Lipidomic analysis of archival pathology specimen identifies altered lipid signatures in ovarian clear cell carcinoma

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Abstract
Cancer metabolism is associated with enhanced lipogenesis required for rapid growth and proliferation. However, the magnitude of dysregulation of diverse lipid species mostly remains to be characterized, particularly in rare cancers such as ovarian clear cell carcinoma (OCCC). Here we implemented a robust sample preparation workflow together with targeted LC-MS/MS to identify the lipidomic changes in formalin-fixed paraffin-embedded specimen from OCCC as compared to uninvolved contralateral ovarian tissue. We quantified 342 lipid species representing 28 lipid classes. We observed differential regulation of diverse lipid species belonging to several glycerophospholipid classes and trihexosylceramide. A number of unsaturated lipid species were increased whereas saturated lipids showed a decrease in OCCC as compared to the controls. We also carried out total fatty acid analysis and observed increase in the levels of several unsaturated fatty acids with concomitant increase in the index of stearoyl-CoA desaturase (SCD) in OCCC. We confirmed the upregulation of SCD, the rate-limiting enzyme for the synthesis of monounsaturated fatty acids, by immunohistochemistry (IHC) assays. Hence, by carrying out mass spectrometry analysis of archival tissue samples, we were able to provide novel insights into the lipidomic alterations of OCCC.

Key words: mass spectrometry, ovarian cancer, lipid profiling, unsaturated fatty acids, desaturation, archival, formalin-fixed
Background
Ovarian clear cell carcinoma (OCCC) shows geographical differences, with higher prevalence in Asia[1]. While being relatively understudied, OCCC is both clinically and molecularly distinct from the more common high-grade serous carcinoma of the ovary (HGSOC) [2]. Advanced OCCCs are more chemoresistant and portend a poorer prognosis compared with advanced HGSOC, and hence represent a significant clinical challenge for oncologists [3]. Better molecular characterization could potentially help in further patient risk stratification and identification of novel targeted therapies. Recent studies have highlighted the importance of dysregulation of lipid metabolism in cancer [4, 5]. Lipidomics studies provide suitable platform for identifications of these molecular alterations [6]. These studies enable identification of lipid species involved in diverse biological processes with a potential to serve as candidate biomarkers for early diagnosis, prognosis or monitoring of therapeutic response in cancer [7, 8]. Thus, highlighting the importance of in-depth lipidomic analysis in cancers for elucidation of potential molecular signatures of carcinogenesis. However, there is currently lack of lipidomics studies in OCCC. Further, formalin-fixed paraffin embedded (FFPE) specimens can be stored easily and permit access to huge collections of previously archived patient material. Few studies have measured metabolite level changes in cancers using FFPE specimens [9-11], but in-depth lipidomics studies are needed for systematic characterization of lipid species in the cancer metabolome. Here, we describe a simple method that can be applied to FFPE specimens in order to extract lipids for downstream lipidomics applications, and applied this for identification of the lipidomics alterations in OCCC.
Materials and Methods

Reagents

MS grade acetonitrile, methanol and 2-propanol were supplied by Fisher Scientific (Waltham, MA). Formic acid, ammonium formate and 1-butanol were supplied by Merck Pte Ltd or Sigma-Aldrich Pte (Singapore). RIPA lysis and extraction buffer and monoclonal antibody for SCD1 (#MA5-27542) were supplied by Thermo Fisher Scientific. Lipid standards consisting of odd chain/non-physiological and deuterated species were purchased from different suppliers. Phosphatidylcholine (PC 13:0/13:0), Phosphatidylethanolamine (PE 17:0/17:0), Phosphatidylglycerol (PG 17:0/17:0), Phosphatidylserine (PS 17:0/17:0), Lysophosphatidylcholine (LPC 13:0), Lysophosphatidylinositol (LPI 13:0), Lysophosphatidylethanolamine (LPE 14:0), Cholesterol (D7), Ceramide-d7 (d18:1-d7/18:0), Glucosyl (β) Ceramide (d18:1/18:1), Sulphatide d18:1/12:0, Lactosyl(β) Ceramide d18:1/18:1, Sphingosine-1-phosphate (Sph-1-P) 17:1, Sphingosine (Sph) 17:1, Sphingomyelin (SM d18:1/12:0), Dihydroceramide (d18:0/8:0) were supplied by Avanti Polar Lipids (Alabaster, AL, USA). Cholesteryl-2,2,3,4,4,6-d6 Octadecanoate was supplied by CDN isotopes (Quebec, Canada). Hexadecanoyl (16,16,16-D3)-L-Carnitine HCl salt was supplied by Larodan Chemicals (Solna, Sweden). Trihexosylceramide 17:0 was supplied by Matreya LLC (PA, USA). Diacylglycerol (DAG) 15:0 15:0 was supplied by Santa Cruz Biotech (Dallas, TX, USA) and Triacylglycerol (TAG) 17:0 17:0 17:0 was supplied by Sigma Aldrich Pte. (Singapore).

