Global Journal of Biotechnology & Biochemistry 10 (1): 32-38, 2015
ISSN 2078-466X

DOI: 10.5829/idosi.gjbb.2015.10.01.1117

Diagnostic Performance of Nuclear Matrix Protein (NMP-52) in Bladder Cancer

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Abstract: Bladder cancer (BC) is one of the most common tumors of genitourinary tract. Therefore, the present study aimed to identification of Nuclear Matrix protein (NMP-52) is a critical step toward accurate diagnosis of BC. Western blot using monospecific anti-NMP-52 antibody was used to identify NMP-52 marker in urine sample from patients with BC, then the NMP-52 was purified. Urine samples were obtained from 40 healthy individuals, 54 cystitis patients and 62 patients with bladder cancer. The NMP-52 levels in the urine samples were measured using ELISA method. The mean urinary NMP-52 level in healthy individuals, cystitis and patients with bladder cancer was 0.72, 3.82 and 17.85 µg/ml, respectively. Area under the rock curve (AUC) of NMP was 0.89. The sensitivity and specificity were 94% and 82% at cutoff value equal 2.2 µg/ml. Our data demonstrated that NMP-52 is a good diagnostic tool for screening BC owing to its high sensitivity and specificity.

Key words: Bladder cancer (BC) · Nuclear matrix protein (NMP-52) · Diagnosis · Urine

INTRODUCTION

Bladder cancer is the second in incidence and mortality cancer of the genitourinary system and estimated to be the ninth most common malignancy globally, accounting for 3.3% of all malignancies [1]. The highest incidence rates of bladder cancer are generally found in industrially developed countries and in areas associated with endemic schistosomiasis, including parts of Africa and the Middle East [2]. Early diagnosis of bladder cancer allows for effective local treatment and optimizes the success of surgical therapy [3]. Owing to the lack of disease specific symptoms, diagnosis and follow up of bladder cancer remain a challenge. Cystoscopy, the gold standard for the detection of bladder cancer, is highly invasive and relatively expensive and many patients with a low grade and stage lesion could undergo cytoscopy less frequently if alternative tests were available for clinical practice, thus limiting its use [4, 5]. Voided urine cytology is a highly specific, non-invasive adjunct to cystoscopy. It has good sensitivity for detecting high grade bladder cancer, but its sensitivity for detection of low grade tumors is only 4% to 31% [6]. Furthermore, the performance of cytology is dependent upon the level of expertise of the cytopathologist, it is relatively expensive and it is not readily available in all countries. Thus, a noninvasive, highly sensitive and specific marker for detecting bladder cancer could decrease the morbidity associated with cystoscopy, improve patient quality of life and decrease costs by substituting a less expensive, noninvasive test for the more expensive endoscopic procedure. The clinical scenarios in which such a test could play a role are in the early diagnosis (voiding symptoms, hematuria and high risk populations) of bladder cancer and the surveillance of patients with previous occurrence of bladder cancer [7]. Nuclear matrix proteins (NMPs) are an important part of the structural framework of the nucleus [8]. It has been

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demonstrated that NMP22 concentration is greater in bladder cancer cell lines than in the urothelium from normal bladder [9, 10]. In this respect, Attallah *et al.* [11] identified a urinary NMP marker at 52 kDa (NMP-52) in the urine of bladder cancer patients. Therefore, this study aimed to evaluate the diagnostics efficacy of NMP-52 assay in urine from patients with bladder cancer.

MATERIALS AND METHODS

Patient's Population: Voided urine samples of 116 patients with renal diseases who underwent cystoscopy as the reference standard for identification of bladder cancer (78 males and 38 females) were enrolled in this study and were collected from Urology and Nephrology Center, Faculty of Medicine, Mansoura University. Biopsy of any suspicious lesion was performed for histopathologic examination. Accordingly, all 116 patients were classified into 2 main groups:

Group I: Bladder cancer patients (n = 62), 40 (64.5%) males and 22 (35.5%) females, with a mean age 45.65±7.39 years (range 32–80). According to TNM classification, 62 bladder cancer patients were included 6 cases with T_1 stage (Tumor invades subepithelial connective tissue), 7 cases with T_2 stage (Tumor invades muscle), 26 cases with T_3 stage (Tumor invades perivesical tissue), 23 cases with T_4 stage (Tumor invades any of the following: prostate, uterus, vagina, pelvic wall, abdominal wall). On the other hand, the 62 patients with bladder cancer were classified into two groups, 6 cases with non-muscle invasive bladder cancer (NMIBC; stage$<T_2$) and 56 cases with muscle invasive bladder cancer (MIBC; stage $\geq T_2$). Bladder cancer patients were also subdivided according to the grade of tumor into 12 cases were (G1) which classified as low grade, 15 cases were (G2) and 35 cases were (G3) which classified as high grade.

Group II: Cystitis patients (n = 54), 38 (70.4%) males and 16 (29.6%) females with a mean age 44.35±7.62 years (range 30-81). These patients included 43 cases with inflammatory smear and 11 cases with haematuria. In addition, 40 apparently healthy volunteers (26 (65%) males and 14 (35%) females, with a mean age 46.5±5.87 years (range 34–58) were used as a control group and included in the study. The Ethical Committee of Mansoura University Hospitals, Mansoura, Egypt approved the present study. Informed consents were obtained from all participants and they were fully informed concerning the diagnostic procedures involved and disease nature. The study protocol conformed to the ethical guide-lines of the 1975 Helsinki Declaration. Urine sample was obtained prospectively from all patients. The collected urine samples were centrifuged at 2500-4000g for 15-20 minutes and separated into supernatant and pellet. The pellet containing the cellular sediment was discarded and the supernatant was used for subsequent analysis or stored at -20°C with no additions or treatment until tested.

Production of Anti-NMP-52 IgG Antibodies: Specific IgG antibodies were produced in four New Zealand white rabbits immunized subcutaneously in three different inoculationsites with the 52-kDa purified marker. In brief, equal volumes (500ml) of the antigen (500mg/ml) and complete Freund's adjuvant (CFA) or incomplete Freund'sadjuvant (IFA) were homogenized together using two Luer-lock syringes connected to three-way stainless-steel valve. Each rabbit was immunized subcutaneously three times, once with antigen in CFA (on day 0) and twice with antigen in IFA (on days 15 and 28) before being killed on day 32. Blood samples were collected from all rabbits at 0, 28 and 32 days of immunization. The sera were separated, purified and stored at -20°C until used. The reactivity of the collected rabbit sera was tested against urine samples of histopathologically diagnosed patients with bladder cancer.

SDS-PAGE and Gel Electro-Elution: Urine samples were subjected to analytical SDS-PAGE at 100 mg/lane, using vertical slabs of 16% polyacrylamide [12]. Molecular weight standards (Sigma Chem Co., St. Louis, MO) were run in parallel. In preparative slab gel electrophoresis, the running condition was adapted to reduce smear of proteins and to enable a long migration distance between bands in the 52 kDa region, according to the pre-stained molecular weight marker. In each run, 250 µL of urine per preparative gel was electrophoresed and a lane from electrophoresed preparative gel stained with Coomassie blue and immunoblotted to identify the 52-kDa band. In the unstained preparative gel, the adjacent band was then cut and the 52-kDa antigen electro-eluted from polyacrylamide gel at 200 V for 3 h in a dialysis bag (Sigma). Forty runs were completed to obtain 1 mg of the 52-kDa antigen. After dialysis, the electro-eluted antigen was concentrated using polyethylene glycol and 40% trichloroacetic acid (TCA), then centrifuged at 6500 *g* for 15 min. The precipitate was washed twice using diethyl ether to remove excess TCA. The excess diethyl ether was

removed by gentle drying and the pellet reconstituted in PBS (pH 7.2). The protein content of a sample of electro-eluted antigen was determined before the remainder was stored at -20°C.

