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Expression of *N-Myc Downstream-Regulated Gene 2* in Bladder Cancer and Its Potential Utility as a Urinary Diagnostic Biomarker

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Initial diagnosis of carcinoma of the urinary bladder remains challenging. *N-Myc downstream-regulated gene 2* (*NDRG2*) has been reported to be closely correlated with cell differentiation and proliferation in various cancers. However, its clinical significance in diagnosis of bladder cancer remains unclear. The purpose of this study was to detect the expression of *NDRG2* and investigate its diagnostic value in bladder cancer.

Material/Methods: We recruited 127 patients with bladder cancer and 97 healthy controls. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) and Western blotting analysis were conducted to measure the *NDRG2* expression levels in urine of patients with bladder cancer, bladder cancer cell lines, and healthy controls. The correlations between *NDRG2* expression and clinicopathological characteristics were analyzed by chi-square test, and the diagnostic value of *NDRG2* was estimated by establishing a receiver operating characteristic (ROC) curve.

Results: The relative *NDRG2* expression were significantly downregulated both at mRNA and protein levels in urine of patients with bladder cancer and in cell lines, and its low expression was distinctively correlated with tumor grade and stage. The ROC curve showed *NDRG2* could be a good diagnostic marker, with an AUC of 0.888, indicating high sensitivity and specificity.

Conclusions: *NDRG2* was decreased in patients with bladder cancer and might be involved in the progression of this malignancy. Moreover, *NDRG2* could be a potential independent diagnostic biomarker for bladder cancer.

MeSH Keywords: **Diagnosis, Oral • Gallbladder Neoplasms • Genes, vif**

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Background

Bladder cancer is a serious health problem worldwide and is the second-most frequent malignancy among all genitourinary tract tumors [1]. Invasive bladder cancer has a high incidence of recurrence, which contributes to the high mortality of this disease [2]. Currently, the combination of cystoscopy and urine cytology is considered to be the "gold standard" for the identification of bladder tumors [3]. However, the diagnosis of bladder remains is challenging, mainly due to limitations of the available methods. For example, cystoscopy is an invasive strategy with high cost, while urine cytology has relatively high specificity but is not sensitive in low-grade bladder cancer [4,5]. Therefore, efforts should be taken to identify noninvasive and highly sensitive and specific diagnostic biomarkers for bladder cancer.

N-Myc downstream-regulated gene 2 (*NDRG2*) is a member of the *NDRG* family, which has 4 members: *NDRG1*, *NDRG2*, *NDRG3*, and *NDRG4* [6,7]. *NDRG2* is located at chromosome 14q11.2 and has been suggested to be a tumor suppressor as well as cell stress-related gene involved in many cellular metabolic processes such as hormone, ion, and fluid metabolism, and in stress responses such as those to hypoxia and lipotoxicity [8–12]. The aberrant expression of *NDRG2* has been described in a variety of human cancer cell lines and primary tumors, including prostate cancer, thyroid cancer, colorectal carcinoma, oesophageal squamous cell carcinoma, and gallbladder carcinoma [13–18]. Previous studies found that *NDRG2* regulates cell proliferation and invasion in bladder cancer [19,20]. However, the diagnostic value of *NDRG2* was never reported.

In the present study, we detected the expression of *NDRG2* in urine of patients with bladder cancer and in cell lines, and analyzed the correlation of *NDRG2* expression with clinicopathological features. In addition, we validated the clinical value of urine *NDRG2* in the early detection of bladder cancer.

Material and Methods

Patients and samples

The study was approved by the Ethics Committee of the Affiliated Luohu Hospital of Shenzhen University and all participants signed written informed consent in advance.

A total of 124 patients who were diagnosed with bladder cancer were enrolled from the Affiliated Luohu Hospital of Shenzhen University. None of the patients had received any chemotherapy or radiotherapy before sampling. We also enrolled 97 healthy controls matched by age and sex; those with a history of bladder diseases were required to undergo cystoscopy to verify their healthy condition. Urine was collected from the healthy individuals to use as the healthy control specimens.

