Cardiolipins Are Biomarkers of Mitochondria-Rich Thyroid Oncocytic Tumors

Jialing Zhang1, Wendong Yu2, Seung Woo Ryu3, John Lin1, Gerardo Buentello4, Robert Tibshirani5,6, James Suliburk4, and Livia S. Eberlin1

Abstract

Oncocytic tumors are characterized by an excessive eosinophilic, granular cytoplasm due to aberrant accumulation of mitochondria. Mutations in mitochondrial DNA occur in oncocytic thyroid tumors, but there is no information about their lipid composition, which might reveal candidate theranostic molecules. Here, we used desorption electrospray ionization mass spectrometry (DESI-MS) to image and chemically characterize the lipid composition of oncocytic thyroid tumors, as compared with nononcocytic thyroid tumors and normal thyroid samples. We identified a novel molecular signature of oncocytic tumors characterized by an abnormally high abundance and chemical diversity of cardiolipins (CL), including many oxidized species. DESI-MS imaging and IHC experiments confirmed that the spatial distribution of CLs overlapped with regions of accumulation of mitochondria-rich oncocytic cells. Fluorescent imaging and mitochondrial isolation showed that both mitochondrial accumulation and alteration in CL composition of mitochondria occurred in oncocytic tumors cells, thus contributing the aberrant molecular signatures detected. A total of 219 molecular ions, including CLs, other glycerophospholipids, fatty acids, and metabolites, were found at increased or decreased abundance in oncocytic, nononcocytic, or normal thyroid tissues. Our findings suggest new candidate targets for clinical and therapeutic use against oncocytic tumors.

Introduction

Oncocytic tumors are a distinctive class of proliferative lesions composed of cells with an aberrant accumulation of mitochondria (1). Tumors composed of oncocytes are particularly common among thyroid neoplasms of follicular cell derivation. Clinically, oncocytic thyroid tumors (also called Hurthle cell neoplasms) have poorer oncologic outcomes than their nononcocytic counterparts and are thus considered an adverse prognostic indicator. The biological mechanisms underlying mitochondria accumulation in oncocytic tumors are not fully understood. Sequencing studies have reported upregulation of genes involved in mitochondrial biogenesis and oxidative metabolism in thyroid oncocytes, including genes from the tricarboxylic acid cycle and cytosolic glycolysis (2). More recently, mutations in the mtDNA disrupt complex I have been described as potential markers of oncocytic lesions (3).

The inner mitochondrial membrane of eukaryotic cells has a complex structure and molecular composition, recognized by the presence of cardiolipins (CL), a unique class of anionic glycerophospholipid located predominantly (if not exclusively) in mitochondria. CLs have a distinctive chemical structure, composed of two phosphatidylglycerols bridged via a glycerol backbone, which displays two negative charges from the phosphate groups and four acyl chains. These complex lipids play multiple structural and functional roles in bioenergetics, mitochondrial signaling, and cellular fate pathways and are associated with individual complexes of the electron transport. Dysregulation of CL expression and composition has been increasingly investigated in biological samples using high-performance liquid chromatography mass spectrometry (HPLC-MS). Lipid analysis by HPLC-MS provides rich and quantitative information from biological samples. Yet, these time-consuming assays require sample homogenization and thus disregard cellular heterogeneity within biological tissue samples and preclude acquisition of spatial information. Recently, HPLC-MS was used to identify CLs and oxidized CL species in a rat model of traumatic brain injury (4). CLs were also identified by HPLC-MS as proliferation markers in prostate cancer cell lines, and differences in CL composition were seen in tissues from 6 prostate cancer patients, although with no statistical significance (5). In clinical research, the ratio of the intensities of CLs and monolysocardiolipins (MLCL), measured in blood by HPLC-MS, has been suggested as a screening test for Barth syndrome (6). Despite the importance of CLs in physiologic function and their role in mediating the pathology of disease states, no studies have investigated their role in human thyroid tumor tissues.

Here, we used MS imaging to chemically characterize the lipid signatures of thyroid tumors. We discovered a rich molecular signature uniquely characterized by a high abundance and diversity of CL species in oncocytic thyroid tumors. Different CL species, including oxidized CLs (ox-CL), adducts of CL, and glycerophosphocholines (PC) or diacylglycerides (DG), and MLCL were identified at high relative abundances in oncocytic tumors when compared with nononcocytic tumors and normal...
thyroid tissues. DESI-MS imaging and optical imaging of stained tissues, even though in a different spatial scale, confirmed that the CL distribution and highest intensity colocalized with regions of oncocyotic tumor cells in thyroid tissues. Fluorescent imaging and mitochondrial isolation studies showed that mitochondrial accumulation and alteration in CL composition of mitochondria occur in oncocyotic tumor cells, thus contributing to the aberrant molecular signatures detected. Statistical analysis showed that CL species were increased in oncocyotic tumors with the highest statistical significance, while other glycerophospholipids (GP) and fatty acids (FA) were also found to significantly discriminate oncocyotic tumors, nononcocytic tumors, and normal thyroid tissues.

