Lysophospholipids Are Potential Biomarkers of Ovarian Cancer

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Abstract

Objective: To determine whether lysophosphatidic acid (LPA) and other lysophospholipids (LPL) are useful markers for diagnosis and/or prognosis of ovarian cancer in a controlled setting. Method: Plasma samples were collected from ovarian cancer patients and healthy control women in Hillsborough and Pinellas counties, Florida, and processed at the University of South Florida H. Lee Moffitt Cancer Center and Research Institute (Moffitt). Case patients with epithelial ovarian cancer (n = 117) and healthy control subjects (n = 27) participated in the study. Blinded LPL analysis, including 23 individual LPL species, was performed at the Cleveland Clinic Foundation using an electrospray ionization mass spectrometry–based method. LPL levels were transmitted to Moffitt, where clinical data were reviewed and statistical analyses were performed. Results: There were statistically significant differences between preoperative case samples (n = 45) and control samples (n = 27) in the mean levels of total LPA, total lysophosphatidylinositol (LPI), sphingosine-1-phosphate (SIP), and individual LPA species as well as the combination of several LPL species. The combination of 16:0-LPA and 20:4-LPA yielded the best discrimination between preoperative case samples and control samples, with 93.1% correct classification, 91.1% sensitivity, and 96.3% specificity. In 22 cases with both preoperative and postoperative samples, the postoperative levels of several LPL, including SIP, total LPA, and lysophosphatidylcholine (LPC) levels and some individual species of LPA and LPC, were significantly different from preoperative levels. Conclusion: LPA, LPI, LPC, and SIP appear useful as diagnostic and prognostic biomarkers of ovarian cancer. (Cancer Epidemiol Biomarkers Prev 2004;13(7):1185–91)

Introduction

The mortality rate for women with ovarian cancer is very high, with an estimated 14,300 deaths from ovarian cancer in 2003 in the United States (1). More than two thirds of patients have late-stage metastatic disease at initial diagnosis with a 5-year survival rate of ~20% to 30% (1-4). Conversely, at early stages, the long-term survival rate approaches 90% (5). There is currently no proven effective method for early detection of ovarian cancer through biomarkers, imaging, or other means. The most common biomarker for ovarian cancer, CA 125, lacks specificity and is elevated in only about 50% of stage I ovarian cancer cases (3, 4, 6). Proteomic patterns derived from surface-enhanced laser desorption/ionization mass spectroscopy analysis have recently shown promise for early ovarian cancer detection (7), but further studies regarding their reproducibility and reliability for early detection and screening are needed.

Lysophosphatidic acid (LPA) has been proposed as a sensitive biomarker (8). However, studies investigating the utility of LPA as a biomarker for early detection of ovarian cancer have yielded conflicting results. Preliminary findings from a study, which included 48 healthy controls and 48 women with ovarian cancer, showed that plasma LPA levels (measured by gas chromatography) were elevated in patients with ovarian cancer (P < 0.001; ref. 8). Importantly, elevated levels were detected in early-stage ovarian cancers compared with controls (8). The study also compared available CA 125 values with LPA levels, and results suggested that plasma LPA may be a more sensitive marker for ovarian cancer, particularly for stage I disease (8). A recent Korean study of only three pairs of samples also showed differences between ovarian cancer cases and controls (9). However, in another study where LPA levels were measured in plasma samples from 32 patients with ovarian cancer and 32 healthy controls using a liquid chromatography/mass spectroscopy assay, results showed no significant elevation in plasma LPA levels in ovarian cancer patients compared with controls, raising questions about the utility of plasma LPA levels for early detection of ovarian cancer (10).
LPA is present in the ascitic fluid of patients with ovarian cancer (11, 12) and may function as an autocrine factor, contributing to ovarian cancer proliferation, cell survival, angiogenesis, and metastasis (13-22). Lyso phosphatidylinositol (LPI), a related lyosphospholipid (LPL) to LPA, has also been found at increased levels in ascites fluid and plasma of ovarian cancer patients compared with controls (23) and has been shown to display signaling properties in cellular systems (24, 25). Thus, LPI may also have utility as a biomarker of ovarian cancer, and data suggest that measuring LPI in addition to LPA may increase the sensitivity and/or specificity of the test (23). Both LPA and LPI represent various subgroups with different fatty acid chains. In addition, the fatty acid chain may link to the glycerol backbone through different chemical linkages resulting in various subclasses [i.e., acyl (LPA), alkyl (A-LPA), and alkenyl (An-LPA)]. Findings of a study to evaluate the discriminating ability of LPA and LPI subgroups for ovarian cancer identification compared with total LPA and LPI suggested that subgroups with unsaturated fatty acid chains may be associated with late-stage or recurrent ovarian cancer (26). Other LPLs that have been proposed to have a biological role in ovarian cancer and be potentially useful as biomarkers of the disease include lyso phosphatidylcholine (LPC), which has also been shown to be elevated in the plasma of ovarian cancer patients (27), and the lysosphingolipid sphingosine-1-phosphate (SIP), which is known to have both extracellular and intracellular signaling properties (28-31).