A single and naturally voided midstream urine sample was obtained from all subjects before cystoscopy. Approximately 50 ml of urine was collected and put on ice immediately, then the samples were centrifuged as soon as possible (not later than 1 h later) at 3000 rpm for 7 min at 4 °C. The clinical and pathologic parameters of bladder cancer patients were recorded and are listed in Table 1. Those individuals smoking at least 1 cigarette per day for over 1 year were defined as smokers. Tumor staging and grading were determined according to TNM and World Health Organization classifications, respectively. Stages T1–T2 were categorized as early stage and T3–T4 were categorized as advanced stage.

Cell lines and cell culture

Human bladder cancer cell lines T24, SW780, and HT1376 and the non-malignant SV-40 immortalized bladder epithelial cell line (SV-HUC-1) were obtained from the American Type Culture Collection (Manassas, VA). All cell lines were maintained in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco, NY) at 37°C in a humidified atmosphere containing 5% CO_2.

RNA extraction and qRT-PCR analysis

Total RNA from urine samples were extracted using RNeasy kit (Qiagen, Valencia, CA). The first chain of cDNA was synthesized by reverse transcription with TaqMan® Reverse Transcription Reagents (Applied Biosystems, Grand Island, NY). GAPDH was used as internal control. The sequences of the primers were: GAPDH, forward-*5'-AGGTCCACCACTGACACGTT-3'* and reverse-*5'-G- CCTCAAGATCATCAGCAAT-3'*; *NDRG2*, forward-*5'-GCCCAGC-GATCCTTAC- CTACC-3'*, and reverse-*5'-GGCTGCCCAATCCATC-CAACC-3'*. RT-PCR reaction was performed using the CFX96 Touch PCR system (Bio-Rad). The relative mRNA expression of *NDRG2* was calculated by $-2^{\Delta\Delta ct}$ method. Each sample was analyzed in triplicate.

Western blotting analysis

Total protein was isolated from the urine samples and cell lines. Then, the protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto PVDF membranes. The membranes were incubated with mouse anti-human *NDRG2* antibody at 4°C for 1 night after being blocked with 5% non-fat milk for 1 h. β-actin was used as the internal control. Horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse secondary antibody (dilution 1: 2000, Santa Cruz) was used to assess the band

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Table 1. Relationship between urinary *NDRG2* expression and clinicopathologic parameters of patients with bladder cancer.

Parameters	Cases (n=124)	*NDRG2* expression		χ^2	*P* values
		Low (n=72)	High (n=52)		
Gender				0.307	0.579
Male	68	41	27		
Female	56	31	25		
Age (years)				2.251	0.134
<50	65	36	29		
≥50	59	36	23		
Tumor size (cm)				0.943	0.332
<3.75	70	38	32		
≥3.75	54	34	20		
Smoking status				1.343	0.247
Non-smoker	86	47	39		
Smoker	38	25	13		
Grade				5.045	0.025
Low	41	18	23		
High	83	54	29		
Tumor stage				8.875	0.003
T1–T2	79	38	41		
T3–T4	45	34	11		

on the membranes. Then, the enhanced chemiluminescence (ECL) system detection solutions (Pierce, NJ) were added, and the final results were obtained using Kodak Digital Science ID software (Kodak, NY). Each sample was analyzed in triplicate.

Statistical analysis

Statistical analyses were all carried out by using the SPSS statistical package (version 18.0) and graphs were plotted using Origin 9.0. Data from all quantitative assays are expressed as the mean±standard deviation (SD). Differences between 2 groups were analyzed using Students' t-test. Associations between *NDRG2* expression and clinical variables were analyzed using the chi-square test. Receiving operating curves (ROC) were made to estimate the diagnostic performance of *NDRG2* in distinguishing patients with bladder cancer from healthy controls. Differences with $P<0.05$ were considered to be statistically significant.