Western Blot: Urine samples separated on SDS-PAGE were electrotransferred on to nitrocellulose membrane (0.45µm pore size, Sigma) in a protein transfer unit [13]. Thenitrocellulose filter was blocked using 5% (w/v) nonfat dry milk dissolved in 0.05 mol/L Tris-buffered saline (TBS) containing 200 mM/L NaCl (pH 7.4), rinsed in TBS and incubated with the anti-NMP-52antibody diluted in blocking buffer with constant shaking. The blots were washed three times (30 min each) in TBS, followed by incubation for 2 h with goat antirabbit IgG alkaline phosphatase conjugate (Sigma) diluted 1: 500 in TBS. After washing three more times with TBS (15 min each), the blots were soaked in substrate (premixed BCIP and NBT in 0.1 mol/L Tris buffer, pH 9.6; ABC Diagnostics, New Damietta, Egypt). The colour reaction was observed within 15 min and dipping the blots in distilled water then stopped the reaction.

Quantitation of Urinary NMP-52 Using ELISA: Voided urine samples were tested for NMP-52 using ELISA procedure. The assay technique is a four-step test. In step 1, diluted urine samples (1:20) in coating buffer (50 mM carbonate/bicarbonate buffer, pH 9.6), were tested (50µl/well) for NMP bound on a 96-well microtiter plate (Costar, Corning Life Sciences, Acton, MA) at 4°C overnight. At the end of the first step, the plate was washed three times using 0.05% (v/v) PBS-Tween 20 (PBS-T20) (pH 7.2) and the free active sites were blocked with 0.5% (w/v) BSA in coating buffer (200µl/well). In step 2, 50µl/well of a highly reactive NMP-52 antibody at dilution 1:250 in PBS-T20 was added separately (50µl/well) and incubated at 37°C for 2 hr. In step 3, 50µ/well of anti-rabbit IgG alkaline phosphatase conjugate (Sigma), 1:500 in 0.2% (w/v) BSA in PBS-T20, was added and incubated for 1hr. The conjugate was bound to a specific anti-NMP-52 bound to urine NMP coating the microwell surface. At the end of step 3, the microwells are washed to remove any unbound conjugate. In step 4, an enzyme detection system composed of nitrophenyl phosphate substrate (50µl/well) was added to the microwell. In the presence of bound conjugate, the p-nitrophenyl phosphate was hydrolyzed, resulting in a colored end product. The reaction was stopped by using 3 M NaOH and the absorbance was read at 490 nm using Σ960 microplate autoreader (Σ960, Metertech, Inc., Taipei, Taiwan). Color intensity was proportional to the amount of bound conjugate and therefore is a function of the concentration 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.

RESULTS

Identification of Nuclear Matrix Protein (NMP) by SDS-PAGE and Western Blot: The anti-NMP-52 antibody identified the reactive epitopes at 52 kDa in all urine from patients with different types of bladder cancer (Fig. 1). The 52-kDa antigen was identified in one of three urine samples from cystitis patients. The reactive epitope 52 kDa purified from urine was analyzed by SDS-PAGE, a single polypeptide band was stained with Coomassie blue, at 52-kDa (Fig. 2). The reactivity of the isolated antigen was confirmed using Western blot (Fig. 3).

NMP-52 Levels among Bladder Patients and Healthy Individuals: As shown in Table 1, the mean urinary level of NMP-52 was appreciably higher in the 62 bladder cancer patients than in the cystitis patients and healthy individuals with extremely highly significant differences ($p<0.0001$) between the different groups. The positive detection rate of urinary NMP-52 in healthy individuals, cystitis patients and bladder cancer patients were 5, 28 and 94%, respectively.

NMP-52 Levels in Bladder Cancer Patients according to Lesions? s Stage and Grade: The positive rate of urinary NMP-52 for different stages of bladder cancer T1, T2, T3 and T4 were 83, 86,100 and 91%, respectively. The NMP-52 levels in urine samples at different bladder cancer stages T1, T2, T3 and T4 were 14.82±4.24, 24.84±7.82, 18.74±5.38 and 15.5±4.56 µg/ml, respectively. However, this study subsequently demonstrated non-significant difference level in different stages (p=0.33).Moreover, the mean level of NMP-52 was 14.82±4.24 µg/ml in urine of patients with (NMIBC; stage$<T_2$) and 18.17±3.24 µg/ml in urine of patients with (MIBC; stage $=T_2$) with non-significant difference (p=0.37) between the two different groups (Table 2). The positive detection rate of urinary NMP-52 for G1, G2 and G3 were 92%, 100% and 91%, respectively. The NMP-52 level in urine samples of G1, G2 and G3 was 10.91±2.43, 34.22±8.83 and 13.21±2.97 µg/ml with non-significant difference between different groups; p=0.57 (Table 2).

