

Sensitive Potential Prognostic Markers for Colorectal Cancer Surgery Screened by UPLC-Q-TOF-MS Combined With Metabolomics Technology: UDP-D-Galactose

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Abstract

Background: Colorectal cancer (CRC) is a common malignant tumor, it is necessary to conduct in-depth research on the pathogenesis, early diagnosis, and prognosis evaluation. The assessment of postoperative prognosis is beneficial to the accurate treatment for patients. Metabolomics can explore the metabolic changes in the process of cancer, to find new markers that can be used for early diagnosis of cancer and evaluation of efficacy.

Method: In this study, ultra-high-performance liquid chromatography quarter time of fly mass spectrometer (UPLC-Q-TOF-MS) technology was used to screen the metabolomics markers of blood samples from 24 CRC patients who underwent surgery and 24 healthy people. To verify the potential markers found above, At the same time, 8 CRC patients and normal control group were included.

Results: UDP-D-galactose, which shown significant concentration changed 2 ~ 3 days after surgery was screened after statistical analysis. Moreover, the analysis of metabolic pathways in tissue samples also fully verified the correlation between UDP-D-galactose and CRC.

Conclusion: UPLC-Q-TOF-MS is a practical method for screening metabolic markers. Accurate monitoring the concentration of UDP-D-galactose in blood can provide important assistance for the treatment of CRC patients.

Background

Colorectal cancer is the third most common cancer worldwide, the fourth leading cause of cancer-related death in men, and the third leading cause of death among women [1]. Among cancer deaths in China, colorectal cancer ranks fifth, and its incidence is still rising [2].

The occurrence of colorectal cancer is the same as many other malignant tumors, it is affected by multiple genes and multiple factors. Therefore, the specific pathogenesis and mechanism of colorectal cancer are still under study. It had been reported [3] that abnormal miR-21 expression is associated with clinical manifestations in patients with CRC. Others [4] proposed that mutations of the tumor suppressor gene Adenomatous Polyposis Coli (APC) are critical in the development of colorectal cancer. Although numerous articles are published each year describing gene mutations or changes in gene expression levels associated with various cancers, gene-based tests are rarely used routinely in clinical settings [5]. Clinically, patients with colorectal cancer often lack typical clinical symptoms [6], and even some patients do not have any symptoms, so it often causes difficulty in diagnosis or even misdiagnosis. Among the current diagnostic methods, colonoscopy is the most authoritative test method. However, colonoscopy is highly invasive and has the disadvantage of being inconvenient and expensive [7]. Although clinical carcinoembryonic antigen (CEA) and fecal occult blood test (FOBT) are commonly used as tumor markers, the sensitivity and specificity are poor, which limit their applications [8]. Therefore, the search for blood molecular biomarkers, the use of blood testing to diagnose and postoperative follow-up treatment of CRC patients is of great significance.

During the development of cancer, some tissue metabolites exhibit regular changes. Systematic application of analytical techniques to assess biological samples from healthy and diseased tissues, or metabolic levels in tissue samples obtained under other different conditions can provide us with indicators for detecting cancer progression [9].

Metabolomics is a new technology developed this year that can be used to quantify changes in small molecules at various pathological, physiological, and external stimuli in biological samples [10]. It can detect and identify different metabolites through a comprehensive analysis of low molecular weight endogenous metabolites in biological samples, revealing the pathways and roles of different metabolites in biological systems under different conditions [11]. The status of patients is monitor and the prognosis and treatment of postoperative colorectal cancer are comprehensively evaluated based on changes reflected from the genome, transcriptome, and proteome [12]. Metabolomics research usually involves multiple steps such as sample preparation, data acquisition, data processing, and statistical analysis. Correct data collection, processing, and analysis are prerequisites for obtaining statistically significant results [13]. With the in-depth study of metabolomics, a variety of detection and analysis methods have been applied to metabolomics, such as nuclear magnetic resonance (NMR), gas chromatography-mass spectrometer (GC-MS), liquid chromatography-mass spectrometer (LC-MS), etc. [14]. In recent years, high-performance liquid chromatography (HPLC) or ultra-high-performance liquid chromatography (UPLC) combined with mass spectrometer (MS) has been widely used due to its high efficiency and sensitivity [15].

In this study, we applied UPLC-Q-TOF-MS technology to analyze the plasma samples from CRC patients before and after surgery and healthy human as well as tissues samples from CRC patients and healthy human. The metabolic markers were screened by principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA). The detailed experimental process was shown in Fig. 1. Preliminary speculation was of great significance and value for the monitoring of colorectal cancer.

Method

Materials

Acetonitrile (ACN) was purchased from Concord Technology Co. Ltd (Tianjin, China). Distilled water was produced by Milli-Q ultra-pure water system (Millipore, Billeria, USA).

