






High level of phosphatidylcholines/lysophosphatidylcholine ratio in urine is associated with prostate cancer

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Abstract

The altered levels of phospholipids (PLs) and lysophospholipids (LPLs) in prostate cancer (CaP) and benign tissues in our previous findings prompted us to explore PLs and LPLs as potential biomarkers for CaP. Urinary lipidomics has attracted increasing attention in clinical diagnostics and prognostics for CaP. In this study, 31 prostate tissues obtained from radical prostatectomy were assessed using high-resolution matrix-assisted laser desorption/ionization imaging mass spectrometry (HR-MALDI-IMS). Urine samples were collected after digital rectal examination (DRE), and urinary lipids were extracted using the acidified Bligh-Dyer method. The discovery set comprised 75 patients with CaP and 44 with benign prostatic hyperplasia (BPH) at Kyoto University Hospital; the validation set comprised 74 patients with CaP and 59 with BPH at Osaka University Hospital. Urinary lipidomic screening was performed using MALDI time-of-flight MS (MALDI-TOF/MS). The levels of urinary lysophosphatidylcholine (LPC) and phosphatidylcholines (PCs) were compared between the CaP and BPH groups. The (PC [34:2] + PC [34:1])/LPC (16:0) ratio was significantly higher ($P < .001$) in CaP tissues than in benign epithelial tissues. The urinary PCs/LPC ratio was significantly higher ($P < .001$) in the CaP group than in the BPH group in the discovery and validation sets.

KEYWORDS

lysophosphatidylcholine, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, phosphatidylcholine, prostate cancer, urine

1 | INTRODUCTION

Lipid metabolism plays an essential role in cell carcinogenesis by affecting the proliferation, migration, differentiation, and motility of cells.¹⁻³ Among lipid categories, phospholipids (PLs) and

lysophospholipids (LPLs) are the most extensively studied.⁴ The expression patterns of several PLs and LPLs reportedly differ in prostate cancer (CaP) cells⁵ and tissues.⁶ Recently, reports have emerged on trained canines being able to detect CaP by sniffing urine samples from these patients.⁷ The odor of the urine perceived by the dogs

Li and Nakayama contributed equally to this study.

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is produced by urinary volatile organic compounds (VOCs), and the differences in VOCs may reflect changes in lipids in the body and their metabolites.^{8,9}

Among the PLs, phosphatidylcholine (PC), which is a class of PLs that incorporate choline as a headgroup, is with highest level in animal membranes as compared with other PLs.¹⁰ A previous study analyzed the distribution of PLs in hepatocellular carcinoma (HCC) tissues using matrix-assisted laser desorption/ionization (MALDI) imaging mass spectrometry (IMS). The results showed that the levels of some PCs, including PC (16:0/16:1), PC (16:0/18:1), and PC (18:0/18:1) were significantly increased in cancerous tissues compared with normal tissues.¹¹ Patel et al¹² analyzed the concentration of serum lipids in patients with CaP and healthy individuals; the results showed that elevated levels of PC (38:5), PC (40:3), and PC (42:4) can be used as serum markers to detect CaP.

Lysophosphatidylcholine (LPC), which is partially hydrolyzed from PC (ie, removal of one fatty acid chain), is the most abundant LPL in blood and a key lipid in various pathophysiological conditions such as inflammation, endothelial activation, and atherogenesis.¹³ In our previous study, we used the high-resolution MALDI-IMS (HR-MALDI-IMS) technique to analyze the distribution of PLs and LPLs in the prostate glands of patients who underwent radical prostatectomy ($n = 31$); the results showed that LPC (16:0) was significantly decreased in cancer tissues than in normal tissues.⁶ The elevated PC/LPC ratio has been introduced as an independent pathophysiological factor in various disorders such as rheumatoid arthritis¹⁴ and Parkinson's disease.¹⁵ Researchers have also reported an increased PC (16:0/16:1)/LPC (16:0) ratio in HCC tissue compared with normal tissue, which was caused by an overexpressed enzyme, lysophosphatidylcholine acyltransferase 1 (LPCAT1) that catalyzes the conversion of LPC to PC.¹¹ The PC/LPC ratio is also useful in MS analyses, as it is easier to calculate than the lipid levels separately.^{16,17}

The MALDI time-of-flight MS (MALDI-TOF/MS) is increasingly used in lipid research because it is rapid, sensitive, tolerates sample impurities well, and provides simple mass spectra without significant analyte fragmentation.¹⁸ More importantly, combined with the imaging system, MALDI-IMS can provide "in-situ imaging" of the molecules in the tissues and offer spatial distribution for a wide variety of molecules within a single tissue section.^{19,20} Among all the studies conducted on the PC/LPC ratio, almost all analytical techniques were based on MALDI.

In this study, we tested the PCs/LPC ratio in urine samples voided after digital rectal examination (DRE), which were revealed to contain biomolecules secreted from prostatic microenvironments that enable the detection of CaP-specific biomarkers.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Ultrapure water, proteomics-grade hydrochloric acid (HCl), high-performance liquid chromatography-grade chloroform (CHCl_3),

methanol (MeOH), acetonitrile (ACN), and isopropanol (IPA) were purchased from Fujifilm Wako Pure Chemical Corp. 9-Aminoacridine (9-AA) was purchased from Sigma-Aldrich. Lipid standards, including LPC (17:1), LPC (16:0), PC (16:0/18:1), and PC (24:0/24:0), were purchased from Avanti Polar Lipids, Inc.

2.2 | Ethics statement

This study was approved by the Ethics Committees of the Kyoto University Graduate School of Medicine and Osaka University Graduate School of Medicine (No. G0052 and No.13397-17, respectively). Informed consent was obtained from all patients for the examinations and experiments. Clinical materials were used after obtaining written informed consent.

