

Midkine Is a Potential Urinary Biomarker for Non-Invasive Detection of Bladder Cancer with Microscopic Hematuria

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Background: To determine the role of Midkine (MDK) in non-invasive detection of bladder cancer (Bca) and the relationship with Ki67.

Methods: Sixty-five Bca patients and 55 non-Bca patients or healthy volunteers were enrolled and voided urine samples were prospectively obtained on the first day of enrollment. Tissue samples were collected by surgery. MDK and Ki67 expressions were analyzed by immunohistochemistry and Western Blot (WB). Specificity and sensitivity of MDK mRNA testing in the detection of Bca were determined by Receiver Operating Characteristic curve (ROC). The relationship between MDK and Ki67 was also assessed.

Results: MDK was overexpressed in Bca tissues than that in the non-cancer tissues. The specificity and sensitivity for MDK mRNA testing in urine in the identification of Bca was 80% and 72.3%. MDK detected 85.7% of high-grade tumors, 87.5% of muscle-invasive tumors and 79.4% of tumors larger than 3 cm in patients without gross hematuria. Microscopic hematuria may even increase the detection rate of Bca by MDK testing. Furthermore, the correlation of MDK and Ki67 was found positive.

Conclusion: MDK was overexpressed in Bca tissues and positively correlated with Ki67. MDK might be a potential biomarker for the detection of Bca, especially for those without gross hematuria but with microscopic hematuria.

Keywords: Midkine, non-invasive detection, bladder cancer, biomarker, Ki67

Introduction

Bladder cancer (Bca) is one of the most common malignancies around the world. Due to its high risk of recurrence and progression, long-term surveillance is required. To date, cystoscopy screening with biopsies and void urinary cytology (VUC) are still the mainstays of confirmation of Bca. However, cystoscopy is an invasive and unpleasant measure for patients. Additionally, although the specificity of >93% is high for VUC, the sensitivity for VUC is dismal at 25–40%, especially for those with low-grade and low-stage tumors.¹ Noninvasive detection and monitoring of Bca with cost-effective signatures are still big challenges. Although several commercially available tests involving urinary biomarkers are now available, none of these assays have reached widespread use owing to insufficient evidence for clinical benefits.

According to the American Urological Association (AUA) and American College of Physicians recommendations, patients with asymptomatic microscopic hematuria should undergo full clinical investigations including cystoscopy.² Due to

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the lack of effective non-invasive tests for Bca suspicion patients with hematuria, results from these currently used tests may lead to an unnecessary work-up for diagnosis. Therefore, new markers with qualities of high sensitive and specificity in confirming Bca presenting with microscopic hematuria are urgently needed.

Midkine (MDK), a 13-kDa heparin-binding growth factor, was originally discovered in embryonal carcinoma cells and involved in the retinoic acid-induced differentiation.³ MDK has been proved to be overexpressed in various types of human cancers including Bca.^{4–9} Down-regulation of MDK expressions by oncolytic virus can achieve antitumor effect in vitro.¹⁰ In addition, elevated MDK levels in blood suggested it could be a biomarker for cancer patients.^{11–14}

Recently, researchers have showed that, by combining with the other markers, MDK was able to detect non-muscle invasive bladder cancer (NMIBC) in high specificity and sensitivity.^{15,16} Furthermore, a combined model with the *MDK* gene test included was able to triage out patients with hematuria but with low probability of urothelial carcinoma.¹⁷ In the present study, we evaluated MDK as a singular bio-marker for detecting Bca, especially for those without gross hematuria. Furthermore, we also accessed the relationships between MDK and clinical characteristics of Bca and Ki67 expressions.

Materials and Methods

Samples

The current study included 65 Bca patients and 55 non-Bca patients and healthy volunteers from the Second Affiliated Hospital of Shantou University Medical College between 2014 and 2015. All the 65 Bca patients were confirmed by biopsies through cystoscopy. Furthermore, the Bca cohort mainly recruited patients without gross hematuria (54/65). Fifty-two of the 65 Bca patients were treated with trans-urethral resection of bladder tumor (TURBt) or radical cystectomy, while the rest of them denied any further intervention after diagnosis. The histological grade and stage were recorded according to

the 2004 World Health Organization grading system and the seventh edition of the tumor-nodes-metastasis (TNM) classification system, respectively. All tumors were predominantly urothelial carcinoma. Among the 55 non-Bca subjects, 26 were healthy volunteers and 29 were patients with benign urological diseases including 12 with benign prostate hyperplasia (BPH) and 17 with urolithiasis. Fifty mL urine samples were prospectively collected on the first day of enrollment and kept frozen at -80°C until analyzed. This study was approved by the Medical Ethics Committee of The Second Affiliated Hospital of Shantou University Medical College and was carried out in accordance with the principles of good clinical practice and the Declaration of Helsinki. Written informed consent was obtained from each patient for surgery and research purposes.