Study population and sample collection

Access to human samples complied with both Chinese laws and the guidelines of the Tianjin Union Medical Center Ethics Committee, and written informed consent was obtained from the patients. 24 blood samples were collected from CRC patients undergoing colorectal resection and that were maintained under fasting conditions in the Department of Colorectal Surgery, the Tianjin Union Medical Center. These patients came from different places in China and had different social environments, dietary practices, and personal habits. The diagnosis of CRC was confirmed in all cases by histopathologic examination

and immunohistochemistry. The preoperative blood samples were drawn at 7:00 o'clock on the second day before hospitalization, and the postoperative blood samples were drawn in 3d after surgery. During 3 h after surgery, the patients were not given any blood transfusion or other transfusion (electrolytes, glucose). Meanwhile, 24 blood samples were also collected from health volunteers. Eight volunteers with anal benign diseases were selected for anal surgery, and specimens of benign anal disease lesions were selected, including hemorrhoids without infection and edema, and normal tissues near the anal fistula. Exclusion criteria were: (1) pregnant or lactating female; (2) metabolic diseases, such as diabetes mellitus (DM), gout, hyperlipidemia (HL); (3) hematopathy, including all types of leukemia and anemia; (4) any symptom of acute diseases during the last 2 weeks, such as: febrile, cough, headache, nausea, vomit, stomachache, diarrhea, hematuria; (5) momentous stress reaction during the last 2 weeks, such as psychic trauma and a large area of empyrosis; (6) use of specific drugs during the last 3 weeks, such as antibiotics (penicillins, quinolones, torlamician), hormone (deoxycortisone, dexamethasone), nonsteroid anti-inflammatory drugs (NSAIDs: acenterine, fragrans, saridon, contac), among others;(7)Excluding acute perianal diseases, such as perianal abscess, incarcerated hemorrhoids, etc. All the information about these CRC patients and were summarized in Table 1.

Sample preparation

The blood samples were kept in room temperature for 30 min, subsequently centrifuged at 3,000 rpm for 10 min at 4°C, the serum samples were stored at -80°C until use. Plasma and colorectum tissue samples were thawed on ice. 200 µL of ACN were added to the 200 µL plasma then vortex-mixed for 1 minute. After centrifugation (12000 r/min, 5 min), the supernatant was taken for UPLC-Q-TOF-MS analysis. 30-50 mg of tissue sample was accurately weighted on ice. To extract all the ingredients, the tissue samples were fixed in 1 mL pure water and grinded in a mortar. 300 µL of ACN were added into 200 µL of the grinding fluid then vortex-mixed for 1 minute, centrifuged (12000 r/min, 5 min) and finally took the supernatant for UPLC-Q-TOF-MS analysis.

UPLC-Q-TOF-MS analysis

A Waters Acquity™ UPLC system (Waters, Milford, MA, USA), including a quaternary pump, an autosampler and a temperature controller, that was used for analyzing the prepared plasma and tissue samples. The chromatographic separation was performed on an ACQUITY UPLC BEHC18 column (100×2.1 mm, I.D., 1.8 µm) purchased from Waters at a flow rate of 0.3 mL/min and 5 µL injection volume. The run time was 13 min for each sample. The target column temperature and target sample temperature were maintained at 45 °C and 5 °C, respectively. Water was used as solvent A and ACN was used as solvent B to conduct the gradient elution. 1% B for the first 0.5 min, 1%-50% B from 0.5 to 2 min, 50%-100% B from 2 to 9 min, 100%-1% B from 9 to 11 min and finally held for two minutes to get ready for the next injection. The column eluent was directed to the mass spectrometry without spilting.

Mass analysis was carried out on a XevoG2 Q-TOF (Waters MS Technologies, Manchester, UK) with ESI source both in positive and negative mode. The desolvation gas rate was set at 800 L/h at a temperature

of 450 °C, the cone gas rate was set at 50 L/h and the source temperature at 120 °C. The capillary voltage and the cone voltage were set at 3000 V and 40 V in the positive ionization mode, and 2000 V and 40 V in the negative ionization mode, respectively. All analyses used the lockspray to ensure accuracy and reproducibility; the leucine enkephalin was used as the lock mass (m/z 556.6306 for positive ion mode; m/z 554.6147 for negative ion mode). Data was collected in centroid mode from m/z 50 to m/z 1000 with a lockspray frequency of 15 s, and data averaging over ten scans.

Statistical analysis

PCA analysis was first used to detect the grouping trend and outliers. OPLS-DA analysis was performed to understand the difference between the two selected groups. Furthermore, variable importance in the projection (VIP) was calculated in OPLS-DA model to screen the potential biomarkers. Compounds whose VIP value was more than 1.5 in negative ion mode and 5.5 in positive ion mode that was considered as potential metabolic biomarkers in tissue samples, whose VIP value was more than 1.5 in negative ion mode and 8.0 in positive ion mode that was considered as potential metabolic biomarkers in plasma samples as well. Besides, the Wilcoxon test was conducted to determine the significance of each metabolite using IBM SPSS Statistics 20 then Graphpad Prism 6 was used to map the data. All univariate tests were two-sided and the significant differences between two groups to be compared were based on the paired test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

Screening and analysis of biomarkers of CRC

The potential metabolic biomarkers were identified by the database of human metabolome database (HMDB, <http://www.hmdb.ca/>) and mapped into their biochemical pathways through metabolic enrichment and pathway analysis was based on database search Kyoto Encyclopedia of Genes and Genomes (KEGG, <http://www.genome.jp/kegg/>) and MetaboAnalysis 4.0 software.