2.3 | Study design and patient selection

The study scheme is presented in Figure 1. Herein, we further analyzed the IMS data from our previous study in the prostate glands of 31 patients with CaP who underwent radical prostatectomy at Kyoto University Hospital (KUHP).⁶ From these data, we selected two major PC peaks detected in the prostate tissues by IMS, PC (34:2) and PC (34:1),²¹ along with one major LPC peak, LPC (16:0), which was reported as a decreased biomarker for CaP,⁶ to build a diagnostic index based on the values of the PCs/LPC ratio. This PCs/LPC ratio was compared between cancerous and normal tissues. The details of patient information and IMS analyses have been described in our previous publication.⁶ Representative HR-MALDI-IMS images were obtained by importing the IMS data (in Image format) into the software *MSiReader* Ver. 1.02.^{22,23} After confirming significant differences in the PCs/LPC ratio values between the cancerous and normal tissues in the prostate gland, we designed and conducted a retrospective study using urine samples other than the tissue cases that were already obtained. This was done according to the standards for reporting of diagnostic accuracy guidelines²⁴ and aimed to explore the potential of the urinary PCs/LPC ratio for use as a non-invasive biomarker to distinguish between benign prostatic hyperplasia (BPH) and CaP.

A total of 281 participants with serum prostate-specific antigen (PSA) levels less than 20 ng/mL were enrolled in this study.²⁵ Among them, 137 participants (90 CaP cases and 47 BPH cases) were recruited from the KUHP as the discovery set. Another 144 participants (85 CaP cases and 59 BPH cases) from the Osaka University Hospital (OUHP) served as the validation set. Eighteen cases in the discovery set and 11 cases in the validation set were excluded because of treatment history, diagnosis of castration resistant prostate cancer or other cancer types (shown in Figure 1). Clinical features including age, serum PSA level, pathological or clinical tumor stage, and Gleason scores of the included patients in the discovery and validation sets are shown in Tables 1 and 2, respectively.

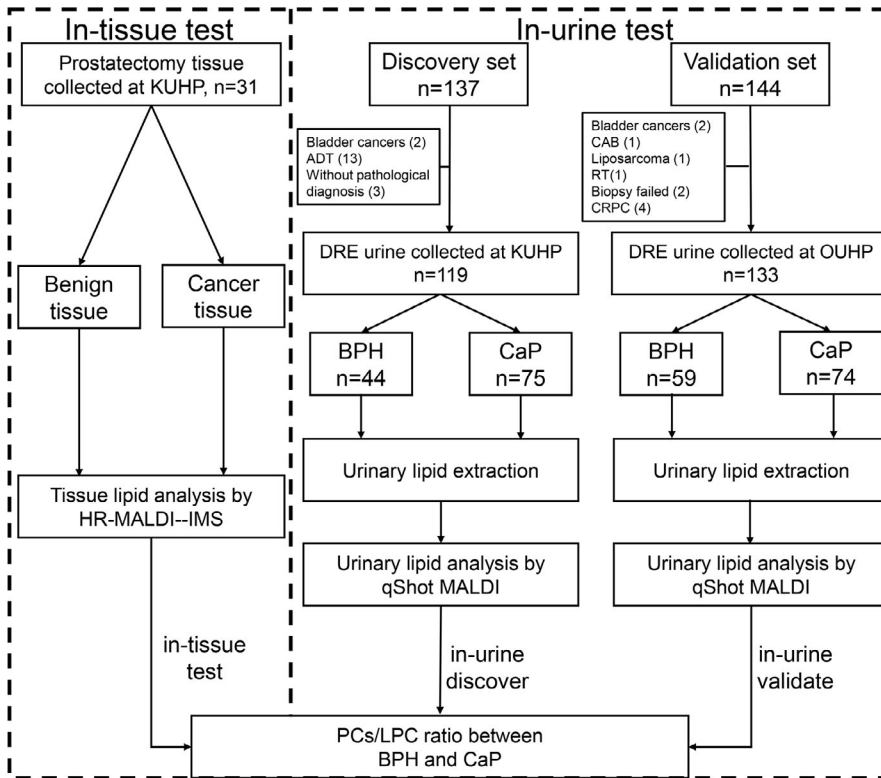


FIGURE 1 Study scheme of the discovery and validation sets. BPH, benign prostatic hyperplasia; CaP, prostate cancer; HR-MALDI-IMS, high-resolution matrix-assisted laser desorption/ionization mass spectrometry; KUHP, Kyoto University Hospital; LPC, lysophosphatidylcholine; OUHP, Osaka University Hospital; PC, phosphatidylcholine; PSA, prostate-specific antigen

TABLE 1 Clinical and pathological characteristics of the discovery set

Category	Subcategory	Total	BPH	CaP
Number of patients		119	44	75
Median age (y)		70	70.5	69
Mean \pm SD preoperative serum PSA (ng/mL)		7.09 \pm 3.74	7.30 \pm 5.02	6.96 \pm 2.77
Pathological GS	3 + 3	--	--	12
	3 + 4	--	--	30
	4 + 3	--	--	17
	4 + 4	--	--	10
	3 + 5	--	--	2
	4 + 5	--	--	4
Pathological stages	pT2a	--	--	13
	pT2b	--	--	1
	pT2c	--	--	47
	pT3a	--	--	14

Abbreviations: BPH, benign prostatic hyperplasia; CaP, prostate cancer; GS, Gleason scores; PSA, prostate-specific antigen; SD, standard deviation.

