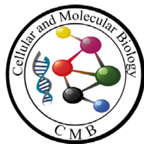

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UHPLC-MS/MS-based central carbon metabolism unveils the biomarkers related to colon cancer

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ABSTRACT

Even though colon cancer ranks among the leading causes of cancer mortality, early detection dramatically increases survival rates. Many studies have been conducted to determine whether altered metabolite levels may serve as a potential biomarker of cancer that affects key metabolic pathways. The goal of the study was to detect metabolic biomarkers in patients with colon cancer using liquid chromatography-mass spectrometry (LC-MS). This study consisted of 30 patients with colon cancer. An analysis of the metabolomes of cancer samples and para-carcinoma tissues was conducted. We identified a series of important metabolic changes in colon cancer by analyzing metabolites in cancerous tissues compared to their normal counterparts. They are mainly involved in the pentose phosphate pathway, the TCA cycle, glycolysis, galactose metabolism, and butanoate metabolism. As well, we observed dysregulation of AMP, dTMP, fructose, and D-glucose in colon cancer. Additionally, the AUCs for AMP, dTMP, fructose, and D-glucose were greater than 0.7 for the diagnosis of colon cancer. In conclusion, AMP, dTMP, fructose, and D-glucose showed excellent diagnostic performance and could serve as novel disease biomarkers for colon cancer diagnosis.

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Introduction

Worldwide, colon cancer is one of the most common types of digestive cancer. The incidence of colon cancer in the United States is the fourth highest and it is the second leading cause of death (1). The number of patients with colon cancer in America is estimated to be more than 1.5 million, with almost 105000 cases reported in 2020 (2). Colon cancer is one of the five most common cancers diagnosed in China and one of the leading causes of cancer-related death (3). There has been a reduction in the incidence rate of colon cancer as a result of widespread colonoscopy testing. As a result of improvements in treatment options, such as chemotherapy, colectomy, and immunotherapy, there is a relative survival rate of approximately 65 percent for colon cancer patients today after five years of diagnosis, in part due to improvements in treatment options (2). The detailed mechanisms of colon cancer development are unknown, despite the fact that microorganisms, dietary factors, and their metabolites are associated with this disease (4). Consequently, it is essential to understand the molecular mechanisms underlying colon oncogenesis.

Tandem mass spectrometry with liquid chromatography (LC-MS/MS) combines high-resolution mass spectrometry as a detection method with liquid chromatography as a separation method (5). Compared to other chromatographic-mass spectrometry techniques, LC-MS/MS is more suited for the analysis of metabolites with low volatility or low thermal stability. In comparison to conventional HPLC columns, ultra-high performance liquid chromatography columns packed with ultrafine particles of 1.7 microns in diameter have at least 10 times the speed and several times the sensitivity of conventional HPLC columns (6). As of the current time, quadrupole-time-of-flight (Q-TOF) mass spectrometry and ultra-performance liquid chromatography have been widely used in research on metabolomics. As a result, protein metabolomics technologies are becoming increasingly important when studying tumor biology, and the field has seen rapid growth in recent years (7).

A variety of cancers have been investigated using LC-MS for metabolomics studies. An in-depth understanding of colon carcinogenesis has been gained by studying the intestinal microbiome, epithelium, and metabolome. Using GC/MS-based serum metabolomics to identify biomarkers for colon cancer prognosis, previous studies have demonstrated that D-mannose and methionine could be useful as possible biomarkers (8). According to another study, calcitroic acid, and glucosyl sphingosine concentrations can predict the development of lymph node metastasis and prognosis of patients with colon cancer (9). There are several cancer-specific metabolisms manifest in colon tissue characterized by Akira Hirayama et al. The pathways involved include glycolysis, the TCA and urea cycles, the pentose phosphate pathway, as well as nucleotide and amino acid metabolism (10). Moreover, Yuping Cai et al. discovered nine metabolites that are specifically associated with colon cancer. These include taurine, glutamate, fructose-6-phosphate, etc (11). At the present time, there are differences between the individual metabolites detected in different studies. This is due to factors such as the sample size, platforms for analysis, and tools for

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statistical analysis. In light of this, further research is still required on metabolomics analysis in colorectal cancer.