Results

The representative TIC chromatograms of both positive and negative ion scanning modes taken from control group as well as CRC group of different retention time were displayed in Fig. S1. According to the peak height data, totally 59,878 peaks of positive ions and 2,244 peaks of negative ions were detected by MassLynx using the same acquiring method mentioned above. Similarly, differences displayed in the chromatograms between the normal group and CRC group were significant. Not only can we find plasma markers of colorectal cancer from these ion peaks, but the levels of these markers were not the same before and after colorectal cancer surgery (Table S1) which have been reported before [16].

PCA variable loading plot was showed in Fig. S2b, f. Each point in the figure represented the ion peak detected by UPLC-Q-TOF-MS in the plasma samples and displayed its retention time. Ion peaks in the center were those with small differences in samples, while those dispersed in the periphery were the ion peaks with statistical significance. PCA scores plot (Fig. S2a, e) showed the separation trend of ESI- and ESI+ model between CRC group and control group which indicated a certain tendency of separation (ESI-

R2X=0.38, ESI+: R2X=0.68). After that, taking the first predicted principal component (abscissa) and the first orthogonal principal component (ordinate) mapped OPLS-DA (Fig. S2c, g). Results showed that the CRC group has a more distinct separation trend from the normal group (ESI-: R2Y=0.99, Q2=0.99; ESI+: R2Y=0.99, Q2=0.99). The above results demonstrated the difference in metabolites between the CRC and normal groups. Finally, by means of VIP value distribution (Fig. S2d, h), ion peaks whose VIP value larger than 8.0 and 1.5 were selected in positive and negative ion modes respectively.

The metabolic profiles of the tissue samples were performed by UPLC-Q-TOF-MS. The representative TIC chromatograms of both positive and negative ion scanning modes taken from control group as well as CRC group of different retention time were displayed in Fig. S3. According to the peak height data, totally 37,375 peaks of positive ions and 1,326 peaks of negative ions were detected by MassLynx using the same acquiring method mentioned above. By comparing the chromatograms, we found that the peak shapes of control group and CRC group were very different, which indicated metabolic differences in tissue samples, and these substances were likely to become potential metabolic markers of tumors.

Here, PCA variable loading plot was showed in Fig. S4b, f. Each point in the figure represented the ion peak detected by UPLC-Q-TOF-MS in the tissue samples and displayed its retention time. Ion peaks in the center were those with small differences in samples, while those dispersed in the periphery were the ion peaks with statistical significance. PCA scores plot (Fig. S4a, e) showed the separation trend of ESI⁻ and ESI⁺ model between CRC group and control group which indicated a certain tendency of separation (ESI⁻: R2X=0.46, ESI⁺: R2X=0.54). After that, taking the first predicted principal component (abscissa) and the first orthogonal principal component (ordinate) mapped OPLS-DA (Fig. S4c, g). Results showed that the CRC group has a more distinct separation trend from the normal group (ESI⁻: R2Y=0.99, Q2=0.94; ESI⁺: R2Y=0.97, Q2=0.92). The above results demonstrated the differences in metabolites between the CRC and normal groups. Finally, by means of VIP value distribution (Fig. S4d, h), ion peaks whose VIP value larger than 5.5 and 1.5 were selected in positive and negative ion modes respectively.

Discussion

Through the VIP values in the OPLS-DA model combined with the HMDB database, the final screening for potential metabolic markers was shown in Table S2. 6 and 11 markers were screened in positive and negative ion mode, respectively. The mean values of plasma biomarkers in all samples were summarized by group of control, preoperative and postoperative. The concentrations of selected metabolites in plasma samples were compared to detect whether there were statistical differences between the selected blood markers before and after surgery. The heatmaps of these 17 substances in the control group, the preoperative group, and the postoperative group are shown in Fig. 2 (Fig. 2a for the positive ion mode and Fig. 2b for the negative ion mode). Although their concentrations changed before and after the surgery, the statistical results show that only one of those 17 substances were statistically significant. This may be caused by the blood samples which were collected 2-3 days after the surgery, when the concentration of metabolites was relatively small. Among them, the statistically significant substance was UDP-D-galactose. By comparing the concentration before and after surgery with that of the control group, we

found that the concentration of the metabolic markers changed in a normal direction after surgery. The results showed that the potential metabolic markers UDP-D-galactose screened by PCA analysis from the control group and CRC group also had statistical differences before and after surgery, which is expected to be a sensitive indicator for judging the operation and prognosis.

Through the VIP values in the OPLS-DA model combined with the HMDB database, the final screening for potential metabolic markers was shown in Table S3, and 19 markers were screened in each positive and negative ion mode. These substances are highly correlated with important biochemical metabolic pathways such as energy metabolism, amino acid metabolism, lipid metabolism and nucleotide metabolism.

The more detailed heat map of the tissue sample between the control group and CRC group is shown in Fig. 3. It is more visualized to see the difference of metabolic markers screened out in tissue samples between two groups. In addition, the results of metabolic pathways are shown in Fig. 4b. We showed the metabolic pathways whose P value were less than 0.05 in Fig. 4a, they are purine metabolism, propanoate metabolism, butanoate metabolism, synthesis and degradation of ketone bodies, citrate cycle (TCA cycle), pyruvate metabolism, valine, leucine and isoleucine degradation, terpenoid backbone biosynthesis, lysine degradation, glycolysis/gluconeogenesis and glyoxylate and dicarboxylate metabolism. Combined with the advanced reports, the relationship between the potential colorectal cancer metabolic markers and cancer were discussed. Finally, we depicted the metabolic pathways of colorectal cancer tissues, as shown in Fig. 5.