A confirmatory diagnosis of CaP was made by histological diagnosis of the prostate glands removed by surgery (in the discovery set at KUHP) or of the prostate specimens obtained by needle biopsy (in the validation set at OUHP). The diagnoses of noncancer subjects (ie, BPH) were established by histological tests of prostate tissues removed by holmium laser enucleation of the prostate, transurethral resection of the prostate, or obtained by needle biopsy.

2.4 | Urine sample collection methods

All urine samples were collected after DRE. DRE urine samples were processed using two different protocols at KUHP and OUHP (details of the protocols are described in Appendix S1). Before proceeding with our studies, we compared the effects of both protocols on urinary PC and LPC concentrations, as well as the PCs/LPC values, which were similar for the two protocols (Figure S1A-D).

TABLE 2 Clinical and pathological characteristics of the validation set

Category	Subcategory	Total	BPH	CaP
Number of patients		133	59	74
Median age (y)		69	69	70
Mean \pm SD prebiopsy serum PSA (ng/mL)		8.51 \pm 3.95	7.54 \pm 2.91	9.29 \pm 4.49
Biopsy GS	3 + 3	--	--	12
	3 + 4	--	--	27
	4 + 3	--	--	17
	4 + 4	--	--	15
	3 + 5	--	--	1
	4 + 5	--	--	1
	5 + 4	--	--	1
Clinical stages	cT1c	--	--	19
	cT2a	--	--	31
	cT2b	--	--	1
	cT2c	--	--	14
	cT3a	--	--	9

Abbreviations: BPH, benign prostatic hyperplasia; CaP, prostate cancer; GS, Gleason scores; PSA, prostate-specific antigen; SD, standard deviation.

2.5 | Extraction of urinary lipids

Digital rectal examination urinary lipids were extracted using the acidified Bligh & Dyer method,²⁶ which was previously described as suitable for extraction of urinary PLs.²⁷ Briefly, 50 μ L of a lipid internal standard (iSTD) mixture containing 20 pmol of LPC (17:1) and 200 pmol of PC (24:0/24:0) was spiked into 1 mL of thawed urine and mixed well in a glass tube. Consequently, 3.75 mL of ice-cold CHCl_3 :MeOH (1:2, v/v) was added and vortexed for 10 minutes. Ice-cold CHCl_3 (1.25 mL) was added and mixed for another 5 minutes. Water (1.25 mL) was then added, followed by mixing for 5 minutes. The mixture was centrifuged for 10 minutes at 2000 \times g, and the lower (organic) phase was transferred to a clean glass tube. Ice-cold CHCl_3 (1.88 mL) and 30 μ L of 6 mol/L HCl were added to the aqueous residue to extract the remaining lipids. The organic phases were combined, washed with 2 mL water, and dried under a N_2 stream. The dried powder was resuspended in 200 μ L of CHCl_3 :MeOH:H₂O (60:30:4.5, v/v/v) and used for MALDI-TOF/MS analysis.

2.6 | Identification of the urinary LPC and PC targets

MALDI-TOF/MS² analyses were performed to identify the lipid targets in the urinary lipid extract. Using 9-AA as the matrix, urinary lipid extract was analyzed in the m/z range of 1-1000 in the positive reflectron mode by AXIMA Performance MALDI-TOF/MS (Shimadzu Kratos Analytical). Peaks with m/z values of 496.3, 758.6, and 760.6, which may have been LPC (16:0), PC (34:2), and PC (34:1), respectively, were selected as the precursors and fragmented by the MS² analyses in collision-induced dissociation (CID)

mode. Fragmented peaks of the precursors were used for identification by the SimLipid[®] version 6.0.5 software package (PREMIER Biosoft International).

2.7 | Testing the reproducible and quantitative abilities of qShot MALDI analysis

In our previous report, we have defined a quick, qualitative, and quantitative analysis of urinary LPLs and PLs using MALDI-TOF/MS, as the "qShot MALDI" analysis.⁴ Briefly, 10 mmol/L of 9-AA dissolved in a mixture of IPA and ACN (60:40, v:v) was used as the matrix; and a specially designed target plate, the μ Focus MALDI plate (Hudson Surface Technology, Inc), was used to form a precise and concentrated distribution of the sample. Meanwhile, iSTDs containing LPC (17:1) and PC (24:0/24:0) were spiked into the sample before extraction to improve the reproducibility between different wells and on different days.

The reproducible and quantitative abilities of the qShot MALDI analysis for detecting urinary PLs and LPLs have been confirmed in our previous studies,^{27,28} and we further tested them on the LPC and PC targets. Briefly, a series of gradient-diluted synthesized LPC (16:0) and PC (34:1) was spiked into 1 mL of urine, followed by the addition of iSTDs and extraction of lipids as described above. The lipid extracts were analyzed using the qShot MALDI analysis on three different days. The standard curves for each target were constructed by plotting the concentrations against the normalized peak intensities (the schematic diagram of the normalization procedure is presented in Figure S2). The coefficient of determination (R^2) of each curve provided an estimate of the linearity and reproducibility of quantitative analysis by the qShot MALDI analysis.

2.8 | The qShot MALDI analyses of the urinary lipids in the discovery and validation sets

For each urine sample in the discovery or validation set, a duplicate lipid extraction was performed, followed by a triplicate MS analysis. The parameters of the batch analysis were set as follows: in the m/z range of 460-1000 in the positive reflectron mode; the power of the laser was fixed at 90, just above the ionization threshold of the lipid species; 100 laser shots per profile and 199 profiles were obtained for each well.

2.9 | Data processing and quality control of the MS analyses

The PCs/LPC ratio in each profile was calculated using homemade software (Section "PCs/LPC Ratio Value Calculation by Homemade Software" in Appendix S1). Analytical quality was determined by calculating the coefficient of variation (CV) of the PCs/LPC ratio values among six different profiles (Microsoft Excel software). The CV values among the six analyses were less than 0.2 (Section "Data Quality Control" in Appendix S1).

