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ORIGINAL ARTICLE

Evaluation of Urine NDRG1 as Noninvasive Biomarker for Bladder Cancer Diagnosis

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SUMMARY

Background: **N-myc downstream regulated gene 1 (NDRG1) was involved in cell differentiation and was recently reported to exert various effects in tumorigenesis. The aim of this study was to assess its diagnostic value in urine as a useful marker for bladder cancer (BC).**
Methods: **In this study, we recruited 119 BC patients, 65 patients with non-cancerous bladder diseases, and 60 healthy volunteers as control. Their urine concentrations of NDRG1, nuclear matrix protein 22 (NMP22), and creatinine (Cr) were measured and relevant clinical information was retrieved from their medical history records.**
Results: **The expression of NDRG1/Cr and NMP22/Cr in urine were significantly higher in BC patients than those in non-cancerous bladder diseases ($p = 0.009$ and $p = 0.023$) and healthy controls ($p = 0.005$ and $p = 0.002$). The level of NDRG1/Cr was significantly associated with pathologic T stage ($p < 0.001$) and pathological grade ($p < 0.001$). The ROC of NDRG1/Cr to diagnose BC was 0.713 (95% CI, 0.630 - 0.797), with a sensitivity of 63.8% and a specificity of 73.4% at a cutoff of 76.3 ng/mg. NMP22/Cr was 0.705 (95% CI, 0.626 - 0.784), with a sensitivity of 64.2% and a specificity of 66.2% at a cutoff of 12.1 ng/mg. NDRG1/Cr in combination with NMP22/Cr shows a ROC of 0.719 (95% CI, 0.632 0.806) with a sensitivity of 64.9% and specificity of 75.9%**
Conclusions: **Urine NDRG1 may be useful in a minimally invasive modality for determining bladder cancer. Predictive value of the two biomarkers was slightly higher than that of routine NMP22 parameter alone.**
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KEY WORDS

NDRG1, NMP22, bladder cancer, biomarker, urine

INTRODUCTION

Bladder cancer (BC), commonly referred to as urothelial cell carcinoma of the bladder, is the 9th most common cause of tumor-related death worldwide [1,2] as well as the first cause of death in genitourinary tumors in China [3]. Two subtypes of BC have been recorded: non-muscle-invasive bladder cancer (NMIBC) characterized by a high recurrence rate and muscle-invasive bladder cancer (MIBC) with poor prognosis [4]. Therefore, early screening and monitoring are essential for tumor detection and improve the treatment of BC. Cystoscopy plays a vital role and remains the criterion and

standard for BC diagnosis and surveillance; however, most patients suffer negative emotions such as phobia and anxiety before cystoscopy because of its uncomfortable experience [5,6]. Some molecular markers that benefit BC diagnosis and monitoring recurrence are commercially available, including nuclear matrix protein 22 (NMP22), signal transducer and activator of transcription (STAT), and kinesin-binding trafficking protein (TRAK) [7]. Other promising protein and nucleic-acid based techniques sourced from voided urine have been developed over the past two decades for noninvasive and high-fidelity diagnosis of BC [8]. However, these biomarkers are not sufficient in the early examination of BC due to the lack of diagnostic sensitivity and specificity. Thereby, alternative noninvasive diagnostic methods with lower cost have been sought. N-myc downstream regulated gene 1 (NDRG1) is the first-discovered member of the NDRG family and has been associated with numerous physiological events, such as cell differentiation, stress-response, development, apoptosis, and lipid synthesis [9]. It has also been demonstrated to potently suppress metastasis in a variety of cancer-types, including cancers of the prostate, pancreas, colon, breast, and bladder [10,11]. Previously, we have shown that the level of NDRG1 protein was significantly increased in tumor tissue of bladder cancer patients and correlated with tumor stage and lymph node metastasis. Its overexpression was an independent predictor of poor clinical outcomes for patients with BC [10].

In the present study, we aimed to investigate level of NDRG1 in urine and determined its diagnostic value in detecting BC. Moreover, a model integrating NDRG1 and NMP22 in the same set of patients provided a unique opportunity to find out whether abnormal expression of the two biomarkers exerts a synergistic effect on BC diagnosis.

