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Original Article

Diagnostic value of combined IQGAP3/BMP4 and IQGAP3/FAM107A expression ratios in urinary cell-free DNA for discriminating bladder cancer from hematuria

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Abstract

Background: Urinary cell-free DNA (ucfDNA) has great potential as a "liquid biopsy" for use in diagnosis of urological cancers. In this study, we compared ucfDNA gene expression levels between patients with bladder cancer (BC) and those with hematuria, and determined whether they could be used as a noninvasive urine-based marker.

Methods: The study cohort of 355 patients included a screening group (40 BC and 41 hematuria controls) and a validation cohort (149 BC and 125 hematuria controls). Expression levels ratios of 1 up-regulated gene (IQGAP3) to those of 7 down-regulated genes were examined in ucfDNA in the screening group to identify ratios that differed significantly between BC and hematuria patients. IQGAP3/BMP4 and IQGAP3/FAM107A ratios were selected and combined to develop a discriminant score (DS) index, which was tested in the validation cohort. Receiver operating characteristic curves and areas under the curve were calculated to evaluate the performance of the DS index.

Results: IQGAP3/BMP4 and IQGAP3/FAM107A ratios in ucfDNA were both significantly higher in BC patients than in hematuria patients (both $P < 0.001$). The DS index had an area under the curve of 0.862, a sensitivity of 71.0%, a specificity of 88.6%, a positive predictive value of 90.3%, and a negative predictive value of 67.2%.

Conclusions: Both IQGAP3/BMP4 and IQGAP3/FAM107A ratios in ucfDNA were significantly higher in patients with BC than in those with hematuria. The DS index exhibits good diagnostic performance as a noninvasive biomarker. © 2018 Published by Elsevier Inc.

Keywords: Urinary cell-free DNA; Biomarker; Discriminant analysis; Bladder cancer; Hematuria

1. Introduction

Bladder cancer (BC) is one of the most common malignant tumors in the genitourinary system and occurs more commonly in men than in women. In 2014, the incidence of BC accounted for 2.8% of all male malignancies in the Korea National Cancer Database [1], while in the United States, approximately 79,000 new cases of BC were diagnosed and 16,870 people died from this malignancy [2]. BC is classified as either nonmuscle invasive BC (NMIBC) or muscle invasive BC

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(MIBC), based on the degree of invasion of BC cells into the bladder muscle layer. Up to 80% of BC cases present with NMIBC, tumors are confined to the mucosa (Ta and Tis) or submucosa (T1) [3,4]. Although NMIBC can be simply controlled by transurethral resection, the recurrence rate is 30% to 70%, with 10% to 15% of cases progressing to MIBC, which has a poor prognosis [4–6]. Therefore, early diagnosis and appropriate treatment are important for patients with BC.

Hematuria is the most common sign of BC, with approximately 90% of cases presenting with painless gross hematuria [7]. Other common etiologies of hematuria include urinary tract infection, urolithiasis, and other genitourinary malignancies (e.g., prostate cancer). Thus, patients with BC presenting with hematuria impose a heavy clinical burden on urologists, because all patients with this symptom require a full work-up to ensure detection of cases with malignant disease. Cystoscopy and adjunct cytology are the current gold standard diagnostic methods, and are used to detect patients with suspected BC and to monitor the risk of tumor recurrence; however, cystoscopy is costly and invasive, causes great discomfort for patients, and can lead to urinary tract infections [8]. Conversely, although cytology is noninvasive, it has poor sensitivity, and false negative results are common [9]. Furthermore, other available screening methods (e.g., fluorescence in situ hybridization and nuclear matrix protein 22) are not sufficiently sensitive to replace cystoscopy [10]. Hence, an ideal noninvasive, urine-based marker with high sensitivity and specificity is needed to supplement or replace cystoscopy for the detection of BC.

In recent years, liquid biopsy has attracted a great deal of attention as a tool to reveal the molecular landscape of tumors [11]. Evaluation of cell-free DNA (cfDNA) is a noninvasive tool for evaluation of tumor-related molecules and is expected to become a method of choice for cancer screening and monitoring of treatment effects [11–13]. CfDNA can be isolated from blood and urine. Currently, blood-based studies are more widespread than those using urine, as blood can potentially be associated with the detection of all tumors [14]; however, there are a large number of nucleic acids, proteins, and inhibitors of other cells in the blood that affect the detection of cfDNA biomarkers. Urinary cell-free DNA (ucfDNA), originating from tumor-derived necrosis or apoptosis, can be directly expelled into the genitourinary tract. Compared with blood, urine sampling is noninvasive and specimens can be collected routinely at any time. Thus, ucfDNA is a particularly attractive monitoring solution, especially for patients with genitourinary cancers.

Currently, there is no consensus on which housekeeping genes are appropriate for use as controls for quantitative assessment of cfDNA. Commonly used internal controls, such as Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-globin, and β-actin, have been used for normalization of ucfDNA expression levels [15]; however, clear variation in the levels of these genes is invariably detected. Concentration-based quantification methods have also been used to calculate cfDNA levels; however, the conditions required for quantitative cfDNA analysis are often difficult to implement in clinical practice. In this study, we used ratios, comprising expression levels of 2 genes (with an up-regulated gene as the numerator and a down-regulated gene as the denominator) as predictive markers, as such ratios do not require correction with an internal control. The aim of this study was to determine whether there was a difference in the levels of 2-gene ratio markers between samples from patients with BC and those with hematuria and to investigate if a model incorporating the validated markers exhibited high diagnostic value for discriminating BC from hematuria.

