

Immunodiagnosis for cervical cancer using antibody-gold nanoparticle conjugate

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ABSTRACT

Immunodiagnosis is an attractive choice for quick and reliable cancer screening. p16^{INK4A} (p16) is a promising biomarkers for cervical cancer. The over-expression of p16 is linked to the oncogenic transformation. Here, monoclonal antibodies (mAbs) specific to p16 were produced by using hybridoma technology for immunodiagnostic purposes. The p16 was expressed in *E.coli* and purified by affinity chromatography. The protein was used for mouse immunization. Hybridomas producing antibodies to p16 were selected by ELISA. The mAbs were purified by protein A and G columns. The specificity and sensitivity of the antibodies were determined by Western blotting and ELISA. The selected anti-p16 mAb with highest sensitivity was conjugated to gold nanoparticles (AuNPs) by non-covalent adsorption. Immunochromatographic (IC) tests by using conjugated anti-p16 mAb and purified p16 were successfully performed for the first time. This method might be further used for early detection of cervical cancer.

Keywords: cervical cancer, immunodiagnosis, monoclonal antibody, p16^{INK4A}

1 INTRODUCTION

Cervical cancer is the second most lethal cancer among women worldwide. Approximately 500,000 new cases and 250,000 deaths per year have been reported. More than 85% of these cases are in the developing countries [1]. A major cause of cervical cancer is Human Papillomavirus (HPV) infection. HPV is a small nonenveloped icosahedral DNA virus which has over 100 genotypes. The high-risk HPVs such as HPV-16 or HPV-18 can cause preneoplastic lesions of cervical intraepithelial neoplasia [2]. The incidence and mortality of cervical cancer can be reduced by practical cancer screening. There are several methods to screen samples for cervical cancer but the most acceptable and widely available one is Papanicolaou test (Pap test) determining abnormal cervical cells. However, the limitation of the Pap test is high false negative rate, because this method needs well-trained pathologist for preparing and analyzing sample slides. Moreover, it usually takes long time to report results.

Immunodiagnosis could provide an alternative for cervical cancer screening. It is a quick and reliable method.

Several biomarkers for cervical cancer have been proposed. p16, a tumor suppressor protein, is a promising biomarker for early diagnosis of squamous cell carcinoma of the cervix [3]. The over-expression of p16 is linked to the oncogenic transformation caused by persistent HPV infection [4]. Also, the level of p16 expression correlates to the stages of abnormal growth of squamous cells on the surface of the cervix [5]. Here, high-affinity monoclonal antibodies against p16 were produced by using hybridoma technology for development of immunochromatographic tests for cervical screening.

2 MATERIALS AND METHODS

2.1 Cloning, Expression and Purification of p16^{INK4A} Protein

The *p16* gene was inserted into *NcoI* and *NotI* sites of pET26b(+) expression vector, fused with six histidine (6His) in this plasmid. The produced plasmid was named pET26-p16. The pET26-p16 plasmid was transformed into *E. coli* Rosetta (DE3) cells (Novagen, USA) and fusion protein expression was induced by isopropyl β -D-thiogalactoside (IPTG) in the presence of tetracycline and kanamycin.

After cells were harvested, they were broken by sonication in lysis buffer (phosphate buffered saline (PBS, pH 7.2), 1 mg/ml lysozyme, and 1 mM phenylmethanesulfonyl fluoride (PMSF)). The lysate was centrifuged for 30 min at 12,000 rpm at 4 °C, and the pellet containing inclusion bodies of the recombinant protein was washed twice with lysis buffer as above. The washed inclusion bodies were stirred in solubilization buffer (50mM Tris-Cl pH8.0, 8M urea, 2mM DTT, and 1mM PMSF) for 4 h at room temperature. The solubilized protein was purified using the HisTrap FF column according to the instruction manual of GE Healthcare.

2.2 Immunization of Mice

To produce monoclonal antibodies against p16, the p16-6His fusion protein was used as an antigen for mouse immunization. Each of four BALB/c mice was immunized intraperitoneally with 50 μ g of the antigen emulsified in complete Freund's adjuvant. Subsequent immunization at 4 week intervals were carried out twice with 50 μ g of the

antigen in Freund's incomplete adjuvant. Mice were bled 10 days after the third injection and the serum was tested by indirect ELISA for reactivity to p16. The mice with high antiserum titer were given a final booster immunization 3 days prior to the cell fusion.