To further explore the potential of LPA, LPI, LPC, and SIP as biomarkers for ovarian cancer detection, we measured plasma LPL levels (including subgroups of LPA, LPI, and LPC) in women with ovarian cancer and healthy controls using an electrospray ionization mass spectrometry method recently developed by Xiao et al. (23). This assay allows simultaneous detection and quantitation of different species of LPL with at least 10 times more sensitivity than the previous gas chromatography method (23).

**Materials and Methods**

**Patients.** All patient-derived biological specimens were collected under protocols approved by the University of South Florida Institutional Review Board, and all participants provided written informed consent.

Whole blood samples were obtained preoperatively in EDTA tubes by routine venipuncture of women undergoing surgery for suspected ovarian cancer in Hillsborough and Pinellas counties, Florida, between December 13, 2000 and October 30, 2002. All women ages 18 to 80 years undergoing surgery for suspected ovarian cancer in the two counties during the defined period were regarded as eligible for entry into the study. No patients who were asked refused to participate. Of the preoperative samples obtained, 45 were from women who were later confirmed to have ovarian cancer or primary peritoneal cancer (ovarian cancer patients; median age 60 years, range 33 to 79). Samples were obtained postoperatively from ovarian cancer patients from the same eligibility pool (n = 94, median age 59 years, range 26 to 80), including 22 patients who had contributed a preoperative sample and 72 who had not. Whole blood samples from control subjects were collected concurrently from healthy women from the same counties who reported no history of cancer, gynecologic disease, oophorectomy or family history of breast/ovarian cancer (n = 27, median age 45 years, range 22 to 79). Whole blood specimens were obtained from a total of 117 ovarian cancer patients, including 18 patients with stage I disease, 11 with stage II disease, 74 with stage III disease, and 14 with stage IV disease. Among the 45 patients for whom a preoperative sample was available, there were 7 patients with stage I disease, 3 with stage II disease, 31 with stage III disease, and 4 with stage IV disease. Cancer diagnosis was confirmed for all cases by review of pathology records by a single ovarian cancer expert. Clinical stage was determined according to International Federation of Gynecologists and Obstetricians criteria (32), and the histologic subtype was evaluated according to the WHO classification (33).