2.10 | Statistics

All statistical analyses in the present study were performed using GraphPad Prism 8 (GraphPad Software Inc) or JMP[®] Pro 13.2.0 (SAS Institute) software, licensed to Kyoto University. The Wilcoxon matched-pair signed-rank test was used to perform statistical analysis of the PCs/LPC ratios in the prostate tissues. The Mann-Whitney U test was employed to identify nonparametric, significant differences in the urinary PCs/LPC ratios and serum PSA values between the CaP and BPH groups. Statistical significance was set at $P < .05$.

3 | RESULTS

3.1 | IMS revealed a higher (PC [34:2] + PC [34:1])/LPC (16:0) ratio in cancer tissues than in normal tissues

The HR-MALDI-IMS was performed on the boundary area of the tissues that contained both cancerous and normal prostate tissue obtained after radical prostatectomy. Representative visualizations of the distributions of LPC (16:0), PC (34:2), and PC (34:1) ions by HR-MALDI-IMS analysis are shown in Figure 2A. The level of LPC (16:0) was lower in cancerous tissues than in benign tissues, whereas PC (34:2) and PC (34:1) were relatively evenly distributed (Figure 2A). These three lipid species combined to build a pathological condition (eg, inflammation, cancer)-related index: the PCs/LPC ratio value. We normalized the spectra acquired from 31 samples using the total ion current and calculated the intensity ratio values of

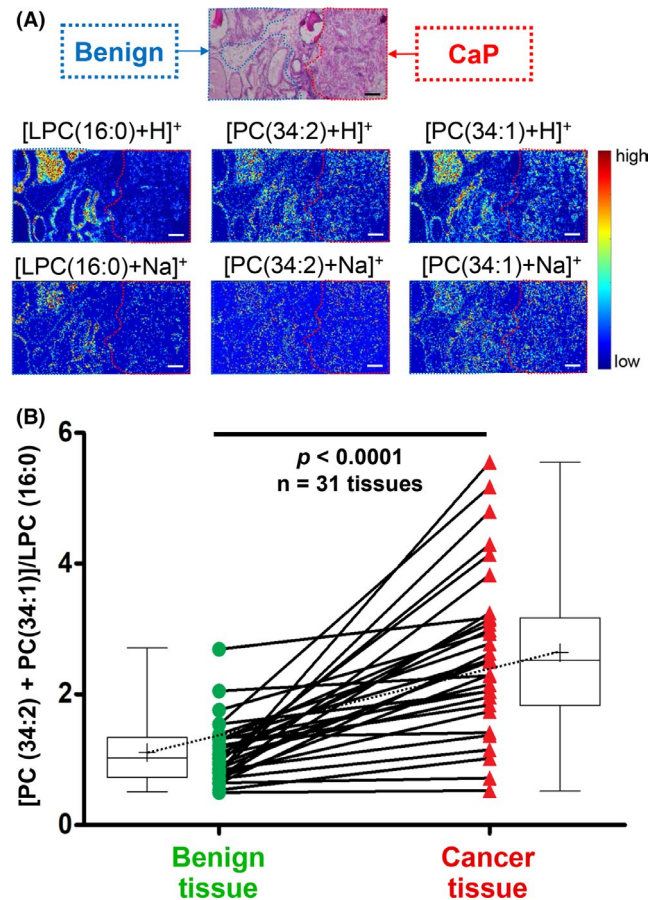


FIGURE 2 Direct in-tissue analysis of the lysophosphatidylcholine (LPC) (16:0), phosphatidylcholine (PC) (34:1), and PC (34:2) lipid species. 9-Aminoacridine (9-AA) matrix-coated tissue was assessed in the positive ionization mode by high-resolution matrix-assisted laser desorption/ionization imaging mass spectrometry (HR-MALDI-IMS) in the mass range of m/z 490-1000. A, Representative H&E staining and mass spectrometry images clearly show the difference in the distribution of LPC (16:0), PC (34:1), and PC (34:2) between the benign and prostate cancer (CaP) tissues. The scale bar on all images is 200 μm . B, The PCs/LPC ratios were compared between the paired benign and cancer tissues in 31 samples, and statistical analyses were conducted using the Wilcoxon signed-rank test

(PC [34:2] + PC [34:1])/LPC (16:0). The PCs/LPC ratios in the cancer tissues were significantly higher than those in the normal tissues. ($P < .0001$, Figure 2B).

3.2 | Identification of urinary LPC (16:0), PC (34:2), and PC (34:1)

Based on the tissue findings, we examined whether urinary LPC and PC species could be detected in the urine. For identification of LPC (16:0), PC (34:2), and PC (34:1), precursor peaks at m/z values of 496.3, 758.6, and 760.6 were analyzed by MALDI-TOF/MS.² The mass spectra of MALDI-TOF/MS² analyses of these precursors are shown in Figure 3A-C, respectively. The SimLipid[®] MS/MS search

engine of the MS² spectra indicated that the peaks at 496.3, 758.6, and 760.6 were (LPC [16:0] + H)⁺ (Figure 3A), (PC [34:2] + H)⁺ (Figure 3B), and (PC [34:1] + H)⁺ (Figure 3C), respectively. This result suggested that the urinary LPC and PC species could be sensitively detected by MALDI analysis. The representative chemical structures of PC (34:2), PC (34:1), and LPC (16:0) are shown in Figure 3D.

