

# Combination role of DAPK methylation in urinary sediment and B ultrasound in evaluating follow-up of urinary bladder cancer

**Fuquan Jiang**

China-Japan Union Hospital of Jilin University

**Changfeng Li**

China-Japan Union Hospital of Jilin University

**Jiansong Han**

China-Japan Union Hospital of Jilin University

**Linlin Wang** (✉ [wanglinlin0427@163.com](mailto:wanglinlin0427@163.com))

China-Japan Union Hospital of Jilin University

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## Research article

**Keywords:** DAPK; B ultrasound in bladder; Urinary sediment; Urinary bladder cancer; Follow-up

**Posted Date:** April 20th, 2020

**DOI:** <https://doi.org/10.21203/rs.3.rs-22264/v1>

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# Abstract

**Background:** Urinary bladder cancer (UBC) is a highly prevalent disease and is associated with substantial morbidity, mortality and cost. This paper aims to explore the combination role of DAPK methylation in urinary sediment and B ultrasound in diagnosing recurrent UBC.

**Methods:** A total of 1021 cases of primary UBC undergone electrocision of bladder tumor through urethra were included and were subjected to follow up every 3 month within 2 years. B ultrasound, DAPK methylation in urinary sediment, examination of exfoliated cells in urine and cystoscopy were performed during the follow up. The data recorded in follow up were subjected to chi-square test and Kappa test. ROC was drawn to evaluate the diagnostic role of each parameter in recurrent UBC.

**Results:** Among the 1021 patients, 115 patients were found with recurrent UBC by cystoscopy and biopsy two years after the operation, and failed to complete the follow up, thus the effective number of follow up was 906. The cystoscopy results were not only consistent with that of B ultrasound (Kappa = 0.785,  $P < 0.05$ ), but also agreed with that of DAPK methylation in urinary sediment and combination of B ultrasound with DAPK methylation (Kappa = 0.517,  $P < 0.05$ , Kappa = 0.593,  $P < 0.05$ ). ROC curve indicated that the area under curve of combination of B ultrasound with DAPK methylation was 0.922 (sensitivity, 92.86%; specificity, 91.63%; Youden index, 0.845) with negative prediction value of 99.4% which suggested that the recurrent risk would be low in case negative results were obtained.

**Conclusion:** Those data supported that combination of DAPK methylation with B ultrasound has high performance in diagnosing recurrent UBC.

## Introduction

Urinary bladder cancer (UBC) is one of the most prevalent cancers in the world with an estimated 430,000 new cases diagnosed in 2012, ranking the sixth most common cancer in the United States with an approximately 74,690 new cases diagnosed in the years of 2014 [1, 2]. UBC is more likely to be primarily diagnosed in individuals older than 65 years and the incidence occurred in those older than 70 years is sevenfold to tenfold [3]. The major risk factors for UBC including exposures to carcinogens, especially tobacco smoking and the incidence of this disease happened in male is three to four times greater than all UBC cases occurring in female [4]. Unfortunately, UBC is a tough disease to be noticed at the first place and most UBC are diagnosed after patients present with macroscopic haematuria, resulting in a higher mortality risk compared with those diagnosed with non-invasive disease [5, 6]. UBC is generally developed into two categories, thus non-muscle-invasive papillary tumors and non-papillary (solid) muscle-invasive tumors, in which the former can be treated by complete resection of tumor with induction and maintenance immunotherapy while radical cystectomy currently remains the standard for the latter [7–9]. All in all, early diagnosis and detection of UBC is of the paramount importance for UBC patients.

