

PSMA mimotope isolated from phage displayed peptide library can induce PSMA specific immune response

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ABSTRACT

Prostate-specific membrane antigen (PSMA) is a cell surface glycoprotein expressed predominantly in prostate secretory acinar epithelium and prostate cancer cells as well as in several extraprostatic tissues. Mouse monoclonal antibody 4G5 specific to the extracellular domain of PSMA was used to screen two phage displayed peptide libraries (9aa linear and 9aa cys library). Three 4G5-reactive phagotopes were identified. Sequence analysis of isolated clones demonstrated that the interaction motif "VDPA/SK" has high homology to 719-725aa on PSMA. Immunohistochemical staining of the prostate cancer sample with the PSMA-mimic phagotope (mimotope) immunized serum antibodies demonstrate that the mimotope isolated from the phage displayed peptide libraries can induce PSMA specific immune response in vivo.

Key words: *PSMA, mimotope, phage displayed peptide library, immunohistochemistry.*

INTRODUCTION

Prostate carcinoma is one of the most common cancer in men[1],[2]. The death rate from prostate carcinoma is also increasing in China. To improve this situation, new

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markers are needed for early and accurate staging and treatment as well as better means to follow disease progress. One such potential marker is prostate specific membrane antigen (PSMA).

PSMA is a type II membrane glycoprotein of Mr ~ 100,000 with folate hydrolase-type and neuropeptidase-type activity[3-5]. PSMA is highly expressed in benign prostate secretory acinar epithelium, prostatic intraepithelial neoplasia and prostatic adenocarcinoma, and evidence suggests that PSMA expression is greatest in high-grade and hormone insensitive cancers. Given its membrane bound character, PSMA has been exploited as a marker for tumor detection and treatment with radioactive isotope or toxin labeled PSMA-specific antibodies. Especially, recent studies demonstrate that the monoclonal antibodies specific for the extracellular domain of PSMA are able to induce the internalization of PSMA[6]. Moreover, PSMA is also consistently expressed in the neovasculature of a wide variety of malignant neoplasms and maybe it is an effective target for Ab-based antineovasculature therapy[7].

With the development of phage displayed peptide library technology, it has been successfully applied to the epitope study and other relevant fields[8-10]. In this report, we employed the phage display peptide library to study the epitope recognized by mAb 4G5 and its immunogenicity to induce PSMA specific antibodies.

MATERIALS AND METHODS

mAb

Prostate membrane specific antigen specific monoclonal antibody 4G5 was from YES Biotech Laboratories Ltd, Canada. The antibody was generated from immunization with crude membrane extract of prostate carcinoma and screened out with synthesized 8-peptide ("ESKVDPSK" derived from the 716-723aa of PSMA sequence).

Phage peptide libraries and bacteria strain

Random phage peptide libraries expressing linear (pVIII 9aa)[11] or circular (pVIII 9aa Cys) [12] nonapeptides fused to pVIII of filamentous bacteriophage fd, wild type phage f1 and bacterial strain DH5aF' were all kindly provided by Dr. Paolo Monaci of IRBM (Istituto di Ricerche di Biologia Molecolare P. Angeletti SPA, Rome, Italy).

Affinity selection

Two rounds of affinity selection were performed as following, 200 μ l mAb 4G5 in coating buffer (50 mM NaHCO₃, pH 9.6) at 2 μ g/ml was incubated in 96-well plate (Immuno plate Maxisorp, Nunc) at 4°C overnight, then blocked with blocking buffer (1 \times PBS, 3% BSA, 0.05% Tween) at 37°C for 1.5 h. 10¹¹ wild type phage f1 in 200 μ l blocking buffer were added to the well and incubated at room temperature for 1 h. The plate was washed extensively with PBST (1 \times PBS, 0.1% Tween), then about 1 \times 10¹⁰ phages from either 9aa linear or 9aa cys circular peptide library in 200 μ l blocking buffer were added to the well and incubated at room temperature for 2 h. After washing, the absorbed phages were eluted and neutralized with Tris-HCL as described[13]. The eluted phages were amplified by infecting DH5aF' and purified with 20% PEG/2.5 M NaCl precipitation. It can also be stored for immunoscreening. The purified phages were used for next round of affinity selection.