In the cytoplasm, glycolysis is performed, PPP occurs in the cytoplasmic component, and the TCA cycle occurs in the mitochondrial membrane. In order to fuel HCC's metabolic activities, glucose and glutamine are recruited by carcinogenic signals. It has been suggested that several mutated oncoproteins have metabolic activities that may increase glucose uptake and lactate production, as a result of aerobic glycolysis. Furthermore, they provide fuel to the TCA cycle by accelerating the metabolism of glutamine (12). A number of tumor suppressors, including p53 and AMPK, act on a variety of nodes to prevent the biosynthesis of cancerous cells. HCC cells are able to synthesize nucleotides, amino acids, and lipids with more carbon intermediates as a result of the TCA cycle and glycolysis. As a branch of glycolysis, PPP supplies R5P and reduces equivalent NADPH, thereby protecting cells from reactive oxygen species (ROS) (13).

The aim of this study was to evaluate metabolic traits associated with colon cancer using LC-MS metabolic profiling. In the first step, we identified the differences in metabolites between 30 adjacent normal tissues and 30 adjacent colon cancer tissues. Alterations in metabolic pathways were then studied. As a final step, we validated the expression of key metabolite and the ROC analysis.

Materials and Methods

Collection of samples

We enrolled colon cancer patients from our hospital's oncology department. The tissues were collected from patients who had colon cancer and their adjunct non-cancerous tissues. As soon as the tumors had been resected, they were immediately placed in liquid nitrogen and then stored at -80°C. Prior to surgical resection, these patients did not receive any radiotherapy or chemotherapy. Informed consent was obtained from all participants, and this study was approved by the hospital's ethics committee.

Metabolite Extraction

After assembling the experimental specimens, the metabolite extraction of each sample is performed according to the SOP requirements for metabolite extraction. The metabolites were measured and analyzed by targeted assays (central carbon metabolism). Then add 500 liters of aqueous solution containing 80% methanol and 0.1% formic acid and vortex before shaking, (cell samples need to be sonicated) stand in an ice bath for 5 min, centrifuge at 15000 rpm, 4°C for 10 min, add 1/2 volume of mass spectrometry grade water to dilute the supernatant to 53% methanol, and place in a centrifuge tube. The supernatant was collected by centrifugation at 15000 g for 20 min at 4°C and injected into the sample for LC-MS analysis(6). An equal volume of samples from each experiment was mixed as QC samples. Rather than the experimental sample, the blank sample contained 53% methanol and 0.1% formic acid, and pretreatment was performed similarly. The samples were analyzed in multiple reaction monitoring mode (MRM).

Metabolite characterization and quantification

Metabolite characterization: Compounds are quantified based on Q3 (daughter ions) and characterized by Q1 (parent ion), Q3 (daughter ion), RT (retention time), DP (declustering voltage), and CE (collision energy). For the characterization of some of these substances, duplicate signals such as isotopic signals need to be removed.

Metabolite quantification: The compounds are quantified based on the peak area of Q3 (daughter ion) using the MRM mode of the triple quadrupole.

Statistical analysis

Using an in-house database with authentic standards, compounds were identified by MS/MS spectra. It was necessary to normalize the processed data before uploading them into SIMCA-P for multivariate analysis, including Pareto-scaled principal component analysis (PCA). Furthermore, differential metabolites were identified using SPSS 13.0 (Chicago, IL, USA). A statistically significant difference was defined as $P < 0.05$. Metabolic pathways were analyzed using the MetaboAnalyst software.

Results

Demographic characteristics and baseline information

We collected the subjects' gender, age, comorbidities, carcinoembryonic antigen (CEA) level, and carbohydrate antigen 199 (CA199). Table 1 summarizes the baseline characteristics of colon cancer patients. We enrolled 30 patients in our study. The average age of all subjects was 67.2 ± 7.78 years old and most of participants were male (22/30). The mean levels of CEA and CA199 were 9.72±15.66 and 24.74±32.47. Diabetes was present in four patients and hypertension was present in four patients.

Metabolic profiling of colon tissues

To determine whether changes in metabolic activity are associated with colon cancer, LC-MS was used to profile 30 pairs of adjunct noncancerous tissues and their corresponding colon carcinoma tissues. Samples for quality control are made by mixing equal amounts of experimental samples. Figures 1A and B show the base peak intensity of the QC sample chromatogram and correlation analysis. The results demonstrated the reproducibility and reliability of the data. The difference between the two groups was observed using principal component analysis (PCA). We can see a separation tendency between the normal and cancer tissues based on the PCA score scatter plots shown in Figures 1 C and D. This suggests that the levels of meta-

Table 1. Baseline information of the subjects included in the study.

Parameters	Participants
Number of subjects	30
Age	67.2±7.78
Male	22
CEA	9.72±15.66
CA199	24.74±32.47
Comorbidity	
Diabetes	4
Hypertension	4
Others	2
Clinicopathologic stage	
I-II	21
III-IV	9

bolites between the two groups are different.