In the positive ion mode, there were 18 different metabolites were screened totally while seven of them were cancer-related metabolites that have been reported: Succinyl-5-aminoimidazole-4-carboxamide-1-ribose 5'-phosphate (SAICAR), riboflavin cyclic-4', 5'-phosphate (cFMN), Dihydroxyacetone phosphate (DHAP), lactosamine, phosphatidylserine, thiamine pyrophosphate, and creatine riboside. Among them, cyclic-4', 5'-phosphate and lactosamine have been reported to be closely related to colorectal cancer.

cFMN has been identified as an endogenous liver compound, probably decomposed by flavin adenine dinucleotide (FAD, also known as active vitamin B2) in alkaline medium. In patients with the methylenetetrahydrofolate reductase (MTHFR) TT genotype, there was a negative correlation between vitamin B2 (also known as riboflavin) intake and colorectal cancer, and the risk of adenoma appeared to be more pronounced [17]. Meanwhile, studies by Robin Myte show a negative correlation between plasma vitamin B2 concentration and CRC risk. This study also demonstrates for the first time that the transcription/translation of RFVT has undergone profound changes in CRC [18]. For lactosamine, studies have shown that the structure of poly-lactosamine is often characterized by different cell types and different stages, and poly-lactosamine may be a potential target for 5-fluorouracil (5-FU) responses in colon cancer cells. Therefore, it may be a novel drug target candidate for the treatment of colon cancer [19]. However, there are no reports on its metabolic regulation until now.

The remaining five substances also have reports on their relationship with tumors. SAICAR is an intermediate of the denovo synthesis pathway of purine nucleotides and a metabolite with higher content

in proliferating cells. It induces pyruvate kinase M₂ (PKM₂) in vitro and in cells, playing an important role in abnormal cell metabolism and sustained proliferation as well [20]. It has also been reported that SAICAR can activate PKM₂ in a dimeric form [21]. At the same time, the interaction of SAICAR-PKM₂ also promoted the survival of cancer cells under glucose limiting condition. DHAP is a biochemical molecule involved in many reaction pathways such as glycolysis and an intermediate product of glycolysis and glycolformulose. This metabolic pathway is highly correlated with tumorigenesis [22]. Phosphatidylserine (PS) is an essential component of all human cells and is present in the inner leaflets of the cell membrane [23]. PS mainly inhibits the biological process, function and malignant transformation of host cells by activating PS receptors on immune cells and inhibiting anti-tumor immune responses [24]. Thiamine pyrophosphate (TPP) is a coenzyme form of vitamin B1. During hypoxic stress, tumor cells adaptively up-regulate thiamine transport. Once ingested, thiamine pyrophosphate kinase-1 (TPK1) promotes rapid phosphorylation of thiamine to TPP [25]. Creatine riboside and N-acetylneuraminic acid (NANA) levels in urine were used as biomarkers of non-small cell lung cancer (NSCLC).

In the negative ion mode, the screened biomarkers were more closely related to colorectal cancer. Seven of them are: Cyclic AMP, caffeoyl aspartic acid, triglyceride (TG), UDP-D-galactose (2-), adenosine monophosphate, diacylglycerol (DG) and Succinic acid. Succinic acid is an important intermediate metabolite in the tricarboxylic acid cycle (TCA) [26], Studies showed significant changes in urinary metabolism in patients with colorectal cancer, as well as differences between patients with early and late colorectal cancer. Aspartic acid and succinic acid decreased in colorectal cancer compared with control group [27]. Adenosine monophosphate (AMP), also known as adenine ribonucleotide, is a unit of RNA and a constituent of NAD and NADP [28]. AMP is an important part in DNA synthesis in cells. The increase of AMP in CRC tissues reflects the rapid division of CRC cells. AMP was significantly different between adenomatous polyps and CRC tumor tissues and was lower in CRC tissues [29]. CA19-9 in serum is a well-known tumor marker and is often used in clinical diagnosis of cancer, especially colorectal cancer. UDP-galactose is necessary for the synthesis of CA 19-9 [30]. For CRC patients, TG levels were significantly higher and significantly different in patients with different TNM stages [31]. In human colon cancer, adenylate cyclase (AC) activity and intracellular cAMP concentration were much lower than dermal fibroblasts. The differences were so large that AC activity and intracellular concentrations of cAMP might be biochemical markers that could be used to distinguish colon cancer from benign cells in tissue culture [32]. The above analysis indicates that all the above substances are closely related to colorectal cancer. More depth and specific research on these metabolites will help to better study of the pathogenesis of colorectal cancer and find more effective treatment methods.

