RESEARCH ARTICLE

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Diagnostic Value of Rabbit-Derived 47 kDa Epitope-Based Antibodies as a Detection and Evaluation Method for Bladder Cancer

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Abstract

Objective: This study aims to generate polyclonal antibodies against the 47 kDa protein using rabbits, and to evaluate both the diagnostic and prognostic potential of this biomarker in patients with bladder cancer. **Methods:** Rabbits were immunized intraperitoneally with epitope antigens of the 47 kDa protein over a period of four weeks. Serum was collected in the fifth week five for antibody isolation. The resulting polyclonal antibodies were employed to detect urinary biomarkers in patients with bladder cancer. Additionally, these antibodies were used to evaluate therapeutic response in patients undergoing trimodal therapy, based on the RECIST (Response Evaluation Criteria in Solid Tumors) criteria, using the dot blot technique. **Results:** The highest density value with the best dilution effectiveness of 18.27 was found at antibody dilution 1/105 and antigen 1/106. From the dot blot analysis, this study found that the sensitivity and specificity of this antibody are 98.7% and 100%, respectively. The 47 kDa protein showed a high significant difference between each cancer stage before and after chemotherapy (PD: 7.91±1.99; SD: -1.00±0.83, PR: -5.80±3.21 CR: -7.95±1.47; p= 0.000), with a negative correlation with RECIST Criteria (r: -0.833, p=0.000). **Conclusion:** The 47 kDa protein demonstrates strong diagnostic performance and may be utilized as a prognostic factor in bladder cancer patients who have undergone trimodal therapy.

Keywords: Anti-body- bladder cancer- diagnostic- prognostic- protein 47 kDa

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Introduction

Bladder cancer is the most common neoplasm of the urinary system. It is the tenth most common cancer in the world. Globally, there were approximately 500,000 new cases and more than 210,000 deaths in 2020 [1]. Men have a higher risk of developing bladder cancer than women with an incidence ratio of 2.2:1. One of the most common types of mullet cancer is Transitional Cell Cancer (TCC) (90%). In addition, there is also squamous cell cancer (SCC) (5%) and adenocarcinoma (5%) [2, 3].

Bladder cancers can be categorized into muscle-invasion (MIBC) and muscle-non-invasion (NMIBC) bladder cancers. Non-muscle invasion bladder cancer is limited to the mucosa and/or with invasion only into the underlying lamina propria, whereas MIBC usually invades deeper layers of the bladder, such as the muscle, bladder wall, or other tissues surrounding the bladder [4]. The distinction between low grade and high-grade urothelial disease has implications relating to risk stratification and patient management [5].

Cytologic examination and histopathologic analysis of bladder biopsies in cystoscopy are the gold standard in making a diagnosis of bladder cancer. In addition, an ELISA test is also being developed to detect bladder cancer [6]. Unfortunately, these tests are still difficult to perform in remote areas due to limited facilities. This condition causes the diagnosis to be delayed, and the cancer will develop into a more advanced stage so that the hope of recovery will be reduced. Early detection is an effort to reduce incidence, prevent progressivity and improve the prognosis of patients with bladder cancer [7]. There is a need for an examination kit that can be used to detect bladder cancer that can be easily used in the region.

An epitope-specific antibody targeting the 47 kDa protein has recently been developed for bladder-cancer screening. The 47 kDa protein is selectively expressed on malignant urothelial cells and is absent in normal bladder epithelium as well as in epithelial tissues from prostate, rectal, and breast cancers. Comparative immunoblot

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analysis showed that a 122 kDa protein is present in both malignant and normal bladder epithelium, a 69 kDa protein is restricted to normal epithelium, and the 47 kDa protein is exclusive to bladder-cancer epithelium [8]. Polyclonal antibodies raised against the 47 kDa epitope demonstrated excellent diagnostic performance, with a sensitivity of 91.7 % and a specificity of 94.4 % for detecting bladder cancer [9].

Current limitations in producing antibodies against the 47 kDa protein stem from reliance on mice models, which require a substantial number of animals to yield sufficient antibody quantities. In this study, rabbits were utilized as an alternative host, allowing for repeated blood collection to facilitate efficient antibody harvesting [9]. The choice of therapy for bladder cancer is largely determined by the extent of muscle invasion. However, conventional assessment methods often lack accuracy and are subject to considerable inter- and intra-observer variability, particularly in tumors with irregular morphology. Given the high diagnostic value of the 47 kDa protein in bladder cancer, it may also serve as a potential marker for evaluating therapeutic response to trimodal therapy. These findings underscore the need for reliable diagnostic and prognostic tools in the management of bladder cancer.