Metabolic alterations of normal and tumor colon tissues

All variables were visualized with the volcano plot if the P value was less than 0.05 and the Log2|FC| was greater than 1. A red mark indicates metabolic upregulation (AMP and dTMP) while a blue mark indicates metabolic downregulation (Fructose and D-glucose) (Fig. 2A, C, and D). An analysis of 29 identified metabolites that are derived from two groups was carried out using a heat map based on hierarchical clustering (Figure 2B). We imported candidate metabolites into MetaboAnalyst 3.0 for enrichment pathway analysis in order to further investigate metabolic changes in the two groups. There are several metabolic pathways shown in Figure 1E, including the pentose phosphate pathway, the TCA cycle, glycolysis, galactose metabolism, butanoate metabolism, etc.

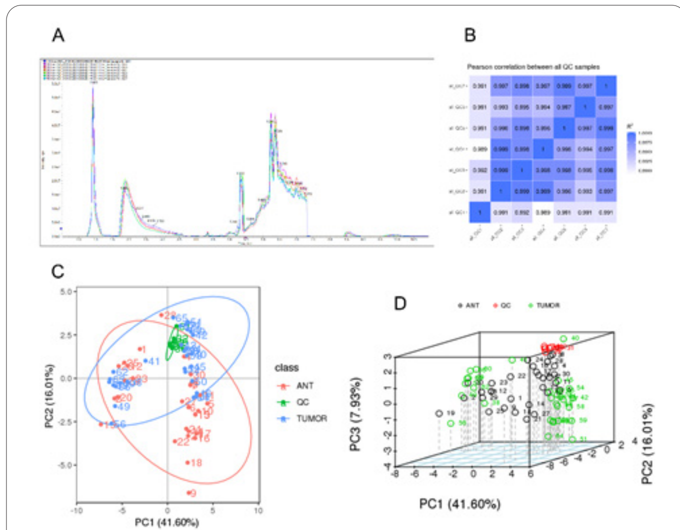

Figure 1. The base peak intensity chromatogram was obtained based on the UHPLC-MS/MS and PCA score plot for normal and tumor colon tissues. (A). The base peak intensity chromatogram of samples was obtained; (B). Correlation analysis was performed. (C-D) Score plot of PCA.

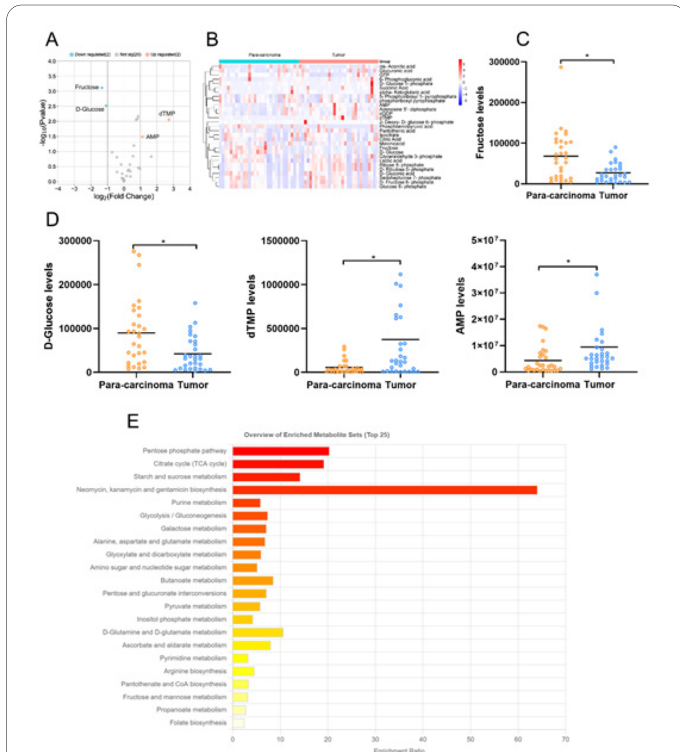

Figure 2. Alterations in the metabolism of normal and tumor colon tissues. Volcano plot analysis (A) and heat map (B) for normal and tumor colon tissues; (C). A bar graph illustrating the difference between normal and tumor colon tissues with respect to metabolic pathways.

Metabolites and the clinical features of colon cancer

An analysis of correlation was conducted to determine the relationship between the differential expression levels of metabolites and the clinical characteristics of colon cancer patients. These findings indicate that fructose, D-glucose AMP, and dTMP are dysregulated metabolites. Figure 3 illustrates a significant positive correlation between dTMP levels and CEA levels.