MS imaging has been extensively used to investigate the lipid profiles of human tumors (7, 8). In particular, ambient ionization MS imaging techniques, such as DESI-MS, allow for direct analysis of tissue sections, in the open air environment, and with minimal sample preparation (9–12). DESI-MS has been used to characterize the lipid profiles of brain (13, 14), gastric (15), breast (16, 17), and other cancers (18). Variations in the relative and total abundance of FA and most abundant complex lipids classes have been described in cancer tissues when compared with normal tissues (18). Yet, as the abundance of CL in cells and tissues is low relative to other lipids, little has been reported on the changes of CL in cancer tissue using MS imaging. Matrix-assisted laser desorption/ionization mass spectrometry has been employed to analyze CL in animal model tissues or cell lines (19–21). Using DESI-MS imaging, five different CL species were identified in MYC-induced lymphoma mouse tissue (22), and a single CL species was detected in normal human gastric epithelial tissue (15). To the best of our knowledge, this is the first study to report a diverse group of CL species as molecular markers of human tumors. As lipid signatures can be readily accessed using ambient MS imaging, we expect this method to be valuable for clinical use (18).

Materials and Methods

For detailed information on materials and methods, please see Supplementary Information.

Banked human thyroid tissues

A total of 45 frozen human thyroid tissue specimens were obtained from Cooperative Human Tissue Network, Baylor College of Medicine Tissue Bank, and Asterand Biosciences under approved Institutional Review Board protocol. A first set of 30 samples included 10 oncocyotic thyroid tumors (8 Hurthle cell adenomas and 2 Hurthle cell carcinomas), 10 nononcocytic thyroid tumors (5 papillary thyroid carcinomas and 5 follicular thyroid adenomas), and 10 normal thyroid tissues. A second set was purchased and analyzed independently, including 5 oncocyotic thyroid tumors (2 Hurthle cell adenomas and 3 Hurthle cell carcinomas) and 10 nononcocytic thyroid tumors (5 papillary thyroid carcinomas and 5 follicular thyroid carcinomas). All tissue samples were sectioned at 16-μm thick sections, and stored in a −80°C freezer until analysis.

DESI-MS imaging

A 2D Omni Spray (Prosolia Inc.) was used for tissue imaging with a spatial resolution of 150 μm. DESI-MS imaging was performed in the negative ion mode from m/z 100 to 1,500 using a hybrid LTQ-Orbitrap Elite Mass Spectrometer (Thermo Scientific). The histologically compatible solvent system dimethylformamide:acetonitrile 1:1 was used for analysis (23). For ion identification, high mass resolution/accuracy measurements and tandem MS analyses were performed in the Orbitrap and the linear ion trap using the same tissue sections analyzed.

Histopathology

The same tissue sections analyzed by DESI-MS were subjected afterwards to hematoxylin and eosin (H&E) staining. Pathologic evaluation was performed by W. Yu using light microscopy. Regions of clear diagnosis of cancer and normal thyroid tissue were assigned in the glass slides.

IHC, immunofluorescence, and confocal microscopy

For IHC, formalin-fixed tissue sections were stained for human mitochondria using primary Human Mitochondria monoclonal antibody MAB1273 (Millipore). All the H&E- and IHC-stained slides were scanned using the Aperio ScanScope imaging platform (Aperio Technologies) with a 20× objective at a spatial sampling period of 0.47 μm per pixel. Whole-slides images were viewed and analyzed by using desktop personal computers equipped with the free ScanScope software. For immunofluorescence, formalin-fixed tissues were stained using Alexa Fluor 488–conjugated anti-mitochondrial antibody MAB1273A4 (Millipore), counterstained and mounted in Prolong Gold Antifade mounting media (Thermo Fisher). Immunofluorescence images were acquired on a Zeiss LSM880 confocal microscope.

Mitochondria isolation and analysis

Mitochondria isolation was carried out following an organelle isolation protocol (24). Quantification was done using BCA Protein Assay Kit (Thermo Scientific). Concentrations were measured by comparing absorbance with standard protein calibration curve created with different concentrations of BSA. Total lipid extraction was carried out by Bligh–Dyer method.

Statistical analysis

Regions of interest in the 2D raw data obtained by DESI-MS were selected, converted to text files, and imported to R language for statistical analysis using the significance analysis of microarrays (SAM) method.