3.3 | Reproducibility and quantitative abilities of the qShot MALDI analysis

After identification of the urinary LPC and PC species, we evaluated the quantitative ability of the qShot MALDI analysis for these species. Because the chemical structure and properties of PC (34:2) and PC (34:1) are similar, we only tested PC (34:1), which was representative of PCs in the quantitative analysis. The standard curve was assessed by plotting the peak area ratio of individual targets to their iSTD concentrations on three consecutive days. The standard curves for spiked LPC (16:0) and PC (34:1) are shown in Figure 4, respectively. Using LPC (17:1) as the iSTD for intensity normalization, a strong correlation was observed between the normalized intensities of LPC (16:0) and the spiked concentrations, with a correlation coefficient (R^2) of .984 (Figure 4A). Similarly, using PC (48:0) as the iSTD, a strong correlation was also observed between the normalized

intensities and the concentrations of spiked PC (34:1), with the R^2 of .990 (Figure 4B). The results also showed good reproducibility on three different days. The results also proved the high accuracy of the qShot MALDI analysis.

3.4 | The urinary (PC [34:2] + PC [34:1])/LPC (16:0) ratio values were higher in patients with CaP than in patients with BPH in the discovery set

After confirmation of the qualification and quantification of the urinary LPC and PC species by qShot MALDI analysis, the urinary PCs/LPC ratio was evaluated in 119 participants (75 CaP cases and 44 BPH cases) who were recruited in the discovery set (Table 1). The representative qShot MALDI spectra of urinary lipids in the discovery set are shown in Figure 5A,B; the peaks corresponding to the LPC iSTD and the LPC target, along with the PC iSTD, and PC targets are indicated in the figures. The PCs/LPC ratios were significantly higher in the CaP group (median = 2.30) than in the BPH group (median = 1.52) in the discovery set (Figure 6A, $P < .0001$). Serum PSA levels of all subjects were evaluated. As shown in Figure 6B, no significant differences were observed between the CaP and BPH groups.

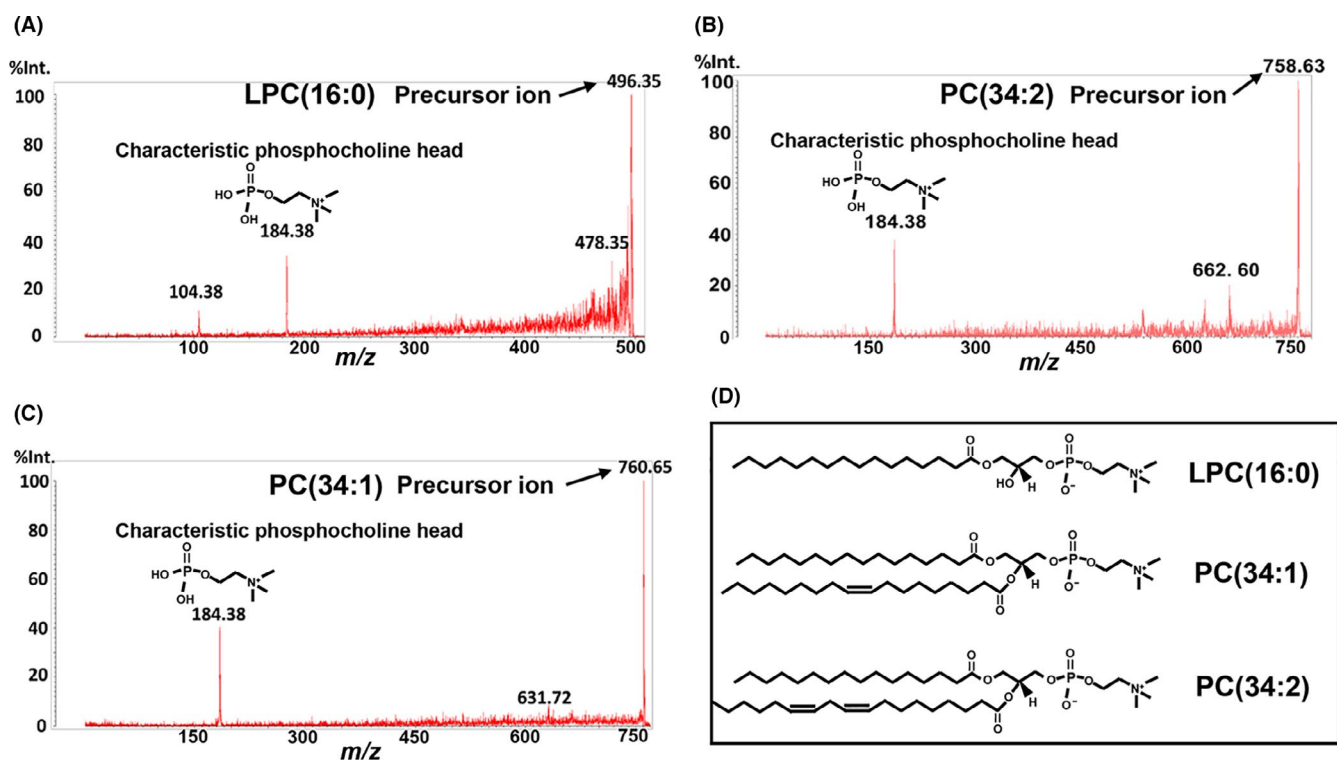


FIGURE 3 Identification of the urinary lysophosphatidylcholine (LPC) and phosphatidylcholine (PC) target species. A, B, C, The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS²) analyses of the precursors (m/z 496.3, 758.6, and 760.6) were performed in collision-induced dissociation (CID) mode. The peaks with an m/z value of 184.4 represent the characteristic phosphocholine head of LPC and PC. The identifications were conducted by the SimLipid[®] software. D, The representative chemical structures of LPC (16:0), PC (34:1), and PC (34:2) lipid species

