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Models including plasma levels of sphingomyelins and phosphatidylcholines as diagnostic and prognostic biomarkers of endometrial cancer

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ABSTRACT

In endometrial cancer, biomarkers for preoperative identification of patients with low risk for disease progression would enable stratification according to the extent of surgery needed, and would avoid the complications that can be associated with radical surgery. A panel of proteins, amino acids, enzymes, and miRNA has been investigated as potential biomarkers for endometrial cancer. At the time of the manuscript submission targeted metabolomics/lipidomics approaches have not been applied to biomarker research in endometrial cancer. Using electrospray ionization–tandem mass spectrometry we quantified 163 metabolites in 126 plasma samples (61 patients with endometrial cancer, 65 control patients). Three single phosphatidylcholines were identified with significantly decreased levels in patients with endometrial cancer. A diagnostic model was defined as the ratio between acylcarnitine C16 and phosphatidylcholine PCae C40:1, the ratio between proline and tyrosine, and the ratio between the two phosphatidylcholines PCaa C42:0 and PCae C44:5; which provided sensitivity of 85.25%, specificity of 69.23%, and AUC of 0.837. Addition of smoking status further improved the constructed diagnostic model (AUC = 0.855). The presence of the major prognostic factors of deep myometrial invasion and lymphovascular invasion were also associated with altered metabolite concentrations. A prognostic model for deep myometrial invasion included the ratio between two hydroxysphingomyelins SMOH C14:1 and SMOH C24:1, and the ratio between two phosphatidylcholines PCaa C40:2 and PCaa C42:6, which provided sensitivity of 81.25%, specificity of 86.36%, and AUC of 0.857. The model for lymphovascular invasion included the ratio between two phosphatidylcholines PCaa C34:4 and PCae C38:3, and the ratio between acylcarnitine C16:2 and phosphatidylcholine PCaa C38:1, which provided sensitivity of 88.89%, specificity of 84.31%, and AUC of 0.935.

1. Introduction

Endometrial cancer (EC) is the fourth most common cancer in women, and its incidence worldwide is rapidly increasing [1]. EC can be divided into two subgroups based on the clinicopathological characteristics [2]. Type 1, or estrogen-dependent endometrioid carcinoma, comprises 80% to 85% of all EC cases that occur in premenopausal and postmenopausal women [3,4]. These are mainly low grade tumors with endometrioid morphology that develop through endometrial hyperplasia and have favorable prognosis [2,5]. On the other hand, type 2 EC usually includes high-grade tumors that evolve from atrophic endometrium *de novo* and occur mainly in postmenopausal women [6].

Obesity, nulliparity, early age at menarche and/or late age at menopause, diabetes, hypertension, estrogen-only hormone-replacement therapy, and tamoxifen use are considered as the main risk factors for developing EC [7,8]. Surgery, and particularly hysterectomy, is still the most important therapy for patients with EC [7] and there remains no consensus regarding lymphadenectomy as a part of this surgery, as it carries additional health risks [9–11]. Hormonal therapy with progestins is an alternative treatment option for younger women who have

Abbreviations: AUC, area under the curve; BMI, body mass index; CI, confidence interval; EC, endometrial cancer; GC, gas chromatography; MS/MS, tandem mass spectrometry; NMR, nuclear magnetic resonance; OR, odds ratio; PC, phosphatidylcholine; PCaa, diacyl phosphatidylcholine; PCae, acyl-alkyl phosphatidylcholine; S1P, sphingosine-1-phosphate; SM, sphingomyelin; SMOH, hydroxysphingomyelin

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low grade tumors and wish to preserve their fertility [12–14].

Despite the high incidence of EC, current diagnosis, prognosis, and treatments remain suboptimal. A less-invasive approach for diagnosis and management of EC is needed. Immense progress has been made in the field of biomarker discovery with the introduction of the so called '-omics' technologies, although there is currently still no reliable biomarker for EC [15–17].

The systematic identification and quantification of metabolic products is known as metabolomics, and this can provide an insight into cellular activities, and reveal phenotypic changes relative to biological function [18,19]. Although metabolic biomarkers of breast, ovarian, liver, pancreatic, cervical, lung, prostate, renal, and brain cancers are gaining diagnostic potential, none of these has been approved for clinical practice to date [20]. To the best of our knowledge, only three studies have examined the diagnostic potential of the metabolic profile in patients with EC [21–23]. The first study evaluated the diagnostic performance of only amino acids in 80 patients with EC, 122 women with benign gynecological diseases, and 240 healthy women. Based on logistic regression analysis, they built a model that distinguished the patients with EC from the healthy women with sensitivity of 60%, specificity of 98.3%, and AUC of 0.94 [21]. In a GC–MS study by Troisi et al. they evaluated the diagnostic performance of metabolomics signature in EC and constructed two models with an accuracy ranging from 62% to 100% [23]. In a recent NMR study Bahado-Singh et al. analysed 181 metabolites and constructed a model for all EC patients (AUC = 0.83) and a model for prediction of early EC (AUC = 0.82) [22].

In the present study, we evaluated the concentration of 163 plasma metabolites in patients with EC and control patients. This targeted approach focused on acylcarnitines, phosphatidylcholines (PCs), and sphingolipids, with the aims to: (i) identify disease-related metabolites and their ratios, and to evaluate their diagnostic potential; (ii) design a diagnostic algorithm that discriminates patients with EC from control patients based on a metabolic phenotype; and (iii) design a prognostic algorithm for patients with EC and with deep myometrial invasion or lymphovascular invasion.

