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The metabolic profiles of pterin compounds as potential biomarkers of bladder cancer—Integration of analytical-based approach with biostatistical methodology

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ABSTRACT

Cancer disease is the second leading cause of death across the world. The analysis of potential biomarkers of cancer can be useful in cancer screening or cancer diagnosis, and may provide valuable information on the disease risk and progression. Pterin compounds have been studied as candidates of potential biomarkers as their elevated levels have been reported in various cancer diseases.

The objective of the study was to compare the profiles of six pterin compounds in urine of 35 healthy subjects and 46 patients diagnosed of bladder cancer with the use of HPLC coupled with fluorimetric detection. The results of the chromatographic analysis together with biostatistical-based approach showed, that the concentrations of pterin compounds in bladder cancer patients were higher as compared to healthy individuals, and statistically significant differences between patients and controls were reported for xanthopterin and isoxanthopterin. Moreover, gender-specific analysis revealed, that the concentrations of pterins in the group of women reached higher values in comparison to men. For metabolites juxtaposed in pairs, namely xanthopterin and isoxanthopterin as well as for neopterin and biopterin, we found significant positive correlations in the group of both, patients and healthy individuals.

We therefore conclude, that chromatographic analysis with simultaneous extensive biostatistical-based interpretation of the metabolite profiles may provide deeper understanding of the relationships between pterin metabolites. The results do not prejudge the possibility of using pterin compounds in the diagnosis of bladder tumors. However the results may have an impact on the study of bladder cancer biomarkers.

1. Introduction

Cancer is the second leading cause of death worldwide. Epidemiological data indicate that early diagnosis greatly increases the chances of patient's recovery. Studies have shown that altered cellular metabolism is characteristic for almost all types of cancer. Application of metabolomics in order to search for differences in metabolic profiles of healthy individuals and cancer patients may provide valuable information towards the diagnosis and therapy of cancer in the future. Metabolomics is a field of knowledge, which aims at determining low molecular weight compounds in tissues and biological fluids such as urine, blood or cerebrospinal fluid, and in various physiological states of the organism as well, e.g., in the course of diseases or drug therapy. Metabolomics utilizes advanced analytical techniques in order to detect, identify and determine the concentrations of metabolites [1–4]. Pterin compounds have been proposed as metabolites of potential diagnostic value [5].

Pterin compounds are a large group of chemical compounds which contain 2-amino-4-hydroksypterin in their structure. The exact role played by all pterin compounds in cell biology has not been fully elucidated. Undoubtedly, the most important and most widely studied representatives of this group of compounds are tetrahydrobiopterin (BH_4) and neopterin (NP). BH_4 is an essential cofactor of three enzymatic reactions: for phenylalanine-4-hydroxylase (PAH), tyrosine-3-hydroxylase, and tryptophan-5-hydroxylase [6], neopterin is a sensitive parameter

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for detecting activation of cellular immune responses in humans. Neopterin enhances the activity of superoxide free radicals in the process of apoptosis of human cells, induces apoptosis independent of nitric oxide and modulates the immune response-dependent on macrophages [7].

So far, no clear explanation on elevated levels of pterin compounds in cancer patients has been given. The authors provide three possible reasons, why changes in metabolic profile of pterin compounds are observed:

(a) increased level of the biosynthesis, which in turn leads to an increased metabolism of pterin compounds,
(b) increased catabolism, without a significant increase in the biosynthesis,
(c) increased excretion of pterins in urine without simultaneous increase in the biosynthesis [6].

However, reports indicating elevated level of tetrahydrobiopterin in blood of patients with various types of cancer indicate, that observed differences between healthy individuals and cancer patients may result from increased synthesis of pterins, which may be caused by their increased metabolism. On the other hand, experiments involving cell cultures indicated an increase in the catabolism of folic acid (or one of its derivatives) in tumor cells, which can also contribute to increased excretion of some pterins in urine. This may also explain foliate deficiency which is observed in many types of cancers [8]. Recently, we observe an increase in the amount of biomarkers of cancer both in the diagnostic and therapeutic treatment. Cancer biomarker is defined as a chemical compound whose concentrations are altered within the course of tumor development. Early detection of tumors, when the tumor is still superficial, could change the treatment option and improve patient prognosis. High costs and constraints such as low sensitivity and specificity of current screening and diagnostic tests, forced researchers to search for alternative biomarkers of bladder tumors. In the literature, many tumor markers potentially useful in the diagnosis and monitoring of patients with bladder cancer, have been described. However, they express a wide range of sensitivity, specificity and different techniques are used to determine their levels in biological fluids. Accordingly, only a small number of markers tested is used in routine diagnostics. Among biomarkers and tests that are already in use, or have great potential in the diagnosis and monitoring of bladder cancer, one can distinguish: urinary cytology, assays to detect hematuria, bladder tumor antigen, test UroVison, nuclear matrix protein 22, survivin, cytokeratin or hyaluronic acid.