Methods

Formalin-fixed paraffin-embedded tissue sections

FFPE tissue were sliced into 20 micron cores from each confirmed clear cell carcinoma (n=14) and contralateral ovarian tissue for lipidomics analysis. The weight of each core was recorded and placed in a labelled 2 ml tube. For immunohistochemistry, standard 4-8 micron slices were mounted onto slides (n=5) each from cancer and corresponding control tissue.

Sample preparation

The FFPE cores were subjected to lipid extraction directly as described previously [9-12] with slight modifications. Briefly, single phase lipid extraction was carried out by using methanol and 1-butanol in 1:1 (v/v), spiked with internal standards as described previously [13-15]. We added 1 ml of extraction
solvent into each tube to completely cover the FFPE core. The samples were then incubated at 70°C for
1 h on a thermal mixer followed by 15 minutes on ice to congeal the wax. The samples were
centrifugation at 14,000 x g for 10 min and supernatants were collected in labelled collection tubes. The
remainder of the samples were kept on ice for 15 min, followed by centrifugation. Again, the
supernatants were collected and added into the respective collection tubes. The supernatants containing
the lipid extract were dried under vacuum and reconstituted in water saturated butanol and methanol
(1:1, v/v) for LC-MS/MS analysis. The remaining pellets were washed with 1 ml of xylene followed by
centrifugation. The xylene was aspirated carefully without disturbing the pellet, and this process was
repeated twice. The pellets were rehydrated by adding 1 ml of 100%, 70% and 50% ethanol in serial
steps of centrifugation and aspiration. The recovered pellets were air dried and solubilized in RIPA lysis
buffer for protein estimation using BCA assays.

For total fatty acid analysis, lipid extracts were subjected to hydrolysis by 0.5 M HCl in acetonitrile-
water 9:1 (by volume) for 45 min at 100°C[16]. After hydrolysis, fatty acids were recovered in
chloroform, dried and reconstituted in water saturated butanol and methanol (1:1, v/v) and analyzed by
LC-MS/MS as described above. Fatty acids were detected in negative mode ESI by measuring precursor
ion in SIM mode and quantitated by normalizing against deuterated internal standard (Oleic acid-d17)
that was spiked-in before extraction.

**LC-MS/MS analysis**

LC-MS/MS analysis of lipid extracts was carried out on 6495A QQQ mass spectrometer interfaced with
an Agilent 1290 series HPLC system (Agilent Technologies). The dried lipid extracts were reconstituted
in 100µl of methanol and water saturated butanol (1:1; v/v) containing 10mM of ammonium formate
and analyzed by using dynamic MRM approach (dMRM). Mass spectrometry settings and MRM
transitions for each lipid class, subclass and individual species were kept as described previously [15].
A step gradient consisting of solvent B (10 mM ammonium Formate in isopropanol/acetonitrile/water
(90/10/1, v/v/v) and solvent A (10 mM ammonium formate in water/isopropanol/acetonitrile (50/30/20,
v/v/v) was used for separation of the lipid species over a total run time of 15 minutes. Isolation widths
were set to unit resolution for both Q1 and Q2. Blanks and pooled QC samples (prepared by pooling of
lipid extracts) were interspersed in the sample sequence to monitor carry over and reproducibility of the lipidomics data.