The common detection approaches for UBC including cystoscopy, urinary cytology, B ultrasound in bladder as well as urinary biomarkers as alternative or adjunct to cystoscopy [10]. Cystoscopy is commonly accepted as a gold standard, but is invasive and relatively expensive, while urinary cytology remains the most used non-invasive method for UBC diagnosis, but is of limited value specifically in low-grade disease [11, 12]. In addition, evidence supported that ultrasonography may be one of the follow-up substitutes for cystoscopy in low risk UBC [13]. Furthermore, a previous study also pointed out that UBC patients had significant increased DAPK promoter methylation than those in normal controls, suggesting DAPK promoter methylation could serve as a biomarker for UBC detection [14]. Although, UBC diagnosis is mainly depended on cystoscopy and urinary cytology, nevertheless both test have certain drawbacks and the existing limitations of cytology and cystoscopy have fostered the research on alternative, minimally invasive, approaches for UBC diagnosis. To this end, we aims to explore the diagnostic value of detecting DAPK methylation in urinary

sediment, B ultrasound in the bladder and urinary cytology in follow up of UBC patients using cystoscopy results as a gold standard.

## **Material And Method**

### **Subjects**

From June 2014 to June 2016, a total of 1021 patients with UBC under surgery in China-Japan Union Hospital were included in our study. All eligible patients were primary UBC patients with complete medical records and shall be excluded if they are complicated with kidney and liver diseases, other urinary system diseases (including renal pelvic carcinoma, ureteral carcinoma, urinary tract carcinoma and renal carcinoma) or other tumors, and if they had unclear pathological diagnosis. All included patients received urinary bladder irrigation chemotherapy at fixed period. The patients with high risk shall receive secondary surgery about 4 to 6 weeks after primary surgery.

### **Follow up**

Follow up was conducted at a regular basis of three month within 2 years of the surgery, including DAPK methylation in urinary sediment, B ultrasound in bladder, detection exfoliated cells in urine and cystoscopy observation. The physician performing each test shall be blind to other detection results. The follow up shall be ended two years after primary surgery or when radical cystectomy is performed in case of invasion UBC.

### **DAPK methylation in urinary sediment**

Urine (50 ml) at the morning of the day of cystoscopy shall be collected and subjected to centrifugation at 2000 rpm /min for 10 min at 4 °C. The sediment of the urine shall be washed in PBS for twice and dissolved in 200 ul of pure water, after that the sediment are to be stored in refrigerator at -80 °C. Tissue and genomic DNA kit (from Tiangen Bio-technology Ltd., Co, Beijing, China) was applied to extract DNA. Meanwhile, ultraviolet spectrophotometer was used to test the purity and content of the extracted DNA ( $A_{260}/A_{280} > 1.8$ ) which shall be preserved in a 1.5 ml centrifuge tube at -20 °C. EpiTect Bisulfite Kits (from Qiagen Corporation, Germany) was utilized to modify genomic DNA using hydrosulphite, which shall be stored at -80 °C and shall be used within one month. The modified genomic DNA shall be subjected to methylated specific polymerase chain reaction (PCR) and non-methylated specific PCR. Upstream primer for methylation: 5'-GGATAGTCGGATCGAGTTAACGTC-3', downstream primer for methylation: 5'-CCCTCCCAAACGCCGA-3', the product of amplification shall be 98 bp. Upstream primer for non-methylation: 5'-GGAGGATAGTTGGATTGAGTTAATGTT-3', downstream primer for non-methylation: 5'-CAAATCCCTCCCAAACACCAA-3', the product of amplification shall be 106 bp. All primers shall be synthesized by Shanghai Sangon Biotech. The reaction system of PCR (25  $\mu$ l) includes 2 ul of modified DNA, 0.5 ul of upstream primer, 0.5 ul of downstream primer, 2.5 ul of 10  $\times$  PCR buffer, 0.5 ul of Taq, 19 ul of ultrapure water. The condition for PCR amplification were pre-denature at 94 °C for 5 min, 40 heating cycle of 94 °C for 60 s, 54 °C for 30 s, 72 °C for 30 s, followed by extension at 72 °C for 10 min. PCR products were subjected to electrophoresis by 2% sepharose gel and observation under a UV lamp (ultraviolet lamp) for photographing.