Analyzing metabolites for colon cancer diagnosis

A ROC analysis was conducted to identify the role of the differential metabolites in colon cancer as potential biomarkers. Based on these results, dTMP and AMP for colon cancer diagnosis had an area under the curve (AUC) of 0.701 and 0.714, respectively, while fructose and D-glucose had an AUC of 0.715 and 0.732, respectively (Figure 4).

Discussion

Even though colon cancer is associated with several ge-

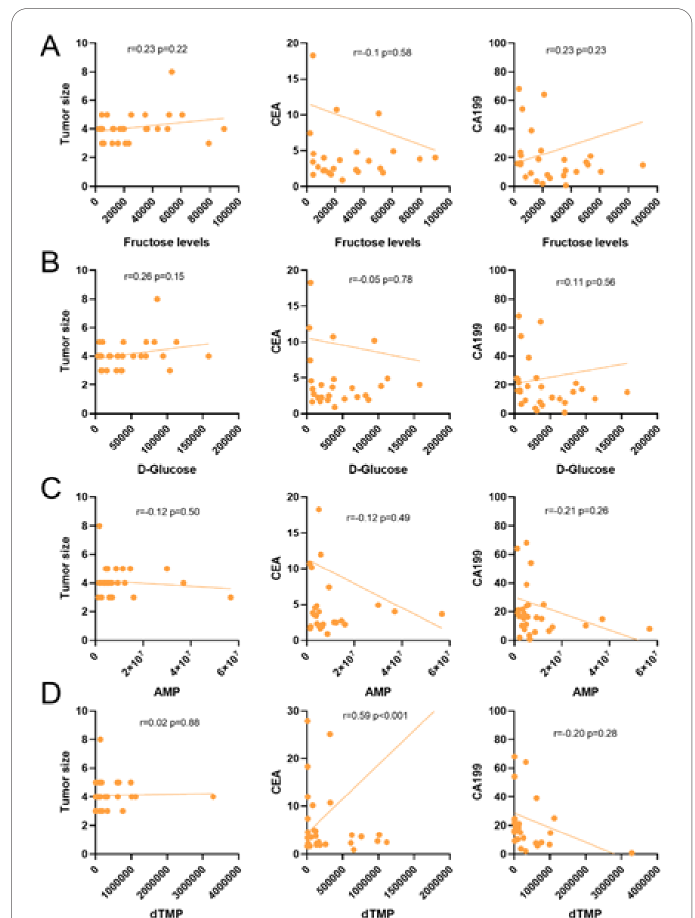

Figure 3. Relationship between metabolite levels and clinical features of colon cancer. (A) Correlation of fructose levels with tumor size, CEA and CA199 levels; (B) Correlation of D-glucose levels with tumor size, CEA and CA199 levels; (C) Correlation of AMP levels with tumor size, CEA and CA199 levels; (D) Correlation of dTMP levels with tumor size, CEA and CA199 levels.

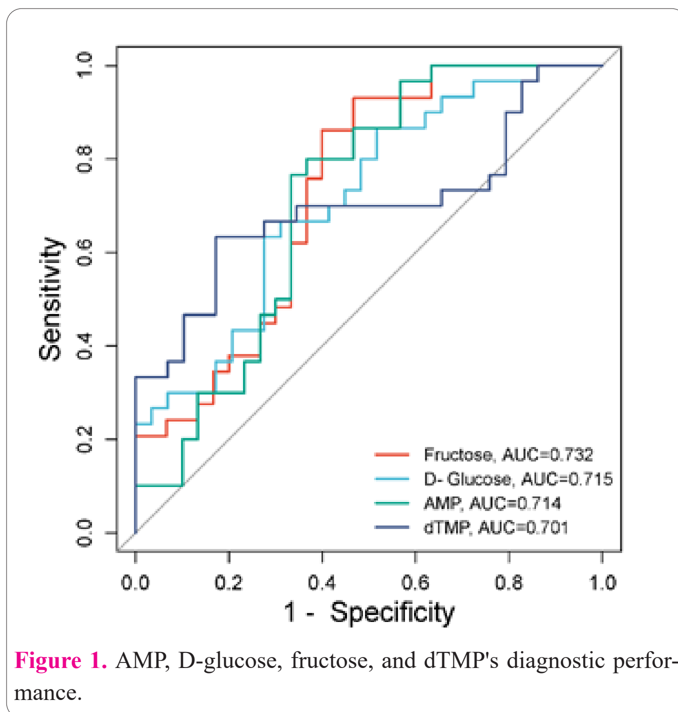

Figure 1. AMP, D-glucose, fructose, and dTMP's diagnostic performance.

netic changes, metabolic reprogramming is also involved in the development and progression of the disease. This study examines the metabolic changes associated with colon cancer by performing LC-MS-based non-targeted metabolomics analysis on 30 pairs of colon cancers and adjacent tissues. We identified 29 differential metabolites based on the analysis of the data, revealing that fructose, D-glucose, AMP, and dTMP are significantly altered in colon cancer. It can be summed up that metabolic profiling and pathway analysis have revealed metabolic changes that are associated with colon cancer.

