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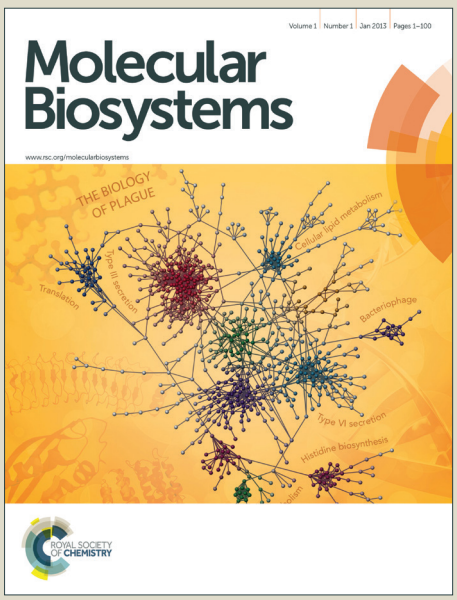

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ARTICLE TYPE

Prediction of advanced ovarian cancer recurrence by plasma metabolic profiling

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Epithelial ovarian cancer (EOC) is the most lethal of gynecologic malignancies due to the high rate of recurrence and poor prognosis. Predicting the prognosis in patients with EOC is clinically challenging, partly because appropriate biomarkers of recurrence have yet to be explored. In this prospective study, pre-treatment plasma samples were collected from 38 patients with stage III or IV EOC who were subsequently followed-up. Ultra-performance liquid chromatography mass spectrometry was used to perform metabolic profiling, which yielded five metabolites that were potential biomarkers for EOC recurrence: L-tryptophan, kynurenine, bilirubin, LysoPC (14:0) and LysoPE (18:2). A combination of these five potential biomarkers strongly predicted recurrence, the area under the curve being 0.91. In summary, the candidate biomarkers identified in this study may both facilitate clinical prediction of EOC recurrence and prognosis and serve as potential therapeutic targets in patients with EOC.

Introduction

Epithelial ovarian cancer (EOC) has the highest case-fatality rate among gynecologic cancers.[1] More than half of women with EOC have advanced-stage disease (stage III or IV) at the time of initial diagnosis.[2] Treatment for advanced ovarian cancer involves cytoreductive surgery followed by standard platinum-based combination chemotherapy. Although overall tumor response rates associated with paclitaxel and platinum range from 70–80%,[3, 4] 50–75% of responders relapse within 18 months after completing first-line therapy and require further systemic therapy.[3] Patients with recurrent EOC are rarely curable and often have only short-term progression-free survival.[5] Because of the high probability of relapse, effective biomarkers are needed to predict progression: such biomarkers would facilitate timely implementation of strategies such as second-line chemotherapy or molecular-targeted therapy.

Metabolomics involves the global quantitative assessment of endogenous metabolites within the context of the immediate environment and takes changes in metabolic reactions into account.[6] Metabolic alterations, a hallmark of tumor cells, play an important role in tumor development. Metabolomics has recently been used to identify biomarkers for diagnosing cancers, predicting their prognosis and clarifying their pathogenesis.[7-10] Utilizing a multi-platform metabolomics approach, Alberice *et al*. identified a panel of urinary biomarkers with the potential to improve prognosis predictions in bladder cancer.[11] Qiu *et al*. also reported that metabolic profiling has strong prognostic and therapeutic potential in colorectal cancer.[12] To date, few studies have used metabolic profiling to investigate prognostic biomarkers of EOC. However, we have previously reported a series of metabolomic studies based on plasma or urinary metabolic profiling relating to the diagnosis and carcinogenesis of EOC.[9, 13-15]

In this prospective study, we used ultra-performance liquid chromatography mass spectrometry (UPLC/MS) to perform metabolomic analysis in 38 advanced EOC patients with the aim of identifying metabolic markers associated with EOC recurrence.

