IJC
International Journal of Cancer

Urinary cell-free microRNA biomarker could discriminate bladder cancer from benign hematuria

Xuan-Mei Piao[1], Pildu Jeong[1], Ye-Hwan Kim[1], Young Joon Byun[1], Yanjie Xu[2], Ho Won Kang[1], Yun-Sok Ha[3,4], Won Tae Kim[1], Jong-Young Lee[5,6], Seung Hwo Woo[7], Tae Gyun Kwon[3,4], Isaac Y. Kim[8], Sung-Kwon Moon[9], Yung Hyun Choi[10], Eun-Jong Cha[11], Seok Joong Yun[1] and Wun-Jae Kim[1]

[1]Department of Urology, College of Medicine, Chungbuk National University, Cheongju, South Korea
[2]Department of Surgery, College of Medicine, Chungbuk National University, Cheongju, South Korea
[3]Department of Urology, School of Medicine, Kyungpook National University, Daegu, South Korea
[4]Department of Urology, Kyungpook National University Hospital, Daegu, South Korea
[5]Department of Business Data Convergence, Chungbuk National University, Cheongju, South Korea
[6]Theragen Etex Bio Institute, Suwon, 443-270, South Korea
[7]Department of Urology, Eulji University Hospital, Daejeon, South Korea
[8]Section of Urologic Oncology and Dean and Betty Gallo Prostate Cancer Center, The Cancer Institute of New Jersey and Robert Wood Johnson Medical School, New Brunswick, New Jersey, USA
[9]Department of Food Science and Technology, Chung-Ang University, Ansung, 456-756, South Korea
[10]Department of Biochemistry, College of Oriental Medicine, Dong-Eui University, Busan, South Korea
[11]Department of Biomedical Engineering, Chungbuk National University College of Medicine, Cheongju, South Korea

The most common symptom of bladder cancer (BC) is hematuria. However, not all patients with hematuria are diagnosed with BC. Here, we explored a novel method to discriminate BC from hematuria under nonmalignant conditions by measuring differences in urinary cell-free microRNA (miRNA) expression between patients with BC and those with hematuria. A multicenter study was performed using 543 urine samples obtained from the National Biobank of Korea, including 326 BC, 174 hematuria and 43 pyuria without cancer. The urinary miR-6124 to miR-4511 ratio was considerably higher in BC than in hematuria or pyuria, and enabled the discrimination of BC from patients with hematuria at a sensitivity of >90% ($p < 0.001$). Conclusively, the proposed noninvasive diagnostic tool based on the expression ratio of urinary cell-free miR-6124 to miR-4511 can reduce unnecessary cystoscopies in patients with hematuria undergoing evaluation for BC, with a minimal loss in sensitivity for detecting cancer.

Key words: noninvasive biomarker, miRNA, bladder cancer, hematuria

Abbreviations: AUC: area under the curve; BC: bladder cancer; MIBC: muscle invasive bladder cancer; miRNA: microRNA; NMIBC: nonmuscle invasive bladder cancer; ROC: receiver operating characteristics; RT-PCR: real-time polymerase chain reaction; VUC: Voided urine cytology

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: International Science and Business Belt Program through the Ministry of Science, ICT and Future Planning ; **Grant numbers:** 2017K000490; **Grant sponsor:** Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education; **Grant numbers:** 2017R1D1A3B03031486; **Grant sponsor:** ChungBuk Bio-International Joint Research Support Project funded by ChungCheongBuk-Do ; **Grant numbers:** CB-BIO-2017-2-011
DOI: 10.1002/ijc.31849
History: Received 14 Jun 2018; Accepted 27 Aug 2018; Online 5 Sep 2018

Correspondence to: Wun-Jae Kim, Department of Urology, College of Medicine, Chungbuk National University, Chungdae-ro 1, Seowon-Gu, Cheongju, Chungbuk 28644, South Korea, Tel.: +82-043-269-6136, E-mail: wjkim@chungbuk.ac.kr

Introduction

Hematuria is a key feature of bladder cancer (BC) in most cases; however, it is also caused by benign diseases such as glomerulonephritis, benign prostate hyperplasia, infection, or urinary calculi.[1,2] Voided urine cytology (VUC) is commonly used for the detection of BC in patients with hematuria after the authorized guidelines.[2] Although cytology is a good method for detecting high grade BC, it is not adequate for low grade BC because of poor sensitivity and overall accuracy. Cystoscopy under local anesthesia is the gold standard method for BC detection. However, this method is invasive, painful and expensive. These issues led to the development of urinary biomarker technologies, although few markers are approved by the FDA for the diagnosis of BC including NMP22, BTA, UroVysion, and ImmuoCyt. These markers are limited by low specificity and high false positive rates in discriminating among BC, hematuria, or pyuria; for instance, false positive NMP22 results are observed in >85% of patients with hematuria.[3,4] Thus, an accurate and noninvasive detection method is urgently needed for discriminating BC among patients with hematuria to reduce the burden of these patients.

What's new?

Noninvasive urinary biomarkers are promising tools for distinguishing nonmalignant hematuria from hematuria associated with bladder cancer during primary patient evaluation. MicroRNAs (miRNAs), which function as oncogenes or tumor suppressors in cancer and are highly stable in body fluids, are emerging markers for this purpose. In the present study, the expression ratio between two urinary cell-free miRNAs, miR-6124 and miR-4511, was found to be significantly higher in patients with bladder cancer than in those with hematuria or pyuria. The findings suggest that noninvasive assessment of miR-6124/miR-4511 expression ratio could help prevent unnecessary cystoscopy procedures when discriminating benign from malignant hematuria.