Conclusion

In this study, we applied UPLC-Q-TOF-MS technology to conduct metabolomics analysis on plasma samples of CRC patients before and after operation, as well as tissue samples of CRC patients and healthy people. We have mapped the metabolic pathway by analyzing related metabolites in tissue samples, which is very important for us to understand and study the metabolic pathogenesis of CRC

deeply. In the study of plasma samples, the concentration of the metabolic marker UDP-D-galactose changed significantly within a short time after surgery, so it is expected to be a very sensitive plasma metabolic marker to evaluate the surgical prognosis.

Abbreviations

CRC: Colorectal cancer, UPLC-Q-TOF-MS: Ultra-high-performance liquid chromatography quarter time of fly mass spectrometer, APC: Adenomatous polyposis coli, CEA: Carcinoembryonic antigen, FOBT: Fecal occult blood test, NMR: Nuclear magnetic resonance, GC-MS: Gas chromatography-mass spectrometer, LC-MS: Liquid chromatography-mass spectrometer, HPLC: High-performance liquid chromatography, UPLC: Ultra-high-performance liquid chromatography, MS: Mass spectrometer, PCA: Principal component analysis, PLS-DA: Partial least squares discriminant analysis, CAN: Acetonitrile, DM: Diabetes mellitus, HL: Hyperlipidemia, NSAIDs: Nonsteroid anti-inflammatory drugs, VIP: Variable importance in the projection, HMDB: Human metabolome database, KEGG: Kyoto encyclopedia of genes and genomes, SAICAR: Succinyl-5-aminoimidazole-4-carboxamide-1-ribose 5'-phosphate, DHAP: Dihydroxyacetone phosphate, FAD: Flavin adenine dinucleotide, MTHFR: Methylenetetrahydrofolate reductase, 5-FU: 5-Fluorouracil, PKM₂: pyruvate kinase M₂, PS: Phosphatidylserine, TPP: Thiamine pyrophosphate, TPK1: Thiamine pyrophosphate kinase-1, NANA: N-acetylneuraminic acid, NSCLC: non-small cell lung cancer, TG: Triglyceride, DG: Diacylglycerol, TCA: Tricarboxylic acid cycle, AMP: Adenosine monophosphate, AC: Adenylate cyclase.

Declarations

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Availability of data and materials

All data are presented in the figures, tables and Electronic Supplementary Material files.

Conflict of Interest

The authors declare that they have no conflict of interest.

Authors' contributions

XZ and LD designed the study. ZZ, WZ and LZ researched relevant references for the study. CX, YL, YY, CC, and ML collected the clinical samples. ZZ and LS performed the experimental work and wrote the manuscript. Both authors have contributed to the data analysis, discussion and interpretations of the results. Both authors have read and approved the final manuscript.

Consent for publication

Not applicable.

Ethics approval and consent to participate

All procedures performed in this study that involved human participants were approved by the Research Ethics Committee of Tianjin Union Medical Center (2016YJ029) and conformed to the ethical standards of the 1964 Helsinki Declaration.

All subjects were over 18 years of age and voluntarily signed informed consent forms.

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Table

Table 1. Clinical information and characteristics of human subjects

	Blood Samples			Tissue samples	
	Control	Preoperation	Postoperation	Control	CRC
Sample size	24	24	21	8	22
Male/female	16/8	15/9	13/8	2/6	13/9
Age (mean, range)	52.13(14~81)	58.75(39-76)	59.48(39-73)	62.38(48~72)	63.09(30~80)
Stage					
TNM-I	-	1(1/0)	1(1/0)	-	1(1/0)
TNM-II	-	13(8/5)	12(7/5)	-	12(9/3)
TNM-III	-	10(6/4)	8(5/3)	-	9(3/6)

