Plasma Lysophosphatidylcholine Levels: Potential Biomarkers for Colorectal Cancer

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ABSTRACT

Purpose

Plasma levels of lysophospholipids were evaluated as potential biomarkers for colorectal cancer (CRC), where a highly reliable and minimally invasive blood test is lacking.

Patients and Methods

Patients with CRC (n = 133) and control subjects (n = 125) were recruited through the Cleveland Clinic. Preoperative plasma samples were analyzed for lysophospholipid levels using liquid chromatography mass spectrometry in a blinded fashion. Participants were randomly divided in a 2:1 ratio into a "training set" (TS) and a "validation set" (VS). Logistic regression models were used in the TS to identify markers that best discriminated between CRC and controls. A cutoff point for the final discriminating model was developed using the receiver operating characteristic curve to achieve 95% specificity. All analyses were then independently validated in the VS.

Results

Plasma levels of several lysophosphatidylcholines (LPCs), including 18:1- and 18:2-LPC, were significantly decreased in CRC patients compared with controls ($P < .001$). A model based on total saturated LPC and the difference between the proportional amounts of 18:2-LPC and 18:1-LPC in the unsaturated LPC fraction was derived from the TS. This model achieved a sensitivity and specificity of 82% and 93%, respectively, in the VS. Overall, 118 (94%) of 125 control subjects and 113 (85%) of 133 CRC cases were correctly identified, including eight (89%) of nine CRC cases with stage T1 disease.

Conclusion

Percentage of 18:1-LPC or 18:2-LPC plasma levels compared with total saturated LPC levels, either individually or in combination, may represent potential biomarkers for CRC.

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INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the second leading cause of cancer-related deaths in the United States.^{1,2} In 2006 alone, approximately 149,000 Americans were diagnosed with CRC and approximately 55,000 died as a result of their disease. When CRC is detected early, the 5-year relative survival rate is 90%. However, only 37% of CRC cases are detected at an early stage, and the 5-year survival rate for patients presenting with distant metastases is only 8% to 9%.^{3,4}

Although colonoscopy has reported sensitivity and specificity values exceeding 95%, the degree of compliance is low, possibly because of the cost and perceived inconvenience and discomfort associated with this test. As a result, many patients still present with late-stage and potentially fatal disease.^{5,6} A

number of biomarkers have been identified for the detection and/or prognosis of colon cancer in colon tissues, feces, and serum.^{7,8} However, none of these markers provide a level of accuracy of detection comparable to colonoscopy. Recently, molecularly based colorectal cancer screening of stool samples has gained great attention.⁹⁻¹⁴ In particular, the results of large-scale studies using the PreGen-Plus (a noninvasive screening test designed to detect DNA alterations found in stool; DNA Direct Inc, San Francisco, CA) compared with fecal occult blood test (FOBT) was recently reported.¹⁵ Although the stool test demonstrated a sensitivity that was four-fold greater than FOBT, the average sensitivity of these tests from nine studies was only approximately 57%.¹⁵ Tests based on detecting colon cancer-specific methylation in fecal DNA gives sensitivities less than 50% when the specificity is 90%.¹⁶ Thus, there

continues to be an important need for the development of a simple, noninvasive, and highly reliable test for the early detection of CRCs.

Certain lysophospholipids have recently been recognized as important cell-signaling molecules.^{17,18} We and others have shown that lysophosphatidic acid (LPA) is an autocrine growth factor that stimulates proliferation, adhesion, migration, invasion, and tumor metastasis of ovarian cancer cells.¹⁹⁻²⁷ We reported that blood LPA levels are elevated in patients with ovarian cancer, including stage I disease.^{27A,27B} Two additional independent studies confirmed our findings,^{28,29} although not all studies have supported these findings.³⁰ To determine whether any lysophospholipids could be used as markers for CRC, we report here analysis of plasma LPCs from 133 CRC patients and 125 unaffected controls using liquid chromatography mass spectrometry (LC-MS) and blinded for disease status.^{31,32}

PATIENTS AND METHODS

Participants

Plasma samples from 133 patients with CRC and 125 unaffected controls enrolled between October 2002 and August 2005 were analyzed for LPCs. All participants were older than 18 years, and cases underwent resection at the Cleveland Clinic (Cleveland, OH) and had pathologically verified CRC. Preoperative blood was drawn from patients with CRC. Controls were not pre-screened by colonoscopy and were spouses or friends of patients attending the Center for Digestive Diseases or employee volunteers of the Cleveland Clinic. All participants provided written informed consent, and the institutional review board of the Cleveland Clinic approved the study.

