Diagnostic accuracy of NMP-52, NMP-22 and urine cytology in the diagnosis of bladder cancer

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Abstract
Bladder cancer (BC) is the most important tumor problem of urologic cancer. Therefore, noninvasive urinary biomarkers were used for diagnosis of BC. However, the new biomarkers failed to reach higher accuracy. The aim of this study was to assess the diagnostic efficacy of nuclear matrix protein-22 (NMP-22), nuclear matrix protein-52 (NMP-52), urinary cytology and to investigate combinations of urine NMP-52 with urinary cytology as noninvasive biomarkers to increase diagnostic performance of bladder cancer at different grades and stages. Overall, there were 156 subjects (62 BC, 54 cystitis patients and 40 healthy volunteers). The NMP-22 and NMP-52 were quantified in urine samples by ELISA. The urinary NMP-52 and NMP-22 were included as noninvasive biomarkers to increase diagnostic performance of bladder cancer at different grades and stages. The sensitivity and specificity for NMP-52 were 94% and 82%, for NMP-22 69% and 80.8% and for cytology 56% and 94.6% respectively and also, both urinary NMP-22 and NMP-52 have extremely significant relation (p<0.0001) to BC vs. healthy individuals and cystitis patients. Moreover, the combination of NMP-52 with urinary cytology could predict all BC stages and grade with 95.6% sensitivity and 94.3% specificity. In conclusion, NMP-52 and urinary cytology in combination improve diagnostic performance for BC detection in different pathological types.

Keywords: Bladder cancer (BC), Diagnosis, NMP-22, NMP-52, Urinary cytology, Urine.

Introduction
The most common malignant cancer of the urinary system is the bladder cancer (BC). It is the second genitourinary cancer in incidence and mortality. In general, in the industrially developed countries and regions associated with endemic schistosomiasis, the incidence rates are highest. The majority (75%-80%) of patients present non-muscle-invasive tumors (NMIBC, stages <T2). Only about 20%-25% of tumors are muscle invasive (MIBC, T2+) or metastatic at diagnosis.

Cystoscopy is the gold standard for the diagnosis and monitoring of BC. It is highly invasive and relatively expensive and many patients in the early stages of the disease undergo clinical examinations, but they undergo cystoscopy less frequently, thus limiting its use. Voided urine cytology is performed to diagnose and discover bladder carcinoma as well as to evaluate the morphological changes in normal cells, it is a non-invasive test with a good sensitivity for detecting high grade BC but the low grade tumors have a low sensitivity that is only 4% to 31%. Investigators are searching to increasing the sensitivity for several urinary tumor markers because urine can be obtained easily and large amounts of sample can be repeatedly obtained with low cost, noninvasive and easy to perform.

Nuclear matrix proteins (NMPs) present the essential part of the nucleus. The level of NMP22 is higher in the case of BC than urothelium in normal bladder. The urinary NMP marker was identified at 52 kDa (NMP-52) in the urine of BC patients. In this study, the NMP-52, NMP-22 and urinary cytology were evaluated alone and as combined with urinary cytology for diagnosis of BC at different stages and grades.

Material and Methods
Patient’s database: Voided urine samples of 156 individuals who underwent cystoscopy as the reference standard for identification of BC (104 males and 52 females) were enrolled in this study and were collected from Urology and Nephrology center, Faculty of Medicine, Mansoura University. Biopsy was performed for histopathologic examination. The participants in this study were classified into 3 groups:

Group I: 62 BC patients including 40 men (64.5%) and 22 women (35.5%), with a mean age of 45.65±7.39 year [range of age 32–80 year].

Group II: 54 cystitis patients (non-cancerous), (38 men (70.4%) and 16 women (29.6%) with a mean age 44.35±7.62 years [range of age 30-81] such as 43 cases with inflammatory smear and 11 cases with haematuria.

Group III: 40 healthy volunteers including 26 men (65%) and 14 women (35%) with mean age 46.5±5.87 years [range of age 34–58 year] were used as a control group.

