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Development of Single Chain Antibodies to P185 Tumor Antigen

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Abstract

Background: The human heavy (VH) and light (VL) chain variable genes are amplified and randomly assembled together and cloned into the minor coat protein gene (g3p) of M13 bacteriophage. The resulting library of scFv is expressed on the phage as g3p fusion protein. The high affinity specific scFv antibodies can be selected against a key antigen using panning process. Our aim was development of scFv antibodies against P185 tumor antigen by recombinant phage antibody system and panning process.

Methods: Antibody engineering technology was applied to lymphocyte mRNA of a non-immune donor and a scFv library was constructed. The library was panned against an immunodominant epitope of P185 which its reactivity had been tested with sera from breast cancer patients. DNA fingerprinting of the scFvs selected the predominated clones. These were then screened by ELISA.

Results: A large library including high repertoires of VH and VL was constructed. DNA fingerprinting differentiated a number of clones. After selection against the immunodominant epitope of P185, nine clones were differentiated including two predominated scFv antibodies. The predominated antibodies were bound to the corresponding epitope and produced a positive ELISA.

Conclusion: The high affinity P185 specific scFv antibodies which were originated from human genes and bound specifically to the P185 epitope are valuable clinical agents and have the potential to be used in cancer immunotherapy in which P185 overexpression and metastasis occurs.

Keywords: scFv; Human recombinant antibody; P185; Cancer immunotherapy

Introduction

P185 is a phosphoglycoprotein that has significant homology with the epidermal growth factor receptor (EGFR). Overexpression of P185, usually in association with gene amplification, occurs in most cancers of epithelial origin.[1,2] Overexpression results in increasing of the aggressiveness of the tumors.[3-5] P185 peptides have been assessed as cancer vaccines in clinical trials of patients with breast and ovarian cancers. Although it has been possible to detect immunological responses, no clinical responses were reported.[6-8] The functional immune responses may be limited by immunological tolerance to self-antigen like P185.[9] Passive immunization could play a prominent role in cancer immunotherapy. Trastuzumab, a monoclonal antibody to P185 has been shown to be effective in the treatment of breast cancer in which P185 overexpression and metastasis occurs.[10-14]

Recent advances in antibody engineering have made possible the production of human recombinant antibodies which offer a potential alternative and provide valuable clinical agents for cancer immunotherapy since the antibodies wont induce anti-mouse antibody response (HAMA) induced by monoclonal antibodies, and are avoid of the risks of serum therapy, such as viral contamination.[15,16] In this technology, genes encoding immunoglobulin variable domains are linked together via a linker peptide and expressed in phage display vectors to produce a library of single chain fragment variable. The recombinant phage

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antibody can be enriched by a panning process.[17] Selection of high affinity binders have been used in tumor immunotargeting approaches.[18-20]

Here we describe the production and selection of anti-P185 tumor antigen.

Materials and Methods

Peptide (KIFGSLAFLPESFDGD) as P185 epitope was synthesized (Isogen, the Netherland) and tested in ELISA with 25 breast cancer patient´s sera. The peptide was diluted to10 µg ml^{-1} in PBS and used at 150 µl to coat Falcon microtest III assay plates for 16 hrs at 4ºC. The plates were washed 3 times with PBS containing 0.05% tween 20 followed by 3 washes with PBS and blocked for 2 hrs at 37ºC with 150 µl of blocking solution (10% v/v foetal calf serum, 2% w/v skimmed milk in PBS). Following washing, the plate was incubated with 150 µl of sera diluted 1:50 in blocking solution at 37ºC for 1.5 hrs (performed in quadruplet). Plate was washed and 150 µl of peroxidase conjugated goat anti-human IgG (Sigma Chemical Co., Pool, UK) diluted 1:4000 was added to each well and incubated at room temperature for 1 hr. After washing, the plate was stained by ABTS buffer (150 µl of 0.5 mg ml^{-1} ABTS in pH of 4.0 citrate buffer with 0.03% (v/v) hydrogen peroxide) for 30 minutes. The absorbencies were measured at 405 nm and the average absorbance for each serum was determined.