2.3 Fusion of Spleen Cells from Immunized Mice and Myeloma Cells

Spleen cells from immunized mice were mixed with myeloma cells. The cell mixture was washed with RPMI-1640 medium and the pellet was collected by centrifugation at 300g for 5 min at room temperature. The spleen and hybridoma cells were fused by the addition of warm 50% polyethyleneglycol (PEG-3000) with gentle stirring for 1 min. After that, 20 ml of warm RPMI-1640 medium was slowly added to the fused cells. The cell suspension was pelleted and resuspended in warm HAT medium to make a cell concentration of $2-4.5 \times 10^5$ cells/ml. The cell suspension was then distributed into each well of 96-well microculture plates. The plates were incubated at 37°C, 5% CO₂ for 7-10 days. After that, hybridoma supernatant was screened for the presence of antibodies against p16. The positive clones were diluted to give approximately one cell per well by a limiting dilution method. Each of the established hybridoma cells producing the antibody were grown in medium supplemented with HAT. Large quantities of antibodies were prepared from cultured supernatants of hybridomas by membrane filtration, ammonium sulfate precipitation, finally purifying using protein A and G columns.

2.4 Indirect ELISA

Immunoplates were coated with 0.1 µg/ml of p16-6His protein and incubated at 4 °C overnight. Plates were washed with PBS containing 0.05% Tween20 (PBST) and blocked with 2% skim milk in PBST for 1 h at 37°C. After washing, diluted serum, individual culture supernatants or purified mAbs were added and plates were incubated at 37°C for 30 min. Plates were washed with PBST and then horseradish peroxidase (HRP) conjugated anti-mouse IgG antibody diluted 1:5,000 with PBST was added and incubated at 37°C for 30 min. After washing, TMB substrate was added and incubated in the dark at room temperature for 30 min. The reaction was stopped with 0.6 M H₂SO₄ and the absorbance at 450 nm was measured by a microplate reader (Bio-tek instrument, USA).

2.5 Measurement of monoclonal antibody affinity

The affinity of the selected monoclonal antibody was determined by using the method developed by Beatty *et al* [6]. Different concentrations of p16-6His recombinant protein were coated on immunoplates. Indirect ELISA using serial dilutions of monoclonal antibodies was

performed. The affinity constant (K_{aff}) of the antibody was measured using the equation: $K_{aff} = (n-1)/2(n[Ab']t-[Ab]t)$, where n is $[Ag]/[Ag']$, $[Ag]$ and $[Ag']$ are antigen concentrations, $[Ab']t$ and $[Ab]t$ are antibody concentrations at the half maximum OD (OD-50) of plates coated with $[Ag']$ and $[Ag]$, respectively.

2.6 Western Blotting

The specificity of selected monoclonal antibody was investigated by Western blotting method. The p16-6His protein was solubilized in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and subjected to SDS-PAGE with a 12% polyacrylamide gel. Alkaline phosphatase (AP) -conjugated anti-mouse IgG (KPL, USA) was used for the secondary antibody. The blot was visualized using the AP development solution (KPL, USA).

2.7 Preparation of the AuNP Conjugate

To prepare the AuNP conjugate, anti-p16 mAb was added to the mixture of AuNP colloid and borate buffer. After incubation for 30 min at room temperature, bovine serum albumin (BSA) was added to the solution to block the AuNP surface. After incubation for 15 min at room temperature, the mixture was centrifuged at 10,000rpm and 4 °C for 30 min. The supernatant was discarded and the pellet was washed twice with IC buffer (Tris-HCl, pH 9.0 and 1% BSA). The final suspension solution was IC buffer.

2.8 Preparation of IC Test Strip

The IC test strip is comprised of a sample pad, nitrocellulose (NC) membrane and an absorbent pad. To prepare test dots, purified p16-6His protein was dotted onto NC membranes and allowed to dry at room temperature. To prepare control lines, anti-mouse IgG (KPL, USA) was dispensed onto the membrane and allowed to dry. The nitrocellulose membranes and pads were assembled with filter papers as sample application pads and absorption pads on a plate within a plastic housing. Here, AuNP-anti-p16 mAb in IC buffer was loaded onto the sample pad. BSA was used as a negative control on the NC membranes.

3 RESULTS AND DISCUSSION

To produce monoclonal antibodies against p16, the *p16* gene was cloned into the pET26b(+) expression vector. The p16 protein was expressed as a fusion protein with 6 histidine tagged by IPTG induction. The fusion protein was mostly expressed in the form of inclusion bodies at high level. The inclusion bodies were solubilized and purified by Ni²⁺ affinity chromatography. Approximately 2 mg of purified p16-6His per litre of cell culture were obtained with purity >90% (data not shown).