Data analysis and quantitation

Peak integration was carried out using MassHunter software (B.10; Agilent Technologies). Manual inspection of raw peaks was carried out to ensure correct peak picking and the peak area data were exported in .csv format for further analysis. Lipid species with an analytical coefficient of variation more than 20% (based on QC) and S/N < 5 were discarded from further analysis. The peak area of each endogenous lipid species was normalized to the corresponding class-specific internal standards as well as protein amounts for quantitation.

Statistical analysis

Statistical analysis was carried out by using MetaboAnalyst software suite[17]. Principal Component Analysis (PCA) was implemented to explain the variance in the data in an unsupervised manner. Univariate analysis was used for Student t-tests (threshold FDR < 0.05) and fold change (cut-off = 2) estimation, both represented as volcano plot. Scatter plots for individual concentrations of representative lipid species were plotted in Graphpad Prism (v 9.1.0) from normalized data. Total fatty acid data were also analyzed in Graph pad Prism by implementing t-tests with a threshold of $p < 0.05$ for significance.

Immunohistochemical staining for SCD and scoring of staining

Surgical resection specimens were fixed with buffered formalin solution, embedded in paraffin, and stained with hematoxylin and eosin. Expression of SCD was determined on tissue sections that were 4 µm thick using antibody clone CD. E10 (Invitrogen) at 1:1000 dilution, a Leica Bond III automated stainer, and Leica Bond DAB polymer Detection kit DS 9800. Appropriate positive and negative controls were included for all staining reactions. The staining intensity was scored by a pathologist (1+ for weak staining, 2+ for moderate staining, 3+ for strong staining), and the h score was calculated in the following manner to take into account the extent as well as intensity of staining: $1^* (\% \text{ area with } 1^+ \text{ staining intensity}) + 2^* (\% \text{ area with } 2^+ \text{ staining intensity}) + 3^* (\% \text{ area with } 3^+ \text{ staining intensity})$. 
Results

Lipidomic profiling of FFPE tissue samples

Targeted LC-MS/MS analysis of lipid extracts from OCCC (n=14) and control samples (n=14) resulted in quantitation of 342 lipid species representing 28 lipid classes. Study subject details are provided in Supplementary Table S1. Details of MRM transitions and internal standards are provided in Supplementary Table S2. Principal Component Analysis (PCA) showed separation between the two sample groups (OCCC vs control tissue) and the variance was evident from PC1 (32.3%) Figure 1A. Top 5 principal components with individual contribution to the total variance are represented in Figure 1B.

Figure 1: Overview of comparative lipidomics analysis OCCC and control samples (A) PCA analysis showing the group variance, PC1 explained 32.3% of variance, circles representing 95% confidence regions (B) Individual variance explained by top 5 principal components.

Differential regulation of lipid species in OCCC

Having observed a noticeable separation between cancer and control samples based on lipid profiles, we carried out further analysis to understand the differential regulation at lipid species level. For this, univariate analysis was carried out to calculate fold-change (OCCC as compared to control) (Supplementary Table S3) and two-sample t-tests (Supplementary Table S4) were implemented.
Volcano plot analysis was carried-out to identify the differential levels of lipid species with a cut-off of fold-change ($\geq 2$) and $p$-value $< 0.05$ (FDR adjusted). We observed differential levels of 43 lipid species, with 38 increased whereas 5 were decreased in OCCC as compared to the controls (Figure 2A). Differential levels of several representative lipid species are provided in Figure 2B. The complete list of lipid species from this analysis are provided in Supplementary Table S5. The lipids with increase in concentration were primarily glycerophospholipid species belonging to the phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE) phosphatidylinositol (PI) and phosphatidylinerine. A few ether-linked phospholipid species belonging to alkenylphosphatidylethanolamine (plasmalogens) were upregulated whereas several alkylphosphatidylcholine and alkenylphosphatidylethanolamine species were downregulated. At the same time, glycerophospholipid species with saturated fatty acids were either unchanged or decreased in cancer. Complex sphingolipid species belonging to the trihexosylceramide (Hex3Cer) class were also upregulated whereas sphingomyelin species with low carbon number and unsaturation (saturated) were downregulated.
Figure 2: (A) Volcano plot showing lipid species that are significantly, $p < 0.05$ (FDR adjusted), increased (red) and decreased (blue) in OCCC as compared to control. (B) Individual dot plots of representative lipid species from several lipid classes showing differential levels in OCCC as compared to control.
Unsaturated glycerophospholipid species are elevated in OCCC