According to TNM classification, the 62 BC patients included 6 cases with T1 stage, 7 cases with T2 stage, 26 cases with T3 stage and 23 cases with T4 stage. On the other hand, 62 patients with BC were classified into two groups, 6 cases with non-muscle invasive BC (NMIBC; stage≤T2) and 56 cases with muscle invasive BC (MIBC; stage≥T2). BC patients were also subdivided according to the grade of tumor into 12 with low grade tumors (G1) and 50 with high...
grade tumors (G2-G3) including 15 cases (G2) and 35 cases (G3).

Sample collection: From all patients, the urine samples have been collected at the first morning. The collected urine samples were centrifuged at 3000-4000g for 15 minutes and separated into supernatant and pellet. The supernatant was stored at -80°C until used for NMP-22, NMP-52.

NMP-52 Assays: The NMP-52 was tested in urine using ELISA. The procedure test includes four steps. In step 1, in coating buffer (50 mM carbonate/bicarbonate buffer, pH 9.6) the urine samples were diluted (1:20), 50µl/well were tested for NMP bound on microtiter plate (Costar, Corning Life Sciences, Acton, MA) at 4°C overnight. After the first step, the plate was washed three times using 0.05% (v/v) PBS-Tween 20 (PBS-T20) (pH 7.2) and blocked the free active sites with 0.5% (w/v) BSA in coating buffer (200µl/well). In step 2, 50µl/well of NMP-52 antibody at dilution 1:250 in PBS-T20 was added (50µL/well) and incubated at 37°C for 2 hr. In step 3, 50µl/well of anti-rabbit IgG alkaline phosphatase conjugate (Sigma), 1:500 in 0.2% BSA (w/v) in PBS-T20, was added and incubated for 1 hr.

The conjugate was bound to a specific anti-NMP-52 bound to urine NMP coating the microwell surface. The microwells in plate are washed. Nitrophenyl phosphate substrate was added (50µl/well) was incubated. In bound conjugate, the color is formed as a result of the hydrolysis of phenylphosphate. The reaction is stopped by adding sodium hydroxide 3 M NaOH and the absorbance was read at 490 nm using microplate autoreader (∑960, Metertech, Inc., Taipei, Taiwan). Color intensity was proportional to the level of NMP-52 present in the urine sample.

A standard curve was generated from dilution series to allow the concentration of NMP-52 in each sample to be measured as a function of the concentration (µg/mL) in urine samples. The sensitivity and specificity of NMP-52 were determined at cut-off 2.2 µg/mL.

There is a proportional relationship among urine antigen concentration and the development of color intensity. In addition, NMP-22 concentration has been calculated from a standard curve. The sensitivity and specificity of NMP-22 were determined at cut-off value 6.5 U/mL.

Urine cytology: Urine cytology was performed using standard techniques. Cytology diagnosis was classified according to the cytopathological: positive, suspicious or negative. To improve the sensitivity and specificity, suspicious samples were considered as positive.

Statistical Analysis: All statistical analyses were done using SPSS; v.21.0 (SPSS Inc., Chicago, IL). Mean ± Standard Error (SE) were performed to express continuous variables. Sensitivity and specificity were then determined.

Results
Diagnostics Performance of NMP-22, NMP-52, urine cytology and combination of NMP-52 and urine cytology for BC detection: The sensitivity of NMP-52 for NMIBC, MIBC, low grade (G1) and high grade (G2-G3) was 83%, 95%, 92% and 94% respectively. For all BC patients, the NMP-52 gave 94% sensitivity and 82% specificity with extreme significance (p<0.0001) in relation to BC vs. healthy individuals and cystitis patients.

The sensitivity of NMP-22 for NMIBC, MIBC, low grade and high grade was 50%, 82%, 33% and 78% respectively. For all BC patients, the NMP-22 gave 69% sensitivity and 80.8% specificity with extreme significance (p<0.0001) in relation to BC vs. healthy individuals and cystitis patients.

