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Serum D-asparagine concentration adjusted for eGFR could serve as a novel screening tool for urothelial carcinoma

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ABSTRACT

The sensitivity of currently available screening tools for urothelial carcinoma (UC) remains unsatisfactory particularly at early stages. Hence, we aimed to establish a novel blood-based screening tool for urothelial carcinoma. We measured serum D-amino acid levels in 108 and 192 patients with and without UC individuals in the derivation cohort, and 15 and 25 patients with and without UC in the validation cohort. Serum D-asparagine levels were significantly higher in patients with UC than in those without UC ($p < 0.0001$). We developed a novel screening equation for the diagnosis of urothelial carcinoma using D-asparagine in serum and estimated the glomerular filtration rate (eGFR). Serum D-asparagine levels adjusted for eGFR exhibited high performance in the diagnosis of UC (AUC-ROC, 0.869; sensitivity, 80.6 %; specificity, 82.7 %), even in early-stage UC (AUC-ROC: 0.859, sensitivity: 83.3 %, specificity: 82.3 %), which were previously misdiagnosed via urinary occult blood or urine cytology. This established strategy combined with urinary occult blood, improves diagnostic ability (sensitivity: 93.7 %, specificity: 70.1 %).

1. Introduction

In 2022, urothelial carcinoma (UC) was reported in 82,290 patients, with 16,710 deaths in the United States [1]. The prognosis of UC depends on the cancer stage at diagnosis, with 5-year survival rates of 86 % and 19 % for stages 1 and 4, respectively [2]; hence, early detection is crucial for managing UC.

Although cystoscopy can accurately diagnose bladder cancer (BCa), it is unsuitable for BCa screening owing to its invasiveness and the need for urologists. Additionally, the current standard screening tools comprising urinary occult blood and urine cytology have poor sensitivity for low-grade or early-stage UC [3]. Other urine-based biomarkers are associated with low specificity [4,5] and their effectiveness is often affected by hematuria and pyuria [3]. Therefore, more accurate and stable biomarkers are required. Recent reports indicate that circulating bacterial DNA is a potential diagnostic tool for various cancer types [6], and UC is strongly associated with the microbiome [7]. Previously, we reported that extracellular vesicles circulating in the bloodstream, which carry Firmicutes DNA, can predict the prognosis of patients with UC treated with immune checkpoint inhibitors [8]. In this study, we hypothesized that specific metabolites produced by the microbiome play crucial roles in UC.

Subsequently, we focused on D-amino acids (DAAs), specific metabolites of bacteria [9]. DAAs have recently been suggested to play biologically significant roles [10,11] and are drawing increasing attention as biomarkers for several diseases [12,13].

The proteins in living organisms comprise 20 amino acid (AA) types; 19 types, excluding glycine, contain L-amino acids (LAAs) and DAAs,

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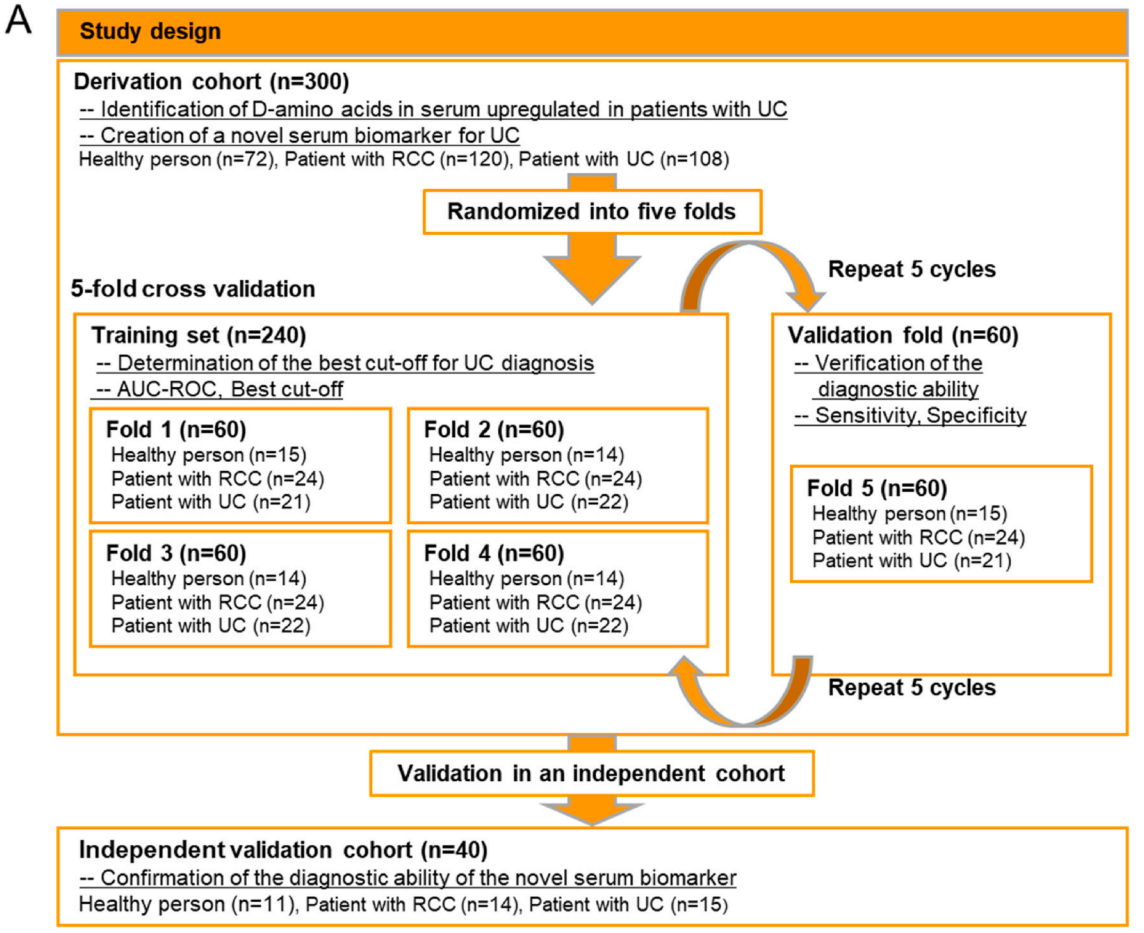