Recent findings indicate the value of microRNAs (miRNAs) as diagnostic markers in many cancers.[5] Studies show that miRNAs play important roles as oncogenes or tumor suppressors.[6] miRNAs are small noncoding RNA molecules that function in RNA silencing and post-transcriptional regulation of gene expression.[7] miRNAs exist in a highly stable status in body fluids, and resistance against degrading enzymes is conferred by enclosure of miRNAs in small vesicles such as apoptotic bodies, microvesicles, or exosomes.[8] Therefore, alterations of urinary miRNAs in cancer patients support their putative role as noninvasive cancer biomarkers. Although urinary miRNAs have been investigated as promising diagnostic and prognostic indicators of malignancy, the technology requires improvement before its clinical application.[9–11] Because no consensus housekeeping miRNAs have been identified to date, alternative markers such as U6 miRNA are used as internal controls.[12–14] However, test results vary according to the used internal control. In our study, we applied the ratio of up- and down-expressed miRNAs, which was not influenced by the unstably expressed housekeeping marker as an internal control. The expression ratio of up- and down-regulated miRNAs in urine were compared between BC, hematuria and pyuria, and the value of using urinary cell-free miRNAs as diagnostic biomarkers of BC was assessed.

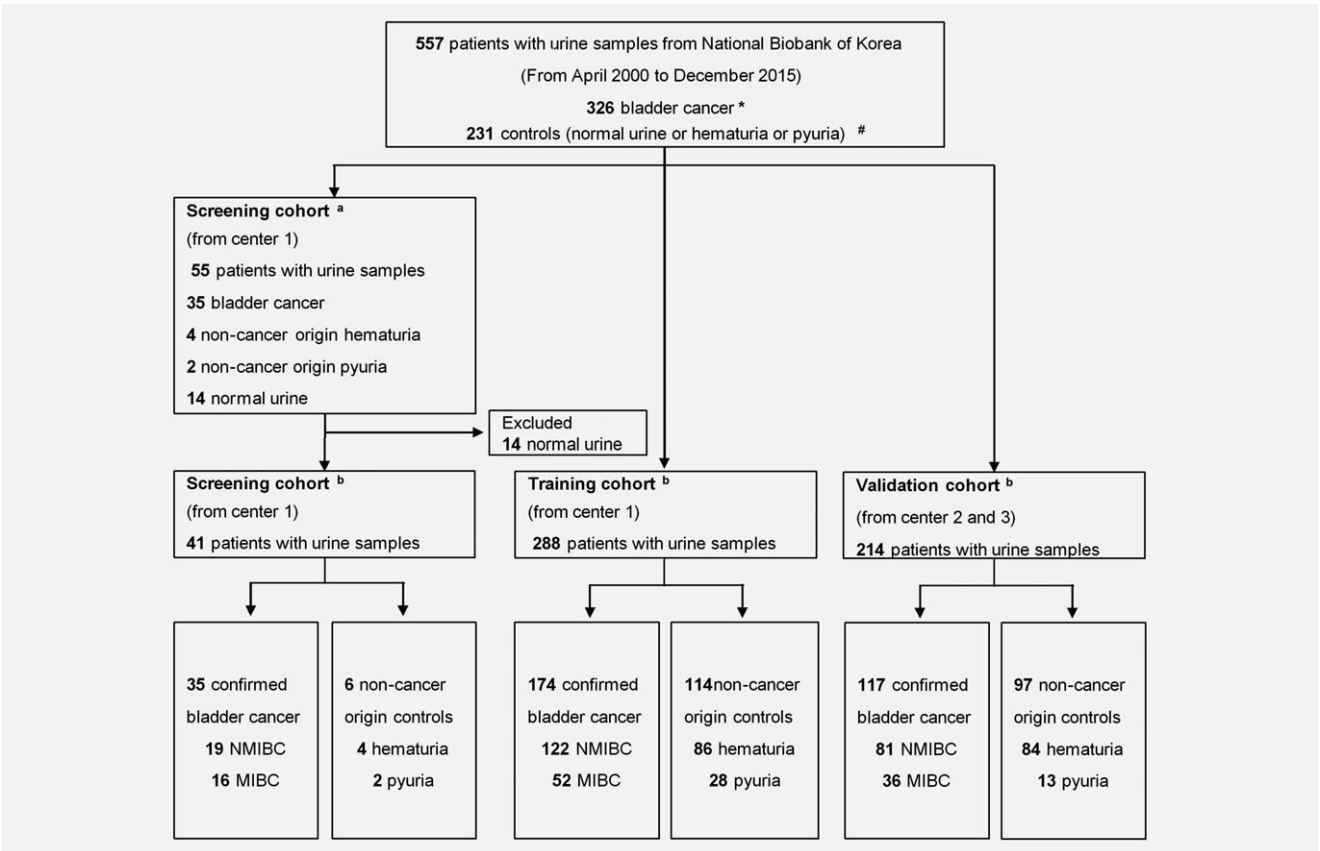

Figure 1. Overview of the study design. *Cases histologically verified as urothelial carcinoma and to reduce confounding factors affecting the analyses, and to delineate a more homogeneous study population, any patients diagnosed with another cancers before or after urothelial carcinoma diagnosis were excluded. #Patients with benign prostate hyperplasia, stress urinary incontinence, urolithiasis, urinary tract infection and patients who referred for hematuria evaluation. [a] miRNA microarray based cohort. [b] Real-time polymerase chain reaction based cohorts. NMIBC, nonmuscle invasive bladder cancer; MIBC, muscle-invasive bladder cancer.