Immunoscreening

After two rounds of affinity selection, DH5aF' cells were infected with eluted phage at a multiplicity of infection (m.o.i.) of 10^{-3} , the mixture was incubated at 37°C for 30 min, then helper phage M13 K07 was added at a m.o.i. of 20-50, and incubated at 37°C for another 15 min. The infected bacteria were centrifuged for 5 min at 3000 g, the supernatant was discarded and bacteria pellet was washed three times in 1ml of LB medium to eliminate the non-absorbed phages. 100μ l of the resuspended pellet with series of dilution (10^{-3} , 10^{-4} , 10^{-5}) were plated on plates containing Ampicillin (100μ g/ml), Kanamycin (50μ g/ml) and IPTG (30μ g/ml). After incubation at 37°C overnight, the plates containing 200-400 separated clones (10 cm plate) were layered with nitrocellulose filters and marked with needle. The filter was took out immediately and blocked with blocking buffer (5% non-fat milk power, $1 \times$ PBS, 0.05% Tween-20, 0.05% N_aN_3) at room temperature for two h. 10μ g mAb preincubated with 25μ l bacterial extract (prepared as described in Ref 13) and 25μ l f1 Phage (2.3×10^{13} /ml) in 5 ml blocking buffer at room temperature for 1 h, were then added to the blocked filters. Filter with mAb mixture was then incubated at room temperature for 1.5 h and then extensively washed with washing buffer ($1 \times$ PBS, 0.05% Tween-20). Then secondary antibody (alkaline phosphatase conjugated Goat anti-mouse Abs, Sigma) diluted to 1/5000 in blocking buffer was incubated with the filter for 1.5 h at room temperature. The filters were then washed as above and developed by incubation with developing solution (330 μ g/ml nitro blue tetrazolium, 165 μ g/ml 5-bromo-4-chloro-3-indolephosphate in 100 mM Tris HCl, 100 mM NaCl, 5mM $MgCl_2$, pH 9.6) at room temperature for 10 min. Reaction was stopped by washing with water.

ELISA

In brief, multi-well plates (Immuno plate Maxisorp, Nunc) were coated overnight at 4°C with the mAb 4G5 at a concentration of 2μ g/ml in 50 mM $NaHCO_3$ pH 9.6. After washing several times with PBS/0.05% Tween-20 (PBST), plates were incubated at 37°C for 60 min with ELISA blocking buffer (5% non-fat dry milk in PBST). 4×10^9 sample phages were diluted in 100μ l blocking buffer and then added to each well and allowed to bind for 1 h at 37°C. The equal amount of wild type phage was used as a negative control. Plates were then washed with PBST and 100μ l/well of goat anti-mouse IgG HRP conjugated antibodies (Sigma, 1/5000 dilution in ELISA blocking buffer) were added. After incubation for 1 h at room temperature, plates were then washed and developed by adding 100μ l substrate TMB buffer and incubated at 37°C in dark for 15 min. Optical density was measured in an ELISA reader at 450 nm.

Competitive ELISA

96-well ELISA plates were coated with 1×10^9 phage/well of f1 or three phage clones. Fixed amount of immune serum (1:2700 dilution) is incubated in each well with increasing amount of competitor f1 (from 0 to 1.5×10^9). After extensively washing, the HRP conjugated secondary antibody (goat anti-mouse IgG) was added. Following development, the optical density value was read at 450 nm.

DNA sequencing

Single stranded DNA of the positive clones was extracted from the amplified phages using DNA purification kit according to the instructions (Promega). Sequencing was performed by the Sanger dideoxy method with T7 sequencing kit (Pharmacia) according to the instructions.

