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Pilot study of plasma creatine riboside as a potential biomarker for cervical cancer

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ABSTRACT

This pilot study aimed primarily to evaluate plasma levels of a novel metabolite, creatine riboside, in patients with cervical cancer (discovery and validation cohorts, $n = 11$ for each) compared with non-cancer subjects (controls, $n = 30$). We found that the pre-treatment plasma creatine riboside level was significantly higher in the discovery cohort than in controls. The cut-off value determined from the discovery cohort distinguished 90.9% of the patients in the validation cohort from controls. Unbiased principal component analysis of plasma metabolites in high-creatine riboside samples demonstrated enrichment of pathways involved in arginine and creatine metabolism. These data indicate the potential utility of plasma creatine riboside as a biomarker of cervical cancer.

1. Introduction

Cervical cancer causes more than 0.3 million deaths annually worldwide; indeed, its mortality ranks fourth among all cancers [1]. The cervical cancer elimination initiative led by the World Health Organization comprises three pillars; i.e., vaccination against human papillomavirus, cervical screening, and treatment [2]. Cervical screening contributes to a huge reduction in cervical cancer mortality in countries with high screening rates. For example, the United Kingdom, where the screening rate in 2011 was 81.5% [3], has seen a 70% reduction in mortality rate since its inception [4]. However, there are still many countries with low screening rates; e.g., in 2011, the rates for Korea and Japan were 11% and 22%, respectively [3]. Poor attendance at traditional cervical screening appointments is due, at least in part, to discomfort and embarrassment associated with internal examination of the cervix [5,6]. This highlights the need to establish physically and mentally less invasive diagnostic tests to assist cervical screening.

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Recent technological advancements in mass spectrometry have enabled high resolution unbiased profiling of small molecules [7]. Compared with traditional cervical screening, mass spectrometry has several advantages; in particular, it is applicable to human biofluids, including blood and urine, thereby enabling non-invasive exploration of cancer biomarkers [7]. Creatine riboside is a novel metabolite discovered by us through untargeted metabolomics profiling of urine samples [8]. In that study, machine learning identified creatine riboside, together with three other metabolites, as a classifier of non-small cell lung cancer (NSCLC) patients (versus non-cancer controls). Subsequent studies suggest that urinary creatine riboside has diagnostic utility not only for NSCLC, but also for hepatocellular carcinoma, intrahepatic cholangiocarcinoma, and adrenocortical cancer [9–11], indicating the potential of this metabolite as a pan-cancer diagnostic marker. Furthermore, most recently we found that creatine riboside is detectable in human blood [12]. However, there is a lack of data regarding blood levels of creatine ribose in patients with cervical cancer; thus, it is not yet clear whether it can be used as a biomarker to assist cervical screening. To address this, we performed a pilot study to analyze plasma samples from patients with cervical cancer, and from ethnicity- and sex-matched non-cancer controls.

2. Materials and methods

2.1. Cases

The discovery cohort, which comprised patients who met the following inclusion criteria, was enrolled prospectively: (i) newly diagnosed and pathologically confirmed squamous cell carcinoma of the uterine cervix; (ii) treated with definitive radiotherapy [13] at Gunma University Hospital from October 2018 to March 2019; and (iii) agreed to collection of plasma and magnetic resonance imaging (MRI) before initiation of radiotherapy (pre-treatment) and at 3 months after completion of radiotherapy.

The validation cohort, which comprised patients who met the following inclusion criteria, were selected randomly at a 1:1 ratio to cases enrolled to discovery cohort: (i) newly diagnosed and pathologically confirmed squamous cell carcinoma of the uterine cervix; (ii) treated with definitive radiotherapy [13] at Gunma University Hospital from 2012 to 2015; and (iii) pre-treatment plasma samples available.

2.2. Controls

From past experience at our institute, it was estimated that approximately 15 cases could be enrolled in this study during the recruitment period. Based on this assumption, ethnicity- and sex-matched non-cancer subjects (aged $\geq$40 years) were selected randomly from Aichi Medical University Biobank (at a 2:1 ratio to the discovery cohort) [9].