Experimental

Sample collection, treatment, and follow-up

This study was approved by the Tumor Hospital Institutional Review Board of Harbin Medical University. Patients with EOC managed by the Department of Gynecology of Harbin Medical University Tumor Hospital (Harbin, China) between August 2009 and December 2012 were enrolled with informed consents, the duration of follow-up being 40 months. Plasma samples were collected from patients prior to them receiving any treatment. EDTA blood samples were centrifuged at 1,000 × *g* for 10 min and the supernatant extracted and frozen at −80°C until analysis. In all, 38 patients with EOC were finally selected based on the eligibility criteria as follows: (1) patients free of metabolic, liver, and kidney diseases and any other cancers; (2) patients had undergone complete cytoreductive surgery and received postoperative intravenous platinum-based combination chemotherapy; (3) presence of FIGO stage III or IV, confirmed by pathological examination.

Two to three weeks later, after complete cytoreductive surgery, patients received 6–8 cycles of intravenous platinum-based combination chemotherapy (at 3-week intervals). The chemotherapy regimen consisted of cisplatin plus paclitaxel or cisplatin, epirubicin and cyclophosphamide, and was in accordance with National Comprehensive Cancer Network guidelines.[16]

Patients were followed-up and disease recurrence or progression recorded on routine hospital flow charts. Examinations performed during follow-up included serum CA-125 concentrations, pelvic magnetic resonance imaging, color Doppler ultrasound and X-rays every 3 months for years 1–2, and at 6-month intervals in years 3–5. Patients were classified into

two groups according to whether or not tumor recurrence had taken place by the end of the study.

Sample preparation and pretreatment

To assess the stability and repeatability of the UPLC/MS systems, five blank and five quality control (QC) samples were used in this study. One blank sample (25% acetonitrile) and one QC sample (prepared by pooling equal volumes of plasma from each of the 38 samples) were run for every 10 samples.

The plasma samples were thawed at 4°C in a refrigerator. After vortexing for 30 s, the plasma samples were centrifuged at 1,000 × *g* for 10 min at 4°C. A volume of 200 μL of supernatant was then mixed with 600 μL of acetonitrile and the mixture vortexed for 1 min. After being stored for 15 min at 0°C, the mixture was centrifuged at 14,000 × *g* for 15 min at 4°C. The supernatant was then transferred into a clean vial and dried in a vacuum rotary dryer. The residue was dissolved in 100 μL acetonitrile/water (1:3, v/v) and vortexed for 5 min. The solutions were then centrifuged at 14,000 × *g* for 15 min at 4°C. Finally, a volume of 10 μL of supernatant was extracted and used for UPLC/MS analysis.

UPLC-quadrupole time of flight mass spectrometry analysis of plasma samples

Plasma metabolic profiling was performed using UPLC-quadrupole time of flight mass spectrometry (QTOF/MS) with a 2.1 × 100 mm (1.7 μm) ACQUITY UPLC BEH C18 column (Waters, Milford, MA, USA). The system used acetonitrile containing 0.1% formic acid (solvent A) and water containing 0.1% formic acid (solvent B) as the mobile phase at a flow rate of 0.3 mL/min at 40 °C. The column was eluted with a linear mobile phase gradient: 1% A for 0–0.5 min; 1–15% A for 0.5–4.0 min; 15–55% A for 4.0–4.5 min; 55–90% A for 4.5–11.5 min; 90–99% A for 11.5–12.0 min; and 99% A for 12.0–15.0 min. After the analytical run, the mobile phase was returned to 1% A in 0.1 min and equilibrated at 1% A for 1 min. Centroid data were collected in both ESI+ mode and ESI− modes on a Q-TOF (Agilent 6520, Agilent Technologies, Wilmington, USA), with the full-scan mode from 50–1000 m/z. The MS capillary voltage was 4000 V in ESI+ mode and 3500 V in ESI− mode, the desolvation gas flow was set at 10 L/min, and the desolvation temperature at 330 °C. To avoid systematic error, samples were analyzed in a randomized sequence.