MATERIALS AND METHODS

Patients and specimens

A total of 244 participants treated at Beijing Friendship Hospital, Capital Medical University, were enrolled in this study. They included 99 patients with NMIBC, 20 patients with MIBC, 65 patients with non-cancerous bladder diseases including bladder calculi and urinary tract infection, and 60 healthy volunteers. Patients with any other types of tumor in their past history or metastasis at diagnosis were excluded. All bladder cancer patients had a primary diagnosis and none of the patients received pelvic irradiation, intravesical or systemic chemotherapy before surgery. Clinical data such as gender, age, TNM stage, and pathological grade were available in all cases. Tumor staging and grading were standardized to the American Joint Committee on Cancer and World Health Organization systems. The tumors were grouped as NMIBC and MIBC by TNM stage. All patients provided informed consent according to the requirements in China Good Clinical Practice and Beijing Friendship Hospital Ethics Committee (Approval Number BJFH-EC/2014-104)

Specimens

Voided urine samples were obtained from all participants and were centrifuged at 4°C and 1,000 x g for 10 minutes within 4 hours of collection. The supernatant was stored at -80°C until analysis. All samples were collected prior to any therapeutic intervention and the levels of creatinine (Cr) were assessed as the internal control. The concentration of Cr was measured by a Beckman Coulter AU5800 (Beckman Coulter, Inc., USA).

Urinary NDRG1 and NMP22 measurements

An enzyme linked immunosorbent assay (ELISA) kit was used to quantify urinary NDRG1and NMP22 concentrations. Urine NDRG1 assay was performed using OKEH ELISA kit (OKEH02359, Aviva Systems Biology, USA) and urine NMP22 was performed using ELISA kit (NUMA1: SEC332Hu, USCN Life Science, Wuhan, China) according to the manufacturer's protocol. The minimum detection concentrations were 0.125 ng/mL for NDRG1 and 0.75 ng/mL for NMP22.

Statistical analysis

All statistical analyses were conducted using SPSS 21.0 software (IBM SPSS Statistics for Windows; IBM Corp, Armonk, NY, USA) and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Nonparametric Mann-Whitney sum rank U test was employed to compare the expression levels of NDRG1 and NMP22 between groups (BC vs. benign bladder lesions and BC vs. normal control). Receiver-operating characteristic (ROC) curve was constituted to assess diagnostic value, the cutoff of NDRG1 or NMP22 in bladder cancer, sensitivity, and specificity according to standard formulas. * $p < 0.5$, ** $p < 0.01$ were considered as statically significant.

RESULTS

Characteristics of studied subject

Participants were all from mainland China and included 119 bladder cancer patients, 65 non-cancerous benign bladder diseases patients, and 60 healthy volunteers. There were 149 males and 95 females. Ages ranged from 41 to 88 years. Table 1 presented clinical and pathological characteristics of all participants in the study. The distributions of gender and age were similar among bladder cancer patients, benign bladder lesion patients and healthy controls ($p > 0.05$ for both).

The expression of NDRG1 and NMP22 were increased in bladder cancer

The level of NDRG1/Cr and NMP22/Cr were compared among bladder cancer patient groups, benign bladder le-

Table 1. Demographic and clinicopathologic characteristics of all participants.

Characteristic	Bladder cancer	Benign bladder lesions	Healthy control	p-values
Total (n)	119	65	60	
Median age (years)	65 (46-84)	62 (41 - 87)	62 (41 - 88)	
Gender (n)				0.816
Male	75 (63.0%)	38 (58.5%)	36 (60.0%)	
Female	44 (37.0%)	27 (36.9%)	24 (40.0%）	
Pathologic T stage		Not applicable	Not applicable	
NMIBC (pTa, pTis, pT1)	94 (78.9%)			
MIBC (pT2-pT4)	25 (21.1%)			
Grade		Not applicable	Not applicable	
Low grade	49 (41.2%)			
High grade	70 (58.8%)			
Recurrence				
No	98 (82.4%)			
Yes	21 (17.6%)			

Table 2. Association between NDRG1 level and clinicopathological characteristics of all participants.

	Bladder cancer		Benign bladder diseases		Healthy control	
	median	p-values	median	p-values	median	p-values
NDRG1/Cr (ng/mg)						
Gender						
Male	69.4 (23.3 - 193.9)	0.294	63.3 (38.4 - 101.2)	0.285	52.1 (23.4 - 86.7)	0.136
Female	74.1 (7.4 - 206.1)		70.5 (51.8 - 136.3)		82.5 (50.9 - 107.3)	
Age (years)						
≥ 65	80.0 (17.6 - 166.3)	0.756	65.0 (50.9 - 103.6)	0.979	70.1 (55.5 - 122.3)	0.081
< 65	65.7 (22.2 - 265.5)		66.6 (37.7 - 117.7)		48.3 (19.0 - 92.2)	
Pathologic T stage						
NMIBC (pTa, pTis, pT1)	52.0 (15.4 - 120.6)	0.000	not applicable		not applicable	
MIBC (pT2-pT4)	277.7 (120.3 - 458.2)					
Grade						
Low grade	27.6 (10.2 - 67.6)	0.000	not applicable		not applicable	
High grade	118.9 (43.5 - 271.8)					

sion groups, and healthy controls. The level of NDRG1/Cr was significantly increased in the BC group compared to benign diseases ($p = 0.009$) and healthy controls ($p = 0.005$). While NMP22/Cr level also showed a significant increase in BC patients compared to benign diseases ($p = 0.023$) and healthy controls ($p = 0.002$) (Figure 1).