2.3. Endpoint definitions

The primary endpoint of this study was the pre-treatment creatine riboside level in patients with cervical cancer versus that in non-cancer subjects. The secondary endpoints were defined to determine the biological basis of this novel metabolite in patients with cervical cancer. To this end, the association between plasma creatine riboside levels and the following parameters were analyzed: (i) the International Federation of Gynecology and Obstetrics 2009 (FIGO) stage; (ii) lymph node involvement; (iii) tumor volume; (iv) plasma tumor markers, i.e., squamous cell carcinoma antigen (SCC) and cytokeratin fragment 19 (CYFRA); and (v) a panel of 99 plasma metabolites, including those involved in the tricarboxylic acid cycle, the urea cycle, nucleotide synthesis, and methionine synthesis (Supplementary Data 1). Tumor volume was calculated based on T2-weighted MRI using the formula: $ABC/2$, where A, B, and C denote the maximum length in a given slice, the width perpendicular to A, and the height perpendicular to A, respectively.

2.4. Metabolomics analysis

Creatine riboside and the metabolites listed in Supplementary Data 1 were assessed using liquid chromatography coupled to a triple quadrupole mass spectrometer (LC-MS/MS; LCMS-8050 system, Shimadzu, Kyoto, Japan). Plasma (100 μl) was added to 1000 μl of 80% methanol in water containing the internal standard (i.e., 10 nM creatine riboside-^{13}C, $^{15}N_2$) [12], and filtered using a Captiva ND Lipid filter plate (Agilent, Santa Clara, CA, USA). The samples were then dried in a vacuum centrifuge and reconstituted in 50 μl of water. Five microliters of each sample were subjected to LC-MS/MS analysis. Creatine riboside was detected and quantitated as described previously [12]. The relative levels of other metabolites were determined using the Method Package for Primary Metabolites (Shimadzu) and a Discovery HS F5-3 column (Sigma-Aldrich). The peak ion intensity for each metabolite was normalized to the sum of peak ion intensity for all detected metabolites.

2.5. Statistical analysis

Differences between two groups were examined using the Mann-Whitney U test. The correlation between two factors was examined using Spearman's correlation test. The diagnostic performance of plasma creatine riboside was examined by receiver operating characteristic (ROC) analysis, in which the cut-off value was determined using the Youden index [14]. The metabolomic profile of plasma samples was examined by principal component analysis (PCA). All statistical analyses were carried out using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant at $p < 0.05$.

2.6. Ethics

The study was conducted in accordance with the principles of the Declaration of Helsinki and approved by the Institutional Ethical Review board of Gunma University Hospital (approval numbers 1109, HS2020-092, and HS2020-256). All participants enrolled in the prospective cohorts provided written informed consent. The opt-out design of the study meant that acquisition of written informed consent from participants in the retrospective cohorts was waived by the Institutional Ethical Review board of Gunma University Hospital.

2.7. Data availability statement

Data were generated by the authors, and are available from the corresponding author upon reasonable request.

3. Results

To test whether the plasma creatin riboside levels are elevated in cervical cancer patients, we prospectively enrolled 11 patients as a discovery cohort (Table 1). We chose advanced cases for this pilot study based on an assumption that a greater tumor burden leads to higher plasma creatine riboside levels. The plasma creatine riboside level was significantly higher in the discovery cohort than in the control group, which comprised 30 ethnicity- and sex-matched non-cancer subjects; median (interquartile range), 96.8 (33.0–123.8) nM versus 18.2 (13.2–24.0) nM, respectively; ($p < 0.0001$) (Fig. 1A). ROC analysis revealed that the area under curve was 0.88 (95% confidence interval, 0.76–1.00) (Fig. 1B). Using a cutoff value of 25.3 nM, plasma creatine riboside showed a sensitivity of 81.8% and a specificity of 83.3% with respect to distinguishing the discovery cohort from the controls.