2. Material and methods

2.1. Study design

A flow diagram of the study design is presented in Fig. 1. DNA targets were selected based on tissue mRNA microarray screens (data not shown). Two-gene expression ratios identified as potential markers were amplified from ucfDNA in a real-time polymerase chain reaction (real-time PCR) screening test. Subsequently, IQ motif-containing GTPase activating protein 3 (IQGAP3)/bone morphogenetic protein 4 (BMP4) and IQGAP3/family with sequence similarity 107 (FAM107A) expression ratios were determined by real-time PCR in the validation cohort. Finally, statistical analysis was used to develop a predictive model for BC detection.

2.2. Study participants and urine sample storage

A total of 355 patients, consisting of screening and validation cohorts, were enrolled in this study. Urine samples were obtained from study participants at Chungbuk National University Hospital between May 2000 and May 2017. Eighty-one patients (40 BC and 41 hematuria) provided samples for the screening test to determine 2-gene expression ratio levels in ucfDNA. First-morning urine samples were obtained before transurethral resection or radical cystectomy, and supernatants separated from them by centrifugation at 1,320 g for 15 minutes were immediately stored at −20°C until use. Tumors were staged according to tumor, lymph node, metastasis classification (2002) and graded by a pathologist using the World Health Organization (WHO) system (2004). Urine sample collection and analysis for this study were approved by the Ethics Committee of Chungbuk National University (GR2010-12-010). All subjects were informed of the purpose of the experiment and provided signed informed consent before entering the study.

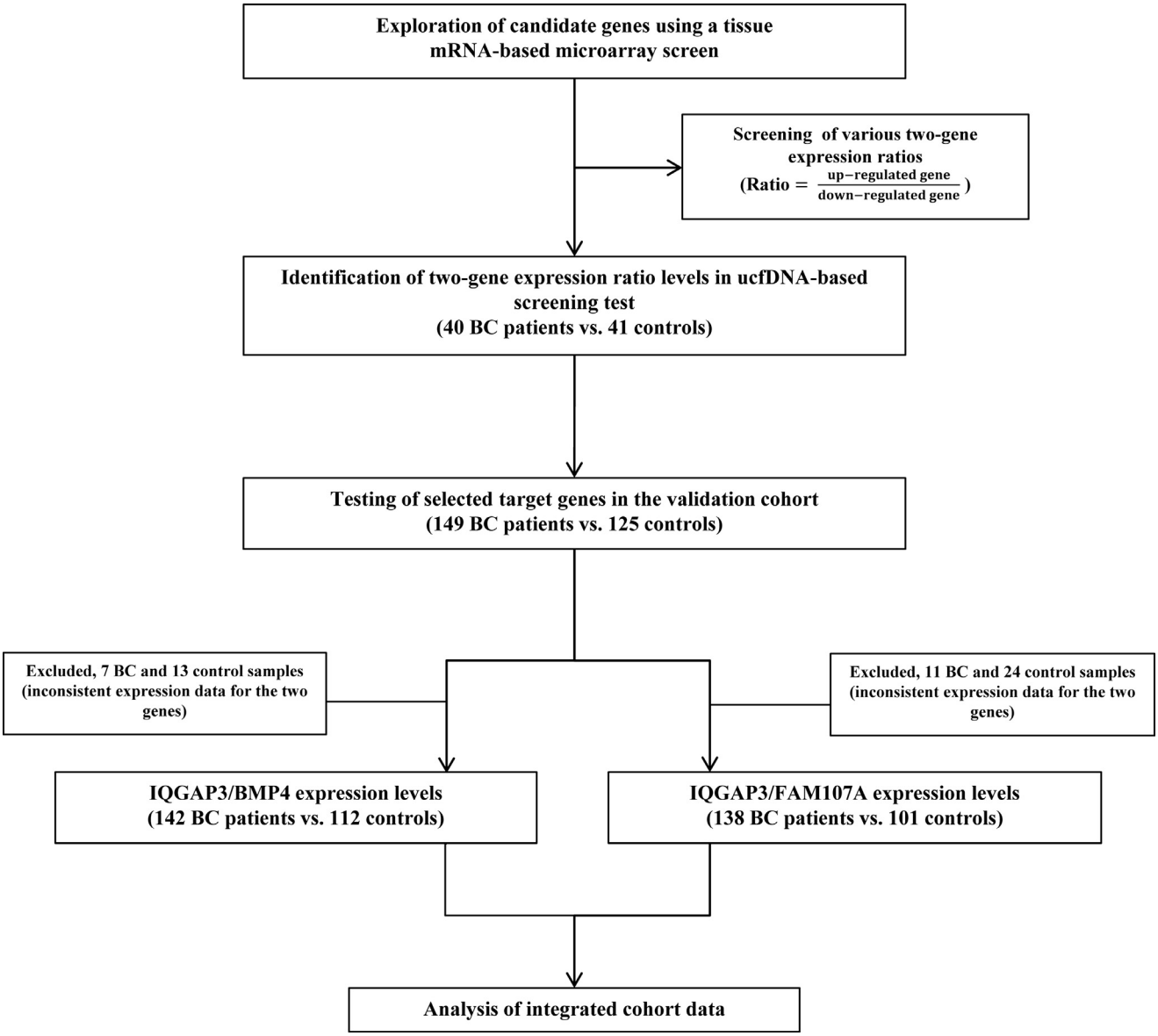

Fig. 1. Flow diagram of the study design.