### **B ultrasound in bladder**

Patients are told to hold their urine to receive transabdominal B ultrasound in bladder by one appointed associate chief physician in B ultrasound department.

### **Cytology observation**

Cytology observation in urine was carried out at routine and each patient is required to check once every day for constant 3 days. Cells are stained by Pap staining method. Cytology shall be considered as positive if tumor cells were found in urine or negative in case of failing to observe any tumor cells.

# Cystoscopy

The cystoscopy was performed by an appointed and experienced physician. Patients shall be in lithotomy position for local anesthesia or intravenous anesthesia. After that, cystoscope was inserted in an angle of 70° to check for tumor recurrence around the bladder. Any unidentified lesions shall be sent for pathological examination by biopsy.

## Statistical analysis

SPSS 22.0 software was used for data analysis. Enumeration data shall be expressed as cases. The results of cystoscopy was considered as gold standard to compare the sensitivity, specificity, Youden index (YI), positive predict value, negative predict value of results of B ultrasound in bladder, DAPK methylation examination in urinary sediment and urinary cytology observation. Pairwise  $\chi^2$  and Kappa test was utilized for comparison. Meanwhile, the results of B ultrasound in bladder and DAPK methylation were combined together. Either of the two results being positive shall be considered as positive and both of the two detections being negative shall be considered as negative. P value of less than 0.05 was regarded as statistical significance.

## Results

### Identification of DAPK methylation in urinary sediment

The collected samples shall be subjected to both methylated specific PCR and non-methylated specific PCR. In case of amplified product for non-methylated primer, the samples shall be regarded as non-methylation. If amplified products for both methylated primer and non-methylated primer were found, the sample shall be considered as partial methylation. Complete methylation in DAPK promoter region shall be identified if only amplified product was found for methylated primer (Fig. 1). Samples with complete methylation or partial methylation are both considered as positive.

## Follow up

Among the 1021 patients, 115 patients failed to complete the 2-year follow up for kinds of reasons, thus the effective number of follow up was 906. B ultrasound found 55 patients with space occupying lesion in the bladder, among which 50 patients were found with tumor recurrence by cystoscopy and biopsy with minimum tumor size of 0.6 cm and the rest 5 patients were diagnosed with cystitis. According to DAPK methylation in urinary sediment, 118 patients were identified as positive and 788 patients were identified as negative. Observation in exfoliated cells in urine found 27 patients with tumor recurrence. The combination of B ultrasound in bladder and DAPK methylation in urinary sediment identified 135 patients as positive (Table 1)

Table 1

Identification of recurrent UBC by B ultrasound in bladder, DAPK methylation in urinary sediment and observation in exfoliated cells in urine

	Cystoscopy	B ultrasound in bladder		DAPK methylation in urinary sediment		Exfoliated cells in urine		Combination of DAPK methylation and B ultrasound in bladder	
		Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Positive	70	50	20	53	17	27	43	65	5
Negative	836	5	831	65	771	0	836	70	766
Total	906	55	851	118	788	27	879	135	771

Note: combination of DAPK methylation and B ultrasound in bladder, either of the inspection results being positive shall be considered as positive for combination inspection and both of the two inspections being negative shall be considered as negative for combination inspection; UBC, urinary bladder cancer.

## Comparisons on UBC recurrence identification

The comparison between cystoscopy and B ultrasound in bladder showed  $\chi^2 = 1.93$ ,  $P = 0.16 > 0.05$ ; and Kappa = 0.785 (Kappa > 0.75 indicates highly agreeable results) and  $P < 0.05$  suggested that these two detections were consistent to greater degree. When the results of cystoscopy and DAPK methylation in urinary sediment were compared, it is found that  $\chi^2 = 13.67$ ,  $P < 0.001$ , and Kappa test showed Kappa = 0.517 (Kappa of 0.4 ~ 0.75 indicates consistency at a low level),  $P < 0.05$ , demonstrating these two detections were consistent to some degree. The detection of cystoscopy and observation of exfoliated cells in urine showed statistical significance with  $P < 0.001$ , but  $\chi^2 = 20.14$  showed these two detections had inconsistent results. Analysis by Kappa test showed Kappa = 0.537,  $P > 0.05$ , implying the consistent part of these two detections may be coincident without statistical significance. The comparison between cystoscopy and combined detection showed  $\chi^2 = 23.24$ ,  $P < 0.001$ , suggesting the inconsistent part of these two detections were significant, and the Kappa test showed Kappa = 0.593,  $P < 0.05$ , indicating these two detections had generally consistent results (Table 2).

