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Metabolomic profiling suggests long chain ceramides and sphingomyelins as a possible diagnostic biomarker of epithelial ovarian cancer

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ABSTRACT

Introduction: Epithelial ovarian cancer (EOC) is a disease with a poor survival rate mostly due to its discovery in late stages. The aim of this study was to investigate the metabolomic profile of ovarian cancer with the intention of identifying and describing novel biomarkers with diagnostic potential.

Material and methods: Targeted serum metabolomic profiling was performed on 15 patients with ovarian cancer, 21 healthy controls and 21 patients with benign ovarian conditions, using HPLC–TQ/MS.

Results: Panel of 49 top performing biomarkers shows separation between EOC and controls with 87% specificity and 87% sensitivity with AUC of 93% (CI 90%–95%). Using only 5 top biomarkers, specificity of 80% and sensitivity of 83% was achieved on extended sample set. Most significant biomarkers belong to sphingolipid classes, especially long chain ceramides and sphingomyelins. Different concentrations of various fatty acid chain lengths ceramides and sphingomyelins are also implying their respective roles in cell proliferation and growth inhibition.

Conclusion: Long chain ceramides and sphingomyelins may serve as a novel biomarker for epithelial ovarian cancer detection and may also offer insight into the role of sphingolipid metabolism in cell proliferation.

1. Introduction

Ovarian cancer (OC) is the leading cause of death among gynecological malignancies with 239,000 new cases and 152,000 deaths worldwide in the year 2012 [1]. Estimations show that 1 in 75 women will develop ovarian cancer in their lifetime and 1 in 100 will die from OC [2]. Poor overall survival rate is a consequence of late detection of the disease. Approximately 75% of women are diagnosed beyond stage I and no efficient screening strategy yet exists to reliably detect early stage disease [1,3].

Currently, beside clinical and imaging evaluation [4], measurement of the serum concentration of cancer antigen CA125 is the most widely used blood biomarker for epithelial ovarian cancer. CA125 is not specific to OC. While increased levels of CA125 are not present in all women with OC, increased levels could also occur in other cancers (pancreatic, breast, bladder, liver, and lung) as well as in benign diseases (uterine fibroids and benign ovarian cysts) [5]. Another commonly used biomarker is HE4 that has proven to be more sensitive, especially in combination with CA125 [6].

A wide spectrum of biological tumor markers is currently being investigated. One of the prominent points of interest is exploring the cancer metabolome which is believed to be an excellent way to reveal the phenotypic changes related to biological function, especially where subtle changes in metabolite concentrations are present [7].

Abbreviations: EOC, Epithelial ovarian cancer; HPLC–TQ/MS, High performance liquid chromatography–triple quadruple/mass spectrometry; AUC, area under curve; OC, ovarian cancer; BMI, body mass index; PLS-DA, partial least square discriminant analysis; VIP, variable importance for a PLS projection; UPLC-MS, ultra-performance liquid chromatography/mass spectrometry; FIGO, International Federation of Gynecology and Obstetrics; WHO, World Health Organization; CT, computed tomography; QC, quality control; CV, coefficient of variation; SBS, sequential backward selection; OOB, out of bag error; MCCV, Monte Carlo cross validation; Cer, ceramide; SM, sphingomyelin; HNSCC, head and neck squamous-cell carcinoma; CerS, ceramide synthase; SMS, sphingomyelin synthase; SMase, sphingomyelinase

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The metabolomics approach using methods such as chromatography coupled with mass spectrometry and/or nuclear magnetic resonance has already revealed significant metabolic variations in biofluids of patients with a variety of cancers [8–11]. Studies on ovarian cancer patients have proposed various biological markers mostly from lipids and amino acid groups [11–13]. Using metabolomic profiling, altered energy utilization associated with glycolysis and beta-oxidation of fatty acids has been noted in metastatic ovarian cancer [13]. Li et al. and Fan et al. successfully used UPLC-MS to differentiate EOC from healthy controls [14,15]. Similarly, Zhang et al. and Buas et al. differentiated between EOC and benign ovarian pathology using similar methods [16,17].

The aim of the study was to identify potential biomarkers for diagnosis of EOC using HPLC–TQ/MS following metabolite extraction from EOC, benign disease and healthy control group patient sera. Analysis of 232 known metabolites was performed in all samples to identify possible perturbations of metabolites in the EOC patient group compared to controls. PLS-DA was used for first line classification of two health groups based on metabolomic profiles and random forest algorithm was used for building a prediction model based over most significant markers, while the markers were also evaluated for their biological significance in cancer progression.

2. Material and methods

2.1. Patients

The study included 15 EOC patients diagnosed at the Clinic of Gynecology and Perinatology, University Medical Centre Maribor. Clinical stages and histological classification based on the criteria of the International Federation of Gynecology and Obstetrics (FIGO) and the World Health Organization (WHO) were established in all cases. Ovarian cancer histopathology was established either with biopsy or post-surgically from tumor cancer tissues.