2.3. Inclusion and exclusion criteria

The inclusion criteria were as follows: (1) BC urine samples were from patients with primary tumors at initial diagnosis; (2) all tumors underwent transurethral resection or radical cystectomy and were histologically verified as urothelial transitional cell carcinomas; (3) control patients (hematuria) were diagnosed as having nonmalignant genitourinary system diseases, including benign prostate hyperplasia , urolithiasis, and stress urinary incontinence. The exclusion criteria were as follows: (1) patients with BC and additional solid tumors or hematological malignancies; (2) not a first diagnosis of BC, or patients had received any other treatments, including radiation therapy, chemotherapy, or biological targeted therapy; (3) patients diagnosed with concomitant carcinoma in situ.

2.4. Extraction of cfDNA from urine

Extraction of cfDNA from urine supernatant samples was performed using the QIAquick gel extraction kit (Qiagen GmbH, Hilden, Germany). Briefly, each frozen urine supernatant aliquot (1 ml) was thawed at room temperature, treated with 500 μl QG buffer (contained in the kit), and incubated at 50°C for 5 minutes. Then, isopropanol (500 μl) was added and samples were vortexed for 10 minutes. Mixtures were then transferred onto the QIAquick column and centrifuged at 19,320 *g* for 30 seconds, and the flow-through was discarded. QG buffer (500 μl) and PE buffer (750 μl) (both contained in the kit) were successively added to the mixture, followed by centrifugation at 19,320 *g* for 30 seconds, and the flow-through was discarded. After the mixture was centrifuged for an additional 2 minutes at

19,320 *g*, the QIAquick column was placed into a new clean microcentrifuge tube (1.5 ml) and 50 μl buffer EB (10 mM Tris·Cl, pH 8.5, contained in the kit) was added to the center of the column membrane and then incubated for 10 minutes prior to centrifugation at 19,320 *g* for 1 minute. Finally, the purified ucfDNA samples were stored at −80°C until required.

2.5. Real-time PCR

UcfDNA levels of IQGAP3, BMP4, and FAM107A were measured by real-time PCR using TB Green Premix Ex Taq (TAKARA BIO INC., Shiga, Japan) in microreaction tubes (Corbett Research; Qiagen), followed by assessment of average cycle threshold (CT) values, determined using a Rotor Gene 6000 instrument (Corbett Research, Mortlake, Australia). Each sample was analyzed in triplicate. Primers were designed from within single exon regions, and their sequences were as follows: (1) IQGAP3 (product, 101 bp) sense, 5′-CTGGAGGAGAGCCTTCGGAATG-3′ and antisense, 5′-AGCTGCTCCACATCGTAGATCT-3′; (2) BMP4 (product, 112 bp) sense, 5′-CCTAGCAAGAGTGCCGTCAT-3′ and antisense, 5′-GGCGCTCAGGATACTCAAGA-3′; and (3) FAM107A (product, 102 bp) sense, 5′-TACTCGGAGATCCAGAGGGAGC-3′ and antisense, 5′-CAGCAGCTTCTTGGGCTTGATG-3′. The total volume of real-time PCR reactions was 10 μl containing TB Green Premix Ex Taq, 10 pmol/μl each primer, and 2 μl purified ucfDNA sample. The real-time PCR conditions for amplification of the 3 gene products were as follows: 1 cycle of 2 minutes at 95°C; 45 cycles of 20 seconds at 95°C (denaturation), followed by 20 seconds annealing (IQGAP3, BMP4, and FAM107A at 58°C, 57°C, and 60°C, respectively), and 20 seconds at 72°C (extension). To determine IQGAP3 ucfDNA content relative to that of BMP4 or FAM107A, the following equations were used: (a) ΔC_T = ucfDNA IQGAP3 C_T − ucfDNA BMP4 C_T (or FAM107A); (b) relative ucfDNA content (ratio) = $2^{-\Delta CT}$; (c) take the natural log(ln) of the ratio = ln $2^{-\Delta CT}$.

2.6. Statistical analysis

The Mann-Whitney U-test was used to compare levels of ucfDNA 2-gene ratios between the BC and hematuria groups, and the Kruskal-Wallis test was used for comparisons of more than 2 groups. The chi-squared test was used to compare categorical covariates, such as sex. Two-group discriminant analysis, using a combination of IQGAP3/BMP4 and IQGAP3/FAM107A ratios, was used to generate log ucfDNA 2-gene ratio coefficients and develop a discriminant scores model (DS index), which was evaluated for BC diagnosis. Wilks' Λ was used to determine the statistical significance of the discriminant power of the DS index. The diagnostic performance of the DS index was evaluated using receiver operating characteristic (ROC) curves, and the area under the curve (AUC) was calculated to determine its diagnostic accuracy. Optimal sensitivity and specificity were measured using Youden Index analysis. For paired categorical variables, a McNemar test was performed to compare the sensitivity between the noninvasive DS index-based diagnostic tool and cytology. Statistical analyses were performed using IBM SPSS Statistics version 24.0 (IBM Corp., Armonk, NY), MedCalc version 18.2.1 (MedCalc Software, Mariakerke, Belgium), and GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA). $P < 0.05$ was considered to indicate significant variation.