Materials and Methods

Production of Polyclonal Antibodies

The antigen used in this study was a 47 kDa protein (originated from Cytokeratin-14 (Gene: CK14)) isolated from epithelial bladder cancer cells obtained from previous study [9]. The determination of antigen in the previous study includes in silico study to narrow the peptide chains into 2 peptides: (1) The lowest Bepipred score (0.53) as Peptide 1, (2) The highest BepiPred score (0.58) as Peptide 2. The BepiPred score indicates the probability of a residue being part of a B-cell epitope. Higher scores suggest a greater likelihood of that residue being an epitope, specifically bind to the antibody, while lower scores indicate a lower probability [10]. The protein was then sent to Proteomic International Australia for sequencing. The Peptide 1 with 108'-136' (DGLLVGSEKV TMQNLNDRLA SYLDKVRAL) and the Peptide 2 with 31' - 55' sequencing (ISSVLAGGSC RAPSTYGGGL SVSSS) were then synthesized at GenScript, New Jersey, USA, (https://www.genscript.com/peptida.html) [9].

Male rabbits were obtained from Bogor Agricultural University, West Java, Indonesia, all rabbits then experienced 1-week acclimatization. The immunogen consisted of epitope peptides with both high and low immunogenicity derived from the 47 kDa protein. These antigens were emulsified with Freund's Complete Adjuvant (FCA) and administered intraperitoneally at a dose of 100 µg in 1 mL of phosphate-buffered saline (PBS). Booster injections were given four weeks at the same dose intraperitoneally. Serum samples for antibody harvesting were collected at the end of the fifth week [9].

Serum Collection

The serum collection and the procedures done to obtain Antibody 1 and Antibody 2 was done in the previous study. Epitope-specific antibodies were obtained from rabbit blood samples. Blood was collected via the peripheral ear vein. Prior to collection, the ear area was shaved to expose the vein and disinfected with an alcohol swab. Blood was drawn using a 4–10 cc syringe and transferred into vacutainer tubes for further processing [9].

Blood was centrifuged, pellet was discarded, supernatant (serum) was taken. Serum was stored at -20^{o}C. Purification of antibodies using chromatography of protein G. Characterization tests (certainty of the nature of epitope-based antibodies) produced were carried out by immunoblotting techniques. Meanwhile, the determination of antibodies produced was carried out using commercial specific immunoglobulin by ELISA technique. The specific immunoglobulin used were IgG-1, IgG-2, IgG-2b, IgG-3, IgA and IgM [9].

Dot Blotting

Dot blotting in this study were divided into three stages. First stage of this study was to determine the best concentration ration between antigen and antibody. Second stage of this study was to determine the detection rate between bladder cancer patients and normal patients. Last stage of this study is to evaluate the expression of the protein 47 kDa expression in pre and post chemotherapy patients of bladder cancer.

Dot Blot was prepared; NC paper was cut to the size according to the number of samples. NC was soaked in distilled water for 30 minutes. NC was mounted on the Dot Blot device, moistened with TBS solution. Samples were dissolved in TBS solution containing 0.02% NaN3, 50 ul was inserted for each hole. Digest slowly, while the faucet on the hose is opened slowly until the sample runs out (or incubated overnight at 4^{o}C. Blocked with 5% TBS-Skim solution, 50 ul for each hole. Overnight incubation at 4^{o}C (cover the hole with aluminium foil). Samples were removed to room temperature, washed with 0.05% TBS-tween 3x5 min.

The liquid is decanted/discarded on tissue. Incubated with primary antibody (Antibody 2), dissolved in TBS-Skim milk 0.05 % at 50 ul for each hole for 1 hour at room temperature, and washed with 0.05% TBS-tween 3x5 minutes. The liquid was decanted/discarded on tissue. Incubated with labelled secondary antibody (streptavidin HRP) dissolved in TBS 50 ul for each well for 1 hour at room temperature. Wash with 0.05% TBS-tween 3x5 min. Liquid was decanted/discarded on tissue. Incubated with SAHRP dissolved in TBS 50 ul for each hole for 40 - 60 minutes at room temperature. Wash with 0.05% TBS-tween 3x5 minutes. Liquid was decanted/discarded on tissue. Incubated with substrate (TMB Western Blue) 50 ul for each hole for 20 min at room temperature in a dark room. Stop the reaction with sterile distilled water 50 ul for each hole.

Dot blot is used to determine the optimal level of antibody that will be used in the therapeutic effectiveness test against polyclonal antibodies based on 47 kDa protein epitopes in the urine of patients suffering from bladder cancer. The dot blot test is based on the antigen-antibody reaction that occurs between the antigens contained in the

patient's urine and the antibodies that have been produced in the first stage of research, calculated the level of antigen-antibody binding in the form of color absorbance in each plate (sample).

