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Development of a Dual-Label Time-Resolved Fluorescence Immunoassay (TRFIA) for Screening of Bladder Cancer based on Simultaneous Detection of BLCA-4 and NMP52 in Urine

Xiaozhu Liu[1], Yinfeng Li[1], Yan Wang[2], Wenqiao Sun[3], Laiqing Li[4] and Licheng Zhang[3*]

[1]*Guizhou Institute of Technology, Guiyang, 550000, China*
[2]*Shangdong Medicine Technician College, Tai'an, 271000, Shandong, China*
[3]*Cancer Center of the 88 Hospital of People's Liberation Army, Tai'an, 271000, Shandong, China*
[4]*Guangzhou Youdi Biotechnology Company, Guangzhou, 510000, Guangdong, China*

Abstract

Bladder cancer is a heterogeneous disease and occupies highest incidence in developed country. Time-resolved fluoroimmunoassays (TRFIA) is a new detection technique with a feature of sensitive, simple and inexpensive. The aim of this study is to establish a dual-label TRFIA for the simultaneous detection of BLCA-4 and NMP52 in urine in a single run. The sandwich immunoassay was used to detect the concentration of BLCA-4 and NMP52 in urine. BLCA-4 and NMP52 in urine were captured by anti-BLCA-4 and anti-NMP52 antibodies immobilized on microtiter wells, and then banded together with another anti-BLCA-4 and anti-NMP52 labeled with europium(III) Sm^{3+} and samarium(III) Eu^{3+} chelates, followed by fluorescence measurement using time resolved fluorometry. To assess the performance of this assay, clinical urine samples were used, and then commercialized kits were compared. The sensitivity for BLCA-4 and NMP52 detection of this assay were 2 U/mL (dynamic range, 5-300 U/L) and 1 μg/ml (dynamic range, 2-150 μg/ml) respectively. The correlation coefficients (R) between the present dual-label TRFIA and commercially available kits were high. R-values were 0.99 for BLCA-4 and NMP52. The cross-reactivity seems not to influence the results. The present dual-label TRFIA, allowing the simultaneous detection ofBLCA-4 and NMP52, has high sensitivity, specificity, and accuracy in clinical sample analysis. Therefore, it has good prospects of application.

Keywords: Bladder cancer; BLCA-4; Dual-label TRFIA; NMP52

Introduction

Bladder cancer is a heterogeneous disease and occupies highest incidence in developed country [1,2]. There are over 70,000 new cases of bladder cancer each year in the United States alone [3]. The incidence of bladder cancer rises with age, peakingbetween age 50 years and 70 years, and is three times more common in men than in women [4]. The high rate of recurrence is the feature of bladder cancer that makes effective detection means is crucial importance for bladder cancer patients.

The conventional method for bladder cancer detection is morphological examination of cytology samples or cystoscopic biopsies [5]. Urine cytology is poorly differentiated tumors and time-consuming practice for both the patient and physician [6,7]. The diagnosis and monitoring of bladder cancer are facilitated by the availability of newer, simpler urine based diagnostic tests [2,8,9]. Recently, a number of molecular markers, such as BLCA-4, NMP52 and chromosomal aberrations, have been studied extensively by various groups [10-13]. BLCA-4 isone of six nuclear matrix proteins that are specifically expressed in bladder cancer [14]. Overexpression of BLCA-4 causes cells to express a more tumorigenic phenotype [15]. ELISA analysis showed that sensitivity and specificity of BLCA-4 for bladder cancer detection was 96.4% and 100% respectively [11,12,16]. High-level of sensitivity and specificity makes BLCA-4 to be a better candidate for detecting bladder cancer. It has been demonstrated that the concentration of NMP52, one of nuclear matrix proteins, was greater in bladder cancer cell lines than in the normal bladder [17]. Evidences proved that sensitivity of NMP52 for bladder cancer test was 94% and specificity was 95% [10,18]. Therefore, NMP52 could be used as a diagnostic tool for screening bladder cancer owing to its high sensitivity and specificity.

At present, many commercialized kits are available to detect urinary levels of BLCA-4 and NMP52. But they need two steps to detect these two markers. In addition, they have some other limitations, such as, time and labor consuming as well as high cost. Time-resolved fluoroimmunoassays (TRFIA) is a new detection technique with a feature of sensitive, simple and inexpensive [19]. TRFIA employs lanthanide chelates as fluorescent labels which offer the advantages of long fluorescence lifetimes and high quantum yields [20]. In a time-resolved manner the specific signal is detected after the disappearance of short-lived, nonspecific background fluorescence. The aim of this study is to establish a dual-label TRFIA for the simultaneous detection of BLCA-4 and NMP52 in urine in a single run (Figure 1).