3. Results

3.1. General characteristics of the study participants

The median ages (interquartile ranges) of 149 patients with BC (105 male and 44 female) and 125 with hematuria (80 male and 45 female) in the validation cohort were 69 (60−75.5) and 66 (53.5−75.5) years, respectively. Among cases with BC, 81 were diagnosed with NMIBC and 68 with MIBC. As shown in Table 1, there were no significant differences in age or sex between cases with BC and hematuria controls in either the screening or validation cohorts (each $P > 0.05$).

3.2. Screening test to determine ratios of candidate gene levels in ucfDNA from patients with BC and hematuria controls

Seven candidate down-regulated genes, AFF3, BMP4, COL6A1, COL6A2, FAM107A, HLA-DRA, and SRPX, were selected using a mRNA-based microarray screen, and their ratios with respect to IQGAP3 (an up-regulated gene) were compared to determine whether they were potentially valuable BC markers. IQGAP3/AFF3, IQGAP3/BMP4, IQGAP3/COL6A1, IQGAP3/COL6A2, IQGAP3/FAM107A, IQGAP3/HLA-DRA, and IQGAP3/SRPX expression level ratios in urine samples from BC and hematuria patients are presented in Fig. 2. Ratios of IQGAP3/BMP4 and IQGAP3/FAM107A in BC patients were significantly higher than those in hematuria patients ($P = 0.03$ and $P = 0.01$, respectively). Therefore, we tested the use of IQGAP3/BMP4 and IQGAP3/FAM107A ratios as diagnostic biomarkers to discriminate patients with BC from those with nonmalignant hematuria.

3.3. IQGAP3/BMP4 and IQGAP3/FAM107A ratios in ucfDNA in the validation cohort

IQGAP3/BMP4 and IQGAP3/FAM107A ratios were evaluated in urine samples from 254 (142 BC and 112 hematuria) and 239 (138 BC and 101 hematuria) patients,

Table 1
Baseline characteristics of the study participants.

Variables (%)	Screening test		*P* value	Validation cohort		*P* value
	BC cases	Hematuria controls		BC cases	Hematuria controls	
No. of patients	40	41		149	125	
Age (y), median (IQR)	70 (63–76.5)	73 (59–79.5)	0.467[a]	69 (60–75.5)	66 (53.575.5)	0.119[a]
Sex			0.241[b]			0.255[b]
Male	30 (75.0)	35 (85.4)		105 (70.5)	80 (64.0)	
Female	10 (25.0)	6 (14.6)		44 (29.5)	45 (36.0)	
Tumor grade						
High	18 (45.0)			91 (61.1)		
Low	22 (55.0)			58 (38.9)		
T stage						
TaM0N0	2 (5.0)			28 (18.8)		
T1N0M0	29 (72.5)			53 (35.5)		
T2N0M0	3 (7.5)			34 (22.8)		
T3N0M0	2 (5.0)			5 (3.4)		
T4 or N1 or M1[c]	4 (10.0)			29 (19.5)		
Hematuria						
Gross		10 (24.4)			38 (30.4)	
Microscopic		31 (75.6)			87 (69.6)	
Cytology						
Atypical cell	8 (20.0)			64 (43.0)		
Positive	10 (25.0)			22 (14.8)		
Negative	15 (37.5)			40 (26.8)		
Unknown	7 (17.5)			23 (15.4)		

BC = bladder cancer; IQR = interquartile range.
[a] A Mann-Whitney U-test was performed for comparison of age between patients with BC and hematuria.
[b] Comparisons of sex frequencies of patients with BC and hematuria were performed using the chi-squared test.
[c] A total of 6 BC patients with M1 were performed palliative transurethral resection of bladder tumor.

respectively, after exclusion of samples generating inconsistent data (Fig. 1). Both gene expression ratios were significantly higher in patients with BC (including those with both NMIBC and MIBC) than in those with hematuria (including gross and microscopic hematuria; both $P < 0.001$; Fig. 3). These data suggest that both IQGAP3/BMP4 and IQGAP3/FAM107A ratios are associated with BC.

3.4. Diagnostic value of combined IQGAP3/BMP4 and IQGAP3/FAM107A ratios for discrimination of patients with BC and hematuria

We performed discriminant analysis using combined IQGAP3/BMP4 and IQGAP3/FAM107A expression level ratios (the DS index) as an independent variable, to evaluate its diagnostic value. The DS index was estimated by calculating as the sum of each score of 2- gene ratio, which was formed using each corresponding coefficient (and adding the constant). The statistical result showed that the discriminant function classified BC from hematuria well (Tables S1–S3).

$$\text{DS index} = -0.932 + 0.329 \times \text{IQGAP3/BMP4} + 1.109 \times \text{IQGAP3/FAM107A}$$

As shown in Fig. 4, the DS index values of patients with BC were significantly higher than those of hematuria controls ($P < 0.001$) and ROC curve analysis indicated good diagnostic performance, yielding an AUC of 0.862, sensitivity of 71.0%, and specificity of 88.6%. DS index values could also discriminate both NMIBC and MIBC from hematuria, with sensitivities of 61.5% and 80.3%, respectively, and specificities of 88.6% for both types of BC. Positive predictive values (PPVs) for discrimination of NMIBC and MIBC from hematuria were 80.0% and 84.1%, negative predictive values were 75.7% and 85.7%, and AUCs were 0.808 and 0.915, respectively (Table 2). Furthermore, the DS index sensitivity values for detection of NMIBC and MIBC among patients with gross hematuria reached 95.4% and 98.5%, respectively, while PPVs were 87.3% and 90.3% (Table 2).