Patient

Participants in this study were divided into two distinct phases: a diagnostic phase and a prognostic phase. The diagnostic phase involved two groups. The first group comprised 77. Subjects were categorized into 10 categories: (1) low-grade bladder cancer, (2) high-grade bladder cancer, (3) localized bladder cancer, (4) metastatic bladder cancer, (5) benign prostatic hyperplasia, (6) urolithiasis, (7) urinary tract incontinence, (8) renal trauma, (9) renal cancer, (10) ureteral and renal pelvis cancer. These categories are used to determine whether the peptide is expressed in non-bladder-cancer patients or not. To make sure that the results are not due to random and unspecific binding with other molecules, we also include negative controls: healthy patients with different ranges of age (>70, 50-70, and <50 years old) and gender (male and female), and several other non-patient negative controls: PBS, PBS-BSA, PBS-antibody 1, PBS-peptide 1.

The second phase of this experimental study focused on prognostic evaluation and included 35 patients with biopsy-confirmed bladder cancer. Subjects were treated with trimodal therapy as as a viable treatment option for patient with MIBC who decline radical cystectomy. This therapeutic approach comprises transurethral resection of the bladder tumor (TURBT) for tumor debulking, followed by a combination of radiotherapy and concurrent chemotherapy [11]. Subjects were categorized into four groups based on the RECIST criteria: progressive disease (PD), stable disease (SD), partial response (PR), and complete response (CR). Inclusion criteria included confirmed histopathological diagnosis and complete clinical staging data. Patients with evidence of metastasis were excluded to minimize potential confounding factors. Clinical staging was performed before and after the administration of trimodal therapy. The expression of the 47 kDa protein was assessed at both time points. Mid-stream urine samples were collected and analyzed for the presence of the 47 kDa protein using dot blotting.

The Research Ethics Committee of the Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia, has approved this research with the number 400/314/K.3/102/7/2024.

Data Analysis

Dot blot images in this study were processed qualitative and quantitatively. Qualitative measurement is processed with direct observation, while quantitative data were processed using ImageJ software. The measurement of this study used the degree of color absorbance in each plate.

After the data were collected, descriptive analysis of the characteristics of the study subjects was carried out, consisting of age, severity of bladder cancer and the amount of 47 kDa protein. The measurement data were processed using SPSS software. The research data obtained was analyzed using Statistical Product and Service Solution software, IBM SPSS Statistics 20 with a significance level or probability value of 0.05 (p=0.05) and a 95% confidence level (α=0.05).

Results

Dot Blot

In this study, the focus was on determining the optimal dosage for binding between antibody and peptide antigen. Based on Figure 1 and Table 1 the first dot blot assay, peptide antigen 1 was diluted from 1/101 to 1/106, with the control placed in the bottom column. Meanwhile, the P1 antibody was diluted from 1/101 to 1/108. The test results show the density and size of the circles (dots), where a larger diameter and higher density indicate a stronger antigen-antibody bond. To obtain objective results, the analysis was performed using ImageJ software. In Figure 1 qualitatively, dots with antibody dilution 1/105 and antigen 1/103, 1/104, 1/105, 1/106 did not shows much different in shade. Based on the analysis in Table 1, the highest density value with the best dilution effectiveness of 18.27 was found at antibody dilution 1/105 and antigen 1/106. This highest density value reflects the best bonding strength. The effectiveness of the assay is determined by the combination of the largest antibody dilution, the smallest antigen dilution, and the highest bond strength. In this study, the most effective combination was found to be 1/105 dilution of antibody and 1/106 antigen.

As seen on Table 2 as patient characteristic in the Dot Blot Analysis of peptide antibody then the results in Figure 2 as qualitative result and Table 3 as quantitative result, analyzed 47 kDa protein levels (with an ROC threshold of 10.35) to distinguish between cancer and normal groups showed a sensitivity of 93.3% and specificity of 93.94%. The high sensitivity means the test is excellent at detecting individuals who are truly sick, thus minimizing cases of undetected disease. High specificity means the test is excellent at ensuring healthy individuals are not misdiagnosed as sick, thus avoiding treatment errors. The positive (PPV) and negative (NPV) predictive values are also high, at 87.5% and 96.88% respectively, indicating that the positive and negative results of the

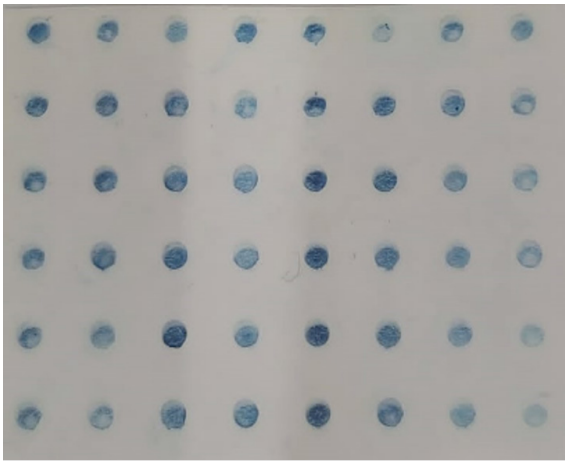

Figure 1. Qualitative Dot Blot Examination of Antibody and Antigen

Table 1. Quantitative Dot Blot Analysis of Antibody and Antigen (processed by J image)

