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Tissue amino acid profile could be used to differentiate advanced adenoma from colorectal cancer

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ABSTRACT

Advanced adenomas are of higher risk to progress to colorectal cancer (CRC), the third leading cause of cancerous death worldwide. Endoscopy-based adenoma removal greatly contributes to arresting the progression of adenoma to CRC. Precise diagnosis, post-polypectomy surveillance and the follow-up clinical decisions predominantly depend on histopathologic inspection of the resected tissues. The common artificial histological inspection is not fully reliable and is only compatible with the *en bloc* removed tissues. An alternative measure ensuring more objective tissue malignance appraisal, which is applicable to various endoscopically acquired sample types are highly appreciated. In this study, we firstly employed capillary electrophoresis–mass spectrometry-based untargeted metabolomic technique to analyze CRC and corresponding paracancerous tissues to narrow the scope of malignancy-related metabolite changes. The primary results implied the most perturbated metabolites by CRC onset were amino acids. Subsequently, a targeted amino acid analysis by ultra-performance liquid chromatography–mass spectrometry indicated 9 amino acids were of different content between advanced adenoma and CRC tissues. Finally, regression analysis of the 9 differential amino acids exhibited that methionine, tyrosine, valine and isoleucine could be used to differentiate CRC from advanced adenomas with good sensitivity and specificity ($p < 0.001$). Area under the receiver operating characteristic curve was 0.991. This study demonstrated the utility of metabolomic analysis in assisting malignance evaluation of colorectal neoplasia and the potential value of amino acids analysis in clinical pathology practice.

1. Introduction

Colorectal cancer (CRC) has a higher incidence especially in the western world. It ranks as the third leading cause of cancer-related death. The National Cancer Institute estimated that, in the United States, about 102,480 colon cancer and 40,340 rectal cancer cases were diagnosed in 2013, of which nearly 50,830 deaths occurred (http://www.cancer.gov/cancertopics/types/colon-and-rectal). The increasingly prevailed westernized diet styles in the developing countries put their citizens at a higher risk to develop CRC [1]. A recent report indicated a clear ascending trend of CRC morbidity in the Chinese population and CRC is thought to be a long-term threat to modern society [2].

CRC usually initiates from benign adenoma, then progresses to advanced adenoma and, finally, to invasive cancer step by step [3]. CRC is characterized by submucosal invasion. Patients carrying advanced adenomas are thought to be at higher risk to suffer from CRC [4]. Resection of adenomas can substantially disrupt the CRC progression. It was estimated that incidence of CRC could be decreased by 76–90% after polypectomy [5]. Although computed tomographic colonography was reported to be superior to optical colonoscopy in colorectal neoplasia screening [4], colonoscopy is still the most favorable approach in clinics because of its unique ability to simultaneous adenomatous polyp detection and removal [6].

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Endoscopic submucosal dissection (ESD) or endoscopic mucosal resection (EMR) are the two popular endoscopy-based adenoma removal strategies, but both of them show limited ability to predict submucosal invasion risk [7]. Polyp diagnosis, post-polypectomy surveillance and the follow-up clinical intervention decisions predominantly depend on histopathologic appraisal of the resected tissues. A recent retrospective study indicated that traditional histopathologic inspection was not sufficiently reliable due to some casual factors, such as the sampling errors and inter-pathologists assessment variations [8]. Additionally, the tissue section preparation is a time-consuming and labor-intensive procedure, although many automatic devices had been developed to improve and standardize the relevant operations. What makes things worse is that piecemeal EMR operation can not provide qualified specimens for histopathological concerns. Therefore, great efforts are needed to improve diagnosis and stratification accuracy of endoscopically removed colorectal neoplasia [8].

Dysregulated metabolism is a hallmark of cancer tissues, which is representative of the well-recognized Warburg effect [9]. Changes taking place at the metabolite levels are more likely to be the initiative factors contributing to tumorigenesis rather than the end-point events resulting from cancer occurrence [9]. A growing body of evidence has demonstrated that metabolic alterations are closely linked to tumorigenesis [10]. For instance, arginine was found to accumulate in CRC tissues. HCT-116 cells lacking sufficient arginine supply tended to suffer from excessive apoptosis [11]. Using other colorectal cell lines, it was found that there was a negative feedback between spermidine metabolism and PI3K pathways in CRC progression [12]. It is evident that tumor tissue metabolite studies can provide meaningful clues to probe oncogensis mechanisms. Additionally, it contributes to finding the cancer's Achilles' heels in the context of developing new therapeutic targets [13,14].

In order to get a more holistic view of the metabolic disorders, metabolomics, a strategy to detect as many metabolites as possible in a single analytic run, has been employed to analyze varied samples from CRC patients. A review summarized 8 independent studies indicated that taurine, lactate, choline, inositol, glycine, phosphocholine, proline, phenylalanine, alanine, threonine, valine and leucine were found to be stably changed in varied CRC samples [15]. Notably, tissue metabolite profiles could be used not only to distinguish CRC of varied T- and N-stages but also to be valuable in 5-year survival prediction [16].