Results

The expression of NDRG2 in urinary samples of bladder cancer patients and its cell lines

To investigate whether *NDRG2* was detectable and altered in urine samples of bladder cancer patients compared with healthy controls, we performed qRT-PCR and Western blotting analysis to detect the expression levels of *NDRG2*at mRNA and protein level, respectively. As shown in Figure 1A and 1B, the relative *NDRG2* expression was significantly lower in patients with bladder cancer than in healthy controls (Figure 1, $P<0.05$). Then, we measured the expression of *NDRG2* in the cell lines of bladder cancer and bladder epithelial cells. The downregulated expression of *NDRG2* at mRNA and protein levels was detected in bladder cancer cell lines T24, SW780, and HT1376 compared to that in SV-HUC-1 cells ($P<0.05$, Figure 2A and 2B).

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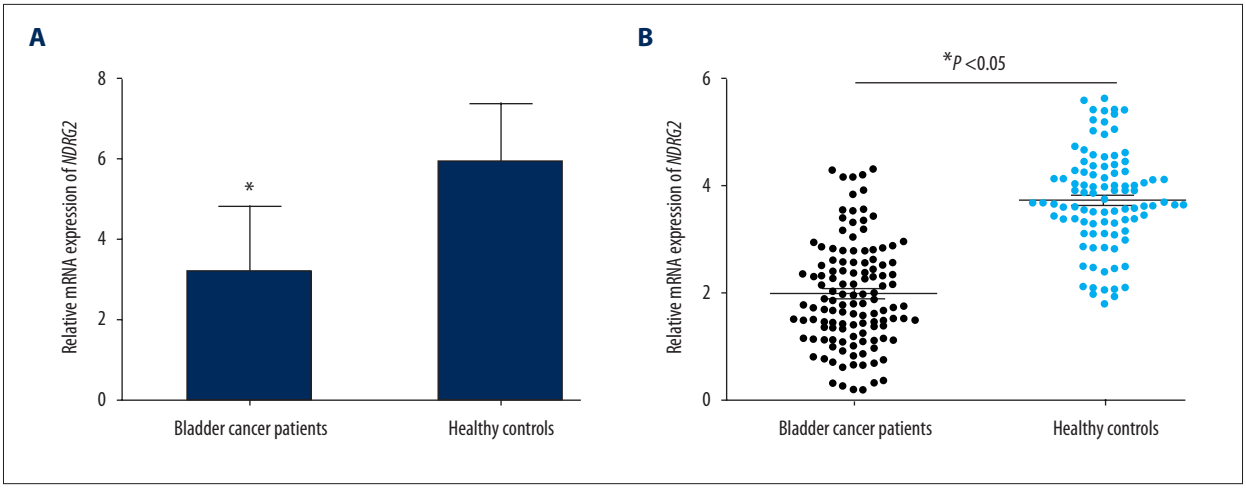

Figure 1. The relative *NDRG2* expression in urinary samples of bladder cancer patients and healthy controls. It was significantly down-regulated in patients with bladder cancer compared with that in healthy controls at mRNA (**A**) and protein (**B**) levels ($P<0.05$).