Table 1. Clinicopathological features of BC patients and controls examined in the urine miRNA expression study

Variables	Screening cohort		Training cohort		Validation cohort	
	BC	control	BC	control	BC	control
No.	35	6	174	114	117	97
Mean age ± SD	66.63 ± 13.06	60 ± 11.47	65.92 ± 12.70	68.02 ± 10.50	66.86 ± 13.13	71.49 ± 8.28
Gender (%)						
Male	29 (82.9)	4 (66.7)	135 (77.6)	92 (80.7)	98 (83.8)	96 (99.0)
Female	6 (17.1)	2 (33.3)	39 (22.4)	22 (19.3)	19 (16.2)	1 (1.0)
Grade (%)						
Low	16 (45.7)		90 (51.7)		63 (53.8)	
High	19 (54.3)		84 (48.3)		54 (46.2)	
Stage (%)						
TaN0M0	8 (22.9)		46 (26.4)		66 (56.4)	
T1N0M0	11 (31.4)		76 (43.7)		15 (12.8)	
T2N0M0	4 (11.4)		18 (10.3)		21 (17.9)	
T3N0M0	2 (5.7)		5 (2.9)		2 (1.7)	
T 4 or N 1 or M1	10 (28.6)		29 (16.7)		13 (11.1)	
Cytology (%)						
Negative	11 (31.4)		54 (31.0)		47 (40.2)	
Atypical urothelial cells	19 (54.3)		69 (39.7)		41 (35.0)	
Positive	3 (8.6)		25 (14.4)		7 (6.0)	
Not tested	2 (5.7)		26 (14.9)		22 (18.8)	
Pyuria only (%)		2 (33.3)		28 (24.6)		13 (13.4)
Hematuria (%)		4 (66.7)		86 (75.4)		84 (86.6)
Microscopic hematuria		3		67		46
Gross hematuria		3		19		38

BC, bladder cancer; RBC, red blood cell; WBC, white blood cell; HPF, high-power field; SD, standard deviation.

Material and Methods

Study design

The workflow and overall study design are shown in Figure 1. Urine samples were obtained from different centers of the National Biobank of Korea between April 2000 and December 2015, and were allocated in chronological order. Urine samples from center 1 were used in the screening and training cohorts, and urine from centers 2 and 3 were used in the validation cohort. Candidate miRNAs were identified by miRNA microarray screening. miRNA microarray profiling on the basis of *p*-value above 10^{-4}, logfold change> ± 3 identified 109 miRNAs have different expression between bladder cancer urine and control urine (hematuria, pyuria, normal urine). We chose the uplinked 27 miRNAs (15 upregulated and 12 downregulated in urine from BC patients) among 109 miRNAs according to the flexible and adaptive test, which results include P, M, A or P (P, present call indicates the quality of measurement is good; A, absent call for bad; M, marginal call for intermediate). Markers were further selected from the 27 candidate miRNAs in a real-time polymerase chain reaction (RT-PCR)-based screening cohort. Subsequently, two potential miRNAs, hsa-miR-6124 (up-regulated in BC urine) and hsa-miR-4511(downregulated in BC urine), were measured in the training cohort. Finally, the performances of both miRNAs as BC detection markers were strengthened in the validation cohort.

Study population and urine samples

The microarray-based screening cohort included urine samples from 35 BC patients and 20 controls (14 normal urine, four hematuria and two pyuria). Of the 35 BC cases, 19 were non-muscle invasive bladder cancer (NMIBC) and 16 were muscle invasive bladder cancer (MIBC). To discriminate BC patients among those with hematuria, normal urine samples were excluded from further RT-PCR analysis. The training cohort consisted of 288 urine samples from 174 primary BC patients, including 122 NMIBC, 52 MIBC and 114 control subjects, which comprised 86 hematuria and 28 pyuria without cancer. There were 90 (51.7%) low grade and 84 (48.3%) high grade BC patients. The validation test included 214 urine samples from 117 primary BC patients (81 NMIBC and 36 MIBC) and 97 control subjects (84 hematuria and 13 pyuria); the BC samples comprised 63 low grade BC (53.8%) and 54 high grade BC (46.2%) (Table 1). Urine samples were collected in the morning, stored at 4 °C, and centrifuged at 2,500 rpm for 15 min. Supernatants were aliquoted into Eppendorf tubes and stored at −20 °C until use. BC samples were obtained from patients

who underwent transurethral resection of bladder tumor or radical cystectomy, and were histologically verified as urothelial carcinoma. To reduce confounding factors that may affect the analyses, and to delineate a more homogeneous study population, patients diagnosed with another cancer before or after urothelial carcinoma diagnosis were excluded. Control urine samples were collected from patients with noncancer origin hematuria or pyuria such as benign prostate hyperplasia, stress urinary incontinence, urolithiasis and urinary tract infection, and patients who were referred for hematuria evaluation.

The collection and analysis of all samples were approved by the Institutional Review Board of Chungbuk National University (GR2010-12-010), and written informed consent was obtained from each subject. The study was performed in compliance with the requirements of the respective institutional review boards.

Microarrays

Total RNA from each sample was extracted using the miRNA Microarray System labeling kit (Illumina, San Diego, CA), and RNA quantity and integrity were examined with the RNA 6000 Pico Chip Kit (Agilent Technologies, Santa Clara, CA) and Agilent 2100 Bioanalyzer. miRNA profiling was performed by the Agilent Human miRNA Microarray Release 16.0 platform, which contains 1,205 human and 144 viral miRNAs.[15] The protocol used to generate microarray gene expression datasets is provided in reference.[16]

miRNA extraction from urine

A Genolution urine miRNA purification kit (Genolution Pharmaceuticals Inc., Seoul, Korea) was used to purify the urine samples. A volume of 500 μL supernatant from each urine sample was added to a tube containing the Genolution proprietary miRNA separation solution and vortexed for 20 sec. Next, 200 μL chloroform was added and vortexed for 10 sec, followed by centrifugation at 13,000 rpm for 10 min at 4 °C. A sample of 650 μL of the top aqueous phase was removed without disturbing the white precipitate and transferred into a new 1.5 mL tube, and 0.8 mL isopropanol was added, followed by centrifugation for 20 min at 15,000 rpm at 4 °C. The solution was decanted by tilting the tube in the opposite direction of the expected RNA pellet, and 500 μL of 70% EtOH was added and the sample was spun for 20 min at 15,000 rpm at 4 °C. After removing the leftover ethanol, the pellet was dissolved in 40 μL RNase-free water and stored at −80 °C until use.

cDNA synthesis of urine miRNA

The Mir-X™ miRNA First Strand cDNA Synthesis Kit (Clontech, TAKARA, Otsu, Japan) was used for synthesis of cDNA from the extracted miRNAs. In each RNase-free 0.2 mL tube, 5 μL mRQ Buffer (2×), 3.75 μL miRNA sample and 1.25 μL mRQ Enzyme were added, and the reagents were mixed well for the thermocycler. The mixture was incubated for 1 h at 37 °C, terminated at 85 °C for 5 min to inactivate the enzymes, and placed on ice. Ninety microliters of ddH2O were added to bring the total volume to 100 μL.