mRNA was prepared from a non immune donor by separation of 20 ml of heparinized blood over Ficoll, extracted by guanidinium thiocyanate and purified on an oligo (dt)-cellulose column (Quick Prep mRNA: Pharmacia. St.Albans. United Kingdom). First-strand cDNA synthesis was performed with a constant region primer for all four subclasses of human IgG heavy chains (HUIgG1-4) using reverse transcriptase (HT Biotechnology, Cambridge, UK). The heavy chain variable domain genes were amplified by primary PCRs with family-based forward (HuJH 1-6) and backward (HuVH1a-6a) primers. A *Sfi*I restriction site was introduced upstream to the VH3a back-generated product. The light chain variable domain genes were amplified by primary PCRs with family based forwards (HUJ_K1-5) and (HUJ_λ1-5) and backwards (HUV_K1a-6a) and (HUλ1-6) respectively. The latter introduced a linker fragment $(Gly_4 Ser)_3$ and a downstream *Not*I site. The heavy and light chains were linked in an assembly reaction. By use of the *Sfi*I and *Not*I restriction enzyme sites, the product was unidirectionally cloned into phagemid vector pCANTAB5 (Pharmacia, UK). The ligated vector was introduced into *E. coli* TG1 by electroporation and rescued by the addition of helper phage M13KO7.

To enrich for P185-specific scFv antibodies, the phage library was panned against the antigenic domain of P185. The peptide was diluted to 10 µg/ml in PBS and used at 4 ml to coat an immunotube (Nunc, Denmark) overnight at 4°C. The tube was washed with PBS. Free binding sites were saturated using blocking solution [10% foetal calf serum (FCS), 2% skimmed milk in phosphate buffered saline (PBS)]. After 2 hrs at 37ºC, tube was washed 4 times with PBST and 4 times with PBS. Phage supernatant [10^9 plaque forming units (pfu) ml^{-1}] was added and incubated for 1 hr at room temperature. After washing, the bound phagemids were eluted by adding log phase TG1 *E. coli* and incubating at 37ºC for 1 hr. The tube was centrifuged and the pellet was plated onto LB agar plates. The resulting phage-transformed *E. coli* was rescued with the helper phage M13KO7. The phage supernatant was used for a second round of panning. Four rounds of panning were performed to select specific scFv antibodies against the peptide.

DNA fingerprinting was carried out to determine whether panning with the peptide succeeded in enriching for specific clones of scFv and to identify the resulting predominant types. It was performed on individual colonies before and after panning. After PCR amplification of the scFv inserts, each product was digested with *Bst*N1 (New England Biolabs, Hitchen, United Kingdom) at 60 ºC for 2 h and run on 2% agarose gel.

Wells of Falcon microassay III assay plates were coated with 150µl of the peptide (10 µg/ml in phosphate-buffered saline). Two sets of control wells, uncoated wells and coated wells with various control peptides (WYSLNGKIRAVDVPK and TMYPNRQP GSGWDSS) were also considered. The plates were washed three times with PBS containing 0.05% tween 20, followed by 3 washes with PBS and blocked for 2 hrs at 37ºC with 150 µl of blocking solution (10% v/v fetal calf serum, 2% w/v skimmed milk). After washing, 150 µl of phage containing supernatant fluid of the selected clones at concentrations 10^3-10^9 PFUml^{-1} was added to the plate and incubated at 37°C for 1.5 hr. The plate was washed and 150 µl of 1:100 dilution of anti fd bacteriophage (Sigma Chemical Co., Pool, UK) in blocking solution was added and incubated at room temperature for 1 hr. After washing, bound phages were

detected with peroxidase conjugated goat anti rabbit IgG (Sigma Chemical Co., Pool, UK) for 1 hour at room temperature. The color reaction was developed using ABTS (Sigma Chemical Co., Pool, UK) for 30 minutes. The absorbance was read at 405 nm and the mean value for scFv antibodies panned against the peptide was calculated.