One of the defining features of the glycerophospholipid species with higher abundance in OCCC was the presence of one or more double bonds in the fatty acid moieties (Supplementary Table S5). Apart from monounsaturated lipid species such as PE 32:1, PE 34:1 and PI 34:1, several polyunsaturated fatty acid containing lipid species were also upregulated in OCCC including phosphatidylethanolamine species (PE 38:4, PE 38:6, PE 40:6) and phosphatidylinositol species (PI 38:4, PI 38:6, PI 40:6). The upregulated plasmalogen species also contained unsaturated fatty acids such linoleic acid.

The fatty acid analysis also showed a substantial increase in monounsaturated (FA 16:1, FA 18:1) and polyunsaturated fatty acids (FA 20:3, FA 22:5, FA 22:6) whereas saturated fatty acid (FA 16:0, FA 18:0) were lower in concentration (Figure 3A) in OCCC as compared to the control (Supplementary Table S6). We also observed an increase in the ratio of 16:1 to 16:0 (Figure 3B), indicating an increase in the fatty acid desaturation in OCCC. Apart from increase in total levels of unsaturated fatty acid, we also observed a decrease in total PC to PE ratio in OCCC (Figure 3C), an indicator of membrane integrity.
Stearoyl-CoA desaturase (SCD) expression in OCCC and control tissue

To follow up on the observations that many unsaturated lipid species were upregulated in OCCC with an increase in desaturation index, we carried out immunohistochemistry (IHC) analysis of the rate limiting desaturation enzyme, SCD. For this analysis, OCCC specimens from different patients and paired unininvolved contralateral ovarian tissue (n=5) from the respective patients were selected on the basis of tissue availability, and examined by immunohistochemistry. Based on the IHC scores, the SCD...
showed increased abundance in all 5 OCCC cases, with heterogeneous protein expression in the individual patients (Figure 4). The IHC scores for these five OCCC patients are provided in Supplementary Table S7.
Figure 4: Immunohistochemistry analysis showing tissue distribution and differential levels of SCD in OCCC and matched controls with an overall upregulation in OCCC.
Alterations in cancer metabolism have been associated with tumor growth, metastasis, resistance to therapy and survival of cancer stem cells [6-8]. Novel information about the role of the lipids in cancer is now emerging [9, 10]. Here we carried out a lipidomic analysis of FFPE specimen from OCCC and matched control samples (n=14). Out of the 342 lipid species profiled in this study, 43 (12%) showed significant changes in abundance in OCCC as compared to the control tissue. The lipid species with increased concentration can be classified into glycerophospholipids (such as phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine), lysoglycerophospholipids (such as lysophosphatidylethanolamine), alkenylphosphatidylethanolamine (PE plasmalogens) and complex sphingolipids (such as trihexosylceramides). Amongst the lipid species with decreased concentration were saturated fatty acid containing phosphatidylcholine and sphingomyelin species. Glycosphingolipids such as trihexosylceramides (including globotriaosylceramide, Gb3) were elevated in OCCC. These lipids are involved in the metastasis and aggressiveness in cancer [18-21]. The general trend in neutral lipids such as diacylglycerol, triacylglycerol and cholesteryl esters was either no change or a marginal decrease (CE 18:2, CE 20:5) in cancer. Glycerophospholipids along with sphingomyelin are the main components of plasma membranes and constitute the bulk of lipids in tissues [22]. Apart from the structural role of these lipids, phospholipids contribute to the formation of other lipids and bioactive species such as lysophospholipids, generated by phospholipases [23]. Due to their central role in the cellular architecture, cancer cells may differentially regulate phospholipid metabolism to support their uncontrolled cell growth and division. Further, plasmalogens contain vinyl ethyl bond at \( sn1 \) position and have been implicated in fighting oxidative stress at the cellular level. Plasmalogens that contain polyunsaturated fatty acids (PUFA) have previously been shown to be involved in resistance to cell death in cancers [24]. In this study, we observed upregulation of PE-plasmalogen species that contain the fatty linoleic acid (C18:2) with two double bonds instead. Further studies could address whether these lipid species play a role in cancer metabolism that is distinct from PUFA-containing species of plasmalogens. These species along with other unsaturated phospholipid species such as PE 32:1, PE 34:1, PI 34:1 and PS 36:1 represent a common signature of enhanced fatty acid desaturation in OCCC. Our total fatty acid analysis further illustrated the overall increase in both monounsaturated
and polyunsaturated lipid species in cancer. Apart from decreased levels of saturated fatty acid
containing lipid species, we also observed a decrease in total levels of FA 16:0 and FA 18:0, two of the
major fatty acids by composition, in OCCC. Further, by using the ratios of FA 16:1 to FA 16:0, we
observed an increase in desaturation index. As increase in fatty acid desaturation may have an impact
on cancer cell architecture and increase membrane fluidity [25]. To gain further insights into the
membrane integrity, we estimated ratio of total phosphatidylyceroline to phosphatidylethanolamine [26]
and observed a decrease in OCCC. These data suggest disruptions in lipid bilayer organization that may
facilitate proliferation and metastasis [27]. Based on our observation that several unsaturated lipid
species were increased in OCCC, we carried out IHC assays for SCD in OCCC. We observed an overall
increase in SCD levels in OCCC. By combining the data from lipidomics, total fatty acid analysis and
IHC, we were able to provide a detailed view of the widespread perturbations in lipid metabolism in
OCCC. These changes were mapped onto the metabolic pathways and provide a global view of lipid
remodeling in OCCC (Figure 5). These molecular alterations may provide suitable avenue for better
understanding and exploitation of metabolic vulnerabilities in OCCC [28]. To the best of our knowledge,
this is the first attempt to characterize lipidomic changes in OCCC, and will help in better understanding
of the role of underlying alterations in lipid metabolism.
Figure 5: Mapping of molecular alteration in OCCC onto lipid metabolism pathways illustrates the diverse nature of these lipid species with an overall increase in fatty acid desaturation.
Conclusions

