RESEARCH ARTICLE

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The Evolution of Polyclonal Antibody from Specific Epitope 47kDA for Detection of Bladder Cancer

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Abstract

Objective: This study will identify specific epitopes from the 47kDa protein as the basis for making polyclonal antibodies to increasing sensitivity and specificity of 47kDa protein as bladder cancer biomarkers. **Method:** The 47kDa protein epitope prediction was carried out using the in-silico method. The epitope with the highest and the lowest value was immunized to the mice for four weeks and was harvested at the fifth weeks. The antibody was tested with the patient's urine using western blotting. Total of 186 participants including in this study. For the first stage (antibody confirmation test) test we have 18 participants, for the second stage (1st antibody diagnosis test) we have 72 participants and for the third stage (2nd antibody diagnosis test) we have 96 participants, consist of total 64 BC patients 48 of healthy individuals and 74 participants with the other diseases. **Results:** Some epitopes from the sequenced protein are candidates for immunization, in the chain 108'-136' (with lowest Bepipred score: 0.53) named as peptide1 and chain 42'-56' (with highest bepipred score: 0.58) named as peptide2. In western blotting test, both antibodies showed detection at 47kDa. When examined with western blot using urine from BC patients, urine from other cancer patients (prostate, kidney, ureter, rectal, breast), and healthy persons, both antibodies were found to only express 47kDa in urine from BC patients. The diagnostic tests showed high sensitivity (91.67%) and specificity (94.44%) inAb2 in predicting bladder cancer. **Conclusion:** The evolution of the polyclonal antibody made from specific epitopes is proven to express specifically on bladder cancer patients and have high sensitivity and specificity to diagnose bladder cancer.

Keywords: Bladder cancer- diagnosis- Epitopes- 47 kDa Protein

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Introduction

Bladder cancer is the ninth-leading cause of malignancy in the world. To date, there have been 430,000 people diagnosed with bladder cancer (Redondo-Gonzalez et al., 2015). Bladder cancer is one of the malignancies that occur in the urinary tract. The most frequent type of bladder cancer is transitional cell carcinoma (TCC), which starts in the cells that form the epithelial lining of the bladder (Wong et al., 2018). The main problem is the lack of accessible simple diagnostic tools that medical professionals in remote areas can use to diagnose BC. Another factor associated with bladder cancer is the high recurrence rate, i.e., 50–70% of newly diagnosed tumors recur within five years, and progression to an invasive disease will occur in 10–20% of patients. Therefore, patients with bladder cancer should undergo life-long surveillance (van Rhijn et al., 2014).

Proteomics is one of the fastest-growing fields of molecular biology and is concerned with an experimental approach using in-silico study to analyze protein expression in cells or organisms by protein isolation and sequencing (Prasetya et al., 2014). In a previous preliminary study, it was found that a protein with a molecular weight of 122 kDa was found in normal bladder epithelial cells and cancer cells in the bladder (Ladner, 2007). In comparison, a protein with a molecular weight of 69 kDa was only found in normal bladder epithelial cells. In addition, a protein with a molecular weight of 47 kDa was only found in bladder cancer cells (Ladner, 2007). The sensitivity value of the immunocytochemical examination using a 47 kDa polyclonal antibody from bladder cancer epithelial cells was 100%, while the specificity was 30%. The sensitivity value of polyclonal antibody of 47 kDA were higher than bladder tumor antigen STAT (BTA-STAT), urinary bladder cancer rapid (UBC Rapid), bladder tumor antigen - TRAK assay (BTA -TRAK), Urinary Bladder Cancer Rapid - immunoradioassay (UBC

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Table 1. Keratin sequence, type I cytoskeletal, red color shows the 47kDA protein sequence obtained from the urine of Protein Carcinoma Bladder.

1	MTTCSRQFTS	SSSMKGSCGI	GGGIGGGSSR	ISSVLAGGSC	RAPSTYGGGL
51	SVSSSRFSSG	GACGLGGGYG	GGFSSSSSSF	GSGFGGGYGG	GLGAGLGGGF
101	GGGFAGGDGL	LVGSEKVTMQ	NLNDRLASYL	DKVRALEEAN	ADLEVKIRDW
151	YQRQRPAEIK	DYSPYFKTIE	DLRNKILTAT	VDNANVLLQI	DNARLAADDF
201	RTKYETELNL	RMSVEADING	LRRVLDELTL	ARADLEMQIE	SLKEELAYLK
251	KNHEEEMNAL	RGQVGGDVNV	EMDAAPGVDL	SRILNEMRDQ	YEKMAEKNRK
301	DAEEWFFTKT	EELNREVATN	SELVQSGKSE	ISELRRTMQN	LEIELQSQLS
351	MKASLENSLE	ETKGRYCMQL	AQIQEMIGSV	EEQLAQLRCE	MEQQNQEYKI
401	LLDVKTRLEQ	EIATYRRLLE	GEDAHLSSSQ	FSSGSQSSRD	VTSSSRQIRT
451	KVMDVHDGKV	VSTHEQVLRT	KN		

IRMA). (Table 1) Nuclear matrix protease-22 (NMP-22) and telomeric repeat amplification protocol (TRAP) were thought to be based on the characteristics of the targeted antigen (Larsen et al., 2006; Proctor et al., 2010; Nugraha, 2011). Alternatively, to increase the specificity of 47 kDa antibodies, they are made using specific epitopes.

The epitope is a particular area in the antigen where the specific antibody binds. This bond will initiate the specific immune response (Nugraha, 2011). In previous research for examining polyclonal antibodies against the 47kDa protein, there was still no identification of specific epitopes that were specifically expressed in bladder cancer patients (Ladner, 2007); hence this study was conducted to identify specific epitopes on the 47kDa protein as the basis for making antibodies for bladder cancer biomarkers. The consideration is that 47 kDa protein is a foreign protein produced by bladder cancer cells that is not generally expressed by normal bladder epithelial cells.