To validate the findings from the discovery cohort, we retrospectively collected the same number (i.e., 11) of patients with cervical cancer for use as a validation cohort (Table 1). The plasma creatine riboside level was significantly higher in the validation cohort than in the controls ($p < 0.0001$) (Fig. 1A). Notably, the cut-off value of 25.3 nM, derived from the analysis of the discovery cohort, distinguished 90.9% (10/11) of patients in the validation cohort from controls.

Patients with cervical cancer in both the discovery and validation cohorts were significantly older than the controls ($p = 0.025$ and 0.030, respectively). Thus, to eliminate age as a confounding factor with respect to outcome, age-matched subgroups were organized by selecting subjects aged 40–59 years. The resultant subgroups consisted of 10 patients with cervical cancer (from the discovery and validation cohorts) and 29 subjects from the control group; median age (range), 51 (40–59) and 45 (40–58) years, respectively ($p = 0.28$). The plasma creatine riboside level was significantly higher in patients with cervical cancer than in controls ($p = 0.0002$) (Fig. 1C).

Previous studies show a positive correlation between creatine riboside levels in urine and tumor tissues from the same patients with lung cancer [9,15], indicating that creatine riboside is derived from tumor tissues. Thus, we explored this using plasma samples from our cohorts. Pre-treatment plasma creatine riboside levels were significantly higher in patients with advanced FIGO stage ($p = 0.0013$) (Fig. 2A). Meanwhile, there was no significant correlation between pre-treatment plasma creatine riboside levels and the status of

Table 1
Patient characteristics.

Characteristic	Discovery	Validation	Controls
n	11	11	30
Ethnicity	Asian	Asian	Asian
Age	58 (40–76)	63 (27–68)	45 (40–64)
FIGO stage			
I	0 (0.0%)	1 (9.1%)	
II	3 (27.3%)	2 (18.2%)	
III	6 (54.5%)	8 (72.7%)	
IVA	2 (18.2%)	0 (0.0%)	
Tumor volume*			
$\leq$30 cm^3	5 (45.4%)	4 (36.4%)	
31–100 cm^3	4 (36.4%)	6 (54.5%)	
>100 cm^3	2 (18.2%)	1 (9.1%)	
Pelvic LN involvement			
Negative	4 (36.4%)	5 (45.4%)	
Positive	7 (63.6%)	6 (54.5%)	
PALN involvement			
Negative	10 (90.9%)	8 (72.7%)	
Positive	1 (9.1%)	3 (27.3%)	
Concurrent chemotherapy			
Yes	9 (81.8%)	10 (90.9%)	
No	2 (18.2%)	1 (9.1%)	

Controls, ethnicity- and sex-matched non-cancer subjects; Discovery, discovery cohort; FIGO, the International Federation of Gynecology and Obstetrics 2009; LN, lymph node; PALN, para-aortic lymph node; Validation, validation cohort. Age is shown as the median (range). *based on T2-weighted magnetic resonance images.

