Exploring predictive molecules of acute adverse events in response to volumetric-modulated arc therapy for prostate cancer using urinary metabolites

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Abstract. Volumetric-modulated arc therapy (VMAT) is a radiotherapy technique used to treat patients with localized prostate cancer, which is frequently associated with acute adverse events (AEs) that can affect subsequent treatment. Notably, the radiation dose of VMAT can be tailored to each patient. In the present study, a retrospective analysis was performed to predict acute AEs in response to a therapeutic high radiation dose rate based on urinary metabolomic molecules, which are easily collected as noninvasive biosamples. Urine samples from 11 patients with prostate cancer who were treated with VMAT (76 Gy/38 fractions) were collected. The study found that seven patients (~64%) exhibited genitourinary toxicity (Grade 1) and four patients had no AEs. A total of 630 urinary metabolites were then analyzed using a mass spectrometer (QTRAP6500+; AB SCIEX), and 234 relevant molecules for biological and clinical applications were extracted from the absolute quantified metabolite values using the MetaboINDICATOR tool. In the Grade 1 acute AE group, there was a significant negative correlation (rs=-0.297, $P<0.05$) between the number of VMAT fractions and total phospholipase A2 activity in the urine. Additionally, patients with Grade 1 AEs exhibited a decrease in PC aa C40:1, a phospholipid. These findings suggested that specific lipids found in urinary metabolites may serve as predictive biomarkers for acute AEs in response to external radiotherapy.

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Introduction

In Japan, the number of patients who have received radiotherapy for cancer has increased since 1995. With the rapidly increasing number of patients with prostate cancer in their 50s and older, as well as an aging society, radiotherapy is expected to be one of the treatment methods used. There were 851 radiotherapy facilities in Japan (1), with intensity-modulated radiotherapy (IMRT), a type of high-precision radiotherapy, being the most commonly used for prostate cancer cases, with over 320 facilities (1). In 2020, 423 facilities were equipped to provide IMRT (2), making it easier for patients to receive treatment. Conversely, for prostate cancer, adverse events (AE) are a significant issue in external beam radiotherapy. Acute AEs include diarrhea, proctitis, cystitis, fatigue, and mild skin irritation, primarily in the gluteal fold (3). Furthermore, the most recent Japanese radiotherapeutic guideline (JASTRO Guidelines 2020) identified dysentery, dermatitis around the anus, rectal bleeding, and frequent urination as AEs (4). Genitourinary toxicity, such as bladder spasms, cystitis, genitourinary fistula, urinary incontinence, genitourinary leak, genitourinary obstruction, genitourinary perforation, prolapse of stoma, renal failure, stricture/stenosis, urinary electrolyte wasting, urinary frequency/urgency, and urinary retention (5), is particularly associated with a decrease in quality of life after treatment initiation. These diagnoses are primarily made by interview and direct observation of the patient. Therefore, no standard biomarkers are known for a priori prediction of them.

A rough treatment plan is developed once the cancer type and pathology are identified within the current radiotherapy strategy. The patient's condition during treatment is monitored through medical examination, blood and urine sampling, and diagnostic imaging; however, little radiobiological information is considered, making it difficult to predict AEs. Thus, there is no definitive method for confirming the response of target tissue cells or organs at risk in real time (6), and it is proposed to incorporate this biological response into radiotherapy strategies.

A well-established theory in which 4R radiobiological concepts are considered is fractionated radiotherapy. Out of these concepts, only 'repair' is considered in the linear-quadratic model (7,8). In clinical practice, assessing the remaining reoxygenation, redistribution, and repopulation (3Rs) in real time has proven difficult. Recent technological advances in molecular biology, as well as increased statistical analysis speed, have enabled more detailed analysis of cancer cells. Furthermore, new theories have emerged that challenge conventional theories of radiation biology. Particularly, cancer cell metabolism remains largely unknown because of the diversity of their characteristics.

Metabolome analysis, which has gained popularity in recent years, can detect metabolite changes using mass spectrometry with high qualitative and quantitative accuracy (9). In this study, metabolomics was performed on urine samples from patients with localized prostate cancer to identify biomarkers predictive of acute AEs.