<table>
<thead>
<tr>
<th>Category</th>
<th>n*</th>
<th>Sensitivity of NMP-52,%</th>
<th>Sensitivity of NMP-22, %</th>
<th>Sensitivity of Urine cytology, %</th>
<th>Sensitivity of NMP-52, + Urine cytology %</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMIBC; stage&lt;T2</td>
<td>6</td>
<td>(5/6) 83</td>
<td>(3/6) 50</td>
<td>(1/6) 16.7</td>
<td>83</td>
</tr>
<tr>
<td>MIBC; stage≥T2</td>
<td>56</td>
<td>(53/56) 95</td>
<td>(46/56) 82</td>
<td>(44/56) 78.6</td>
<td>96.4</td>
</tr>
<tr>
<td>Low grade (G1)</td>
<td>12</td>
<td>(11/12) 92</td>
<td>(4/12) 33</td>
<td>(2/12) 17</td>
<td>92</td>
</tr>
<tr>
<td>G2</td>
<td>15</td>
<td>(15/15) 100</td>
<td>(11/15) 73</td>
<td>(4/15) 27</td>
<td>100</td>
</tr>
<tr>
<td>G3</td>
<td>35</td>
<td>(32/35) 91</td>
<td>(28/35) 80</td>
<td>(29/35) 83</td>
<td>94.3</td>
</tr>
<tr>
<td>High grade (G2-G3)</td>
<td>50</td>
<td>(47/50) 94</td>
<td>(39/50) 78</td>
<td>(33/50) 66</td>
<td>94</td>
</tr>
</tbody>
</table>

n* = number of BC patients; G=grade; NMIBC= non-muscle-invasive tumors; MIBC= muscle invasive
Although several urinary markers have shown higher sensitivity and specificity for cytology in all types of all BC were 56% and 94.6 respectively (table 1 and table 2).

According to this result, NMP-52 test had better sensitivity and the urine cytology had better specificity. Remarkably, when NMP-52 was combined with urinary cytology for detection of BC at different stages or grades, the sensitivity and specificity reached to the highest values of 95.6% and 94.3% respectively as shown in table 1 and table 2.

### Discussion

The most common malignant cancer of the urinary system is the BC which is considered as heterogeneous disease as well as very aggressive disease. Many urinary markers for diagnostics of bladder cancer have been investigated such as urinary NMP22 and NMP-52 as bladder tumor antigen. The diagnostic utility for NMIBC is limited because the sensitivity of NMP-22 was 46%-48% for stages T2. Recently, many investigational urine markers have been described in reviews. Although several urinary markers have shown higher sensitivity, most suffer from low specificity.

Urinary cytology is a non-invasive test compared to cystoscopy. The sensitivity was 83% for high grade of BC but the specificity was relatively low for detection of low grade tumors. It is only 17% and the specificity was excellent; 94.6% whereas the sensitivity and specificity for NMP-22 were 50-80% and 80.8% respectively (p<0.0001). In addition, the NMP-52 gave a sensitivity of 83% for NMIBC (stage<2) and 92% for low grade and It gave a sensitivity of 95% for MIBC (stage≥T2) and 94% for high grade (p<0.0001). These results are similar to other studies where the NMP-52 was used for diagnostic of BC with a sensitivity of 87% and a specificity of 83%.

The explanation of these results would be that the concentration of NMP-52 is twenty-five times higher in urine from bladder cancer than in urine from normal bladder and five times than in urine from cystitis patients because the NMP-52 and the NMP-22 are probably released from nuclei of tumor cells during apoptosis. In present study, the NMP-52 test gave higher sensitivity and the urine cytology has the higher specificity. Therefore, the combinations between NMP-52 and cytology were used to increase the sensitivity and the specificity for BC detection for both low grade and high grade but the higher grade tumors were associated with higher sensitivity and specificity than lower grade tumors because low grade tumors undergo less apoptosis than more advanced tumors.

The average sensitivity could be increased by using combinations of NMP-52 with urine cytology for all BC patients to 95.6% and the range sensitivity for BC at different stage and grade (83-100%). The sensitivity increased for the combination of ImmunoCyt with urinary cytology from 78.1% to 89.1%. Also, the combination increased sensitivity for the NMP22 with urinary cytology from 85% to 91%. The advantage of this study is that the specificity remains high and unchanged, it was 94.3% at the combination of NMP-52 with cytology.

Conversely, also the sensitivity is relatively high in the combination of NMP-22 with cytology (range 91–94%), although the specificity dropped to 28-31%. Future researches are needed to improve biological markers at different stages and grade of BC. Our research results highlight the improved bladder cancer diagnosis through the combined use of NMP-52 and urine cytology.

### Conclusion

The combination of NMP-52 and urine cytology is a good diagnostic tool for BC in different pathological types of BC.

### References


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