		AB							
		$1/10^1$	$1/10^2$	$1/10^3$	$1/10^4$	$1/10^5$	$1/10^6$	$1/10^7$	$1/10^8$
AG	$1/10^1$	12,435	12,486	19,751	19,225	10,841	9,477	12,785	
	$1/10^2$	12,674	16,481	10,776	9,218	14,409	17,934	8,508	
	$1/10^3$	17,136	15,881	12,228	12,044	20,277	12,707	6,731	2,996
	$1/10^4$	3:56	4,989	2,759	2,665	5,286	3,749	1,964	0.453472222
	$1/10^5$	14,932	14,723	16,542	14,579	0.9	12,432	4,501	1:33
	$1/10^6$	12,972	11,639	19,659	17,327	18:27	12,223	6:56	1:35

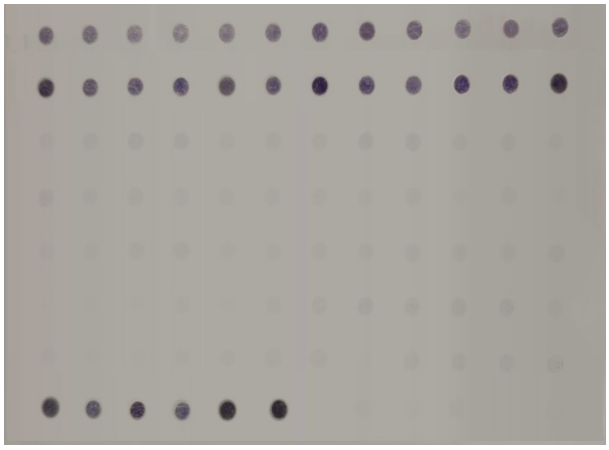

Figure 2. Qualitative Results of Dot Blot Examination of Rabbit Peptide 2 Antibody to Patient Urine Antigen

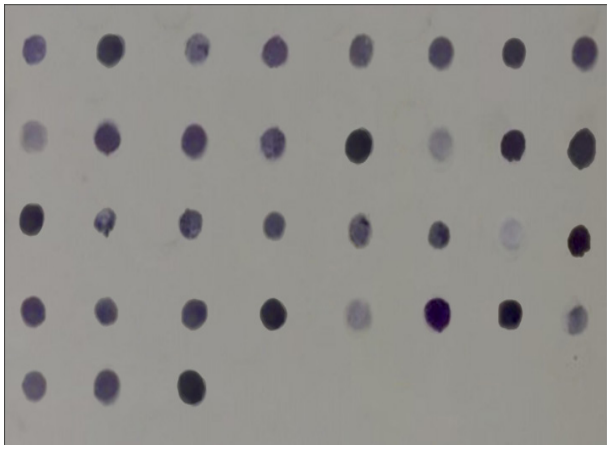

Figure 3. Dot Blot Assay of Pre- Trimodal Therapy Subjects, Blue Mark Indicates the Density of 47 kDa Protein Expression Processed J image

test are highly reliable in predicting cancerous or normal status as put in Table 4.

Based on Table 5, During the sample collection period, there were 35 subjects who agreed to participate in this study. The subjects were divided into 4 groups based on the RECIST criteria. Subjects were intervened with trimodal therapy and the same between the four groups.

Based on the data presented in the Figure 3 as 47 kDa protein expression processed J image with Supplementary Table 1 shown a quantitative result, the highest pre-trimodal therapy expression level of the 47 kDa protein was observed in sample D3 (28.8 units), while the lowest was found in sample B6 (10.5 units). According to the RECIST criteria prior to trimodal therapy, the average 47 kDa protein levels were as follows: 25.20 units in 9 patients classified as having progressive disease (PD), 19.02 units in 5 patients with stable disease (SD), 20.50 units in 12 patients with partial response (PR), and 12.23 units in 6 patients with complete response (CR).

Based on the data presented in the Supplementary Table 2 that was quantitative version of Figure 4, the highest post-chemotherapy expression level of the 47 kDa protein was observed in sample D3 (35.3 units), while the lowest was found in sample B6 (3.2 units). According to the RECIST criteria following trimodal therapy, the average 47 kDa protein levels were as follows: 33.11 units in 9 patients with progressive disease (PD), 18.02 units in 5 patients with stable disease (SD), 14.70 units in 12 patients with partial response (PR), and 4.28 units in 6 patients with complete response (CR).