We identified differential levels of several lipid species in ovarian clear cell carcinoma as compared to the controls, and an overall increase in total levels of unsaturated fatty acids. We confirmed the upregulation of stearoyl-CoA desaturase, by using immunohistochemistry assays. From our lipidomics analysis, we demonstrate the feasibility of using archival pathology specimen to identify alteration in lipid metabolism in cancer.

List of Abbreviations

FFPE: Formalin-fixed paraffin-embedded, SCD: Stearoyl-CoA desaturase, OCCC: Ovarian clear cell carcinoma, IHC: Immunohistochemistry, PUFA: Polyunsaturated fatty acid, PE: Phosphatidylethanolamine, PS: Phosphatidyserine, Hex3Cer: Trihexosylceramide.
Declarations

Ethics approval and consent to participate

All subjects gave their informed consent for inclusion before they participated in this study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the respective Institutional Review Boards, DSRB for National University Hospital (NHS-DSRB 2013/00705) and NUS-IRB (N-20-006E) for National University of Singapore.

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Author Contributions

SAM, SBJW, AKB, DSPT and MRW conceived and designed the study. SAM and CWLE carried out sample preparation, LC-MS/MS and data processing. SAM, KN, BB and SJ were involved in data analysis and representation. SBJW carried out sectioning of FFPE tissue specimen and IHC. SAM and SBJW wrote the manuscript and all authors agreed and contributed to the final version of the manuscript.

Competing interests

The authors declare that there are no conflicts of interest.

Consent for publication

All authors have given their consent for this publication.

Availability of data and materials

The data that support the findings of this study are including as supplementary information. Data and information about reagents/methods are also available from the authors upon reasonable request.

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NA
Supplementary Information

Supplementary Tables

Supplementary Table S1: Clinical characteristics of the study participants.

Supplementary Table S2: Targeted lipidomics method details.

Supplementary Table S3: Lipid species with a two-fold change between OCCC and control.

Supplementary Table S4: Significant lipid species from two sample t-tests with \( p \)-value (FDR adjusted) < 0.05.

Supplementary Table S5: Details of the lipid species from Volcano plot analysis with at least two-fold change and \( p \)-value (FDR adjusted) < 0.05.

Supplementary Table S6: Details of fatty acids in individual samples represented as percent of the total concentration.

Supplementary Table S7: IHC scores of OCCC and paired control specimen (n=5).
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