Results

Molecular characterization of CLs in oncocyotic tissue

Negative ion mode DESI-MS was used to analyze a total of 45 human thyroid samples, including 15 oncocyotic thyroid tumors (10 Hurthle cell adenomas and 5 Hurthle cell carcinomas), 20 nononcocytic thyroid tumors (10 papillary thyroid carcinomas, 5 follicular carcinomas, and 5 follicular thyroid adenomas), and 10 normal thyroid tissues. The mass spectra obtained from m/z 100 to 1,500 (Supplementary Fig. S1) presented high relative abundances of several molecular ions commonly characterized as lipid species in the negative ion mode DESI mass spectra of human tissues, including FA and GP, such as glycerophosphoinositols (PI), glycerophosphoethanolamines (PE), and glycerophosphoserines (PS). Normal thyroid tissue displayed high relative abundances of [PI(20:4/18:0)] (m/z 885.548), [PS(20:3/18:0)] (m/z 812.544), [PS(18:1/18:0)] (m/z 788.544), [PE(20:4/18:0)] (m/z 766.538), [PE(18:2/18:1)] (m/z 742.538), and phosphatidic acids (PA) (18:1/18:0) (m/z 701.512), which are lipid ions commonly

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detected from mammalian tissues (Fig. 1, bottom). In contrast, the mass spectra obtained from oncocytic tumor samples showed a very distinct and reproducible profile with abnormally high relative abundances of a series of doubly charged ions in the mass range from m/z 590 to 760 and m/z 1,000 to 1,200 (Fig. 1, top). The spectra were remarkably rich in molecular diversity, and unlike what commonly observed in human cancer tissues by DESI-MS imaging. It should be noted that ions with two negative charges are characterized by m/z corresponding to half the molecular weight of the ion. Doubly charged ions are easily recognized by MS, as the 13C isotope peak is at a 0.5 m/z difference from the 12C isotope peak (Fig. 1, inset on top), whereas for singly charged ions, the 13C peak is observed with 1 m/z difference. Doubly charged lipid ions are unusually detected at high intensities from human tissues by DESI-MS analysis and stand out from other FA and GP ions, which are most commonly observed as singly charged species in the negative ion mode.

Using high mass accuracy measurements and tandem MS analysis via collision-induced dissociation and higher energy collisional dissociation, we identified these doubly charged ions as a diverse group of CL species. Structural elucidation using lithium adducts was also explored and provided confirmatory structural information (Supplementary Fig. S2; ref. 25). CLs have been previously investigated by electrospray ionization and tandem MS and present key fragment ions that enable structural characterization (26–28). For example, tandem MS experiments of doubly charged molecular ion m/z 724.483 yielded fragment ions corresponding to 18:2-carboxylate anion (m/z 279.233), 18:1-carboxylate anion (m/z 281.249), 20:2-carboxylate anion (m/z 307.264), lyso-PA fragments (m/z 415.225, m/z 417.241, and m/z 461.249), a doubly charged ketene (m/z 593.371) arising from loss of the 18:2-fatty acyl substituent, and a fragment ion at m/z 1169.737 produced by neutral loss of FA(18:2), indicating that the molecular ions correspond to CL(20:2/18:2/18:1/16:2 or 18:2/18:2/18:2/18:1) (Supplementary Fig. S3). High mass accuracy measurements agree with the exact mass (m/z 724.4867) of the proposed molecular formula (C₈₁H₁₄₄O₁₇P₂) with a mass error of −1.7 ppm. Note that isomerism of the double bonds in the FA chains of GP complicates precise structural assignment, which is why acyl chains are only tentatively assigned. Furthermore, several combinations of the four acyl chains at different positions in the CL structure are possible; thus, the exact configuration cannot be