Blood Processing, Lipid Extraction, and Analysis of LPC

Blood samples were collected in EDTA-containing tubes and centrifuged at $1,750 \times g$ for 15 minutes at room temperature. Plasma samples were aliquoted into siliconized eppendorf tubes (Slick Clickseal tubes; United Lab Plastics, St Louis, MO; or SafeSeal microcentrifuge tubes; PGC Scientifics, Frederick, MD) and frozen at -80°C until use. Lipids were extracted as described previously with minor modifications.^{31,32} All extraction procedures were performed in 15-mL glass disposable centrifuge tubes. Plasma (20 μL) was diluted with $1 \times$ phosphate-buffered saline (PBS) to 0.5 mL. After the addition of 10 μL of 12:0 LPC (100 $\mu\text{mol/L}$) as an internal standard and 2 mL of MeOH/chloroform (2:1), samples were vortexed for 2 minutes, and incubated on ice for 15 minutes. Chloroform (1 mL) and H_2O (1 mL) were added to separate the phases and samples were vortexed for 2 minutes before centrifugation ($1,750 \times g$ for 10 minutes at 4°C). The lower phase was transferred to a new glass tube. A second round of extraction was achieved by adding 1 mL chloroform to the remaining upper phase and processed as previously. The lower phase extracts were combined and the solvent evaporated under nitrogen at room temperature. The dried lipids were resuspended in 100 μL of MeOH for MS analyses. Samples (20 μL) were directly delivered into the electrospray ionization (ESI)-MS ion source through an LC system (Waters 2690; Waters Corp, Milford, MA) with an auto sampler. The mobile phase was MeOH/water/AmOH (90:10:0.1, v/v/v). The flow rate was 0.2 mL/min, and 5 minutes for each sample.

MS analyses were performed using a Quattro Ultima triple quadrupole ESI-MS (Micromass Inc, Beverly, MA) with the Masslynx data acquisition system. The instrument was equipped with a Z-spray ionization source, and was operated in the positive ion ESI mode. The nebulizer gas and desolvation gas were nitrogen and the collision gas was argon. Typical operating parameters were as follows: capillary voltage 3.5 kV; cone voltage, 80 V; hex 1, 35 V; Aperture, 0 V; hex 2, 0.5 V; source temperature, 120°C ; desolvation temperature, 250°C ; cone gas flow, 109 L/h; desolvation gas flow, 715 L/h; resolution, 12; ion energy 1, 1; entrance, 50; collision, 22; exit, 90; ion energy 2, 3; and multiplier, 600 V. Parent scanning and MS/MS analyses were performed to detect and confirm the structures of all LPCs in blood samples. Multiple

reaction monitoring (MRM) mode was used for quantification. Monitoring ions were at m/z 440 (the parent ion) -184 (the product ion) for 12:0-LPC (as an internal standard), 496-184 for 16:0-LPC, 520-184 for 18:2-LPC, 522-184 for 18:1-LPC, 524-184 for 18:0-LPC, 544-184 for 20:4-LPC, 568-184 for 22:6 LPC. The dwell time in the MRM mode was 100 ms.

Quantitative analysis was performed as described previously.³¹ We established standard curves for each LPC form by mixing different concentrations (5 to 300 $\mu\text{mol/L}$) of a particular form of LPC with the same concentration (100 $\mu\text{mol/L}$) of an internal standard 12:0-LPC (All standard LPC forms were purchased from Avanti Polar Lipids [Birmingham, AL]), and then performing ESI-MS analyses. The peak intensity ratios (an LPC form/internal standard) versus the concentration ratios (an LPC form/internal standard) were plotted and fitted to a linear regression. Data processing was highly automated by using the MS software.

