GENERATION OF MONOCLONAL ANTIBODY AGAINST HUMAN ANDROGEN RECEPTOR WITH SYNTHETIC PEPTIDE

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ABSTRACT

Objective: Preparation of anti-human androgen receptor(hAR) monoclonal antibody (McAb). Methods: Four cells lines of hybridoma secreting specific monoclonal antibodies against AR were first established by fusion SP2/0 cell with spleen cell from BALB/c mice immunized with the coupling complex of hAR-KLH. Results: Paraffin-embedded sections of 45 prostate cancers were detected. There was an overall concordance of 91% using Immunohistochemistry between AR polyclonal antibody from Zymed and hAR-N McAb selfmade. Conclusion: The results show that the McAb obtained in this study would be a useful tool to detect the AR status in prostate cancer.

Key words: Prostate cancer, Monoclonal antibody, Androgen receptor, Immunohistochemistry

Prostate cancer is one of the common malignant tumor in man. The incidence of the disease has been growing in the past few years.[1] The metastasis of patients is the chief cause of death.[2] The level of AR expression in prostate cancer is an important index to estimate its prognosis and provide scientific basis for make clinical cure scheme. Sometimes, the relation between AR and other tumor cause people's attention.[3-4] At present, the method of convention test AR is DCC and IHC method at home and abroad. DCC method require amount tissue, exist isotope pollution, specific equidded, restrict its application and extend.[5] IHC method can overcome above-mentioned shortcoming operate simple and easy, specific effective easy extend. But we need reagent anti-hAR McAb is not found at home. It is most costly AR polyclonal antibody from Zymed restrict development of scientific research and popularize of clinical test. For reach replace abroad reagent, we studied anti-hAR McAb and compared with AR polyclonal antibody from abroad. The result showed that made-oneself AR McAb can applicated for clinical test.

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MATERIALS AND METHODS

Peptide Synthesis, Animat and Agent

The peptide (hAR-N AA301-320) was synthesized by professor Lu Yong-jun at center of Life Science Beijing University. Female BALB/c mice six-week-old purchased from Beijing medical University for immunize and ascites production. hAR polyclonal antibody, Biotin-anti-mouse IgG, Strepavdin/ horseradish peroxidase (Zymed Co).

Coupling Methods

All peptides were coupled to immunogenic carrier protein to enhance antigen presentation (*in vivo*) or to be used in screening.

Coupling hAR-N and Keyhole Limpet hemocyanin (KLH): The KLH was dissolved in 0.1M PBS pH6.0 at a concentration of 10 mg/ml^{-1} and stirring; The m-Maleimidobenzonyl-N-hydro-xysuccinim ide (MBS) was dissolved in dimethylformamide at a concentration of 30 mg/ml^{-1}. The MBS was added in KLH. After stirring, the mixture was purified with gel filtration column (SephadexG-25) to remove excess MBS. The KLH-MBS mixed with synthetic peptide 5 mg was stirred for 3 hours at 4℃. The diluted hAR-N with a concentration of 1 mg/ml^{-1} stored in -20℃.

Conjugates of peptides and protein for screening were made using bovine serum albumin (BSA) and for immunization purposes using KLH. The conjugates were produced with the aid of various coupling agents (MBS<GA) and designated as KLH-MBS-SPxx or BSA-GA-Spxx.

Preparation of Anti-hAR-N McAb

The peptide-KLH were emulsified with equal complete Freund's adjuvant and injected into 6-week-old female BALB/c mice subcutaneously by 25μg for each. The mice were boosted very 2 weeks with the same dose of conjugate and adjuvant for four times. The first time is complete Freund's adjuvant, the rests are incomplete Freund's. The final one was boosted via the tail vein. A spleen cell suspension was prepared 4 days after the last boost. Spleen cells and SP2/0 cells in logarithmic growth were fused at a ratio of 5:1 in 40% polyethylene glycol 4000. The fused cells (10^5/well in 0.2 ml) were cultured in Dulbecco's Modified Essential Medium (DMEM-H) containing 1 $\mu g/ml^{-1}$ Azaserine and 0.1mM hypoxanthine, 15% fetal calf serum (Fcs), 2mM glutamine, 0.1 mg/ml^{-1} streptomycin $100E/ml^{-1}$ penicillin, 1mM sodium pyruvate, and 5.10^{-5}M β-mercaptoethanol. After 1 week of culture, the azaserine was discontinued and the Fcs concentration was redued to 10%. Selected cell cultures were subcloned by limiting dilution at a density of 5 cells/well to obtain specific hAR-N McAb.

Anti-hAR-N McAb Screening

Indirect ELISA method:

PVC-microtiter plates were coated overnight at 4 ℃ with 50 μl PBS containing 1 $\mu g/ml^{-1}$ protein, peptide conjugated to BSA with a reagent not used in the immunogen. Plates were sealed with 1% gelatin in PBS for 30 minutes incubation at room temperature. The hybridoma supernatant were added with 50μl for 1 hour incubation at 37℃. The plates were washed three times with PBS containing 0.05% Tween-20; The Horseradish peroxides (HRP) conjugated to rabbit-anti-mouse IgG (diluted 1: 1000) 50 μl/well were used for 40 min incubation at room temperature, then washed 6 times again; 3,3',5,5'-Triphenylmethy-lene Benzedrine (TMB) was used as substrate. After 30 min the absorbance was read at OD450nm against normal mouse serum response as a blank.