In the current study, an untargeted metabolomic analysis based on capillary electrophoresis–mass spectrometry (CE–MS) was firstly employed to profile the metabolite difference between CRC and the corresponding paracancerous tissues. Then, the mainly affected metabolites and corresponding metabolic pathways were screened to guide the subsequent targeted metabolomic analysis of the CRC and advanced adenoma tissues using a new set of samples. Finally, differential metabolites between the CRC and advanced adenoma samples were subjected to regression analysis. The statistic model were evaluated in view of specificity and sensitivity for advanced adenomas and CRC discriminating. The aim of this study was to explore an alternative measure to assess malignancy status of the polypectomy-removed tissues.

2. Experimental

2.1. Reagents and chemicals

Methanol (HPLC grade), chloroform (HPLC grade), 98% formic acid and ammonium acetate (HPLC grade) were purchased from Merck (Darmstadt, Germany), J&K Chemical (Ulsan, Korea), and Tedia (Fairfield, OH), respectively. Ultrapure water was produced by Milli-Q system (Millipore, Billerica, MA).

2.2. Sample collection and preparation

All samples were collected from Dalian University Affiliated Xinhua Hospital. The inclusion of the samples in this study was under the survey of Clinical Ethic Committee of the hospital. Before sample collection, informed consent was acquired from every patient. All the patients denied any inherited CRC history. Only specimens confirmed without any discrepancy amid three independent pathologists were included in this study.

The first set of samples included 4 rectal cancer, 7 colon cancer and the corresponding paracancerous tissues. They were collected through surgical operation and immediately immerged in liquid nitrogen when they were resected from the patients. The second set of samples included 22CRC samples and 10 advanced adenoma samples. The adenoma samples were collected through either ESD or EMR as recommended [7]. For simplicity, advanced adenoma was called as adenoma hereafter, except mentioned specifically.

2.3. CE–MS analysis

The first set of samples were finely pestled in liquid nitrogen. Each sample powder was collected in a 15 mL centrifuge tube containing 1 mL ice-cold methanol, 1 mL chloroform, 400 μL Milli-Q water and commercial internal standards (50 μM methionine sulfone and camphor-10-sulfonic acid) from HMT Inc. (ToKyo, Japan). After triplicate 5 s-sonication (ultrasonic power of 300 W) in ice bath, every sample was subjected to centrifugation at 12,000 × *g* for 15 min at 4 °C. Each 800 μL of the upper aqueous phase was pipetted out and equally dispensed into two 5-kDa cutoff filters (Sartorius Stedium, Germany) and centrifugally filtered for 4 h to remove the contaminated solid debris. Filtrate was lyophilized by vacuum freeze-dried system (Labconco, Cansas, MO). Each middle protein layer was dried to a constant weight. For CE–MS analysis, every lyophilized sample was dissolved in proper volume of dissolving reagent (HMT) proportional to corresponding dry weight. The dissolving reagent contained 3 internal standards (1 mM 3-aminopyrrolidine dihydrochloride and trimesic acid). About 10 μL aliquot of each reconstructed sample was utilized for subsequent CE–MS Analysis. Quality control (QC) samples were prepared by mixing equal aliquot of each filtrate sample. Standard solution samples (HMT) and QCs (injected with an interval of 3–4 real samples) were run to evaluate instrument performance. The instrumental analysis was conducted using an Agilent CE G7100A (Santa Clara, CA) system equipped with an Agilent G6224A TOF mass spectrometry as proposed previously [17].

2.3.1. Cationic mode analysis

Metabolites were separated in a fused-silica capillary (50 μm i.d. ×80 cm total length) using 1 M formic acid as the running electrolyte. The sheath liquid with delivering rate of 10 μL/min was 0.1 μM hexakis phosphazene in methanol/water (50% v/v) solution. The capillary temperature was constantly maintained at 20 °C and the sample tray temperature was held at 5 °C. Sample injection time was 3 s at 50 mbar (~3 nL). The positive voltage was set to 30 kV. The capillary voltage was 4 kV. Drying gas flow rate of heated nitrogen (300 °C) was 7 L/min. The skimmer, fragmentor, octapole radio frequency voltage, and nebulizer pressure were 50 V, 105 V, 650 V, and 5 psi, respectively. Exact mass data were acquired at a rate of 1.5 cycle/s and the scanning m/z range was 60–1000.

2.3.2. Anionic mode analysis

A fused-silica capillary preconditioned with a commercial buffer (H3302-1022, HMT) was used for separation. The background electrolyte was 50 mM ammonium acetate solution (pH 8.5). Sample injection was achieved by 50 mbar pressure lasted for 25 s (~25 nL). The applied negative voltage, fragmentor and capillary voltages