Polyclonal antibody from 47 kDa protein of bladder cancer is sensitive and specific for detection of bladder cancer

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Abstract This study aims to investigate whether the protein isolated from bladder cancer in an Indonesian population can produce the polyclonal antibody for clinical markers of bladder cancer. The participants in this study are bladder cancer patients and healthy persons who were approved for midstream portion urine collection. The inclusion criteria included bladder cancer patients who obtained hematuria examination, urine cytology, and initial therapy with transurethral resection of the bladder; a healthy volunteer who was approved for midstream portion urine collection but without hematuria history, bladder stones, and signs or symptoms of tractus urinarius infection. The procedure consisted of tissue preparation, tissue processing, isolation of membrane cell protein, monitoring of protein in membrane cell, production of polyclonal antibodies, and dot blot technique. A protein with 122 kDa molecular weight is present in epithelial cells of bladder cancer and the normal bladder. A protein with 69 kDa molecular weight is only present in the epithelial cell bladder of normal individuals. In addition, a protein with molecular weight of 47 kDa is only present in epithelial cells of bladder cancer. The minimal ratio of polyclonal antibody with antigen is 1:6400 of the antibody and 1:40 of antigen. Subsequently, this concentration was applied to detect proteins with 47 kDa only in several cancer tissues. Positive results in bladder cancer, but negative results in prostate cancer, rectal cancer, and breast cancer were found. The polyclonal antibody produced from bladder cancer can be used for the detection of bladder cancer.
Polyclonal antibody from 47 kDa protein is sensitive and specific to detect bladder cancer and become an alternative biomarker for diagnosis and surveillance of bladder cancer. Copyright © 2014, Taiwan Genomic Medicine and Biomarker Society. Published by Elsevier Taiwan LLC. All rights reserved.

Introduction

Bladder cancer is a malignancy in the seventh position of incidence in men and women. In a developed country, such as the United States, this malignancy is in 4th position for men and 10th position for women, and remains increasing. In Indonesia, a 10 year study in Cipto Mangunkusumo Hospital on 340 patients, had a 15% increase per year with an average age of 54 years. The transitional carcinoma is frequency of histopathologic feature (80.66%) and 60% of patients usually diagnosed at locally advanced and advanced stage. The delay of patients to seek medical treatment or difficulty of the medical practitioner to obtain the diagnosis remains a problem in developing countries. This local problem increases efforts to provide a simple diagnostic tool to be available in urban or rural health facilities.

The pattern of a specific polypeptide using a proteomic approach is a useful tool for detection of urothelial carcinoma. This technique was applied to identify a new biomarker for new diagnostic tools. Proteomic approach focuses on the difference in the protein pattern in bladder cancer compared to a healthy bladder. Finally, a proteomic approach would be available for polyclonal antibody production then applied as clinical markers of bladder cancer. As far we know, there is no study to investigate the protein pattern of bladder cancer in the Indonesian population. Therefore, this study aimed to investigate whether the protein isolated from bladder cancer in the Indonesian population can produce polyclonal antibodies for clinical markers of the bladder cancer diagnostic tool. Besides, this study was also conducted to determine the effectiveness of polyclonal antibody to detect bladder cancer.

Materials and methods

Patients

The participants in this study were bladder cancer patients and healthy persons who agreed to participate in this study and provide a midstream portion of urine collection. The inclusion criteria included bladder cancer who performed hematuria examination, urine cytology, and initial therapy with transurethral resection of the bladder. A healthy volunteer was approved for midstream portion urine collection but without hematuria history, bladder stone, and signs or symptoms of tractus urinarius infection. This research has been approved by Research Ethics Committee Faculty of Medicine University of Brawijaya, Malang, Indonesia.

Tissue preparation

Bladder cancer tissue was obtained by transurethral resection of the bladder. Bladder epithelial cells were also obtained from healthy volunteers. All specimens were cold packed in 0.9% NaCl then delivered to the Biomedical Laboratory, Faculty of Medicine, University of Brawijaya, Malang, East Java, Indonesia.