Immunohistochemistry

Paraffin-embedded section of prostate tissue with glandular hyperplasia were dewaxed and dewatered with graduated ethanol. The section were incubated with 0.3%H_2O_2-methyl alcohol for 30 min; They were sealed with 1% gelatin in PBS for 30 minutes incubation at room temperature; The slides were incubated overnight with appropriate dilutions of hybridoma supernatant in PBS (pH7.8) at 4℃. After several washing steps, the reactivity was visualized using strepavdin/horseradish peroxidase conjugated to horse anti-mouse immunoglobulin (Zymed Co) diluted 1:500 in PBS. Diaminobenzidine and hydrogen peroxide were used as substrate. Control slides were incubated with pre-immune sera or PBS.

The Purity of the McAb

They were purified by PEG immunoprecipitation.

Determination of the Subclass of McAb

Double agar diffusion was used.

Sauiquantitative Assay

The immunohistochemical staining slides samples were analyzed by HI-Ci real color image (Beijing aeronautical engineering Institute) system. The positive cells contain brown particles. AR is predominantly located in the nuclei of the immunopositive cell stained by hAR-N McAb. The assay include (1) the content of positive matter, (2) the average grey degree of positive matter, (3) the average light density of positive matter, (4) the integral light density of positive matter. The product of stained grey degree and content of positive matter is the Immunoreactive Score(IRS). On the basis of statistical need, averge value of IRS is divided into five grades: 0 is grade 1(−); 1-5 is grade 2(±); 6–10 is grade 3(+); 11–20 is grade 4(++); >20 is grade 5(+++)

Table 1. The positivity, density and IRS ($\bar{x}\pm s$) of AR expression in prostate cancer

Antibodies	Antigen repair	Prostate cancer							
		N	−	±	+	++	+++	positivity	IRS ($\bar{x}\pm s$)
AR polyclonal antibody from Zymed	+	45	4	1	12	20	8	89%	11.716±8.662
hAR-N McAb self made	−	45	3	0	11	21	10	93%	12.865±6.361
hAR-N McAb self made	+	45	3	1	12	21	8	91%	11.816±5.66

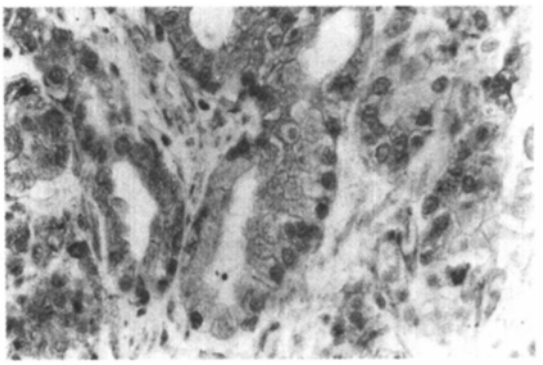

Fig. 1. Immunohistochemistry of self-made anti-hAR-N McAb(Prostate cancer) SP method×400

RESULTS

After fusion and subcloning, the hybridoma cells of anti-hAR McAb were obtained four cell lines of hybridoma secreting specific monoclonal antibody against AR (55, 141, 207, 257) were derived from different wells in the original 96-well fusion plates. The titre of ascites fluid of 55, 141 were $1:10^5$; and 207, 257 were $1:10^4$.

Characterization of hAR Specific Monoclonal Antibodies

The immunoglobulin subclass of four McAbs were identified as IgM by immunoprecipitation assays. Specificity for the human androgen receptor was determined by the cross-reactive response to other antigen (PR, BSA, HSA) in immuno-precipitation assays. The four McAbs showed a positive response with the hAR only.

ImmunohistoChemical Analysis

Prominent nuclear staining of AR was seen in prostatic cancers cells. Paraffin-embedded sections of 45 prostate cancers were analysed. There was an overall concordance of 91% using IHC between AR polyclonal antibody from Zymed and our hAR-N McAb in this study ($r=0.86$, $P<0.0001$). There was an overall concordance of 92% using IHC between antigen repair and nonrepair ($r=0.85$, $P<0.0001$).

DISCUSSION

In this study, a synthetic peptide (SP) containing 20 aminoacids (AA301-320) situated in the N-terminal part of the hAR was used. It was highly hydrophilic. There was no homology with other steroid receptors. hAR-N McAb self made was highly specific. Paraffin-embedded sections of 45 prostate cancer were examined. There was an overall concordance of 91% using IHC between AR polyclonal antibody from Zymed and hAR-N McAbs in our work. The results show that the McAbs are widely applicable in histochemical techniques and that they do not crossreact with other steroid receptor. These properties demonstrated that they are useful as a diagnostic tool. In previous studies, there are many methods to determine steroid receptors for example DCC and IHC, DCC needs specific equipment. It costs more need more amount of tissue and exists isotope contamination. It could not accurately show the location and function of receptor. However, the simple, highly sensitive and specific AR McAb immunohistochemistry can easily overcome the shortcoming above-mentioned.

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