The field of proteomics has emerged in the post-genomics era and is concerned with identifying all proteins (14). Proteomics is the analysis of protein interactions and connections from a holistic point of view with the purpose of revealing the rules that govern the functions of proteins and the functions of cells (7). Non-targeted metabolomics is one of the most powerful methods for quantifying metabolites in biological systems, thus maximizing the information obtained from metabolites. Considering that small molecule metabolites constitute a large component of biological samples and have a large dynamic range of concentrations in a wide range of sizes, chromatography-mass spectrometry is one of the most important tools available for understanding metabolomics (15).

It has been found that a number of approaches have been used to detect biomarkers in colon cancer, including gut microbiota analysis and metabolome analysis. In order to obtain biofluids, less invasive collection techniques are used in order to collect them as opposed to tissues (16). Despite the fact that the biofluid profiles may provide some clue to a certain extent about the state of the body, the biofluid profiles are easily affected by many external factors, such as diet, drugs, and lifestyle habits. The use of tissue samples in several studies was beneficial since it may reduce the effects of the disease on non-tumors and/or the body as a whole, thus emulating the phenotype that is most apparent in humans (17).

It has been shown that metabolomic studies of colon cancer tissues showed that the biosynthesis of steroid hormones, the biosynthesis of terpenoid hormones, the bile metabolic process, the metabolization of short chains of fatty acids, fructose, mannose, galactose, glycolysis, gluconeogenic metabolism, and pyruvate differed between colorectal cancer tissues (18-20). However, in spite of these findings, we observed that in colon cancer patients, metabolic changes in metabolites associated with the pentose phosphate pathway, the TCA cycle, glycolysis, galactose metabolism, and butanoate metabolism are evident. It can be seen from the present findings that colon cancer's differential metabolites follow glycolysis, gluconeogenesis, and purine metabolism closely. As per the study, the MYC gene mediates the cell metabolism of cancerous cells and increases the proliferation of cancerous cells in order to promote their growth. Several of the metabolic pathways related to mitochondrial biogenesis are also regulated by MYC, which is also reflected in our results regarding MYC and mitochondrial biogenesis (21). In other studies, there has also been a report that glucose and metabolites in the TCA cycle undergo metabolic changes (22). The differential metabolites of CRC also include some metabolites derived from gut bacteria, such as Trp and butanoic acid. It has been suggested that gut microbes may play a role in CRC carcinogenesis as the deregulation of these metabolites suggests. According to the study, 16S rRNA gene sequencing provided some evidence that may help in proving the presence of CRC and some unique microbes were found to be responsible (23).

ROC analyses demonstrated that fructose, D-glucose, AMP, and dTMP had better prediction utilities and could be used in clinical practice. Based on the differential metabolites identified in the metabolomics analysis, diagnostic models were developed for colon cancer. The results of various studies have suggested that CEA and CA199 have low specificity, primarily due to the fact that they are altered in not only patients who suffer from gastrointestinal cancers, but also in patients who suffer from other cancers (24). The term "metabolomics" refers to a method for the identification of high-specificity markers for metabolites within a particular disease by means of comprehensive analyses of altered metabolites. In light of the fact that CRC can be easily affected by a wide variety of factors, it may not be possible to create a test that is sensitive and specific enough, suggesting that the use of multiple metabolite markers may represent a more comprehensive view of the patient's condition. A number of metabolites have been reported to be altered in a discrepant manner in patients with CRC in previous metabolomics studies. A number of studies have reported higher levels of glycine in cancer patients compared with healthy control individuals (25), whereas a study noted lower levels of glycine in cancer patients (26). Comparing the colon cancer tissues to the para-carcinoma tissues in this study, AMP was found to be upregulated in the colon cancer tissue and dTMP was found to be downregulated. Previous studies have produced inconsistent results.

It can be concluded from our study that mass spectrometry-based metabolomics has the potential to provide novel insights into metabolic profiling of colon cancer by using tissue samples collected using mass spectrometry. To verify the validity of the results of this study, further studies should be conducted due to the limited sample size.

Interest conflict

The authors report no conflict of interest.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgements

Not applicable.

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