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Research Paper

Urine metabolic fingerprinting using LC–MS and GC–MS reveals metabolite changes in prostate cancer: A pilot study☆

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ABSTRACT

Prostate cancer (CaP) is a leading cause of cancer deaths in men worldwide. The alarming statistics, the currently applied biomarkers are still not enough specific and selective. In addition, pathogenesis of CaP development is not totally understood. Therefore, in the present work, metabolomics study related to urinary metabolic fingerprinting analyses has been performed in order to scrutinize potential biomarkers that could help in explaining the pathomechanism of the disease and be potentially useful in its diagnosis and prognosis. Urine samples from CaP patients and healthy volunteers were analyzed with the use of high performance liquid chromatography coupled with time of flight mass spectrometry detection (HPLC-TOF/MS) in positive and negative polarity as well as gas chromatography hyphenated with triple quadruple mass spectrometry detection (GC-QqQ/MS) in a scan mode. The obtained data sets were statistically analyzed using univariate and multivariate statistical analyses. The Principal Component Analysis (PCA) was used to check systems' stability and possible outliers, whereas Partial Least Squares Discriminant Analysis (PLS-DA) was performed for evaluation of quality of the model as well as its predictive ability using statistically significant metabolites. The subsequent identification of selected metabolites using NIST library and commonly available databases allows for creation of a list of putative biomarkers and related biochemical pathways they are involved in. The selected pathways, like urea and tricarboxylic acid cycle, amino acid and purine metabolism, can play crucial role in pathogenesis of prostate cancer disease.

Abbreviations: AMDIS, Automated Mass Spectral Deconvolution and Identification Software; BMI, body mass index; BSTFA, N,O-bis(trimethylsilyl)trifluoroacetamide; CaP, prostate cancer; CE-TOF/MS, capillary electrophoresis with time of flight mass spectrometry detection; CV, coefficient of variation; ESI, electrospray ionization; FPSA/tPSA, ratio of free to total PSA; GC, gas chromatography; GC–MS, gas chromatography–mass spectrometry; GC-QqQ/MS, gas chromatography hyphenated with triple quadruple mass spectrometry detection; GC-TOF/MS, gas chromatography with time of flight mass spectrometry detection; HPHA, hydroxyphenylhydroxypropionic acid; HPLC-TOF/MS, high performance liquid chromatography coupled with time of flight mass spectrometry detection; HR-MAS, magic angle spinning magnetic resonance spectroscopy; LC, liquid chromatography; LC–MS, liquid chromatography–mass spectrometry; MFE, molecular feature extraction; MSGUS, MS group useful signal; NIST, National Institute of Standards and Technology; NMR, nuclear magnetic resonance; PCA, Principal Component Analysis; PFTBA, pentafluorotributylamine; PLS-DA, Partial Least Squares Discriminant Analysis; PSA, prostate-specific antigen; PSAD, density of PSA; PSAV, velocity of PSA; QA, quality assurance; QCs, quality control samples; RNA, ribonucleic acid; TCA, tricarboxylic acid; TMCS, trimethylchlorosilane; tRNA, transfer ribonucleic acid; TRUS, transrectal guided ultrasound; UHPLC–MS, ultra-high performance liquid chromatography–mass spectrometry.