Materials and Methods

Patients

Participants in this study were divided into three different stages, stage one consists of 18 patients; four bladder cancer patients and other cancer patients (two patients of prostate cancer, two patients of kidney cancer, one patient of ureteral cancer, two patients of rectal cancer, and two breast cancer patients) who agreed to participate and were willing to provide a midstream portion of urine. The inclusion criteria for bladder cancer patients in this study were as follows: experiencing hematuria, having a urine cytology report, and undergoing initial therapy with transurethral resection of the bladder. The inclusion criteria for another cancer patient are those who have had their diagnosis confirmed by pathological findings, and the study included four healthy individuals if their anamnesis, physical examination, urine, and abdominal ultrasound revealed no abnormalities.

The participants for stage two of the study (diagnostic test for 1st antibody) consisted of 72 participants including 30 bladder cancer patients with hematuria (localized disease, metastatic disease, low-grade TCC, and high-grade TCC), six patients with hematuria caused by BPH (Benign Prostate Hyperplasia), six patients with kidney stones, six patients with urinary tract infections, six patients with kidney cancer, three patients with ureteral cancer, and 18 healthy normal persons (with various ages and genders). All the patients who were included in this study were the patients that came to the hospital, some were admitted, and some were polyclinic patients who came to check up on their condition based on the control schedule (Table 2).

The participants for the stage three study (diagnostic test for second antibody) consist of 96 participants including 30 bladder cancer patients with hematuria (localized disease, metastatic disease, low-grade TCC, and high-grade TCC), six patients with hematuria caused by BPH, 6 patients with kidney stones, 6 with urinary tract infections, 6 with kidney trauma, 6 patients with kidney cancer, 6 patients with ureteral cancer, and 24 healthy normal persons (with various ages and genders) who agreed to participate and were willing to provide a midstream portion of urine, detail sample characteristics shown in Table 3 and 4.

The Research Ethics Committee of the Faculty of Medicine, University of Brawijaya, Malang, Indonesia, has approved this research with the number 400/099/K.3/302/2021.

47 kDa Protein Sequence Determination

Prior to sequencing by mass spectrometry, the 47-kDa protein preparation process was carried out as follows: The 47 kDa protein required for sequencing is 10 to 100 picomoles. The liquid sample was first dissolved in acetonitrile or propanol 45%. Samples were supplied on 40 mm^2 of PVDF (polyvinylidene difluoride) membrane. Alkylation was performed to identify cysteine. A solution of the alkylating agent was prepared. Some commonly used alkylating agents for DNA and RNA include formaldehyde, dimethyl sulfate (DMS), and ethylene oxide (EtO). The concentration of the alkylating agent will depend on the specific agent and the desired level of modification. Incubate the DNA or RNA sample with the alkylating agent. We performed the alkylation of our DNA/RNA samples using formaldehyde as the alkylating agent. The concentration of formaldehyde was determined based on preliminary experiments to achieve the desired level of modification. We used an Eppendorf Research plus adjustable volume pipette for precise measurement and delivery of the alkylating agent to our samples.

The samples were then incubated with the alkylating agent in a Thermo Scientific Heratherm Compact Incubator at a temperature of 37°C for a duration of 2 hours. The incubation was performed in 1.5mL Eppendorf Safe-Lock Tubes to ensure the integrity of the samples during the process. Post incubation, the samples were purified to remove excess alkylating agent and its by-products using the Qiagen RNeasy Mini Kit following the manufacturer's instructions. The length of the incubation time will depend on the specific agent and the desired level of modification. Neutralize the reaction with an appropriate quenching agent; this may include options such as sodium borohydride, ammonium chloride, or sodium thiosulfate. Proceed to purify the post-reaction DNA or RNA using a method suitable for the specific sample, which could involve techniques like column chromatography or ethanol precipitation. Once purified, the DNA or RNA is ready for sequencing. In this context, consider utilizing the Oxford Nanopore MinION Sequencer, a third-generation sequencing (TGS) technology. Unlike its predecessors, the MinION Sequencer identifies DNA bases by measuring changes in electrical conductivity as DNA strands pass through a biological pore. This sequencing approach, despite having higher error rates at the base level, can greatly improve assembly quality, particularly in handling long repeats, which often pose a challenge for short reads. As such, the MinION Sequencer is not only portable and affordable but also capable of producing high-quality de novo assembly and structural variation detection for human-sized genomes, making it highly suitable for real-time applications and genome surveillance in resource-limited settings (4).

The 47-kDa protein was also freed from primary amines such as tris or glycine. Dialysis was carried out to keep the salt concentration under 2 mM. The protein must be transferred to the PVDF (polyvinylidene difluoride) membrane by electroblotting to remove the glycine used during SDS-PAGE then the membrane was washed 4 or 5 times to remove tris or glycine altogether using Thermo Fisher Scientific product. The amino acid composition was determined by hydrolyzing a 47 kDa protein using 6 M hydrochloric acid for 24 hours at 100°C. Amino acids that have been hydrolyzed are separated using chromatography, and the amounts of each amino acid are counted using the ninhydrin method. The absorbance was measured to determine the number of amino acids. The N-terminal amino acids were sequenced using the Sanger, fluoro 2,4-dinitrobenzene (FDBN), or dansyl chloride methods. C-terminal sequencing was carried out using carboxypeptidase enzymes. Disulfide bonds that could hinder sequencing were reduced by adding beta-mercaptoethanol, followed by iodoacetic acid. Specific protease enzymes were added to break down large polypeptides into smaller polypeptides with 15–25 amino acids.

SDS-PAGE gels were used to separate proteins. Put the protein up for electroblotting on a sturdy surface, such PVDF membrane. Proteins attached to the PVDF membrane were fed into an automated protein sequencer. Modifies the N-terminal amino acid when the protein on the solid support is exposed to Edman's reagent. The modified N-terminal amino acids were hydrolyzed under acidic conditions and identified using chromatography. Automated amino acid sequencers are created based on this method. The amino acid sequence will be analyzed using mascot sequence matching software based on UniProt database (RRID:SCR_002380).