Materials and Methods

Materials

The BLCA-4 and NMP52 standards were supplied by Abcam (Cambridge, MA). The monoclonal antibody (McAbs) against BLCA-4 and NMP52 were got from Hotgen Biotechnical Corporation. BSA was obtained from Merck (Darmstadt, Germany). Sm^{3+}- and Eu^{3+}-labeling kits were purchased from PerkinElmer (Norwalk, CT, USA). Enzyme-linked immuno sorbent assay kits for BLCA-4 and NMP52

***Corresponding author:** Licheng Zhang, PhD, Cancer Center of the 88 Hospital of People's Liberation Army, Humen Road 6, Tai'an, 271000, Shandong, China, Tel: +8602022056980; Fax: +8602022056980; E-mail: zhang88cn@sohu.com

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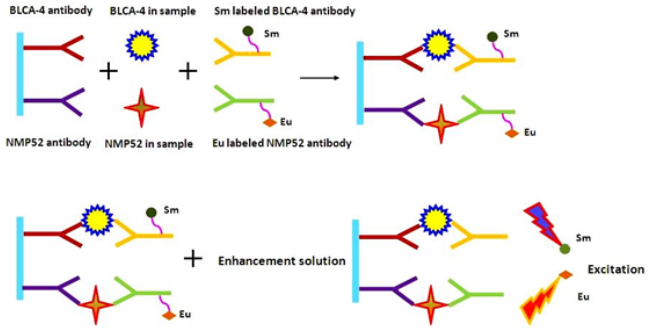

Figure 1: Schema of the dual-label TRFIA for BLCA-4 and MNP52 detection.

were purchased from Roche Diagnostics GmbH (Mannhein, Germany). Centrifugal filters with molecular weight cut off 10 KDa and 60 KDa were purchased from Millipore (Bedford, MA). Sephadex G50 column was obtained from GE Healthcare (Uppsala, Sweden). Tris andTriton X-100 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Other reagents were of analytical grade and were supplied by Beijing Reagent Corporation (Beijing, China).

Coating of the micro well plates

Monoclonal antibody for BCLA-4 and NMP52 were diluted to final concentration of 1 mg/L with 50 mmol/L carbonate (pH 9.6) buffer. Then, 100 µL of the diluted monoclonal antibody for BCLA-4 and NMP52 was added to each well for double coating followed by incubation at 4°C overnight. After coating, the coating buffer was discarded, the plates were washed 3 times with PBS containing 0.05% Tween-20 (v/v) then blocked with blocking buffer (50 mmol/L Tris-HCl containing 3% BSA, m/v, pH 8.0) overnight at 4°C. The blocking bufferwas discarded. The plate was vacuum dried and stored at -20°C until use.

Labeling antibody with Sm^{3+} and Eu^{3+} chelates

Antibody labeling with Sm^{3+} and Eu^{3+} chelates were carried out using Sm^{3+} and Eu^{3+} labeling kits according to the manufacturer's instructions. In brief, 1 mg of McAbs(BLCA-4) was washed 3 times using labeling buffer (50 mmol/L Na_2CO_3, pH 9.0) and collected through centrifugal filters. Then, it was suspended in 250 µL of labeling buffer, and mixed gently with 500 µg of Sm^{3+} chelates in 250 µL of the same buffer. The mixture was incubated overnight at room temperature, and collected using Sephadex G50 column equilibrated with 50 mmol/L Tris-HCl buffer (pH 7.8) containing 9 g/L NaCl. Store the conjugated McAbs(BLCA-4)Tris-HCl buffer (50 mmol/L, 0.1% BSA, m/v, pH 7.8) at 4°C. McAbs (NMP52) was labeled with Eu^{3+} by the same procedure used for Eu^{3+}.

Assay procedures

We first added 25 µL of standards or clinical urine samples into the coated wells, and then added 200 µL of Sm^{3+} labeled BCLA-4 and Eu^{3+} labeled NMP52 antibody (1 µg/mL). The plate was incubated at room temperature for 1 h. After 4 times of washing, we put 200 µL of enhancement solution (15 µmol/L β-NTA, 50 µmol/L TOPO and 0.1% Triton X-100, v/v) into each well, gently shake the plate for 5 min and read the fluorescence values by fluorescence readings (Auto DELFIA 1235, PerkinElmer).