FIGURE 4 Quantification of the urinary lysophosphatidylcholine (LPC) and phosphatidylcholine (PC) species by the qShot matrix-assisted laser desorption/ionization (MALDI) analysis. A, B, Assessment of the reproducibility and quantitative reliability of our lipid extraction and analysis system for detecting LPC and PC species in the urine. Results show that good linearity and reproducibility were achieved

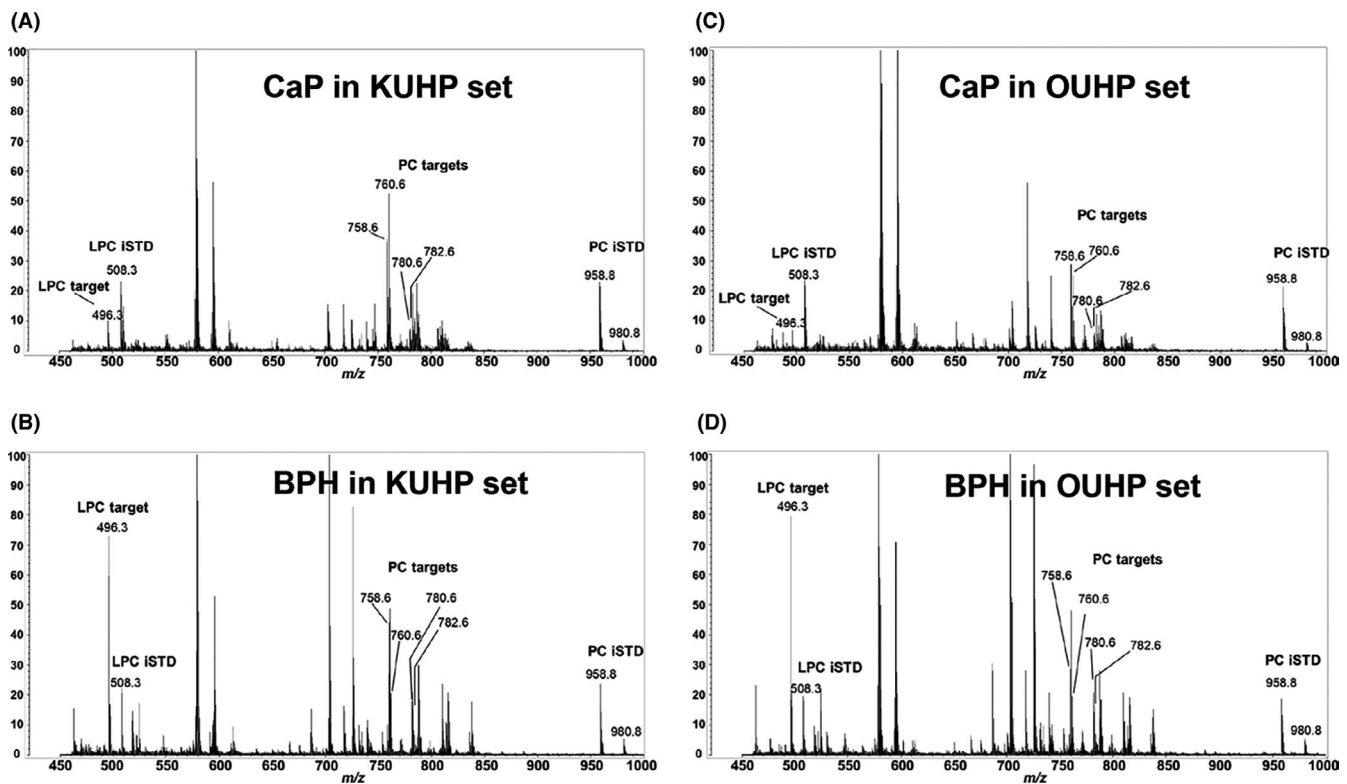
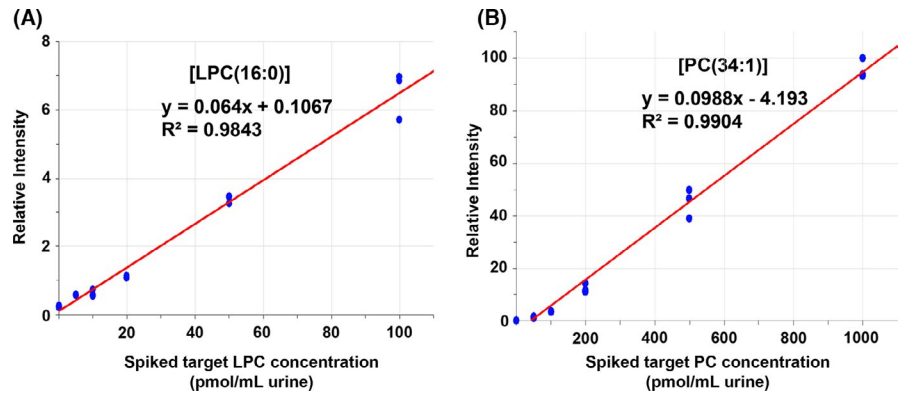


FIGURE 5 Representative qShot matrix-assisted laser desorption/ionization (MALDI) spectra of the lipids in the digital rectal examination (DRE) urine sample. Urinary lipids were extracted using the acidified Bligh & Dyer method. The qShot MALDI spectra were acquired in positive ionization mode in the m/z range of 460-1000, using 9-aminoacridine (9-AA) as the matrix. A, B, Representative mass spectrometry (MS) spectra of prostate cancer (CaP) and benign prostatic hyperplasia (BPH) patients in the Kyoto University Hospital (KUHP)/discovery set, respectively C, D, Representative MS spectra of the lipids in the DRE urine samples of CaP and BPH patients from the Osaka University Hospital (OUHP)/discovery set, respectively

3.5 | The urinary (PC [34:2] + PC [34:1])/LPC (16:0) ratio values were higher in patients with CaP than in patients with BPH in the validation set

External validation of the findings from the discovery set was performed using DRE urine samples collected at another clinical center (OUHP). A total of 133 participants (74 CaP cases and 59 BPH cases) were included in the study. The representative qShot

MALDI spectra of urinary lipids in the validation set are shown in Figure 5C,D. Comparisons of the urinary PCs/LPC ratio and serum PSA levels were performed between the CaP and BPH groups. Significantly higher PCs/LPC ratio values were observed in the CaP group (median = 2.52) than in the BPH group (median = 1.45), with a P -value of .0001 (Figure 6 C). No significant differences were found in the serum PSA levels in the CaP and BPH groups in the validation set (Figure 6D).