Real-time polymerase chain reaction

RT-PCR was performed using a Rotor-Gene 6000 instrument (Qiagen, Hilden, Germany) to amplify urine miRNAs. Microreaction tubes (Qiagen, Hilden, Germany) containing SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA) were used for the RT-PCR reactions. The after forward primers were used for amplifying miRNAs: miR-6124: 5′-GGGAAAAG-GAAGGGGGAGGA-3′ (20 base pairs); miR-4511: 5'-GAA-GAACUGUUGCAUUUGCCCU-3′ (22 base pairs). After thawing the reagents and template cDNA, the PCR reaction was carried out in a final volume of 10 μL containing 5 μL of 2× QuantiTect SYBR Green PCR master mix, 0.2 μL of 10× miScript universal primer, 0.2 μL of 10 pmol forward primer, 2 μL template cDNA, and 2.6 μL RNase-free water. RT-PCR conditions were as follows: 45 cycles of 3 min at 95 °C and 20 sec at 60 °C, followed by one cycle of 60 sec at 95 °C, 30 sec at 55 °C, and 30 sec at 95 °C. The melting program was performed at 70–99 °C at a heating rate of 1 °C per 5 sec. Spectral data were captured and analyzed by using Rotor-Gene Q software 2.3.1.49. All samples were run in triplicate.

Statistical analysis

The Robust Multiarray Average, in the R package, was used to perform global correction, quantile normalization, and median Polish summarization. *P*-values (t test) were calculated from bead mRNA signal intensities.[17] The Chi-squared test was used to identify expression differences between BC and noncancer controls, and to compare our marker to VUC. receiver operating characteristics (ROC) curves were used to identify the optimal cutoff point for BC diagnosis that yielded the highest combined sensitivity and specificity. The Mann–Whitney *U* test was used to examine the expression level of urinary miRNA in BC *versus* control urine. Statistical analysis was performed using IBM SPSS Statistics ver. 20.0 (IBM Co., Armonk, NY), MedCalc software ver. 15.8 (MedCalc Software, Mariakerke, Belgium) and GraphPad Prism 7 (GraphPad Software, San Diego, Calif). Results with *p* values less than 0.05 were considered to be significant.

Results

Selection from miRNA microarray data

miRNAs differentially expressed in BC and control urine were extracted using a miRNA microarray assay, as described in the materials and methods section. The profiling analysis on the basis of *p*-value comparison, fold changes, and the flexible and adaptive test for gene sets[18] identified 27 miRNAs out of 109 miRNA lists with the potential to separate BC from controls (noncancerous patients with normal urine, hematuria, or pyuria). The relative expressions of these candidate miRNAs (15 upregulated and 12 downregulated in urine from BC patients) were then estimated using RT-PCR to determine their diagnostic ability (data not shown).

Confirmation by RT-PCR in the screening cohort

The expression ratio of urinary miR-6124 to miR-4511 was considerably higher in the BC groups ($p < 0.01$) than in the control groups (hematuria and pyuria) in the screening cohort (Fig. S1A, Supporting Information). In addition, the expression ratio of urinary miR-6124 to miR-4511 in NMIBC and MIBC urine was similar, and significantly higher than that in the control groups ($p < 0.05$ and <0.01, respectively) (Fig. S1B, Supporting Information). Therefore, miR-6124 and miR-4511 were selected and used for further analysis.

Validation in the training and validation cohorts

Figure 2 summarizes the expression ratio of urinary miR-6124 to miR-4511 in BC patients compared to controls (hematuria and pyuria) in the training and validation cohorts. The expression ratio of urinary miR-6124 to miR-4511 in NMIBC and MIBC was similar in both training and validation cohorts, which was significantly higher than that in the control groups including gross hematuria, microscopic hematuria and pyuria ($p < 0.05$ each).

The expression ratio of miR-6124 to miR-4511 was then plotted in ROC curves to assess the sensitivity and specificity when testing for BC. In the training cohort, the urinary miR-6124 to miR-4511 ratio could discriminate NMIBC and MIBC from hematuria with a sensitivity of 91.0% and 90.4%, respectively [area under the curve (AUC): 0.803 and 0.761] ($p < 0.001$ each) (Fig. 3 and Table S1, Supporting Information). In the validation cohort, ROC analysis showed that the sensitivity and specificity of urinary miR-6124 to miR-4511 ratio for detecting BC was 91.5% and 76.2%, respectively (AUC: 0.888) ($p < 0.001$), compared to that of patients with hematuria (Fig. 4 and Table S1, Supporting Information). The sensitivity increased to 94.0% ($p < 0.001$) for detecting BC among gross hematuria patients, and the sensitivity for discriminating NMIBC and MIBC from gross hematuria was 92.6% and 97.2%, respectively ($p < 0.001$ each). Nevertheless, the expression ratio of urinary miR-6124 to miR-4511 was not significantly related to clinical stage or grade subsets (data not shown).

Diagnostic power of urinary miR-6124 to miR-4511 ratio compared to VUC in detecting BC

The clinical application of a new diagnostic tool requires re-determination of the cutoff value according to the clinical situation. In our study, 284 BC patients were distinguished from 206 noncancerous patients with hematuria or pyuria derived from multicenter cohorts (training and validation cohorts) with a sensitivity of 78.5% and specificity of 70.9% (AUC: 0.810) ($p < 0.001$) (Fig. S2, Supporting Information) using the optimal cutoff value of 1.9111. Table S2, Supporting Information, shows the sensitivity and specificity for detecting BC obtained with a cutoff value of 1.9111. The sensitivity and specificity were higher than 70.0% in all groups.

