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Plasma metabolic profiling on postoperative colorectal cancer patients with different traditional Chinese medicine syndromes

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ABSTRACT

Objectives: This study aims to investigate the metabolic profiles of postoperative colorectal cancer (PCRC) patients with different traditional Chinese medicine (TCM) syndromes and to discuss the metabolic mechanism under PCRC progression and TCM syndrome classification.

Methods: Fifty healthy controls (HC) and 70 PCRC patients, including 10 Dampness and heat syndrome (DHS), 33 Spleen deficiency syndrome (SDS), 19 Liver and kidney Yin deficiency syndrome (LKYDS) and 8 with non-TCM syndrome (NS) were enrolled. Plasma metabolic profiles were detected by Gas chromatography-mass spectrometry (GC–MS) and analyzed by principal component analysis (PCA) and partial least squares-discriminate analysis (PLS-DA). Furthermore, pathway enrichment was analyzed based on KEGG and DAVID databases and metabolic network was constructed via metaboanalyst and cytoscape.

Results: The top-3 metabolites with higher abundance in PCRC compared with HC were terephthalic acid (165.417-fold), ornithine (24.484-fold) and aminomalonic acid (21.346-fold). And the cholesterol (0.588-fold) level was decreased in PCRC. L-Alanine, 1, 2-ethanediamine, urea, glycerol, glycine, aminomalonic acid, creatinine and palmitic acid were specifically altered in the DHS, while D-tryptophan was exclusively changed in SDS, and L-proline, 1, 2, 3-propanetricarboxylic acid, D-galactose and 2-indolecarboxylic acids in LKYDS.

Conclusions: The plasma metabolic profiles were perturbed in PCRC patients. Increased levels of terephthalic acid might indicate high risk of relapse and elevated ornithine may contribute to the post-operational recovery or may raise the susceptibility to PCRC recurrence. The metabolic profiles of DHS, SDS, LKYDS and NS were almost separately clustered, indicating the possibility of explaining TCM syndromes classification using metabolomics. Furthermore, creatinine and aminomalonic acid alternation might correlate with the formation of DHS, while D-tryptophan may associate with SDS and D-galactose and 1, 2, 3-propanetricarboxylic acid may relate to LKYDS. As numbers of patients in each TCM syndrome are small, further study is needed to verify those results.

1. Introduction

Colorectal cancer (CRC) ranked the second highest morbidity in females and third in males. 1.4 million cases and 693.900 deaths were estimated globally in 2012 [1]. Surgical treatment is the mainstay for 80% CRC patients without metastatic disease, but over 40% of patients in the stage II or III may encounter postoperative progression.[2,3] In some cases with residual cancer, tumor size increased fast after operation.[4,5] Screening programs could help to stratify those patients who have a tendency to relapse and personalized approach may be able to improve the survival of postoperative CRC (PCRC) patients.

Traditional Chinese medicine (TCM) has been applied in cancer treatment for a long time. TCM treatment benefits cancer patients in terms of modulating immunity, enhancing efficacy, reducing adverse effects and abrogating drug resistance etc. and represents a promising complementary approach in CRC prevention and treatment.[6,7] An effective TCM therapy is based on accurate TCM syndrome differentiation, which summarized the pathological essence of patients' clinical

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manifestations based on holism concept and guided the individualized treatment. But its biological material basis and molecular classification remain obscure.

Metabolomics is a systematic method to capture the alternations of entire profile of small molecules. It is the ultimate station of gene expression and protein synthesis and is sensitive to the environmental exposure, which is an effective method to reveal the essence of TCM syndrome. Studies using metabolomics methods to unveil the mechanism of TCM syndrome and to explore the action of Chinese herbs have been conducted and pleasant discovery has been obtained.[8,9] Gas chromatography-mass spectrometry (GC–MS) was the primary method to examine the metabolic profile due to its high sensitivity.[10,11] Metabolic alternations were also closely correlated with CRC development.[12] But no study has been carried out to discuss the metabolic profiling alternations of Dampness and Heat Syndrome (DHS), Spleen Deficiency Syndrome (SDS) and Liver and Kidney Yin Deficiency Syndrome (LKYDS) in PCRC patients.

The study was conducted to investigate the underlying metabolic mechanisms of PCRC and to explore the metabolic basis for TCM syndromes including DHS, SDS and LKYDS using GC–MS.

2. Methods

2.1. Sample collection

This study was approved by the Official Ethics Committee in Shuguang Hospital, which is affiliated to Shanghai University of TCM. The research protocol was granted by respective institutional review boards. All participators have signed the informed consent. CRC was diagnosed according to the *World health organization classification of tumors of the digestive system* without metastatic lesions based on laboratory examination.[13] The TCM syndromes were identified by the consensus of 3 chief or deputy physicians, according to the *The Guiding Principles for the Clinical Study of New Drugs for Use in Traditional Chinese Medicine*.[14] Patients with severe cardiovascular, renal, hematopoietic system disease, psychological problems, gastrointestinal obstruction and those who have taken drugs that may influence the results were excluded. Fifty healthy control (HC) and 70 CRC patients who have received the scheduled surgery resection (10 with DHS, 33 with SDS, 19 with LKYDS and 8 with non-TCM syndrome (NS)) were enrolled. Morning fasting peripheralvenous blood samples were taken and plasma was collected within 2 h and stored at −80 °C.

