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Preparation and Characterization of Monoclonal Antibody Against Truncated Recombinant Nuclear Matrix Protein (NMP22)

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Abstract

Background: Bladder cancer is a major worldwide health problem. Diagnosis of acute and chronic bladder carcinoma is based on the detection of a number of tumor markers such as nmp22 (nuclear matrix protein 22). Nmp22 is one of the tumor markers used for detecting the recurrence of bladder cancer.
Objectives: The aim of this study was to develop hybrid cell producing monoclonal antibodies (MAbs), specifically for nmp22 using hybridoma technology.
Materials and Methods: Complete and incomplete adjuvant with recombinant truncated nmp22 antigens were emulsified and injected to BALB/c mice. The spleen was removed and the splenocytes were fused with sp2/0 myeloma cells. Characterization of the produced mAb was carried out.
Results: As a result of the fusion, hybridoma cells were produced and exhibited high- titer antibodies. By testing of colons based on the Enzyme Linked Immunosorbent Assay (ELISA), 135 hybridoma colons were selected, out of which eight high titer clones including 54D-6F-2E-36-7H-2A-8E-1D-6D were detected and one of the clones named FPR92 was selected, and the produced mAb was further characterized. The produced antibody belongs to the IgG class and its light chain was kappa. With respect to affinity, the mAb was included as high affinity $3 \times 10\text{-}7$ reacting with NMP22 recombinant protein .The western blot of cancerous bladder tissue showed the presence of 40 and 55 kD proteins as major bands that reacted with this mAb.
Conclusions: Based on a double limiting dilution protocol, a type of monoclonal antibody, named FPR92 was produced and characterized.

Keywords: Nmp22, Hybridoma, mAb, FPR92, Characterization

1. Background

In Iran, bladder cancer has been found as the most common urological cancer among the general population (48.3%) (1, 2). It is the 4th most common cancer in males and it is amongst the top five newly diagnosed cancers in males (3, 4). Bladder cancer has been ranked second in the classification of genitourinary malignancies. Most comparative studies have shown that molecular biomarkers have high sensitivity for prediction of outcomes of bladder cancer that could help clinicians provide individualized prognostic actions (5). Noninvasive urine-based biomarkers, including nuclear matrix protein 22 (NMP22) (6, 7), with methods such as fluorescence in situ hybridization (FISH) (8), bladder tumor antigen (BTA) and immunocyt detection of bladder cancer, have generated better-quality and diagnostic accuracy (9). The NMPs are an essential part of a three-dimensional network of the nucleus that localizes to the spindle poles during mitosis (9, 10). The NMP22 regulates the distribution of chromatids to daughter cells (9, 11). By serving as an attaching point for enzymatic machines, it plays significant roles in DNA replication, transcription, RNA processing and gene expression. This antigen is released from dying urothelial cells and can be measured in the urine. There are some variations in NMP composition in human tumors such as breast, prostate, colon, cervical, renal, and head and neck cancer (12). Because tumor markers can be measured by immunologic assay tests, these have potential applications to present objective measurable results (13). Currently, beneficial monoclonal antibodies comprise over 30% of biopharmaceuticals in clinical trials (14). Also, monoclonal antibodies are applied for research components and preparing diagnostic kits such as enzyme-linked immunosorbent assay (ELISA) and

immunofluorescence of numerous diseases (15). Monoclonal antibody based tests have been considered to measure levels of fragmented NMP22 from bladder tissues with great specificity and can be created in large quantities (16). They allow measureable assessment of the forms of the mitotic apparatus in voided urine for recurrence of NMP-22 as a marker of tumor relapse. Thus, these biological reagents (monoclonal antibody) have been used extensively as probes for studying tumor markers and characterization of a wide range of tumors (17).

2. Objectives

The aim of this study was to generate a series of monoclonal antibodies with basic protocols for mouse immunization, cell fusion and selection using a truncated recombinant antigen (nmp 22).

3. Materials and Methods

3.1. Source of Immunogen

Recombinant truncated nmp22 was provided by the Tarbiat Modares university, school of medical sciences, and department of medical biotechnology. In this antigen, the 300 amino acids of N terminal was cloned and expressed in *E. coli* BL21.