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1. Introduction

Prostate cancer (CaP) is the second most frequently occurring type of cancer and the third leading cause of cancer death in men [1,2]. One of reasons for such high mortality is late detection of CaP related to its histological differentiation, latent character as well as non-specificity of the available diagnostic methods [3,4]. Currently, the main marker used in the diagnosis of CaP is a prostate-specific antigen (PSA) which has low diagnostic specificity, particularly in the range 4–10 ng/ml (25–40%) that leads to over-diagnosis (numerous samples with false positive results) [5]. In addition, the clinical significance of derivatives markers of prostate specific antigen like PSAD (density of PSA), PSAV (velocity of PSA), FPSA/tPSA (ratio of free to total PSA) as well as other markers of CaP such as human kallikrein 2, PCA 3 gene or micro RNA is still under investigation [6–8]. Transrectal guided ultrasound (TRUS) biopsy performed several times (twelve or even more times) can also be applied but its invasiveness for false negatively selected patients is questionable. Besides, the pathogenesis of prostate cancer development is not totally explained. According to the current knowledge, the major risk factors include age, race, and occurrence of CaP in the family line. Besides, genetic alterations caused by epigenetic factors can also play a significant role [9,10]. Lifestyle, rich-fatty diet or environmental factors can affect the human genome and initiate processes of carcinogenesis [11]. Ambiguous is also a role of steroid hormones, which on the one hand, demonstrates a protective effect on prostate gland but on the other hand can stimulate the development of CaP [12,13]. All these factors cause that deeper understanding of the processes affecting CaP is urgently needed. Therefore, in this work we applied comprehensive analysis of urine metabolic fingerprinting study for the sake of searching of potential biomarkers that may help in understanding pathological processes related to CaP. Urine as biological material was chosen due to its non-invasiveness, ease of collection and transportation as well as stability during the short and long term storage which was proven by many studies [14,15]. Searching for possible biomarkers for prostate cancer in the field of metabolomics has been taken widely concerning targeted as well as untargeted approach. The studies can be performed using either NMR technique or other separation techniques involving mass spectrometry detection. Kumar et al. [16] presented application of high resolution NMR technique for determination of metabolites in prostate tissue extracts, prostatic fluid, seminal fluid, serum and urine. Moreover, Decelle et al. [17] proposed high-resolution magic angle spinning applied as determination technique in prostate cancer tissues. Although, NMR technique is very powerful in metabolites' qualification, this technique is less sensitive in comparison to mass spectrometry detection. Regarding mass spectrometry detection, application of metabolomics in prostate cancer disease was mainly related to targeted analyses. Szymańska et al. [15] and Struck et al. [18] determined urinary nucleosides as possible prostate cancer markers using capillary electrophoresis coupled with DAD detection and LC-ESI-QqQ/MS detection, respectively. For determination of possible cancer markers in prostate tissue, capillary electrophoresis coupled with time of flight mass spectrometry was proposed by Kami et al. [19]. Besides, liquid chromatography coupled with tandem mass spectrometry detection was applied for determination of sarcosine level in urine samples of prostate patients by Jiang et al. [20]. Giskeødegard et al. [21] proposed spermine and citrate as possible biomarkers of prostate cancer aggressiveness determined using magic angle spinning magnetic resonance spectroscopy (HR-MAS). Regarding untargeted metabolomic study Zhang et al. [22] determined urinary compounds and Zhou et al. [23] was focused on plasma lipids as potential CaP markers using LC–MS/MS technique. Moreover serum metabolomic untargeted approach using LC–MS and GC–MS revealed that level of 1-stearoylglycerol can be elevated in CaP patients [24].

In the present work, liquid chromatography coupled with time-of-flight mass spectrometry detection as well as gas chromatography hyphenated with triple quadrupole mass spectrometry detection were applied for urinary metabolic fingerprinting study in terms of searching for possible prostate cancer biomarkers. To the best of author's knowledge, our report is the first regarding usage of these two complementary analytical techniques in urinary untargeted study of CaP disease. The advantage of applying different analytical techniques like LC and GC is the possibility of determination of broader set of metabolites than using only one technique. High performance liquid chromatography technique allows for determination of polar and non-polar compounds whereas gas chromatography technique focuses on volatile compounds and these which undergo derivatization process. Thanks to the applied approach, the obtained sets of metabolites will be treated as representative ones for searching of possible prostate cancer markers and explaining the pathogenesis of the disease.

2. Experimental

2.1. Sample collection

Urine samples were collected from Department of Urology at the Medical University of Gdańsk, Poland. 32 prostate cancer patients and 32 healthy volunteers matched to age (64.2 ± 8.1 in cancer group; 52.9 ± 10.7 in healthy group) and BMI (28.5 ± 4.9 in cancer group; 27.0 ± 5.1 in healthy group) were involved in the project after signing their informed consents. The studies were performed in accordance with the principles embodied in the Declaration of Helsinki and executed according to the Ethical Committee of the Medical University of Gdansk (number of consent: NKEBN/660/2003). The group of healthy men did not undergo medication at the time of sample collection and declared the healthy status. After urine collection, samples were immediately frozen and stored at −80 °C. Directly before analysis, urine samples were thawed at room temperature.

2.2. Sample preparation for LC–MS analysis

Urine samples after thawing at room temperature were vortex-mixed for 1 min and centrifuged at 4000 × *g* for 10 min. Subsequently the supernatant was diluted in deionised water 1:2 (v/v) from Milli-Q water system (Millipore Inc., Bedford, MA, USA) and then centrifuged at 4000 × *g* for 15 min. After centrifugation, the samples were filtered directly to HPLC vials using 0.2 μm nylon filters (Agilent Technologies, Waldbronn, Germany).

2.3. Sample preparation for GC–MS analysis

Samples were prepared with earlier optimized procedure. Urine samples were thawed at room temperature for 1 h. The first step was addition of 30 units (50 μl) of urease (Sigma-Aldrich, USA) solution (600 units/ml; 0.0085 g/ml in Milli-Q water) to 200 μl of urine. Next, the sample incubation in 37 °C for 30 min was applied. The aim of this sample treatment was to decompose and remove excess amount of urea [25]. Next, 800 μl of cold methanol (kept for 30 min in −80 °C) and 10 μl of pentadecanoic acid (Sigma-Aldrich, USA, 1 mg/ml in methanol) were added to urine samples. Then the samples were vortex-mixed for 5 min and centrifuged at 4000 × *g* for 15 min. 200 μl of supernatants were transferred into glassinserts in GC vials and evaporated to dryness (QuatroMiVac Concentration, GeneVac, Great Britain) in 30 °C for 1 h 30 min. Next, 30 μl of methoxyamine (Sigma-Aldrich, Switzerland) in pyridine (Sigma-Aldrich, Germany) in concentration of 15 mg/ml was added