

hEGFfr-PEII-scFv gene cloning into Pichia pastoris

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Abstract. Human acidic fibroblast growth factor (aFGF) is a member of the family of structurally related heparin-binding growth factors. aFGF has important roles both in normal biological activities and in tumorigenesis, indicating that aFGF could be a target for the cancer treatment. To explore the relationship between aFGF and tumors growth, a single-chain variable fragment (scFv) antibody specific for aFGF was generated. This scFv may block the activity of aFGF which leads to tumor cell growth inhibition. However, anti aFGF-scFv as a medicine might be harmful for the normal cells because the aFGF is not a tumor specific antigen. In other hand, we have found that the scFv interacts with its antigen in the cytoplasm but delivering proteins with therapeutic potential activity into cells is difficult. Thus, it has been difficult to utilize such proteins as therapeutic drugs. In this work, we want to test a strategy for cancer targeting and intracellular delivery of anti aFGF-scFv by generating a fusion protein consisting of anti aFGF-scFv, a protein translocation domain (*Pseudomonas* exotoxin A domain II, PEII) and a ligand which can bind with cancer cells (human epidermal growth factor receptor binding fragment, hEGFfr). In previous study the fusion gene (*hEGFfr-PEII-scFv*) encoding for this fusion protein was cloned into a prokaryotic expression vector. The fusion gene (*6 His*Tag-hEGFfr-PEII-scFv*) derived from the pET-28a(+) recombinant plasmid was successfully cloned into a *Pichia pastoris* expression system.

Keywords: acidic fibroblast growth factor, single-chain variable fragment, gene cloning, fusion gene, *Pichia pastoris*

1. Introduction

Tumor immunotherapy is a relatively new treatment modality and comprises active specific immunotherapy, nonspecific immunostimulation with cytokines, adoptive cell transfer, monoclonal antibodies (mAbs) and other methods designed to elicit anti-tumor immunity [1]. In mAbs field there is a range of applications, however, in which the Fc-mediated effects are not required. To remove the Fc domain, IgGs have been dissected into constituent domains, initially through proteolysis and later genetically engineered into their monovalent (Fab, scFv, single variable V_H and V_L domains) or bivalent fragments (Fab₂, diabodies, minibodies, etc). Single-chain Fvs are a popular format in which the variable heavy V_H and variable light V_L domains are joined with a flexible polypeptide linker preventing dissociation [2].

Abbreviations: aFGF, acidic fibroblast growth factor; scFv, single-chain variable fragment; hEGF, human epidermal growth factor; hEGFfr, human epidermal growth factor receptor binding fragment; PEII, *Pseudomonas* exotoxin A domain II; mAbs, monoclonal antibodies; IgG, immunoglobulin class G; Fab, antigen binding fragment; Fc, fragment crystalline; V_H, variable domain of heavy chain; V_L, variable domain of light chain; ADP, adenosine diphosphate; DNA, deoxyribonucleic acid; PCR, polymerase chain reaction; μM, micromolar; μl, microliter; AOX, alcohol oxidase; Mut⁺, methanol utilization plus; Mut^s, methanol utilization slow; HIS4, histidinol dehydrogenase.

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Growth factors and their receptors are suitable target antigens for generating mAbs such as vascular epidermal growth factor; human basic fibroblast growth factor and human acidic fibroblast growth factor (aFGF). aFGF belongs to a large family consists of at least 23 different factors. It is a single-chain anionic polypeptide built of 154 amino acid residues. aFGF is expressed in almost all tissues and plays important roles in a variety of normal and pathological processes [3].

In order to deliver the functional domain of a selected protein from the outside to the inside of intact cells, a carrier is needed. Cell Permeable Peptides are carriers with small peptide domains that can freely cross cell membranes. Some studies have shown that *Pseudomonas* exotoxin A domain II (PEII) which is involved in translocation of the *Pseudomonas* toxin across membranes may be used for translocation of other protein across the human cell membrane. *Pseudomonas* exotoxin A is made up of three domains namely; Domain Ia is composed of residues 1-252 and it is responsible for cell recognition. Domain II, residues 253-364, it is likely to be involved in translocation of the toxin across membranes. Domain Ib, residues 365-404. Domain III, residues 405-613, it catalyzes the ADP-ribosylation of elongation factor 2, which arrests protein synthesis and results in cell death [4].

For cancer targeting, some ligands specific for cancer cells or overexpressed in these cells are needed such as the human epidermal growth factor receptor binding fragment (hEGFfr). Human epidermal growth factor is a polypeptide of 53 residues with three looped regions from residues 1-20, 14-31, and 32-53 [5]. Komoriya et al found that the linear and cyclic forms β -loop peptide (residues 20-31) were only active receptor binding [6]. Several studies have used the hEGF or the hEGFfr linked with different molecules such as human angiogenin Pthalocyanines; and Doxorubicin as a cancer targeting molecule.