The European Association of Urology now recommends dose-escalated IMRT or volumetric-modulated arc therapy (VMAT) as standard therapies for prostate carcinoma because of the lower toxicity compared to 3D-conformal radiotherapy (3D-CRT) (10). VMAT may be the first option (11) and is widely accepted as the gold standard for prostate radiotherapy (12). With the use of modern RT (IMRT, VMAT), greater precision was achieved when compared to conventional RT (13). Various studies have established IMRT as the standard of care for external beam RT for prostate cancer, with a lower rate of acute and late RT-induced toxicities compared to 3D-CRT (14). To optimize planning for VMAT, which has fewer acute and late complications, the AUA/ASTRO guideline 2022 recommended the use of highly conformal radiotherapy such as IMRT, VMAT, and stereotactic body radiotherapy, in conjunction with published target and normal tissue dose objectives (15).

The high radiation dose rate causes DNA damage in cancer cells as a direct or indirect reaction mediated by ROS. The most serious damage is caused by DNA single-strand breaks and double-strand breaks. Several AEs are caused by chronic oxidative stress, which impairs the nuclear function of DNA repair mechanisms (16). However, there are different types of AEs based on symptoms, frequency, and severity.

Identifying and predicting metabolites that respond to acute AEs in external beam radiotherapy would help to maintain radiotherapy safety and quality of life, as well as improve treatment selection (i.e., optimization). This study sought to identify a predictive biomarker from urinary metabolites for AEs during VMAT in localized prostate cancer and to optimize this radiotherapy in preparation for an increase in target patients.

Materials and methods

Study population. The current study included 11 patients with localized prostate cancer who received VMAT at Hirosaki University Hospital between June 2021 and March 2022 were enrolled (Fig. 1). All of the patients were Asians from across eastern Japan. The key characteristics examined included age, T stage, Gleason score, prostate-specific antigen (PSA), National Comprehensive Cancer Network (NCCN) risk classification, and fraction with acute AEs. The acute AEs highlighted in this study were classified using the Common Terminology Criteria for Adverse Events Version 5.0 from the U.S. Department of Health and Human Services (https://ctep.cancer.gov/protocolDevelopment/electronic_applications/ctc.htm#ctc_50).

Patient urination. The participants were patients with localized prostate cancer who underwent VMAT (76 Gy/38 fractions) at Hirosaki University Hospital between June 2021 and March 2022. Urine was self-collected using Uro Catch II (ATLETA Corp., Ltd., Osaka, Japan) early in the morning at rest and via midstream catch (10 ml). Self-urination samples were collected daily from the first to the last day of irradiation (Fig. 1). Urine samples were collected and stored in a freezer (MY BIO VT-208HC, Nihon freezer Corp., Ltd., Tokyo, Japan) at -80˚C, to ensure metabolite stability until mass spectrometry analysis. Acute AEs such as urinary frequency during the irradiation period were documented during the patient's medical interview.

Metabolomics. Urine samples were thawed to room temperature. A total of 630 metabolites from 14 small molecules and 12 different lipid classes were analyzed using the MxP® Quant 500 kit (Biocrates Life Sciences AG, Innsbruck, Austria) according to the manufacturer's instructions. Approximately 10 μl of urine was pipetted on a 96-well plate with internal standards and dried under a nitrogen stream using a positive-pressure manifold (Biotage AB, Uppsala, Sweden). Then, 50 μl of 5% phenyl isothiocyanate solution was added to each well to derivatize amino acids and biogenic amines. After 1 h of incubation at room temperature, the plate was dried again. To extract metabolites, 300 μl of 5-mM ammonium acetate in methanol was pipetted into each filter and incubated for 30 min. The extract was eluted into a new 96-well plate via a positive-pressure manifold. To conduct further LC-MS/MS analyses, the 150-μl extract was diluted with an equal volume of water. For FIA-MS/MS analyses, a 10-μl extract was diluted with 490-μl FIA solvent (Biocrates). LC-MS/MS and FIA-MS/MS measurements were performed following dilution. For chromatographic separation, an ExionLC AD (AB SCIEX, Framingham, Massachusetts, USA) system was connected to a SCIEX QTRAP 6500+ mass spectrometry system in electrospray ionization mode. Data were generated using the Analyst (AB SCIEX) software suite and transferred to the MetIDQ software (using the Recipe Urine QC), where they were further processed and analyzed. All metabolites were identified using isotopically labeled internal standards and multiple reaction monitoring through optimized MS conditions provided by Biocrates. For quantification, a seven-point calibration curve or one-point calibration was used depending on the metabolite class. Urine samples were processed with no prior preparation. Furthermore, in each well (except for the blank), an internal standard (creatinine) was added before urine was pipetted onto the plate. Metabolite concentrations were adjusted for creatinine content. Biologically and clinically relevant 293 metabolic indices were determined using the MetaboINDICATOR tool (Biocrates). Each metabolite was given absolute quantitative values. The collected metabolome data was registered to the integrated metabolome data repository (MetaboBank; MTBKS242 and MTBKS243),