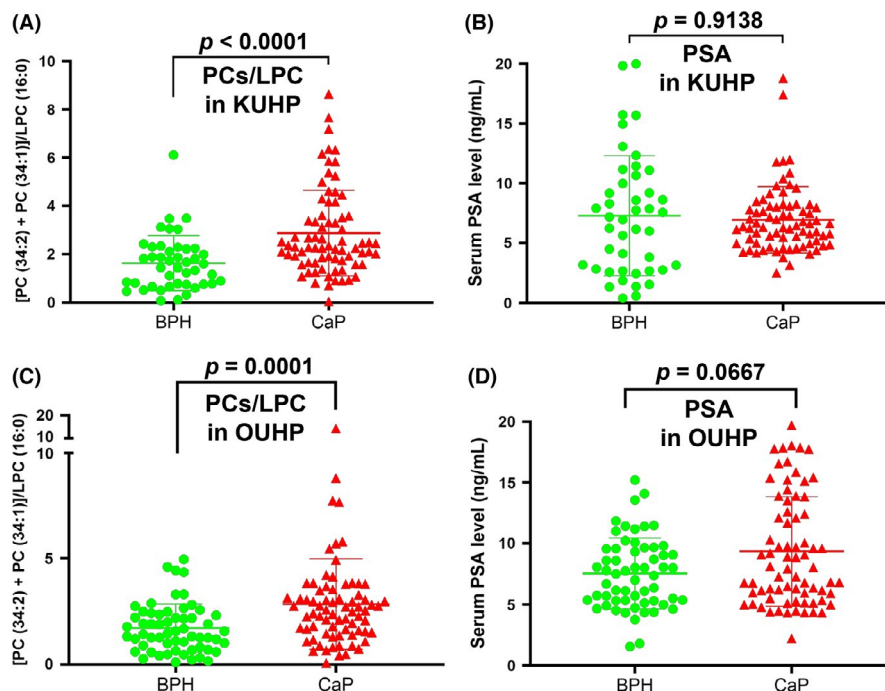


FIGURE 6 Scatter plots of the urinary phosphatidylcholines/lysophosphatidylcholine (PCs/LPC) ratios and serum PSA levels in the Kyoto University Hospital (KUHP)/discovery and Osaka University Hospital (OUHP)/validation sets. The PCs/LPC ratios in the digital rectal examination (DRE) urine samples and serum PSA levels were compared between the benign prostatic hyperplasia (BPH) and prostate cancer (CaP) groups in the KUHP/discovery and OUHP/validation sets. Results show that the PCs/LPC ratios were significantly higher in the CaP group than in the BPH group in both KUHP/discovery (A) and OUHP/validation sets (C). However, PSA was not significantly different between BPH and CaP in either the KUHP/discovery (B) or OUHP/validation set (D). Statistical analyses were conducted using the nonparametric Mann-Whitney *U*-test

3.6 | The association between urinary (PC [34:2] + PC [34:1])/LPC (16:0) ratio values and clinical features of CaP

In this study, we also evaluated the relationship between the urinary PCs/LPC ratio and the clinical features of CaP (ie, pathological or clinical stages, Gleason scores, and serum PSA levels). However, no significant association was observed between the urinary PCs/LPC ratio and clinical features in terms of CaP stage, Gleason score, and serum PSA level (Figure S3, S4, and S5).

4 | DISCUSSION

The rapid and reliable qShot MALDI analysis of the urinary PCs/LPC ratio conducted in this study can be completed within a few minutes, and the batch analysis of samples can be easily achieved. We first evaluated urinary PC (34:2)/LPC (16:0) or PC (34:1)/LPC (16:0) ratios; however, the differences between these ratios were not significant; therefore, we focused on the (PC [34:2] + PC [34:1])/LPC (16:0) ratio (data not shown). We found that the (PC [34:2] + PC [34:1])/LPC (16:0) ratio values detected in urine samples were significantly higher in CaP patients than in the noncancerous controls. The PCs/LPC ratio values between the discovery and validation sets showed similar tendencies (ie, the median

values in the CaP groups were 2.30 and 2.52 in the discovery and validation sets, respectively, and the median values in the BPH groups were 1.52 and 1.45 in the discovery and validation sets respectively). Meanwhile, decreased PCs/LPC ratio values were observed in urine samples collected after prostatectomy ($n = 6$, shown in Figure S7). Therefore, the PCs/LPC ratio is a reliable urinary marker for CaP.