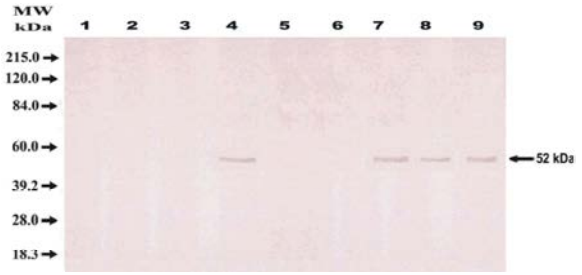

Fig. 1: Immunoblots of NMP antigen in urine from patients with bladder cancer, cystitis patients and healthy individuals

Lanes (1-3): urine samples from healthy individuals, Lanes (4-6): urine samples from cystitis patients, Lanes (7-9): urine samples from patients with bladder cancer, Molecular weight markers (M) was included myosin (215.0 kDa), phosphorylase B, (120.0 kDa), bovine urine albumin (84.0 kDa), Ovalbumin (60.0 kDa), carbonic anhydrase (39.2 kDa), trypsin inhibitor (28.0 kDa) and lysozyme (18.3 kDa)

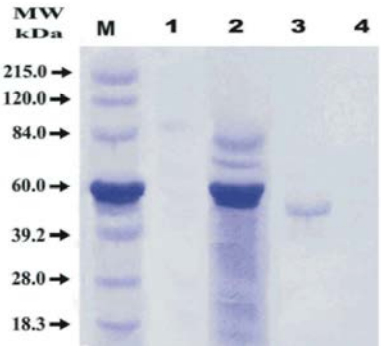

Fig. 2: SDS-PAGE analysis of the 52 kDa purified NMP

Lane 1: Urine sample of healthy individual as a negative control. Lane 2: Urine sample of bladder cancer patient as a positive control. Lane 3: Trichloroacetic acid (TCA) precipitate fraction of purified 52-kDa. Lane 4: Trichloroacetic acid (TCA) supernatant fraction. Molecular weight marker (M) was included myosin (215.0 kDa), phosphorylase B, (120.0 kDa), bovine urine albumin (84.0 kDa), ovalbumin (60.0 kDa), carbonic anhydrase (39.2 kDa), trypsin inhibitor (28.0 kDa) and lysozyme (18.3 kDa)

Table 1: Urinary NMP-52 levels (µg/ml) in healthy individuals, cystitis and bladder cancer patients

Group	n*	Mean ± SE (µg/ml)	Positivity %
Healthy individuals	40	0.71±0.1	5
Cystitis patients	54	3.82±0.64	28
Bladder cancer patients	62	17.85±2.95	94

n*= number of cases

Table 2: Urinary NMP-52 levels (µg/ml) in bladder cancer patients according to lesions?'s stage and grade

Category	Mean ± SE (µg/ml)	Positivity %
T1	14.82±4.24	83
T2	24.84±7.82	86
T3	18.74±5.38	100
T4	15.5±4.56	91
NMIBC; stage < T_2	14.82±4.24	83
MIBC; stage ≥ T_2	18.17±3.24	95
G1	10.91±2.43	92
G2	34.22±8.83	100
G3	13.21±2.97	91
Low grade (G1)	10.91±2.43	92
High grade (G2-G3)	19.52±3.59	94