Protein 47 kDa Analysis

Based on the Supplementary Table 3, the 47 kDa levels in patients with RECIST PD showed a significant increase from a pre trimodal therapy mean of 25.2 units to a post trimodal therapy mean of 33.11 units ($p = 0.000$). In contrast, patients with RECIST SD had no significant change in 47 kDa levels, with means of 19.02 pre-chemotherapy and 18.02 post-chemotherapy ($p = 0.055$). Patients with RECIST PR exhibited a significant decrease

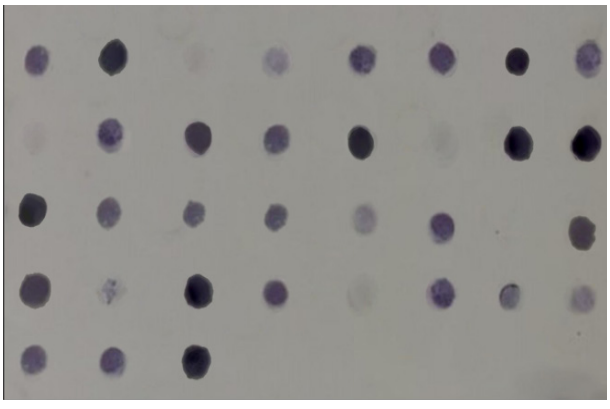

Figure 4. Dot Blot Assay of Post-Trimodal Therapy Subjects, Blue Mark Indicates the Density of 47 kDa Protein Expression Processed J image

Table 3. Quantitative Dot Blot Analysis of Antibody and Antigen in Rabbits (processed by J image)

	1	2	3	4	5	6	7	8	9	10	11	12
A	14.3	16.3	17.4	14.4	15.8	19.4	29.5	30.2	28.1	23.3	27.5	28.5
B	33.2	30.2	29.9	30.2	33.4	33.7	51.28	44.3	38.2	48.6	44.3	51.2
C	7.8	8a.2	7.9	7.5	3.5	4.9	3.1	4.4	3.7	9.1	7.9	7.6
D	10.3	9.4	8.2	6.7	5.6	6.2	5.6	6.3	5.6	8.4	6.8	8.7
E	6.3	5.8	2.3	4.8	7.5	6.9	7.5	7.8	5.7	8.4	6.7	5.4
F	2.5	1.3	2.5	6.9	5.3	5.1	6.5	2.1	6.9	2.6	7.5	4.2
G	3.2	3.4	5.9	4.5	2.3	3.2	8.9	6.7	3.2	2.5	7.8	4.9
H	36.8	29.3	44.3	38.2	44.7	55.4	1.5	0.4	0.3	1.2	1.4	0.5

Table 2. Patient Characteristics in the Dot Blot Analysis of Peptide 2 Antibody

	1	2	3	4	5	6	7	8	9	10	11	12
A	low grade bladder cancer	low grade bladder cancer	low grade bladder cancer	low grade bladder cancer	low grade bladder cancer	low grade bladder cancer	high grade bladder cancer	high grade bladder cancer	high grade bladder cancer	high grade bladder cancer	high grade bladder cancer	high grade bladder cancer
B	localized bladder cancer	localized bladder cancer	localized bladder cancer	localized bladder cancer	localized bladder cancer	localized bladder cancer	metastatic bladder cancer	metastatic bladder cancer	metastatic bladder cancer	metastatic bladder cancer	metastatic bladder cancer	metastatic bladder cancer
C	BPH	BPH	BPH	Urolithiasis	Urolithiasis	Urolithiasis	UTI	UTI	UTI	Renal trauma	Renal trauma	Renal trauma
D	BPH	BPH	BPH	Urolithiasis	Urolithiasis	Urolithiasis	UTI	UTI	UTI	Renal trauma	Renal trauma	Renal trauma
E	renal cancer	renal cancer	renal cancer	renal cancer	renal cancer	renal cancer	ureteral and renal pelvis cancer	ureteral and renal pelvis cancer	ureteral and renal pelvis cancer	ureteral and renal pelvis cancer	ureteral and renal pelvis cancer	ureteral and renal pelvis cancer
F	Male >70yo	Male >70yo	Male >70yo	Male >70yo	Male 50-70yo	Male 50-70yo	Male 50-70yo	Male 50-70yo	Male <50yo	Male <50yo	Male <50yo	Male <50yo
G	Female >70yo	Female >70yo	Female >70yo	Female >70yo	Female 50-70yo	Female 50-70yo	Female 50-70yo	Female 50-70yo	Female <50yo	Female <50yo	Female <50yo	Female <50yo
H	low grade bladder cancer	low grade bladder cancer	high grade bladder cancer	high grade bladder cancer	localized bladder cancer	localized bladder cancer	negative control-antibody1 only	negative coontrol-PBS	negative control- BSA	negative control-peptide 1 only	negative control-antibody1 only	negative control-PBS

Table 4. Diagnostic Test Results of Phase 1 Study

	Sensitivity	Specificity	PPV	NPV
47 kDa level (ROC 10.35 threshold) to predict Group (Cancer and normal)	93.3%	93.94%	87.5%	96.88%

* CI 95%, Confidence interval 95% ; PPV, Positive Predictive Value; NPV, Negative Predictive Value