There are two possible origins of urinary PCs and LPCs: one possibility is that the PCs and LPCs are filtrated from the bloodstream; the other possibility is that they are exfoliated from the urinary tract. The human urine metabolome is thought to be a subset of the human serum metabolome.^{29,30} Thus, altered PCs and LPCs in the serum of patients with CaP may cause changes in those of the urine. Previous studies have used plasma or serum as sources of CaP lipid biomarkers. For example, using serum obtained from 57 patients with CaP and 76 healthy individuals, the levels of two PC species, ie, PC (40:3) and PC (42:4), were found to be significantly increased in the CaP group.¹² However, contradictory findings have been reported in different studies. For example, using plasma as the sample, the levels of three sphingomyelin (SM, with a chemical structure similar to PC) species, SM (34:1), SM (36:2), and SM (36:1), were found to be increased in the CaP group³¹; however, the levels of the same three SM species were found to be decreased in the CaP group in another study.³² These results indicate that analysis of plasma or serum for CaP biomarker screening is more complicated than that of urine; any

systemic or local disorders at other sites may influence the lipid levels in the serum or plasma.

Biomaterials (eg, cells, fluids, and exosomes) exfoliated from the urinary tract may also contribute to the altered levels of urinary PCs and LPCs in patients with CaP. As the prostate gland is anatomically connected to the lower part of the urinary tract, various types of cells and fluids can be shed from the gland into the urinary tract, including tumor cells and prostate fluids, which can be washed out during urination. A recent study collected exfoliated CaP cells from the urine, which offers a specific and sensitive detection of CaP.³³ These prostate cells in the urine may offer a cellular origin for lipids such as PCs and LPCs. Similar to the cells, fluids containing lipids derived from the prostate gland can also be secreted into the urine, especially after DRE. As shown in Figure S6, the concentration of urinary LPC (16:0) was significantly increased after DRE; however, this did not happen for urinary PC (34:2) and PC (34:1). The reason for this may be that the levels of urinary PC (34:2) and PC (34:1) in our samples were higher than that of LPC (16:0), and the effect of DRE on the concentration of urinary PC (32:2) and PC (34:1) was not as significant as that on LPC (16:0). Urinary exosomes, first identified in 2004,³⁴ were thought to originate from the cells of organs involved in reproduction and urine excretion. A previous study performed a lipidomic analysis of urinary exosomes from 15 patients with CaP and 13 healthy individuals. The results showed that some exosomal lipid species were significantly different between the two groups. Among them, the levels of urinary PC (34:1) and PC (34:2) were higher in the CaP group than in the control group.²¹

As summarized in a review,³⁵ LPC (16:0) was reported as the third-most downregulated biomarker in cancer research. A total of six types of cancers in breast, colon or the rectum, liver, kidney, prostate, and thyroid showed significantly decreased levels of LPC (16:0) compared with the noncancerous control samples. In the case of PCs, PC (34:1) was reported as the most frequently upregulated biomarker in cancer research. It was reported as increased in breast cancer, colorectal cancer, gastric cancer, lung cancer, CaP cases, and thyroid cancer cases. Meanwhile, PC (34:2), which has a similar bio-function but a higher degree of unsaturation in the fatty acid chains (ie, one more carbon double-bond) than PC (34:1), was reported as increased biomarker in cases of esophageal cancer, gastric cancer, lung cancer, CaP, and thyroid cancer.

In this study, the increased (PC [34:2] + PC [34:1])/LPC (16:0) ratios in the tissue and DRE urine samples of CaP patients are in agreement with the dysregulation of PL and LPL metabolism observed in cancer cells. There are two possible reasons for the increased PCs/LPC ratio in cancers such as CaP. First, it is partly caused by a loss of LPC (16:0), which is triggered by the elevated demand for lysophosphatidic acid (LPA) in cancer cells.³⁶ In cells, the conversion of LPC (16:0) to LPA occurs via activation of lysophospholipase D (lysoPLD), which hydrolyzes LPLs to produce LPA.³⁷ Its expression has been reported to be much higher in CaP tissue than in benign epithelium and significantly correlated with the potential for PSA recurrence after surgery.³⁸ Second, the

change in conversion between LPCs and PCs may also be affected by a remodeling pathway (Lands' pathway) in cancer cells.³⁹ In the Lands' pathway, LPC is produced by the hydrolysis of PC by an enzyme called phospholipase A2 (PLA2), while PC can be produced by the addition of a fatty acid to LPC using LPCATs.⁴⁰ Although some previous study indicated that PLA2 activity is increased in CaP cells,⁴¹ PLA2 was also reported to be highly active in BPH, and no significant difference of PLA2 levels in serum was observed between patients with BPH or CaP.^{42,43} Considering that the conversion of PC from LPC is catalyzed by LPCATs, some previous studies may offer a possible explanation for the elevated PCs/LPC ratio in cancers. In HCC tissues and cells, the PC (16:0/16:1)/LPC (16:0) ratio has been reported to be significantly higher because of the elevated activation of LPCAT1, which is a key enzyme in the PC and LPC remodeling pathway.¹¹ Moreover, the increased expression of LPCAT1 correlated with the progression of CaP,⁴⁴⁻⁴⁶ suggesting that increased expression of LPCAT1 may also increase the PC/LPC ratio in CaP tissue. Further mechanistic studies are required to determine whether changes in PCs/LPC ratio expression and the activities of lysoPLD, PLA2, and LPCAT are correlated.

In summary, the changed levels of PCs and LPCs were observed in the tissues and body fluids of cancer patients, including in patients with CaP. The major advantages of liquid biopsy using urine over serum and tissue biopsy are that it is a truly noninvasive method with no limitations in the sample volume, a relatively low background complexity. As shown in our research, a potential biomarker found in the tissue can be detected and validated in the urine by qShot MALDI analysis, which is a rapid and sensitive tool for lipid detection. Unfortunately, the (PC [34:2] + PC [34:1])/LPC (16:0) ratio might not be directly applicable as a clinical diagnostic tool for CaP in the present form. However, our trials have shed light on a possible route to biomarker exploration and validation in a rapid, noninvasive, and easy way.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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