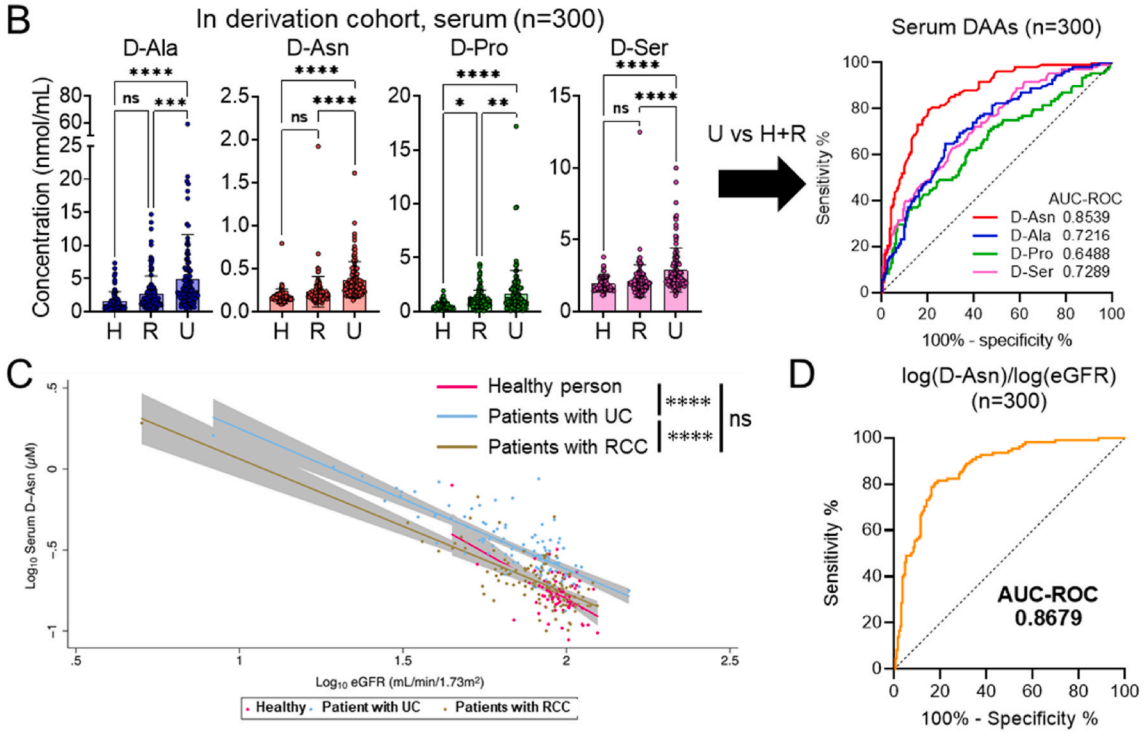

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Fig. 1. D-asparagine is increased in the serum of patients with urothelial carcinoma. (A) The study design: The derivation cohort included 300 participants (72 healthy persons, 120 patients with RCC, and 108 patients with UC). Independent validation cohort included 40 participants (11 healthy persons, 14 patients with RCC, and 15 patients with UC). The participants in the derivation cohort were assessed by five-fold cross validation method. The determined cut-off value in the derivation cohort was revalidated in an independent validation cohort. **(B)** Left panel: The bar plots showed individual D-amino acids (DAAs) in the serum of participants with each cancer type in the derivation cohort. H: healthy person (n = 72). R: patients with RCC (n = 120). U: patients with UC (n = 108). The one-way ANOVA with the Tukey-Kramer post hoc test was used; ns = not significant. *p < 0.05. **p < 0.01. ***p < 0.001. ****p < 0.0001. The error bar showed mean ± SD. Right panel: The ROC curve corresponding to each DAA was used to compare the UC and non-UC individuals in the derivation cohort; the AUC-ROC for each DAA was depicted. D-Asn: D-asparagine. D-Ala: D-alanine. D-Pro: D-proline. D-Ser: D-serine. **(C)** The scatter plots presenting $\log_{10}$(eGFR) on the X-axis and $\log_{10}$(D-Asn) on the Y-axis in the derivation cohort. The ANCOVA was performed; ns = not significant. ****p < 0.0001. **(D)** The ROC curve of $\log_{10}$(D-Asn)/$\log_{10}$(eGFR) was used to compare the UC to participants with non-UC in the derivation cohort; the AUC-ROC was described in the figure.

representing the mirror-image enantiomers [14]. In the laboratory and space, DAAs and LAAs exist in equal proportions [15]. However, it has long been believed that only LAAs exist in mammals and DAAs are either absent or present only in minimal amounts that have no biological activity [16]. Recently, advanced measurement techniques and equipment [17,18] validated DAAs have been shown to exist in the body [10,11]. Hence, in this study, we aimed to investigate whether measuring DAAs is a useful screening tool for patients with UC.