47 kDa Protein Epitopes Determination

After the 47kDa protein had been sequenced, the next step was to identify epitope candidates using the in-silico method (Bepipred 2.0). This step begins with multiple sequence alignment using ClustaIX 2.0.12 software RRID:SCR_018393. This step examines the different protein sequences and determines one representative for each type. The sequence is uploaded by clicking load sequence. Then click Alignment > Complete Alignment to align the sequence. It provides an integrated environment for performing multiple sequence and profile alignments and analyzing the results. The sequence alignment is displayed in a window on the screen, and a versatile coloring scheme has been incorporated to highlight conserved features in the alignment. The software allows users to cut-and-paste sequences to change the order of the alignment, select a subset of sequences to be aligned, and select a sub-range of the alignment to be realigned and inserted back into the original alignment. An alignment quality analysis can be performed, and low-scoring segments or exceptional residues can be highlighted. Clustal X has two modes: Multiple Alignment Mode and Profile Alignment Mode. Multiple Alignment Mode allows for the alignment of a set of sequences, while Profile Alignment Mode allows for the alignment of two alignments, or profiles, and can be used to add a new sequence to an old alignment or to use secondary structure to guide the alignment process. The software also includes features for indicating gaps in old alignments and scrolling through profiles.

Proteosomal cleavage is predicted by using the NetChop server, which can be freely accessed via the internet from the homepage address. To predict the 47 kDa proteosomal cleavage protein, the NetChop service needs protein sequences as input data. The outcomes of NetChop's prediction of proteosomal cleavage are shown as peptide sequences and the predicted cleavage score for each peptide sequence. In our study, we employed the NetChop server, a bioinformatics tool, to predict the proteosomal cleavage sites within our 47 kDa protein of interest. The amino acid sequence of the 47 kDa protein was initially obtained through a combination of protein sequencing methods, including Sanger sequencing and the fluoro 2,4-dinitrobenzene (FDBN) and dansyl chloride methods. The obtained sequence data was then prepared for input into the NetChop server. The protein sequence was inputted into the NetChop server in the required format, which is the standard one-letter amino acid code. It is important to note that all non-standard characters and spaces were removed from the sequence to ensure correct interpretation by the NetChop algorithm.

Transporter antigen processing binding was predicted using the TAPPRED server, which can be freely accessed

via the internet from the homepage address. The predicted results of TAP binding from the TAPPred server are represented as peptide sequences along with their positions and affinity prediction scores for each peptide sequence as seen in the result section.

The 47 kDa protein T cell epitope prediction was based on the most significant binding score. The prediction results consist of two MHC classes, namely MHC Class I and MHC Class II. SVMHC predicts T cell epitopes with 47 kDa protein amino acid sequence input data in FASTA format. Prediction of epitopes in MHC class I is done through the site1 index (). Then the sequence is copied to the toolbox used or uploaded using browse. Data was submitted to the server to see the results.

The 47 kDa protein B cell epitope was predicted via the BepiPred server and the DiscoTope server. The B-cell epitope is an antigen-specific region with high interaction with B-cell lymphocytes. As a result, B cells can produce antigen-specific antibodies and memory cells. B cell epitopes are divided based on hydrophilicity, accessibility, and the -turn region. To predict B cell epitope, one can use the method provided by the immune epitope database (IEDB) web server (http://tools.iedb.org/main/bcell/) with the default thresholds: BepiPred with threshold 0.229 (Jespersen et al., 2017), surface accessibility Emini with threshold 1.000 (Emini et al., 1985), antigenicity Kolaskar-Tongaonkar with threshold 1.015 (Kolaskar and Tongaonkar, 1990), hydrophilicity Parker with threshold 2.068 (Parker et al., 1986), Karplus, and flexibility. Schulz with a threshold of 1.002 and Beta Chou and Fasman with a threshold of 1.017 (Ferlay et al., 2010), accessed via the sites http://ailab.cs.iastate.edu/bcpred/predict.html and http://tools.immuneepitope.org/tools/bcell/iedb.input. Sequences are copied to the toolbox provided. To see the results, click Run Prediction.

The predicted T-cell epitope sequences were determined based on the peptide scores predicted by the SVMHC, NetChop, and TAPPRED servers. The designed epitope sequence is a peptide sequence in FASTA format. The epitope sequence was determined by combining peptides based on the predicted B and T cell epitope results. The epitope used for the combination was the T cell epitope (MHC-I and MHC-II), and the B cell epitope had the highest binding score (high binders).

Template searches are performed via the CPHModels server, which is connected directly to the PDB database. A template search was performed for the 47 kDa protein.

Modeling the epitope's tertiary structure based on the template was carried out through the Swiss-PdbViewer/DeepView program. Structural residues can be seen through the Control Panel menu. Attaching the epitope structure to the template is done through the Magic Fit menu. The attached residues can be seen through the alignment menu. The visualization of the tertiary structure of the attached epitope is carried out by moving the tertiary structure of the template so that the tertiary structure of the vaccine is visible. The repair of the tertiary epitope structure was carried out in two stages. The first stage is repairing the epitope structure in the DeepView viewer by fixing overlapping residues. In comparison, the second stage is via the Swiss-Model server, which sends the structure of each epitope that has been repaired in DeepView to the Swiss-Model server in the form of a PDB for structure optimization by selecting the Optimise Mode menu on the Swiss-Model server.

The epitope's tertiary structure is evaluated by looking at the overlapping residues and analyzing the Ramachandran plot of the epitope structure via the Ramachandran Plot menu in the Deep View program. A comparison of the tertiary structure of each epitope with the protein database in the form of a PDB was performed via FeatureMap3D on the Center for Biological Sequence Analysis (CBS) website. The homepage address can be accessed at http://cbs.dtu.dk/services/FeatureMap3D.