**Sample Collection.** LPA is produced and released by activated platelets during coagulation and therefore is a normal constituent of serum, but it is present only at very low levels in whole blood or fresh platelet-poor plasma from healthy individuals (8). To prevent platelet activation and phospholipase activity, whole blood samples were collected via routine venipuncture in EDTA-containing tubes. Because LPLs are metabolites and levels may change during incubation, it is important that sample processing be as consistent as possible across all samples for comparison. We collected samples from multiple locations in the two study counties and processed (centrifugation and aliquoting) all samples at the University of South Florida. H. Lee Moffitt Cancer Center and Research Institute (Moffitt). After blood drawing, samples were immediately chilled for transport to Moffitt by being placed in a Styrofoam container accompanied by a frozen pack for overnight delivery. This system allowed centrifugation within 16 to 28 hours after blood drawing. Samples appear stable for measurement of LPL when processed according to this protocol (Y. Xu, personal communication). Centrifugation was at 3,000 × g for 20 minutes after which the plasma was immediately aliquoted per each 0.5 mL into coated micro-Eppendorf tubes and immediately frozen at −70 °C. Samples were batch shipped on dry ice by overnight delivery to the Cleveland Clinic Foundation for analysis. Shipped samples were identified by a unique sample number only, without identifiers or any indication of the subject’s status as ovarian cancer patient or control. The samples were maintained at −70 °C until preparation for mass spectrometry analysis. No personnel at the Cleveland Clinic Foundation had knowledge of the subjects’ disease status at any time. Laboratory data were transmitted according to each unique sample number to Moffitt where all statistical analyses were performed.

**LPL Analysis.** Lipids were extracted as described previously with minor modifications (23, 34). To 0.5 mL plasma, 2 mL of MeOH/chloroform (2:1) and 0.1 mL of 6 N HCl were added. Samples were vortexed for 1 minute and incubated on ice for 10 minutes. Chloroform (1 mL) and H2O (1 mL) were added to separate the phases. Samples were vortexed for 0.5 minute prior to centrifugation (2,000 × g for 10 minutes). The lower phase was
transferred to a new glass tube. To the upper phase left in the original tube, 1 mL of chloroform was added to extract more lipids and the tube was centrifuged (2,000 \( \times g \) for 10 minutes). The lower phase was transferred into the same tube (with the lower phase extract), and the solvent was evaporated under nitrogen at 30°C. The dried lipids were resuspended in 50 \( \mu L \) of solvent (MeOH/chloroform 2:1), vortexed, and applied to a TLC plate. Two standards (18:1-LPA and 18:1-LPC) were applied to help in identifying the “LPA band” and “LPC band” on each TLC plate. The TLC plates were developed in the solvent system (chloroform/MeOH/ethyl alcohol 65:35:5.5) until the solvent front was 1.5 inch from the top of the plate. The lipids from the “LPA band” and “LPC band” were eluted with 2 mL of MeOH/chloroform (2:1) twice. The lipid solutions were dried under nitrogen at 30°C, and lipids were resuspended in 100 \( \mu L \) of MeOH for mass spectrometry.

Mass spectrometry analyses were performed using a Quattro Ultima triple quadrupole electrospray mass spectrometer (Micromass, Inc., Beverly, MA) with the MassLynx data acquisition system. A Waters 2690 (Waters, Milford, MA) autosampler was used to introduce the samples into the electrospray ionization source. The mobile phase used for all experiments was MeOH/H\(_2\)O (9:1 v/v), and the flow rate was 100 \( \mu L/\min \). The injection volume was set to 20 \( \mu L \) per sample for all experiments. The positive or negative ion mode with multiple reaction monitoring was used to quantitatively analyze the positively or negatively charged phospholipids. The collision energies were 70 eV in the negative mode and 50 eV in the positive mode. Nitrogen was used as both drying and nebulizing gas at flow rates of 500 and 50 L/h, respectively. The electrospray ionization probe capillary was held at 3 kV for the positive mode and +3 kV for the negative mode, and the cone voltage was set at 35 V in positive mode and +50 V in negative mode. The source and desorption temperatures were 100°C and 200°C, respectively.

