in Figure 1D. The metabolic profiles of the patients tended to be closer to those of the HVs, suggesting that the levels of some metabolites recovered to the levels similar to HVs after surgery. Peaks with variance importance
in projection (VIP >1.0) in the PLS-DA model were selected, and the Kruskal-Wallis test was performed to identify the metabolites with significant differences between the groups.

After comparing the differences in metabolites among the CC, RC, and HV groups by the Kruskal-Wallis test, there were 10 metabolites with significant differences preoperatively and 13 metabolites with significant differences postoperatively ($P < .05$; Table 2). The differential metabolites, such as 2, 3-butanediol, 2-hydroxybutyric acid, isoleucine, L-alanine, and L-proline, were not identical before and after surgery, indicating that changes in the metabolic profiles were associated with the outcome of surgical treatment.

3.3 | Pathway analysis of differential metabolites

The related pathways for the differential metabolites were analyzed to reveal the potential contributors of the metabolic changes before and after surgery (Figure 2). According to the results of the pathway impact analyses, seven metabolic pathways were screened out. These pathways were related to biosynthesis, energy supply, and oxidative stress response, such as arginine and proline metabolism pathway, and starch and sucrose metabolism pathway. It is known that the pattern of energy metabolism changes markedly when cancer occurs, resulting in tremendous metabolic alterations to related small molecules, such as carbohydrates and amino acids. As proline acts as an important provider of ATP and is related to the TCA cycle and urea cycle, the arginine and proline metabolism pathway was highlighted in this study.

3.4 | Potential assessment of metabolites for diagnosis of CC and RC

The differential metabolites in the preoperative RC, CC, and HV groups were regarded as potential biomarkers and subjected to
TABLE 2 The differential metabolites between CC, RC, and HV groups before/after surgery

| Metabolite                  | Retention time (min) | $P$ value | $P$ value |
|-----------------------------|----------------------|-----------|-----------|
|                            | preop                | postop    |           |
| 2,3-Butanediol             | 7.51$^a$             |           | .00613    |
| 2-Aminobutanoic acid       | 11.27$^a$            | .01693    | 1.54E-08  |
| 2-Hydroxybutyric acid      | 9.96$^a$             |           | .00218    |
| 3-Hydroxybutyric acid      | 13.13$^a$            | .000123   | .007597   |
| 3-Hydroxypryidine          | 10.67$^a$            | 3.09E-09  |           |
| Butanoic acid              | 7.26$^a$             | 5.44E-12  |           |
| D-Fructose                 | 28.46$^a$            | .0006573  | 1.33E-06  |
| D-Glucose                  | 28.97$^a$            | .0004288  | 5.59E-06  |
| D-Mannose                  | 28.75$^a$            | .06038    | 9.23E-08  |
| Glycine                    | 9.86$^a$             |           |           |
| Isoleucine                 | 11.41$^a$            | .01992    |           |
| Lactic acid                | 8.07$^a$             | .81       | .01688    |
| L-Alanine                  | 9.27$^a$             |           |           |
| L-Proline                  | 14.75$^a$            | 6.98E-07  |           |
| L-Tryptophan               | 34.59$^a$            | .0007671  |           |
| Urea                       | 13.25$^a$            | .01023    |           |
| Uric acid                  | 32.87$^a$            | .001717   | 5.49E-11  |

$^a$Based on both of NIST database and elution time of DICP GC/MS database.  
$^b$Only based on NIST database.

ROC analyses to identify their potential roles in early diagnosis. Paired comparisons were performed to eliminate the metabolites with no significant differences between the HV group and the patient groups. The area under the curve (AUC) was 0.805 for combination of d-glucose and d-mannose for CC diagnosis, and 0.889 for combination of 2-aminobutanoic acid, 3-hydroxypryidine, d-glucose, d-mannose, isoleucine, l-tryptophan, urea, and uric acid for RC diagnosis. In clinical practice, CEA and CA-199 are commonly used as tumor markers. In this study, the AUC for CEA and CA-199 was only 0.800 and 0.722 for CC diagnosis, and 0.744 and 0.765 for RC diagnosis, respectively. Combining the metabolite markers with protein markers effectively improved the diagnostic accuracy, and the AUC reached 0.936 and 0.937 for CC and RC diagnosis, respectively (Figure 3).