Epitope sequence validation with the database used BLASTp against the human protein database the homepage address can be accessed here.

Chimera software (RRID:SCR_004097) was used to separate the native ligand bundle, or a protein without a ligand, ignoring the presence of water in the pdb extension. Ligands were generated by utilizing the MarvinBean Suite to obtain the 3D chemical structure of each compound. Proteins and ligands are prepared to become ready-to-use files using the AutoDock and openbabel conversion facilities in PyRx. Additionally, the docking validation process is used to determine the native ligand's 3D conformation to the receptor by determining the coordinates of the structure's center of mass and the gridbox's size in angstroms (Vina) or points (AutoDock). Ligand docking data analysis was performed to produce binding energy values in units of kcal/mol. The value of the binding energy used is the one that makes the value go negative; the value is minus 12, so the bond strength can be determined. For each set of data tested, the two best ones were taken. The collected test ligand data was compared with the native ligand. Protein-ligand interaction data was displayed using PyMOL and PLIP to see the residues involved. The molecular prediction of docking between antigens (ligands) and antibodies in humans (receptors) was carried out using ArgusLab 4.0.1 software. If there is more than one predicted epitope, the epitope with the highest and lowest immunogenicity levels will be used.

Production of polyclonal antibodies

Thirty-five male mice, 6–8 weeks old Mus Musculus, of the BALB/C strain were obtained from the Integrated Research and Testing Laboratory, Gadjah Mada University, Yogyakarta, Indonesia. Conducted according to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines, explanation of the ethical review permissions obtained for the animal work and the national/international regulations adhered to, which is in Saiful Anwar General Hospital ethical commission, Malang, East Java, Indonesia. It also included Detailed information about the animals used, including species, strain (BALB/c), sex, age or weight range, and housing conditions. Detailed description of the procedure for producing polyclonal antibodies. This would include the type of antigen used, the method of immunization, the adjuvant used, the number of boosts given, the period between immunizations, and the method of collecting

the antisera.

Antigens used were epitope with the highest and lowest levels of immunogenicity derived from 47 Kda protein. The antigen in the syringe was emulsified with Freund's Complete Adjuvant (FCA) (Sigma-Aldrich, Singapore) and administered intraperitoneally at a dose of 100 mg in 100 mL phosphate-buffered saline (PBS). Booster injections were performed in Weeks 2–4 by using antigen emulsified with Incomplete Freund's Adjuvant (IFA) at the same dose. The serum was taken one week after the last booster.

Serum collection

Male mice was euthanised with high dose ketamine 1-2 cc (50mg/cc) before the serum was collected. Later the blood was drawn from the heart of five mice, the blood was then collected in sterile tubes, and put in an incubator set at 37 °C for 30 minutes in a tilted position. It was then centrifuged for 5 minutes at 10,000 rpm after being kept at a temperature of 40 °C in the refrigerator for 10 minutes. Blood clots were removed from the clear liquid zone and placed in sterile tubes and kept at 20°C.

Western Blotting

Western Blotting Method used the Towbin method. The first SDS-PAGE procedure was done to see the protein band that was formed. A separating and a stacking solution were made, then pour the separating solution into the gel cassette. After it hardens pour the stacking solution into the gel cassette, then add DD H2O to adjust the volume. Place the mold (comb) into the gel cassette and wait for the stacking gel to harden. Remove the gel cassette from the gel casting and insert it into the chamber. Pour the running buffer until the gel cassette is completely submerged. RSB was added in a ratio of 1:1 before running the sample, then heated in boiling water for ± 5 minutes. A sample of 15 µl was taken and put into the gel well. Samples were processed at 200 volts for 30-40 minutes. The gel was taken and stained with coomassie blue dye solution for ± 2 hours. The background stain is totally removed after ± 4 hours of incubation, at which point the staining solution is discarded and replaced with a destaining solution. Observations were made of the protein bands that developed.

After the protein sample completed SDS-PAGE, the gel was transferred to nitrocellulose (NC) paper using a semi-dry blotter produced by Biored. 300 mA of electricity is utilized for 120 minutes. After that, it was marked to determine the molecular weight and stained with 2% ponceau dye containing up to 3% TCA for 30 minutes to determine if the sample protein had been transferred to nitrocellulose paper. Paper made of nitrocellulose is cut based on the wells. The NC paper is rinsed with dH2O in order to get rid of the ponceau colorphos. Then it was blocked with TBS containing 3% albumin at pH 7.4 plus 1% BSA soaked overnight at 4°C. Washing was done twice; each process included shaking for 10 minutes using TBS liquid pH 7.4 containing Tween 20 0.05°A, incubation with a primary antibody (anti-protein XXX which is antibody 47 kDa) concentration of 1/100 for 2 hours, washing with TBS-Tween 0.05% A for 5 minutes two times, and addition of a secondary antibody, namely IgG-anti-mouse conjugated with alkaline phosphatase labeled biotin with a concentration of 1/1000 in TBS pH 7.4 and 1% BSA and protected against the effects of light. It is incubated for 2 hours, then washed for 2x5 minutes using 0.05% PBS-Tween. Incubated with streptavidin HRP for 40 minutes, washed with 0.05% TBS-Tween for 2x10 minutes, and dripped with TMB substrate as a dye. If a band with a bluish color formed, the reaction was stopped using distilled water. The results were dried and immediately recorded.