Evaluation of the assay

We evaluated the sensitivity of the assay using a serial standard dilutions of BLCA-4 (0 U/mL, 5 U/µg, 10 U/µg, 25 U/µg, 50 U/µg, 100 U/µg, 200 U/µg, 300 U/µg), and NMP52 (0 µg/ml, 1 µg/ml, 5 µg/ml, 10 µg/ml, 20 µg/ml, 40 µg/ml, 80 µg/ml, 150 µg/ml). First, we assessed the averages (X) and standard deviations (SD) from twenty independent experiments (n=20). Next, we analyzed the low, median and high grade of clinical samples to calculate the accuracy of the assay. Then, we evaluated the intra- and inter- assay variations from ten independent experiments (n=10). Finally, we estimated the recovery using 5 urine samples at different analyte levels. The recoveries (%) were established using the equation: Recovery=100 × (measured value/original concentration). For the specificity tests, we evaluated different concentrations of thyroid hormones (TH), luteinizing hormone (LH), testosterone, estradiol and cortisol and follicle stimulating hormone (FSH).

Comparison of TRFIA with commercialized kits

We compared the TRFIA method with BCLA-4 and NMP52 enzyme-linked immuno sorbent assay kits as parallel tests. The appropriate controls were within the ranges provided by the manufacturer for all runs. We analyzed 175 clinical urine samples, 85 from Zhujiang Hospital (Guangzhou, China) and 90 from Jinan Hospital (Jinan, China). The enzyme-linked immuno sorbent test was carried out according to the manufacturer's instructions.

We constructed the receiver operator characteristic (ROC) curves, and calculated the area under the curve (ROC– AUC) with a 95% confidence interval using 360 samples from Zhujiang Hospital. Sensitivity and specificity were calculated independently using voided urine samples of patients with pathologically confirmed bladder cancer. Subgroups of bladder cancer were assessed from the histological evidences (subtype, stage and grade of the tumor).

Preparation of urine samples

This study approved by the ethics committee of Zhujiang Hospital (REC number: GDYY205427C) and Jinan Hospital (REC number: SDYY306236C). Urine samples from patients with pathologically confirmed bladder cancer, cystitis patients and normal controls were collected from Zhujiang Hospital and Jinan Hospital. All samples were morning voiding urines, bar coded, and stored in the -20°C freezer. Infected urine samples were from patients excluded from urologic tumors.

Statistical analysis

The data were shown as mean ± standard deviation (mean ± SD). Pearson's linear regression was used to present the linearity and correlations. Urine samples analyzed using TRFIA method and a commercial ELASA kit were compared using the paired Student's-test by SPSS 19.0 (Chicago, IL, USA), and $P<0.05$ was considered as statistically significant.

Results

Demographic data of patients

A total of 360 patients (240 males and 120 females) and 40 healthy individuals were enrolled in this study.54 (15%) patients were diagnosed as cystitis, 126 (35%) patients were diagnosed as benign disease and 180 (50%) patients were diagnosed malignant bladder cancer. The histopathological grade of bladder cancer patients were Ta stage (n=25, 8%), T1 stage (n=21, 7%), T2 stage (n=98, 32%), T3 stage (n=80, 26%) and T4 stage (n=82, 27%) refer to the WHO classification system.

The standard curves plotted for BLCA-4 and NMP52 were obtained

and shown in Figure 2. The working concentration ranges for BLCA-4 and NMP52 were 5-300 U/ml and 2-150 μg/ml respectively. The resulting calibration plots exhibited well-defined linear relationships between the analyte concentrations and fluorescent intensities. Both calibration plots exhibited well-defined linear relationships between the concentration and fluorescent intensity. The sensitivity for BLCA-4 detection was 2 U/ml and for NMP52 detection was 1 μg/ml.

Accuracy and precision assay

Three clinical samples with low, median and high concentrations of BLCA-4 and NMP52 were analyzed to calculate the accuracy and precision of this assay. As shown in Table 1, for BLCA-4 detection, the inter-assay CVs ranged from 6.9% to 7.4%, and intra-assay CVs ranged from 5.1% to 6.7%. For NMP52 detection, the inter-assay CVs ranged from 6.6% to 8.5%, and intra-assay CVs ranged from 5.8% to 6.3%. Therefore, the low CVs (less than 10%) demonstrating this assay as a good performance.