None of the patients were involved in any specific oncological treatment such as surgery, chemotherapy or radiotherapy prior to sample collection. Pre-treatment staging procedures included physical examination, laboratory workup, ultrasound and abdominal CT scanning and chest X-rays. In addition, bone scintigraphy, brain and thoracic CT imaging were performed as necessary.

Altogether 99 samples were collected from patients without known ovarian or uterine abnormalities and patients with benign gynecological disease such as endometriosis and ovarian cysts. Case control matching was performed based on BMI and age to select 21 patients without known ovarian or uterine abnormalities as a control group and additionally 21 patients with benign gynecological diseases. Women included in the control group were mostly patients undergoing diagnostic evaluation for pelvic floor dysfunction. All patients were examined by a gynecologist prior to sample collection and the ultrasound examination was performed in every case. All patients and controls were received and treated at the Clinic of Gynecology and Perinatology, University Medical Centre Maribor, in the years 2014–2017. The study was approved by the national Ethics Committee (approval No. 37/04/14) and all the patients gave their written informed consent for study participation.

The age of participants, menopausal status, additional diseases, use of prescription or over the counter drugs, smoking and alcohol use were registered at the time of sample collection while histopathological results were acquired additionally after the surgery or biopsy.

2.2. Sample collection

Serum samples from EOC patients, healthy controls and patients with benign diseases were collected prior to any treatment or surgery after minimum of 8 h fasting, avoiding smoking, alcohol and medication. From each participant 5 mL of whole blood was collected using BD Vacutainer Plus tubes with spray-coated silica. Extracted whole blood was centrifuged at 2000xG for 10 min at temperature of 4 °C. Serum samples were separated into four 500 µL portions and stored at −80 °C within 4 h from whole blood collection.

Serum samples were stored at −80 °C until thawed for analysis and were only thawed once at 4 °C using an ice bath. Quality control (QC) pool was combined from all samples subjected to experiment and prepared alongside individual samples. All samples (prepared in duplicate), QC samples and blank standards were prepared as one analysis set and analyzed during one analysis run.

2.3. Sample analysis

Based on literature and our previous unpublished results, 232 known metabolite transitions were selected and targeted with 4 different analytical methods in dedicated AB Sciex TQ and QTRAP 4500 mass spectrometers that were coupled with Nexera X2 HPLC System (Shimadzu) comprising a pump, auto sampler, controller and oven. Blank samples and QC samples were analyzed every 10 samples for evaluating stability of the system over the long run and applying normalization for the samples.

2.4. Analysis in detail

Analytical samples were injected in four dedicated instruments for four different platforms using Shimadzu 20/30 AD 4500 coupled to Triple Quad/QTRAP (Sciex, Madrid, Spain).

Separation of the C18 polar metabolites was performed on a ACQUITY UPLC BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 50 mm attached to VanGuard Acquity UPLC BEH C18 1.7 µm for the column safety. The mobile phases were aqueous solution (phase A) and acetonitrile (phase B) both complemented with 0.1% (v/v) formic acid and the gradient method 98% for 1 min and from 98% to 2% in 9 min (held 4 min).

Lipid analysis was performed on a ACQUITY UPLC BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 100 mm attached to VanGuard Acquity UPLC BEH C18 1.7 µm for the column safety. The mobile phases were: phase A - 40% water, 60% acetonitrile, 10 mM ammonium formate, 0.1% formic acid and phase B - 10% acetonitrile, 85% isopropanol, 5% water, 10 mM ammonium formate, 0.1% formic acid. The gradient method was as follows: 85% of phase A for 1 min and the percentage of A changes to 70% in 2 min, then goes to 52% in 0.5 min, and goes to 18% in 8.5 min. Then A changes to 1% in 0.5 min where is held additional half minute.

Amide separation was performed on a ACQUITY UPLC BEH Amide Column, 130 Å, 1.7 µm, 2.1 mm × 50 mm attached to VanGuard Acquity UPLC BEH C18 1.7 µm for the column safety. The mobile phases used phase A - 70% water, 30% acetonitrile, 10 mM ammonium formate, 0.1% formic acid and phase B - 1 95% acetonitrile, 5% water, 10 mM ammonium formate, 0.1% formic acid. The gradient method was 80% of phase A for 2 min and from 80% to 20% in 2 min (held 4 min).

FIA (flow injection analysis) was performed using an injection volume of 2 µL and a flow rate of 0.36 mL/min. The mobile phase was 100% Isocratic with run-time 1 min.

Multiquant Software was used to extract the areas of 232 known compound peaks from all 4 analytical methods.

2.5. Data analysis

The complete workflow for mass spectrometry based significant metabolite selection can be seen on Fig. 1.

2.6. Data processing

For targeted profiling, peak areas for detected metabolites were integrated using MultiQuant Software (AB SCIEX). Peak integrals were