LPA and other negatively charged LPLs were analyzed in the negative mode with the monitoring ions at m/z 378-79 (parent ion-product ion) for S1P, 381-79 for 14:0-LPA, 393-79 for 16:0-An-LPA, 395-79 for 16:0-A-LPA, 409-79 for 16:0-LPC, 421-79 for 18:0-An-LPA, 423-79 for 18:0-LPA, 433-79 for 18:2-LPA, 435-79 for 18:1-LPA, 437-79 for 18:0-LPA, 571-79 for 16:0-LPI, 599-79 for 18:0-LPI, SPC, 496-79 for 16:0-LPC, 510-79 for 18:0-An-LPA, 512-79 for 14:0-LPI, 520-79 for 16:0-An-LPC, and 524-79 for 18:0-LPC, respectively. All lipids with the phosphorylcholine group (positively charged) were analyzed in the positive mode. Monitoring ions were at m/z 378-79 for SPC, 496-184 for 16:0-LPC, 510-184 for 17:0-LPC, 520-184 for 18:2-LPC, 524-184 for 18:0-LPC, 544-184 for 20:4-LPC and 568-184 for 22:6-LPC, respectively. The dwell time in the multiple reaction monitoring mode was 0.11 millisecond, and the scan delay was 0.02 second.

**Statistical Analysis.** Categorical variables were analyzed using \( \chi^2 \) tests or Fisher’s exact tests depending on sample size. Continuous variables, including univariate comparisons for quantitative variables between normal and cancer cases, were compared using the Student’s \( t \) tests or the Wilcoxon rank sum test depending on the distribution of the variable of interest. Adjustment for potential confounding variables, such as the stage at diagnosis, was carried out by using general linear modeling or ANOVA methods, as appropriate. Stepwise logistic regression analysis was used to determine the statistical significance of LPA, LPI, LPC (and their subspecies), and S1P. All statistical significance testing was two sided, and \( P < 0.05 \) was considered to be statistically significant. \( P \) values in the 0.01 to 0.05 range should be interpreted with caution because of multiple testing issues. Statistical analyses were performed using SAS software (SAS Institute Inc., Cary, NC).

**Results**

The ages, stages, grades, histologic subtypes, and treatment status of the 117 ovarian cancer patients who participated in the study are shown in Table 1. A total of 166 samples were analyzed including 27 from healthy controls, 45 obtained preoperatively from women with ovarian cancer, and 94 obtained postoperatively from women with ovarian cancer, with 22 patients having both preoperative and postoperative samples.

There were statistically significant differences between preoperative case samples (\( n = 45 \)) and control samples (\( n = 27 \)) in the mean levels of several individual LPA species, the combination of 16:0-LPA/20:4-LPA, total LPA, total LPI, and SIP (Table 2). The best discrimination between samples obtained preoperatively from ovarian cancer patients and those from healthy controls was achieved by the combined levels of 16:0-LPA and 20:4-LPA, with 93.1% correct classification, 91.1% sensitivity, and 96.3% specificity (Fig. 1). Receiver operating characteristic curves (35) were examined, and a cutoff 16:0-LPA/24:0-LPA level of 0.62 \( \mu \)mol/L was identified as optimizing the sensitivity and specificity of the assay (Fig. 1). All patients with preoperative samples had

| Table 1. Clinical data for patients with ovarian cancer (\( n = 117 \)) |
|------------------------|------------------------|------------------------|------------------------|
| Characteristics        | Stages I and II (\( n = 29 \)) | Stages III and IV (\( n = 88 \)) | Percentage (\( n = 117 \)) |
| Age (y), median (range) | 60 (32-77)             | 59 (26-80)             |                          |
| Stages                 | I                      | II                     | III                     |
|                        | 18 –                   | 11 –                   | –                       |
| Grades                 | 1                      | 2                      | 3                       |
|                        | 10 11                  | 8 21                   | 11 55                   |
| Ungraded               | 0                      | 1                      |                          |
| Histologic types       | Serous                 | Endometrioid           | Mixed                  |
|                        | 12 61                  | 11 7                   | 0 8                    |
|                        | 2 2                    | 3 4                    | 3 4                    |
|                        | 2 2                    | 1 2                    | 0 2                    |
| Treatment status       | Preoperative           | Postoperative          |                          |
|                        | 10 35                  | 19 53                  |                          |
| Percentage             |                          |                        | 38.5                    |
|                        |                          |                        | 61.5                    |
The mean (SD) values for total LPI in the plasma samples obtained preoperatively from patients with stages I to IV ovarian cancer were 2.98 (1.57), 4.58 (2.71), 4.25 (2.81), and 2.96 (0.33) μmol/L, respectively, compared with 1.51 (0.79) μmol/L for the controls (Table 2).