Table 5. Demographic Characteristic of Phase 2 Study

Basic Demographic	N	RECIST								P-Value
		PD		SD		PR		CR		
		n	%	n	%	n	%	n	%	
Age (Years)										
31 - 40	1	0	0.0	1	20.0	0	0.0	0	0.0	0.226
41 - 50	2	0	0.0	1	20.0	1	7.1	0	0.0	
51 - 60	9	4	40.0	1	20.0	2	14.4	2	33.3	
>60	23	6	60.0	2	40.0	11	78.5	4	66.7	
Gender										
Male	33	8	80.0	5	100.0	14	100.0	6	100.0	0.099
Female	2	2	20.0	0	0.0	0	0.0	0	0.0	
T										
2a	4	0	0.0	0	0.0	1	7.1	3	50.0	0.003
2b	11	0	0.0	3	60.0	5	35.7	3	50.0	
3b	7	1	10.0	2	40.0	4	28.5	0	0.0	
4a	9	7	70.0	0	0.0	2	14.4	0	0.0	
4b	4	2	20.0	0	0.0	2	14.4	0	0.0	
N										
N0	6	1	10.0	0	0.0	2	14.4	3	50.0	0.019
N1	4	1	10.0	0	0.0	1	7.1	2	33.3	
N2	16	3	30.0	2	40.0	11	78.5	0	0.0	
N3	9	5	50.0	3	60.0	0	0.0	1	16.7	

* PD, Progressive Disease; SD, Stable Disease; PR, Partial Response; CR, Complete Response.

from 20.5 to 14.7 units (p = 0.000), and those with RECIST CR also showed a significant reduction from 12.23 to 4.28 units (p = 0.000). These results indicate that 47 kDa protein levels significantly change after chemotherapy in patients with increase in PD and decrease in PR, and CR, but remain stable in patients with SD.

The correlation analysis between pre- and post-chemotherapy 47 kDa levels in Supplementary Table 4, showed a strong positive correlation in the PD group (r = 0.755, p = 0.019), indicating that higher pre-treatment levels were associated with higher post-treatment levels. In contrast, the SD, PR, and CR groups exhibited very strong negative correlations (r = -0.969, -0.857, and -0.992 respectively, all $p < 0.05$), suggesting that higher pre-chemotherapy 47 kDa levels were associated with greater decreases after chemotherapy in these groups.

The correlation analysis in Supplementary Table 5 revealed a strong and significant negative relationship

Table 6. Pre-Chemotherapy Quantitative Results Based on Dot Blot Test Images of Pre-Chemotherapy Subjects

	1	2	3	4	5	6	7	8
A	T2bN3	T4aN2	T2aN1	T2bN0	T2bN2	T3bN2	T4aN1	T3bN2
	17:05	22:06	12:04	16:04	16:07	19:05	23:04	20:02
B	T2bN1	T3bN2	T4aN0	T3bN1	T4bN3	T2aN0	T2aN1	T4aN2
	12:03	21:04	22:04	18:04	27:05:00	10:05	26:04:00	28:05:00
C	T4aN3	T4aN2	T2aN2	T2bN2	T4aN2	T2bN0	T2bN3	T2aN0
	24:05:00	14:08	19:02	26:04:00	13:04	16:08	9:06	24:06:00
D	T3bN3	T3bN3	T2bN3	T4aN3	T4bN2	T2bN0	T4aN2	T4bN2
	22:06	17:04	28:08:00	27:05:00	11:02	25:04:00	28:05:00	14:03
E	T2bN2	T3bN2	T4bN3					
	20:02	18:04	22:06					

Table 7. Post-Chemotherapy Quantitative Results Based on Dot Blot Test Images of Pre-Chemotherapy Subjects

	1	2	3	4	5	6	7	8
A	T2bN3	T4aN2	T2aN1	T2bN0	T2bN2	T3bN2	T4aN1	T3bN2
	15:08	30:06:00	4:03	11:02	15:08	18:01	33:21:00	15:04
B	T2bN1	T3bN2	T4aN0	T3bN1	T4bN3	T2aN0	T2aN1	T4aN2
	4:05	17:05	29:56:00	14:02	37:05:00	3:02	30:01:00	36:06:00
C	T4aN3	T4aN2	T2aN2	T2bN2	T4aN2	T2bN0	T2bN3	T2aN0
	30:08:00	13:04	14:06	18:03	10:02	17:02	3:02	23:02
D	T3bN3	T3bN3	T2bN3	T4aN3	T4bN2	T2bN0	T4aN2	T4bN2
	30:08:00	6:07	35:03:00	19:02	3:08	16:05	12:04	10:05
E	T2bN2	T3bN2	T4bN3					
	15:04	14:02	30:06:00					

Table 8. Comparison of Protein 47 kDa Expression between Pre and Post Chemotherapy