Statistical Methods

The data analysis was conducted in three stages. Participants were first stratified by cancer status and randomly assigned in a 2:1 ratio to a training set (TS; $n = 172$; case = 89, control = 83) or validation set (VS; $n = 86$; case = 44, control = 42). The TS was analyzed as described below. All analyses were then repeated in the VS. Absolute and relative levels (%) of individual LPC forms within the total plasma LPC and within the saturated and unsaturated fractions were evaluated. In addition, the pairwise ratios of LPC forms were also evaluated. Nonparametric methods and logistic regression models were used to analyze the data. Fisher's exact test and the χ^2 test were used to assess the associations between cancer status and categorical demographic characteristics such as gender and race. Spearman rank correlations were used to assess the associations between individual lysophospholipids and age. The Wilcoxon rank sum test was used to compare lysophospholipid levels between groups in univariate analyses. Multivariate analyses to assess multiple lysophospholipids simultaneously were conducted using logistic regression models with stepwise variable selection, where all factors assessed were initially considered as potential candidates. Terms for factors such as age, sex, and race that might be associated with lysophospholipid levels and/or cancer status were also included as covariates in these models. Changes in the Akaike information criteria (AIC) served as the basis for model selection, and the Hosmer-Lemeshow test was used to assess goodness of fit. Once a final model was defined, a cutoff point for determining cancer status was chosen empirically from the associated receiver operating characteristic (ROC) curve using an arbitrarily predefined specificity of 95%.

All tests of statistical significance were two-sided and there were no adjustments for multiple comparisons. P values less than .05 were considered to be statistically significant. All data analyses were performed using SAS (version 8, SAS Institute, Cary, NC) and StatXact 6.0 (Cytel Software Corp, Cambridge, MA).

RESULTS

Patient Demographics

Table 1 summarizes the demographic data (age, race, and sex) for cases and controls both overall and separately for the TS and VS, respectively. Overall, 56% of participants were male, 83% were white, and the median age was 57 years. Although patients were consecutively recruited, males were over-represented in the CRC group (Fisher's exact test $P = .001$) as shown in Table 1. Further, CRC patients tended to be older than controls (Wilcoxon rank sum $P < .001$) and were predominantly white (χ^2 test $P < .001$), reflecting the patient population served at this institution. Although the CRC and control groups differed with respect to these factors, there were no statistically significant differences between CRC patients or controls in the TS and VS. To take into account the differences in demographics between cases and controls, analyses were also performed on a subset of 60 CRC cases and 60 controls matched for age and sex (and 95% with

Table 1. Descriptive Characteristics of Study Participants

Analysis Set	No.	Age (years)		Race (%)				Sex (%)	
		Mean	SD	White	African American	Asian	Other	Male	Female
CRC									
Training	89	62.0	14.1	93	3	1	2	64	36
Validation	44	62.9	10.5	91	7	0	2	70	30
Overall	133	62.3	13.0	92	5	1	2	66	34
Controls									
Training	83	46.3	15.4	76	8	11	5	45	55
Validation	42	45.4	16.6	64	5	19	12	48	52
Overall	125	46.0	15.8	72	7	14	7	46	54
Total	258	54.4	16.5	83	6	7	4	56	44
Substudy population									
Matched CRC	60	56.3	12.7	92	5	2	2	55	45
Matched controls	60	55.8	12.5	88	5	5	2	58	42

Abbreviations: SD, standard deviation; CRC, colorectal cancer.

matched race/ethnicity; Table 1). Table 2 summarizes CRC tumor characteristics. Overall, 92 (69%) of 133 of CRC cases had rectal tumors. The majority (56%) of tumors were T3 (72 of 128) and most (68%) were N0 (87 of 128). Tumor stage was not identified for five patients. As with demographics, there were no statistically significant differences with respect to disease characteristics between subjects in the TS and VS.

