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Determination of low-molecular-weight organic acids in non-small cell lung cancer with a new liquid chromatography–tandem mass spectrometry method

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ABSTRACT

As compared to other classes of metabolites, determination of organic acids is an underrepresented field in cancer research and till now there has been a lack of appropriate analytical procedure for determination of serum levels of organic acids potentially associated with cancer development. The aim of the study was to develop a new rapid liquid chromatography–tandem mass spectrometry method for the quantification of six low-molecular-weight organic acids in human serum and to apply this method in an analysis of samples collected from non-small cell lung cancer (NSCLC) patients and a matched control group. The samples were prepared by solid phase extraction (Clean-up CUQAX, UCT). Chromatography was conducted on a Synergi Hydro–RP column (Phenomenex) and a gradient run of 15 min. Detection was performed using a negative multiple reaction monitoring mode. The calibration ranges were as follows: 0.24–38.42 μmol/L for 2-hydroxybutyric acid, 0.09–17.23 μmol/L for fumaric acid, 0.08–15.13 μmol/L for glutaric acid, 0.11–2.22 mmol/L for lactic acid, 0.39–30.98 μmol/L for pyroglutamic acid, and 0.08–16.93 μmol/L for succinic acid. Mean relative recovery range was 85.99–114.42% and the determined intra- and inter day coefficients of variation were ≤14%. Among the studied acids, pyroglutamic acid showed the best discriminating potential and enabled to identify accurately NSCLC patients and control subjects regardless of the cancer stage. Further investigations of serum organic acids may allow us to better understand the underlying mechanisms involved in NSCLC and develop novel means of its detection and treatment. The developed method may be also a valuable tool to study metabolic changes associated with other types of cancer.

1. Introduction

Analysis of tumor-related changes in human metabolome has become a rapidly expanding approach in cancer research in the recent years [1,2]. Cancer is a complex disease that affects multiple metabolic pathways and therefore it is a suitable object of metabolomic studies. Apart from their extensive cognitive significance, metabolomic investigations offer also a practical importance as they may contribute to a design of new tools for cancer diagnosis or monitoring therapy. Cancer metabolome is reflected not only in the affected tissues but in the biological fluids as well [1,3]. An important group of metabolites correlated with a tumor presence are low-molecular-weight organic acids. Alterations in their concentration levels were reported in different types of cancer [4–6].

Lactic acid is one of the most important organic acids during cancerogenesis. Many tumor cells produce and secrete large amounts of lactic acid as a consequence of altered glucose metabolism [7]. It was found that lactate is a pro-inflammatory mediator and plays also a critical role in tumor growth, angiogenesis, and metastasis [8–11]. Determination of organic acids in gastric cancer tissues revealed significantly increased levels of lactate and crucial Krebs cycle components, such as fumaric acid and succinic acid compared with normal tissues [12,13]. Elevated levels of lactic acid and fumaric acid were also found in serum of lung cancer patients in a non-targeted metabolomic study [4]. Moreover, serum metabolite profiles from patients with esophageal cancer demonstrated increased levels of pyroglutamic acid and lactic acid [14]. Another low-molecular-weight organic acid that requires special

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attention in cancer research is 2-hydroxybutyric acid. Significant alteration in the serum level of 2-hydroxybutyric acid was reported in metabolomic analysis of colorectal cancer and thus this metabolite was incorporated into a colorectal cancer prediction model [15]. Glutaric acid seems to be also worth investigation in terms of better characterization of carcinogenesis [16]. This acid is related to tryptophan metabolism, which has been recognized as an important factor in immunobiology of cancer [17,18]. Studies focused on serum metabolomic profiling in other types of tumors, such as pancreatic [19], hepatocellular [20] and prostate [21] tumors also revealed significantly altered levels of some organic acids. Therefore, it may be supposed that measurements of organic acids in blood of cancer patients may facilitate identification of tumor-associated metabolic alterations that may result in a development new diagnostic tools and treatment strategies.

A number of studies indicated the changes in small molecule organic acid profiles as a characteristic feature of cancer metabolome. However, many of the above-cited reports were performed by applying a non-targeted metabolomic analysis, which allows for identification of metabolites without their accurate quantification. Although the organic acids may apparently play important role in carcinogenesis, there are few methods allowing for quantitative analysis of this class of metabolites in human serum or plasma. Some of them focus on determination of only one organic acid – methylmalonic acid that is a marker of vitamin B12 deficiency, and utilize liquid chromatography–mass spectrometry (LC–MS) techniques [22–25]. A methodology for determination of plasma levels of selected organic acids presented by Hilton et al. [26] was also based on LC–MS and enabled investigation of metabolic acidosis and malnutrition. Sriboonvorakul et al. [27] developed a method for quantitative analysis of low-molecular-weight organic acids in human plasma using LC–MS and solid phase extraction technique. However, that methodology allows for determination of eight organic acids responsible for acidosis in malaria. Therefore, there is no suitable method for precise and accurate determination of organic acids involved in cancerogenesis in human serum. Development of appropriate methodology is necessary to obtain reliable information regarding changes in serum organic acid profiles that were observed in global metabolic profiling studies.