3.5. Comparison of the diagnostic value of the noninvasive DS index for detecting BC with that of cytology

Selecting the most appropriate cut-off value is vital for effective clinical application of a diagnostic test. The optimal sensitivity and specificity for detecting BC were determined using a cut-off value of 0.095, which achieved sensitivities and specificities $>70.0\%$ for all groups (Table 3). Comparison of the diagnostic performance of the DS index (using this 0.095 cut-off value) with that of cytology showed that the sensitivity of the

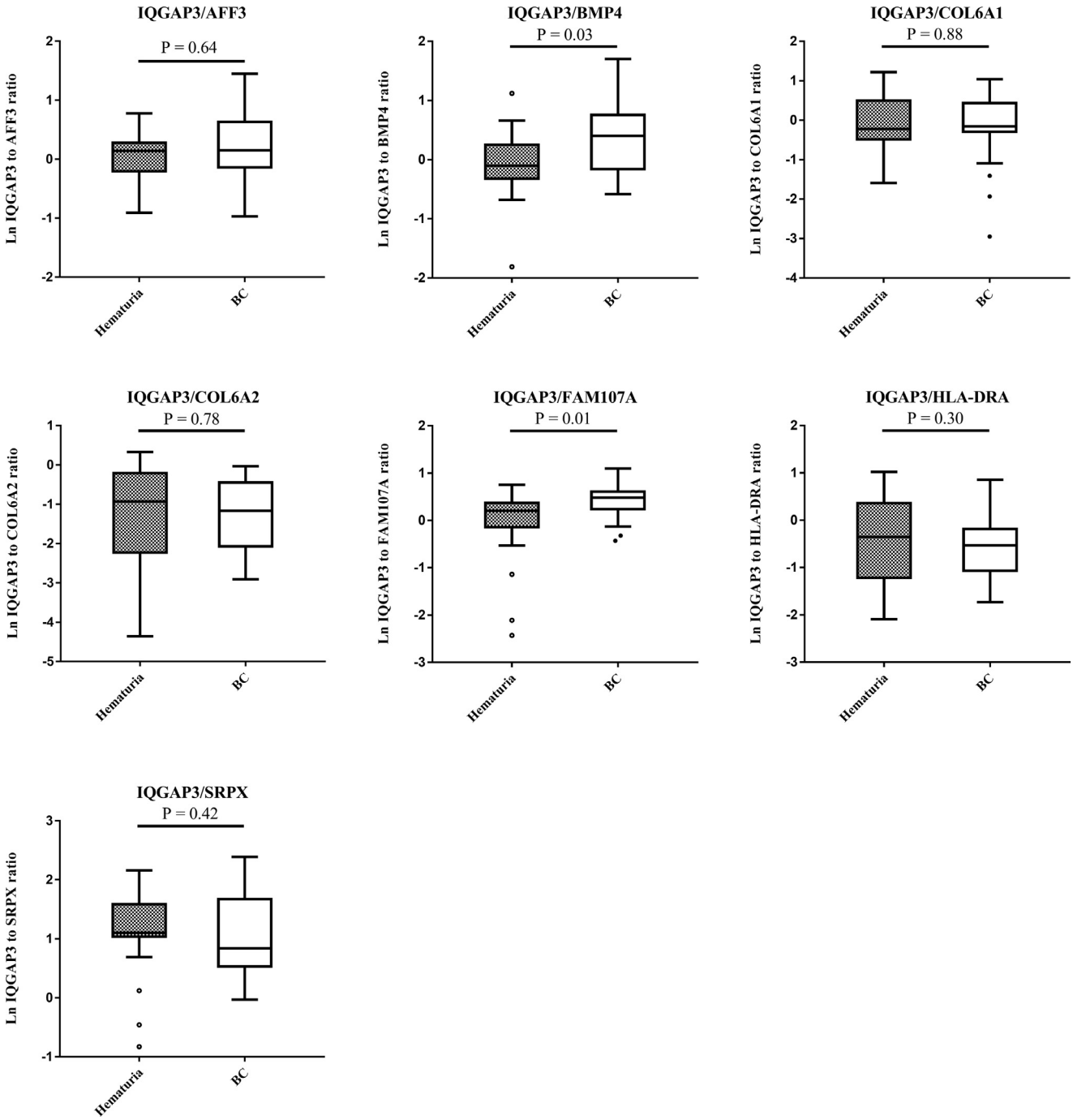

Fig. 2. Comparison of 2-gene expression level ratios in ucfDNA between patients with BC and hematuria by real-time polymerase chain reaction. Two-gene expression ratios using the up-regulated gene IQGAP3 as a numerator and various down-regulated genes (as indicated on the figure) as the denominator. BC = bladder cancer; UcfDNA = urinary cell-free DNA.

DS index (80.9%) was significantly higher than that of cytology (19.0%; $P < 0.0001$; Table 4).

4. Discussion

In this study, we determined the IQGAP3/BMP4 and IQGAP3/FAM107A ratios in ucfDNA in patients with BC and hematuria and developed a diagnostic model, the DS index, to discriminate patients with BC from those with hematuria, using a combination of these 2 ratios. The DS index has high power to discriminate BC from nonmalignant hematuria and has potential as a valuable noninvasive diagnostic tool for BC.