Phospholipid Levels in Patients and Controls

Preliminary analyses of negatively-charged lysophospholipids (such as lysophosphatidic acid and lysophosphatidylinositol) and positively-charged lysophospholipids, as well as PC and sphingomyelin were performed in a subset of 12 CRC cases and 12 controls. Different LPC forms showed most promise as markers for CRC based on this pilot screening set and were analyzed further in the full population.

In the full study sample set, LPC 16:0 was the predominant form (ie, the form with the highest levels) in all but one participant (a control). On average, it comprised 47% of total plasma LPC in CRC patients, and 45% in controls. LPC 20:4 was the second most abundant form in CRC patients (50%), compared with only 10% of controls, whereas LPC 18:2 was the second most abundant form in controls (88%), compared with 21% of CRC patients. There were no statistically significant differences in these LPC forms with respect to tumor location, tumor stage, nodal status, or differentiation.

Plasma LPC Levels Significantly Reduced in the CRC Cases

The CRC group showed significantly lower plasma levels of total LPC, saturated LPC, unsaturated LPC, and LPC forms 16:0-, 18:0-, 18:1-, 18:2-, total 18-LPC, and 20:0-LPC, compared with controls in both the TS and VS (Table 3). CRC patients, however, tended to have higher ratios of saturated to unsaturated LPC than did the controls. Similar results were seen with respect to relative amounts of the different LPC forms and ratios of LPC forms (data not shown). As with demographics, there were no statistically significant differences in these parameters between CRC patients or between controls in either the TS or VS.

Considering each marker individually, the percentage of 18:2-LPC in the total plasma LPC and the proportion of unsaturated LPC comprising 18:2-LPC were the best single discriminators of cancer status in the TS. Using a cutoff of 12.1% with a fixed specificity of 95% (ie, classify as cancer if 18:2-LPC was < 12.1% of the total plasma LPC), the sensitivity was 64%. Similarly, classifying a subject as having CRC if 18:2-LPC comprised less than 27.5% of the total unsaturated LPC yielded a sensitivity of 54%. Using multiple logistic regression models, 18:1-LPC and total saturated LPC were seen to have discriminatory power that was independent of 18:2-LPC. In particular, by considering total saturated LPC and the proportion of unsaturated LPC comprising 18:2-LPC minus the proportional amount of 18:1-LPC, sensitivity was increased to 87% in the TS while still maintaining a high specificity (95%). On the basis of these results, a final model was derived from the TS as follows: Consider a subject to have CRC if $[(\%18:2\text{-LPC} - \%18:1\text{-LPC}) \text{ in unsaturated LPC} + 0.053 \times \text{total saturated LPC}]$ is less than 16.8.

Table 2. Clinical Characteristics of Colorectal Cancer Cases

Factor	Training Set		Validation Set		Overall	
	No.	%	No.	%	No.	%
Primary site						
Colon	22	25	16	36	38	29
Rectum	65	73	27	61	92	69
Other*	2	2	1	2	3	2
T-stage						
T1, T2	37	42	16	36	53	40
T3, T4	49	55	26	59	75	56
Unknown	3	3	2	5	5	4
N stage						
N0	59	66	28	64	87	65
N1, N2, N3	27	30	14	32	41	35
Unknown	3	3	2	5	5	4
Differentiation						
Well, moderate	66	74	32	72	98	74
Poor	13	15	8	18	21	16
Unknown	10	11	4	9	14	11

*Rectosigmoid (n = 1), colon and rectum (n = 1), and rectum and cecum (n = 1).