Immunization of mice with selected phage

The identified positive phage clones were amplified in DH5aF' and purified by PEG/NaCl precipitation. Then the phages were resuspended in $1 \times$ PBS at a concentration of 5×10^{12} phage particles/ml. Ten to twelve-week old male C57BL/6 mice were immunized by i. p. 250μ l of positive phage clones emulsion (1:1 with CFA for the primary injection, IFA for the boost injections),

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using wild type phage f1 as control. The immunization was performed at week 0, 4 and 7 and bled at days 10 after second and third injection.

Absorbing immunized serum with wild type phage f1

The immune serum was absorbed with wild type f1. 20 ml serum was diluted 1 ml and mixed with 1 ml PBS containing 1×10^{13} f1 phage particles. Then the mixture was centrifuged at 200,000 g for 2 h, The supernatant was collected and used in immunohistochemical staining at 1:200 dilution.

Immunohistochemical staining

All the slides of prostate cancer, lung cancer, stomach cancer and colon cancer were prepared routinely. Endogenous peroxidase activity was quenched by 30 min incubation in 3% hydrogen peroxide. After washing, the slides were blocked with 5% normal goat serum for 30 min. The f1-absorbed mimotope immunized serum diluted in 1:200 were added to the slides as first layer antibody and incubated at 4 °C overnight. Immunized serum with wild type phage f1 was used as negative control. After washing extensively with $1 \times$ PBS 0.05% Tween-20, HRP conjugated goat anti-mouse IgG was added and incubated at 37 °C for 1 h. After washing with PBS tween, the slides were developed by adding the substrate DAB and H_2O_2 according to the manufacture' s instructions. The reaction was stopped by washing with water. Slides were counterstained lightly with Harris hematoxylin, dehydrated through a graded series of ethanol to xylene and coverslipped with permount.

RESULTS

Affinity selection

Two rounds of affinity selection were performed before the immunoscreening. After each round of affinity selection, the percentage of the blue clones was calculated from every eluted pool of phage, and the titer of the phage was counted (Tab 1). From both libraries, the increase of the phage titer and percentage of blue clones were observed, indicating the enrichment of specific ligand during the affinity selection. The increase of phage titers was in parallel with an increase of phages binding to 4G5 in ELISA. And low binding was observed with equal amounts of wild-type phage f1 (Fig 1).

Tab 1. Eluted phage titer after affinity selection with mAb 4G5.

Phage library	Affinity selection			
	Round 1		Round 2	
	Percentage of blue clones ¹	Titer	Percentage of blue clones	Titer
9aa library	45%	1.4×10^4	83%	2.3×10^6
9aa cys library	42%	8.4×10^3	76%	6.7×10^5

1. Percentage of blue clones can be used as a parameter indicating the enrichment of functional phage peptide[11].
2. Titer indicates the number of phages eluted after affinity selection. In each round of affinity selection, the number of input phages was 1×10^{10} .

Immunoscreening and sequence analysis.

After the second round of affinity selection, the eluted phages were plated on the LB plates. After the development of the filters, for the 9aa library, 10% of the clones on the

filter were positive, while for the pool from 9aa cys library, only 1% of the clones were positive. 9 positive clones from 9aa library and 2 clones from 9aa cys library were randomly picked out. After amplification and purification with PEG/NaCl precipitation, ELISA was performed. All the clones showed specific binding to the mAb (Fig 2). DNA sequencing results indicate the c1, c3 and c7 are from a single clone, which is referred as clone No. 1; c2, c4, c5, c6, c8 and c9 are from another single clone, which is referred as clone No. 2; cc1 and cc2 are also from one single clone, which is referred as clone No. 3. Sequence analysis of the three clones shows high homology with the 719-725aa of PSMA (Tab 2). From the sequence analyses of all the isolated clones, combining with their reactivity with the mAb, the conserved motif sequence is “VDPA/SK”, which is very close to the PSMA sequence from 719-725aa in extracellular part.