3.2. Immunization

Eight-week-old female BALB/c mice were immunized with recombinant nmp22. A solution containing 15 μg of recombinant nmp22 antigen was prepared in phosphate-buffered saline (PBS: 10 mM K2HPO4, 10 mM KH2PO4, 0.15 mM NaCl, pH 7.2) and mixed with equal volumes of Freund's complete adjuvant (Sigma). The stability and quality of the emulsion was carefully checked. This solution (0.5 mL) was given by intra peritoneal injections. Second and third injections were carried out using Freund's incomplete adjuvant (Sigma) at three weekly intervals. A booster comprised of 30 μg of nmp22 antigen in 100 μL PBS without adjuvant was injected four days before fusion (18). Blood samples were collected for estimating the immune response by ELISA. Furthermore, 100 μL of recombinant antigen NMP22 (1 μg/mL in BPS) was coated in ELISA wells. The wells were washed with PBS buffer and blocking was carried out by 0.1% skim milk solution for one hour at 37°C. The content of the wells were removed and the plate was washed three times with phosphate buffered saline and 0.05% Tween-20 (PBST) buffer. In the following 100-μL mouse serum was added to the wells at 1/500, 1/1,000 and 1/2,000 dilutions. Also the normal mouse serum was added at a concentration of 1/500 to the wells as negative control. After a one-hour incubation, the plate was washed three times with PBST buffer. One hundred microliters of 1: 2000 dilution of horseradish peroxidase (HRP)-conjugated antimouse IgG antibody was added to the wells and was incubated for one hour at 37°C. The 100-μL substrate was then added and after 10 minutes the reaction was stopped with 100 μL of 2 M HCl and the absorbance was read on a micro plate reader at 450 nm.

3.3. Cell Fusion and Antibody Production

Lymphocytes of spleen cells were used for antibody source. Spleen cells were suspended in 10 mL of the serum-free RPMI and washed five times in the same buffer. SP2/0-Ag14 myeloma and spleen cells were mixed in a 50-mL conical centrifuge tube at 1: 2 ratio. Lymphocytes from the spleen cells were fused with SP2/0-Ag14 myeloma cell in the presence of 50% polyethylene glycol (PEG) (Merck). Polyethylene Glycol was added slowly over a one-minute period while gently stirred and kept without any stirring for another one minute. After centrifugation (8000 g, five minutes, 4°C), the fusion suspension was diluted by adding 4 mL of RPMI medium (Gibco) containing 15% Fetal Calf Serum (FCS), 20% Hypoxanthine-Aminopterin-Thymidine medium (HAT) and antibiotics (plasmocin, penicillin and streptomycin) drop by drop over a period of two minutes. The fusion suspension was then diluted with 40 mL of the same media at a rate of 10 mL per minute. The suspension was plated on 96-well plates that were covered with feeder cells (120 μL per well) and incubated overnight at 37°C, 5% CO_2 and 95% humidity. After eight to nine days, 50 μL of RPMI medium containing 15% FCS, 5% HAT and antibiotic (plasmocin, penicillin and streptomycin) was added to each well. After six to seven days, the wells were screened for presence of efficient antibody by using indirect ELISA (18).

3.4. Calculation of the Affinity of FPR92 Monoclonal Antibody

The supernatant of colons was prepared by culturing in RPMI medium with minimum concentration of FCS, followed by antibody purification from FPR92 clone supernatant. Protein concentration was assayed using the Bradford method. Different concentrations of recombinant antigen NMP22 (1, 0.5, 0.25 and 0.125 μg/mL in BPS) were coated in ELISA wells separately, followed by 45 minutes of blocking with 5% skimmed milk-PBS at 37°C. Furthermore, 1: 4000, 1: 2000, 1: 1000 and 1: 500 dilutions of purified antibodies of FPR92 clone were prepared in PBS and added to ELISA wells. Next, the plate was washed three times with PBST buffer and incubated with 100 μL of 1: 2000 dilution of horseradish peroxidase (HRP)-conjugated antimouse IgG antibody, and gentle shaking carried out

for one hour at 37°C. After five times of washing, 100 μL of substrate was added to the wells and after 10 minutes the reaction was stopped with 100 μL of 2 M HCl and the absorbance was read on a micro plate reader at 450 nm.

