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Rapid and comprehensive 'shotgun' lipidome profiling of colorectal cancer cell derived exosomes

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ABSTRACT

There is an increasing recognition of the role that cancer cell derived exosomes play in intercellular signaling upon fusion or uptake with a target cell, including immune system evasion, tumor growth and metastasis. To date, however, although exosomal membrane and cargo lipids are expected to play a pivotal role in exosome biogenesis and secretion, as well as in fusion or uptake and target cell functional response, the detailed characterization of cancer cell derived exosome lipids across a range of different cancers has not yet been broadly explored. Here, a simple and straightforward lipidome analysis strategy consisting of optimized sample extraction and novel sample derivatization techniques, coupled with high-resolution 'shotgun' mass spectrometry and 'targeted' tandem mass spectrometry methods, is demonstrated for the rapid identification of >520 individual lipids in 36 lipid classes and sub classes from exosomes secreted by the colorectal cancer cell line, LIM1215. Relative quantification and comparison of exosome versus cellular lipid profiles reveals significant enrichment of certain lipid classes, as well as substantial lipid subclass remodeling and changes in abundance of individual lipids, including sphingolipids, sterol lipids, glycerolipids and glycerophospholipids, and particularly plasmalogen- and alkyl ether-containing glycerophospholipids. This analysis strategy therefore provides a platform for comprehensive lipidome profiling across a wide range of cancer cell or tissue derived exosomes, that will facilitate subsequent functional studies aimed at elucidating the role of specific cellular or exosome lipids in the onset and progression of colorectal cancer, or to identify specific lipid(s) that could serve as effective diagnostic or prognostic disease biomarkers.

1. Introduction

The disruption of lipid metabolism pathways involved in the dynamic regulation of membrane structure, energy homeostasis and signaling can affect critical cellular processes including cell proliferation, differentiation and mobility, and are known to be associated with the onset and progression of various diseases, including cancer [1–8]. For example, changes in the compositions and/or abundance of certain lipid classes including glycerolipids, glycerophospholipids, sphingolipids and sterol lipids, or their bioactive signaling lipid metabolites, have been shown to be correlated with malignancy and metastatic capacity in numerous human cancers [9–14]. Therefore, elucidating the molecular compositions and functions of these lipids is expected to significantly increase our understanding of colon cancer biology, as well as

Abbreviations: BHT, butylated hydroxytoluene; CE, cholesterol ester; Cer, ceramide; Chol, cholesterol; $^{13}C_1$-DMBNHS, $^{13}C_1$-*S,S'*-dimethylthiobutanoylhydroxysuccinimide ester; DG, diradylglycerol; DMF, N,N-dimethylformamide; FCS, fetal calf serum; HCD, higher-energy collisional dissociation; Hex-Cer, hexosylceramide (cerebroside); LC, liquid chromatography; LPC, lysoglycerophosphocholine; LPE, lysoglycerophosphoethanolamine; LPG, lysoglycerophosphoglycerol; LPS, lysoglycerophosphoserine; MG, monoradylglycerol; MS, mass spectrometry; MUFA, monounsaturated fatty acid; nESI, nano-electrospray ionization; O-, alkyl ether lipid; P-, plasmalogen lipid; PBS, phosphate buffered saline; PC, glycerophosphocholine; PE, glycerophosphoethanolamine; PG, glycerophosphoglycerol; PI, glycerophosphoinositol; PS, glycerophosphoserine; PUFA, polyunsaturated fatty acid; SM, sphingomyelin; MS/MS, tandem mass spectrometry; TEA, triethylamine; TEM, transmission electron microscopy; TG, triradylglycerol.

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enable the identification of diagnostic or prognostic biomarkers [15–17], or novel therapeutic targets, for the disease.

There is also an increasing recognition of the role that cancer cell derived exosomes (40–100 nm vesicles formed within multivesicular bodies and secreted from parent cells upon fusion of late endosomes with the cellular membrane) can play in cell–cell communication and in effecting phenotypic changes upon uptake or fusion with a recipient cell [18–24]. For example, numerous reports indicate that exosome mediated intercellular transfer of protein, mRNA and miRNA can facilitate a range of processes including immune system evasion, the promotion of angiogenesis, tumor growth, migration and cancer metastasis [25–27]. This inter-cellular communication role of exosomes is especially important since cancer cells exhibit increased secretion of exosomes relative to non-malignant cells [23,24].

Exosomal membrane lipids have also been shown to play a pivotal role in exosome formation and secretion, fusion and uptake, and in target cell functional response [28–34]. Numerous studies have clearly demonstrated that exosome lipid compositions are different to those of the parent cells, with significant enrichment observed in multiple lipid classes including cholesterol, sphingomyelin, phosphatidylserine, ganglioside, and saturated phosphatidylcholine and phosphatidylethanolamine molecular lipid species. [28,29,35–39]. Exosomes have also been shown to contain the necessary functional components of active lipid metabolism, including enzymes (e.g., phospholipases) and bioactive signaling lipids (e.g., prostaglandins and leukotrienes), in addition to the above complex lipid precursors of lipid metabolism contained within the exosomal membrane [33,34].