The urinary miRNA biomarker was compared to VUC in the multicenter cohorts (Table S3, Supporting Information). The sensitivity of VUC increased from 7.8% to 25.0% in correlation with increasing grade of BC. The sensitivity of the urinary miRNA biomarker was significantly higher than that of VUC ($p < 0.001$) and it could detect BC in any grade with a sensitivity of >70.0%.

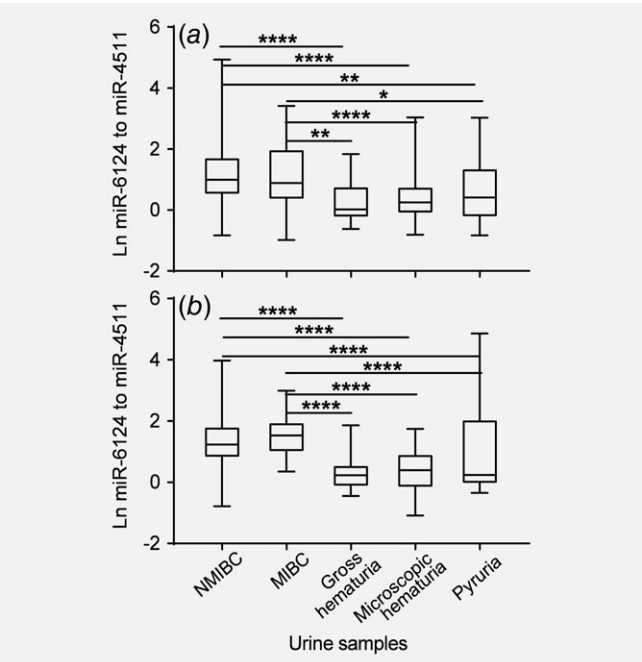

Figure 2. Expression ratio of urinary miR-6124 to miR-4511 of different urine samples in different cohorts. (*a*), training cohort. (*b*), validation cohort. BC, NMIBC, and MIBC indicate bladder cancer, nonmuscle invasive bladder cancer and muscle invasive bladder cancer, respectively. *p* value was determined by Mann–Whitney test. $*p < 0.05$, $**p < 0.01$, and $****p < 0.0001$.

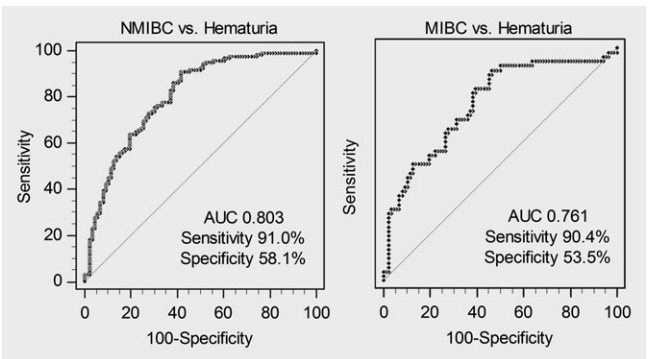

Figure 3. Receiver operating characteristic curve for discrimination of BC from from hematuria in the training cohort. AUC, area under the curve. NMIBC and MIBC indicate the nonmuscle invasive bladder cancer and muscle invasive bladder cancer, respectively.