Figure 1.
Comparison of DESI-MS results for oncocytic thyroid tumor, nononcocytic thyroid tumor, and normal thyroid tissues. To aid visualization, representative negative ion mode DESI mass spectra of oncocytic tumor (top), nononcocytic tumor (middle), and normal thyroid tissue (bottom) mass spectra are shown from m/z 590 to 1,500, where CL species (red) and other GPs are detected (for full m/z range, please see Supplementary Fig. S1). Inset in top spectrum shows the mass spectrum of doubly charged CL ion in the mass region from m/z 723 to m/z 729, with a 0.5 mass difference between each peak (characteristic of doubly charged ions).
assigned by our method. In total, 31 CL molecular ions were identified and characterized, except for two CL ions that present insufficient fragment ion intensity. The singly charged CL molecular ions were also observed from m/z 1,400 to 1,500 at high relative intensities in the oncotic tumor when compared with nononcocytic and normal thyroid tissues. Several ox-CL species were also identified in the oncotic tumors mass spectra. For example, tandem MS experiments of doubly charged molecular ion m/z 677.414 yielded oxidized carboxylate anion (9:1-OOH; m/z 187.099), 18:2-carboxylate anion (m/z 279.234), oxidized lyso-PA from 9:1-OOH at m/z 323.092, and lyso-PA at m/z 415.228, indicating that the CL molecular species is ox-CL(18:2/18:2/18:2/9:1(0OH); Supplementary Fig. S3; ref. 29). Altogether, 17 different ox-CLs were identified in oncotic tumor tissues, previously unreported in human tissues. Note that these oxidized species were also observed in oncotic tissues when no voltage was applied in the DESI source (30). Most interestingly, this oxidation effect was specific to CL, as other polyunsaturated GPs at similar relative abundances were not detected in their oxidized forms by our method. Thus, our data indicate that the detected ox-CL are endogenous molecules present in oncotic tissues.

An uncommon series of doubly charged peaks from m/z 1,000 to 1,200 were observed in high relative abundance in oncotic tumors when compared with nononcocytic and normal thyroid tissues. These peaks were identified using a series of tandem MS experiments as a combination of CL with DG (m/z 1,000–1,100) or, more predominantly observed, with PC (m/z 1,100–1,200), with no bridging or other additional atoms. The ion m/z 1,102.262, for example, was identified as CL + PC (106:12) using MS2 and MS3 experiments (Supplementary Fig. S3). The chemical structures of many fragment ions include structural components of both PC and CL molecules, which indicates that these species are strongly bound, through what we hypothesize to be an ionic bond within a concatenated structure. To confirm the chemical composition of these ions, we performed DESI-MS analysis on a mixture of CL and PC standards and observed the formation of these doubly charged species, which presented identical fragmentation pattern (Supplementary Fig. S4) to those observed in tissue. PCs were not observed in the negative ion mode in our experiments; thus, it is interesting to observe these molecules bound to CL species. We have not found previous reports on the observations of these doubly charged molecular ions from tissue samples.

In total, 28 CL species, 17 ox-CL, 2 MLCL, 27 CL + DG were identified in oncotic tumors. The results for a representative set of these species are shown in Table 1, whereas the full list is found in Supplementary Table S1 (note that only 131 isotopes were included for each molecule). Note that two MLCL species were identified in oncotic thyroid tumor tissue, although at a lower relative intensity when compared with the

### Table 1. Representative CL species identified using high mass resolution/high mass accuracy measurements and tandem MS analyses