Results

The peptide was reactive against the breast cancer patient's sera. Of the 25 patient's sera, 48% (12) were reactive against the peptide. The average absorbance and standard deviation for the peptide in ELISA with the patient's sera were 0.453 and 0.034 respectively

A diverse library with large repertoires of VH and VL was produced which was shown by *Bst*N1 fingerprinting of PCR-amplified scFv inserts. Each colony gave unique fingerprinting (Figure 1).

After four rounds of panning against the peptide, 20 colonies were PCR amplified and *Bst*N1 fingerprinted. nine different antibody types were differentiated and two fingerprint patterns were predominated containing type I (Lanes 1, 3, 8, 10, 16, 20) and type II (Lanes 5, 9, 12, 15, 19). The other types were type III (Lanes 2, 6), type IV (Lane 4), type V (Lane 7), type VI (Lanes 11, 14), type VII (Lane 13), type VIII (Lane 17) and type IX (Lane 18). The patterns of scFv clones are shown in Figure 2.

The scFv antibodies were tested in ELISA. The predominated scFv produced positive ELISA. The absorbance was more than 4 times of baseline readings (Table 1). There was no positive reaction with control peptides. The baseline readings were from the wells with no peptide.

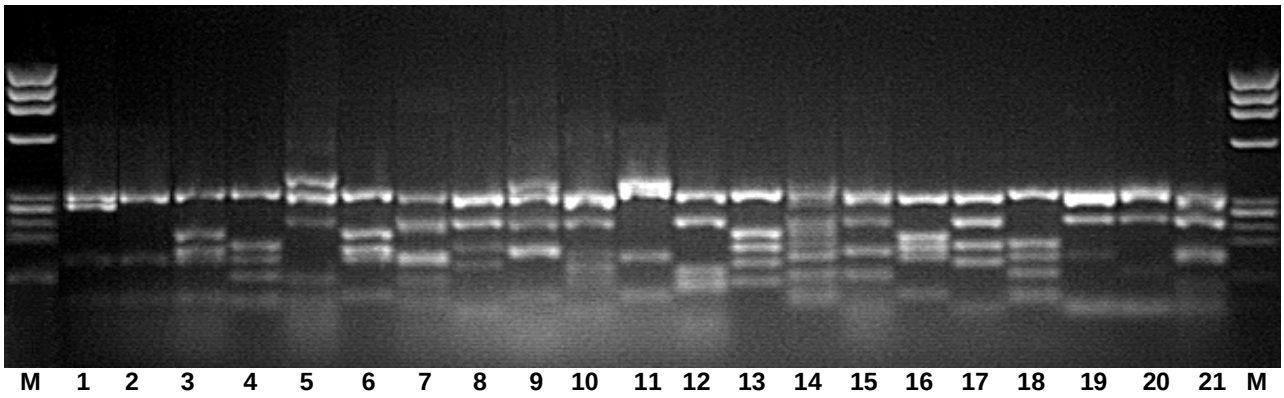

Fig 1: Fingerprinting pattern of un-panned library showing a diverse library with large repertoires of VH and VL. Each colony gave unique fingerprinting.