In 22 cases with both preoperative and postoperative samples, the postoperative levels of total LPA, total LPC, 22:6-LPA, 18:0-LPA, the combination of 20:4-LPA/22:6-LPA, 20:4-LPC, and 18:2-LPC were significantly lower than preoperative levels (P = 0.03, 0.05, 0.02, 0.04, 0.03, 0.02, 0.003, and 0.03, respectively; Table 3). Of these LPLs, 18:0 LPA, 18:2 LPC, and total LPC levels also showed statistically significant differences between preoperative case samples (n = 45) and all postoperative case samples (n < 0.05). There were no statistically significant differences in mean LPL levels between postoperative samples obtained prior to initiation of chemotherapy versus postchemotherapy.

### Discussion

Ovarian cancer is a disease associated with a high mortality mainly because it currently escapes detection at early stages. Identification of an effective biomarker for early detection would improve survival. This study reports statistically significant differences in LPL levels between preoperative samples of ovarian cancer patients and those of healthy controls. The study also confirms that statistically significant elevations in LPL levels are present in patients with early-stage disease. Thus, the findings support the utility of LPL, especially LPA, as...
biomarkers for early detection of ovarian cancer. The study is the first to report significant postoperative changes in specific LPL levels. Further study is needed to determine whether some LPLs may return to baseline after successful treatment and/or have utility as biomarkers of recurrence. The study also contributes data toward determination of the best combinations of markers and cutoff values for clinical use.

Although our conclusions are still preliminary because our study sample is small and not ideal for demonstrating the value of LPL for screening, our findings regarding the utility of LPL as biomarkers of ovarian cancer are critically important because the two previous studies showed conflicting results (8, 10). To ensure the validity of our data, only investigators at Moffitt had access to clinical data, and the investigators performing LPL measurements at Cleveland Clinic Foundation were blinded to the case versus control status of the samples. All statistical analyses were performed at Moffitt.

The reason for the discrepancy between the findings of the two prior studies with interpretable results regarding the utility of LPA as a biomarker for detection of ovarian cancer is unclear. There were many methodological differences between the two studies, including differences in sample collection, processing, and lipid analyses (8, 10). Our experience suggests that it is critical to maintain consistency of procedures for all samples to be compared, including the time and temperature prior to and during centrifugation, sample storage vials (see below), extraction solvents and methods, establishment of standard curves, and mass spectroscopy methods. The following example demonstrates the importance of these aspects. Prior to analyzing the samples included in this report, we analyzed a batch of samples (n = 33) that showed lower overall LPL levels than anticipated among both cases and controls, with less separation than anticipated between levels of cases and those of controls. These findings prompted a review of procedures. Our review identified that the type of micro-Eppendorf tubes used for storage after centrifugation was critically important. If the tubes were not siliconized or prelubricated, as much as 90% of negatively charged LPLs were absorbed into the tube walls. Further analysis was performed, including paired storage of identical samples using coated and uncoated tubes, with the resulting differences in LPL levels analyzed. The analysis confirmed that the difference in tubes accounted for the differences in levels observed; therefore, data from these samples were not included in the analyses (data not shown). The following suggestions are offered for future investigations of LPL: we recommend use of SafeSeal microcentrifuge tubes (catalogue 505-201, PGC Scientific, Frederick, MD) for plasma storage and use of glassware only (not plastic ware), except for the storage tubes mentioned above.

Figure 1. 16:0-LPA/20:4-LPA levels (µmol/L) in preoperative case samples and controls.

Figure 2. Total LPA levels (µmol/L) in preoperative case samples and controls.
References


Acknowledgments

We thank JoAnn Runk for administrative assistance; Li Song for technical assistance; Judith A. Betts, R.N., and Jennifer Permutt Wey, M.S., for genetic counseling assistance; Margaret (Peggy) R. Maggiacomo, B.A., and Tricia Hollie, M.S.P.H., for data management assistance; and the patients for everything.
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