<table>
<thead>
<tr>
<th>Measured m/z</th>
<th>Lipid classa</th>
<th>Tentative attribution</th>
<th>Exact m/z</th>
<th>Mass error (ppm)b</th>
<th>Proposed formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>592.364</td>
<td>MLCL</td>
<td>CL(54:5)</td>
<td>592.364</td>
<td>0.2</td>
<td>C55H90O15P2</td>
</tr>
<tr>
<td>609.414</td>
<td>ox-CL</td>
<td>20:4/18:2/16:0/9:1(0H)</td>
<td>609.414</td>
<td>0.2</td>
<td>C55H90O15P2</td>
</tr>
<tr>
<td>677.411</td>
<td>ox-CL</td>
<td>18:2/18:2/18:2/9:1(0H)</td>
<td>677.411</td>
<td>0.1</td>
<td>C55H90O15P2</td>
</tr>
<tr>
<td>678.419</td>
<td>ox-CL</td>
<td>20:2/18:2/19:1(0H)</td>
<td>678.419</td>
<td>0.3</td>
<td>C55H90O15P2</td>
</tr>
<tr>
<td>689.429</td>
<td>ox-CL</td>
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<td>689.429</td>
<td>0.2</td>
<td>C55H90O15P2</td>
</tr>
<tr>
<td>690.435</td>
<td>ox-CL</td>
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<td>690.435</td>
<td>2.8</td>
<td>C55H90O15P2</td>
</tr>
<tr>
<td>697.428</td>
<td>ox-CL</td>
<td>CL(55:6-6)</td>
<td>697.427</td>
<td>1.1</td>
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<tr>
<td>697.464</td>
<td>CL</td>
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<td>697.463</td>
<td>0.5</td>
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</tr>
<tr>
<td>701.493</td>
<td>CL</td>
<td>18:1/18:1/18:0/14:0</td>
<td>701.495</td>
<td>0.5</td>
<td>C55H90O15P2</td>
</tr>
<tr>
<td>706.487</td>
<td>CL</td>
<td>18:2/18:1/18:1/15:0</td>
<td>706.487</td>
<td>0.3</td>
<td>C55H90O15P2</td>
</tr>
<tr>
<td>710.471</td>
<td>CL</td>
<td>18:2/18:2/18:2/18:1</td>
<td>710.471</td>
<td>0.1</td>
<td>C55H90O15P2</td>
</tr>
<tr>
<td>723.479</td>
<td>CL</td>
<td>18:2/18:2/18:2/18:2</td>
<td>723.479</td>
<td>0.1</td>
<td>C55H90O15P2</td>
</tr>
<tr>
<td>724.485</td>
<td>CL</td>
<td>18:2/18:2/18:2/18:2</td>
<td>724.487</td>
<td>1.7</td>
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<tr>
<td>732.482</td>
<td>ox-CL</td>
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<td>730.484</td>
<td>2.8</td>
<td>C55H90O15P2</td>
</tr>
<tr>
<td>735.478</td>
<td>CL</td>
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<td>735.479</td>
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<td>736.487</td>
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</tr>
<tr>
<td>737.494</td>
<td>CL</td>
<td>18:2/18:2/18:2/18:2</td>
<td>737.495</td>
<td>0.1</td>
<td>C55H90O15P2</td>
</tr>
<tr>
<td>738.502</td>
<td>CL</td>
<td>18:2/18:2/18:2/18:2</td>
<td>738.502</td>
<td>0.2</td>
<td>C55H90O15P2</td>
</tr>
<tr>
<td>745.491</td>
<td>ox-CL</td>
<td>CL(74:8)</td>
<td>745.491</td>
<td>0.7</td>
<td>C55H90O15P2</td>
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<tr>
<td>1,034.755</td>
<td>CL + DG</td>
<td>CL + DG(108:9)</td>
<td>1,034.756</td>
<td>2.9</td>
<td>C55H90O15P2</td>
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<td>1,035.760</td>
<td>CL + DG</td>
<td>CL + DG(108:8)</td>
<td>1,035.764</td>
<td>3.0</td>
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<tr>
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<td>CL + DG</td>
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<td>1,047.764</td>
<td>2.0</td>
<td>C55H90O15P2</td>
</tr>
<tr>
<td>1,102.259</td>
<td>CL + PC</td>
<td>CL + PC(106:12)</td>
<td>1,102.260</td>
<td>0.5</td>
<td>C55H90O15P2</td>
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<tr>
<td>1,117.282</td>
<td>CL + PC</td>
<td>CL + PC(104:8)</td>
<td>1,117.283</td>
<td>1.6</td>
<td>C55H90O15P2</td>
</tr>
</tbody>
</table>

aCL = cardiolipin (X:Y) denotes the total number of carbons and double bonds in the fatty acid chains.
bMass errors were calculated on the basis of the exact monoisotopic m/z of the deprotonated form of the assigned molecules.
other CL species detected. In contrast to oncocytic thyroid tumors, the mass spectra obtained from nononcocytic thyroid tumors showed high relative abundances of PI(20:4/17:0) at m/z 871.536, PI(20:4/16:0) at m/z 857.520, PI(18:2/16:0) at m/z 833.518, and PE(18:1/16:1) at m/z 700.530 (Fig. 1), as well as many other GPs and FAs. CL species were also observed in nononcocytic tumors but at lower relative intensities than that observed in oncocytic thyroid tumors, whereas oxidized species were either undetectable or at much lower abundance using our method. In normal thyroid tissues, although the mass spectra total ion counts were similar to those observed from tumor tissues, all ox-CL species identified in oncocytic tissues were undetectable by DESI-MS (Supplementary Fig. S5). Although the DESI-MS imaging results provide a qualitative assessment of the changes in lipid abundances, multivariate statistical analysis of the individual ions was performed to evaluate whether the observed changes are significant (results shown later in the article).

CL distribution correlates with oncocytic cells and mitochondria accumulation in tissues