Data processing and statistical analysis

The raw data were transformed into mzdata-format files using MassHunter Qualitative Analysis Software (Agilent Technologies). The XCMS package in R platform was then used for preprocessing, including retention time alignment, matched filtration, peak detection and peak matching.[17, 18] The parameters in the XCMS package were set as follows: xcmsSet (method = 'centWave', peak width = c[5,20]); group (bw = 10); rector (method = 'obiwarp'); the other parameters were set at default values. After preprocessing, the CAMERA package was used to annotate isotope peaks, adducts and fragments in the peak lists.[18, 19] After isotopic peaks had been excluded, there were 2511 ions in ESI+ mode and 2111 ions in ESI− mode for subsequent statistical analysis.

Welch's *t*-test was used to determine the significance of each metabolite ($p < 0.05$). Orthogonal partial least squares-discriminant analysis (OPLS-DA) was performed to discriminate the performances of metabolites between patients with and without recurrent EOC.[20, 21] The variable importance in the projection (VIP) values (VIP > 1.0) was used for the selection of potential biomarkers.[8] Based on leave-one-out cross-validation, the random forest model and area under the receiver operating characteristic (AUC) analysis were used to evaluate predictive performance.[22, 23] Statistical analysis was performed in the R platform,[24] with the exception of OPLS-DA which was analyzed using SIMCA-P (version 11.5; Umetrics, Malmö, Sweden).[21]

Results

Clinical characteristics of patients

Relevant patient characteristics are displayed in Table 1. In all, 38 patients with EOC, including 36 (94.74%) with stage III and two (5.26%) with stage IV disease, were enrolled in this study. Lymph node metastases were identified in 14 patients (36.84%). The histological types of tumor comprised 23 (60.53%) serous ovarian carcinomas and 15 (39.47%) cases of other histological types. Three patients (7.89%) had well-differentiated, nine (23.68%) moderately differentiated, and 26 (68.42%) poorly differentiated tumors.

Table 1 Detailed demographic and clinical characteristics of EOC patients

Characteristics	Total (N=38) (%)	Without recurrence (N=12) (%)	With recurrence (N=26) (%)	*p*-value[a]
Age(years)				1
< 50	15(39.47)	5(41.67)	10(38.46)	
>= 50	23(60.53)	7(58.33)	16(61.54)	
Serum CA-125 level				0.3158
< 35	1(2.63)	1(8.33)	0(0)	
>= 35	37(97.37)	11(91.67)	26(100)	
Lymph node metastasis				0.7281
Absent	24(63.16)	7(58.33)	17(65.38)	
Present	14(36.84)	5(41.67)	9(34.62)	
FIGO stage				0.5377
III	36(94.74)	11(91.67)	25(96.15)	
IV	2(5.26)	1(8.33)	1(3.85)	
Histology type				0.2964
Serous	23(60.53)	6(50)	17(65.38)	
Others	15(39.47)	6(50)	9(34.62)	
Histology differentiation				0.5412
Well differentiated	3(7.89)	0(0)	3(11.54)	
Moderately differentiated	9(23.68)	4(33.33)	5(19.23)	
Poorly differentiated	26(68.42)	8(66.67)	18(69.23)	

[a] *p*-values were derived from two-tailed Fisher's exact test.

Table 2 Identified metabolites showing statistically significant changes between patients with and without recurrent EOC

Compound	mode	Retention time (min)	Measured mass (Da)	ppm	*p*-value[a]	VIP[b]	Pathway
L-tryptophan[c]	ESI+	4.6182	205.0972	0.00	0.00452	1.46	Tryptophan metabolism
Kynurenine[c]	ESI-	3.6747	207.0774	0.48	0.00046	1.2	Tryptophan metabolism
Bilirubin	ESI+	8.2201	585.2706	0.34	0.00187	1.09	Porphyrin metabolism
LysoPC(14:0)[d]	ESI+	8.0965	490.2912	1.63	0.03039	1.34	Glycerophospholipid metabolism
LysoPE(18:2)	ESI-	8.8917	476.2793	2.10	0.00459	1.24	Lysophospholipid metabolism

[a] *p*-values were derived from two-tailed Welch's *t*-test. [b] VIP was calculated based on OPLS-DA model. [c] This compound was verified using a reference standard. [d] This metabolite was an adduct compound.