Quantitative Polymerase Chain Reaction (Q-PCR)

The sample urine was thawed and total RNA was isolated using TRIZAL reagent (Invitrogen) according to manufacturer's instructions. RNA concentration was quantified and integrity was checked with electrophoresis by observing the 28S and 18S RNA bands. Reverse transcription of RNA was carried out with the Prime Script TM RT Kit (TaKaRa). The sequences of primers are listed in Table 1. PCR was performed with Premix Ex Taq TM kit (TaKaRa) in a final reaction mixture of 25 μL containing 2 μL cDNA, 12.5 μL 2 \times Premix Ex Taq, 0.5 μL each 10 μM forward and reverse primer, 1 μL TaqMan probe and 8.5 μL ddH₂O.

All samples were analyzed in duplicate and the values of sample copies were obtained after quantitative amplification and normalized to GAPDH using $2^{-\Delta\text{CT}}$ method, where $\Delta\text{CT} = \text{CT}(\text{MDK}) - \text{CT}(\text{GAPDH})$. Each sample was measured in triplicate.

Immunohistochemistry (IHC)

The sample tissues were paraffin-embedded and cut into 4 μM . Immunohistochemical staining was performed as

Table 1 The Sequences of MDK and GAPDH Primers

	MDK	GAPDH
Forward primer	5' cctgcaactggaagaaggag3'	5'tgttcgcatgggtgtgaac3'
Reverse primer	5' ctggcactgagcattgtagc3'	5' tgagtccttcacgatacca3'
Probe	5' ttggagccgactgcaagtacaagtttg3'	5' acagcctcaagatcatcagcaatgcctc3'

previously described.¹⁸ Monoclonal mouse IgG anti-MDK antibody (1:100, Santa Cruz) and polyclonal rabbit IgG anti-Ki67 (1:100, Santa Cruz) were used in IHC staining.

IHC staining was reviewed and scored separately by two pathologists blinded to the clinical parameters. The staining intensity was graded (negative = 0; weak = 1; moderate = 2; strong = 3), and percentage of positive-staining cells was counted (<5% = 0; 6–25% = 1; 26–50% = 2; 51–75% = 3; >76% = 4). The total score was obtained by multiplying the intensity by the proportion (intensity score × proportion score). Positive was defined as staining index ≥4, whereas negative was defined as staining index <4. The cutoff of Ki67 labeling index was 15%.¹⁹ Those with the nuclear staining of tumor cells >15% was defined as positive, and those with the nuclear staining of tumor cells ≤15% was defined as negative.

Immunocytochemistry

Cells were plated onto slides and fixed with 4% paraformaldehyde. The primary antibodies MDK (Santa Cruz) and Ki67 (Santa Cruz) were used at 1:50 and 1:100 work solutions. The fluorescein mouse and rabbit IgG antibody (1:1500, Life technologies) were used as labels for immunofluorescence assay. After immune-labeling, cells were stained with DAPI (Sigma) and viewed with fluorescent microscopy (Nikon, Japan). Photograph was then taken and analyzed by Image J software.

Western Blot (WB)

Frozen tissues or cells were lysed by lysis buffer and protein concentrations were measured by BCA protein assay kit (Beyotime). Total protein was separated by SDS-PAGE using a 12% polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane (Millipore). The membrane was blocked and incubated with the MDK antibody (1:500, Santa Cruz), followed by incubation with a secondary antibody (1:1500; Santa Cruz). Blots were detected with ECL detection reagent (Bio-Rad). The images were quantified by Alpha Ease FC (Alpha View).

Statistical Analysis

SPSS 17.0 for Windows software system (SPSS Inc., Chicago, IL) was used for the statistical analyses. The χ^2 or Fisher's exact tests were used to analyze the categorical data. Univariate ANOVA analysis and the least significant difference test or the Mann-Whitney *U*-test or the Kruskal-Wallis test were used to compare the means of more than two independent groups. The cut-off values for

the urine MDK mRNA levels to predict the presence of Bca were determined using ROC curves. The Spearman test was used to investigate the correlation between 2 quantitative variables. Differences or correlations with *P* values <0.05 were considered statistically significant.

Results

MDK Was Overexpressed in Bladder Cancer

We first investigated the MDK expressions in Bca and non-cancer tissues by IHC (Figure 1). Our results showed that 86.5% (45/52) of Bca tissues stained positive for MDK, which was significantly higher than 26.7% (4/15) of non-cancer tissues positive for MDK (*P*<0.01, Table 2). However, the differences of MDK expressions in Bca patients with high grade or low grade, muscle invasive or non-muscle invasive, were not statistically significant (*P*=0.656 and *P*=0.578, respectively, Table 2).

Similar to the results of IHC, our WB (Figure 2A) also showed that MDK protein expressions in the high and low-grade Bca were significantly higher than that in the adjacent tissues (0.62±0.31 and 0.38±0.25 vs 0.03±0.01, all *P*<0.05). MDK expression of high-grade Bca was 1.63 fold higher than that of low-grade Bca, it was not statistically significant (*P*=0.099, Figure 2B).