2. Patients and methods

2.1. Participants

This cross-sectional, observational, and retrospective cohort study included a derivation cohort and an independent validation cohort. A total of 340 patients who underwent surgery at Osaka University Hospital between 2016 and 2022 were included in this study; patients were randomly included in a derivation cohort (300 patients) and an independent validation cohort (40 patients). The derivation cohort included 72 living kidney donors (healthy persons), 120 patients with renal cell carcinoma (RCC), and 108 patients with UC. The independent validation cohort included 15 and 14 patients with UC and RCC, respectively, as well as 11 healthy individuals. This study complied with the Declaration of Helsinki and the Ethical Guidelines for Medical Research Involving Human Subjects. This study complied with all the relevant ethical regulations and was approved by the Ethics Committee of Osaka University (Permit No 13397-20). Written informed consent was obtained from all the participants. Detailed information was mentioned in supplementary materials.

2.2. Sample preparation for two-dimensional high-performance liquid chromatography

Samples were prepared for two-dimensional high-performance liquid chromatography (2D-HPLC) following previously reported methods with some modifications [17,18]. For this purpose, 20-fold volumes of methanol were added to the sample, and an aliquot (10 μL) of the supernatant acquired from the methanol homogenate was placed in a brown tube and used for 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD) derivatization (0.5 μL of the plasma was used). After drying

Table 1
Participant characteristics of derivation and validation cohort.

	Discovery cohort (n = 300)			Validation cohort (n = 40)		
	Healthy person	Patient with RCC	Patient with UC	Healthy person	Patient with RCC	Patient with UC
	(*n* = 72)	(*n* = 120)	(*n* = 108)	(*n* = 11)	(*n* = 14)	(*n* = 15)
Age (y), median (range)	65.5 (26–82)	67.5(27–88)	73 (47–90)	70 (39–80)	63 (38–78)	73 (44–86)
Sex, *n* (%)						
Male	37 (51.4)	87 (72.5)	81 (75.0)	4 (36.4)	10 (71.4)	12 (80.0)
Female	35 (48.5)	33 (27.5)	27 (25.0)	7 (63.6)	4 (28.6)	3 (20.0)
Cancer site, *n* (%)						
Bladder	–	–	101 (93.5)	–	–	40 (100.0)
Upper urinary tract	–	–	7 (6.5)	–	–	0 (0.0)
Urinary occult blood, *n* (%)						
Negative	65 (90.3)	79 (65.8)	31 (28.7)	8 (72.7)	7 (50.0)	5 (33.3)
Positive	3 (4.2)	31 (25.8)	59 (54.6)	2 (18.2)	5 (35.7)	7 (46.7)
Boarder	3 (4.2)	6 (5.0)	5 (4.6)	1 (9.1)	0 (0.0)	3 (20.0)
Unknown	1 (1.4)	4 (3.3)	13 (12.0)	0 (0.0)	2 (14.3)	0 (0.0)
Urine cytology, *n* (%)						
Negative	3 (4.2)	15 (12.5)	21 (19.4)	1 (9.1)	3 (21.4)	7 (46.7)
Positive	0 (0.0)	1 (0.8)	60 (55.6)	0 (0.0)	0 (0.0)	8 (53.3)
Boarder	1 (1.4)	1 (0.8)	25 (23.1)	0 (0.0)	0 (0.0)	0 (0.0)
Unknown	68 (94.4)	103 (85.8)	2 (1.9)	10 (90.9)	11 (78.6)	0 (0.0)
Pathological T stage *n*, (%)						
Ta	–	–	30 (27.8)	–	–	4 (26.7)
T1	–	81 (67.5)	32 (29.6)	–	9 (64.3)	3 (20.0)
pT2 ≤	–	39 (32.5)	46 (42.6)	–	5 (35.7)	8 (53.3)
N stage *n*, (%)						
N0	–	115 (95.8)	93 (86.1)	–	13 (92.9)	12 (80.0)
N1	–	5 (4.2)	15 (13.9)	–	1 (7.1)	3 (20.0)
M stage *n*, (%)						
M0	–	105 (87.5)	105 (97.2)	–	10 (71.4)	14 (93.3)
M1	–	15 (12.5)	3 (2.8)	–	4 (28.6)	1 (6.7)
Pathological grade, *n* (%)						
Low grade	–	93 (77.5)	68 (63.0)	–	11 (78.6)	9 (60.0)
High grade	–	27 (22.5)	40 (37.0)	–	3 (21.4)	6 (40.0)
eGFR (ml/min/1.73m2), median (range)	93.9 (44.7–124.6)	86.6 (5.0–124.2)	81.2 (8.3–155.2)	91.6 (46.2–115.2)	87.4 (7.0–114.2)	71.9 (21.7–100.6)

Abbreviations: RCC: renal cell carcinoma, UC: urothelial carcinoma, eGFR: estimated glomerular filtration ratio.
Pathological grade: UC: WHO/ISP classification, RCC: Fuhrman grading; 1,2 = Low, 3,4 = high.