CfDNA is a topic of emerging interest in the field of cancer diagnosis. Relative to the extensive research into cfDNA in circulating blood, little is known regarding

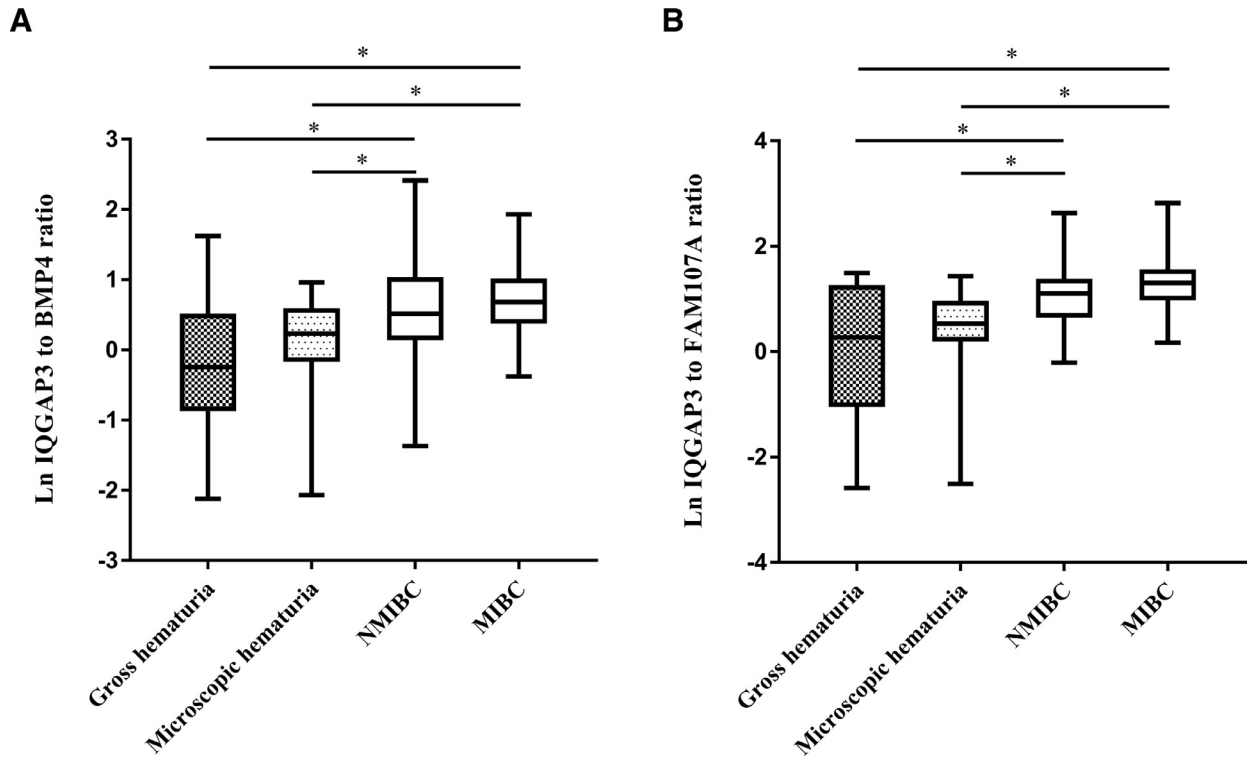

Fig. 3. IQGAP3/BMP4 and IQGAP3/FAM107A ratios in ucfDNA from patients with BC and hematuria. (A) IQGAP3/BMP4. (B) IQGAP3/FAM107A. NMIBC = nonmuscle invasive bladder cancer; MIBC = muscle invasive bladder cancer; UcfDNA = urinary cell-free DNA; $*P < 0.001$.