3.5. Screening of Hybridoma

The screening of hybridoma supernatant was performed by indirect ELISA. Wells of ELISA plates were coated with 100 μL of nmp22 antigen at a concentration of 1 μg/mlL in PBS buffer and incubated at 37°C overnight. The wells were washed with PBS buffer and blocking was carried out by 0.1% skim milk solution for one hour at 37°C. The contents of the wells were removed and the plate was washed three times with PBST buffer (18).

Supernatants of the hybridoma from each culture was added to the wells (100 μL per well) and incubation was done at 37°C for one hour. Washing with PBST was performed for five times, then 100 μL of proper dilution of anti-mouse antibody labeled with HRP was added to each well and incubated for one hour at 37°C. The supernatant of SP2/0 was used as a negative control. At the end of the incubation time, the wells were washed three times with PBST and 50 μL of Tetramethylbenzidine (TMB) solution was added to each well as substrate. Stopping of the reaction was performed by adding 50 μL of 1N HCl and absorbance was detected at 450 nm (19).

3.6. Cloning by Limiting Dilution

Sub cloning by limiting dilution that resulted in single cell secreting monoclonal antibodies was performed. The number of hybridoma cells was calculated until it reached four, two and one cell per well in 10% FCS-RPMI. The sizes of the produced hybridoma clones were checked and the numbers of wells including single hybridoma clone were determined 4, 6, 10 and 14 days after cultivation. The plate was incubated at 37°C in a 5% CO_2 incubator. Single hybridoma clone-producing antibody was assessed by indirect ELISA, as described in previously. Finally, hybridoma clone-producing antibodies were selected with desired affinity (18, 19).

Each result was confirmed by triplicate repeats.

3.7. Purification

Purification of MAbs was performed using protein G-Sepharose column affinity chromatography. The hybridoma supernatant was precipitated at 40% to 50% (NH4)2SO4 saturation, whereas other proteins persisted in the solution. After centrifugation, the supernatant was removed and the precipitate was dissolved in phosphate buffer (PB) and the solution was dialyzed against PB buffer overnight, with three changes. Purification was performed by protein G-based immunoaffinity chromatography in which the acidic elution buffer (0.1 M glycine-HCl pH 2.8) was added and fractions were collected from the column. The acidic condition separated the antibody from the immobilized protein G and the IgG was improved in its purified state (18).

3.8. Immunoprecipitation of the Antigen and Blotting

Native Numa (nuclear mitotic apparatus protein) was extracted by immunopercipitation of Numa from a bladder cancer cell line (HN-5 gifted by department of genetic, Tarbiat Modares university). Bladder cancerous cells were lysed with lysis buffer (containing 20 mM tris, 100 mM EDTA, 100 mM EDTA in 150 mL NaCl, triton X 100, 2.5 mM phosphate, 1 mM β glycerol, 1 mM Na3PO4, 1 μg/mL Leupeptin, Tris 187.5 mM. pH = 6.8) (20). Cell lysates were centrifuged (12000 g for 10 minutes at 4°C) to remove cell debris, and protein concentration was assessed by the Bradford method. Furthermore, 300 μL of lysate was incubated with 12 μg of G100 (Sigma) treated by anti Numa polyclonal antibody (produced against truncated antigen of Numa, gifted by TMU) for one hour at 4°C. Immunoprecipitates were washed three times with the same lysis buffer and resuspended in the sample buffer containing glycine buffer pH 2. The resulting solution was centrifuged and the supernatant was removed; the pellet was run on 15% tris-glycine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and transferred to nitrocellulose membranes for immunoblotting analysis, using the produced monoclonal antibody (21). A similar procedure was applied for cancerous tissues obtained from a bladder cancer patient. Briefly, the tissue was collected and 1 mg was weight out, treated with the mikrodismembrator apparatuses (5 minutes at 20 rpm), added to PBS (10 mM pH 7), centrifuged and measured for protein content. Finally 10 μg was used for electrophoresis.