Table 2
The risk factors for urothelial carcinoma in derivation cohort.

	Derivation cohort (n = 300)					
	Univariate analysis			Multivariate analysis		
	OR	95 % CI	p value	OR	95 % CI	p value
Age (y), BCO						
<67	1			1		
≥67	3.29	1.95–5.55	<0.0001	1.53	0.68–3.45	0.3069
Sex						
Female	1			1		
Male	1.65	0.97–2.79	0.0638	1.14	0.53–2.48	0.7343
Urinary occult blood						
Boarder-Negative	1			1		
Positive	7.38	4.23–12.87	<0.0001	7.23	3.54–14.81	<0.0001
Serum D-Ala (nmol/mL), BCO						
<2.42	1					
≥2.42	4.83	2.91–8.01	<0.0001	3.11	1.37–7.07	0.0067
Serum D-Asn (nmol/mL), BCO						
<0.234	1			1		
≥0.234	15.34	8.54–27.54	<0.0001	9.64	4.15–22.40	<0.0001
Serum D-Pro (nmol/mL), BCO						
<1.24	1			1		
≥1.24	3.33	1.96–5.65	<0.0001	0.86	0.37–2.01	0.7296
Serum D-Ser (nmol/mL), BCO						
<2.17	1			1		
≥2.17	3.83	2.33–6.30	<0.0001	1.00	0.47–2.12	0.9928
eGFR (ml/min/1.73m2), BCO						
≥89.5	1			1		
<89.5	3.65	2.14–6.23	<0.0001	0.77	0.31–1.90	0.5714

Abbreviations: BCO: best cut-off, D-Ala: D-alanine, D-Asn: D-asparagine, D-Pro: D-proline,D-Ser: D-serine, eGFR: estimated glomerular filtration ratio.

the solution under reduced pressure, 20 μL of 200 mM sodium borate buffer (pH 8.0) and 5 μL of fluorescence labeling reagent (40 mM NBD in anhydrous MeCN) were added and heated for 2 min at 60 °C. An aqueous solution (75 μL) of 0.1 % (v/v) trifluoroacetic acid was added; 2 μL of the reaction mixture was subjected to 2D-HPLC. Detailed information was mentioned in supplementary materials.

2.3. Determining amino acid enantiomers using 2D-HPLC

AA enantiomers were quantified using a 2D-HPLC platform (DASH 27B3X00322000001; KAGAMI, Ibaraki, Osaka, Japan) using a shape-fitting algorithm as previously described [17–19]. Briefly, the NBD derivatives of the AAs were separated from numerous intrinsic substances using a reversed-phase column (Singularity RP column, 1.0 mm i.d. × 50 mm; KAGAMI, Ibaraki, Japan) through gradient elution; aqueous mobile phases containing MeCN and formic acid was used. The fractions of the target AAs were automatically collected using a multi-loop valve and transferred to the enantioselective column (Singularity CSP-001S, 1.5 mm i.d. × 75 mm; KAGAMI) to further determine the D- and L-forms. The mobile phase was a mixed solution of MeOH and MeCN supplemented with formic acid, and the fluorescence of NBD-AAs was detected at 530 nm with excitation at 470 nm. The fluorescence detector uses two photomultiplier tubes to cover the high and low ranges and enables the simultaneous and accurate measurement of abundant L-AAs and trace D-AAs in human serum and urine samples. The blood and urine levels of D-AAs were determined in the micromolar range or as a ratio to L-AAs [9,14].

2.4. Five-fold cross-validation

The derivation cohort was randomly divided into five equal-sized folds to ensure that the proportion of patients with cancer and living kidney donors in the folds was consistent; in each iteration of the cross-validation scheme, four folds were used as the training sets, and the remaining one served as the validation fold. The model was trained on combined training folds, incorporating data from four folds. To assess its performance, the trained model was evaluated based on a validation fold not used during the training phase. This process was repeated five times with each of the five folds serving as the validation set. Performance metrics were calculated for each iteration, and the results were averaged for an overall assessment of the model's performance.

2.5. Statistical analyses

Statistical analyses and quantifications were performed using JMP Pro (v. 16.0.0; SAS Institute, Cary, NC, USA), SPSS (v. 29.0.1; IBM Corp, Armonk, N.Y., USA), and GraphPad Prism (v. 9.2.0; GraphPad Software, La Jolla, CA, USA) software. Statistical significance was set at $p < 0.05$. Detailed information was mentioned in supplementary materials.