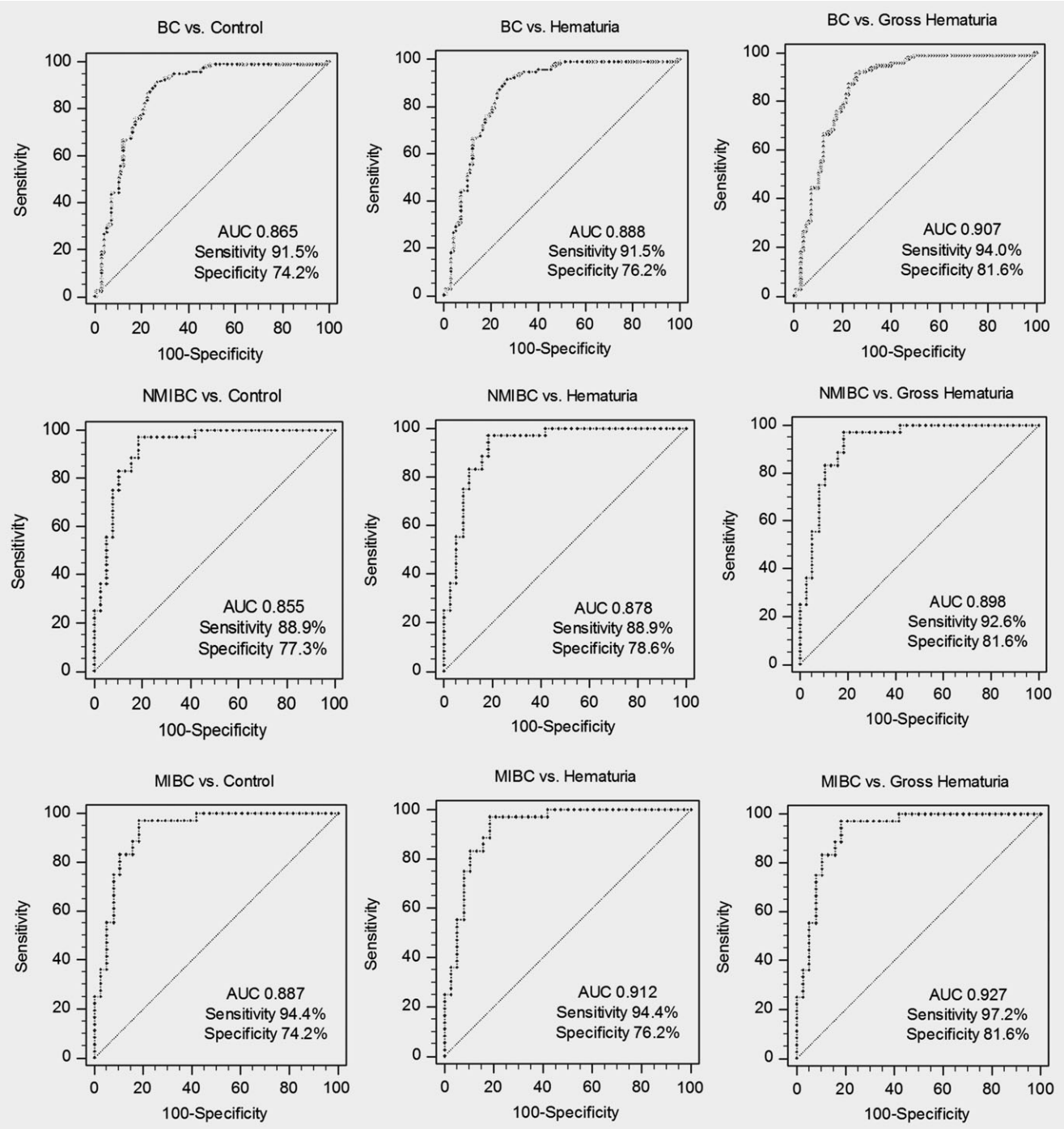

Figure 4. Receiver operating characteristic curve for discrimination of BC from control in validation cohort. AUC, area under the curve. BC, NMIBC, and MIBC indicate the bladder cancer, nonmuscle invasive bladder cancer, and muscle invasive bladder cancer, respectively. Control indicates hematuria and pyuria.

Diagnosis of BC depending on cutoff-related scores from urinary miR-6124 to miR-4511 ratio

To discriminate BC from hematuria according to the cutoff ranges of miR-6124 to miR-4511 expression ratio, a scoring system was used to analyze the relationship between the scoring system and sensitivity and specificity. Figure 5 and Table S4, Supporting Information, show the scores from 1 to 10 according to the cutoff ranges for discriminating BC from hematuria, which indicated that patients with a high score have a higher risk of BC. A score of 10, which corresponded

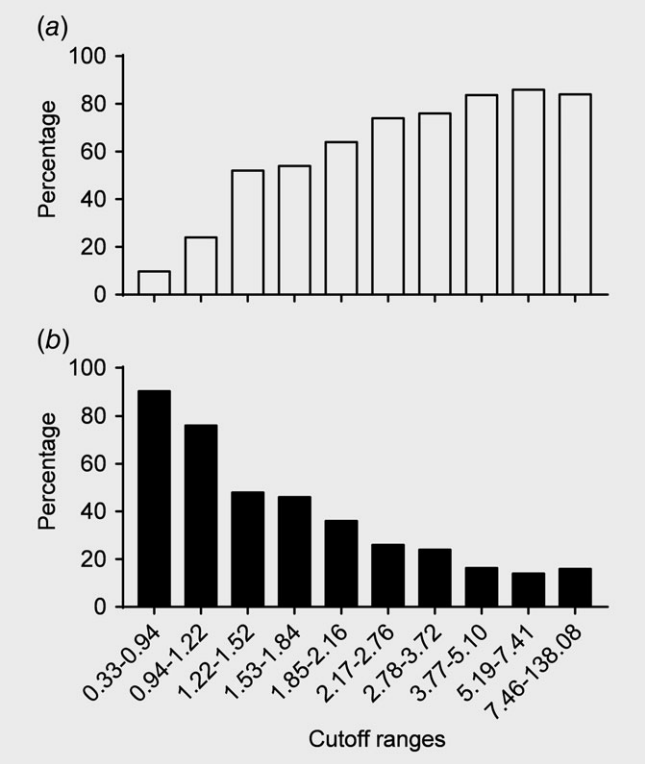

Figure 5. Distribution of cutoff ranges according to expression ratio of urinary cell-free miR-6124 to miR-4511 in bladder cancer diagnosis. (*a*), Bladder cancer. (*b*), Control (hematuria+pyuria).

to a cutoff range of >7.46, indicated that 84% of patients with BC were detected, whereas a score of 1, which corresponded to a cutoff range of <0.94, indicated that 90.2% of patients with hematuria could avoid being misdiagnosed as BC.

Discussion

Our study identified urinary miRNAs as a promising noninvasive biomarker for diagnosing BC among patients with hematuria. compared to previous urine biomarkers, miRNAs are relatively stable and can be easily extracted from circulating body fluids, small biopsies, frozen samples and even formalin-fixed/paraffin-embedded tissues.[11,19–21] In addition, miRNAs can be measured by small RNA isolation, PCR, and next-generation sequencing. Because of these special features, miRNAs are potential diagnostic markers.

Current guidelines support the use of VUC with cystoscopy for the diagnosis of BC.[22] Cystoscopy is an invasive approach associated with the risk of infection and trauma. Although VUC is noninvasive, its sensitivity is limited, especially in low grade BC.[23] Many urinary biomarkers have been tested in trials for many decades; however, none of these markers are currently used for the diagnosis of BC because of poor sensitivity and high false positive rates.[24]

Most miRNA studies use U6, the endogenous control RNA, to normalize miRNA expression; however, its use as a housekeeping marker is controversial.[10,20] We therefore designed a new method by measuring the expression of upregulated and downregulated miRNAs and investigated whether the ratio of up- to downregulated miRNAs in urine differed between patients with BC and controls (hematuria and pyuria). The results showed that the expression ratio of urinary miR-6124 to miR-4511 was higher in patients with BC than in those with nonmalignancy-related hematuria. ROC analysis showed that the urinary miRNA ratio detected the presence of BC with a sensitivity of >90% in both training and validation cohorts, whereas VUC identified only 12.3% of NMIBC and 16.7% of MIBC patients. Additionally, we have tried to assess NMP22 levels in our study. However, in this multicenter cohort, NMP22 were test in only 120 BC patients, among them, 25 patients (20.83%) showed positive result and 95 patients (79.17%) showed negative results (data not shown).