MDK mRNA Could Be Used as a Biomarker for the Detection of Bca Characteristics of Study Subjects

Table 3 presents the clinical and pathological characteristics of the subjects. The Bca set comprised 54 patients without gross hematuria and 11 with gross hematuria. The control group included 26 healthy volunteers and 29 patients with benign urological diseases. There were no significant differences in sex and age between the two groups (*P*>0.05). However, the different smoking habits showed statistically significant (*P*=0.027).

Association of Urinary MDK mRNA Levels with Bca Presence

Figure 3 shows the urinary concentration of MDK mRNA of Bca and Non-cancer groups. The mean urinary level of MDK mRNA was significantly higher in the Bca subjects without gross hematuria than that in the controls (0.223 (0.30) vs 0.050(0.08), *P*<0.01). However, there was no significant difference between the subjects of the Bca group with gross hematuria and those in the non-cancer

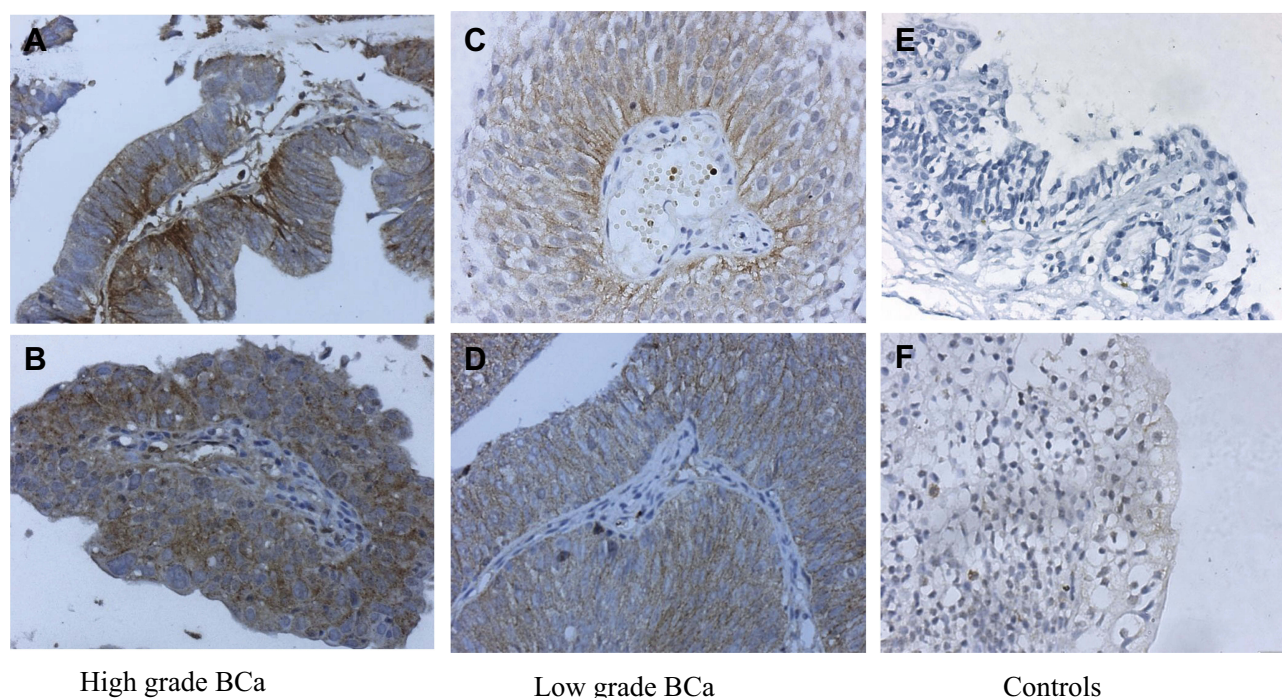


Figure 1 Immunohistochemical staining of MDK in cancerous and benign tissues. **A-D** showing the high expressions of MDK in high- and low-grade Bca samples. **E** and **F** showing the low expressions of MDK in biopsies taken from glandular cystitis and chronic cystitis. Scale bar: 100 μ m (all $\times 40$).

group (0.116(0.29) vs 0.050(0.08), $P=0.554$). Additionally, the MDK mRNA expression in the Bca patients with gross hematuria did not show significant differences in comparison with those without gross hematuria (0.116(0.29) vs 0.223(0.30), $P=0.283$).

Based on our data, the appropriate cut-off value of urinary MDK mRNA for predicting the presence of Bca was 0.116. At this cut-off value, the sensitivity, specificity, positive predict value, negative predict value and

the accuracy of the urinary MDK mRNA for identifying Bca patients were 72.3%, 80%, 81%, 71% and 75.8%, respectively. However, when subdivided into two groups of all the Bca patients, those with gross hematuria was shown with difficulty in identification of tumor by MDK mRNA level (AUC=0.65, $P=0.15$) while those without gross hematuria exhibited a slight improvement in diagnosis (AUC=0.785, $P<0.01$) This may imply that the MDK mRNA test for confirmation of Bca is superior in patients without gross hematuria.