Recovery assay

In the present study, 5 clinical urine samples with known concentrations of BLCA-4 and NMP52 that spiked with different amounts of BLCA-4 and NMP52 standards were performed. In clinical samples, the initial concentrations of BLCA-4 were 10.4 U/mL, 24.6 U/mL, 51.5 U/mL, 102.7 U/mL, 263.8 U/mL, and for NMP52, the initial concentrations were 5.6 μg/ml, 15.4 μg/ml, 42.1 μg/ml, 88.9 μg/ml, 135.7 μg/ml. For BLCA-4, the average recoveries ranged from 95.1% to 108.7%, and for NMP52, the average recoveries ranged from 94.6% to 105.2% (Table 2). These data indicated that the present dual-labeled TRFIA was independent of urine interferent.

Specificity assay

To detect the specificity of this assay, some potential various concentrations of interferents were analyzed. No cross-reactivity was determined among these molecules (Table 3). The data suggested that the present dual-labeled TRFIA has high affinity and specificity for BLCA-4 and NMP52.

Comparison with commercialized kits

To carry out the parallel tests, 80 clinical samples were analyzed using the present dual-label TRFIA and Roche ELISA methods. The results observed that high correlations between the two methods were obtained. For BLCA-4, the linear equation was shown as y=0.971x-7.845, and the correlation coefficient was 0.99 (P<0.001) (Figure 3). For NMP52, the linear equation was shown as y=0.863x-0.732, and the correlation coefficient was 0.99 (P<0.001). These observations indicated that the present dual-label TRFIA was a reliable method for the simultaneous detection of BLCA-4 and NMP52 in clinical samples.

Clinical urine sample analysis

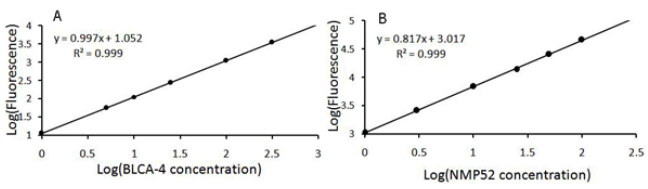

Figure 2: Calibration plots for BLCA-4 (A) and MNP52 (B) detection.

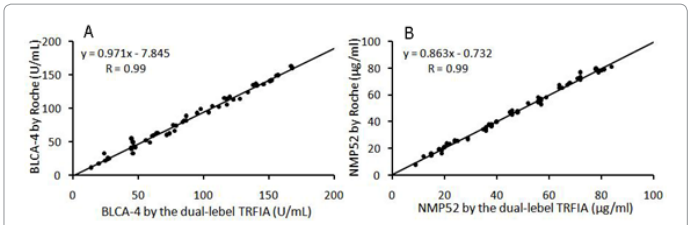

Figure 3: Correlation of BLCA-4 (A) and MNP52 (B) results between TRFIA and ELISA.

Significant differences of BLCA-4 and NMP52 urine levels between benign and malignant cases for the whole group were found (Table 4). In benign patients, the median BLCA-4 level was 37.2 U/mL, and was much less than that of malignant patients (100.1 U/mL, <0.01). The median NMP52 levels in benign patients and malignant patients were 9.2 μg/ml and 43.7 μg/ml respectively, which were significant differences within the two groups. There were significant differences for BLCA-4 and NMP52 between low grade (Ta-T2) and high grade (T3, <0.01) of tumors. In stage T3 tumors, levels of BLCA-4 and NMP52 were lower than those in stage T4 tumors in urine. However, there was no difference exist among low grade (Ta-T2) (>0.05).

Discussion

Bladder cancer is the second most common genitourinary malignant disease in the USA [21,22]. The high risk of recurrence makes effective and sensitive detection means to be very importance for bladder cancer patients [9,23]. Conventional methods for bladder cancer detection such as morphological examination of cytology samples or cystoscopic biopsies, is time-consuming and poor discrimination. Here, we developed a dual-label TRFIA for bladder cancer determination in a single run using BLCA-4 and NMP52 as biomarkers.

TRFIA represent an ultrasensitive technique using lanthanide elements and their chelates as the tracer with unique fluorescence properties. Compared with other immunoassay methods, such as, radioimmunoassay (RIA), enzyme immunoassay (EIA) and chemiluminescence immunoassay (CLIA), TRFIA is safe, highly sensitive, and has a wide dynamic range (19). Furthermore, the labeling procedure is very easy and produces labelled compounds with high specific activity and good stability with minimal influence on immunoreactivity and other bioactivities. Therefore, TRFIA has better performance in urine sample analysis than traditional fluoro immunoassays. In addition, TRFIA has been widely used in various fields, such as microbiology molecular biology, drug analysis and clinical research [24].