Table 2

Analysis on identification of recurrent UBC by cystoscopy, B ultrasound in bladder, DAPK methylation in urinary sediment and observation in exfoliated cells in urine

		B ultrasound in bladder	DAPK methylation in urinary sediment	Exfoliated cells in urine	Combination of DAPK methylation and B ultrasound in bladder
Cystoscopy	$\chi^2$	1.93	13.67	20.14	23.24
	$P$	0.16	< 0.001	< 0.001	< 0.001
	Kappa	0.785	0.517	0.537	0.593
	$P$	0.041	0.046	0.061	0.041

Note: UBC, urinary bladder cancer.

## Evaluation on diagnosing recurrent UBC

ROC curve was drawn to assess the above detections on diagnosing recurrent UBC by area under curve (AUC), sensitivity, specificity and YI ( $YI = \text{sensitivity} + \text{specificity} - 1$ ) (Table 3 and Fig. 2). The AUC, sensitivity, specificity and YI for combined detection were respectively 0.922, 92.86%, 91.63% and 0.845. The combined detection for diagnosing recurrent UBC was superior to other detections. The negative predict value of combined detection was 99.35%, indicating the risk of recurrent UBC was low if combined detection was negative.

Table 3  
Evaluation on diagnosing recurrent UBC by B ultrasound in bladder, DAPK methylation in urinary sediment and observation in exfoliated cells in urine

	AUC	Sensitivity (%)	Specificity (%)	Youden index	Positive predict value (%)	Negative predict value (%)
B ultrasound in bladder	0.854	71.43	99.04	0.708	90.91	97.65
DAPK methylation in urinary sediment	0.840	75.71	92.22	0.679	44.92	97.84
Exfoliated cells in urine	0.693	38.57	100	0.386	100	95.11
Combination of DAPK methylation and B ultrasound in bladder	0.922	92.86	91.63	0.845	48.15	99.35

Note: Youden index = sensitivity + specificity - 1; Positive predict value = cases of true positive / (cases of true positive + cases of false positive) × 100%; Negative predict value = cases of true negative / (cases of true negative + cases of false negative) × 100%; UBC, urinary bladder cancer, AUC, area under curve.

## Discussion

UBC is still the most common malignancy of the urinary tract in both male and female and cystoscopy currently is believed to be the optimal approach for the detection and monitoring of both primary tumor and follow-up of patient after resection [15]. Nevertheless, the false negative results associated with cystoscopy can range from 10 to 40% [16], thus in this study, we included 1021 patients who had diagnosed with primary UBC and analyzed their 2 years follow-up data to assess the optimal diagnostic tool for diagnosing recurrent UBC. By using the cystoscopy results as gold standard, we compared the diagnostic accuracy of B ultrasound in bladder, DAPK methylation in urinary sediment, urinary cytology and combination of B ultrasound in bladder with DAPK methylation in urinary sediment. The findings in our study supported that the combination of B ultrasound in bladder with detection of DAPK methylation in urinary sediment has a high performance in diagnosing recurrent UBC.