Among patients with hematuria, only approximately 10% are diagnosed with BC; therefore, researchers have investigated methods to discriminate patients with BC from those with nonmalignant hematuria. However, a clinically applicable method has not been reported, and most are limited by low sensitivity or specificity, whereas some are too complex for clinical use.[9,25] A study proposed measuring the expression of IGFBP5, HOXA13, MDK, CDK1 and CXCR2 in a voided urine sample (genotypic), and used age, gender, frequency of gross hematuria and smoking history (phenotypic) data from 587 patients with gross hematuria to develop predictive models for BC.[26] Another study investigated a high-throughput target bisulfite sequencing assay, UroMark, to probe cancer epigenetic alterations in urinary sediments.[27] They designed a 150 loci panel using a genome-wide DNA methylation profiling assay for the detection of BC in urinary sediment cells. And CxBladder as well as ASSUREMDx test were evaluated as available test to aid in the decision for cystoscopy. CxBladder assay was to quantify mRNA levels of HOXA13, CDC2, IGFBP5, MDK and CXCR2 genes; and ASSUREMDxBladder test was to analyze gene mutation as well as DNA methylation of TWIST1, ONECUT2 and OTX1 genes. Although the data showed high sensitivity and specificity, there were too many variables, which resulted in a complicated and costly assay. compared to previously reported assays, the test proposed in our study is unique for several reasons.[9,26,27] Our urinary miRNA biomarker could discriminate BC in patients with hematuria by estimating the expression levels of only two miRNAs in a large number of multicenter clinical samples (326 BC samples, 174 hematuria and 43 pyuria). This method is a simpler and cost-effective assay. Analysis of both training and validation cohorts estimated the majority of BC with a positive predictive value of 78.5% and a negative predictive value of 70.4% using the expression ratio of urinary miR-6124 to miR-4511.

Unlike the serum PSA test or histopathological Gleason score, which is used for the detection of prostate cancer, a scoring system for BC screening or diagnosis according to

different cutoff ranges has not been reported. In our study, the sensitivity and specificity for discriminating BC among patients with hematuria were 78.5% and 74.1%, respectively, at a single cutoff value of 1.9111 (Table S2, Supporting Information). To date, there are no available noninvasive biomarkers with sensitivity and specificity of >70.0%. However, a precise method with a high sensitivity is needed as an alternative to invasive cystoscopy for BC diagnosis. Our study attempted to find an efficient method using a scoring system based on the cutoff ranges of the expression ratio of two miRNAs. The scoring was subdivided into 10 cutoff ranges for the accurate diagnosis of BC, and the results showed that 85.0% of BC patients were detected at a cutoff range of >5.19, namely, at scores of 9–10. In addition, only 9.8% of patients with hematuria were misdiagnosed with BC at a score of 1, which corresponded to a cutoff range of <0.94.

Before referral for cystoscopy, patients are stratified by undergoing urinary tests such as VUC or NMP22; however, because of the low sensitivity and high false positive rates of these tests, most patients require cystoscopy for accurate diagnosis.[28] Our study identified a promising urinary biomarker for BC diagnosis that could discriminate BC among patients with hematuria, which may minimize the need for invasive procedures and reduce unnecessary cystoscopy rates.

BC, especially NMIBC, tends to recur at a rate of 30–70% despite the successful removal of tumors by transurethral resection and the progression rate is 10–30%.[29–33] Therefore, not only the diagnosis but also patient monitoring is essential for the early detection of these events. Urine cytology combined with cystoscopy every 3 months for the first 2 years after the diagnosis of primary BC and at a reduced interval in subsequent years is currently the standard of care at most institutions.[33] This may become a burden for patients who need to undergo several costly and invasive procedures per year. Noninvasive and cost-effective procedures such as BTA stat, BTA TRAK and NMP22 are currently under development to detect tumor recurrence.[34] However, these markers are not sensitive enough to replace cystoscopy, and avoiding unnecessary cystoscopies by an accurate, cost-effective and simple urinary test remains an unresolved issue. A follow-up study is necessary to validate our urinary miRNA marker for predicting the recurrence and progression of primary BC.

Acknowledgements

Urine samples were provided by the Chungbuk National University Hospital and Kyungpook National University Hospital, members of the National Biobank of Korea, which is supported by the Ministry of Health, Welfare, and Family Affairs. Subjects were also obtained from Eulji University Hospital. All samples derived from the National Biobank of Korea were obtained with informed consent under Institutional Review Board-approved protocols.

Author contributions

X.M. P, P. J, S.J.Y and W.J. K designed the study and all experiments; X.M. P performed all experiments; Y.H. K, Y.J. B, Y. X, Y.S. H, S.H. W, T.G. K and S.K. M collected patient samples; X.M. P, H.W. K and W.T. K assisted with data collection; X.M. P, J.Y.L, I.Y. K, E.J. C and S.J.Y analyzed the data; W.J. K and W.T. K provided funding; X.M. P, S.J. Y and W.J. K wrote the paper.

Conflict of interest

There are no conflict of interest to be reported.