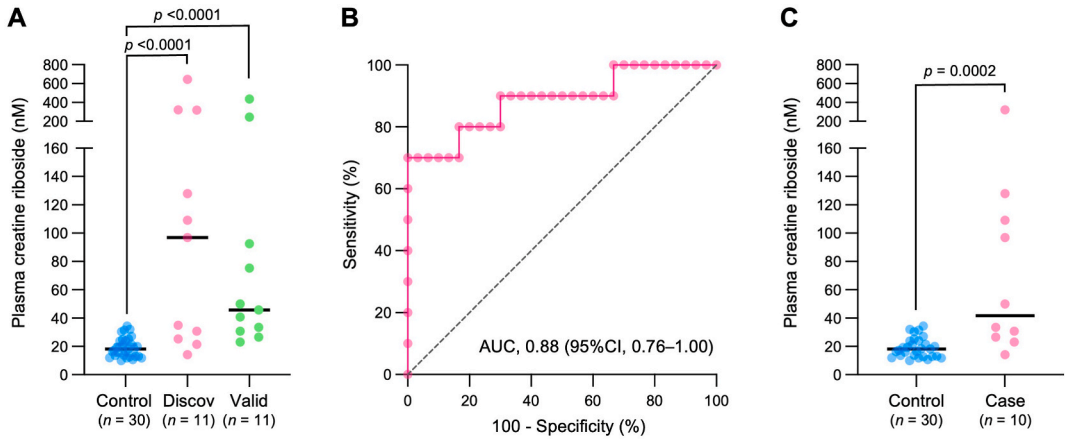

Fig. 1. Plasma creatin riboside levels are elevated in patients with cervical cancer. (A) Discovery cohort (Discov) or validation cohort (Valid) versus non-cancer controls. (B) ROC analysis of the discovery cohort versus controls. (C) Age-matched cases (Discov plus Valid) versus controls. AUC, area under the curve; CI, confidence interval. Black bars, median. *P* values, Mann–Whitney *U* test.

lymph node involvement (Fig. 2B). Consistent with the findings in Fig. 2A, plasma creatine riboside levels showed a significant and strong correlation with the volume of the primary tumor ($p = 0.0084$, $r = 0.54$) (Fig. 2C). Furthermore, at 3 months post-treatment, when the tumor volume decreased in all patients, plasma creatine riboside levels decreased in 81.8% (9/11) of patients in the discovery cohort (Fig. 3). These data suggest that the plasma creatine riboside detected in patients with cervical cancer is derived from primary tumor tissues. By contrast, there was no significant correlation between pre-treatment plasma creatine riboside levels and the tumor markers SCC or CYFRA (Supplementary Data 2).

Finally, we sought to explore the biological basis of creatine riboside metabolism by analyzing the metabolite profiles of plasma samples from the discovery cohort. Seventy-three percent (72/99) of the plasma metabolites analyzed were detected in all cases at both pre- and 3 months post-radiotherapy (Supplementary Data 1). Interestingly, samples with high and low levels of creatine riboside (dichotomized by the median) were separated nicely by an unbiased PCA of all metabolites analyzed in this study (Fig. 4A). Furthermore, in that analysis, creatine riboside showed the greatest loading for the first principal component (i.e., 0.92, Table 2), whereas the loading for SCC and CYFRA was almost negligible (i.e., 0.035 and 0.025, respectively). Five of the top ten metabolites showing high loading were involved in arginine and creatine metabolism; in addition, one of the ten metabolites (i.e., guanosine) contained ribose (Fig. 4B).

4. Discussion

Creatine riboside was first discovered in urine obtained from patients with lung cancer in 2014 [8]. This metabolite was also detected in urine from patients with intrahepatic cholangiocarcinoma [10] and adrenocortical cancer [11] in 2017 and 2019, respectively. Nevertheless, at those times, the lack of a standard compound made inter-study comparison of creatine riboside levels