To explore the relationship between aFGF and tumors growth, a single-chain variable fragment (scFv) antibody specific for aFGF was geneted. This scFv may block the activity of aFGF which leads to tumor cell growth inhibition. However, anti aFGF-scFv as a medicine might be harmful for the normal cells because the aFGF is not a tumor specific antigen. On the other hand, we have found that the scFv interacts with its antigen in the cytoplasm but delivering proteins with therapeutic potential activity into cells is difficult. Thus, it has been difficult to utilize such proteins as therapeutic drugs. Therefore, the therapeutic application of proteins could be achieved by the development of delivery vectors that are capable of the efficient delivery of various proteins into cells. In this work, we want to test a strategy for cancer targeting and intracellular delivery of anti aFGF-scFv by generating a fusion protein consisting of anti aFGF-scFv, a protein translocation domain (PEII) and a ligand which can bind with cancer cells (hEGFfr). To obtain such soluble protein we have used *Pichia pastoris* expression system for protein production. The cloning procedure and cloning results of fusion gene in *Pichia pastoris* cells is described in this study.

2. Materials and methods

2.1. The generation of pPIC9K recombinant plasmid

The *His*Tag-hEGFfr-PEII-scFv* fusion gene was amplified using the pET-28a(+)-hEGFfr-PEII-scFv as a DNA template in PCR and the following primers: **Yeast-pPIC9K-forward (primer F):** 5'-GCCTACGTACATCATCATCATCACAG-3', **Yeast-pPIC9K-reverse (primer R):** 5'-TTCCTAGGCTATGAGGAGACGGTGAC-3', creating an *Avr* II upstream site and a downstream *Sna*B I site (underlined), respectively. PCR reaction system was as follows (50 μ l): 25 μ l Premix-Taq, 1 μ l (20 μ M) Primer F, 1 μ l (20 μ M) Primer R, 1 μ l (10 ng) pET-28a(+)-hEGFfr-PEII-scFv, 5 μ l DMSO and 17 μ l ddH₂O. PCR reaction parameters were as follows: heating 95°C for 5 min and 35 cycles of denaturation at 94°C for 50 sec, annealing at 55°C for 40 sec, and extension at 72°C for 90 sec, a final extension at 72°C for 10 min. The amplified fragment contains 1191 bp in length. PCR amplified product was analyzed by using 1% agarose gel electrophoresis and was purified from the gel by using the DNA gel extraction kit. Both purified gene fragment and pPIC9K empty vector were treated with both *Avr* II and *Sna*B I enzymes separately. Incubation at 37°C for 16 hours. The digested gene was linked into the digested plasmid by the T4 DNA ligase at 16°C for overnight.

2.2. Transformation

The recombinant vector was transformed into *Escherichia coli* DH5 α competent cells coating on LB agar plates containing 50 mg/ml Ampicillin. Positive transformants were selected after performing a bacterial PCR and double enzyme digestion. The positive clones were subjected to DNA sequencing to confirm the correct reading frame and to confirm that the ATG is in the proper context for eukaryotic translation initiation. It is recommended to linearize the recombinant vector and the parent vector which will be transformed into GS115 and will be used as a background control for expression, in such a manner to generate both Mut⁺ and Mut^S recombinants; in our work we have used the *Sac* I enzyme for insertion at AOX1 and to generate GS115 Mut⁺. The reaction system is as follows (200 μ l): \leq 20ug DNA, 20 μ l 10X NEBuffer 1, 2 μ l BSA, 1 μ l *Sac* I and up to 200 μ l ddH₂O. Incubate the reaction at 37°C overnight (~14 hours). Analyze a small portion of the digest by agarose gel electrophoresis to confirm complete digestion of the fragment. The number of transformants and frequency of targeting will be reduced if digestion is not complete. Resuspend DNA pellet in 10-20 μ l of TE buffer and store it at -20°C until ready to transform. In general electroporation provide the highest efficiency of transformation for most researchers (10³ to 10⁴ transformants per ug DNA). Pulse the cells according to the parameters for yeast (*Saccharomyces cerevisiae* 1.5 kV, 200 Ω 25 μ F; Bio-Rad Gene Pulser) suggested by the manufacturer of the specific electroporation device being used. Incubate the plates at 30°C until colonies appear. Screen for Mut⁺/Mut^S phenotypes.