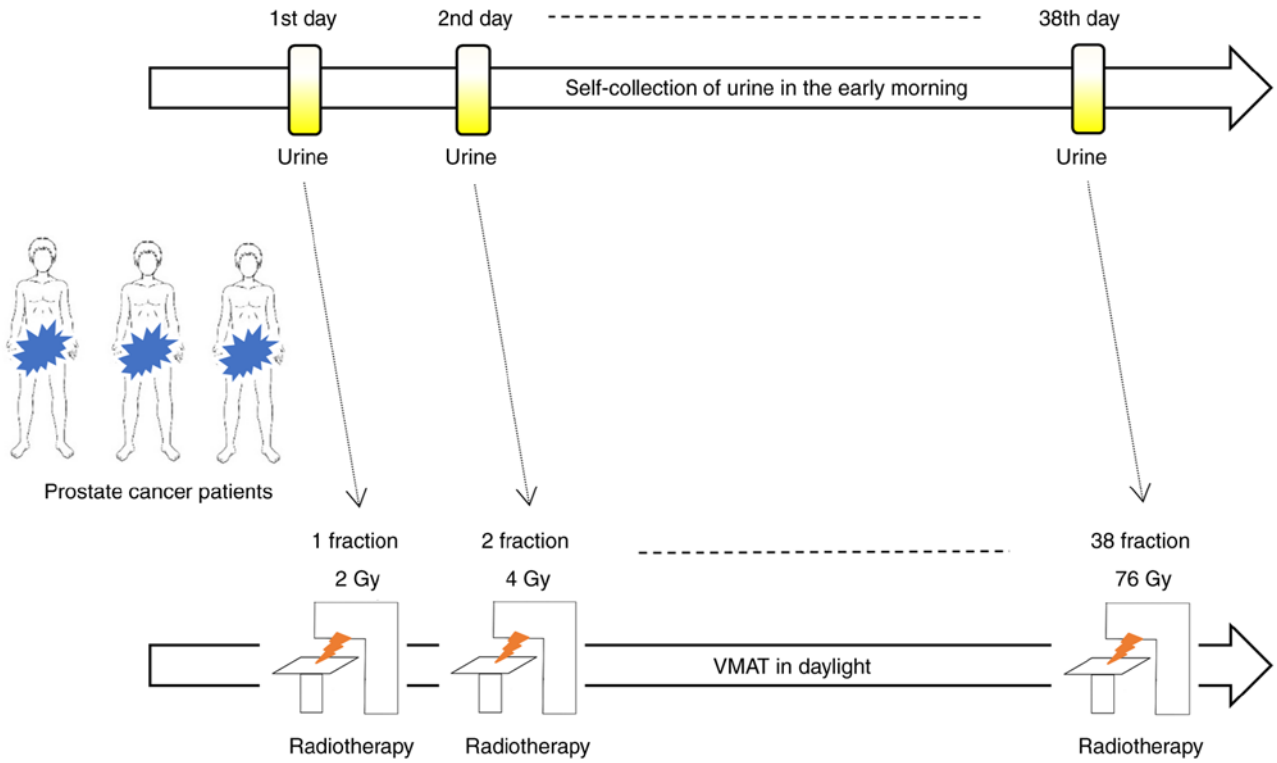

Figure 1. Schematic image of self-collection of urine and fraction of VMAT in patients with prostate cancer. VMAT, volumetric-modulated arc therapy.