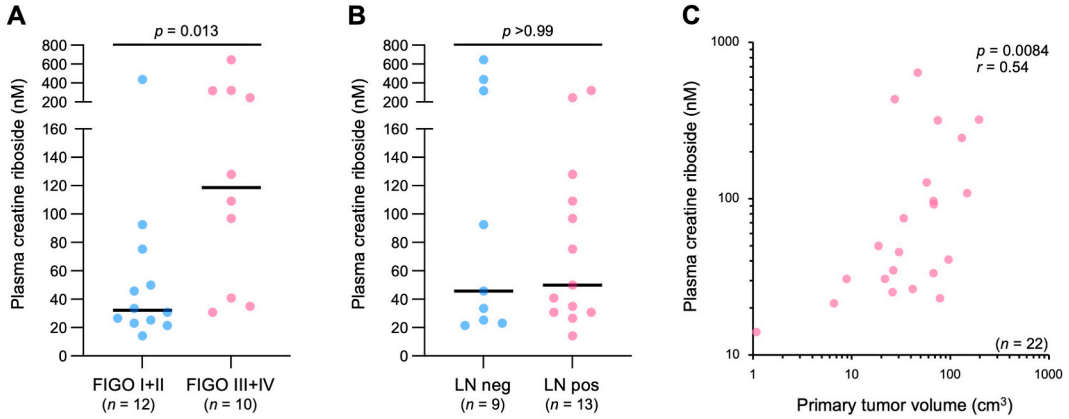

Fig. 2. Plasma creatin riboside levels stratified by (A) FIGO stage or (B) pelvic/paraaortic lymph node involvement in patients with cervical cancer (discovery cohort plus validation cohorts). *P* values, Mann–Whitney *U* test. (C) Correlation of plasma creatin riboside levels with primary tumor volume. *p* and *r* values, Spearman's correlation test.