Urinary NDRG1 levels were correlated with BC patient characteristics

Increasing TNM stage was associated with increasing frequency of NDRG1 alterations ($p < 0.05$). In addition, NDRG1 expressions were also correlated with TNM stage and tumor grade ($p < 0.001$) (Table 2). However, NMP22 analysis indicated that the level of NMP22 was

Table 3. Association between NMP22 level and clinicopathological characteristics of all participants.

	Bladder cancer		Benign bladder diseases		Healthy control	
	median	p-values	median	p-values	median	p-values
NMP22/Cr (ng/mg)						
Gender						
Male	13.4 (5.7 - 23.4)	0.225	9.6 (6.1 - 13.7)	0.128	7.5 (3.8 - 15.4)	0.163
Female	19.1 (7.3 - 45.3)		15.1 (6.5 - 23.3)		12.7 (5.9 - 31.4)	
Age (years)						
≥ 65	14.8 (9.1 - 29.2)	0.229	11.9 (7.8 - 14.8)	0.823	8.0 (1.7 - 18.1)	0.225
< 65	11.9 (5.6 - 23.1)		10.0 (6.0 - 19.7)		8.0 (6.5 - 15.9)	
Pathologic T stage						
NMIBC (pTa, pTis, pT1)	13.9 (6.5 - 25.2)	0.755	not applicable		not applicable	
MIBC (pT2-pT4)	13.1 (4.1 - 76.7)					
Grade						
Low grade	13.4 (6.0 - 20.4)	0.376	not applicable		not applicable	
High grade	13.8 (6.8 - 30.0)					

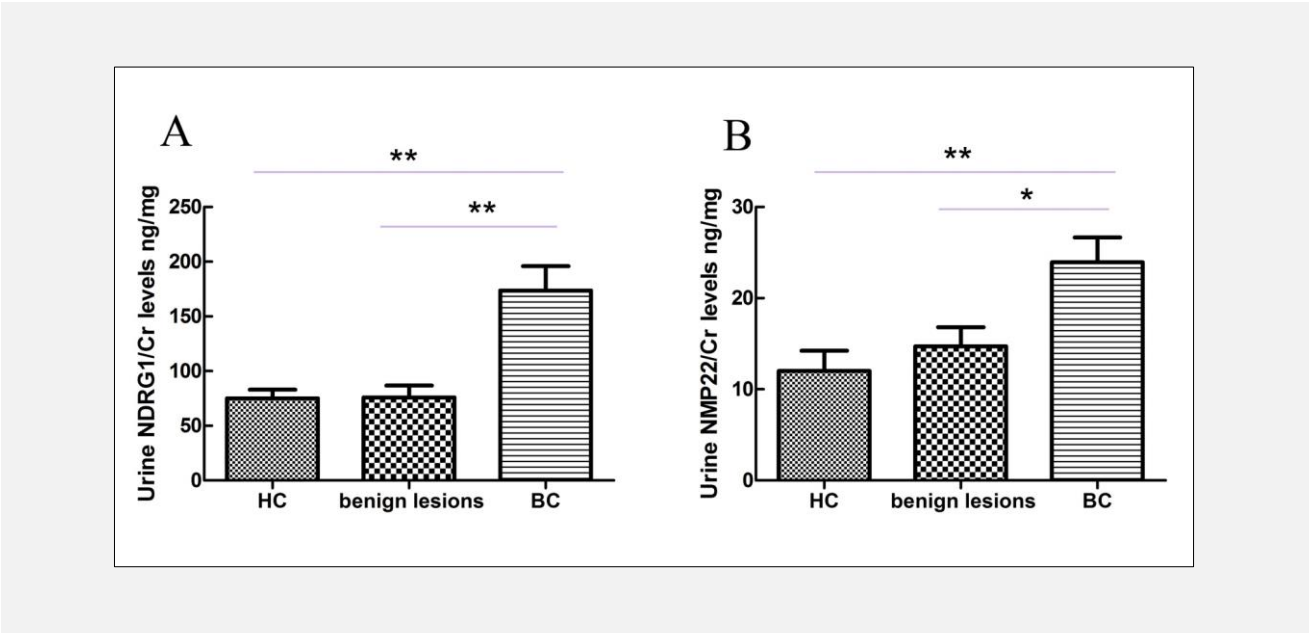

Figure 1. Comparisons of urinary NDRG1/Cr and NMP22/Cr levels among health groups, benign lesions, and BC.