Table 2 IHC of MDK in BCa and Non-BCa Tissues

	n	MDK		P
		Positive	Negative	
Non-BCa	15	4(26.7)	11(73.3)	<0.01
BCa	52	45(86.5)	7(13.5)	
Grade				0.656
High grade	12	10(83.3)	2(16.7)	
Low grade	40	35(87.5)	5(12.5)	
Stage				0.578
MIBC	8	8(100)	0(0)	
NMIBC	44	37(84.1)	7(15.9)	

Note: P values were calculated by Chi-squared test.

Abbreviations: IHC, immunohistochemistry; BCa, bladder cancer; Non-BCa, non bladder cancer; MIBC, muscle-invasive bladder cancer; NMIBC, non-muscle invasive bladder cancer.

Diagnostic Performance of MDK for Detecting Bca Patients Without Gross Hematuria

Therefore, we further analyzed the ROC curves generated using the Bca patients without gross hematuria and the control group (Figure 4). As shown in Table 4, at a specificity of 80%, MDK showed higher sensitivity for detecting high-grade, muscle-invasive and large volume (≥ 3 cm) tumors than that for low-grade, non-muscle-invasive and small volume (< 3 cm) tumors. MDK detected 85.7% of high-grade tumors, 87.5% of muscle-invasive tumors and 79.4% of tumors larger than 3 cm. Interestingly, while microscopic hematuria had minor effect on AUC (0.796 vs 0.798) in Bca patients without gross hematuria, the accuracy rate (ACC) for those without microscopic hematuria was only 65.7%, which was

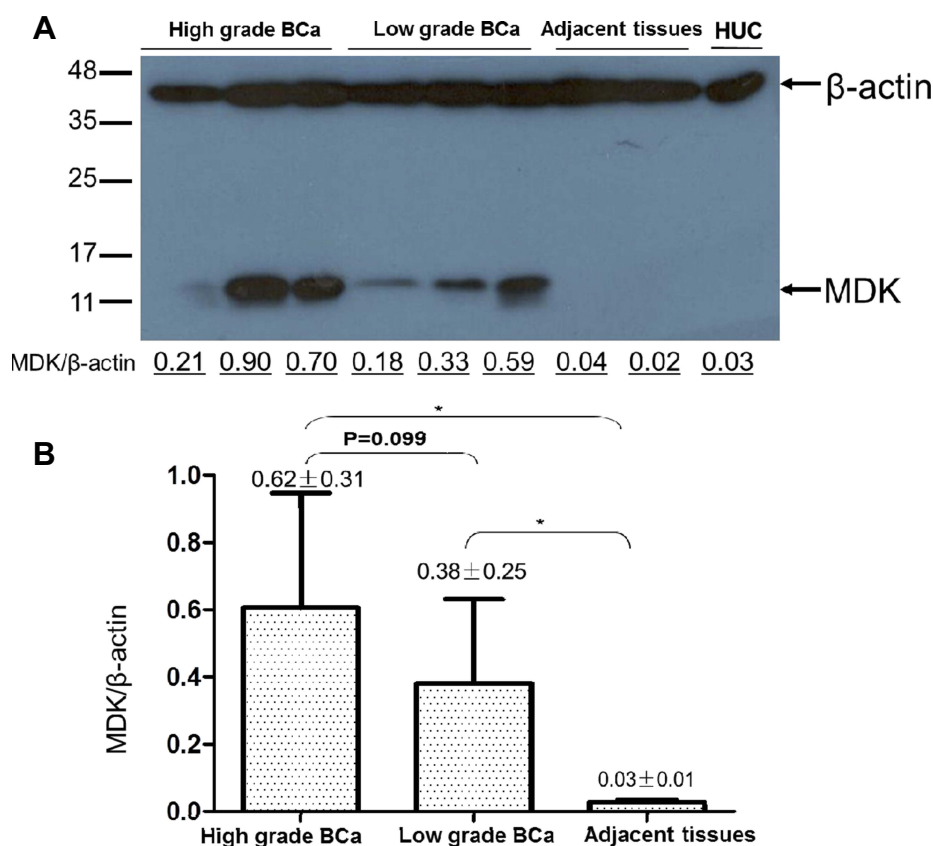


Figure 2 (A) MDK protein was high in Bca tissues and low in the adjacent tissues and human urothelial cells (HUC). **(B)** By LSD test, we found the differences of MDK protein expressions between the tumor and non-tumor groups were statically significant, while those between the high- and low-grade cancer groups were not. Values were presented as mean \pm S.D. (n in high-, low-grade Bca and adjacent tissues were 6, 16 and 3, respectively) of three independent experiments carried out in triplicate (*P<0.05).

15.1% lower than that for those with microscopic hematuria (81.8%). Furthermore, Bca patients with infections but without gross hematuria also showed higher probability in the detection of MDK than those without infections and gross hematuria (under 80% specificity and 85% sensitivity, AUC 0.832 vs AUC 0.771).