Figures

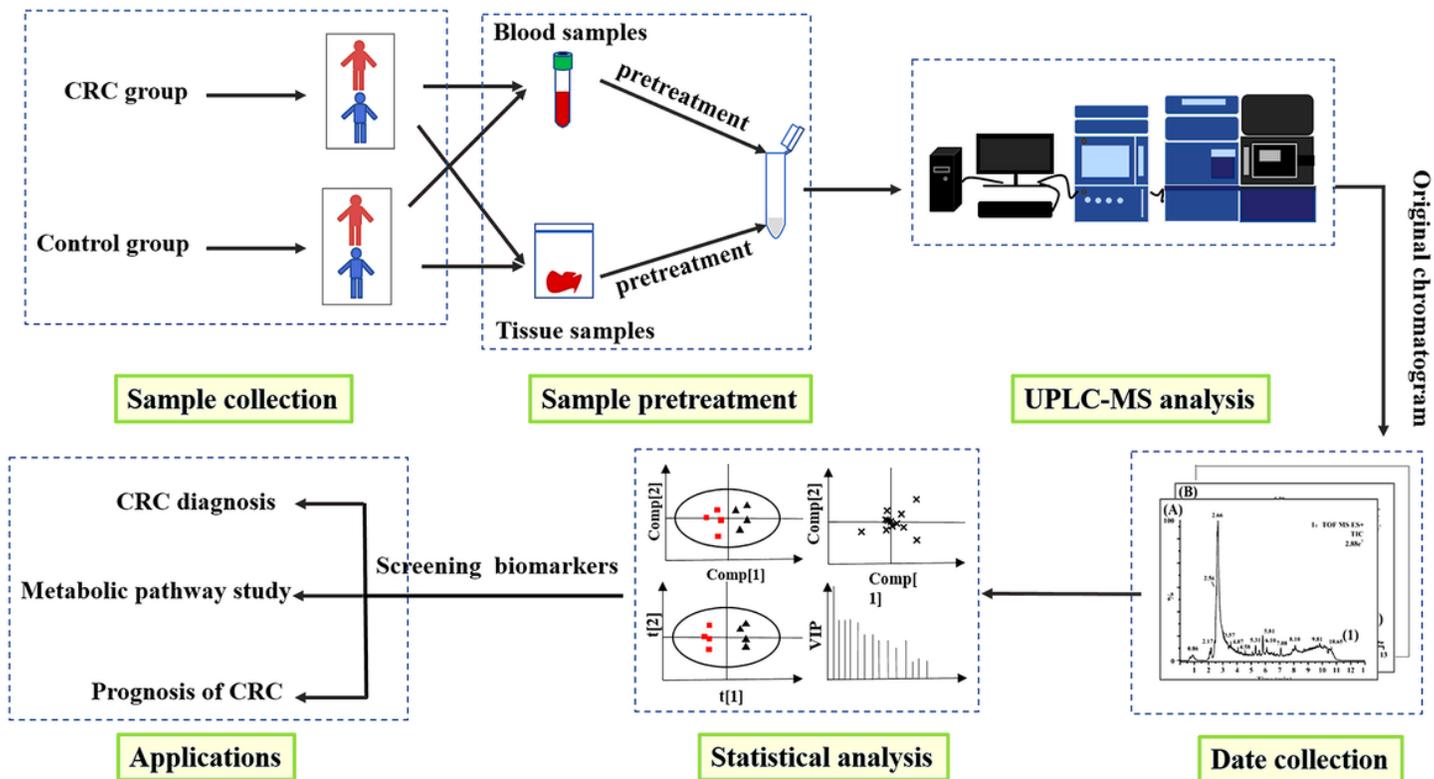


Figure 1

The overall flowchart of application of metabolomic method in this study.