3. Results

3.1. D-asparagine is increased in the serum of patients with urothelial carcinoma

The study design is depicted in Fig. 1A. The participant characteristics are shown in Table 1. We measured twenty-two DAAs using the serum of the participants in the discovery cohort (72 healthy individuals, 120 patients with RCC, and 108 patients with UC). Only D-alanine, D-asparagine, D-proline, and D-serine could be detected in human serum. All four DAAs which could be detected in human serum showed significantly higher levels in patients with UC than in patients without UC (healthy person vs. patients with UC; D-alanine, D-asparagine, D-proline, D-serine $p < 0.0001$; patients with RCC vs. patients with UC; D-alanine, $p = 0.0006$; D-asparagine, D-serine, $p < 0.0001$; D-proline, $p = 0.0087$, Fig. 1B). Furthermore, we measured the above-mentioned twenty-two DAAs in urine of matched patient samples (14 healthy individuals, 29 patients with RCC, and 17 patients with UC) (Supplementary Fig. S1A, B). Comparison of the effectiveness of serum and urine DAA levels in UC diagnosis revealed the best score for serum D-asparagine level (AUC-ROC, 0.8539; best cut-off value, 0.234 nmol/mL; sensitivity, 79.6 %; specificity, 79.7 %, Fig. 1B, Supplementary Fig. S1A, B). The independent risk factors for UC included high level of serum D-asparagine (<0.234 vs.≥0.234; HR:9.64, 95%CI: 4.15–22.40, p