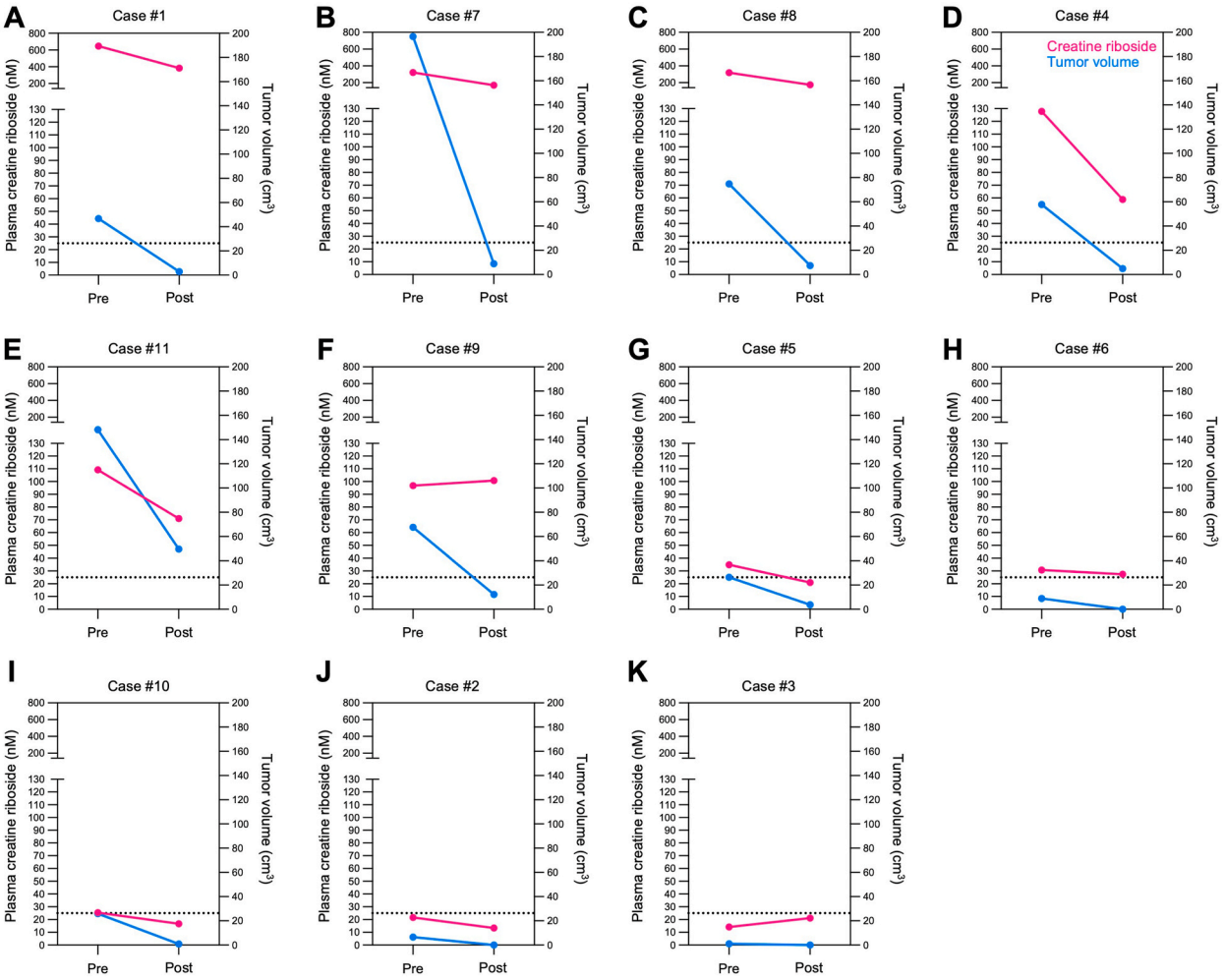

Fig. 3. Kinetics of plasma creatine riboside and tumor volume between pre-treatment (Pre) and post-treatment (3 months; Post) in individual patients (A–K) with cervical cancer. Dashed lines indicate a value of 25.3 nM on the left-hand Y axes, i.e., the best cutoff that distinguishes cases from controls based on ROC analysis (see Fig. 1B).

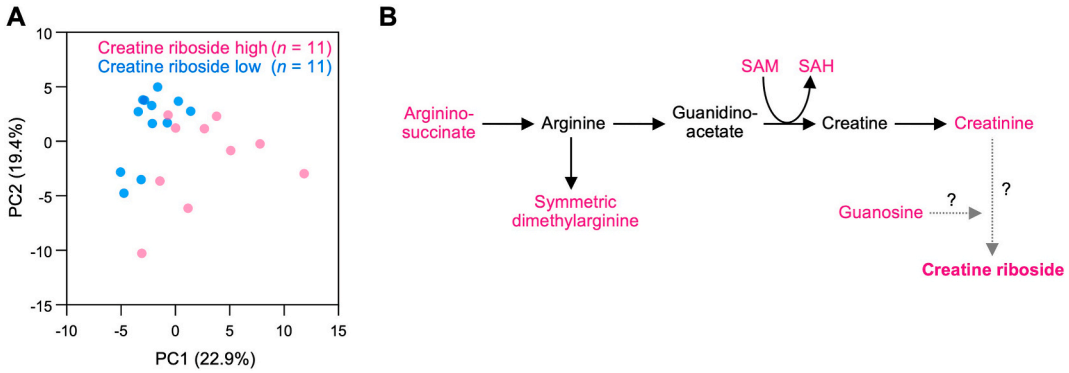

Fig. 4. Principal component (PC) analysis of 75 metabolites detected in 22 plasma samples from the discovery cohort. (A) Score plot. (B) Pathways involved in arginine and creatine metabolism. The top ten metabolites showing high loading for PC1 are indicated in pink. Dashed lines indicate putative unelucidated pathways responsible for creatine riboside metabolism. SAM, *S*-adenosylhomocysteine; SAH, *S*-adenosylmethionine. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Top ten metabolites showing high loading in principal component analysis.

Metabolite	KEGG ID	PC1 loading
Creatine riboside	not listed	0.92
Guanosine	C00387	0.90
Isocitric acid	C00311	0.89
Creatinine	C00791	0.86
Acetylcarnitine	C02571	0.86
S-Adenosylhomocysteine	C00021	0.85
Argininosuccinic acid	C03406	0.84
Symmetric dimethylarginine	C03626×	0.84
S-Adenosylmethionine	C00019	0.82
Kynurenine	C00328	0.77

KEGG, Kyoto Encyclopedia of Genes and Genomes; PC1, first principal component.