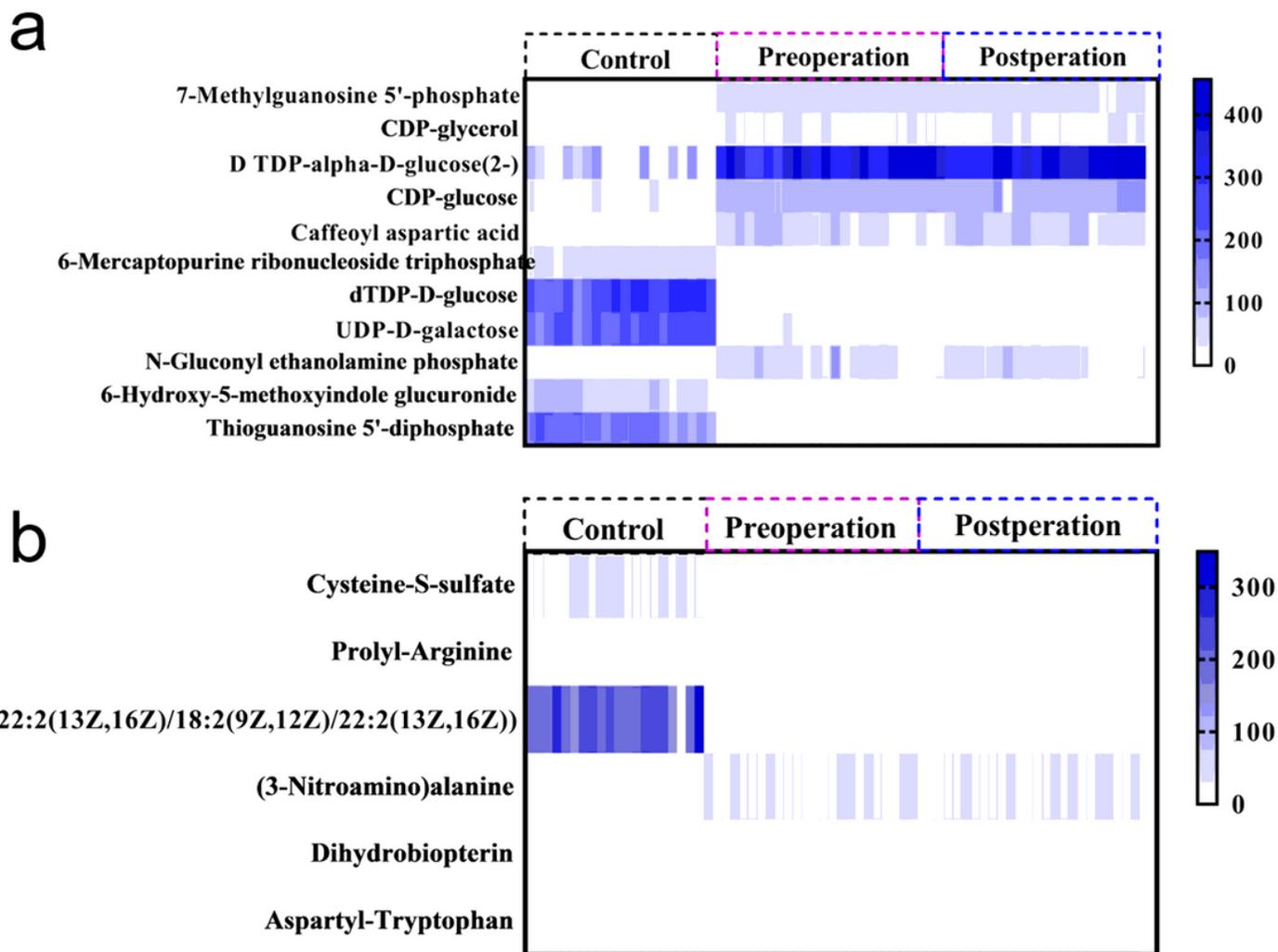


Figure 2

Heatmaps of the markers screened by ESI+(a) and ESI-(b) ion mode in the control group, the preoperative group, and the postoperative group.

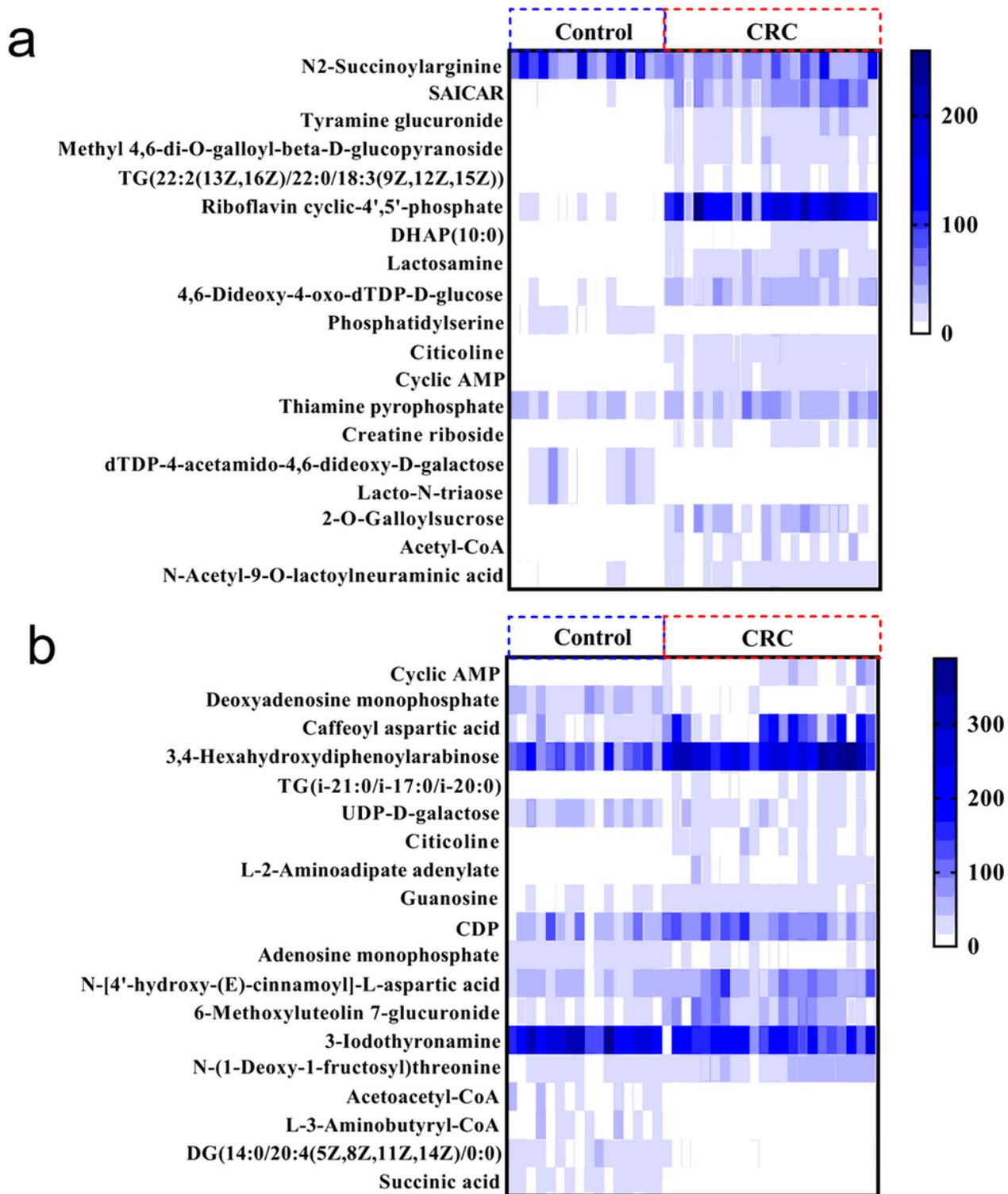


Figure 3

Heatmaps of the markers screened by ESI+(a) and ESI-(b) ion mode in the control group and the CRC group of tissue samples.