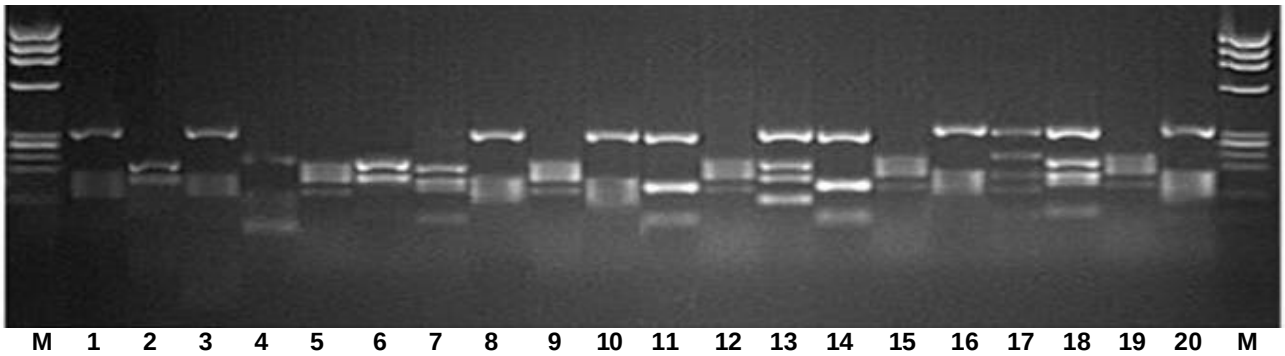

Fig 2: Fingerprinting pattern after panning against the immunodominant epitope of P185. Predominated types containing: type I (Lanes 1, 3, 8, 10, 16, 20) and type II (Lanes 5, 9, 12, 15, 19). The other types: type III (Lanes 2, 6), type IV (Lane 4), type V (Lane 7), type VI (Lanes 11, 14), type VII (Lane 13), type VIII (Lane 17) and type IX (Lane 18).

Table 1: ELISA results of the selected scFv against P185 peptide.

scFv type	Average absorbance(S.D.)	Baseline absorbance
I	0.553 (0.034)	0.129
II	0.601 (0.019)	0.137
III	0.292 (0.071)	0.116
IV	0.118 (0.022)	0.113
V	0.178 (0.045)	0.162
VI	0.305 (0.110)	0.181
VII	0.137 (0.058)	0.139
VIII	0.189 (0.006)	0.144
IX	0.174 (0.167)	0.158

Discussion

To develop a source of anti-P185 recombinant antibodies we describe in this study the production, isolation and assessment of specific anti-P185 single chain fragment variable antibodies. Antibody engineering technology was used to produce a library of scFv antibodies. The genes encoding immunoglobulin variable domains were amplified and randomly assembled together in an assembly reaction and expressed in phage display vector the resulting library of scFv was electroporated into *E. coli* and rescued using helper phage M13KO7. A large library with high repertoires of VH and VL was produced. To select the specific scFv antibodies against P185, the phage library was panned against the immunodominant epitope of P185 (KIFGSLAFLPESFDGD). *Bst*N1 DNA fingerprinting of PCR-amplified scFv inserts showed that before panning the library was highly heterogeneous. After four rounds of panning against the immmunodominant epitope of P185, nine antibody types were differentiated containing 2 predominated scFv (types I and II) which showed the successful panning in enriching the scFv antibodies. The predominated scFv reacted specifically with the peptide and produced positive ELISA. The nonpredominated scFv may be due to antibodies that bind to immunotube during the panning process.

It has been shown that the epitope (KIFGSLAFLPESFDGD) has important role in P185 signaling. Binding to this epitope could inhibit the signaling and change the tumor metastasis.[21] Antibodies to this important epitope could contribute in cancer immunotherapy both in binding to the site that participate in signaling and metastasis and in passive immunization to enhance anti-tumor immune responses.[21-23]

Immunologic targeting of the oncoprotein P185 with monoclonal antibodies is an important component of current therapeutic strategies for patients with locally and systemically advanced breast cancer. In some clinical trials, it has been shown that intravenous Trastuzumab is effective as a single-agent and in combination with chemotherapy.[10,12,14] It has been reported that combined therapy significantly improves the median time to disease progression and survival time in patients with metastatic breast cancer overexpressing the P185 receptor compared with chemotherapy alone.[24,25] Therefore binding to P185 domain would lead to blocking the P185 signaling and inhibiting the proliferation of tumor cells. The specific scFv antibodies to P185 described here offer the possibility for development a new biologic targeted agent for immunotherapy of cancers overexpressing P185 since these engineered specific antibodies are high affinity binders and don't produce problems of HAMA (Human Anti-Mouse Antibody) response and serum therapy.

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Conflict of interest: None declared.

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