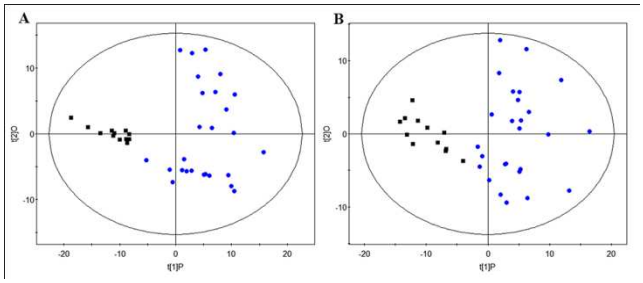

Fig.1 (A) OPLS-DA score plot for discriminating patients with recurrence (●) and without recurrence (■) in ESI+ mode. (B) OPLS-DA score plot for discriminating patients with recurrence (●) and without recurrence (■) in ESI- mode.

Plasma metabolomic profiles

An initial unsupervised principal components analysis (PCA) was performed to ensure a grouping of QC samples that indicated stability of the analyses.[25] The PCA, which was performed on all the samples, revealed that the QC samples were tightly clustered in PCA score plots (data not shown), indicating the robustness of our metabolic profiling platform.

All the statistically significant ions (Welch's *t*-test, $p < 0.05$) based on the ESI+ and ESI− modes were subjected to further analysis. A supervised OPLS-DA model was conducted to distinguish differentiations between patients with and without recurrent EOC. The OPLS-DA score plot revealed a clear separation between the groups with and without recurrence in both ESI+ mode (Fig.1A) and ESI− mode (Fig.1B). The OPLS-DA models contained one predictive and one orthogonal component in ESI+ mode (R2X = 0.44, R2Ycum = 0.775, Q2cum = 0.545), and one predictive and one orthogonal component in ESI− mode (R2X = 0.383, R2Ycum = 0.742 Q2cum = 0.49). To avoid overfitting, permutation tests with 100 iterations were performed.[26] The results showed all the permuted Q2cum were lower than the original values (data not shown), which assured the validity of our supervised models.