2. Material and methods

2.1. Study design and sample source

Patient enrollment took place from June 2012 to December 2014 at the Department of Obstetrics and Gynaecology, University Medical Centre Ljubljana, Ljubljana, Slovenia. In this prospective case–control study, 126 women were enrolled who underwent surgical treatment. These women were stratified into those with EC (n = 61) and the control group of women with prolapsed uterus or myoma (n = 65). Within a week prior to surgery, morning blood samples were collected, and additional information regarding the patient life-style, and gynecological and clinical status was obtained.

The blood collection and sample processing was carried out strictly according to a detailed standard operating procedure. Blood samples of 6 mL were obtained from the median cubital vein by venipuncture, using tubes with K_2EDTA anticoagulant (BD Vacutainer; Becton, Dickinson and Company, Franklin Lakes, NJ, USA, #367864). The samples were inverted 10 times for sufficient mixing with the anticoagulant, and then placed immediately at +4 °C. The samples were centrifuged at 3213 × *g* for 10 min at 4 °C. The plasma was aspirated and aliquotes of 200 µL were taken and stored at –80 °C in 1.8 mL cryotubes (Nalge Nunc International, Roskilde, Denmark, #375418) at the Institute of Biochemistry, Faculty of Medicine, University of Ljubljana.

The present study was approved by the National Medical Ethics Committee of the Republic of Slovenia, and all of the participants signed their written informed consent before being included in this study.

2.2. Metabolite quantification

Targeted metabolomics measurements were performed in the Metabolomics Platform of the Genome Analysis Center, Helmholtz Zentrum München, Germany. The plasma samples were thawed at room temperature, vortexed, and centrifuged at 2750 × *g* for 5 min at 4 °C. Out of 10 µL plasma, 163 metabolites were quantified using the metabolomic Absolute*IDQ*™ p150 Kit (BIOCRATES Life Sciences AG, Innsbruck, Austria) and FIA-ESI-MS/MS (flow injection-electrospray ionization-tandem mass spectrometry). The method of Absolute*IDQ*™ p150 Kit has been proven to be in conformance with the EMEA-Guideline "Guideline on bioanalytical method validation ("July 21st 2011") [24], which implies proof of reproducibility within a given error range. The assay procedures of the Absolute*IDQ*™ p150 Kit as well as the metabolite nomenclature have been described in full detail previously [25,26].

Sample handling was performed by a Hamilton Microlab STAR™ robot (Hamilton Bonaduz AG, Bonaduz, Switzerland) and a Ultravap nitrogen evaporator (Porvair Sciences, Leatherhead, U.K.), beside standard laboratory equipment. Mass spectrometric analyses were done on an API 4000 triple quadrupole system (Sciex Deutschland GmbH, Darmstadt, Germany) equipped with a HTC PAL auto sampler (CTC Analytics, Zwingen, Switzerland) controlled by the software Analyst 1.6.2. Data evaluation for quantification of metabolite concentrations and quality assessment was carried out using the Met*IDQ*™ software package. Quantification of metabolites was done based on one or more appropriate internal standards for each metabolite class. For amino acids and hexoses, an own isotopically labelled internal standard have been used for each metabolite. Each metabolite was quantified separately using internal calibration (direct comparison of the MRM-intensities of the analyte to the respective internal standard).

Metabolite concentrations are reported as µmol/L. Five reference plasma samples have been measured with each batch of samples and were used to determine the reproducibility of the assay.

2.3. Metabolites pannel

The panel of 163 metabolites included 41 acylcarnitines, 14 amino acids (13 proteinogenic, and ornithine), hexoses (sum of hexoses; 90%–95% glucose), 15 sphingolipids, and 92 glycerophospholipids (15 lysophosphatidylcholines [lysoPCs], 77 PCs).

The glycerophospholipids were divided according to the presence of ester (acyl) or ether (alkyl) bonds in the glycerol moiety. Here, a single letter (acyl, a; alkyl, e) indicates a single fatty acid or fatty alcohol residue on the *sn-1* position of the glycerol moiety, as denoted by the prefix 'lyso' (*e.g.*, lysoPCs). Two letters (diacyl, aa; acyl-alkyl, ae) indicate that the *sn-1* and *sn-2* positions on the glycerol moiety are each bound to a fatty acid or fatty alcohol residue (*e.g.*, PCaa, PCae). The lipid side chain composition is abbreviated as C*x:y*, where *x* is the number of carbons in the side chain and *y* is the number of double bonds.

Sphingomyelins (SM*x:y*) and hydroxysphingomyelins (SMOH*x:y*) constitute the sphingolipid group of metabolites, according to the same lipid chain composition. Acylcarnitines are L-isomers that are abbreviated based on the fatty acid that is bound (C*x:y*), with the exception of DL-carnitine, which is abbreviated as C0. Amino acids are listed according to their three-letter abbreviations.

With the use of electrospray ionisation–MS/MS technology, the exact positions of the double bonds in these complex lipids and the configuration of the carbon atoms in different fatty acid or fatty alcohol side chains cannot be determined. The metabolite nomenclature has been previously described in detail [27], and the full list of the metabolites followed is given in Supplementary Table S1.