The objective of this study was to carry out the chromatographic analysis of six pterin compounds with the use of HPLC coupled with fluorescence detection, in order to determine the metabolic profiles of six pterin compounds in urine of healthy individuals and patients diagnosed with bladder cancer with simultaneous evaluation of the relationship between pterin metabolites, as well as between pterin metabolites and clinical outcome.

2. Methods

2.1. Chemicals and Materials

Deionized water, purified with Direct-QUV (Millipore, France) system was used for all aqueous solutions. Acetonitrile and methanol of HPLC-grade were purchased from Chempur (Piekary Śląskie, Poland). Phosphoric acid was purchased from Sigma-Aldrich (Switzerland), sodium hydroxide from P.P.H. Stanlab (Lublin, Poland). Disodium hydrogen phosphate and monosodium phosphate were purchased from POCH S.A (Gliwice, Poland).

The pterin standards: L-biopterin, D-neopterin, pterin, pterin 6-carboxylic acid, isoxanthopterin, xanthopterin, were purchased from Dr B. Schircks Laboratories (Jona, Switzerland).

2.2. Instrumentation

LC analysis was performed with Dionex (USA) Ultimate 3000 system (Dionex, USA) equipped with fluorescence detector (Shimadzu, Japan). The fluorescence detection wavelengths were established at $\lambda_{ex}/\lambda_{em}$ = 280/444. Chromatographic column LiChrospher C_8 60 RP select B 250 mm × 4.0 mm, 5 μm (Merck, Darmstadt, Germany) was used.

2.3. Urine collection and sample preparation

Urine samples from 35 healthy individuals and 46 bladder cancer patients from the Department of Urology, Medical University of Gdansk, Poland, were collected after completion of informed consents. In both groups, urine was collected after night break. Control group consisted of individuals with no cancer diagnosed or any other pathological changes within the genitourinary system.

The mean age for healthy volunteers was 54.8 (±14), and the group consisted of 20 men and 15 women, who declared healthy status. The mean age for bladder cancer patients was 70.1 (±12), and the group involved 22 men and 24 women.

Pterins excreted in urine are found at different oxidation levels. In order to determine the total pteridine content in urine sample, it is necessary to transform different oxidation states of pterins into the highest oxidation state. For this purpose the urine samples (400 μl) were transferred into 25-ml volumetric flask. Next, 30 μl of 2 M NaOH solution and 60 μl of I_2/I^- solution were added. The mixture was maintained in the dark for 40 min at 4 °C. Then, 15 ml of buffer was added, the sample pH was adjusted to 7 with diluted phosphoric acid. The solution was finally diluted with buffer to a final volume of 25 ml. The sample was filtered through a nylon filter before injection.

2.4. Chromatographic method

The chromatographic analysis was carried out using the method proposed by Kośliński et al. [9]. The chromatographic system consisted of C_8 LiChrospher 60 RP select B 250 mm × 4.0 mm, 5 μm chromatographic column, fluorescence detector with ex/em 280/444 wavelengths. The mobile phase contained, A: methanol and B: phosphate buffer (pH 7; 10 mM). The proportion of the mobile phase was 5% A, 95% B; isocratic elution, flow rate: 0.5 ml/min and injection volume was 20 μl. This method allowed for the separation of six pterin compounds, (pterin acid, neopterin, xanthopterin, isoxanthopterin, biopterin and pterin), during separation time of 16 min. Determined concentrations of six pterin compounds were further normalized against creatinine and expressed as nmol analyte per mM creatinine ratio.

Fig. 1 presents the results obtained during chromatographic analysis of the urine sample on LiChrospher C_8 column (Merck, Germany).

2.5. Statistical analysis

The normalized data on six pterin concentrations in the group of bladder cancer patients and healthy individuals, were subjected to statistical analysis. Non-parametric Wilcoxon sum rank test was adopted in order to check whether there are differences in pterin concentrations between bladder cancer patients and controls. Additionally, gender-related effect regarding pterin profiles in patients and controls was also evaluated.