Results

Sequencing Determination and Equalizing sequencing

In the 47 kDa protein sequencing performed by the sequencing method, the following sequences were obtained:

1. 42' APSTY GGGLS VSSSR 56'

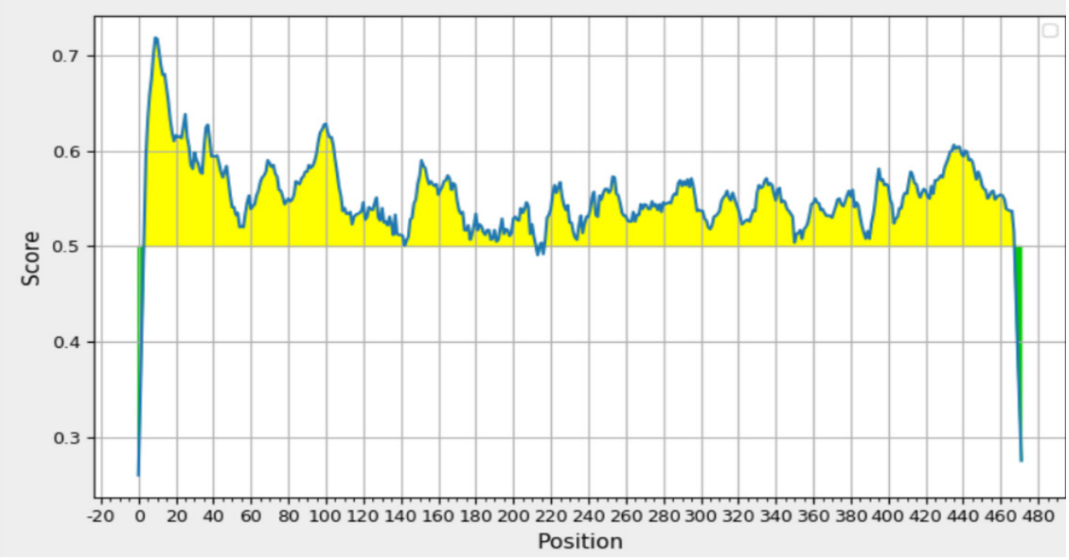

Figure 1. Prediction Result of Bepipred 2.0; the yellow color on the graph indicates that this amino acid can play a role and is predicted as an epitope region, while the green color represents a non-epitope region. The average result is 0.551

Table 2. Antigenity Prediction Results of Amino Acid Sequences.

Start	End	Peptide	Amino Acid Counts	Bepipred 2.0 Score
31	55	ISSVLAGGSCRAPSTYGGGLSVSSS	24	0:58
108	136	DGLLVGSEKVTMQNLNDRLASYLDKVRAL	30	0:53
221	240	LRRVLDELTLARADLEMQIE	20	0:54
366	387	YCMQLAQIQEMIGSVEEQLAQL	21	0:54
398	417	YKILLDVKTRLEQEIATYRR	20	0:55

Table 3. Sample Characteristic for Ab1 Diagnostic Test

Sample characteristic	Number (percentage) (n=72)
Kidney cancer	6 (8.33%)
Bladder cancer : Low-grade	6 (8.33%)
Bladder cancer : Localized	8 (11.1%)
Bladder cancer : High-grade	6 (8.33%)
Bladder cancer : Metastasis	5 (6.91%)
BPH	5 (6.91%)
Urinary tract stone	6 (8.33%)
Urinary tract infection	6 (8.33%)
UTUC	3 (4.17%)
Healthy men >70 years old	4 (5.56%)
Healthy men 50-70 years old	4 (5.56%)
Healthy men <50 years old	1 (1.39%)
Healthy women >70 years old	4 (5.56%)
Healthy women 50-70 years old	4 (5.56%)
Healthy women <50 years old	1 (1.39%)
<50 years old	8 (11.11%)
50-70 years old	32 (44.44%)
>70 years old	32 (44.44%)
Gender	
Men	36 (50%)
Women	36 (50%)

Table 4. Sample Characteristic for Ab2 Diagnostic Test

Sample characteristic	Number (percentage) (n=96)
Kidney cancer	6 (6.25%)
Bladder cancer : Low-grade	8 (8.33%)
Bladder cancer : Localized	8 (6.33%)
Bladder cancer : High-grade	8 (6.33%)
Bladder cancer : Metastasis	6 (6.25%)
BPH	6 (6.25%)
Urinary tract stone	6 (6.25%)
IUrinary tract infection	6 (6.25%)
Kidney trauma	6 (6.25%)
UTUC	6 (6.25%)
Healthy men >70 years old	4 (4.17%)
Healthy men 50-70 years old	4 (4.17%)
Healthy men <50 years old	4 (4.17%)
Healthy women >70 years old	4 (4.17%)
Healthy women 50-70 years old	4 (4.17%)
Healthy women <50 years old	4 (4.17%)
Age	
<50 years old	32 (33.33%)
50-70 years old	32 (33.33%)
>70 years old	32 (33.33%)
Gender	
Men	48 (50%)
Women	48 (50%)