Lung cancer remains one of the major challenges in contemporary oncology. Although its incidence rate is similar to other malignant tumors, such as prostate cancer and breast cancer, lung cancer is characterized by 4–5 times higher death rate and it has been the main cause of malignant tumor-related deaths for years [28,29]. This high lung cancer mortality rate is caused mainly by poor clinical manifestation at its early stage and low effectiveness of diagnostic tools [30]. Broadening the knowledge of altered levels of some classes of metabolites, including organic acids, may contribute to better understanding of the nature of lung cancer and may allow for better identification of patients at the earlier stage of the disease. It may be also useful in monitoring anti-cancer treatment.

The aim of the current study was to develop a new rapid liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for determination of low-molecular-weight organic acids and to apply it in an analysis of serum samples collected from non-small cell lung cancer (NSCLC) patients and a matched control group. The newly developed method was fully validated and allowed for accurate and precise measurement of a panel of organic acids that were found to be associated with a development of many tumors. The obtained organic acid profiles of NSCLC patients were subjected to various statistical tests to check their correlation with clinical data. To the best of our knowledge, this is the first study presenting targeted quantitative analysis of organic acids in the serum of NSCLC subjects.

2. Material and methods

2.1. Chemicals and reagents

Deionized water (18.2 MΩ cm^{-1}) was obtained from Direct-Q (Merck Millipore, Darmstadt, Germany) water purifying system. LC–MS grade methanol, glacial acetic acid and formic acid were purchased from Sigma Aldrich (St. Louis, MO, USA). All organic acids (with appropriate internal standards in brackets): succinic acid (succinic-d6 acid), fumaric acid (fumaric-d2 acid), lactic acid (L-lactic-d3 acid), pyroglutamic acid (succinic-d6 acid), glutaric acid (2-ethyl-2-hydroxybutyric acid), and 2-hydroxybutyric acid (methylmalonic-d3 acid) were obtained from Sigma Aldrich (St. Louis, MO, USA).

2.2. Patients and samples

The patients were recruited at the Department of Thoracic Surgery, Poznan University of Medical Sciences, Poland. Ninety serum samples from the patients with biopsy-confirmed NSCLC were collected. Sampling was carried out before initiation of the cancer treatment. The tumor staging was performed according to the 7th edition of the TNM system (tumor size, node involvement, metastasis presence) [31]. Demographic and clinical data of the subjects are included in Table S1. Sixty two control samples were collected from volunteers undergoing a routine periodic medical examination and any subjects with chronic metabolic diseases or cancer were excluded. The control group was matched to the studied group in terms of sex, age, ethnic origin and BMI (Table S1). All serum samples were collected after overnight fasting to S-Monovette tubes (Sarstedt, Nümbrecht, Germany) containing a clotting activator according to the manufacturer's instruction. After blood clot formation, the supernatant was divided into aliquots and immediately frozen at −20 °C for up to 6 h. Then, the samples were transferred to −80 °C and stored until use. The research project was approved by the Bioethics Committee of Poznan University of Medical Sciences (Decision no. 200/13). All participants were acquainted with the purpose of the study and signed a written informed consent.

2.3. LC–MS/MS instrumentation

LC–MS/MS analyses were performed using Agilent 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA) coupled with a QTRAP 4000 triple quadrupole mass spectrometer (Sciex, Framingham, MA, USA). Electrospray ionization (ESI) TurboV ion source operated in negative ion mode was used. Data were acquired and processed under the control of Analyst 1.5.2 software (Sciex, Framingham, MA, USA).

Chromatographic separation was accomplished by an application of a Synergi Hydro – RP column (4 μm, 150 × 2.0 mm, 80 Å,Phenomenex, Torrance, CA, USA) under ambient temperature. Separation was performed using the mobile phase composed of 0.2% formic acid in water (mobile phase A) and 0.2% formic acid in methanol (mobile phase B) at a flow rate 0.25 mL/min. The following gradient elution was applied: 0–3 min 3% phase B, 3–9 min linear increase from 3% to 50% phase B, followed by a hold at 50% phase B for 1 min, 10–10.5 min 50%–3% mobile phase B, 10.5–15 min 3% phase B. Injection volume was always constant and equal to 10 μL. After each injection, the needle was flushed by mobile phase A to avoid contamination of the next sample.

All results were generated in a multiple reaction monitoring (MRM) mode. MS source parameters were as follows: ion spray voltage (IS): −4500.0 V; nitrogen curtain gas (CUR) 40.0 psi; temperature (TEM): 600 °C; nitrogen as ion source gas, (GS1, GS2): 40.0 psi and 50.0 psi, respectively. To achieve the highest level of