At present, many of the bladder tumor markers from serum and urine were reported and some of them had been developed for disease diagnosis. For example, Shahrokh et al. combined results from 10 centers and 2542 patients to develop internally validated nomograms to predict bladder cancer recurrence [25]. In this model, NMP22 had independent predictive power to predict bladder cancer recurrence and progression. BTA-stat tests detect complement factor H and a complement-factor H-related protein in urine. Cheng et al. had identified the binding sites of 2 monoclonal antibodies that are used in the BTA-stat tests [26]. However, the emerging evidences found that there were many disadvantages in some bladder tumor markers. Investigations revealed that NMP22 has no predictive value for bladder cancer recurrences, the specificity of NMP22 ELISA was low (between 60% and 80%) and a high false-positive rate among patients with inflammatory conditions, renal or bladder calculi, foreign bodies, bowel interposition and protein urea [25,27-

	BLCA-4			
	Concentration	**Mean (U/ml)**	**Standard deviation (SD)**	**Variable coefficient (CV)**
Inter-assay	low	14.9	1.1	7.4%
	median	142.8	9.9	6.9%
	high	286.5	21.0	7.3%
Intra-assay	low	13.8	0.9	6.5%
	median	150.1	10.1	6.7%
	high	290.6	14.8	5.1%
	NMP52			
	Concentration	**Mean (μg/ml)**	**Standard deviation (SD)**	**Variable coefficient (CV)**
Inter-assay	low	9.4	0.8	8.5%
	median	80.8	6.1	7.6%
	high	145.5	9.6	6.6%
Intra-assay	low	11.1	0.7	6.3%
	median	77.9	5.1	6.5%
	high	138.6	8.1	5.8%

Table 1: Accuracy and precision of the dual-label TRFIA (n=10).

Samples	BLCA-4		Recovery (100%)	NMP52		Recovery (100%)
	Original concentration (U/mL)	**Measured Value (U/mL)**		**Original concentration (μg / mL)**	**Measured Value (μg /mL)**	
1	10.4	11.3	108.7	5.6	5.3	94.6
2	24.6	24.2	98.4	15.4	16.2	105.2
3	51.5	53.8	104.5	42.1	43.9	104.3
4	102.7	105.2	102.4	88.9	86.1	96.6
5	263.8	250.9	95.1	135.7	136.2	103.7
Mean ± SD			101.8 ± 4.2			100.9 ± 3.9

Table 2: Recoveries of the dual-label TRFIA (n=10).

Compound	% Cross-reactivity
Thyroid Hormones	0.3
Luteinizing Hormone	0.1
Testosterone	0.05
Follicle Stimulating Hormone	0.1
Cortisol	0.01
Estradiol	0.02

Table 3: Cross-reactivities of the dual-label time-resolved fluorescence immunoassay (n=10).

	Number	BLCA-4 (U/mL)		NMP52 (μg/ml)	
		Median	**Range**	**Median**	**Range**
Normal control	40	8.5	3.1-13.8	1.5	1.1-1.8
cystitis	54	32.2	20.5-43.8	4.0	3.3-4.6
Benign	126	37.2	24.8-49.6	9.2	6.2-12.2
Malignant	180	100.1	29.7-170.5	43.7	12.6-74.8
Ta	25	40.5	26.3-54.6	12.1	8.4-15.8
T1	21	44.9	30.0-59.7	14.4	9.8-18.9
T2	98	51.9	35.9-67.8	18.6	13.7-23.4
T3	80	123.6	77.6-169.5	49.8	17.8-81.8
T4	82	107.3	60.2-154.3	26.1	11.5-40.6

Table 4: Urinary BLCA-4 and NMP52 levels in clinical samples.

29]. Also, low sensitivity been found in BTA-stat tests, ranges from 8% to 89%, especially, for low-grade tumors detecting [30]. The specificity of BTA-stat tests is low among patients with benign urologic conditions, especially those that cause hematuria and small recurrent tumors [26,31].

In this study, we developed a TRFIA for bladder cancer detection using novel urinary biomarkers BLCA-4 and NMP52. Well-defined linear relationships between the analyte concentrations and fluorescent intensities with broad detection ranges were revealed in this assay. High correlations with commercial ELISA kits made this assay as a good alternative to detect bladder cancer. Moreover, the urinary level of BLCA-4 and NMP52 form the clinical sample analysis was correlated with the stages of tumors.

In summary, the present dual-label TRFIA, allowing the simultaneous detection of BLCA-4 and NMP52, has high sensitivity, specificity, and accuracy in clinical sample analysis. It is helpful for the early diagnosis and prognosis monitoring of bladder cancer patients. Furthermore, it also could lower the economic burden to the bladder cancer patients. Therefore, it has good prospects of application.

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