Table 3. Comparison of Plasma LPC Levels Between CRC Cases and Controls

LPC Forms	μM									
	Training Set					Validation Set				
	CRC (n = 89)		Controls (n = 83)		Wilcoxon Rank Sum P	CRC (n = 44)		Controls (n = 42)		Wilcoxon Rank Sum P
	Mean	SD	Mean	SD		Mean	SD	Mean	SD	
16:0-LPC	108.0	34.9	129.8	35.0	< .001	111.4	32.8	142.2	41.1	< .001
18:0-LPC	23.9	7.7	31.5	9.7	< .001	25.1	6.6	33.8	10.3	< .001
18:1-LPC	21.0	8.1	28.2	9.0	< .001	22.0	7.3	31.9	11.5	< .001
18:2-LPC	25.7	10.1	50.5	16.9	< .001	27.2	9.5	58.4	20.3	< .001
Total 18:-LPC	70.7	24.1	110.2	31.3	< .001	74.2	21.5	124.0	36.7	< .001
20:0-LPC	0.6	0.4	0.9	0.6	.004	0.6	0.3	0.9	0.6	.04
20:4-LPC	29.5	12.3	29.7	13.4	1.00	26.4	8.1	29.4	13.5	.50
22:6-LPC	19.4	8.2	21.5	11.2	.41	20.5	9.4	21.7	13.0	.99
Saturated LPC	132.5	42.2	162.2	43.8	< .001	137.1	38.6	176.9	50.7	< .001
Unsaturated LPC	95.7	30.0	129.8	36.8	< .001	96.1	25.9	141.5	46.1	< .001
Saturated:unsaturated LPC ratio	1.45	0.43	1.29	0.29	.04	1.46	0.36	1.31	0.32	.05
Total LPC	228.2	64.8	292.0	73.7	< .001	233.2	59.2	318.4	88.7	< .001

Abbreviations: LPC, lysophosphatidylcholine; CRC, colorectal cancer; SD, standard deviation.

Applying this final model to the VS yielded sensitivity and specificity of 82% and 93%, respectively. The discriminatory ability of this model in the VS is shown in Figure 1. Overall, 113 (85%) of 133 CRC cases were classified correctly, including eight (89%) of nine T1 stage and 35 (80%) of 44 T2 stage CRC. Of the 20 cancers that were

misclassified, 16 had rectal cancer (17% of all rectal cancers studied) and four had colon cancer (11% of all colon cancers studied). Four patients were T3N+, five were T3N0, one was T2N+, eight were T2N0, one was T1N0, and stage was unknown for one patient. Seven controls (6%), all of whom were 55 years of age or older, were misclassified.

Further adjustment for age, race, and sex in the multivariate logistic regression model did not materially alter the results. Moreover, when analysis was restricted to a matched (by age and sex) set of 60 cases and 60 controls, very similar results were obtained with the model correctly classifying 85% of CRC cases (51 of 60) and 90% of controls (54 of 60).

DISCUSSION

We report here that plasma levels of different LPC forms, including 18:1 and 18:2, are significantly reduced in CRC patients compared with unaffected controls, suggesting that these lipids may represent potential biomarkers for CRC. When different plasma LPC forms were incorporated into a multivariate analysis, CRC could be distinguished from unaffected controls in the VS with 93% specificity and 82% sensitivity. Of particular importance, our final model, which was based on the total plasma levels of saturated LPC and the difference between the relative amount of 18:2- and 18:1-LPC in the unsaturated LPC fraction, correctly classified eight (89%) of nine T1 stage CRC cases, implying that these lipids may be sensitive markers to detect early-stage CRC.

Mortality resulting from CRC is largely preventable if the disease is detected early, and colonoscopy is currently the gold standard for CRC screening. Currently, there is no satisfactory and convenient minimally-invasive screening method that is highly specific, sensitive, and reliable for the detection of early CRC.³³ Thus, the identification and validation of such biomarkers remain critical for early CRC intervention. Our previous studies have shown that lysophosphatidic acids may be promising diagnostic markers for certain cancers,^{27A,27B} and

Fig 1. Multiple lysophosphatidylcholine (LPC) forms as markers for colorectal cancer (CRC) in the validation set (VS). Plasma levels of (18:2-LPC - 18:1-LPC) as percentages of unsaturated LPC from CRC cases (C) and unaffected controls (H) are plotted against total saturated LPC levels in each sample. The equation (18:2-LPC-18:1-LPC)% in the unsaturated LPC fraction + 0.053 × total saturated LPC = 16.8 is also plotted (the slanted line in the figure). This VS comprises 86 participants.

results presented here suggest that LPC plasma levels may represent a useful biomarker for the early detection of CRC.