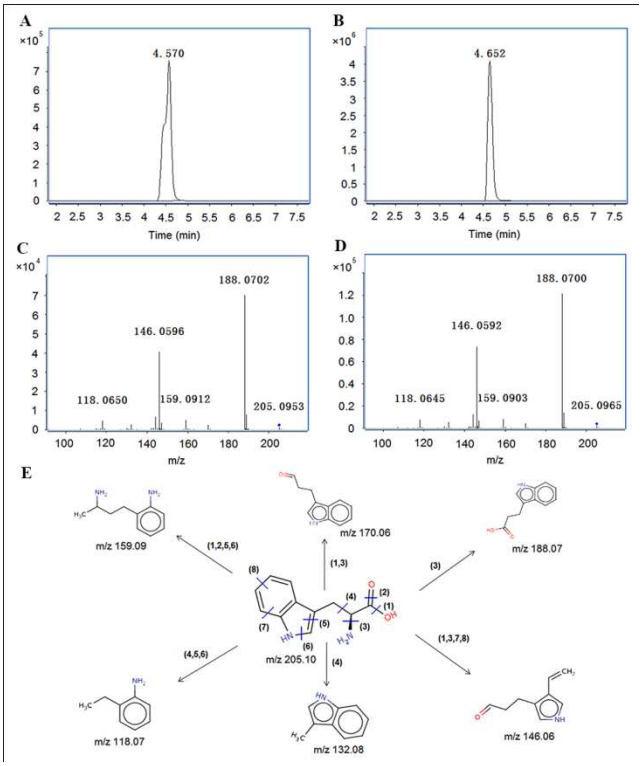

Fig.2 The identification information of L-tryptophan in ESI+ mode. (A) The extracted ion chromatographic peak at m/z 205.0972 in sample. (B) The extracted ion chromatographic peak in the reference standard of L-tryptophan. (C) The MS/MS spectrum of ion of m/z 205.0972 at 4.57 min. (D) The MS/MS spectrum of the reference standard of L-tryptophan. (E) The possible fragment structure of L-tryptophan.

Identification of metabolic biomarkers

Following VIP values with a threshold of 1, differential metabolites were selected as potential biomarkers for subsequent identification. Structure identification was carried out as described in our previous study.[27] The quasi-molecular ions were first identified based on peak lists and annotation results. The accurate mass data of the monoisotopic ions were then used to search online databases (HMDB, METLINE, and MassBank). MS/MS experiments were then performed to confirm the chemical structures of the metabolites identified in our study. A total of five metabolites were successfully identified as metabolic biomarkers of EOC recurrence (Table 2). Among these metabolites, L-tryptophan, LysoPC (14:0) and LysoPE (18:2) were decreased in EOC patients with recurrence, whereas kynurenine and bilirubin were increased. L-tryptophan and kynurenine were further confirmed by standard references. The MS/MS spectra of L-tryptophan in our plasma sample matched

those of the reference standard (Fig.2).

Prognostic potential of metabolic biomarkers

AUC analysis was performed to evaluate the predictive performance of the biomarkers. As expected, the five metabolites were able to discriminate between patients with and without recurrent EOC (Fig.3). The AUC values were as follows: L-tryptophan (AUC = 0.80), kynurenine (AUC = 0.79), bilirubin (AUC = 0.76), LysoPC (14:0) (AUC = 0.77), and LysoPE (18:2) (AUC = 0.82). Remarkably, combining these five biomarkers provided an AUC of 0.91, which suggests strong potential for predicting EOC recurrence.

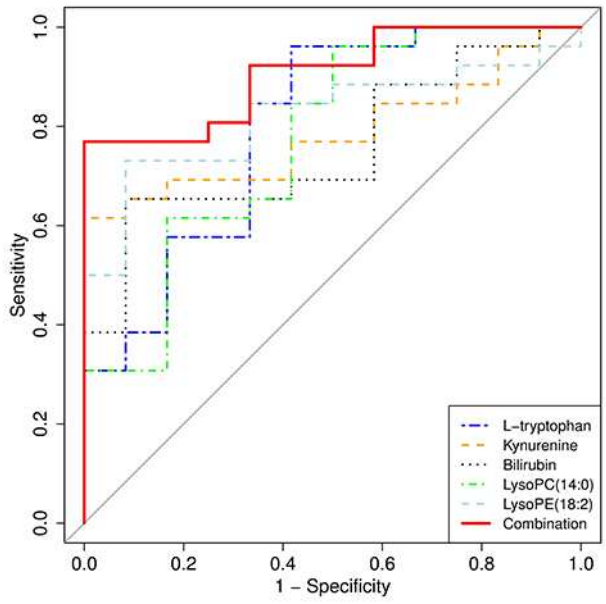

Fig.3 Receiver operator characteristic (ROC) curves for prediction with five candidate biomarkers: L-tryptophan (AUC=0.80), kynurenine (AUC=0.79), bilirubin (AUC=0.76), LysoPC (14:0) (AUC=0.77), LysoPE (18:2) (AUC=0.82), combination (AUC=0.91).