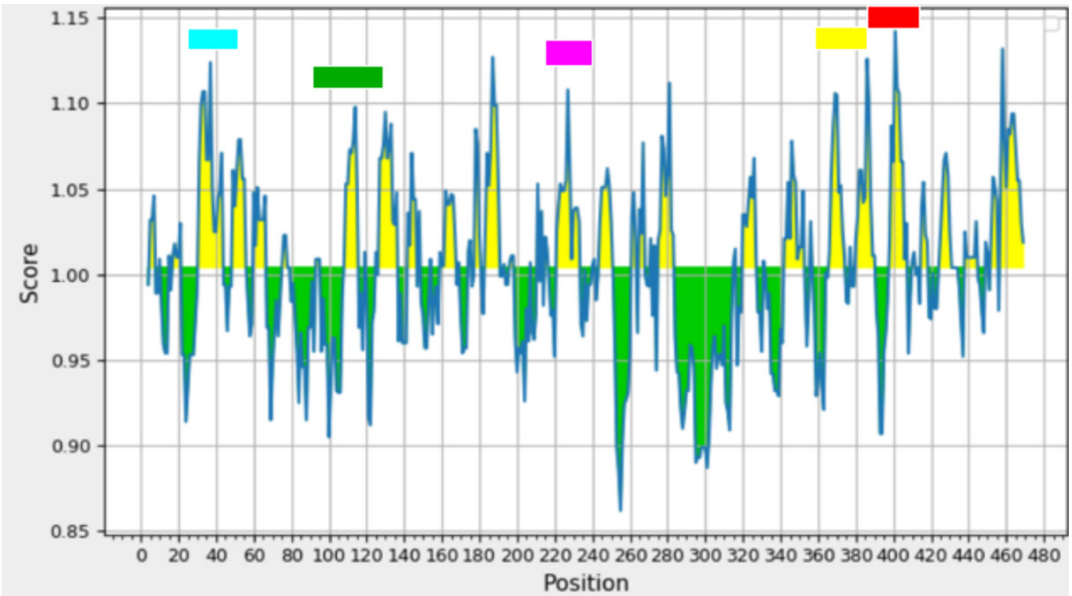

Figure 2. Antigenicity Prediction Results of Kolaskar & Tongaonkar

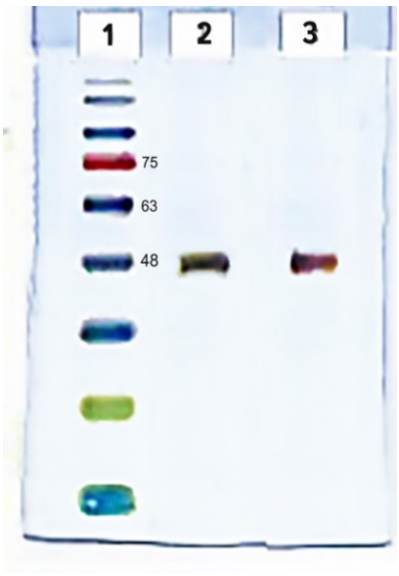

Figure 3. 47kDA Antibody Test Results Using 47kDa Protein as Antigen. Column 1: markers, column 2: Antibody peptide 1 (Ab1), column 3: Antibody peptide 2 (Ab2).

2. 117' VTMQN LNDRL ASYLD KVRAL EEANA DLEVK IRDWY QR 153'

3. 161' DYSPY FK 167'

4. 176' ILTAT VDNAN VLLQI DNARL AADDF RTKYE TELNL RMSVE ADING LRRVL DELTL AR 232'

5. 252' NHEEE MNALR GQVGG DVNVE MDAAP GVDLS RILNE MRDQY EK 293'

6. 301' DAEEW FFTKT EELNR EVATN SELVQ SGKSE ISELR RTMQN LEIEL QSQLS MKASL ENSLE ETKGR 265

7. 406' TRLEQ EIATY RRLLE GEDAH LSSSQ FSSGS QSSR 469'

8. 460' VVSTH EQVLR 469'

The matching process was carried out with the mascot from the results of the sequenced pieces obtained from the extraction. Obtained a series of sequences that match Keratin, type I cytoskeletal obtained index score of 908 and sequence coverage of 56%.

Table 5. Sensitivity and Specificity Diagnostic Result on Ab 1 and Ab 2

	Sensitivity	Specificity
Ab 1	83.33%	90.91%
Ab 2	91.67%	94.44%

Epitopes Mapping

Epitope mapping prediction was carried out using the Immune Epitope Database (IEDB) B Cell Linear Epitope Prediction program (iedb.org). At IEDB there are several predictions of epitopes with approaches such as Bepipred.

BepiPred-2.0 is based on a random forest algorithm trained on epitopes annotated from antibody-antigen protein structures. This new method outperformed other methods for sequence-based epitope prediction, both on epitope data collected from solved 3D structures and on a large collection of linear epitopes downloaded from the IEDB database (Figure 1).

Antigenicity is the ability of a foreign protein to stimulate a host's immune response. Based on the antigenicity prediction results of Kolaskar and Tongaonkar, there are several candidate peptides (Figures 2-3) with an average score of 1,004, a minimum of 0,862, and a maximum of 1,142. Each color describes the location of the peptide (Table 4). This study used antigens that were sequenced at 108'-136 (peptide 1) and 31-55' (peptide 2) (lowest score of Bepipred and highest score of Bepipred). Then this sequencing of epitopes was made from synthetic peptide by GenScript, USA and then immunized on mice.

47kDa Antibody Evaluation Activation Test with Control

47kDa antibody testing used 47kDa protein as an antigen. This test used the western blotting method. In the test, three well columns were obtained: (1) markers; (2) antibody from peptide 1 (Ab1); and (3) antibody from peptide 2 (Ab2). In columns 2 (Ab1) and 3 (Ab2), we only obtained expression at a molecular weight of 47 kDa. This result shows that Ab1 and Ab2 are specific for 47 kDa protein (as antigen).