3.9. Western Blotting

Western blot analysis was performed with a Bio-Rad apparatus (running buffer: 0.1% (w/v) sodium dodecyl sulfate (Merck, Darmstadt, Germany), 191.8 mM glycine (pH 8.2) and 5% (v/v) 2-mercaptoethanol (Merck)). For Western blotting the gel was run at room temperature at constant voltages (100 V (for 50 minutes. The protein was transferred to the nitrocellulose membrane with 0.2 pm pore size. The nitrocellulose membrane was washed with TBST (tris-buffered saline with Tween 20: 50 mM Tris, 150 mM NaCl and 0.05% Tween 20, pH 7.4) and was blocked with 5% skim milk in TBST for one hour. The membrane was washed and incubation was done overnight with 5 mL (3 ug /mL) of produced monoclonal antibody. After washing with TBST

(three times for 10 minutes), a second antibody-HRP label was added to nitrocellulose and incubated at 37°C for 45 minutes. Finally, DAB /H_2O_2 was added as the substrate (18, 22).

3.10. Determination of Antibody Class and Subclass

Antibody class determination kit (Roche) was used for isotype and light chain determination. For this purpose based on company instruction, the dilution of 1: 20 of single clone supernatant was prepared. One hundred microliters of supernatant was added to the indicator solution. After 10 minutes the results were read on the isostrip.

3.11. Statistical Methods

The data were statistically analyzed using SPSS version 16 software. Statistical significance was set at $P < 0.05$.

4. Results

4.1. Mouse Polyclonal Antibody Titer

Table 1 shows the results of the titration of polyclonal antibody against recombinant protein NMP22. As it is clear from the results, a high difference exists between the reaction for normal mouse serum and the serum of immunized mice.

4.2. The Results of Hybridoma Formation

Initially, 135 clones were obtained from the first fusion step. The optical density of antibody production was between 2.5 and 3, and some times over 3. They were selected and monitored continually for antibody production. Table 2 shows the results of the first screening.

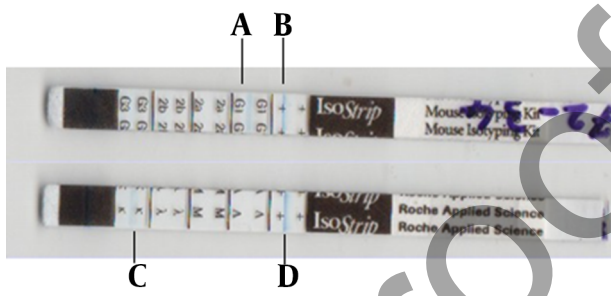
4.3. Screening the Best Clones for Further Investigation

After a few days, clones were screened with indirect ELISA using NMP 22 as the antigen. The optical densities of 15 clones was found to be appropriate for limiting dilution and their OD at 450 nm was found to be stable and their macroscopic investigation showed proper viable cells (Table 3).

4.4. Determination of the Class and Subclass and Light Chain Type of Monoclonal Antibody

Figure 1 shows the immunochromatography of the strip test for class and subclass determination of the antibody (clone FRP92). As indicated, the antibody is of type IgG2 containing kappa light chain.

Figure 1. Detection of the Class and Subclass of MAbs (FPR92) Produced in This Study



A band shows the class antibody (IgG), C band shows type of light chain (kappa), and B and D bands were the controls.

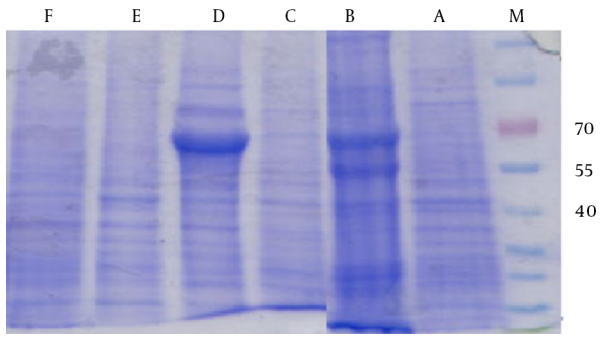
4.5. Characterization of Monoclonal Antibody

The results of cross-reactivity of different recombinant proteins with the MAb (FRP92) are shown in Table 4. The recombinant protein used in these studies was obtained from Abcam.