2D DESI-MS experiments were performed to examine the spatial distribution of the molecular ions detected from thyroid tissues (Fig. 2A). In particular, we were interested to investigate whether the spatial distribution of CL ions colocalized with specific histologic features in oncocytic tumor tissues. Figure 2B shows the DESI-MS images obtained for selected molecular ions for an oncocytic sample, a nononcocytic sample, and a normal thyroid tissue sample (additional imaging results are shown in Supplementary Fig. S6). Optical images of the same tissue section that were H&E stained after DESI-MS are also presented (23). On a microscopic scale, all oncocytic tumors analyzed showed characteristic histologic features with enlarged cells of high cytoplasmic volume that accommodates the increased number of mitochondria. In many samples, regions predominantly composed of cancer cells were observed adjacent or within regions defined as regions of fibrosis tissues. These tissue regions (hundreds of micrometers) were spatially assigned by pathologic evaluation and were discernable in the DESI-MS images (spatial resolution of 150 μm). In oncocytic tumors, the molecular distribution of CL species was colocalized, homogeneous, and remarkably high within the regions of oncocytic tumor cells and was in lower intensities in fibrosis regions in sample, as shown for m/z 738.502 and m/z 723.479 (Fig. 2B). Similar spatial distribution was observed for other CL, ox-CL, CL + PC, and CL + DG molecular ions (Fig. 3). In particular, all ox-CL showed highly similar spatial distributions with increased relative intensities observed in regions with accumulation of oncocytic thyroid tumor cells and absence in adjacent normal cells or fibrosis. In all normal thyroid tissue analyzed, common patterns of the cellular organization were observed, with spherical colloid surrounded by a single layer of follicular cells and scattered parafollicular cells. The molecular images obtained for normal tissues showed lipid signal colocalized with follicular cells (Fig. 2B). Colloid did not show lipid profiles and are thus seen as dark regions in the DESI-MS ion map.
images. Nononcocytic follicular and papillary thyroid carcinoma showed typical histologic patterns and displayed a homogenous molecular distribution of the most abundant molecular ions within regions of tumor cell.

To evaluate whether CL distribution correlates with regions of mitochondrial accumulation in oncocytic tumors, we performed IHC with an anti-mitochondrial antibody in tissues sections adjacent to those imaged by DESI-MS. Positive staining for mitochondria was observed for all thyroid tumors analyzed, whereas negative (weak) staining was observed for all normal thyroid tissues. As expected, strong mitochondrial staining are seen in all oncocytic tissues (Supplementary Fig. S7). Spatial agreement was observed between regions of strong mitochondrial staining in oncotypic tissues and regions of high relative intensities of CL species in DESI-MS images. Papillary thyroid carcinoma that presented higher relative abundances of CL also showed mitochondria staining by IHC.

Mitochondria accumulates in oncotypic thyroid tumor cells

To further investigate the mitochondrial distribution within the cells of thyroid tissues, we performed immunofluorescence staining with an anti-mitochondrial antibody (green) and nuclear DAPI staining (blue) in adjacent sections of thyroid tissues (Fig. 4A). Confocal microscopy images obtained for oncotypic tumors show high density staining of mitochondria. A punctate staining pattern showcases accumulation of mitochondria within the cellular cytoplasm in oncotypic tumors. Nononcocytic follicular carcinoma shows less pronounced accumulation of mitochondria in scattered cells, lower than that observed for oncotypic tumors, and significantly higher than normal tissue. To provide a quantitative assessment of mitochondrial in thyroid tissue, tissues were homogenized and mitochondria was isolated following a standard organelle isolation protocol (Fig. 2A; ref. 24). Quantification of the total protein content in the isolated mitochondria pellet by BCA assay shows that there is on average 8.8 mg of protein/g of tissue sample, whereas oncotypic tumor tissues contained on average 22.2 mg of protein/g of tissue sample and nononcocytic thyroid tumors contained 15.6 mg of protein/g of tissue sample (Fig. 4B). The changes in mitochondrial protein content between groups were found to be statistically significantly ($P < 0.001$ using a one-way ANOVA test).

Alteration in CL composition occurs in the mitochondria of oncotypic human tumor cells

To investigate whether the abnormally high relative abundance and diversity of CL species detected from oncotypic tissues were solely related to the accumulation of mitochondria per oncotypic cell or were also associated to an alteration in the CL composition of the mitochondria membrane, we diluted the isolated mitochondrial pallets to the same concentration (3 mg protein/g of tissue) for all tissues, performed a lipid extraction, and analyzed them using the same conditions used for DESI-MS imaging of tissue sections. The mass spectra obtained showed a higher relative intensity of CL species from the mitochondria isolated from oncotypic tumors when compared with nononcocytic tumors and normal thyroid tissues (Supplementary Fig. S8). To compare the CL abundance within the samples, we normalized the total ion counts of CL species to the total lipid counts in the spectra obtained from isolated mitochondria. The average normalized value was 0.081 for oncotypic tumors, 0.037 for nononcocytic tumors, and 0.002 for normal tissue (Fig. 4C), which allows discrimination between these groups with statistical significance ($P < 0.001$ using a one-way ANOVA test). These results confirm that besides mitochondria accumulation, an alteration in the CL composition of the mitochondrial membrane occurs in oncocytic thyroid cells. These biological phenomena collectively contribute to the abnormally high relative intensities and diversity of CL species detected directly from oncotypic tumor tissue in our DESI-MS imaging experiments.