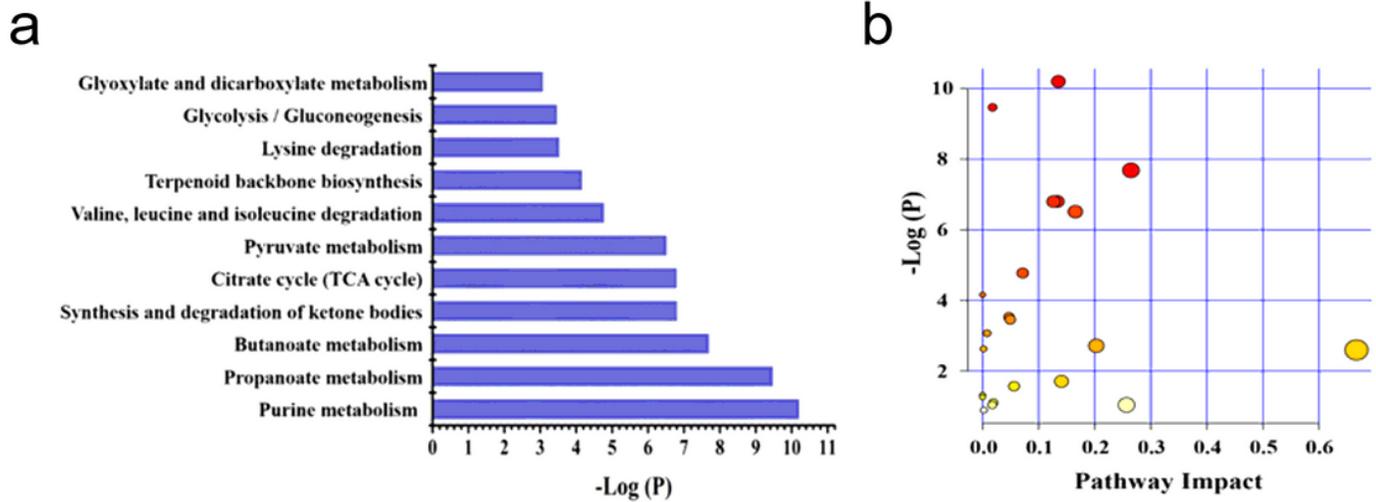


Figure 4

Log P value map(a) and metabolic pathway analysis chart(b) of candidate markers.

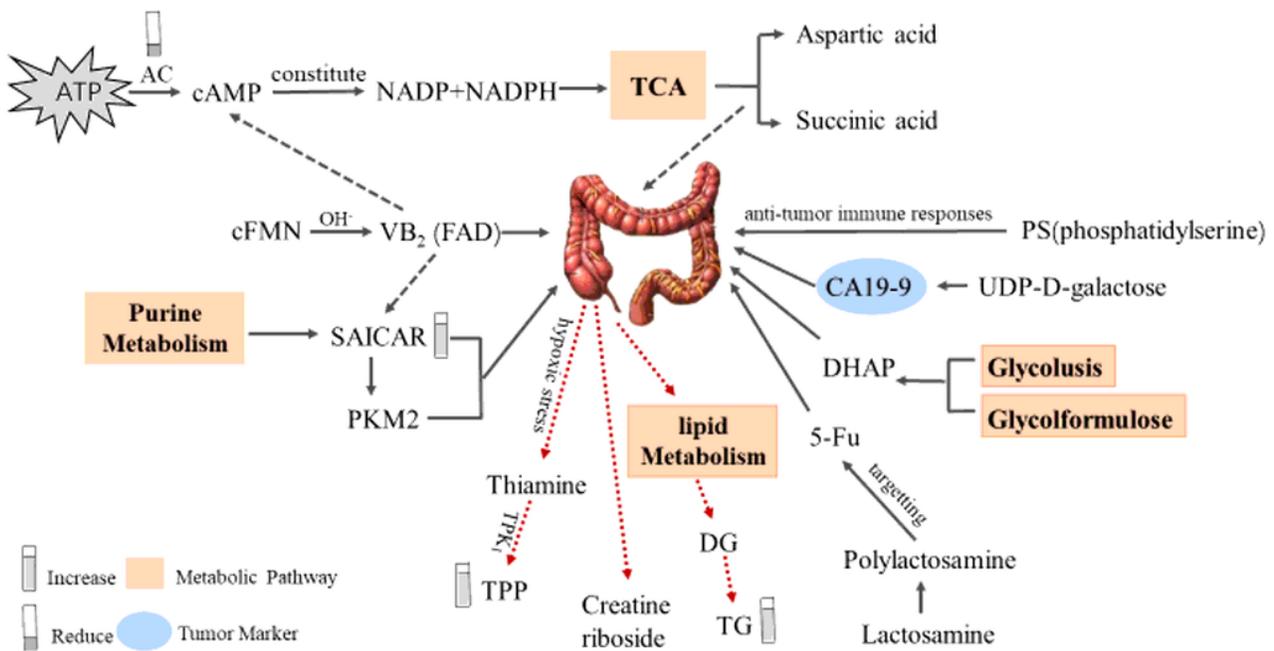


Figure. Metabolic pathways of colorectal cancer metabolic markers in tissue samples

Figure 5

Metabolic pathways of colorectal cancer metabolic markers in tissue samples.

Supplementary Files

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- [ElectronicSupplementaryMaterial.docx](#)