MDK Test for Triage Out Non-Bca Patients Without Gross Hematuria

To further determine the MDK test in non-Bca differentiation, we analyzed the ROC curves generated by 55 non-Bca patients and 54 Bca patients without gross hematuria (the positive control group). As shown in Table 4, we found that micro hematuria and infection had no effect on AUC (AUC ranges from 0.778 to 0.798). Furthermore, the MDK test for triage out non-Bca patients can reach a sensitivity of 75.9% for those with micro hematuria or infection, which is higher than 72.2% for those without micro hematuria or infection, under the specificity of 80%.

The Relationship Between MDK and Ki67 in Bca

Ki67 is a cell cycle and tumor growth marker. The mitogenicity of MDK may indicate its important role in tumor cell proliferation.²⁰ Therefore, we try to determine the relationship between Ki67 and MDK in Bca. By immunofluorescent assay, we found that MDK mainly expressed in cytoplasm of Bca cell line 5637 (Figure 5). Interestingly, Ki67 staining, meanwhile, was found located in nucleus of those MDK-positive cells (Figure 5). By applying Ki67 IHC staining in Bca tissues (Figure 6), we analyzed the association of expressions of MDK and Ki67 by spearman test. Of the 52 specimens, 31(59.6%) and 21(40.4%) were classified as Ki67 positive and Ki67 negative, respectively. Moreover, we detected 30 Ki67 positive cases of the 45 MDK-positive group (66.7%) and 1 of the 7 MDK-negative group (14.3%). According to our research, we found that Ki67 labeling index was positively associated with MDK overexpression ($r=0.364$, $P=0.008$) in our Bca cohort.

Table 3 Clinical and Pathological Data of BCa and Non-BCa Cohorts

	Bca		Non-Bca		P
	Without GH*	With GH*	Healthy Controls	Benign Disease	
Total (n)	54	11	26	29	
Age (mean, range)	62.7(37–86)	68.7(55–85)	55.2(36–76)	63.6(41–84)	0.102*
Gender					
Male (%)	46(85.2)	11(100.0)	16(61.5)	25(86.2)	0.096**
Female (%)	8(14.8)	0(0.0)	10(38.5)	4(13.8)	
Smoking habit					
Smoker (%)	35(64.8)	7(63.6)	11(42.3)	13(44.8)	0.027**
Non-smoker (%)	19(35.2)	4(36.4)	15(57.7)	16(55.2)	
Size					
≥3cm	34	6	n/a	n/a	–
<3cm	20	5			
Stage					
Non-muscle invasive	46	9	n/a	n/a	–
Muscle invasive	8	2			
Grade					
High Grade	14	2	n/a	n/a	–
Low Grade	40	9			

Notes: GH* stands for gross hematuria. *P value was calculated by independent-samples *T* test. **P value was calculated by chi-square test.

Discussion

In the present study, we found that MDK was overexpressed in Bca tissues when compared with that in non-cancer tissues. This is consistent with the previous research by O'Brien et al.⁹ Interestingly, although overexpression of MDK was found to be associated with the

development of Bca and predicted a poor outcome in a short period follow-up, there was no significant difference in expressions between muscle invasive and non-muscle invasive tumors.⁹ Similarly, the MDK expression was not correlated with grade or stage in our study. Considering we only included 52 cases for analysis, bias should be taken into consideration. Therefore, the relationship between MDK and clinical grade and stage in Bca need to be further exploited in a larger cohort.

MDK has been identified as a promising cancer biomarker detected in serum in a variety of malignant tumors.^{12,13,21–23} In Bca, Andrew et al. found that the test of mRNA of MDK in urine was more sensitive in detecting high stage/high grade Bca than that of IGHBP5 and HOXA13.¹⁶ In combining with other genes, MDK test showed great improvement of Bca detection.¹⁵ Moreover, Kavalieris et al have proven that a combined genotypic-phenotypic model including the MDK gene testing in urine can triage out 80% of patients with microhematuria but without urothelial carcinoma.¹⁷ Clinically, although microscopic hematuria was only presented in 4% of patients with Bca, there was still plenty of patients with this manifestation were referred to further urological evaluation.²⁴ Therefore, urine markers with high sensitivity and

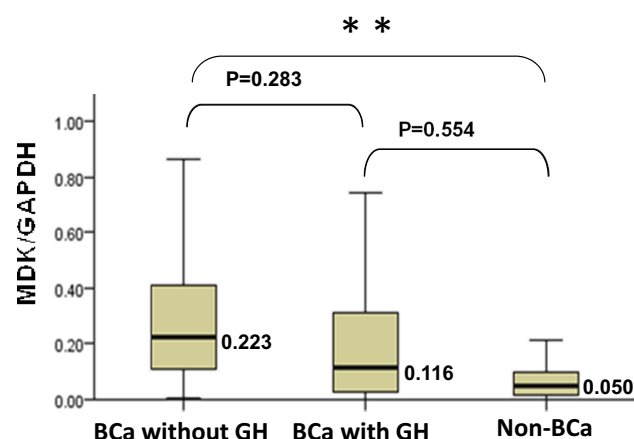


Figure 3 MDK mRNA expressions in urine samples. By Kruskal–Wallis test, we found MDK mRNA expression was much higher in BCa patients without gross hematuria (GH) than that in the control group. The results were presented as mean \pm SD, n in Bca without GH, Bca with GH and Non-Bca groups were 54, 11 and 55, respectively (**P<0.01).