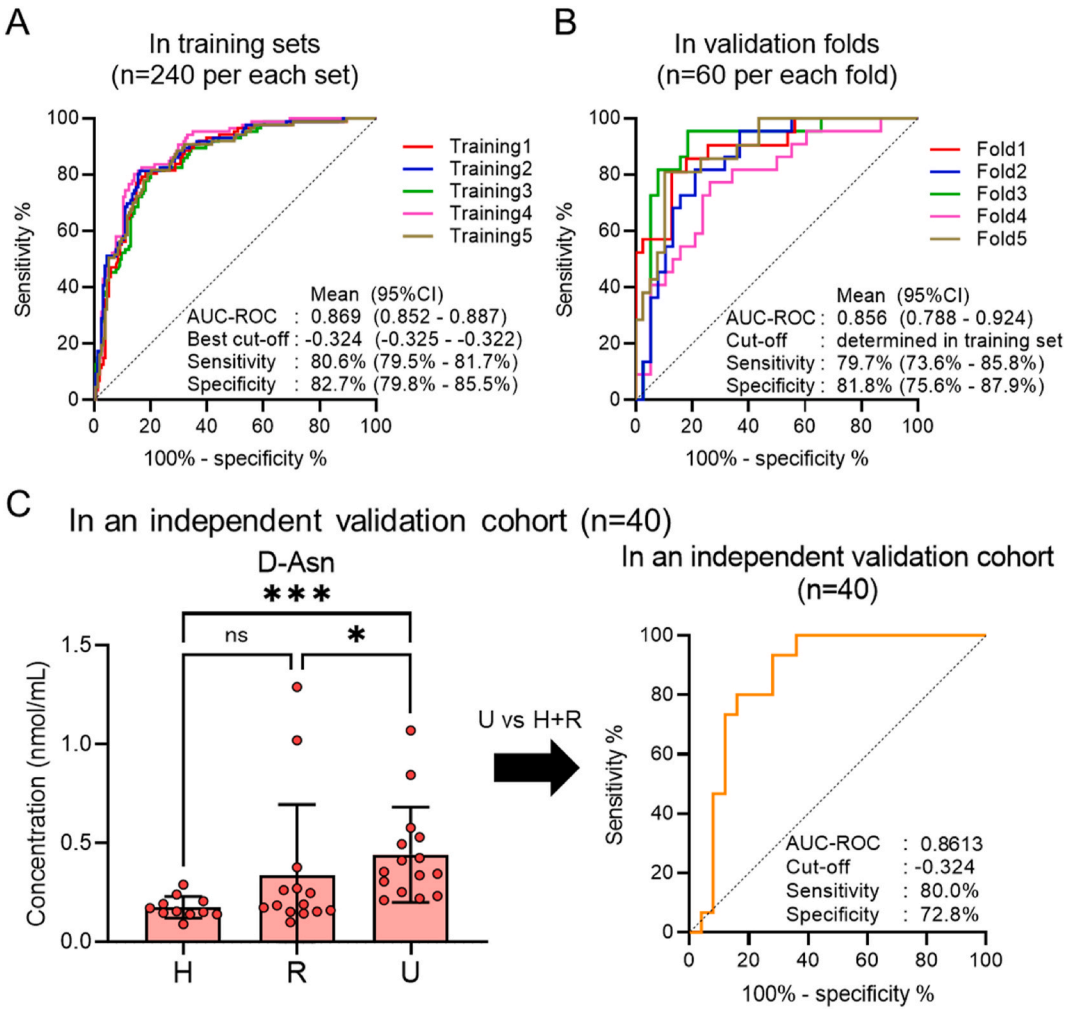

Fig. 2. The $\log_{10}$(D-Asn)/$\log_{10}$(eGFR) ratio accurately diagnosed UC in independent and two distinct cohorts. (A) The ROC curve of $\log_{10}$(D-Asn)/$\log_{10}$(eGFR) was used to compare the UC and non-UC individuals in each training set from the derivation cohort. The AUC-ROC, optimal cut-off value, sensitivity, and specificity were described in the figure. Data were expressed as the mean ± 95 % confidence interval (95 % CI). **(B)** The ROC curve of $\log_{10}$(D-Asn)/$\log_{10}$(eGFR) was used to compare the UC and non-UC individuals from each validation fold in the derivation cohort. The cut-off value was determined in training sets. The AUC-ROC, sensitivity, and specificity were described in the figure. The mean ± 95 % CI was presented. **(C)** Left panel: The bar plots showed serum D-asparagine levels in participants with or without UC in an independent validation cohort. D-Asn: D-asparagine. H + R: healthy person (n = 11) plus patients with RCC (n = 12). U: patients with UC (n = 15). The Kruskal-Wallis with Dunn's post hoc test was used; ns = not significant. *p < 0.05. ***p < 0.001. The error bar showed mean ± standard deviation. Right panel: The ROC curve of $\log_{10}$(D-Asn)/$\log_{10}$(eGFR) was used to compare the UC and non-UC individuals in an independent validation cohort. The AUC-ROC, cut-off value determined in derivation cohort (−0.324), sensitivity, and specificity were described in the figure.

< 0.0001, Table 2). The serum D-asparagine levels exhibited a significantly negative correlation with eGFR (D-asparagine, $r^2 = 0.485$, Supplementary Fig. S1C), which is comparable to a previous report [12]. Moreover, ANCOVA reflected significantly higher levels of serum D-asparagine, adjusted for eGFR, in patients with UC than in patients without UC (healthy vs. UC, RCC vs. UC, p < 0.0001; healthy vs. RCC, p = 0.2000, Fig. 1C). From these results, we created a diagnostic equation using serum D-asparagine level and eGFR ($\log_{10}$[D-Asn]/$\log_{10}$[eGFR] ratio) and successfully established a more accurate diagnostic biomarker than serum D-asparagine level alone (AUC-ROC, 0.8679; sensitivity 80.1 %; specificity, 82.3 %, Fig. 1D).

3.2. The $\log_{10}$(D-Asn)/$\log_{10}$(eGFR) ratio could accurately diagnose UC in independent and distinct two cohorts

We evaluated the diagnostic ability of $\log_{10}$(D-Asn)/$\log_{10}$(eGFR) ratio for UC using two independent cohorts. Based on the 5-fold cross-validation method (Supplementary Table 1), in the derivation cohort, we demonstrated that $\log_{10}$(D-Asn)/$\log_{10}$(eGFR) ratio exhibited high diagnostic ability for UC (AUC-ROC, 0.869 [0.852–0.887], optimal cut-off value, −0.324 [−0.325 to −0.322], sensitivity, 80.6 % [79.5 %–85.5 %], and specificity, 82.7 % [79.8 %–85.5 %], Fig. 2A). In the training set, each cut-off value was validated in each validation fold (AUC-ROC, 0.856 [0.788–0.924]; cut-off value, determined in each paired training set; sensitivity, 79.7 % [73.6%–85.8 %]; specificity, 81.8 % [75.6%–87.9 %]) (Fig. 2B, Supplementary Fig. S2A). In an independent validation cohort, the determined mean cutoff value (−0.324) was revalidated, and the $\log_{10}$(D-Asn)/$\log_{10}$(eGFR) ratio consistently exhibited high diagnostic performance for UC (AUC-ROC, 0.861; sensitivity, 80.0 %; specificity, 72.8 %) (Fig. 2C, Supplementary Fig. S2B).

3.3. Applied uses of $\log_{10}$(D-Asn)/$\log_{10}$(eGFR) ratio

Patients with UC were evaluated separately for each pathologic T stage (pTa, pT1, and over pT2) in the derivation cohort. Serum D-asparagine levels did not differ between T stages, and patients with early-stage (pTa) UC showed significantly higher levels of serum D-asparagine than non-UC participants (Fig. 3A). Based on the previously