Lipids are molecular markers of oncotypic tumors

To evaluate whether the changes in relative abundance of the molecular ions observed in DESI mass spectra and images obtained were statistically significant, we applied SAM statistical analysis to our complex DESI-MS imaging dataset. Mass spectral data were extracted from regions of interest of a single sample.
predominant histologic composition for the first set of 30 samples investigated (i.e., cancer cells or normal follicular cells). SAM identifies whether the change in the abundance of a molecular ion (m/z value) is statistically significant between the three different phenotypes by computing a contrast value that measures the average change in the peak intensity for that m/z between the groups (31). Repeated permutations were used to determine whether the change is significantly related to the phenotype and to estimate the percentage of molecular ions identified by chance, the FDR. The mean intensity value for all samples for a certain m/z was set to zero, so that the contrast values obtained represent the mean fold increase (positive contrast) or decrease (negative contrast) for the groups when compared with the overall mean intensity value. From all the ions detected (m/z 100–1,500) for all the samples analyzed, 219 different molecular ions were selected with FDR < 5%. As expected, ions corresponding to LECL, ox-CL, CLs, CL + PC, and CL + DG presented the most significant changes in average abundances between the three groups by SAM analysis. For example, the singly charged CL(20:4/18:2/18:2/16:0 or 18:2/18:2/18:2/18:2) detected at m/z 1,447.975 presented the highest contrast values of –1.927 for normal tissue, –0.845 for nononcocytic tumors, and +2.772 for oncocytic tumors (FDR = 0). Figure 5A shows the overall trend in contrast values obtained for the CL species selected by SAM (FDR < 5%). As observed, all CL species present positive values for oncocytic tumors, which demonstrates that these lipids are significant for discriminating oncocytic tumors from nononcocytic tumors and normal thyroid tissues. Box plots for selected ions are shown in Fig. 5B. Highly reproducible results that corroborate these findings were obtained in a set of 15 independent thyroid tumors analyzed (Supplementary Fig. S9). The remaining GPs selected by SAM (FDR < 5%), including PI, PE, and PG, presented no clear trends in contrast values within the three groups.

Discussion

Accumulation of CL-rich mitochondria is a fundamental characteristic of oncocytic tumors. Mutations in mitochondrial DNA have been previously described in oncocytic thyroid tumors, yet, little is known on their lipid composition. In this study, we used DESI-MS to image and chemically characterize the lipid composition of thyroid tumors. We discovered a novel molecular signature in oncocytic tumors characterized by an abnormally high abundance of CL species. DESI-MS imaging and IHC experiments confirmed that the spatial distribution of these molecular ions overlapped with regions of accumulation of mitochondria-rich oncocytic cells. Fluorescence imaging confirmed that the oncocytic tumors

Figure 4.
Mitochondria accumulation and changes in mitochondrial CL composition occur in oncocytic tumors. A, confocal images for oncocytic tumor (O1 and O2), nononcocytic tumor (NO1 and NO2), and normal (N1 and N2) thyroid tissues stained with anti-mitochondrial antibody (green) and DAPI nuclear staining (blue). B, quantification of the total protein content of isolated mitochondria pallet obtained from normal thyroid tissues (N), nononcocytic thyroid tumors (NO), and oncocytic thyroid tumors (O; *, P < 0.001). C, normalized CL intensities of the isolated mitochondria pallets obtained from normal thyroid tissues (N), nononcocytic thyroid tumors (NO), and oncocytic thyroid tumors (O) at the same concentration (5 μg protein/g of tissue; **, P < 0.001). P < 0.001 was considered as significant.
investigated presented high accumulation of mitochondria when compared with nononcocytic and normal thyroid tissue.

Using high mass accuracy, high mass resolution, and tandem MS experiments, we identified 101 different CL-containing molecular ions directly from oncocytic thyroid tissues. MS imaging of this large amount and diversity of CL species is unprecedented in untreated human tissues. Among these, two MLCL, which are intermediate molecules in CL remodeling (32), were detected in oncocytic thyroid tumor tissues. In addition, 54 intriguing doubly charged molecular ions composed of CL bound to PCs or DGs were seen at high relative abundances in oncocytic tumors when compared with nononcocytic or normal thyroid tissues. The mitochondrial inner membrane of eukaryotic cells has a unique composition of GPs, predominantly composed of PCs, CLs, and PEs, although the exact percent composition of lipids in human mitochondria is not known (33). Thus, the detection of CL + PC molecular ions, although unexpected, is not surprising considering the composition and spatial proximity of these molecules in the inner mitochondria membrane of oncocytic tumors.