References

1. Kelly JD, Fawcett DP, Goldberg LC. Assessment and management of non-visible haematuria in primary care. *BMJ* 2009;338:a3021.
2. Davis R, Jones JS, Barocas DA, et al. Diagnosis, evaluation and follow-up of asymptomatic microhematuria (AMH) in adults: AUA guideline. *J Urol* 2012;188:2473–81.
3. Budman LI, Kassouf W, Steinberg JR. Biomarkers for detection and surveillance of bladder cancer. *CUAJ* 2008;2:212–NaN.
4. Atsü N, Ekici S, Öge Ö, et al. False-positive results of the NMP22 test due to hematuria. *J Urol* 2002; 167:555–8.
5. Yun SJ, Jeong P, Kim W-T, et al. Cell-free microRNAs in urine as diagnostic and prognostic biomarkers of bladder cancer. *Int J Oncol* 2012;41: 1871–8.
6. Croce CM. Causes and consequences of microRNA dysregulation in cancer. *Nat Rev Genet* 2009;10:704–14.
7. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004;116:281–97.
8. Kosaka N, Iguchi H, Ochiya T. Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis. *Cancer Sci* 2010; 101:2087–92.
9. Miah S, Dudziec E, Drayton R, et al. An evaluation of urinary microRNA reveals a high sensitivity for bladder cancer. *Br J Cancer* 2012;107: 123–8.
10. Hanke M, Hoefig K, Merz H, et al. A robust methodology to study urine microRNA as tumor marker: microRNA-126 and microRNA-182 are related to urinary bladder cancer. *In Urol Oncol-Semin Ori Elsevier* 2010; 28:655–1.
11. Kim SM, Kang HW, Kim WT, et al. Cell-free microRNA-214 from urine as a biomarker for non-muscle-invasive bladder cancer. *Korean J Urol* 2013;54:791–6.
12. Xiang M, Zeng Y, Yang R, et al. U6 is not a suitable endogenous control for the quantification of circulating microRNAs. *Biochem Biophys Res Commun* 2014;454:210–4.
13. Zhou X, Zhang X, Yang Y, et al. Urinary cell-free microRNA-106b as a novel biomarker for detection of bladder cancer. *Med Oncol* 2014; 31:1–7.
14. Wang J, Zhang X, Wang L, et al. Downregulation of urinary cell-free microRNA-214 as a diagnostic and prognostic biomarker in bladder cancer. *J Surg Oncol* 2015;111:992–NaN.
15. Wang H, Ach RA, Curry B. Direct and sensitive miRNA profiling from low-input total RNA. *RNA* 2007;13:151–9.
16. Takeshita N, Hoshino I, Mori M, et al. Serum microRNA expression profile: miR-1246 as a novel diagnostic and prognostic biomarker for oesophageal squamous cell carcinoma. *Br J Cancer* 2013;108:644–52.
17. Irizarry RA, Hobbs B, Collin F, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* 2003;4:249–64.
18. Huang J, Wang K, Wei P, et al. FLAGS: a flexible and adaptive association test for gene sets using summary statistics. *Genetics* 2016;202:919–29.
19. Xi Y, Nakajima G, Gavin E, et al. Systematic analysis of microRNA expression of RNA extracted from fresh frozen and formalin-fixed paraffin-embedded samples. *RNA* 2007;13:1668–74.
20. Yun SJ, Jeong P, Kang HW, et al. Urinary MicroRNAs of prostate cancer: virus-encoded hsv1-miRH18 and hsv2-miR-H9-5p could be valuable diagnostic markers. *Int Neurourol J* 2015;19:74–84.
21. Westermann AM, Schmidt D, Holdenrieder S, et al. Serum microRNAs as biomarkers in patients undergoing prostate biopsy: results from a

prospective multi-center study. *Anticancer Res* 2014;34:665–9.
22. Oosterlinck W, Lobel B, Jakse G, et al. Urology EWGoO. Guidelines on bladder cancer. *Eur Urol* 2002;41:105–2.
23. Raitanen M-P, Aine R, Rintala E, et al. Differences between local and review urinary cytology in diagnosis of bladder cancer. An interobserver multicenter analysis. *Eur Urol* 2002;41:284–9.
24. Lotan Y, Shariat SF, Schmitz-Dräger BJ, et al. Considerations on implementing diagnostic markers into clinical decision making in bladder cancer. *In Urol Oncol-Semin Ori Elsevier* 2010;28:441–8.
25. Ramakumar S, Bhuiyan J, Besse JA, et al. Comparison of screening methods in the detection of bladder cancer. *J Urol* 1999;161:388–94.
26. Kavalieris L, O'Sullivan PJ, Suttie JM, et al. A segregation index combining phenotypic (clinical characteristics) and genotypic (gene expression) biomarkers from a urine sample to triage out patients presenting with hematuria who have a low probability of urothelial carcinoma. *BMC Urol* 2015;15:23.
27. Feber A, Dhami P, Dong L, et al. UroMark—a urinary biomarker assay for the detection of bladder cancer. *Clin Epigenetics* 2017;9:8.
28. Lotan Y, Roehrborn CG. Sensitivity and specificity of commonly available bladder tumor markers versus cytology: results of a comprehensive literature review and meta-analyses. *Urology* 2003;61:109–8.
29. Delnero A, Esposito N, Curro A, et al. Evaluation of urinary level of NMP22 as a diagnostic marker for stage pTa-pT1 bladder cancer: comparison with urinary cytology and BTA test. *Eur Urol* 1999;35:93–7.
30. D'Hallewin M-A, Baert L. Initial evaluation of the bladder tumor antigen test in superficial bladder cancer. *J Urol* 1996;155:475–6.
31. Ellis WJ, Blumenstein BA, Ishak LM, et al. Clinical evaluation of the BTA TRAK assay and comparison to voided urine cytology and the bard BTA test in patients with recurrent bladder tumors. *Urology* 1997;50:882–7.
32. Ianari A, Sternberg C, Rossetti A, et al. Results of bard BTA test in monitoring patients with a history of transitional cell cancer of the bladder. *Urology* 1997;49:786–9.
33. Johnston B, Morales A, Emerson L, et al. Rapid protection of bladder cancer: a comparative study of point of care tests. *J Urol* 1997;158:2098–101.
34. Poulakis V, Witzsch U, De Vries R, et al. A comparison of urinary nuclear matrix protein-22 and bladder tumour antigen tests with voided urinary cytology in detecting and following bladder cancer: the prognostic value of false-positive results. *BJU Int* 2001;88:692–701.