The levels of NDRG1 (A) and NMP22 (B) were measured between groups of BC and benign lesions, BC and healthy controls. The results in all groups are presented as the mean and SD. Significance ($^{*}p < 0.05$; $^{**}p < 0.01$) was assessed using the Mann-Whitney test.
NMIBC - non-muscle-invasive bladder cancer, MIBC - muscle-invasive bladder cancer.

not related to bladder cancer, TNM stage, or tumor grade ($p > 0.05$). Gender and age were not associated with individual marker alterations ($p > 0.05$) (Table 2 and Table 3).

Diagnostic value of NDRG1 and NMP22 in bladder cancer

The ROC curve based on the ELISA results was plotted to evaluate the potential of urinary NDRG1 as a non-in-

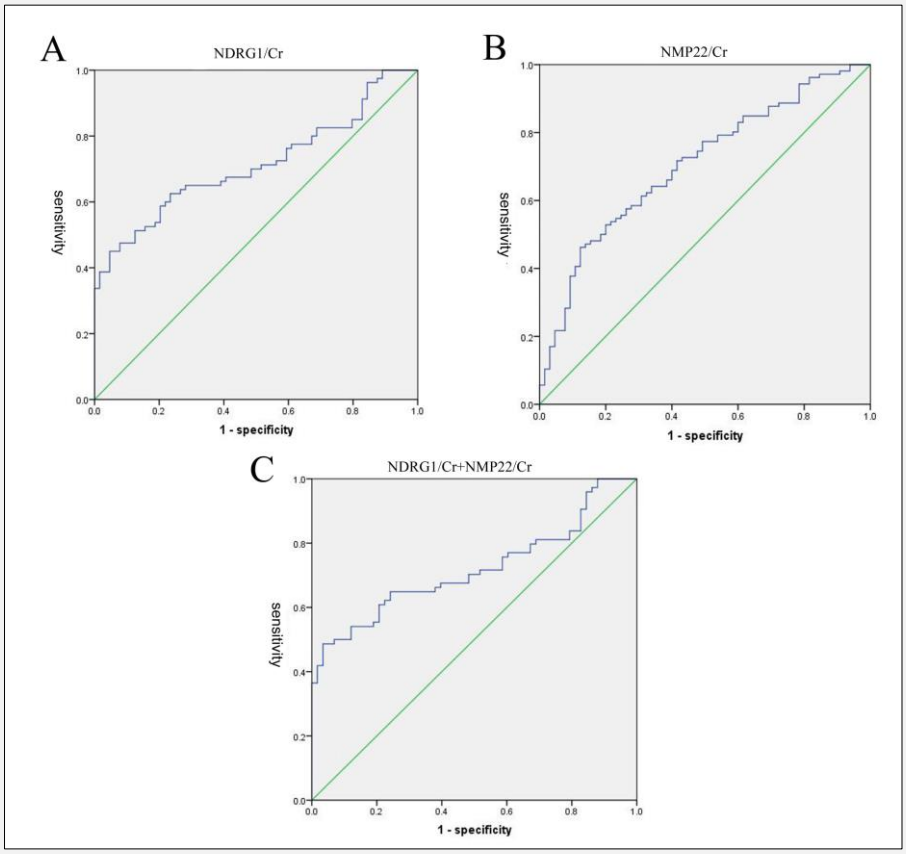

Figure 2. Diagnostic performance of NDRG1 and NMP22 for BC.

A: ROC curve analysis using NDRG1. B: ROC curve analysis using NMP22. C: ROC curve analysis using the combination model NDRG1 and NMP22.
ROC - Receiver operating characteristic.

vasive biomarker for the diagnosis of BC (Figure 2). The cutoff values for NDRG1/Cr and NMP22/Cr were 76.3 ng/mg and 12.1 ng/mg, respectively. The ROC of NDRG1/Cr to diagnose bladder cancer was 0.713 (95% CI, 0.630 - 0.797), with a sensitivity of 63.8% and a specificity of 73.4%. The AUC of NMP22/Cr was 0.705 (95% CI, 0.626 - 0.784), with a sensitivity of 64.2% and a specificity of 66.2%. The ROC of NDRG1/Cr+ NMP22/Cr was 0.719 (95% CI, 0.632 - 0.806), with a sensitivity of 64.9% and a specificity of 75.9%.