Clone No.	OD ₄₅₀
1	1.29
2	2.59
3	2.08
4	0.99
5	2.28
6	1.39
7	2.20

Table 1: The binding activity of anti-p16 mAbs by indirect ELISA.

The purified p16-6His protein was used as an immunogen for the production of mouse monoclonal antibody by hybridoma technology. Seven clones were subcloned for three cycles by limiting dilution to obtained 1 cell per well. Large quantities of antibodies were prepared from cultured supernatants of hybridomas by membrane filtration, ammonium sulfate precipitation, finally purifying using protein A and G columns. Purified mAbs were tested for reactivity to p16-6His protein by indirect ELISA (Table 1). Finally, mAb clone 2 was chosen for further mAb characterization because of its highest sensitivity among the seven obtained. To ensure the specificity of the selected mAb to p16 protein, Western blotting was performed and GST-p16 protein (S. Tapaneeyakorn, unpublished data) was used to examine cross reactivity of the mAb to 6His. The antibody reacted specifically with a single band that co-migrates with purified p16-6His (Figure 1A) or GST-p16 (Figure 1B). Moreover, it exhibited no cross reactivity with the 6His fragment.

The affinity of the chosen antibody was determined by indirect ELISA using serial dilutions of monoclonal antibody and purified p16-6His. The affinity constant of

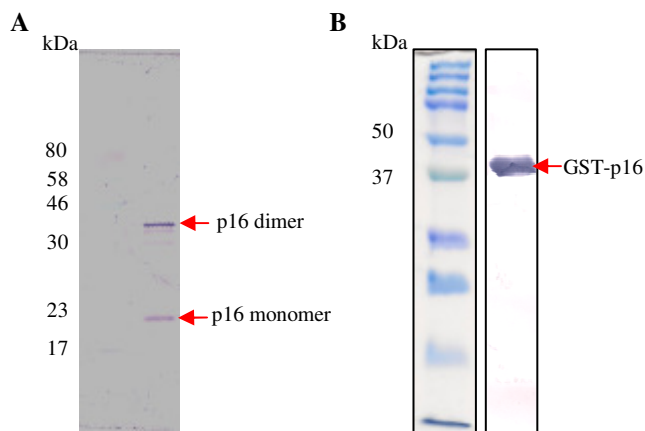


Figure 1: Western Blot analysis of p16-6His and GST-p16 with anti-p16 mAb.

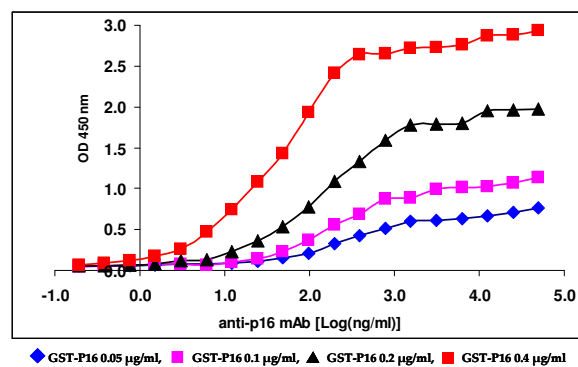


Figure 2: Affinity constant of anti-p16 mAb.

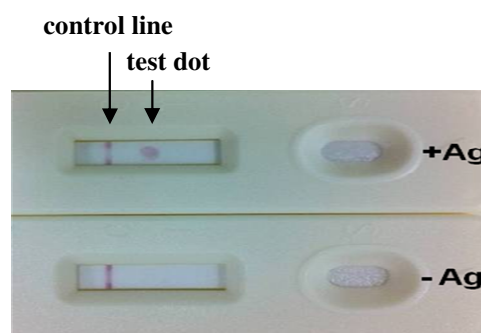


Figure 3: Immunochromatographic tests by using gold nanoparticles conjugated to anti-p16 mAb as a detector and purified p16 as an antigen.

the antibody was found to be $0.56 \times 10^9 \text{ M}^{-1}$, indicating a high affinity binding of antigen-antibody complex and the antibody can detect the antigen at the concentration lower than $0.05 \mu\text{g/ml}$ (Figure 2).

The selected anti-p16 mAb was conjugated to AuNPs by non-covalent electrostatic van der Waals' forces. IC tests by using conjugated anti-p16 mAb and purified p16 were successfully performed (Figure 3). This method might be further used for early detection of cervical cancer.

4 CONCLUSION

The monoclonal antibody with high affinity and specificity for p16 protein from this study can be used for immunodiagnosis of cervical cancer.

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