Statistical analysis. Using R (Ver. 4.2.0), statistical analysis was conducted, as well as correlation analysis (Spearman's rank correlation) was performed between these metabolic indices and acute AEs or metabolic indices and fraction (physical quantity). Receiver operating characteristic (ROC) curves were produced using MetaboAnalyst (17) on metabolic indices that showed a significant correlation with the number of irradiations. The paired samples Student's t-test was run on fractions ranging from 0 to 29 to look for metabolites associated with various metabolic indicators with and without AEs. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Patient characteristics. We identified 11 patients who had localized prostate cancer and underwent VMAT. Four patients (median age, 69 years) had no confirmed AEs, while the remaining seven patients (71 years) experienced genitourinary toxicity (GU, Grade 1). The clinical characteristics of the study population are shown in Table I. With a median PSA of 7.23 ng/ml, T2b-T3b of 72.7%, and a median total Gleason score of 7 (6-9), NCCN high-risk constituted 54.5% (n=6) of the study population.

Indicator of lipid metabolism. It has been reported that the phospholipase A2 activity (PLA2 activity) in healthy adults is approximately 1.21 (18). This was higher values than the fraction zero (F=0) of AEs (0.48±0.18) and F=0 of non-AEs (0.58±0.38) in the current data. In seven patients with acute AE(+), Spearman's rank correlation revealed a significant correlation between the fraction and the PLA2 activity index (rs=-0.297, $P<0.05$) in Fig. 2 and Table II. There was no significant correlation found with the absence of AEs (Fig. 3 and Table II). Because PLA2 activity is represented in the MetaboINDICATOR as lysoPC a Cxx:x/PC ax Cxx:x, we chose to focus on lysophosphatidylcholine (lysoPC) and phosphatidylcholine (PC) as metabolites linked to radiation-induced AEs.

ROC analysis of metabolic indicators. The ROC curves for the PLA2 activity index in each fraction are shown in Fig. 4 and Table III. The cutoff value of the PLA2 Activity index was 0.178 in 29 fractions, resulting in an area under the curve (AUC) of 0.75. Fraction 0 also had a high AUC of 0.75; however, fractions 9 and 19 did not have an AUC greater than 0.5, indicating a random prediction. Thus, the PLA2 activity index demonstrated high specificity and sensitivity as a biomarker in fractions 0 and 29 for predicting the occurrence of AEs.

Lipid evaluation classification in metabolomics. PLA2 activity was found to be correlated with fractions through correlation analysis. The use of this index as a biomarker necessitates measuring the entire series of lysoPCs and PCs. Thus, we explored surrogate markers for PLA2 activity by performing paired samples Student's t-test for metabolites associated with the PLA2 activity index. Phosphatidylcholine with diacyl residue sum C40:1 (PC aa C40:1) was significantly reduced in the presence of AEs ($P<0.01$). However, there was no significant difference without AEs (Fig. 5). This suggests that a reduction in PC aa C40:1 at 29 fractions is indicative of an AE. In the post-treatment serum data, PSA could be used as a known indicator of tumor activity. PSA levels in the AE(+) population decreased after radiotherapy (Table IV).

Table I. Clinical characteristics of the study population.

Characteristic	Adverse event (-) (n=4)	Adverse event (+) (n=7)
Age, years		
Range	59.0-73.0	61.0-75.0
Median (IQR)	69.0 (65.8-70.8)	71 (69.0-71.5)
T stage		
cT1c	2	1
cT2a	1	0
cT2b	1	2
cT3	0	3
cT3a	0	1
Gleason score		
3+3	2	0
3+4	1	1
4+3	0	2
4+4	1	0
4+5	0	1
5+3	0	1
5+4	0	2
PSA, ng/ml		
Range	4.4-27.2	4.4-26.4
Median (IQR)	7.1 (6.3-12.2)	12.1 (4.8-21.1)
NCCN risk classification		
Low-risk	1	0
Intermediate-risk	2	2
High-risk	1	5
Fraction with adverse event		
Median (IQR)	-	29 (21.5-30.5)
Ethnicity	Asian (Japanese)	Asian (Japanese)

NCCN, national comprehensive cancer network.