The initial finding in our study showed that the detection of B ultrasound in the bladder has highly consistent results with that of cystoscopy, which in certain way proved that ultrasonography may also be a reliable way to diagnose UBC. This study also found that the AUC of ultrasonography was 0.854 with a sensitivity of 71.43% and a specificity of 99.04%. This is in accordance with observations in a other study that modern sensitive transducers have made much improvement on the imaging of urinary tract rendering transabdominal ultrasonography (US) in visualizing intraluminal filling defects in the bladder than it was in the past [17]. Frankly, it has to be admitted that although ultrasonography represents a valuable surveillance tool in UBC diagnosis, its accuracy is still behind that of cystoscopy. There are many factors that may affect the accuracy of UBC diagnosis, including operator's skill, amount of abdominal fat and bladder distension during procedure. So, ultrasonography may prove a useful adjunct to cystoscopy as a screening test for UBC.

Methylation of tumor suppressor gene promoter leads to transcription inactivation and is involved in tumorigenesis [14]. DAPK gene is closely associated with cancer genesis by inducing the suppression of cell proliferation and the product of DAPK gene is considered to be a positive mediator of apoptosis [18, 19]. In a previous study, the percentage of DAPK

methylation in UBC patients was 64.3% [18]. In this study, we detect the DAPK promoter methylation in urinary sediment in included UBC patients. ROC curve demonstrated that the AUC for DAPK methylation was 0.840, sensitivity of 75.71, specificity of 92.22 and YI of 0.679. Moreover, in this study, the detection of ultrasonography was also analyzed with combination of DAPK methylation in urinary sediment, and the AUC, sensitivity, specificity and YI of the combination were all the greatest among all the applied approaches, respectively of 0.922, 92.86%, 91.63% and 0.845. Those results supported that the combination of ultrasonography with DAPK methylation detection was superior to other single approach.

We also assess the performance of urinary cytology in diagnosing UBC and the results were far less than ultrasonography and DAPK methylation detection. The ROC showed that the AUC was only 0.693, sensitivity of 38.57%, but with a high specificity of 100%. This observation was consistent with previous description that cytology, however, remains the preferred bladder tumor marker for specificity [20].

## **Conclusion**

Based on above data, it may be concluded that the combination of DAPK methylation in urinary sediment and B ultrasound in the bladder seems to be the better approach to improve the accuracy of UBC diagnosis compared with single approaches. However, we should also bear in mind that the clinical research shall be authentic and rigorous, therefore, more studies involving a larger number of included patients shall be required. In addition, as racial/ethnic differences may be a risk factor that influences therapy surveillance [21, 22], we should also consider those racial/ethnic disparities when choosing optimal diagnostic tools for patients with different ethnic background.

## **Declarations**

### **Ethics approval and consent to participate**

This study was conducted based on the protocols proposed by the commitment of China-Japan Union Hospital. All patients signed written informed consents and had the right to know about the experiment prior to the study.

### **Consent for publication**

Not applicable.

### **Funding**

Not applicable.

### **Authors' contributions**

FQJ is the guarantor of integrity of the entire study; FQJ contributed to the study concepts, study design, and definition of intellectual content, CFL contributed to the literature research, JSH contributed to the manuscript preparation and LLW contributed to the manuscript editing and review; FQJ contributed to the clinical studies; JSH and LLW contributed to the experimental studies and data acquisition; CFL contributed to the data analysis and statistical analysis. All authors read and approved the final manuscript.

### **Acknowledgments**

Not applicable.

### **Conflicts of interest**

The authors declare that they have no competing interests.

