Plasma Lysophosphatidylcholine Levels: Potential Biomarkers for Colorectal Cancer

Zhenwen Zhao, Yijin Xiao, Paul Elson, Haiyan Tan, Sarah J. Plummer, Michael Berk, Phyu P. Aung, Ian C. Lavery, Jean P. Achkar, Li Li, Graham Casey, and Yan Xu

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. 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%.

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. A number of biomarkers have been identified for the detection and/or prognosis of colon cancer in colon tissues, feces, and serum. 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. 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. 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%. Tests based on detecting colon cancer-specific methylation in fecal DNA gives sensitivities less than 50% when the specificity is 90%. 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

**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 (χ² 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

**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 χ² 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).
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 (i.e., 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% (i.e., classify as cancer if 18:2-LPC was \(\leq 12.1\%\) 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

\[
\left(\frac{\%\text{18:2-LPC} - \%\text{18:1-LPC}}{\%\text{unsaturated LPC}}\right) < 16.8.
\]

### Table 1. Descriptive Characteristics of Study Participants

<table>
<thead>
<tr>
<th>Analysis Set</th>
<th>Age (years)</th>
<th>Race (%)</th>
<th>Sex (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>White</td>
</tr>
<tr>
<td><strong>CRC</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Training</td>
<td>89</td>
<td>62.0</td>
<td>14.1</td>
</tr>
<tr>
<td>Validation</td>
<td>44</td>
<td>62.9</td>
<td>10.5</td>
</tr>
<tr>
<td>Overall</td>
<td>133</td>
<td>62.3</td>
<td>13.0</td>
</tr>
<tr>
<td><strong>Controls</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Training</td>
<td>83</td>
<td>46.3</td>
<td>15.4</td>
</tr>
<tr>
<td>Validation</td>
<td>42</td>
<td>45.4</td>
<td>16.6</td>
</tr>
<tr>
<td>Overall</td>
<td>125</td>
<td>46.0</td>
<td>15.8</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>258</td>
<td>54.4</td>
<td>16.5</td>
</tr>
<tr>
<td><strong>Substudy population</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Matched CRC</td>
<td>60</td>
<td>56.3</td>
<td>12.7</td>
</tr>
<tr>
<td>Matched controls</td>
<td>60</td>
<td>55.8</td>
<td>12.5</td>
</tr>
</tbody>
</table>

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

### Table 2. Clinical Characteristics of Colorectal Cancer Cases

<table>
<thead>
<tr>
<th>Factor</th>
<th>Training Set</th>
<th>Validation Set</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td><strong>Primary site</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td>22</td>
<td>25</td>
<td>16</td>
</tr>
<tr>
<td>Rectum</td>
<td>65</td>
<td>73</td>
<td>27</td>
</tr>
<tr>
<td>Other*</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><strong>T-stage</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1, T2</td>
<td>37</td>
<td>42</td>
<td>16</td>
</tr>
<tr>
<td>T3, T4</td>
<td>49</td>
<td>55</td>
<td>26</td>
</tr>
<tr>
<td>Unknown</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td><strong>N stage</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>59</td>
<td>66</td>
<td>28</td>
</tr>
<tr>
<td>N1, N2, N3</td>
<td>27</td>
<td>30</td>
<td>14</td>
</tr>
<tr>
<td>Unknown</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td><strong>Differentiation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well, moderate</td>
<td>66</td>
<td>74</td>
<td>32</td>
</tr>
<tr>
<td>Poor</td>
<td>13</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>Unknown</td>
<td>10</td>
<td>11</td>
<td>4</td>
</tr>
</tbody>
</table>

*Rectosigmoid (n = 1), colon and rectum (n = 1), and rectum and cecum (n = 1).
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/H11001, five were T3N0, one was T2N/H11001, 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

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

<table>
<thead>
<tr>
<th>LPC Forms</th>
<th>Training Set</th>
<th>Validation Set</th>
<th>Wilcoxon Rank Sum P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:0-LPC</td>
<td>108.0 ± 34.9</td>
<td>111.4 ± 32.8</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>18:0-LPC</td>
<td>23.9 ± 7.7</td>
<td>25.1 ± 6.6</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>18:1-LPC</td>
<td>21.0 ± 8.1</td>
<td>22.0 ± 7.3</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>18:2-LPC</td>
<td>25.7 ± 10.1</td>
<td>27.2 ± 9.5</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>Total 18-LPC</td>
<td>70.7 ± 24.1</td>
<td>74.2 ± 21.5</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>20:0-LPC</td>
<td>0.6 ± 0.4</td>
<td>0.6 ± 0.3</td>
<td>.004</td>
</tr>
<tr>
<td>20:4-LPC</td>
<td>29.5 ± 12.3</td>
<td>26.4 ± 8.1</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>22:6-LPC</td>
<td>19.4 ± 8.2</td>
<td>20.5 ± 9.4</td>
<td>.99</td>
</tr>
<tr>
<td>Saturated LPC</td>
<td>132.5 ± 42.2</td>
<td>137.1 ± 38.6</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>Unsaturated LPC</td>
<td>95.7 ± 30.0</td>
<td>96.1 ± 25.9</td>
<td>.461</td>
</tr>
<tr>
<td>Saturated:unsaturated LPC ratio</td>
<td>1.45 ± 0.43</td>
<td>1.46 ± 0.36</td>
<td>.05</td>
</tr>
<tr>
<td>Total LPC</td>
<td>228.2 ± 64.8</td>
<td>233.2 ± 59.2</td>
<td>&lt; .001</td>
</tr>
</tbody>
</table>

Abbreviations: LPC, lysophosphatidylcholine; CRC, colorectal cancer; SD, standard deviation.
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 cancer. LPC and sphingophosphocholine (SPC) are substrates of lyosphospholipase D (lyso-PLD) or autotaxin (ATX). Overexpression of lyso-PLD/ATX has been shown to lead to increased cancer cell growth and motility through the production of LPA. Overexpression of lyso-PLD/ATX has been observed in several cancers, including glioblastoma, thyroid carcinomas, and renal cell carcinoma. 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 lyosphospholipids. 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.

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.

**AUTHOR CONTRIBUTIONS**

Conception and design: Zhenwen Zhao, Yijin Xiao, Graham Casey, Yan Xu

Financial support: Graham Casey, Yan Xu

Administrative support: Yan Xu

 provision of study materials or patients: Michael Berk, Ian C. Lavery, Jean P. Achkar, Yan Xu

Collection and assembly of data: Haiyan Tan, Yan Xu

Data analysis and interpretation: Zhenwen Zhao, Yijin Xiao, Paul Elson, Phyu P. Aung, Li Li, Yan Xu

Manuscript writing: Zhenwen Zhao, Yijin Xiao, Paul Elson, Haiyan Tan, Michael Berk, Phyu P. Aung, Ian C. Lavery, Jean P. Achkar, Graham Casey, Yan Xu

Final approval of manuscript: Zhenwen Zhao, Yijin Xiao, Paul Elson, Haiyan Tan, Michael Berk, Phyu P. Aung, Ian C. Lavery, Jean P. Achkar, Graham Casey, Yan Xu

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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34. Reference deleted

35. Reference deleted