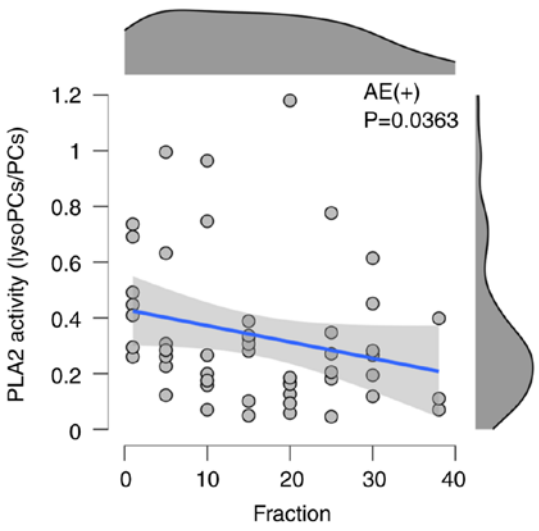

Figure 2. Spearman rank correlation between fraction and PLA2 activity in patients with AEs. AE, adverse event; PLA2, phospholipase A2.

Table II. Spearman rank correlation between fraction and PLA2 activity in with/without AE groups.

AE group	Lower confidence limit	rs-value	Upper confidence limit	P-value
AE(+)	-0.531	-0.297	-0.0202	0.0363
AE(-)	-0.658	-0.291	0.188	0.227

AE, adverse event; PLA2, phospholipase A2. rs, correlation coefficient, the definition of a significant coefficient is P<0.05.

Table III. Receiver operating characteristic analysis between fraction and PLA2 activity.

Fraction	AUC	Cut-off	Specificity	Sensitivity
F0	0.75	0.698	0.8	0.9
F9	0.429	0.377	0.5	0.7
F19	0.482	0.124	0.5	0.7
F29	0.75	0.178	0.8	0.8

AUC, area under the curve; PLA2, phospholipase A2.

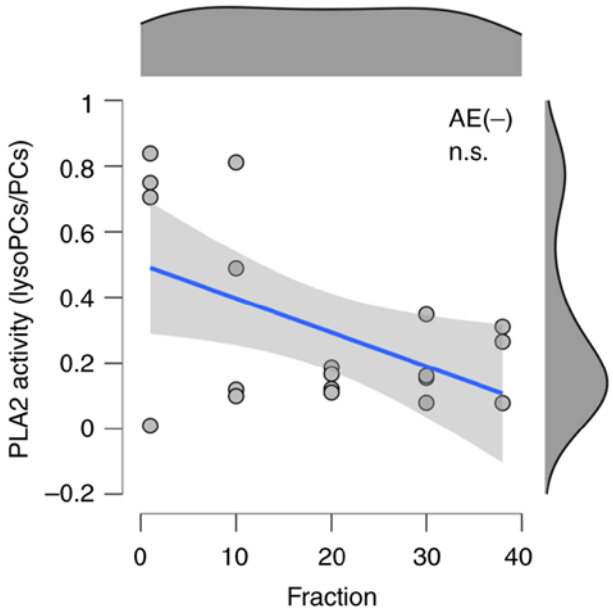

Figure 3. Spearman rank correlation between fraction and PLA2 activity in patients without AEs. AE, adverse event; PLA2, phospholipase A2.

Discussion

This study aimed to explore metabolites that predict acute AEs in patients with localized prostate cancer who underwent VMAT with urine. Patient analysis of acute AE was conducted at every outpatient visit during the VMAT course for approximately 2 months. While numerous urinary toxicity biomarkers have been reported, we discovered a

Table IV. The relationship between adverse events and serum PSA in each patient.

Variable	Pt.1	Pt.2	Pt.3	Pt.4	Pt.5	Pt.6	Pt.7	Percentage
Urinary frequency/urgency	+	+	+	+	+	+	+	100.0
Urethritis	-	+	-	-	-	-	-	14.3
Urodynia	+	+	+	+	+	+	+	100.0
Slow stream	+	+	+	+	+	+	+	100.0
PSA, ng/ml								
Pre-RT	0.04	0.03	0.22	0.01	0.03	0.024	4.4	
Post-RT	<0.01	0.01	0.01	0.01	0.01	<0.01	<0.1	

Pt, patient; RT, radiotherapy.