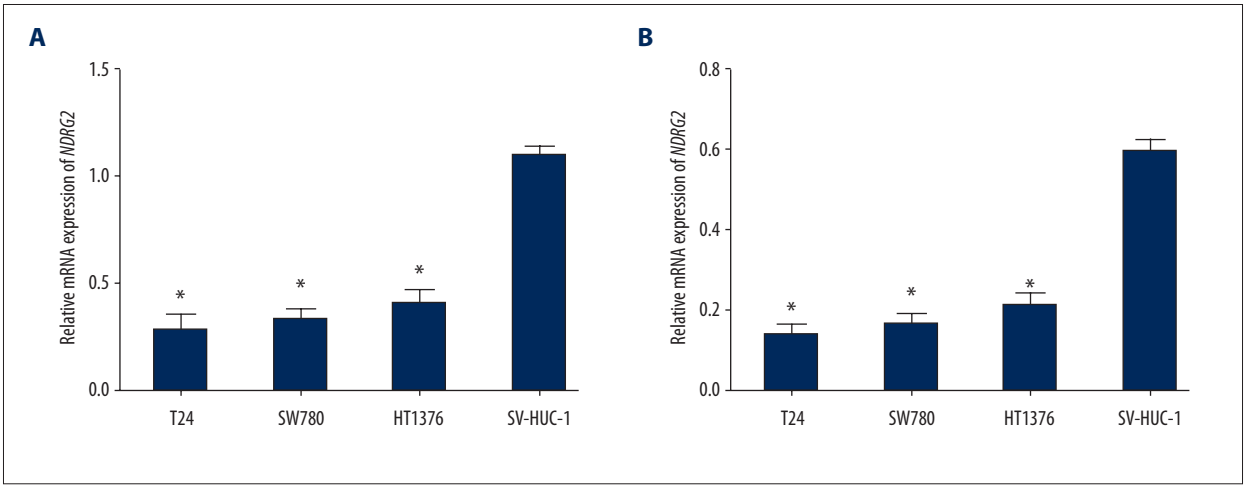

Figure 2. The relative *NDRG2* expression in cell lines of bladder cancer patients and bladder epithelial cell line. It was significantly lower in bladder cancer cell lines T24, SW780, and HT1376 than in SV-HUC-1 cells at mRNA (**A**) and protein (**B**) levels ($P<0.05$).

Relationships between NDRG2 expression and clinical pathological characteristics of patients with bladder cancer

To assess the correlation of *NDRG2* expression with clinicopathologic data, the patients with bladder cancer were categorized into low and high groups on the basis of the mean value. The chi-square test revealed that low *NDRG2* expression was significantly associated with grade ($P=0.025$) and tumor stage ($P=0.003$), but it had not relationship with other parameters, including sex, age, tumor size, and smoking status ($P>0.05$, Table 1).

The diagnostic value of NDRG2 in bladder cancer

The potential clinical utility of *NDRG2* in discriminating patients with bladder cancer from healthy controls was assessed. ROC curves showed that *NDRG2* had a high diagnostic value, with an AUC of 0.888, with a sensitivity of 85.5% and a specificity of 81.4% (Figure 3). The ideal cutoff value of *NDRG2* was 4.840.

Discussion

We assessed the expression levels of *NDRG2* in urine samples from 124 bladder cancer patients and 97 healthy controls, as well as in human bladder cancer cell lines T24, SW780, and HT1376 and non-malignant SV-40 immortalized bladder epithelial cells (SV-HUC-1). The results showed that the relative *NDRG2* expression was significantly downregulated in the urine of patients with bladder cancer and bladder cancer cell lines compared with that in controls, both at mRNA and protein levels. Our results agree with most of the published literature,

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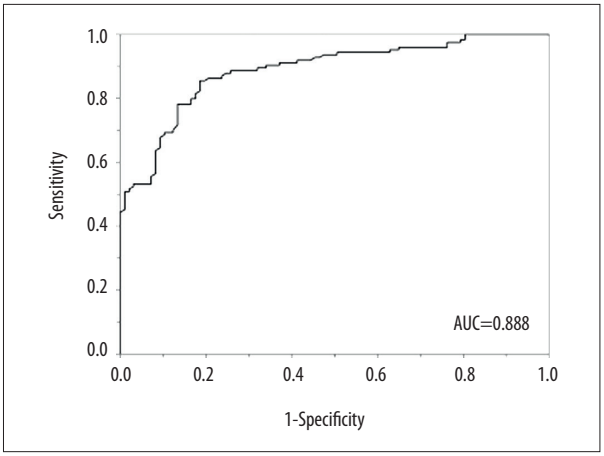

Figure 3. A receiver operating characteristic curve was established with the expression of *NDRG2* from urinary samples of bladder cancer patients (n=124) and healthy controls (n=97). The area under the curve of *NDRG2* was 0.888 (95% confidence interval: 0.845–0.930).

and show that *NDRG2* might be a tumor suppressor in bladder cancer. Further analysis indicated that NDRG2 might be a diagnostic biomarker for bladder cancer.