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## Figures

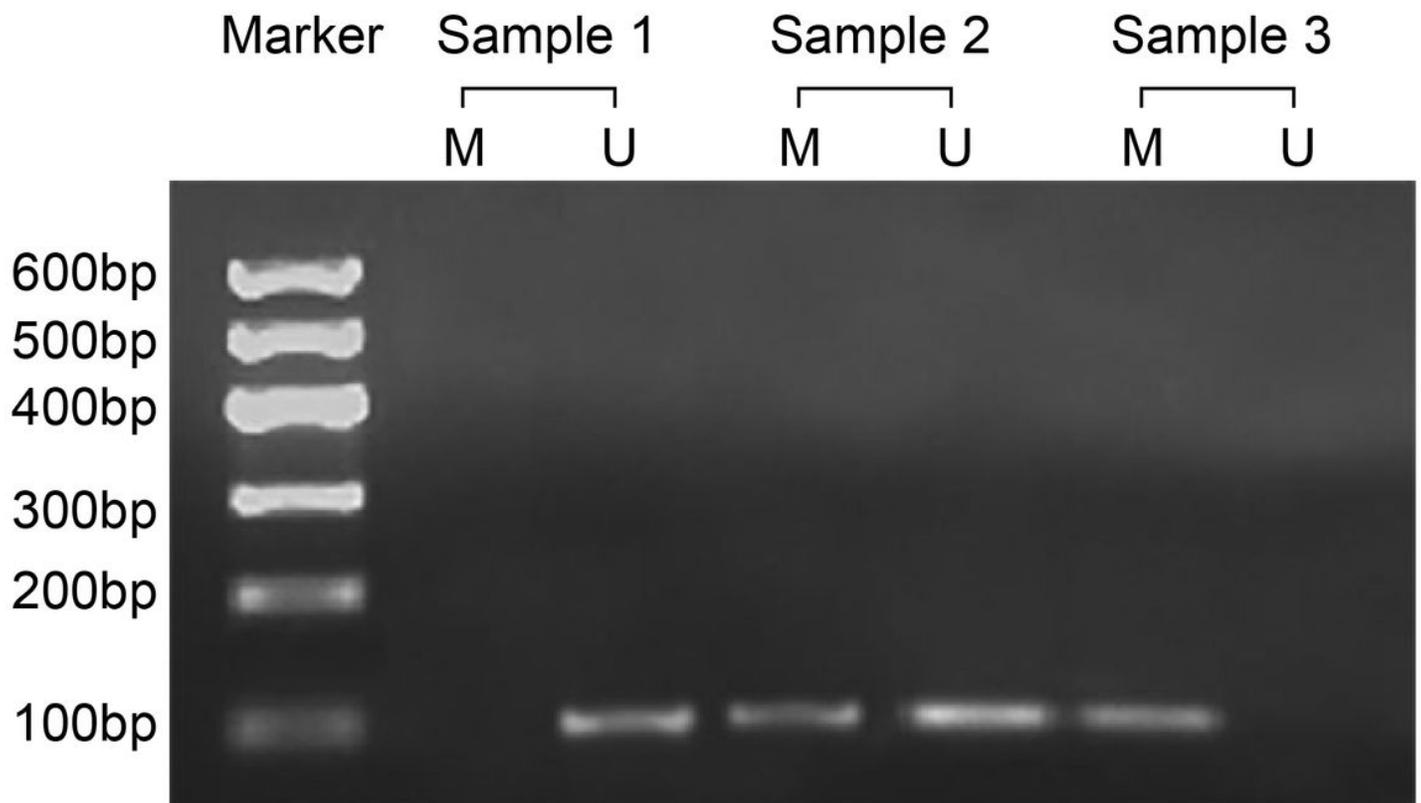
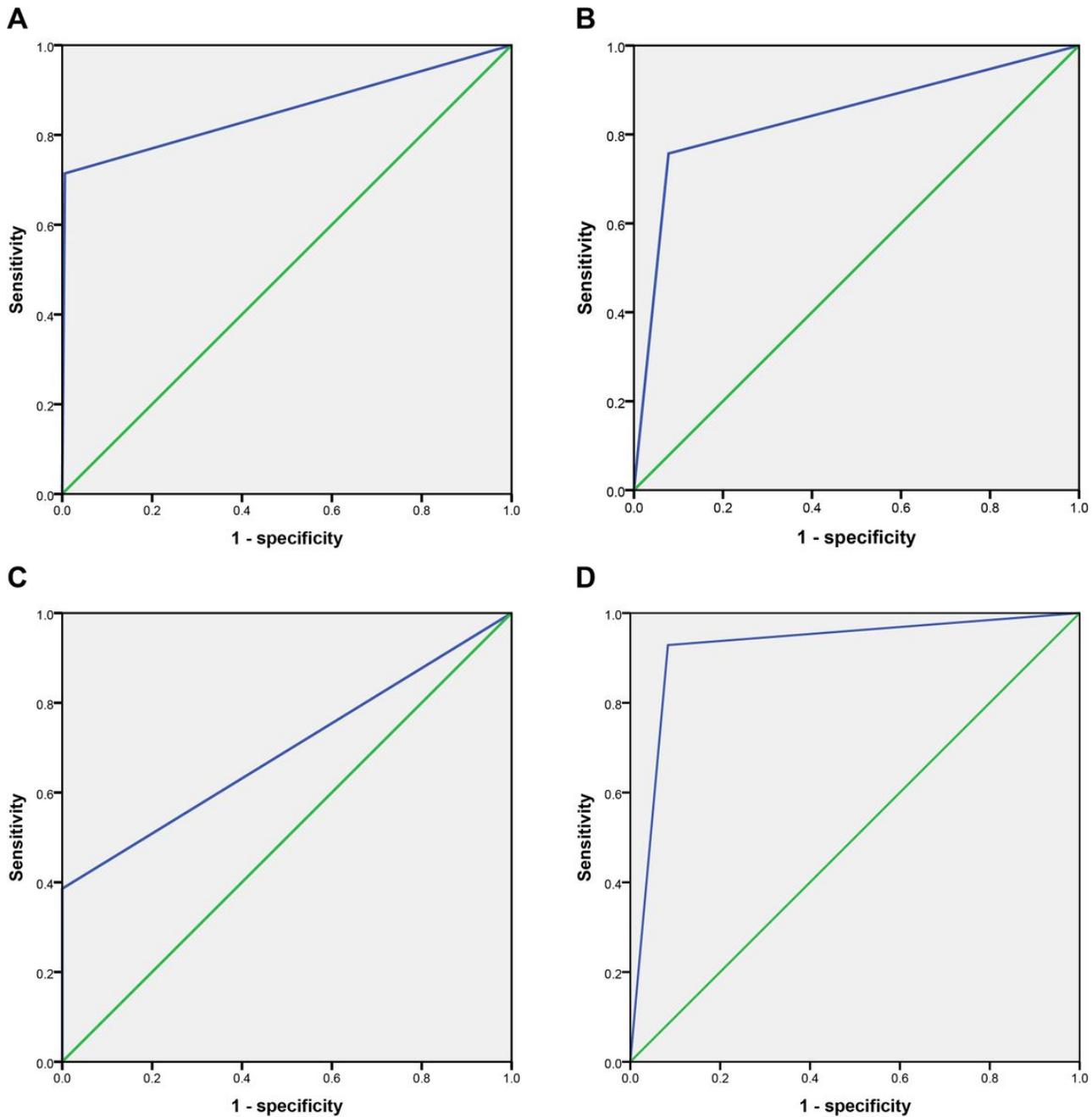


Figure 1

Identification for DAPK methylation in urinary sediment Note: M, methylated band; U, non-methylated band; sample 1, non-methylation; sample 2, partial methylation; sample 3, complete methylation.



**Figure 2**

ROC curves for diagnosing recurrent UBC by B ultrasound in bladder, DAPK methylation in urinary sediment and observation in exfoliated cells in urine Note: ROC, receiver operating characteristic; UBC, urinary bladder cancer; A, ROC curve of diagnosing recurrent UBC by B ultrasound in bladder; B, ROC curve of diagnosing recurrent UBC by DAPK methylation in urinary sediment; C, ROC curve of diagnosing recurrent UBC by detection exfoliated cells in urine; D, ROC curve of diagnosing recurrent UBC by combination of B ultrasound in bladder and DAPK methylation in urinary sediment.