4.6. SDS-PAGE and Western Blotting to Determine the Molecular Weight of Antigen

To determine the molecular weight of antigen in bladder cancer tissue, SDS-PAGE (10%) and Western blotting were performed. The results are shown in Figure 2. Bands of approximately 40 - 50 KD for antigen were obtained. The results of western blot analysis of cell line lysate (MCF-7, HpG2, MDA-MB-468, HN5, and A431) and tumor tissue bladder were also shown.

Figure 2. SDS-PAS of Tissue and Cell Lysates



M: MW marker, B : tumoral bladder tissue, C: HN5, D: HpG2, E: MDA-MB-468, F: MCF-7.

4.7. Calculating the Affinity of FPR92

The concentration of purified antibody was measured by the Bradford method (555 ug/mL equivalent to 333 nM).

Table 1. Mouse Polyclonal Antibody Titer After Injection With the Recombinant Protein NMP22

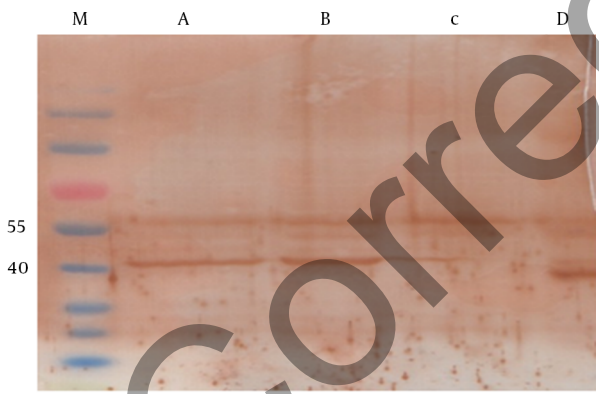
Mice Number	Serum Dilution			
	1/500	1/1000	1/2000	Normal Mouse Serum 1/500
1-(OD450 nm)	3.5	3.5	3.2	1.1
2-(OD 450 nm)	3.5	3.5	3.5	1.1

Table 2. Classification of Obtained Clones, Based on the Reaction With the Recombinant Antigen

Absorption	The Number of Clones
High absorption > 3 $OD_{450\ nm}$	25
Absorbing between 2.5 to 3 $OD_{450\ nm}$	47
Absorbing between 2 to 2. 5 OD_{450nm}	73
SUM	135

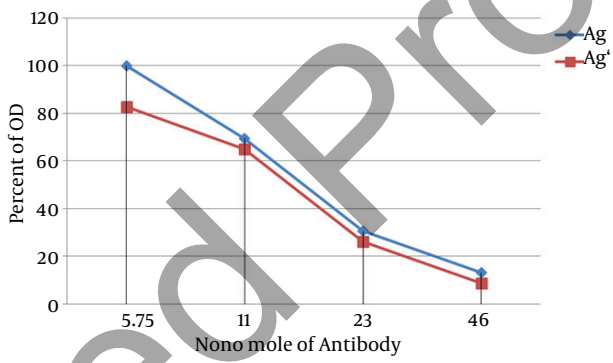
Different concentrations of the antigen was coated and reacted with different concentrations of the antibodies. The results are summarized in Figure 3. The affinity was calculated as 3 × 10-7 M-1 for the clone named FRP92.

Figure 3. Western Blot Analysis



M: weight marker, A: MCF-7, B: MDA-MB-468, C: HN5, D: bladder tumor tissue (detected with the monoclonal Ab produced in this study). Note the bands at the region of 40 and 55 KDs. Ab concentration : [10^{-7} M^{-1}]