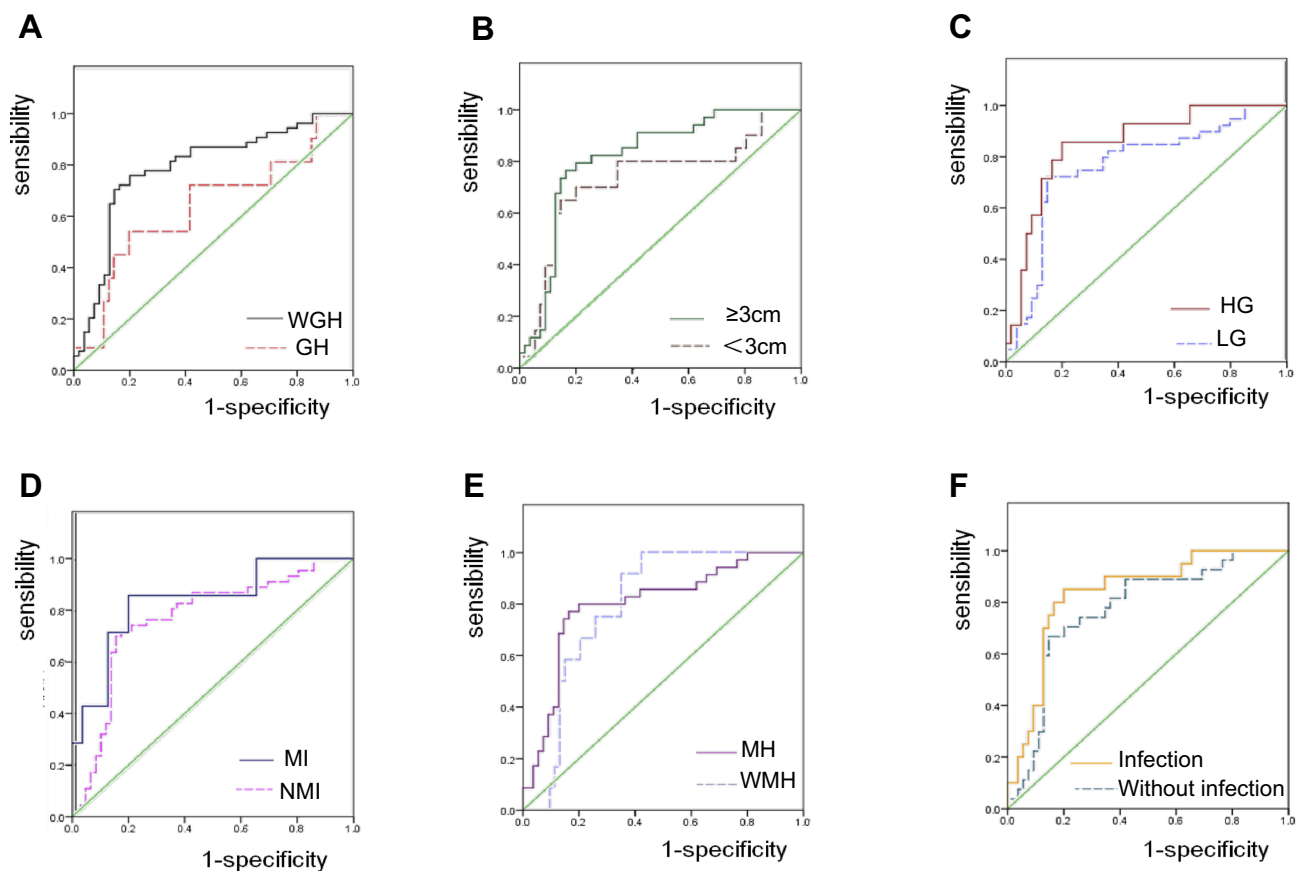


Figure 4 ROC curves for the detection of Bca (A) with gross hematuria (GH) and without gross hematuria (WGH), (B) larger than 3cm and smaller than 3cm, (C) in high grade (HG) and low grade (LG), (D) of muscle invasive (MI) and non-muscle invasive (NMI), (E) with micro hematuria (MH) and without micro hematuria (WMH), (F) with infection and without infection.

specificity in distinguishing between malignant and benign causes for patients with microhematuria are urgently needed. However, a multiplex test also means more time and money consuming. Thus, we tried to determine whether MDK could be used singly as a urine marker for Bca detection in patients without macrohematuria.