To date, however, the functional (i.e., causative) roles of exosome derived lipids in malignant and metastatic cancer cell transformation, and exploration of their potential to serve as biomarkers of colorectal cancer malignancy and metastatic capacity, remains poorly understood. Indeed, only a few studies have been performed to date to systematically characterize the diverse range of hundreds, or potentially thousands, of individual molecular lipid species that may be present within cancer cell secreted exosomes (i.e., the lipidome), or to quantitatively monitor the global exosome lipid profile alterations occurring as a function of cancer cell or tissue molecular phenotype, or malignancy and metastatic potential. One notable example is the recent report by Llorente et al. who used a combination of different lipid extraction strategies coupled with a series of 'targeted' positive and negative ionization mode precursor ion scanning and neutral loss 'shotgun' (i.e., direct infusion) tandem mass spectrometry (MS/MS) and liquid chromatography (LC)–MS/MS methods, to perform an in depth lipidomic study of exosome compositions from PC-3 prostate cancer cells. From this study, an overall 8.4-fold enrichment of lipids per mg of protein in exosomes was observed, along with significant enrichment and remodeling of phosphatidylserine, cholesterol, sphingomyelin and glycosphingolipids compared to their parent cells, and the identification and quantitative analysis of 280 individual lipid species [39], at the molecular lipid level [40].

The development of high-resolution/accurate mass spectrometry instrumentation [41–44], and optimized lipid extraction protocols [45], has facilitated additional recent improvements for direct 'shotgun' lipidomic analysis of a broad range of both non-polar and polar lipid classes and their individual lipids within crude lipid extracts, across at least three orders of magnitude ion abundance, without need for complex liquid–liquid extraction or chromatographic fractionation techniques prior to mass spectrometry analysis, and with limited sample consumption requirement. These strategies are further enhanced by the use of functional group selective derivatization reactions that result in the resolution of isobaric mass overlap of several common lipid classes and their subsequent unambiguous assignment, as reported previously by our group [44,46]. These benefits are particularly attractive for exosome lipidome analysis, where limited sample availability is an ever present challenge. Here, therefore, we have combined each of these different strategies, i.e., optimized sample extraction, sample derivatization and high-resolution 'shotgun' mass spectrometry and 'targeted' tandem mass spectrometry (MS/MS), for the rapid identification and determination of normalized lipid abundance changes for a wide variety of lipid classes, sub classes and individual lipid species from exosomes secreted by the colorectal cancer cell line, LIM1215 [47].

2. Materials and methods

2.1. Materials

Ammonium formate was purchased from Alfa Aesar (Ward Hill, MA). Iodine, N,N-dimethylformamide (DMF), and triethylamine (TEA) were from Jade Scientific (Westland, MI). Internal standard lipids $PC_{(14:0/14:0)}$, $PE_{(14:0/14:0)}$, and $PS_{(14:0/14:0)}$ were purchased from Avanti Polar Lipids (Alabaster, AL). Ammonium bicarbonate, isopropanol, methanol, and water were from J.T. Baker (Phillipsburg, NJ). Chloroform was from EMD Chemicals (Billerica, MA). All solvents used were high performance liquid chromatography grade, and all lipid extraction and storage solvents contained 0.01% butylated hydroxytoluene (BHT) from Sigma Aldrich (St. Louis, MO). $^{13}C_1$-S,S′-dimethylthiobutanoylhydroxysuccinimide ester ($^{13}C_1$-DMBNHS) was synthesized as previously described [44,46]. RPMI 1640 cell culture medium and Penicillin–Streptomycin were from GIBCO, Life Technologies (Grand Island, NY). Fetal calf serum (FCS) was from SAFC® Bioscience (St. Louis, MO).

2.2. LIM1215 cell culture

The human colorectal cancer LIM1215 cell line was cultured in RPMI 1640 medium supplemented with 10% FCS and 100 Units/mL of Penicillin–Streptomycin in 150 cm^2 BD Falcon™ tissue culture flasks at 37 °C in 5% CO_2. 1.48×10^7 cells, containing 2976 μg of protein, were collected then lyophilized and stored at −80 °C prior to lipid analysis.

2.3. Isolation of secreted exosomes from LIM1215 cells

LIM1215 cells were grown to ~80% confluent (~5×10^8) in Integrid™ 150 mm cell culture dishes then washed three times to remove residual FCS. Subsequently, cells were cultured for 24 h in RPMI 1640 media supplemented with 10% exosome depleted FCS. The cell viability was more than 98% as determined by Tryphan Blue assay. The conditioned media from the plates was then collected and centrifuged at 500*g* for 10 min. A subsequent spin at 2000*g* for 20 min was performed to remove floating cells and cell debris followed by 10,000*g* for 30 min. The supernatant was further subjected to ultracentrifugation at 100,000*g* in a Beckman SW-28 rotor for 1 h at 4 °C. Finally, pellets were washed with PBS to remove residual traces of media and centrifuged again at 100,000*g* for 1 h at 4 °C to isolate exosomes. The exosome preparation containing 230 μg of protein was then lyophilized and stored at −80 °C prior to lipid analysis.

2.4. Protocol for monophasic lipid extraction from LIM1215 cells and secreted exosomes

(1) Lyophilized cell pellets containing 2.9 mg of cellular protein, or lyophilized exosome pellets containing 230 μg of exosomal protein, were combined with 1.0 mL of ice-cold 40%