2.2. Sample preparation and GC–MS analysis

Firstly, samples were frozen in −20 °C. Then they were centrifuged at 14 000 × *g* for 10 min and supernatant was obtained for derivatization as follows. 100 ul of plasma were transferred to an Eppendorf tube and 10ul of 4-Chloro-L-phenylalanine (0.3 mg/mL) and heptadecanoic acid (1 mg/mL) respectively were added as internal standards. Then, 300ul of the mixture of methyl alcohol and chloroform with 3:1 ration were added, vortexed for 30 s and then standing in −20 °C for 10 min to precipitate protein. After that, 300ul of supernatant was collected into the glass vial and dried with N_2, incubated in 30 °C for 90 min with shaking. Finally, those samples were standing for 60 min.

One μL of derivatized sample was transferred. The temperatures of injection port and ion source (electron impact) was 280 °C and 230 °C respectively. 99.999% high pruityhelum flowed with a rate of 1 mL/min constantly. Temperature programming of DB-5MS capillary column (Agilent J&W Scientific, Folsom, CA, USA) was listed in table S1. MS scan ranges from 30 *m/z* to 550*m/z*. Raw data were extracted by the Agilent MSD and processed by the R package XCMS (http://meltin.scripps.edu/download/). Metabolites were identified according to NIST library (http://nistmassspeclibrary.com) and data were normalized to the total peak area of each sample before further analysis.[15,16]

2.3. Statistical analysis

Clinical parameters of continuous variables were calculated by One way-anova or Nonparametric tests and categorical variables by Chi-square test. Normalized metabolomics data were imported into the Simca-P (version 11.0, Umetrics, Umea˚, Sweden). Principal component analysis (PCA) and partial least squares discriminate analysis (PLS-DA) models were constructed to overview the distribution of samples. Metabolites with variable importance in the projection values (VIP) over 1.0 were selected as Differently altered metabolites (DMs) between groups. Metabolic correlations were determined based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Ahttp://www.kegg.jp/kegg/tool/map_pathway.html) and metaboanalyst (http://www.metaboanalyst.ca) and constructed by Cytoscape.

3. Results

3.1. Clinical parameters of PCRC patients with different TCM syndromes

The characteristics of clinical parameters including sex, age, alpha fetoprotein (AFP), carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9), CD3, CD4 and NK + CD56 in PCRC patients with DHS, SDS, LKYDS and NS were analyzed. As shown in Table 1, all parameters among DHS, SPDS, LKYDS and NS have no statistical significance ($P > 0.05$).

3.2. Plasma metabolic profiles of PCRC patients with different TCM syndromes and HC

The profiles of metabolites and clinical parameters were analyzed by PLS-DA. PCRC and HC revealed distinct clustering (Fig. 1A) and PCA results were displayed in Fig. S1. R^2Y in the PLS-DA is to evaluate the proportion of variance that the model could explain and Q^2Y indicates the predictability of the model. The model in Fig. 1 has 2 components and its R^2Y and Q^2Y were 0.943 and 0.930 respectively. Eight identified endogenous metabolites were differently altered in PCRC compared to

Table 1
Clinical parameters of PCRC patients with different TCM syndromes (Mean ± SD).

Parameters	Mean ± SD				P value
	DHS	SDS	LKYDS	NS	
Sex (M/F)	12/7	16/17	7/3	5/3	0.56
Age (Year)	68.86 ± 8.57	61.77 ± 11.27	62.92 ± 10.84	59.57 ± 10.81	0.433
APF (ng/m)	2.81 ± 0.54	3.25 ± 2.62	2.77 ± 1.03	2.42 ± 0.79	0.793
CEA (μg/L)	5.30 ± 11.53	3.53 ± 2.66	2.09 ± 1.22	3.03 ± 0.64	0.066
CA199 (kU/L)	16.83 ± 17.53	21.97 ± 30.31	15.73 ± 6.69	22.39 ± 27.95	0.629
CD3	61.31 ± 11.76	60.97 ± 9.85	61.53 ± 6.86	69.64 ± 13.41	0.465
CD4	37.06 ± 10.67	34.8 ± 5.55	34.13 ± 5.37	34.14 ± 9.39	0.890
CD4/CD8	1.35 ± 0.58	1.19 ± 0.41	1.14 ± 0.25	1.07 ± 0.51	0.485
NK + CD56	22.14 ± 9.76	23.3 ± 9.09	20.76 ± 7.93	17.02 ± 6.42	0.478