Tissue processing

Five grams of tissue were cut into little pieces then inserted into 10-mL sterile tubes, 5 mL phosphate buffer saline was added and then centrifuged at 5000 rpm for 15 minutes at 4°C. The supernatant then discharged, the phosphate buffer saline which contains 1.5mM EDTA and 0.5mM dithiothreitol, pH 7.4, was added to the sediment. The suspension was replaced into a homogenizer tube. These tubes were then homogenized at 1000 rpm for 1 minute. The suspension was then placed in a falcon tube and subsequently centrifuged at 5000 rpm for 15 minutes at 4°C. The supernatant discharged, then 2 mL phosphate buffer saline, pH 7.4, was added through the tube wall slowly. The white layer at the surface of the sediment was then replaced with a new tube and centrifuged at 1000 rpm for 15 minutes at 4°C. The sediment was then suspended with 10 mL phosphate buffer saline 500 rpm for 5 minutes at 4°C. The supernatant was discharged and the sediment was evaluated using Giemsa staining.

Isolation of membrane cell protein

The isolation of the membrane cell protein was done according to the previous method with modification. The sediment of cancer epithelium was added to 2 mL of phosphate buffer saline which contains 0.05% N-Octyl β glucopyranoside (NOG) then vortexed for 1 minute. The supernatant was centrifuged at 5000 rpm for 15 minutes at 4°C. The supernatant was then suspended with 5 mL phosphate buffer saline 500 rpm for 5 minutes at 4°C. The supernatant was discharged and the sediment was evaluated using Giemsa staining.
mini slab gel was applied 4% with tracking gel. Coomassie brilliant blue was chosen for colour material.

**Observation specific protein**

Observation of specific protein was carried out using protein pattern in Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) results.

**Production of polyclonal antibodies**

Male mice, 6–8 weeks old, BALB/C strain were obtained from Integrated Research and Testing Laboratory, Gadjah Mada University, Yogyakarta, Indonesia. Antigens used were specific protein subunits from bladder cancer. The antigen in the syringe was emulsified with Freund’s Complete Adjuvant (FCA) (Sigma-Aldrich, Singapore, Singapore) intraperitoneally with a dose of 100 mg in 100 mL phosphate buffered saline (PBS) 10. Booster injections were performed in Weeks 2–4 by using antigen emulsified with Incomplete Freund’s Adjuvant (IFA) with the same dose. The serum was taken 1 week after the last booster.

**Serum collection**

Blood was collected from the heart. Blood was taken from five mice, after that it was collected in sterile tubes and placed in the incubator with temperature of 37°C in a tilted position for 30 minutes. Then it was stored in a refrigerator with a temperature of 4°C for 10 minutes and then centrifuged at 10,000 rpm for 5 minutes. The clear liquid zone was separated from blood clots, put in sterile tubes, and stored at −20°C.

**Dot blot**

Dot blot is used as a function serological specificity to detect reactions between antigens and antibodies. Nitrocellulose was cut by measuring 7.5 × 11 cm and inserted between two pieces of metal blotter apparatus. Then it was mounted on the dot blot, incubated overnight at a temperature of 40°C until completely absorbed into the nitrocellulose membrane. Further Tris buffered saline (TBS) blocking was carried out with blocking buffer (containing 50mM Tris Base, 0.2M NaCl, 5% skim milk, pH 7.4). The next stage in the membrane, 50 mL of the primary antibodies specific to 47 kDa (1:1000–1:12800 dilution) was spilled, incubated for 2 hours at room temperature and placed on a shaker. The solution was removed, and then washed three times with TBS-0.05% Tween-20, Affymetric, Santa Clara, CA, USA. The secondary antibody with 1:2500 dilution was added. Again it was washed three times with TBS 0.05% Tween-20 and chromogenic substrate (BCIP-NBT) was added.