Bladder cancer is a heterogeneous disease with unpredictable clinical course. There are 2 predominant histological types: transitional cell carcinoma and squamous cell carcinoma. The major risk factors for bladder cancer include cigarette smoking and chronic infection with *Schistosoma haematobium* [21]. Numerous biomarkers and histopathological factors, such as tumor stage, tumor grade, and lymph node, have been investigated as important biomarkers of bladder cancer [22,23]. However, these biomarkers cannot provide sufficient specificity and sensitivity to detect the whole spectrum of bladder cancer encountered in routine clinical practice [24].

NDRG2 is highly expressed in skeletal muscle tissue both at the gene level and as a phosphoprotein [7,25]. Previous studies have shown that *NDRG2* is highly expressed in the adult brain, salivary glands, and muscle, but it rarely occurs in bone marrow, leukocytes of peripheral blood, and thymus [26–29], suggesting that it may play important functions in different tissues. Decreased expression of *NDRG2* has also been found in some types of human cancer and tumor cells. For example, Li et al. revealed that protein and mRNA expression levels of *NDRG2* were significantly downregulated in astrocytomas [30]. Downregulated *NDRG2* has also been detected in tissue specimens from clear cell renal cell carcinoma and cancer cell lines [31]. *NDRG2* is a potential tumor suppressor gene, and abundant data demonstrates close associations of *NDRG2* with cell proliferation, metastasis, and apoptosis [32,33].

We further explored whether *NDRG2* was involved in the development of bladder cancer, finding that low expression of *NDRG2* was associated with grade and tumor stage, but we found no relationship between *NDRG2* expression and other clinicopathological features, including sex, age, and tumor size. These data suggest that *NDRG2* expression levels are closely associated with the development and progression of bladder cancer. Moreover, we plotted the ROC curves and showed the performance of the prediction mode in bladder cancer patients and healthy controls. The results indicated that the AUC of *NDRG2* levels was 0.888; in addition, the sensitivity and specificity were 85.5% and 81.4%, respectively, indicating it might be a potential biomarker for diagnosis of bladder cancer.

In recent years, many studies have been performed to search for diagnostic biomarkers for bladder cancer. Eissa et al. reported that survivin and TIMP-2 could be considered as urine markers in early detection of bladder cancer. Survivin showed 78.6% sensitivity and 95.3% specificity in the diagnosis of bladder cancer, while TIMP had 93% sensitivity and 83.7% specificity [3]. Another study concluded that VEGF was an accurate urinary biomarker for bladder cancer, with 83% sensitivity and 87% specificity, and an AUC of 0.886 [4]. *NDRG2* showed 85.5% sensitivity and 81.4% specificity in the diagnosis of bladder cancer, and the AUC was 0.888. Although *NDRG2* showed no advantages compared with the 2 molecules in the diagnosis of bladder cancer, it was suggested as a potential biomarker. Future analysis should be performed to confirm our findings.

The current study has a few limitations. Since tumors result from the functions of environmental and genetic factors, the *NDRG2* gene may be one of several factors involved in the carcinogenesis of bladder cancer. Two or more factors should be considered in future research, which makes the result much more accurate. In addition, the sample size was relatively small. Larger-scale experiments should be performed to verify the outcome.

Conclusions

In conclusion, attenuated *NDRG2* level may play pivotal roles in the occurrence, development, and progression of bladder cancer. The detection of *NDRG2* level may be a useful biomarker in the diagnosis or prediction of clinical outcomes of patients with bladder cancer. Results of the present study may provide novel approaches for targeted molecular therapy for patients with bladder cancer, although further study with larger samples is needed to validate and optimize our findings.

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