Remarkably, we identified 17 different ox-CLs in oncocytic tumors. Oxidation of other abundant polyunsaturated phospholipids was not observed in our experiments, which indicates that this phenomenon is primarily occurring for CL, likely due to mitochondria dysregulation, which is known to occur in oncocytic thyroid tumors (2). CL oxidation has been implicated in degenerative diseases and reported in neuronal tissue that has suffered traumatic brain injury (4). Oxidative stress has been largely connected to tumorigenesis. In the mitochondrial inner membrane, CLs are found in association with the components of the electron transport chain (ETC), which generates reactive oxygen species. The proximity of the FA chains of CL to the various ETC complexes makes it a likely target of oxidative damage (29). Furthermore, redistribution of mitochondrial membrane CLs and accumulation of CL oxidation products through interactions with cytochrome c are required stages in the cellular apoptotic program, a process known to occur in tumor progression (34). Recently, ox-CLs were detected in PC-3 prostate cancer cell lines, although their content did not correlate with the proliferation of cells (5). We have also detected ox-CLs in human
kidney oncocytic tumors (unpublished), further suggesting their importance in oncocytic neoplasms, although more rigorous studies are needed to determine their biological role in human tumors.

This is the first study to report an abnormal expression and composition of lipids, such as CLs, in human thyroid tumor tissues. Through immunofluorescence imaging, DESI-MS imaging, and mitochondria isolation experiments, we demonstrated that this aberrant CL signature is related to: (i) accumulation of mitochondria in oncocytic tumors; and (ii) dysregulation (increased abundance and diversified structures) of CL composition in mitochondria membrane of oncocytic tumor cells. Gene expression profiling studies have suggested a profound modification of energy metabolism in oncocytic tumors (2). Genes coding for subunits of the respiratory chain enzymes, glycolytic enzymes, and energy metabolism enzymes involved in glycolysis, the tricarboxylic acid cycle are overexpressed in oncocytic tumors, many of which are directly involved in lipid biogenesis and metabolism. Our results further suggest that dysregulation of mitochondria and lipid metabolism are relevant in oncocytic tumors and provide novel molecular information for deciphering the biological mechanisms involved in these tumors.

The thyroid is the main hormonal regulator of lipid biogenesis and mitochondrial function (35). Nevertheless, no previous studies have investigated the lipid signatures of thyroid tumors. We show that lipids are molecular markers of oncocytic tumors with statistical significance. Although direct MS imaging does not provide a quantitative assessment of molecules in tissues, using SAM, 219 distinct molecular ions (FDR < 5%), including various lipids and metabolites, were found at increased or decreased relative abundance in oncocytic, nononcocytic, or normal thyroid tissues. Besides CL, significant changes in FA abundances were also observed using statistical analysis. This rich lipid signature is characteristic and diagnostic of oncocytic phenotypes. Although our sample size is not sufficient for discriminating adenomas and carcinomas within the oncocytic tumor group, our pilot study gives further rationale to explore this problem using the molecular information obtained by ambient ionization MS. Importantly, these unprecedented findings provide possibilities for new therapeutic targets for oncocytic tumors.

As lipid signatures can be readily accessed from tissue samples using ambient ionization MS, we expect this method to be valuable for diagnosis of thyroid cancers and clinical use (18). Nondestructive DESI-MS can be adapted for fine needle aspiration biopsy analysis, the gold-standard method for preoperative diagnosis of thyroid lesions. With further increase in sample size and analysis of different tumor types, we expect to identify unique molecular signatures in various types of thyroid neoplasia to enhance diagnosis of nodules (especially those deemed indeterminate) and thus overcome current limitations of thyroid cytology.

Our work showcases the power of ambient ionization MS for CL imaging in biological tissues and is relevant to a variety of applications. Dysregulation of mitochondria occurs in many pathologies besides cancer. Lipids and their oxidized counterparts have been increasingly appreciated as important molecular markers and investigated to uncover biological pathways in disease (36–38). Further studies will be performed to extensively investigate alterations in small metabolites, FAs, CLs, and other GPs in thyroid tumors.

Disclosure of Potential Conflicts of Interest

J. Zhang and L.S. Eberlin have ownership interest (including patents) in a patent. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: J. Zhang, J. Suliburk, L.S. Eberlin
Development of methodology: J. Zhang, J. Suliburk, L.S. Eberlin
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Zhang, W. Yu, S.W. Ryu, J. Lin, G. Buentello, J. Suliburk, L.S. Eberlin
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Zhang, S.W. Ryu, R. Tibshirani, L.S. Eberlin
Writing, review, and/or revision of the manuscript: J. Zhang, W. Yu, J. Suliburk, L.S. Eberlin
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Zhang, G. Buentello, J. Suliburk, L.S. Eberlin
Study supervision: L.S. Eberlin

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