Discussion

Metabolomics has been used to identify novel biomarkers with potential applications in early detection, diagnosis and prognosis of cancer. Previous biomarker studies on EOC have focused mainly on the discovery and validation of diagnostic biomarkers. To the best of our knowledge, this is the first study to use metabolomics to identify plasma biomarkers for predicting EOC recurrence.

A non-targeted metabolomic approach was used to screen for potential biomarkers of EOC recurrence by investigating the metabolic signatures of plasma samples from EOC patients. Five metabolites were finally identified as potential biomarkers for EOC recurrence and patients with and without recurrent EOC could be distinguished using this panel of metabolites (AUC = 0.91). The accuracy with which these metabolites were classified further confirms the value of plasma metabolomics for investigating predictors of EOC recurrence.

In this study, L-tryptophan levels were lower in patients with recurrent EOC than in those without recurrent EOC, whereas the opposite trend was found for kynurenine. Given that kynurenine is the direct breakdown product of L-tryptophan in a reaction catalyzed by indoleamine 2,3-dioxygenase (IDO),[28, 29] this finding indicates enhanced activity of IDO. Tumor cells were recently shown to express IDO and to escape immune surveillance by degrading local tryptophan, which suppresses the proliferation of T cells and natural killer cells.[30-33] Okamoto *et al*. have shown that IDO is a marker of poor prognosis in ovarian cancer, as confirmed in tumors from chemoresistant patients and chemoresistant cell lines, suggesting that IDO correlates with chemosensitivity of ovarian cancer.[34] Muller *et al*. have reported that a combination of an IDO inhibitor and paclitaxel induces significantly greater suppression of breast tumor growth compared with paclitaxel alone.[35] The relationship between IDO and chemotherapy resistance may further confirm that the tryptophan metabolic pathway is disturbed in patients with relapsed EOC, which could have important implications for individualized treatment of EOC in the clinic.

LysoPCs and LysoPEs were found to be disturbed in several diseases such as cancer, diabetes and cerebrovascular disease.[36-38] Studies have reported that LysoPCs and LysoPEs were shown to be up-regulated in diabetes and cerebrovascular disease,[37, 38] while our study demonstrated that LysoPC (14:0) and LysoPE (18:2) were both down-regulated in patients with recurrent EOC. We have previously found that decreased plasma concentrations of LysoPC (14:0) and LysoPE (18:2) are highly-represented metabolic characteristics in EOC patients.[9, 14] It has been suggested that down-regulation of lysophospholipids reflects abnormal activity of specific cell-surface G protein-coupled receptors, which could result in initiation of tumor growth and survival pathways, suggesting the likelihood of cell proliferation, progression and metastasis in patients with EOC.[39-41] Alterations in phospholipid metabolism may therefore play important roles in the development of EOC. Bilirubin, a degradation product of heme,[42] had accumulated in patients with recurrent EOC. This is consistent with a recent finding that high serum bilirubin concentrations adversely affect survival in patients with malignant ascites.[43] Further research is needed to validate and elucidate the relationship between bilirubin and EOC prognosis.

In this study, the biomarkers associated with metabolic dysfunction in EOC recurrence mainly involved tryptophan metabolism and phospholipid metabolism. Despite some of our biomarkers were also observed in other diseases, they have yet to be explored as prognostic biomarkers for EOC recurrence. Extending our previous metabolomics studies on EOC, the current study provided additional information concerning the recurrence of EOC, which could potentially facilitate the medical management of this disease and improve its clinical outcomes. Limitations of our study included its small sample size, which may have precluded identification of changes in certain metabolites. Future studies with larger independent cohorts are necessary to validate and complement the current findings.

Conclusions

In conclusion, the results of this plasma metabolomic study have provided a new tool for predicting relapse and prognosis in patients with EOC. Monitoring of the potential biomarkers identified in this study could facilitate detection of recurrence. Overall, our findings suggest that plasma metabolomics could

improve the prediction of likelihood of recurrence of EOC and have the potential to shape individualized treatments in the future.

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