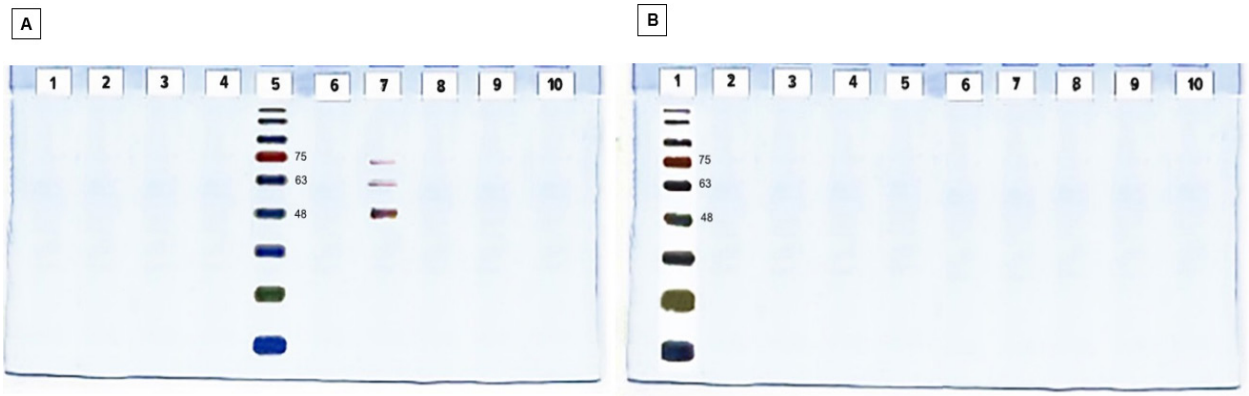

Figure 4. Results of Westenbloting Ab 1, (A) Column 1-4 urine from healthy person, column 5 markers, column 6 crude urine from bladder cancer patients, column 7 urine precipitate from bladder cancer patietns, column 8 crude urine from bladder cancer patients with concentration 1/100, column 9 urine precipitate from bladder cancer patients with concentration 1/100. (B) Westernbloting results comparison among various urine of cancer patients column 1 markers, column 2-3 rectal cancer, column 4-5 prostate cancer, column 6-7 kidney cancer, column 8-9 breast cancer, column 10 ureteral cancer

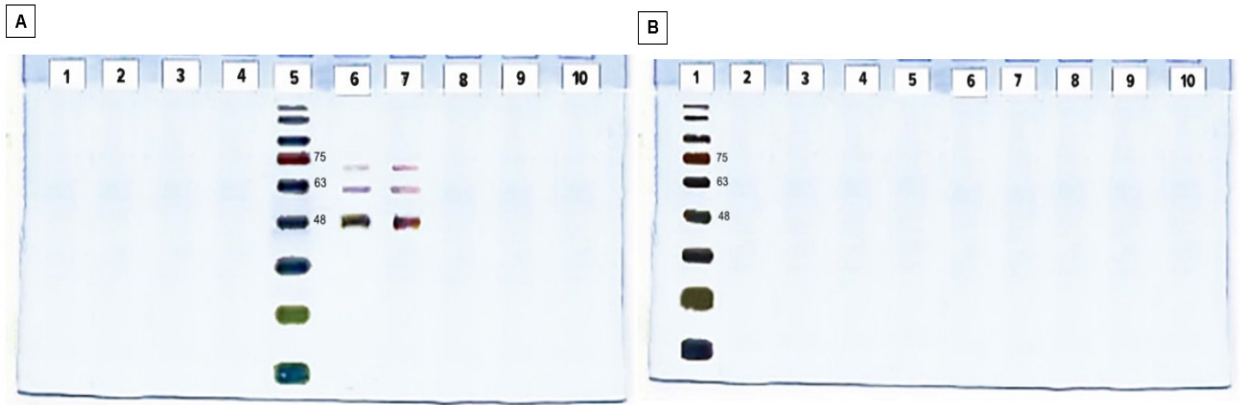

Figure 5. Results of Westenbloting Ab 2, (A) Column 1-4 urine from healthy person, column 5 markers, column 6 crude urine from bladder cancer patients, column 7 urine precipitate from bladder cancer patietns, column 8 crude urine from bladder cancer patients with concentration 1/100, column 9 urine precipitate from bladder cancer patients with concentration 1/100. (B) Westernbloting results comparison among various urine of cancer patients column 1 markers, column 2-3 rectal cancer, column 4-5 prostate cancer, column 6-7 kidney cancer, column 8-9 breast cancer, column 10 ureteral cancer

Western blotting test of 4kDa Antibody Evolution with Urine several patients

Two types of antibodies have been made; antibody from peptide 1 (Ab1) with a length of 30 peptides and antibody from peptide 2 (Ab2) with 24 peptides. These two antibodies were then subjected to western blotting testing of the patient's urine.

Figure 4 shows Ab1 expression in column 7 (urine from a bladder cancer patient with precipitate) at 47 kDa. Excretion is described as a band with almost the same thickness as the markers for 47 kDa-weight protein (column 5). In this test, there are no expressions in the other columns (urine from a healthy person and urine from other cancer patients).

Figure 5 shows Ab2 expression in column 6 (crude urine from bladder cancer patients) and column 7 (urine precipitate from bladder cancer patients) at 47 kDa. We obtained nearly the same thick expression as the markers for 47 kDa weight protein (column 5). No expression was found compared to normal urine (columns 1-4), diluted urine from bladder cancer patients (columns 8 and 9), and urine from various cancers (rectal, breast, prostate, kidney, and ureter).

This result indicates that the urine precipitate from bladder cancer patients can react with Ab1 and Ab2 and is specific for 47 kDa. This study also showed that Ab2 could bind to the urine antigen of crude bladder cancer patients and its precipitate, so Ab2 was more specific and could bind directly to urine compared to Ab1.

Sensitivity and specificity diagnostic test on Ab1 and Ab2

In order to detect the sensitivity of polyclonal antibodies to bladder cancer-specific proteins, polyclonal antibodies were reacted with antigen in serial concentrations. The minimal ratio of polyclonal antibodies to antigen is 1:107 for the antibody and 1:40 for the antigen. Subsequently, this concentration was applied to detect proteins of 47 kDa only in several cancer tissues. In polyclonal evolution, specific expression was found in bladder cancer. No expression was found in normal patients' urine or in the urine of other cancer types (rectal, prostate, renal, breast, ureteral).

The results of the sensitivity and specificity tests show that the Ab 2 marker for predicting bladder cancer has a higher sensitivity (91.67%) than Ab 1 (83.33%). As for the results of the specificity test, it shows that the Ab 2 marker for predicting bladder cancer has a higher specificity value (94.44%) than Ab 1 (90.91%) (Table 5).

Discussion

The incidence of bladder cancer is relatively high in developed countries due to rapid industrial development. Along with that, bladder cancer rates worldwide are also increasing (Zhu et al., 2018). The clinical picture of bladder cancer is gross hematuria or microscopic hematuria. About 80% of bladder cancers have been diagnosed as non-muscle-invasive bladder cancer (Witjes, 2021). However, the recurrence rate of NMIBC is as high as 60% within one year after the first diagnosis (Woldu et al., 2017). The gold standard for diagnosing bladder cancer is cystoscopy and tissue retrieval for histopathological examination (Zhu et al., 2018). This method is invasive, hindering the initial diagnosis of bladder cancer.