Altered ratios of LPC forms have been reported previously in ovarian cancer³⁶ and myeloma.³⁷ LPC and sphingophosphocholine (SPC) are substrates of lysophospholipase D (lyso-PLD) or autotaxin (ATX).³⁸⁻⁴¹ Lyso-PLD/ATX converts LPC and SPC to lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P), respectively, both of which have been implicated in cancer development.^{17,18,21,22,42} Furthermore, lyso-PLD/ATX has been shown to lead to increased cancer cell growth and motility through the production of LPA.⁴³ Overexpression of lyso-PLD/ATX has been observed in several cancers, including glioblastoma, thyroid carcinomas, and renal cell carcinoma.⁴³⁻⁵⁰ In CRC, lyso-PLD/ATX was found to be overexpressed in 16 of 48 CRC cases compared with uninvolved colon epithelium (unpublished data), suggesting a mechanism for reduced plasma LPC levels at least in some CRC cases. Taken together, these observations suggest that the reduction in plasma LPC seen in CRC patients may reflect pathophysiological changes in CRC development. However, further studies will be needed to confirm the relationship between ATX and plasma LPC levels.

Our assays rely on quantitative measurements of individual LPC forms, which differ from each other by only 2 to 72 Da in their molecular weights. Unlike protein/peptide factors, it is much harder to develop reliable and specific antibodies against lysophospholipids. LC-MS is the best and perhaps the only way to accurately determine the levels of each individual LPC form simultaneously in minimal amounts of blood. Although MS-based methods are generally thought to be inconvenient and impractical for clinical usage due to the relatively high cost, this concept is rapidly changing as the remarkable progress in MS-based methodology in recent years has made it more affordable and suitable for clinical samples.^{51,52}

There are several limitations to our study. First, control subjects in our study were not screened by colonoscopy and may include patients with undiagnosed CRC. The number of undiagnosed CRC in the control group, if any, however, would be extremely low, and therefore would not alter our results significantly. It is also possible that there might be patients with polyps included as controls. However, even if there were a small number of misclassified polyp patients in the control group, it would serve only to bias away from our model. Secondly, we do not have information with regard to family history of colorectal cancer, and thus were unable to control for its potential effect in our analysis. Finally, we only included patients with pathologically confirmed colorectal cancer. It would be interesting to conduct

similar analysis in a large number of patients with adenomatous polyps, which would have important implication for the early detection and prevention of CRC. To this end, further studies of cases with adenomatous polyps and controls with negative screening colonoscopy and information on family history are warranted.

In summary, our results suggest that reduced plasma LPC levels may represent useful biomarkers for CRC. We believe that our test has a better specificity and sensitivity than any currently available minimally invasive test for CRC. It is particularly noteworthy that our test also correctly detected eight of nine early stage (T1) CRC cases. To this end, further validation of the clinical significance of LPC levels as a first-line screening method for CRC and the detection of adenomas is warranted.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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Other: Zhenwen Zhao [conducted lipid analysis, manuscript preparation], Yijin Xiao [established original lipid MS analyses method, performed analysis for samples with old method], Paul Elson [performed all statistical analysis, manuscript preparation], Haiyan Tan [sample collection, organization, data management], Sarah J. Plummer [sample collection, organization, data management], Michael Berk [recruiting subjects, sample collection, organization, data management], Phyu P. Aung [conducted ATX-related research], Ian C. Lavery [oversaw clinical aspects of projects, provided clinical samples], Jean P. Achkar [oversaw clinical aspects of projects, provided clinical samples], Graham Casey [overall experiment design, organization, manuscript preparation], Yan Xu [initiated project, obtained funding]

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