	Pre	Post	P-value
PD	25.2±2.65	33.11±2.98	0.000
SD	19.02±3.32	18.02±3.06	0.055
PR	20.5±5.36	14.7±2.98	0.000
CR	12.23±2.75	4.28±1.30	0.000

* PD, Progressive Disease; SD, Stable Disease; PR, Partial Response; CR, Complete Response

Table 9. Correlation of Protein 47 kDa Expression Between Pre- and Post-Trimodal Therapy

	PD	SD	PR	CR
Pre Trimodal Therapy	25.20±2.65	19.02±3.32	20.50±5.36	12.23±2.75
Post Trimodal Therapy	33.11±2.98	18.02±3.06	14.70±2.98	4.28±1.30
Delta 47 kDa	7.91±1.99	-1.00±0.83	-5.80±3.21	-7.95±1.47
Correlation coefficient	0.5243	-0.969	-0.857	-0.992
P Value	0.019	0.000	0.000	0.000

* PD, Progressive Disease; SD, Stable Disease; PR, Partial Response; CR, Complete Response

Table 10. Corellation of Protein 47 kDa Expression Between Pre- and Post- Trimodal Therapy with RECIST Criteria

	Correlation coefficient	P Value
Pre Trimodal Therapy with RECIST	-0.663	0.000
Post Trimodal Therapy with RECIST	-0.915	0.000
Delta 47 kDa with RECIST	-0.883	0.000

between 47 kDa levels (pre-trimodal therapy, post-trimodal therapy, and delta changes) and RECIST status, with higher 47 kDa values observed in patients with PD and the lowest values in patients with CR ($p < 0.05$).

Discussion

Bladder cancer shows marked gender differences in terms of incidence, presentation and outcome. Epidemiologic data consistently show that men have a three to four times higher incidence of bladder cancer than women worldwide. Nonetheless, women tend to present with more advanced disease and experience worse clinical outcomes [12, 13].

CK14 has been extensively utilized as a biomarker for the detection and classification of malignancies. The expression of CK14 serves as a significant indicator of basal/squamous-like (BASQ)-type MIBC, due to its high rate differentiation. Several mechanisms are believed to facilitate the release of CKs fragments into the circulatory system in cases of bladder cancer, including epithelial differentiation processes that encompass abnormal mitosis, proteolytic degradation, and the spillover from cells undergoing proliferation, apoptosis, or neoplasm [3]. Some studies has indicated that CK14 expression can help predicting and identifying bladder epithelial metaplasia, which can lead to bladder carcinomas, particularly in squamous cell carcinoma (SCC) of the bladder. In the context of bladder cancer, the presence of CK14 in urine may heighten the suspicion of SCC, which is generally associated with a poorer prognosis [14]. Prior studies have reported consistent findings, demonstrating that CK14 expression is most pronounced in SCC of the bladder and diminishes as the squamous cell component decreases [15].

A 47 kDa protein is specifically expressed in the epithelial tissue of bladder cancer. Research by Prasetya et al. demonstrated that this protein was exclusively detected in bladder cancer epithelium and was absent in prostate, rectal, and breast cancers, as well as in normal bladder epithelial tissue. In comparison, a 122 kDa protein was present in both cancerous and normal cell, a 69 kDa protein was found only in normal bladder epithelium, and the 47 kDa protein was uniquely expressed in bladder cancer epithelial cells [16, 17]. These sequencing findings are supported by a study conducted by previous study, which demonstrated the formation of 47 kDa protein-specific antibodies for detecting bladder cancer using rat models. The resulting epitopes specifically bound to two peptide

sequences: peptide 1 (amino acids 31–55) 'ISSVLAGGSC RAPSTYGGGL SVSSS' and peptide 2 (amino acids 108–136) 'DGLLVGSEKV TMQNLNDRLA SYLDKVRAL' [9]. In this study, rabbit specimens were used to enhance the affinity of polyclonal antibodies, thereby improving the sensitivity and specificity of the antibodies.

Rabbits are the most commonly used laboratory animals for polyclonal antibody production due to several advantages. Their suitable body size and the easy access to the peripheral ear vein and central auricular artery allow for the convenient collection of large blood samples. The use of specific pathogen-free rabbits ensures a healthy immune response focused on the administered antigens. Additionally, rabbits exhibit strong immune responses to a wide range of antigens, possess a single predominant IgG isotype, and benefit from well-established methods for immunoglobulin production and purification, along with the wide availability of secondary reagents—further supporting their role in efficient polyclonal antibody production [18].

Research by Saito, M. et al. supports the use of rabbits in producing polyclonal antibodies in detecting gastrointestinal stromal tumor (GIST) cancer and shows a better comparison than using mice [19]. Another study by Vilches, Moure, et al. also showed the superiority of using rabbit monoclonal antibodies over mice in canine specimens [20]. The study by Qi, Z. et al. describes the first attempt to test subunit vaccines and EV76 vaccines using a rabbit model and showed better model enhancement of expression compared to mice [21] . In addition, the use of rabbits in this study also showed a better comparison of the amount of antibodies in binding 47 kDa protein antigens which may affect the cost effectiveness of the production of this model.