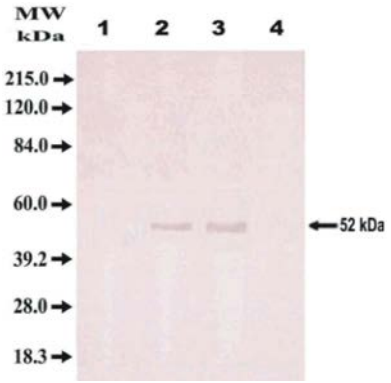

Fig. 3: Immunoblot analysis of the 52-kDa purified NMP

Purified NMP was resolved in 12% SDS-PAGE and electrobloted onto NC. Lane 1: Urine sample of healthy individual as a negative control. Lane 2: Urine sample of bladder cancer patient as a positive control. Lane 3: Trichloroacetic acid (TCA) precipitate fraction of purified 52-kDa. Lane 4: Trichloroacetic acid (TCA) supernatant fraction. Molecular weight marker (M) was included myosin (215.0 kDa), phosphorylase B, (120.0 kDa), bovine urine albumin (84.0 kDa), ovalbumin (60.0 kDa), carbonic anhydrase (39.2 kDa), trypsin inhibitor (28.0 kDa) and lysozyme (18.3 kDa)

Diagnostics Performance of NMP-52: The area under the ROC curve for bladder cancer*vs.* Cystitis patients and healthy individuals detection using urinary NMP-52 was 0.89 (Fig. 4). The present study demonstrated 94% sensitivity of NMP-52 for bladder cancer detection and 82% specificity. In addition, the positive predictive value was 77%, negative predictive value was 95% and the efficiency of this assay was 87%. The ROC analysis indicated that the optimal test characteristics of urinary NMP-52 at 2.2µg/ml. But when healthy individuals were excluded the area under the ROC curve for bladder cancer detection was decreased to 0.82 (Fig, 5) with 94% sensitivity and 72% specificity. Moreover, When cystitis

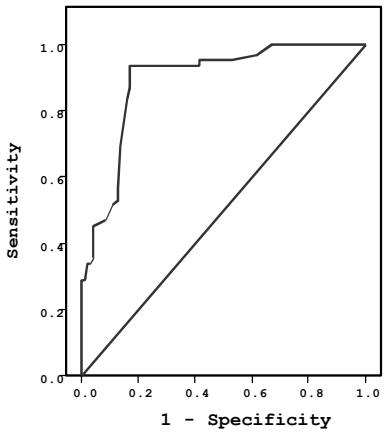

Fig. 4: ROC curve for detection bladder cancer against cystitis patients and healthy individuals (AUC=0.89; P<0.0001)

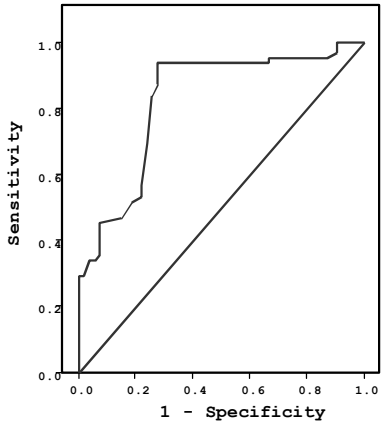

Fig. 5: ROC curve for detection bladder cancer against cystitis patients (AUC=0.82; P<0.0001)

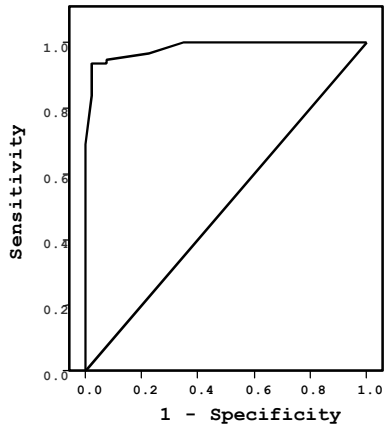

Fig. 6: ROC curve for detection bladder cancer against healthy individuals (AUC=0.98; P<0.0001)

patients were excluded the area under the ROC curve for bladder cancer detection using NMP-52 was increased to 0.98 (Fig. 6) with 94% sensitivity and 95% specificity.