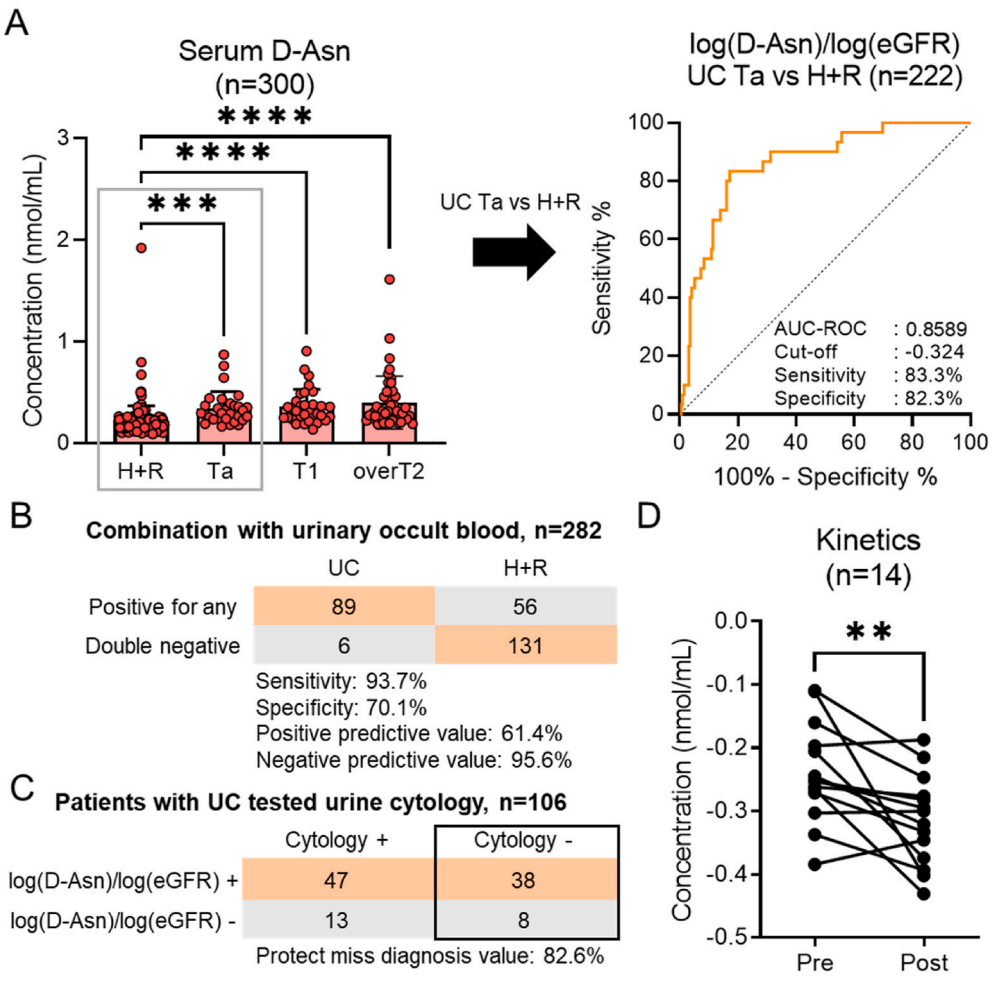

Fig. 3. Applied uses of $\log_{10}$(D-Asn)/$\log_{10}$(eGFR) ratio. (A) Left panel: The bar plots showed serum D-asparagine levels in patients with each pathological T-stage (pT-stage) of UC and non-UC participants in the derivation cohort. D-Asn: D-asparagine. H + R: healthy persons (n = 72) plus patients with RCC (n = 120). Ta: patients with pTa UC (n = 30). T1: patients with pT1 UC (n = 32). over T2: patients with pT2 or over pT2 UC (n = 46). The one-way ANOVA with Dunnett's post hoc test was used as H + R was the control; ***p < 0.001. ****p < 0.0001. The error bar showed mean ± SD. Right panel: The ROC curve of $\log_{10}$(D-Asn)/$\log_{10}$(eGFR) was used to compare patients with pTa UC to participants with non-UC in the derivation cohort. The AUC-ROC, cut-off value determined in derivation cohort (−0.324), sensitivity, and specificity were described in figure. **(B)** The 2 × 2 table for UC diagnosis using $\log_{10}$(D-Asn)/$\log_{10}$(eGFR) ratio and urinary occult blood combination. A positive test for the $\log_{10}$(D-Asn)/$\log_{10}$(eGFR) ratio or urinary occult blood was considered positive (upper line: positive for any), whereas a negative test for both was considered negative (lower line: double negative). The cut-off value determined in the derivation cohort (−0.324) was used. UC: patients with UC (n = 95). H + R: healthy person (n = 71) plus patients with RCC (n = 116). **(C)** The 2 × 2 table of patients with UC tested urine cytology (all patients had UC, n = 106). Upper line: positive for $\log_{10}$(D-Asn)/$\log_{10}$(eGFR) ratio. lower line: negative for $\log_{10}$(D-Asn)/$\log_{10}$(eGFR) ratio. Left column: patients with UC correctly diagnosed based on urine cytology. Right column: patients with UC misdiagnosed based on urine cytology. The cut-off value determined in the derivation cohort (−0.324) was used. **(D)** The kinetics of $\log_{10}$(D-Asn)/$\log_{10}$(eGFR) ratio pre- and post-radical surgery for bladder cancer (n = 14). A comparison was performed using the two-tailed paired Student's t-test; **p < 0.01.

determined cutoff value (−0.324), the $\log_{10}$(D-Asn)/$\log_{10}$(eGFR) ratio exhibited higher performance in diagnosing early-stage UC than the urinary occult blood ($\log_{10}$[D-Asn]/$\log_{10}$[eGFR] ratio, AUC-ROC, 0,8589; sensitivity 83.3 %; specificity, 82.3 %; urinary occult blood, sensitivity 48.0 %; specificity, 81.8 %) (Fig. 3A, Supplementary Fig. S3A). We further demonstrated the diagnostic ability of the $\log_{10}$(D-Asn)/$\log_{10}$(eGFR) ratio in combination with urinary occult blood or urinary cytology. While analyzing the efficacy of the $\log_{10}$(D-Asn)/$\log_{10}$(eGFR) ratio combined with urinary occult blood, participants were classified as UC-positive if at least one of the two indicators was positive and UC-negative if both indicators were negative; this combination increased the sensitivity to 93.7 % while maintaining a specificity of 70.1 % (Fig. 3B, Supplementary Fig. S3B). We focused on misdiagnoses of patients with UC while analyzing the efficacy of the log10(D-Asn)/log10(eGFR) ratio combined with urine cytology; the $\log_{10}$(D-Asn)/$\log_{10}$(eGFR) ration demonstrated the potential to correctly reclassify 82.6 % of UC patients missed on urine cytology-based diagnosis (Fig. 3C, Supplementary Fig. S3C). Finally, we evaluated the kinetics of the $\log_{10}$(D-Asn)/$\log_{10}$(eGFR) ratio before and after radical surgery for UC (Supplementary Table 2), which revealed that $\log_{10}$(D-Asn)/$\log_{10}$(eGFR) ratio was significantly reduced after radical surgery (p = 0.0066, Fig. 3D).