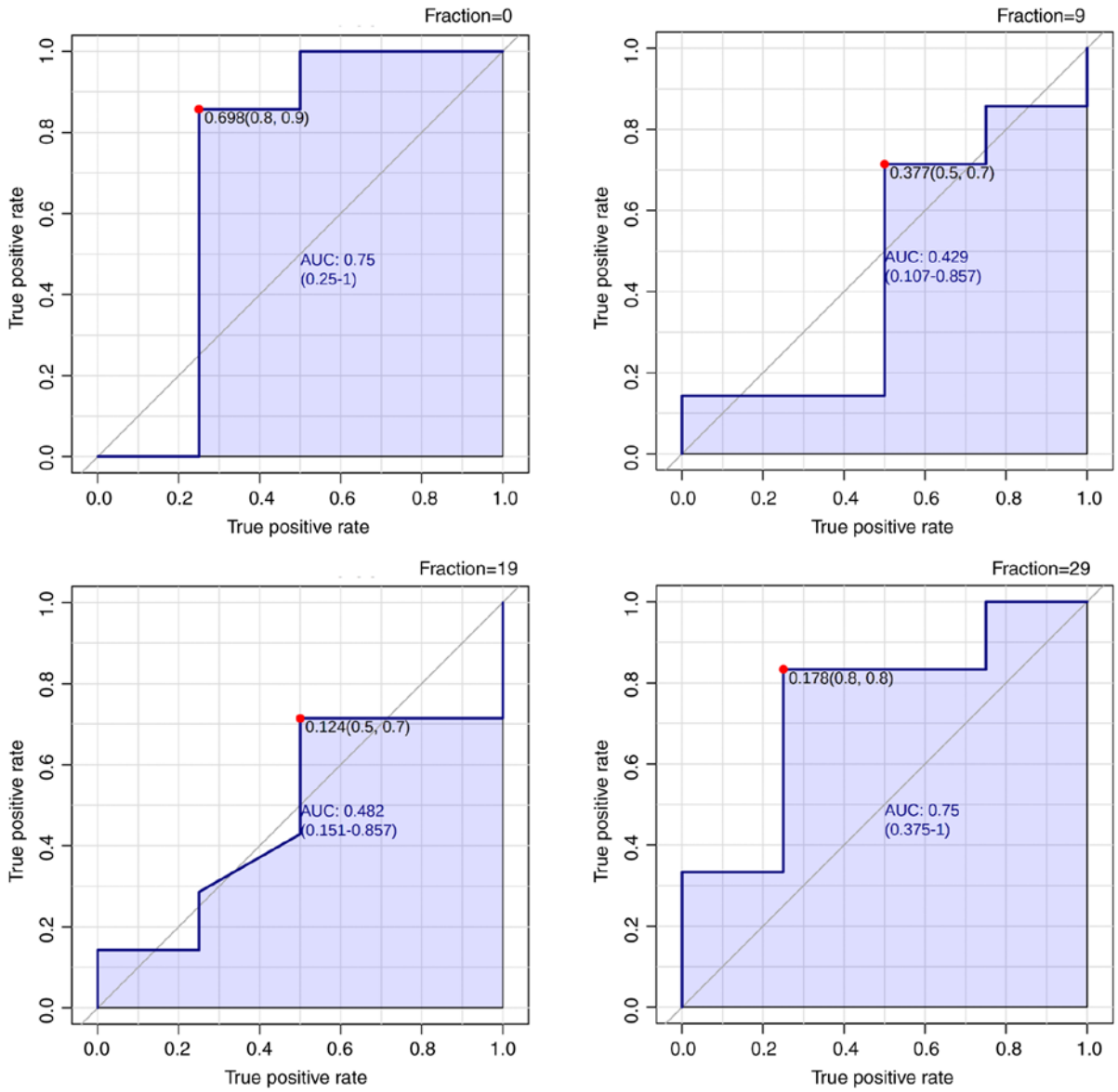

Figure 4. The ROC curves for the index of PLA2 activity in each fraction. The AUC (values in parentheses indicate confidence intervals) and cut-off values (values in parentheses represent specificity and sensitvity) are shown in the figure. ROC, receiver operating characteristic; AUC, area under the curve; PLA2, phospholipase A2.