Figure 4. Determination of Affinity of Antibody



Calculated to be 3 × 10^{-7} M^{-1}

5. Discussion

Nmp22 is a member of the intermediate filament proteins families. This protein is released in different cells when apoptosis is taking place. Monoclonal antibodies produced against nmp22, can be used in diagnostic immunohistochemistry, in detection of carcinoma of the bladder and bladder carcinoma metastasis (23, 24). The described experiment using the standard hybridoma technique (25), for specific MAbs production and detection of proteins of interest can be easily reproduced. However, it is not always straight-forward to obtain MAbs that have all respected properties. In this study, a nmp22 recombinant protein was injected to balb/c mouse, while our attempt to use bladder tissue to raise the antibody failed (26). A number of researchers have used tissue for generation of antibody against the respective antigen (27), however for the reasons not clear for us it was not possible to use these antigens in case of nmp22 antigen. Four weeks after the first injection with the recombinant protein, we obtained a high titer polyclonal antibody. Only two weeks after the first fusion, 135 clones were obtained; their optical densities were between 2.5 to 3.5, measured using a direct ELISA system. However, only 15 healthy clones remained after the first limiting dilution. These clones were selected for a second limiting dilution. At the end, we only selected eight clones with stable optical densities of over 3. One of the clones named FPR92 was selected and further characterized. To detect the class and subclass of monoclonal antibodies produced by the clone FRP92, antibody isostrip kits were used showing that the antibody was of IgG2a κ type. For antibody purification we performed ammonium sulfate precipitation. After dialysis of the product, further purification was done by using G protein Sepharose column chro-

Table 3. Absorption of Fifteen Selected Clones that Reacted With the Recombinant Protein nmp22

Clone Number	OD450 nm	Clone Number	OD450 nm	Clone Number	OD450 nm
123	2.8	98	2.3	77	1.9
122	2.9	90	2.7	56	2.4
120	3	89	3	44	3
100	2	79	2	33	2.3
99	2.8	78	2.4	22	3

Table 4. Optical Densities, When the Antibody Reacted With Different Recombinant Antigens at the Same Concentration (1 ug /mL)

Coated Protein	Nmp22	P2	CD147	Ck19	P6	BSA	P5
OD450 nm	3.5	0.2	0.4	0.26	0.2	0.2	0.2

matography. The concentration of antibody purified in this manner was found to be 550 micrograms per milliliter. In order to detect the reactivity of the antibody with the antigen of interest obtained from tissue, we performed an immune-precipitation experiment where the tissue extract reacted with the polyclonal antibody. The product purified by centrifugation was separated from antibody conjugated sepharose using glycine buffer, centrifuged, electrophoresed and blotted using the monoclonal antibody produced in this study (FRP92) as the detector antibody. Bands at the region of 40 KD - 55 KD in both bladder tissue extract as well as some cell line extracts were observed. It has been shown by other researches (9), that other proteins of the same filamentous protein subfamily (such as cytokeratin, bcla and KI67) exhibit a molecular weight of 40 - 50 KD and sometimes a homology of more than 70% is found in between these proteins and the protein nmp22. In some other studies it has already been reported that nmp22 was degraded during cell death rising to many fractions in which the 40 KD protein is predominant (9). Our experience with this protein (nmp22) showed that even freeze towing will degrade and produce various small fragments. The cross-reactivity experiment of FPR92 with various antigens, including BSA, CK antigen (CK 19) and recombinant antigens, such as P5 of muc1, P6 (related proteins in skeletal intermediate), p8 (EFGR of cell surface antigens), P6 and CD147 was performed showing minimal cross reactions with the antibody as compared to antigen nmp 22. Furthermore in the present study, the antibody was reacted with bladder tumor tissue extracts and cell lines such as MCF7, MDA-MB-468, and HepG2 extracts in Western blot analysis. The results of Western blotting indicated the presence of two distinct bands at molecular weights of 40 and 55 KD, respectively, which has been reported by other researches (9).

Eight out of 15 antibodies showed significant binding towards the recombinant protein (nmp22) with optical density ranging from 2.5 to 3.5 in indirect ELISA. The affinity of antibodies towards nmp22 was calculated to be 3×107. Compared with other antibodies reported by other workers in our lab, such as antibodies produced against MUC1 (3×108) (18), melatonin (109) (19) and antibodies produced against lung cancer tissue by others (7×107) is comparable. In conclusion, the newly generated antibody reacted with truncated recombinant Numa and with the Numa expressing bladder cell lines and bladder cancer tissue extract.

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Footnotes

Conflict of Interests: None to declare.

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