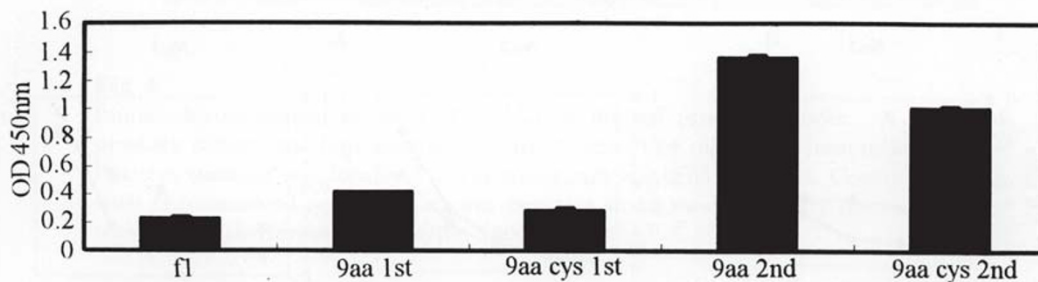


Fig 1.

ELISA results of the binding activity of the selected phage pool after amplification from two rounds of affinity selection

9aa 1st, 9aa 2nd and 9aacys 1st and 9aacys 2nd refer to the amplified phage pool after first round and second round selection from 9aa linear peptide library and 9aa cys circular library respectively. Equal amount of f1 was used as control.

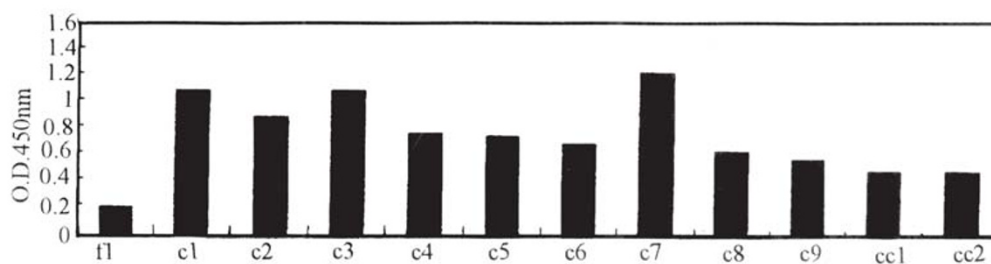


Fig 2.

ELISA results of the binding activity of single clones identified from 9aa and 9aa cys library after immunoscreening. Clones c1-c9 were from 9aa library, clones cc1 and cc2 were from 9aa cys library, and f1 was used as control.

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Tab 2. Sequence analysis of the isolated clones

PSMA peptide	716-725aa	E S K V D P S K A W
No 1 (x3) from 9aa library		V D P G K Y N K Y
No 2 (x6) from 9aa library		E G P A K G F K L
No 3 (x2) from cys library		G C Y E A P S K A A K C

Immunogenic mimicry of PSMA epitope

Three phage clones and wild type f1 were used to immunize the C57BL/6 mice respectively. Ten days after the third immunization, all the immunized mice were bled, and ELISA was performed to identify the titers of the immunized serum. Competitive ELISA was employed to analyze the specificity of the immunized serum (Fig 3).