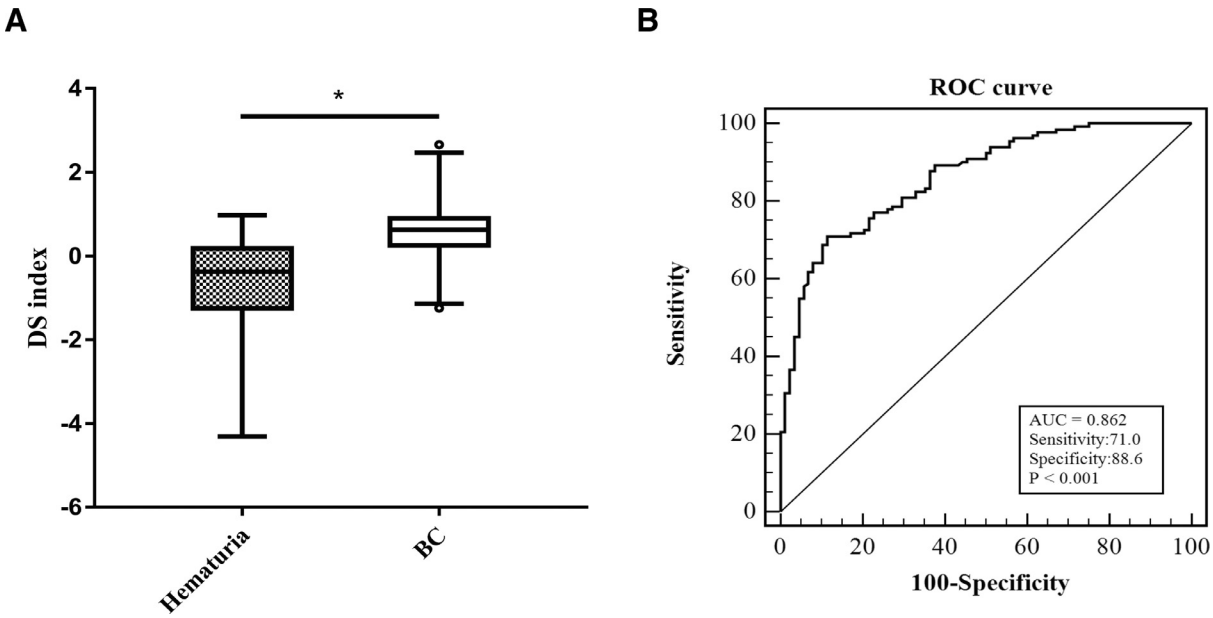

Fig. 4. DS index[a] levels and ROC curves for patients with BC and hematuria. (A) Comparison of DS index levels between patients with BC and hematuria. (B) ROC curve analysis of the discrimination of patients with BC and hematuria using DS index levels. [a]DS index is a discriminant scores model developed using the combined *IQGAP3*/*BMP4* and *IQGAP3*/*FAM107A* gene expression ratios. BC = bladder cancer; ROC curve = receiver operating characteristic curve. $*P < 0.001$.

ucfDNA. Since the majority of ucfDNA originates from the necrosis or apoptosis of exfoliated urinary tract cells, it is a potentially important noninvasive biomarker, particularly for urologic neoplasms [16]. Bryzgunova et al. reported that approximately 3×10^6 urethral epithelial cells can flow into the urine over a 24-hour period under normal circumstances [17]. In urinary tract diseases, particularly bladder or prostate tumors, which are in direct contact with urine,

Table 2
ROC curve analysis of ucfDNA expression ratios using the DS index[a].

Group	AUC	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	*P* value[b]
BC						
vs. hematuria	0.862	71.0	88.6	90.3	67.2	< 0.001
vs. gross hematuria	0.851	90.8	70.8	94.4	58.6	< 0.001
NMIBC						
vs. hematuria	0.808	61.5	88.6	80.0	75.7	< 0.001
vs. gross hematuria	0.816	95.4	62.5	87.3	83.3	< 0.001
MIBC						
vs. hematuria	0.915	80.3	88.6	84.1	85.7	< 0.001
vs. gross hematuria	0.886	98.5	70.8	90.3	94.4	< 0.001

AUC = area under the curve; BC = bladder cancer; MIBC = muscle invasive bladder cancer; NMIBC = non-muscle invasive bladder cancer; NPV = negative predictive value; PPV = positive predictive value; ROC = receiver operating characteristic; UcfDNA = urinary cell-free DNA.
[a] DS index is a discriminant scores model developed using the combined IQGAP3/BMP4 and IQGAP3/FAM107A expression level ratios.
[b] A Mann-Whitney U-test was used to compare ucfDNA expression ratio levels between the BC and nonmalignant hematuria groups.

Table 3
DS index[a] ROC analysis according to the optimal cut-off value.

ROC analysis	BC		NMIBC		MIBC	
	vs. hematuria	vs. gross hematuria	vs. hematuria	vs. gross hematuria	vs. hematuria	vs. gross hematuria
Sensitivity at the optimal cut-off (0.095)	80.9%	80.9%	72.3%	72.3%	89.4%	89.4%
Specificity at the optimal cut-off (0.095)	70.5%	75.0%	70.5%	75.0%	70.5%	75.0%

BC = bladder cancer; MIBC = muscle invasive bladder cancer; NMIBC = non-muscle invasive bladder cancer; ROC = receiver operating characteristic.
[a] DS index is a discriminant scores model developed using the combined IQGAP3/BMP4 and IQGAP3/FAM107A expression level ratios; ROC analysis results and optimal cut-off values for DS index in NMIBC or MIBC vs. hematuria and gross hematuria.

Table 4
Comparison of the sensitivity of bladder cancer detection methods.

Detection method	NMIBC			MIBC			Total
	Overall	Low grade	High grade	Overall	Low grade	High grade	
Cytology	18.1%	13.6%	25.0%	20.4%	22.2%	20.0%	19.0%
DS index[a]	72.3%	71.1%	74.1%	89.4%	75.0%	91.4%	80.9%
OR (95% CI)[b]							2.21 (0.47–10.41)
P value[b]							< 0.0001

CI = confidence interval; MIBC = muscle invasive bladder cancer; NMIBC = non-muscle invasive bladder cancer; OR = odds ratio.
[a] DS index is a discriminant scores model developed using the combined IQGAP3/BMP4 and IQGAP3/FAM107A expression level ratios.
[b] A McNemar test was used to compare the detection performance of the DS index with that of traditional cytology. *P* values < 0.05 were considered statistically significant.

tumor cells may also be released into the urine, indicating that the detection and analysis of ucfDNA in BC has potential clinical value.

IQGAP proteins comprise a newly discovered eukaryotic family, contain multiple protein interacting sequences, and regulate various cellular processes, including cytokinesis, extracellular signal migration, cell proliferation, and cytoskeletal dynamics [18–21]. There are 3 IQGAP genes in humans: IQGAP1, which is described as an oncogene; IQGAP2, a potential tumor suppressor [18,21]; and IQGAP3, which was isolated in 2007 and is thought to be overexpressed in malignant disease [22–24]. Kumar et al. reported that BC, as well as lung, liver, esophageal, breast, and colorectal cancers, exhibits significantly higher IQGAP3 mRNA expression levels relative to normal tissues [25]. Similarly, our previous study also identified significantly higher IQGAP3 expression levels in patients with BC than in controls with nonmalignant disorders, based on mRNA microarrays (data generated by real-time PCR). Moreover, IQGAP3 levels in ucfDNA were also significantly higher in patients with BC than in controls (including healthy tissue and hematuria controls) based on analysis using PicoGreen and RiboGreen, with adjustment for

reference genes [26]; however, in our subsequent investigations, we observed that ucfDNA concentrations detected by PicoGreen were unstable, leading to difficulties in normalizing levels of ucfDNA genes, indicating that the concentration of ucfDNA extracted from urine was too low for reliable measurement using PicoGreen. Consequently, a new approach for monitoring ucfDNA levels was necessary.