4. Discussions

In this study, we demonstrated the potential of serum log10(D-Asn)/log10(eGFR) ratio as a screening tool of UC. Several blood-based liquid biopsy methods, including circulating tumor cells, circulating tumor DNA, cell-free RNA, exosomes, metabolomics and proteomics have been reported to be useful for diagnosing UC (AUC-ROC, 0.899 [0.711–0.970], sensitivity, 86.2 % [22.6 %–97.6 %], and specificity, 88.0 % [66.7 %–100 %]) [20]. Most of these studies compared patients with UC and only healthy control. In our study, results demonstrated that serum D-asparagine levels were significantly elevated in patients

with UC than in those without UC, including not only healthy controls but also patients with RCC. The diagnostic ability of patients with UC from only healthy control using serum log10(D-Asn)/log10(eGFR) ratio showed particularly high performance both in derivation cohort (AUC-ROC, 0.916; sensitivity, 79.6 %; specificity, 93.1 %) and validation cohort (AUC-ROC, 0.958; sensitivity, 80.0 %; specificity, 100 %). Our study highlights that serum log10(D-Asn)/log10(eGFR) ratio presents a noninvasive high-accuracy adjuncts to traditional urine-based biomarkers, such as urinary occult blood and urine cytology, which are often associated with low sensitivity in detecting low-grade or early-stage UC [3]. Interestingly, serum D-asparagine levels were elevated in all T stages of UC, including early-stage UC. Hence, serum log10(D-Asn)/log10(eGFR) ratio is a potentially valuable tool for early detection of UC, which is advantageous over urinary occult blood. The serum log10(D-Asn)/log10(eGFR) ratio combined with urinary occult blood indicates significantly improved detection rates and reduced false negatives. Similarly, serum log10(D-Asn)/log10(eGFR) ratio reclassified 82.6 % of UC cases misdiagnosed by urine cytology alone. The significant reduction in post-radical surgery, serum log10(D-Asn)/log10 (eGFR) ratio further underscores its potential as a biomarker for monitoring disease progression and treatment response. Hence, future studies can validate the utility of this approach in the postoperative longitudinal follow-up of non-muscle invasive BCa. While our findings are promising, this study has limitations, including the need for studies including larger, external and across diverse population validation cohorts, and elucidation of the biological significance of D-asparagine in UC. Moreover, the study has not completely ruled out potential confounders between patients with UC and control cases.

5. Conclusions

In conclusion, serum log10(D-Asn)/log10(eGFR) ratio represents a promising biomarker for the diagnosis of UC. Its high diagnostic accuracy and the ability to enhance existing screening methods underscores its potential as a valuable tool for the early detection and management of UC.

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Availability of data and materials

The data supporting these findings are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Akinaru Yamamoto: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing. **Atsunari Kawashima:** Conceptualization, Funding acquisition, Project administration, Resources, Writing – review & editing. **Shinsuke Sakai:** Writing – review & editing. **Masashi Mita:** Formal analysis, Project administration, Resources, Writing – review & editing. **Nesrine Sassi:** Writing – review & editing. **Shunsuke Inoguchi:** Writing – review & editing. **Yuki Horibe:** Writing – review & editing. **Akihiro Yoshimura:** Writing – review & editing. **Masaru Tani:** Writing – review & editing. **Liu Yutong:** Writing – review & editing. **Yohei Okuda:** Writing – review & editing. **Toshiki Oka:** Writing – review & editing. **Toshihiro Uemura:** Writing – review & editing. **Gaku Yamamichi:** Writing – review & editing. **Yu Ishizuya:** Writing – review & editing. **Takuji Hayashi:** Writing – review & editing. **Yoshiyuki Yamamoto:** Writing – review & editing. **Taigo Kato:** Writing – review & editing. **Koji Hatano:** Writing – review & editing. **Yoichi Kakuta:** Writing – review & editing. **Ryoichi Imamura:** Writing – review & editing. **Shiro Takahara:** Writing – review & editing. **Tomonori Kimura:** Data curation, Writing – original draft, Writing – review & editing. **Norio Nonomura:** Supervision, Writing – review & editing.

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.

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Appendix A. Supplementary data

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Abbreviations

2D-HPLC	two-dimensional high-performance liquid chromatography
AA	amino acid
ANCOVA	analysis of covariance
AUC	area under the curve
BCa	bladder cancer
DAAs	D-amino acids
eGFR	estimated the glomerular filtration rate
LAA	L-amino acids
NBD	4-fluoro-7-nitro-2,1,3-benzoxadiazole
RCC	renal cell carcinoma
ROC	receiver operating characteristic
SD	standard deviation
UC	urothelial carcinoma

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