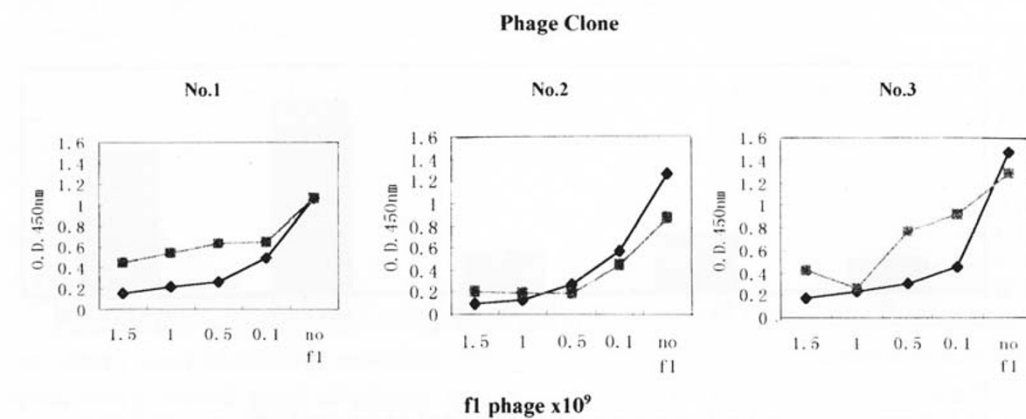


Fig 3.

Results of competitive ELISA, testing the specificity of phage-immunized serum.

(- ◆ - coated with wild type phage f1).

(- ■ - coated with phage clone 1, 2, 3 respectively)

When the f1-reactive antibodies in the phage peptide immunized serum were sufficiently absorbed with f1, and if the absorbed serum still has significant reactivity to the corresponding phagotope, it indicates the existence of phage displayed peptide specific antibodies in the immunized serum. In comparison of the reactivity of all three clones, immunized serum from both clone No.1 and clone No.3 showed significant phagotope specificity but result from No.1 clone seemed to be more consistently and was selected to check its specificity to PSMA on the slides with immunohistochemical staining, and f1 immunized serum was used as negative control. At the same time, several other human cancer samples such as lung cancer, colon cancer and stomach cancer are also tested and no positive staining was observed on the epithelial cells of these samples (Data not shown). However, positive staining was localized on the malignant epithelial cells of human pros-

tate cancer tissue, while, no positive staining was observed on the f1-immunized serum stained slides (Fig 4).

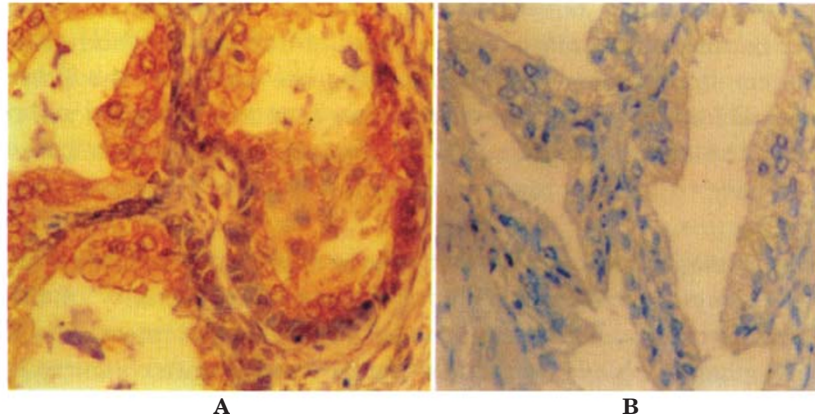


Fig 4.

Immunohistochemical staining of PSMA in human prostate cancer. **A.** Slide of prostate cancer was immunostained with f1 absorbed mimotope-immunized serum. Positive staining was localized to the malignant epithelial cells. **B.** Control staining with f1-immunized serum was negative in all cases. Lightly counterstaining was done with Harris hematoxylin. (400 ×).

DISCUSSION

In our experiment, we did not perform the biopanning with the mixture of 9aa linear and 9aa cys peptide library. Since in many cases, when these two libraries were mixed together in the selection, only the linear peptide clones can be isolated from the last identification (unpublished data, Dr. Paolo Monaci), possibly because the growth and amplification efficiency of the linear phage peptide clones is much higher than that of the circular phage peptide clones. After several rounds of elution and amplification, the ratio of the linear phage peptide clones will dramatically increase in the selected pool, while the positive clones from circular phage peptide library will be lost at last. From our experimental results we can see the enrichment of the positive linear phage peptide is much faster than that of the circular phage peptide library.