DISCUSSION

Bladder cancer, a highly aggressive and heterogeneous disease, is the most common malignancy of the urinary tract [14]. Cystoscopy is an integral part in the diagnosis of transitional cell carcinoma of the urinary bladder. Standard practice for early detection of recurrences includes cystoscopy and urine cytology at regular intervals after the diagnosis [15]. However, cystoscopy is invasive expensive and uncomfortable for the patients. Urinary cytology is limited by the ability to diagnose well-differentiated lesions. It has good sensitivity for detecting high grade bladder cancer, but its sensitivity for detection of low grade tumors is only 4% to 31% [6]. Furthermore, urinary tract infections, stones or previous radiotherapy can result in false-positive cytology [16]. Examiner variability in interpreting urine cytology further confounds the use of cytology. Due to the above reasons and the fact that 60–70% of cases are well differentiated, urine cytology cannot be used to replace cystoscopy [17]. Several urinary markers for transitional cell carcinomas have been investigated, including urinary NMP22, bladder tumor antigen, fibrinogen degradation products and telomerase. The advent of noninvasive urine-based markers as well as other novel modalities has yielded improved diagnostic accuracy however it failed to reach higher sensitivity and specificity [18]. Here in, we evaluated urinary NMP-52 as a diagnostic marker for bladder cancer patients. NMP-52 kDa was identified in urine using specific anti-NMP-52 antibodies and Western blot. In contrast to the normal urine, intense immunoreactive bands were appeared in the urine of patients for different types of bladder cancer. These results further suggest that NMP-52 could be potential biomarkers for diagnosis of bladder cancer. So next, we purified the target antigen and developed a direct ELISA for the quantitation of NMP-52 separately in urine of bladder cancer.

Our results revealed a marked increase in the mean ± SD (µg/ml) of urinaryNMP-52 in BC patients vs. the cystitis patients or healthy individuals (P<0.0001). The most likely explanation for these results would be that NMP-52 is probably released from the nuclei of the tumor cells during apoptosis [11]. The NMP-52 level in urine samples of low grade (G1) and NMIBC is lower than high grade (G2-G3) and MIBC. But we could not differentiate between low and high grade depending on the level of NMP-52due to the extremely wide range of values in both groups. It is unlikely that for the individual patient the NMP-52 test can not differentiate between both low grade

or NMIBC and high grade or MIBC [19]. However the levels of urinary NMP-52 also increase with disease aggressiveness and tumor burden [17]. NMP-52 concentration is 25 times greater in urine from patients with bladder cancer than in urine from normal bladder and five times than in urine from cystitis patients. The most explanation for these results would also be that NMP-52 is probably released from the nuclei of tumor cells during apoptosis [11, 20]. The best performances were observed in patients with stage T3 disease. We also obtained the best results in this category, demonstrating a positive detection rate of 100% compared with 83%, 86%, 91% for the T1, T2 and T4, respectively. Traditionally, detection of low grade tumors has been the most challenging for noninvasive assays. When grade1 and NMIBC assayed, the NMP-52 assay detected 92% and 83%, respectively.

In the present study, urinary NMP-52 was extremely highly significant in relation to bladder cancer vs. healthy individuals and cystitis patients ($p<0.0001$) and the area under the ROC curve (AUC) for bladder cancer detection was 0.89, using a cut-off value of 2.2 μg/ml in the screening group yielded a sensitivity, specificity and positive and negative predictive values of 94%, 82%, 77% and95%, respectively. If healthy individuals were excluded the AUC and the specificity decreased to 0.82, 72%. Because NMP-52 protein is released from dead and dying urothelial cells, many benign conditions of the urinary tract (such as stones, infection, inflammation and hematuria) carry these proteins as well and cystoscopy can cause a false positive test result [7]. If false-positive results due to cystitis patients were excluded, then the AUC and the specificity improved to 0.98 and 95%, respectively and the positive predictive value to 97%. NMP-52 was only better than cytology in its sensitivity, with similar specificity and positive and negative predictive values. Reporting in a similar population of 'at risk' patients [21].

CONCLUSION

Urinary NMP-52 levels are significantly higher in patients with bladder cancer than in individuals negative for tumors, the diagnosis of bladder cancer by NMP-52 using ELISA technique in urine samples is non-invasive, low coast, simple, sensitive and very specific in either low or high stage and also in low or high grade of bladder cancer. So that NMP-52 is a good diagnostic tool for bladder cancer.

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