**Immunohistochemical analysis**

Sections obtained from paraffin-embedded tissues were placed in an antigen retrieval solution (DAKO, Glostrup, Denmark), followed by peroxide and protein blocking.

**Results**

Fig. 1 shows the difference of morphology between normal epithelium and bladder cancer.

The profile of SDS-PAGE showed the difference of molecular weight in bladder cancer compared to healthy tissue as shown in Fig. 2. A protein of 122 kDa molecular weight is present in the epithelial cells of bladder cancer and normal bladder. A 69 kDa protein is only present in
epithelial bladders of normal individuals. In addition, a 47 kDa protein is only present in epithelial cells of bladder cancer.

In order to detect the sensitivity of polyclonal antibodies from bladder cancer specific protein, polyclonal antibody was reacted with antigen in serial concentrations. The minimal ratio of polyclonal antibody with antigen is 1:6400 of the antibody and 1:40 of antigen. Subsequently this concentration was applied to detect proteins of 47 kDa only in several cancer tissues as shown in Fig. 3.

In order to detect the specificity of polyclonal antibody from bladder cancer specific protein, we tested the antibody in several cancer tissues, including prostate cancer, rectal cancer, and breast cancer. We found positive results in bladder cancer, but negative results in prostate cancer, rectal cancer, and breast cancer as seen in Fig. 4.

Discussion

Transitional cell carcinoma of the bladder is a common cause of bladder cancer in the developing world. This type of bladder cancer has been often present at an advanced stage so early diagnosis is warranted. Urinary cytology is widely used for screening for bladder cancer, it is sometimes difficult to judge cytology specimens, particularly for low-grade cancers. Therefore new, noninvasive methods for bladder cancer detection would open new possibilities in diagnosis and monitoring, as well as in screening of groups at risk. An ideal test to monitor bladder cancer should be objective, noninvasive, easy to administer and interpret, and have high sensitivity and specificity. Soluble molecular markers secreted in urine could serve as urinary markers for bladder cancer detection, depending on their efficiency to
provide early detection capabilities and insight into the appropriate treatment response and tumor recurrence. A previous study found several antigens in urothelium tissue. Fradet et al. showed 25 kDa was expressed as surface glycoprotein of bladder cancer. Perk et al. found 17 kDa was lower expressed or negatively expressed in normal urothelium. In this study we found that a protein with 122 kDa molecular weight is present in epithelial cells of bladder cancer and normal bladder. A protein with 69 kDa only presents in epithelial bladder of normal individuals. In addition, a protein with 47 kDa only presents in epithelial cells of bladder cancer. This finding indicated that 47 kDa of protein is the specific protein expressed in epithelial cell of bladder cancer. Therefore, this protein is a good candidate for polyclonal antibody production for clinical markers of the bladder cancer diagnostic tool.

The perfect urinary marker of bladder cancer should reliably detect all tumors for the well-being of the patients and at the same time have a very high specificity to minimize false positive results. This is especially true when considering screening in general, but also when considering screenings of high risk groups and patients under suspicion of bladder cancer. In order to detect the sensitivity of polyclonal antibody from a bladder cancer specific protein, polyclonal antibody was reacted with antigen in serial concentrations. The minimal ratio of polyclonal antibody with antigen is 1:6400 of the antibody and 1:40 of antigen. Subsequently this concentration was used to detect protein with 47 kDa only in several cancer tissues. Positive results were found in bladder cancer, but negative results were found in prostate cancer, rectal cancer, and breast cancer. This finding indicates that polyclonal antibody produced from 47 kDa protein subunits is sensitive and specific to detect bladder cancer.

Conclusion

Polyclonal antibody obtained from 47 kDa protein subunit is sensitive and specific to detect bladder cancer and become an alternative biomarker for diagnosis and surveillance of bladder cancer.

Conflicts of interest

The authors declare that they have no conflicts of interest regarding the publication of this article.

References