We found that MDK mRNA was upregulated in urine of Bca patients in compared with those in the control group. Similar to the previous studies,^{15,16} we also found that the higher urinary MDK mRNA level was associated with the increased risk of Bca presence and the more malignant phenotypes. Furthermore, we observed that while gross hematuria showed a negative effect on the diagnosis of Bca, microscopic hematuria may increase the sensitivity of detecting Bca by MDK testing. According to our research, MDK might be a potential urine marker in screening Bca patients without gross hematuria but with microscopic hematuria.

The presence of inflammatory cells may reduce the diagnostic accuracy of many Bca screening tests, including

NMP22, BTA-Stat and even urine cytology.^{25,26} In our study, infection was not an obstacle for detecting Bca by MDK mRNA test, even though studies have shown that inflammation may affect the expression of MDK.²⁷ According to different cut-off values, the sensitivity and specificity of NMP22 immunoassay could range from 47–100% and 60–90%.²⁸ Our research suggested that MDK, in compared with NMP22, had an equivalent performance in Bca discrimination, especially for those without gross hematuria. However, further validations by multicenter clinical trials are needed.

MDK was involved in many biological processes including induction of chemotaxis, angiogenesis, and enhancement of fibrinolytic activity and inhibition of apoptosis. Mototsugu et al found that MDK overexpression was associated with angiogenic activity of Bca cells in vivo.²⁹ Recently, researchers found that SP1 can promote the proliferation of glioma cells by MDK. However, the biological role of MDK in Bca has yet to be determined. The proliferation marker Ki67 has been correlated

Table 4 Diagnostic Performance of MDK for Detecting Bca

	n	AUC	SN	SP	SN Under 80%SP
BCa	65	0.760	72.3	80	72.3
With gross hematuria	11	0.640	–	–	–
Without gross hematuria	54	0.785	75.9	80.0	75.9
≥3cm	34	0.813	76.5	83.6	79.4
<3cm	20	0.733	65.0	85.5	70.0
High grade	14	0.843	85.7	80.0	85.7
Low grade	40	0.765	70.0	85.5	72.5
MIBC	8	0.857	87.5	80.0	87.5
NMIBC	46	0.772	71.7	83.6	73.9
Microhematuria	35	0.796	77.1	83.6	80.0
Without microhematuria	12	0.798	100.0	58.2	66.7
Infection	20	0.832	85.0	80.0	85.0
Without infection	27	0.771	66.7	85.5	70.4

Notes: All 55 non-BCa samples were negative controls. Seven urine samples of BCa patients without gross hematuria were missing.

Abbreviations: BCa, bladder cancer; Non-BCa, non-bladder cancer; MIBC, muscle-invasive bladder cancer; NMIBC, non-muscle invasive bladder cancer; AUC, area under the curve; SN, sensitivity; SP, specificity.

with poor prognosis in Bca.^{19,30} In our study, we found that MDK and Ki67 were co-occurrence in the same 5637 cells by immunofluorescence staining. We speculated that

this may suggest a close relationship between MDK and Ki67. According to our IHC results, we found that Ki67 was positively associated with MDK overexpression.