Generally, many researchers perform three rounds or more of biopanning in the affinity selection before picking out single clones for identification. However, we just performed two rounds of affinity selection which is sufficient for the isolation of positive clones. In fact, how many rounds of affinity selection is appropriate depend on the aim of the selection and the target used for the selection. In our case, we used the purified mAb as the target for affinity selection. The enrichment of target-specific phage peptide with mAb is much faster than that of the selection with complicated target such as serum

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antibodies. Thus, less rounds of selection is sufficient for the isolation of positive clones. On the other hand, the aim of our experiment is the epitope location of the PSMA. Generally, only 4-6 amino acids are critical for the interaction between the antigen and antibody. In this case, trying to pick out the interaction motif from a series of different antibody-reactive phage peptides is very important. Therefore, keeping the variety of the isolated positive clones is critical for this point. However, higher rounds of selection will result in the enrichment of the dominant clone (Dr. Li Hua, personal communication). On the contrary, we have to identify many more clones in order to keep the variety of the positive clones.

After the immunization of mice with three mimotopes displayed on phage, we did not use the synthesized peptide to test the binding activity of the mimotope immunized serum antibodies, because to some extent, the interaction between the antibody and phage displayed peptide depends on the peptide's micro environment which is provided by the phage particle during the affinity selection, in this case the free peptide will lose the conformation which can be recognized when it was displayed on phage particle[14]. Therefore, we employed the competitive ELISA to test the mimotope specificity of immune serum. From the competitive ELISA results, we observed that both clone No.1 and No.3 induced significant mimotope specific response. Many researchers have demonstrated that filamentous phage is an excellent immunogen. The easily induced immune response in mice is T-cell dependent and undergoes class switching from IgM to IgG[14]. However, the induction of mimotope-specific antibodies is a much more difficult process and varies considerably from one mimotope to another mimotope. This may be reasonable since the immune system can differentiate different antigens and produces very different responses.

Comparing the conserved sequence motif "VDPA/SK" derived from No.1, 2 and 3 phage clones with the PSMA sequence "ESKVDPSK" which was used in the screening process of mAb 4G5, we found they are of high homology, and the "VDPSK" may play a major role in the interaction between the PSMA and mAb 4G5. In addition, the homology of the peptide to the real antigen may be further promoted through an in vitro evolution strategy (Zhu et al submitted[15]). From our results it showed that if we have not any data about the mAb reactive antigen, the deduced peptide or oligonucleotide from the interaction motif can be used as a probe for the identification of the mAb specific antigen. In fact, since the establishment of monoclonal antibody technology, many monoclonal antibodies have been developed which are specific for many different antigens. However, many of these mAb-specific antigens have not been identified yet, such as many cell specific mAbs. In this case, in addition to its successful and extensive application in the epitope mapping of antigen, phage displayed peptide library technology can be a very effective way worthy to be tried for the identification of antigen, even this strategy is limited to the identification of the linear epitope at present.

Our experimental results have demonstrated that 4G5 specific epitope is located at 719-723aa of PSMA, which belongs to extracellular of PSMA. The highly prostate-specific and membrane-bound character of PSMA makes it an ideal target for clinical diagnostic

and therapeutic applications in the treatment and management of prostatic carcinoma. Especially, the antibody induced internalization of PSMA which has been identified recently possibly indicate the biological function of PSMA *in vivo*. Evidently, the characterization of this PSMA extracellular domain specific mAb will be very useful in the antibody targetting strategy for the diagnosis and therapy of prostate cancer.

Recent researches showed PSMA not only overexpressed in prostate cancer, but also in the neovasculature of a variety of malignant neoplasms, indicating that PSMA may play an important role in the genesis and development of other cancer. The mimotopes screened out presumably might be used as vaccine to induce PSMA specific immune responses *in vivo*, and maybe hopefully inhibit the development of the cancer.

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