Accurate bladder tumor markers will be useful for screening high-risk populations and for monitoring patients with a history of bladder cancer to help identify early recurrence and prevent disease progression. The Food Drug Association (FDA) has currently approved 6 urinary tests to be used in conjunction with cystoscopy for diagnosis and surveillance. These include BTA stat (Polymedco), BTA TRAK (Polymedco), NMP22 enzyme linked immune-sorbent assay (ELISA) (Matritech), NMP22 BladderChek Test (Alere), uCyt (Scimedx), and UroVysion (Abbott Molecular). Urine cytology has high sensitivity and specificity for detecting high-grade urothelial cancer but is less sensitive for detecting low-grade tumors [22].

The 47 kDa protein has emerged as a significant biomarker for bladder cancer, especially in evaluating treatment response. In a previous study [9], it was found that the use of a polyclonal antibody targeting the 47 kDa protein has shown a sensitivity of 91.67% and specificity of 94.44% in urine samples from bladder cancer patients. These values indicate that this marker is highly effective in identifying true positive cases while minimizing false positives, which is important for diagnosis and treatment evaluation [17]. Traditional diagnostic methods such as urine cytology have shown lower sensitivity, especially in low-grade tumors, where sensitivity can be as low as 4%-31%. In contrast, the 47 kDa protein shows a significantly improved detection rate, making it a good parameter to assess treatment response over time [17]. These results are aligned with this study which also showed a high sensitivity and specificity.

NMP-22 is another urinary marker used in bladder cancer detection. This marker shows moderate sensitivity (approximately 70%-80%) but lower specificity compared to the 47 kDa protein [23-25]. When compared with other commonly used antigen detection biomarkers, BTA-STAT, UBC-Rapid, BTA-TRAK, UBC IRMA, the sensitivity and positive predictive value of immunocytochemical technique using 47 kDa polyclonal antibody is higher than these biomarkers [26]. Thus, proving protein 47 kDa has a high significance in detecting bladder cancer patients.

The ability to detect changes in 47 kDa protein levels may provide insight into the tumor's response to therapy. A decrease in protein levels in urine may indicate effective treatment, while stable or elevated levels may indicate disease progression or resistance to therapy. The non-invasive nature of the urine cytology test allows for frequent monitoring without subjecting the patient to invasive procedures such as cystoscopy [17]. It is also shown in this study that subject with different degrees of progressivity showed different expression of protein 47 kDa. Higher degrees of severity showed a significant higher expression, while lower degrees of severity showed lower expression. This same expression is also shown in pre and post chemotherapy subjects. Thus, a certain activity of protein 47 kDa correlates with the disease progression.

This study has several limitations that need to be noted. First, the control group in this study only included healthy individuals and did not include patients with other micturition complaints, such as hematuria. This may limit the generalizability of the findings, as 47 kDa protein expression in patients with other urological disorders that may have similar patterns was not considered. Secondly, the RECIST categorization used in this study made the number of samples for each category tends to be small and uneven, which might limit the statistical power and generalizability of the findings. Thirdly, this study did not evaluate the relationship between bladder tumor volume and 47 kDa protein expression, even though tumor volume could potentially be a factor that affects the level of expression. The absence of this analysis limits further understanding of the relationship between protein expression and tumor progression. Fourthly, the use of the dot blot method in this study still has limitations, as it is only able to identify protein expression in general without providing precise quantitative results. Therefore, further research with a more comprehensive design is needed to overcome these limitations.

Conclusion, the 47 kDa protein detected in urine demonstrated strong diagnostic performance as a specific biomarker for bladder cancer, detected by rabbit-derived 47 kDa epitope-based antibodies. In trimodal therapy, 47kDa change after chemotherapy, it found significantly increase in patient with progressive disease, significantly decrease in patient with partial response and complete response, but remain stable in patients with stable disease of bladder cancer.

Author Contribution Statement

TNB: Conceptualization, Methodology, Data curation, Visualization, Project administration, Supervision; KPS: Conceptualization, Methodology, Writing-original draft, Supervision, Validation; HS: Conceptualization, Methodology, Investigation, Writing-original draft, Resources; SH: Conceptualization, Formal analysis, Resources, Data curation, Writing-original draft; BW: Writing-original draft, Methodology, Writing – review & editing Methodology; CA: Formal analysis, Writing – review & editing Methodology

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General

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Ethical Declaration

This study was approved by the health research ethical commission from General Hospital Saiful Anwar Malang with approval number 400/314/K.3/102/7/2024.

Data Availability

The corresponding author can obtain the study's data on reasonable written request.

Conflict of Interest

All the authors declare that there are no competing interests of this study.

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