specific parameter for the detection of AE: the PLA2 activity index, which reacts during the phase of 30 fractions (60 Gy as cumulative dose) in the VMAT course (6 and 7 weeks from initial fraction) (Figs. 2, 3, and 5). In this study, radiotherapy was performed according to JASTRO Guidelines (4), and urine samples were collected at regular intervals. This

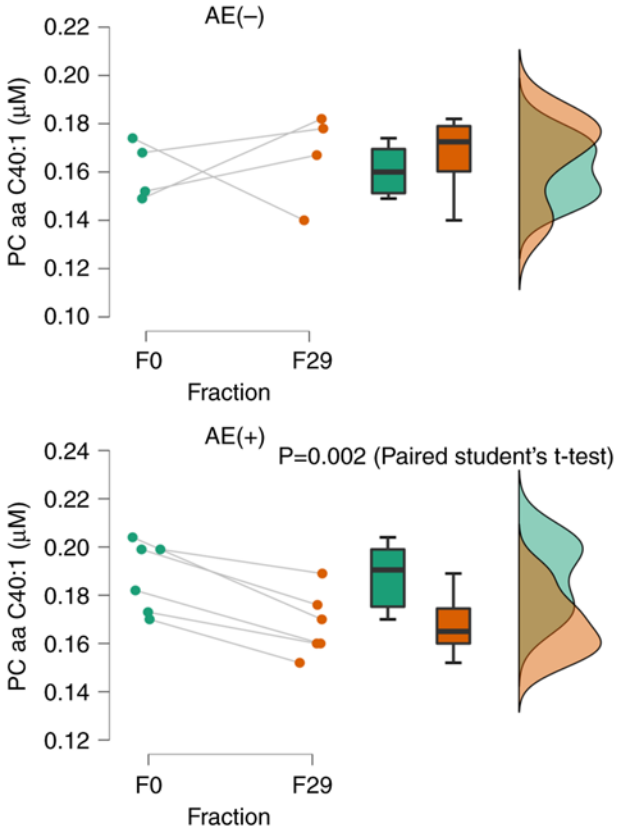

Figure 5. The urinary concentration of PC aa C40:1 between fraction 0 and fraction 29. The paired Student's t-test was performed with and without AEs. AE, adverse events.

type of analysis has not been reported, but post-treatment serum data (PSA) can be used as a known indicator of tumor activity. PSA levels decreased after radiotherapy, indicating an antitumor effect. It is critical to monitor PSA concentrations in peripheral blood, but invasive blood sampling is difficult to perform frequently. Therefore, urine has the advantage of being simple to collect and useful for tracking markers over time during treatment. PLA2 activity is calculated using the lysoPCs to PCs ratio, and Zhai *et al* found that PLA2 activity upregulation is associated with both inflammatory and noninflammatory types of osteoarthritis (19). According to reports dating back more than 30 years, extracellular phospholipase A2 expression has been linked to inflammation-related diseases (20). In this study, we discovered for the first time a negative correlation between Grade 1 AE by VMAT and PLA2 activity. LysoPC and PC are produced by various metabolic pathways and degraded by enzymes. AE can reflect these metabolites directly or indirectly. Wegener *et al* reported the highest acute GU toxicity with grades 1 and 2 in weeks 7 and 8 of radiotherapy (21). Spratt *et al* found that the GI and GU scores in grades 2 and 3 gradually increased during treatment, plateauing after 5 weeks and peaking at 7 weeks (22). According to the reports of these radiotherapy-related AEs, we believe that the significant decrease in the maximum GU score and PLA2 activity in fraction 30 is accurately reflected. The AUC calculated from the ROC generated by the current predictors is a measure of their accuracy. According to the AUC values, test accuracy can be classified as perfect (AUC=1), highly accurate (AUC=0.9-1), moderately accurate (AUC=0.7-0.9), less accurate (AUC=0.5-0.7), and noninformative (AUC=0.5) (23). According to this AUC guideline, the predictors developed in the current study for investigating predictive molecules of AE events were moderately accurate (Fig. 4). Alicikus *et al* found that when using IMRT to localize prostate cancer, the presence of acute Grade >2 GU toxicity predicted the development of late Grade >2 GU toxicity using a multivariate analysis (24). Zelefsky *et al* found that the presence of acute gastrointestinal (GI) and GU symptoms during treatment conferred a 5-fold and 3-fold increase in the risk of late GI and GU toxicities, respectively, in 1,571 prostate cancer patients who had a long follow-up after receiving three-dimensional, conformal radiotherapy or IMRT (25). In contrast, our findings for GU toxicity clearly revealed that manifesting GU symptoms prior to radiotherapy initiation is a strong predictor of acute GU toxicity, as 94% of patients with a Grade 2 before radiotherapy also scored a Grade 2 as the maximum acute GU score (26).