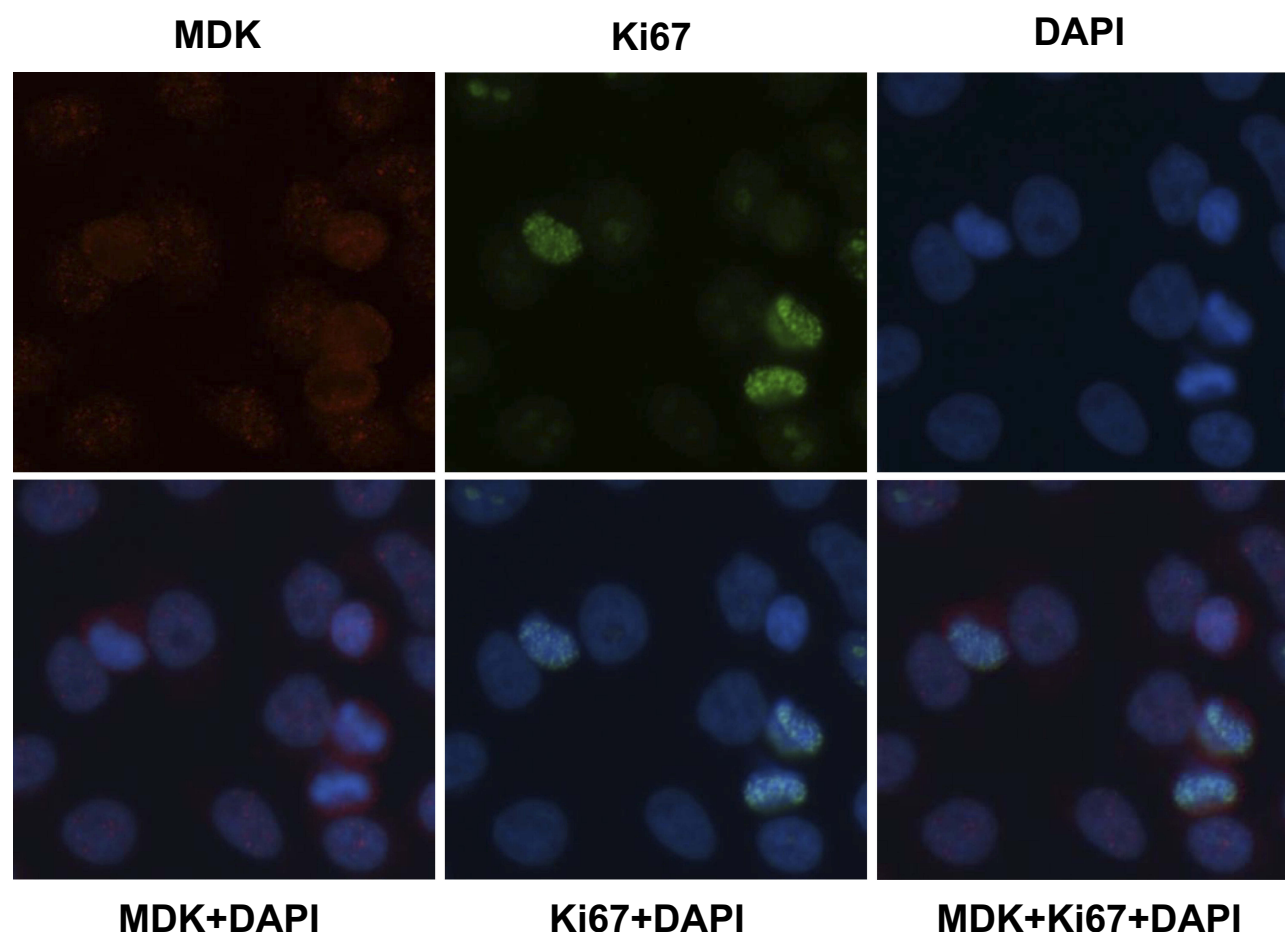


Figure 5 Immunofluorescence staining for MDK and Ki67 in 5637 cells. Scale bar: 100 μ m (all \times 400).

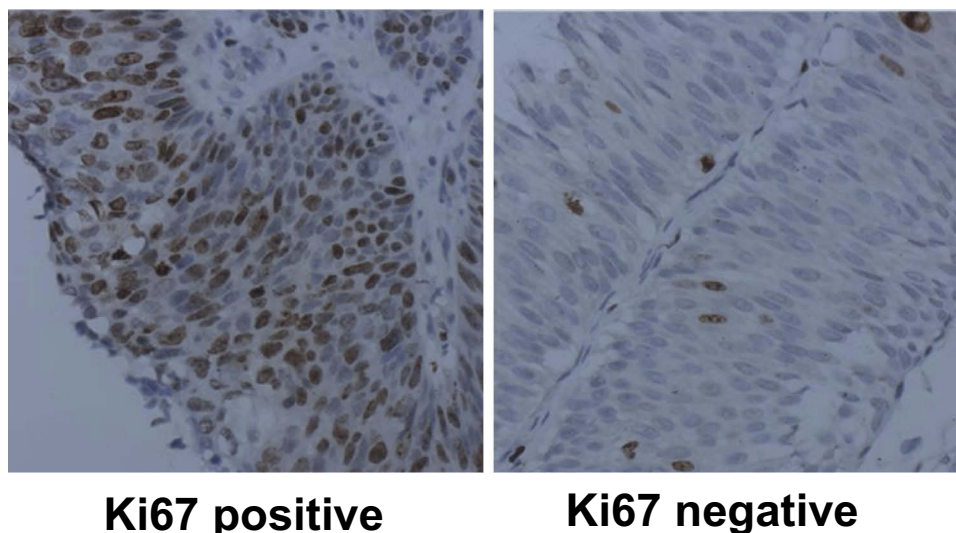


Figure 6 Positive (left) and negative (right) staining of Ki67 in Bca tissues. Ki67 was mainly expressed in nucleus of the tumor cells. Scale bar: 100 μ m.

However, further explorations are expected to uncover the relationship between MDK and Ki67 in vitro.

Conclusions

Taken together, the present study confirmed that MDK was overexpressed in Bca tissues and positively correlated with Ki67; MDK might be a potential biomarker for the detection of Bca, especially for those without gross hematuria but with microscopic hematuria. These results presented here need to be further validated and explored to determine the role of MDK in Bca.

Abbreviations

MDK, midkine; Bca, bladder cancer; VUC, void urinary cytology; AUA, American Urological Association; NMIBC, non-muscle invasive bladder cancer; TURBt, trans-urethral resection of bladder tumor; BPH, benign prostate hyperplasia; WB, Western Blot; IHC, Immunohistochemistry; Q-PCR, quantitative polymerase chain reaction.

Ethics Approval and Consent to Participate

This study was approved by the Medical Ethics Committee of The Second Affiliated Hospital of Shantou University Medical College and was carried out in accordance with the principles of good clinical practice and the Declaration of Helsinki. Written informed consent was obtained from each patient for surgery and research purposes.

Data Sharing Statement

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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