Urine cytology is an alternative way to detect bladder cancer. Urine cytology examines morphological changes in exfoliated cells from the urinary tract to assess abnormalities (Moreira et al., 2018). In high-risk urothelial carcinoma, the sensitivity is as high as 86%, but in low-risk, it is 20–50% (Zhu et al., 2018). In addition to diagnostic tools, urine cytology is used for post-trimodal therapy evaluation. The evolution of 47kDa polyclonal antibodies can increase the sensitivity and specificity of the diagnosis of bladder carcinoma.

The epitope is part of the antigen where specific antibody would bind. This antigen-antibody binding initiates an immune response. Generally, an epitope consists of an amino acid sequence five to six amino acids long. An antigen can contain more than one or more

epitopes to which an antibody can bind. Then different antibodies can bind to a single antigen at one time (Larsen et al., 2006; Proctor et al., 2010; Nugraha, 2011).

Based on the arrangement of amino acids, the epitope is divided into continuous and discontinuous epitopes. Continuous epitopes are linearly ordered based on amino acids, whereas discontinuous epitopes are present in particular conformations and folded in different conformations (Nugraha, 2011). Physiologically, epitopes are classified as B-cell epitopes and T-cell epitopes. The B-cell epitope is part of the antigen that binds to the B-cell antibody. A T-cell epitope is the peptide fragment that has been processed by an antigen-presenting cell (APC) with protein molecules from the major histocompatibility complex (MHC) that are recognized by recipient T cells (Amanna et al., 2006; Larsen et al., 2006; Proctore et al., 2010; Nugraha, 2011).

Intact proteins have traditionally been used as immunogens for antibody production (Liew et al., 2021). However, using intact proteins causes several side effects and low binding affinity, so the antibodies formed have weak bonds with antigens (Colwill et al., 2011). Each step provides distinct advantages over traditional methods using whole proteins or synthetic peptides combined with immunogenic carriers that harm the host. The selection of a peptide that has a high in silico affinity will increase antibody production and antibody ability. In addition, the epitope that has been predicted tends to be hydrophilic, can be reacted with solvents, and is flexible (Yang and Yu, 2009).

An epitope antibody, or B-cell epitope, is a molecular structure in the antigen that binds specifically to the antibody (Colwill et al., 2011). The identification of B-cell epitopes in antigenic proteins can have protective potential that can be used to increase specific antibodies. On this basis, it can be developed as a vaccine, a diagnostic, or an immunotherapy. This can be achieved by analyzing the affinity of an epitope with an antibody so that it can provide an immune response (Arnon et al., 1971; Sela-Culang et al., 2013). The choice of the epitope is believed to control the ability and effect of antigen and antibody binding. An example is the smallpox vaccine, which is the only vaccine that can completely reduce infectious diseases in the human population (Arnon et al., 1971). The epitopes used in this vaccine are specific, so the antibodies produced are specific and will form specific memory B cells so that they can protect against the smallpox virus.

Linear B cell epitope consists of sequential peptide residues, while conformational B cell epitope consists of additional dissolved atoms derived from peptide residues that do not have to be sequential. Antibodies against linear B cell epitopes can recognize denatured antigens, whereas denatured antigens will not be recognized by conformational B cell epitopes (Pellequer et al., 1994). The linear B cell epitope consists of peptides that substitute for antigens for immunization and antibody production. Therefore, despite being in the minority, the linear B-cell epitope has received significant attention. Linear B-cell epitopes were predicted from antigen primer sequences using sequence-based methods. Early computational methods for B-cell epitope prediction are based on simple amino acid trend scales that describe the physicochemical features of B-cell epitopes (Pellequer et al., 1994; Sanchez-Trincado et al., 2017). Currently, available bioinformatics software for predicting linear B-cell epitopes using trend scales includes PREDITOP and PEOPLE (Sanchez-Trincado et al., 2017). In this study, two peptides were predicted to bind specifically to antibodies: peptide 1: 31' ISSVLAGGSC RAPSTYGGGL SVSSS '55 and peptide 2: 108' DGLLVGSEKV TMQNLNDRLA SYLDKVRAL '136.

A previous study about the profile of SDS-PAGE showed a difference in molecular weight in bladder cancer compared to healthy tissue (Prasetya et al., 2014). A protein of 122 kDa molecular weight is present in the epithelial cells of bladder cancer and normal bladders. A 69 kDa protein is only present in the epithelial bladders of normal individuals. In addition, a 47 kDa protein is only present in the epithelial cells of bladder cancer. The results of the polyclonal antibody evolution (made from epitopes) we have conducted have the same results as previous studies. Where polyclonal antibodies 1 and 2 can only detect urine from Ca bladder patients at 47 kDa.

In conclusion, the evolution of the polyclonal antibody (made from epitopes) that has been made is proven to express specifically on Ca bladder protein. Antibody peptide 1 can react with crude urine protein and its precipitate, so that peptide 1 antibody can be used directly in urine. However, peptide-2 antibodies can react with crude urine and urine precipitates. Both antibodies have high sensitivity and specificity to diagnose bladder cancer

Author Contribution Statement

Taufiq Nur Budaya, Happy KP, Widodo, Sumarno RP, conceptualized the study. Taufiq Nur Budaya collected the data used for the analysis, drafted the manuscript. Taufiq Nur Budaya, Happy KP, Widodo, Sumarno RP reviewed and approved the final draft of the manuscript.

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Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by Ethics Committee of Saiful Anwar Hospital Number 400/099/K.3/302/2021

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patient(s) to publish this paper

Data availability

Data availability has already addressed to deposit into repository of Figshare under the Open License CC-BY 4.0

Conflict of intereset

The authors declare no conflict of interest

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