Both the preRT baseline IPSS score of >15 (P<0.001) and acute GU toxicity (P<0.001) predicted late GU toxicity. The RTOG study 94-06 showed an excellent toxicity profile with a dose escalation of up to 79.2 Gy, with the use of 3D-CRT, with ≤3% of patients experiencing a Grade 3 GI or GU acute toxicity, and 85% of patients experiencing no late toxicity or Grade 1 toxicity (22). According to the above reports, the discovery of a biomarker that predicts Grade 1 AEs based on PLA2 activity is extremely important because it allows us to prepare for severe AEs of Grade 2 or higher. This marker can be used to reconsider treatment regimens to prevent AEs when there is a negative correlation in the number of fractions vs. PLA2 activity with continued urine collection. According to a recent paper, hyperbaric oxygen therapy can be used to prevent AEs during radiotherapy (27). Combining these can be considered a new measure against AEs. The limitation of this study is the small sample size used to analyze the prediction of acute AE in other grades (more than 2) (28,29), and lack of healthy group as control. However, it is encouraging that despite the small sample size, the predictor of treatment delay was moderately accurate. Future studies with larger sample sizes may enable the identification of predictive molecules for acute AEs. Detection of PLA2 activity is associated with inflammatory diseases. Kartikasari *et al* explained that the tumor microenvironment is an environment of chronic inflammation (30). Interestingly, Zhao *et al* reported that plasma LysoPC [20:2] and LysoPC [20:3] decrease depending on radiation exposure doses and suggested that it is involved apoptosis (31). It is suggested that there are two regulation pathways by lysoPCs production, one is inflammatory pathway, the other is radiation induces apoptotic pathway. These may be involved in the increase or decrease in LysoPCs and AE, which determines PLA2 activity levels, but the details remain unknown. There is currently no information the relationship between these pathways, metabolites and impact of genitourinary toxicity (Grade 1). Furthermore, the analysis of the biological function, these pathways associated with the identified metabolites, and their relationship to genitourinary toxicity will be clarified by basic experiments using a cell line model.

In conclusion, this study demonstrated the significance of genitourinary metabolite biomarkers in predicting

radiotherapy toxicity using urine metabolomics. In patients undergoing VMAT for localized prostate cancer, the surrogate marker PC aa C40:1 for PLA2 activity was found to predict genitourinary (Grade 1) acute AEs at approximately 30 fractions. Larger sample sizes are expected to improve accuracy even more in future validation studies.

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Availability of data and materials

The metabolomics data generated in the present study may be found in the MetaboBank under accession numbers MTBKS242 and MTBKS243 or at the following URLs: https://mb2.ddbj.nig.ac.jp/study/MTBKS242.html and https://mb2.ddbj.nig.ac.jp/study/MTBKS243.html.

Authors' contributions

HO, SaM, and YT designed the study, prepared the manuscript draft and substantively participated in the manuscript revision. ShM, HY, NK, MS, FK, MN, YH and MA analyzed both the patient and biological data. SaM and MA supervised the study, critically reviewed the manuscript, and provided final approval for the version to be submitted and published. All authors have read and approved the final version of the manuscript. HO, YT and SaM confirmed the authenticity of all the raw data.

Ethics approval and consent to participate

This study was approved by the Medical Ethics Committee of Hirosaki University Graduate School of Medicine (Hirosaki, Japan; approval no. 2020-075) to ensure the welfare and privacy of the donors. Following a detailed verbal explanation regarding the content of this study, written informed consent was obtained.

Patient consent for publication

All patients and their families provided both written and oral informed consent for publication.

Competing interests

The authors declare that they have no competing interests.

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