DISCUSSION

Among all urine detection for bladder cancer, urine-cytology is a well-accepted non-invasive procedure. However, doctors and patients are all disappointed at its low sensitivity (only 35 - 50% in low grade BC, 60 - 90% in high grade BC) [12]. The results will be dramatically affected by the observer's experience [13]. Tumor markers such as NMP22 and BTA are known to play a role in the diagnosis of bladder cancer and have been approved by the U.S. Food and Drug Administration (FDA) [14]. However, these molecular markers that predict the growth and recurrence of BC are not routinely used in clinical practice. A meta-analysis of urine test performance reported the sensitivity of the quantitative NMP22 test was 0.69 (95% CI, 0.62 - 0.75), and the specificity was 0.77 (95% CI, 0.70 - 0.83) (19 studies). The results of the qualitative NMP22 test were also not as accurate, with a sensitivity of only 0.58 (95% CI, 0.39 - 0.75) and a specificity of 0.88 (95% CI, 0.78 - 0.94)[14]. In our study, the results showed that the ROC of NMP22/Cr was 0.705, with a sensitivity of 64.2% and a specificity of 66.2%, which is very close to the results of other previous studies. There is another

limitation for NMP22. Atsu et al. have reported that pyuria and hematuria significantly affected the accuracy of urinary NMP22 [15]. Similarly, Miyake et al. found benign cells of the urinary system may also cause a false positive NMP22 [16]. However, urine sample is easily obtained, and many patients would prefer to use a less invasive approach. Therefore, we tried to find another feasible biomarker in the diagnosis of BC using urine sample.

In the present study we investigated the expression of NDRG1 in BC patients as it is a non-invasive and cost-effective indicator. Our previous studies have shown that NDRG1 was upregulated in BC tissue. This time we studied NDRG1 in BC urine sample. Our results have shown an elevation of NDRG1/Cr as well as NMP22/Cr in BC patients' urine samples, as compared to non-cancerous bladder diseases and healthy controls. Moreover, diagnostic value of urine NDRG1/Cr in BC was also investigated using ROC curve, and the results suggested that NDRG1/Cr might be a potential biomarker for the detection of BC. Consequently, urinary NDRG1/Cr had better diagnostic performance than urinary NMP22/Cr for detecting BC. We next proceeded to analyze the combined diagnostic value of alterations in the two biomarkers. Another ROC curve was created using NDRG1/Cr+ NMP22/Cr. This time we found a slight increase in both sensitivity and specificity.

In previous studies, NDRG1 was reported to be associated with metastasis and prognosis of multiple cancers. For example, the NDRG1 protein was overexpressed in human hepatocellular carcinoma (HCC) samples and was associated with vascular invasion and poor survival [17,18]. High NDRG1 was also directly associated with shorter progression-free survival (PFS) and overall survival (OS) of patients with cervical adenocarcinoma [19]. We have found that NDRG1 overexpression in BC tissues was associated with increased cell proliferation, migration, and invasion and decreased apoptotic cell numbers [10]. In this study, we found patients with MIBC had higher NDRG1/Cr levels than those with NMIBC. NDRG1/Cr level was also higher in patients with high-grade disease than in those with low-grade disease. These results imply that NDRG1 may be closely associated with bladder cancer occurrence as well as metastasis and prognosis.

This study employed two significant biomarkers in the diagnosis of bladder cancer. Although neither of them is precise, they still cannot replace cystoscopy. However, physicians would provide several different diagnostic options for individual patients when they really fear the cystoscopy test. High-risk BC patients need to have a routine cystoscopy follow-up. Further prognostic data will be collected from our bladder cancer patients to verify our assumption. Use of a non-invasive urine test as a replacement will definitely be marvelous news. Our results should be further validated by multicenter clinical trials with more patients. Further studies are needed to elucidate the individual mechanisms of the markers and their association to each other. This may lead to an understanding of how these markers contribute to tumor recurrence and progression and facilitate clinical decision making.

CONCLUSION

In conclusion, the level of NDRG1/Cr in urine is higher in bladder cancer patients than patients with benign bladder lesions and healthy controls. It may serve as a noninvasive biomarker for the diagnosis of BC. Further studies, including cancer prognosis and multicenter studies are required.

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Declaration of Interest:
There is no conflicts interest to declare.

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