In this study, we used a 2-gene expression ratio method that did not require correction based on internal controls. Ma et al. discovered that HOXB13 was overexpressed in patients with breast cancer recurrence who were treated with tamoxifen therapy, while IL17BR was down-regulated in those patients, and suggested that a HOXB13/IL17BR 2-gene expression ratio can be a predictor of breast cancer recurrence [27,28]. Moreover, Yan et al. selected TSPAN13 (an up-regulated gene) and S100A9 (a down-regulated gene) as candidates in prostate cancer and compared the expression ratio of these 2 genes in tissue and urine nucleic acids between patients with prostate cancer and benign prostate hyperplasia, thereby demonstrating the potential for use of ucfDNA 2-gene expression ratios as diagnostic biomarkers [29]. These studies showed that 2-gene expression ratios, consisting of 1 up-regulated and 1 down-regulated gene, can maximize value and be tested in tissue and urine samples from patients with BC as a diagnostic marker. In the current study, we identified IQGAP3 (an up-regulated gene) as the numerator, whereas BMP4 and FAM107A (down-regulated genes) were used as denominators.

BMP4, a member of the BMP family, regulates stem cell regeneration and differentiation, and participates in vascular and bone formation [30]. BMP4 has differing effects in various malignant tumors, and its expression levels vary in normal and cancerous tissues. Alarmo et al. reported that BMP4 mRNA is overexpressed in gastrointestinal, lung, and ovarian cancers, whereas low expression was detected in prostate and BC [31]. Consistent with these findings, our data indicated that BMP4 mRNA expression was significantly lower in BC than in normal tissue ($P < 0.01$, data not shown). Therefore, we evaluated BMP4 as a down-regulated gene in our 2-gene expression ratio method to determine the reliability of ucfDNA for BC diagnosis.

FAM107A (also referred to as DRR1 and TU3A), a "family with sequence similarity 107" gene, is also considered a tumor suppressor because of its low expression levels in several types of cancer, including renal cell carcinoma [32], nonsmall cell lung cancer [33], and prostate cancer [34]. There are no previous reports of evaluation of the significance of FAM107A expression in BC. In our previous study, we first discovered that mRNA expression of FAM107A was lower in BC than in normal tissue using tissue microarrays ($P < 0.01$, data not shown); therefore, we also included FAM107A in our assay as a candidate down-regulated gene.

The majority of individuals with BC present as outpatients with painless hematuria; however, less than 5% of patients with hematuria are diagnosed with BC [35]. Thus, a noninvasive diagnostic tool for distinguishing BC from hematuria with other causes is urgently required. Laimonis et al. developed predictive models for detecting BC based on IGFBP5, HOXA13, MDK, CDK1, and CXCR2 expression data from voided urine samples, along with age, sex, frequency of gross hematuria, and smoking history, using data from 587 patients with gross hematuria. Although this model is highly sensitive, it is too complex and costly for implementation in clinical practice. In this study, we defined a clinical diagnostic model (DS index) using discriminant analysis of IQGAP3/BMP4 and IQGAP3/FAM107A ratios. This detection method is not only simple and inexpensive, but also has good diagnostic ability, with an AUC of 0.862, sensitivity of 71.0%, specificity of 88.6%, a PPV of 90.3%, and a negative predictive value of 67.2%.

To assess the diagnostic value of DS index, we compared its results with those of cytology. In the present study, the sensitivity and specificity for discriminating BC from hematuria were 80.9% and 70.5%, respectively, at the optimal cut-off value (0.095). The DS index also had superior diagnostic value relative to cytology. Additionally, all sensitivity and specificity values for comparisons of BC subgroups with hematuria were $>70\%$, suggesting that the novel DS index potentially represents a good biomarker for BC. In addition, in order to investigate whether pyuria affected the BC detection, we compared the DS index between BC and hematuria subgroup according to the presence or absence of pyuria in hematuria, and the ROC curve analysis revealed that the AUCs for detecting BC were 0.880 (BC vs. hematuria with no pyuria), and 0.837 (BC vs. hematuria with pyuria), respectively (data not shown).

This study had several limitations. First, it was a single-center study with a relatively small sample size. Hence, a larger, multicenter study will be required to validate our findings. Second, although we compared results using these new noninvasive biomarkers with those of cytology, we only compared the sensitivity of the 2 methods, as there were no cytology data for our control group. Third, the sensitivity results of cytology in our study were extremely low compared to those represented in published literature, as the lack of clear guidelines for cytological results reported as "atypical cell" in clinical practice made it difficult to manage such cases. Therefore, evaluation of a correlation between cytologic and the corresponding histologic interpretations will be needed to solve this problem.

5. Conclusions

IQGAP3/BMP4 and IQGAP3/FAM107A expression level ratios in ucfDNA from patients with BC are both significantly higher than those in individuals with hematuria.

The noninvasive biomarker, DS index, developed in this study exhibits good diagnostic performance.

Conflict of interest

The authors declare no conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.urolonc.2018.10.023.

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