4 | DISCUSSION

In recent years, increasing numbers of studies have employed metabolomics to identify CRC markers.17-20 However, to the best of our knowledge, this is the first study to investigate the differences of metabolite markers between CC and RC patients. In the present metabolomics study on preoperative samples, RC patients exhibited more changes in metabolites than CC patients (Table 3). Furthermore, nine metabolites differed significantly between CC and RC patients including 2-aminobutanoic acid, 3-hydroxypryidine, d-mannose, glycine, isoleucine, lactic acid, l-tryptophan, urea, and uric acid.

For example, isoleucine differed significantly between the HV and RC groups, and between the RC and CC groups ($P < .05$), but showed no significant difference between the HV and CC groups. Isoleucine is a branched-chain amino acid that can provide additional energy when the body consumes large amounts of energy. It may play an important role in the balance of body energy. These findings suggest that there may be differences in metabolic activity between the two cancers during the mid-differentiation stage, which may partly explain there are different metastatic patterns between CC and RC in different stage.

Diagnostic models for CC and RC were established based on the differential metabolites identified in the metabolomics analysis, and ROC analyses showed that the resultant prediction utilities were better than those of CEA and CA199, which are commonly used in clinical practice. The specificity of CEA and CA199 is poor, because they are not only altered in patients with gastrointestinal cancer but also in patients with other cancers such as breast cancer and lung cancer. Metabolomics is a technique that allows comprehensive exploration of altered metabolites in a particular disease, thus identifying metabolite markers with high specificity for the disease under investigation. Not a single marker might be sufficient to create a test with appropriate sensitivity and specificity for CRC as it is easily affected by a variety of factors, suggesting that a combination of multiple metabolite markers can better reflect the real state of patients.
Mirnezami et al and Chan et al found that glycine was elevated in CC patients was opposite to that in RC patients, which is inconsistent with the previous studies. The previous CRC studies did not compare patients with CRC compared with healthy controls, while Ma et al provided some evidence from 16S rRNA gene sequencing and found some unique microbes for the occurrence of CRC.

There are several limitations to the present study. First, two carbohydrates were combined to establish a diagnostic model for CC, which may be affected by high-sugar diets or other in vitro intakes. Second, the examined samples were all from cancer patients and were not compared with inflammatory samples and benign tumor samples. Third, the small sample size may have introduced potential interference. Fourth, a previous study found that early-stage CRC patients were easier to distinguish than mid-stage or late-stage patients, while mid-stage patients were the most difficult. As the patients in the present study were all in the middle-differentiation period, we were unable to assess whether the candidate metabolite markers are suitable for early screening. In future studies, it will be necessary to include more samples to verify our findings.

In conclusion, a GC-MS-based metabolomics study was performed to identify the different metabolic characteristics of patients with CC and RC. Significantly changed metabolites were selected as potential markers for disease diagnosis. Combinations of metabolite markers, comprising d-glucose and d-mannose for CC diagnosis, and 2-aminoobutanoic acid, 3-hydroxypryidine, d-glucose, d-mannose, isoleucine, l-tryptophan, urea, and uric acid for RC diagnosis, showed a better predictability than CEA and CA199. Furthermore, metabolic differences between CC and RC patients were identified, which may assist in distinguishing the two types of cancers.

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CONFLICT OF INTEREST
None of the authors have any commercial or other association that might pose a conflict of interest.

ORCID
Qianxia Wu https://orcid.org/0000-0002-8442-6476

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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