impossible; in addition, creatine riboside was only detected in urine samples. In 2020, we detected creatine riboside in blood samples (i.e., serum), in which creatine riboside levels were approximately 10-fold lower than in urine [12]. In that study, we also performed quantitative measurement of creatine riboside using a synthetic compound as a standard. By employing these methods in the present study, we demonstrate for the first time that patients with cervical cancer have elevated plasma levels of creatine riboside. The data indicate the potential utility of plasma creatine riboside as a diagnostic biomarker for locally advanced cervical cancer. We also found that plasma creatine riboside levels decreased at 3 months post-radiotherapy, in line with tumor shrinkage in most cases; this is the first study to report to measure the kinetics of plasma creatine riboside levels along a treatment course in patients with non-surgically treated cancer. These data indicate the potential utility of creatine riboside for post-treatment surveillance. None of the 22 patients analyzed in this study developed recurrence during the follow-up period of 3 months. Nevertheless, post-radiotherapy recurrence of cervical cancers occurs at later time points [12]. From this perspective, the post-treatment kinetics of plasma creatine riboside should be investigated over a longer period to elucidate its utility as a post-treatment surveillance marker for recurrence. In addition, in this study, we chose to analyze locally advanced cases to maximize the possibility of detecting tumor-derived metabolites in plasma. Thus, the diagnostic ability of plasma creatine riboside should be tested further using earlier stage cases.

We found a strong correlation between plasma creatine riboside levels and primary tumor volume. Previously, we reported a correlation between urinary- and intratumoral-creatine riboside levels in patients with lung cancer [15]. Haznadar et al. reported that urinary creatine riboside levels correlate with tumor size in patients with lung cancer [9]. These data support the notion that creatine riboside is derived from malignant tumors. Despite robust evidence for creatine riboside as an oncometabolite, the biological properties of this molecule are largely unknown. Thus, the reason for the poor correlation between plasma creatine riboside levels and those of conventional tumor markers of squamous cell carcinoma of the uterine cervix (i.e., SCC and CYFRA) remains unclear, warranting further research.

This study shows for the first time the results of metabolic profiling of cancer patient-derived biofluids with a focus on creatine riboside. The data showing that an unbiased PCA separated samples according to creatine riboside level indicate the presence of a given specific metabolic status associated with creatine riboside. Although the pathways responsible for creatine riboside metabolism are largely unknown, the results of the present study imply involvement of pathways that metabolize arginine to creatine and creatinine. In addition, nucleoside classes (e.g., guanosine) may play a role as provider of ribose. The creatine–phosphocreatine system plays a key role in cellular energy buffering and transport, and the system is deregulated in a subset of cancers [16]. Meanwhile, arginine is a semi-essential amino acid, meaning that defects in arginine synthesis are a common metabolic vulnerability in cancers (known as arginine auxotrophy) [17]. These pathways can be a therapeutic target for creatine riboside-enriched tumors. Metabolic flux analysis using a heavy labeled compound should be considered to elucidate the details of creatine riboside metabolism.

Clinical implementation of plasma creatine riboside measurement is not considered technically difficult. The standard compound is highly stable in water, and is commercially available elsewhere; measurement utilizes conventional LC-MS/MS systems [12]. Thus, the following should be clarified to facilitate clinical application of plasma creatine riboside for the diagnosis of cervical cancer: (i) verification of the outcome of this study in a larger cohort; (ii) elucidation of the pharmacokinetics and pharmacodynamics of creatine riboside; and (iii) determination of the biological properties of cancers with high or low creatine riboside levels.

It should be noted that a limitation of this study is the difference in age between the cases and controls, which might have affected the results. The influence of age on plasma creatine riboside has not been elucidated, warranting further research. Another limitation is that we were unable to collect samples from early stage patients. Thus, the utility of plasma creatine riboside for early detection of cervical cancer should be studied in the future.

In summary, we demonstrate for the first time that cervical cancer patients show elevated levels of plasma creatine riboside. The data indicate the potential utility of plasma creatine riboside levels as a diagnostic biomarker to assist in cervical cancer screening by detecting advanced cases, warranting validation in a larger cohort.

Author contribution statement

Takahiro Oike: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.
Naoto Osu: Analyzed and interpreted the data.

Yuya Yoshimoto; Kazuhiro Yoshikawa: Contributed reagents, materials, analysis tools or data.
Hideru Obinata: Performed the experiments.
Curtis Harris: Contributed reagents, materials, analysis tools or data.
Tatsuya Ohno: Analyzed and interpreted the data; Wrote the paper.

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e16684.

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