2.3. *In Vivo* screening of multiple inserts

Using sterile technique, add 200 μ l YPD medium to each microtiter well of 96 well plates. Inoculate each well of the first set of plates with a single His⁺ transformant using a sterile toothpick and stirring to resuspend cells. Inoculate the second set of microtiter plates with 10 μ l from the first set of microtiter plates by using a multi-channel pipette and incubate the second set of plates overnight at 30°C. Take the third set of plates and resuspend the cells in each well by pipetting up and down with a multi-channel pipette set on 100 μ l volume. Spot 10 μ l from each well on YPD plates (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) containing Geneticin[®] at a final concentration of 0, 0.5, 1.5 and 3.0 mg/ml. Spot in a regular pattern using the multi-channel pipette or a grid underneath the plate. Let the liquid soak in, then incubate plates at 30°C, and check after 2, 3, 4, or 5 days for Geneticin[®] resistant clones.

2.4. PCR analysis of *P. pastoris* integrants

2.4.1. Identification of integrations into *P.pastoris*

The total genomic DNA isolated from yeast cells using glass beads was used for the PCR analysis and the following primers **5' AOX1 sequencing primer:** 5'-GACTGGTTCCAATTGACAAGC-3' and **3' AOX1 sequencing primer:** 5'-GCAAATGGCATTCTGACATCC-3'. Set up PCR reactions as follows (25 μ l): 12.5 μ l (5 U/ μ l) premix Taq enzyme, 1 μ l (0.1 ug/ μ l) 5' AOX1 Primer, 1 μ l (0.1 ug/ μ l) 3' AOX1 Primer, ~1 ug DNA template, 2.5 μ l DMSO and up to 25 μ l ddH₂O. For amplification controls, use 100 ng of recombinant plasmid (positive control) and 100 ng of the appropriate plasmid without insert (negative control). Load thermocycler and run the following program: start heating at 95 °C for 5 min, and 33 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 90 sec, a final extension at 72°C for 10 min. Analyze 10 μ l on a 1X TAE, 0.8 % agarose gel.

3. Results and analysis

3.1. Construction of *hEGFfr-PEII-scFv* for yeast expression

In this study, the *His*Tag-hEGFfr-PEII-scFv* fusion gene was PCR amplified from plasmid pET-28a(+)-hEGFfr-PEII-scFv. The PCR product was confirmed to contain 1191 bp as expected by 1% agarose gel electrophoresis (**Fig 1A**). The gene fragment and the pPIC9K plasmid were digested with *Avr* II and *Sna*B I, below is the double-enzyme digestion result of the pPIC9K plasmid as expected by 1% agarose gel electrophoresis (**Fig 1B**). After transformation into DH5 α cells the positive clones were determined by the double-enzyme digestion using the recombinant pPIC9K-His*Tag-hEGFfr-PEII-scFv plasmid (**Fig 1C**) extracted from the transformant cells followed by sequencing.

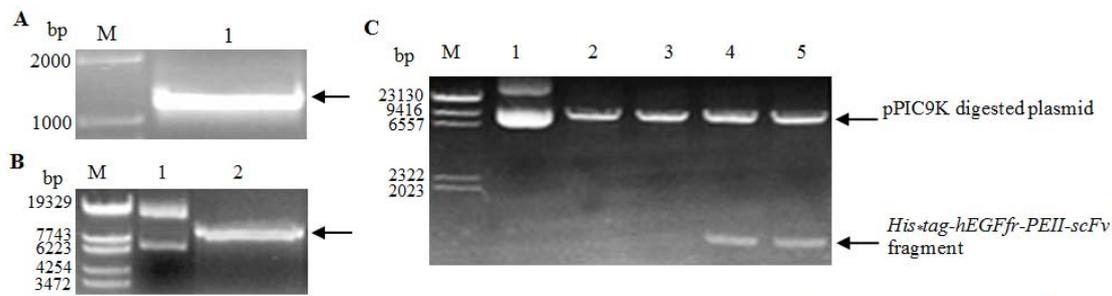


Figure 1. Construction of *hEGFfr-PEII-scFv* for yeast expression **A**, *His-tag-hEGFfr-PEII-scFv* gene amplification result. M, DL-2000 marker; 1, *His-tag-hEGFfr-PEII-scFv* gene indicated by arrow. **B**, double enzyme digestion of pPIC9K plasmid using *SnaB* I and *Avr* II enzymes. M, λ -Eco T14 digest marker; 1, undigested plasmid; 2, pPIC9K digested plasmid indicated by arrow. **C**, Double enzyme digestion result of the recombinant pPIC9K-*His-tag-hEGFfr-PEII-scFv*. M, λ -Hind III digest marker; 1, negative control; 2, 3, negative clones; 4, 5, positive clones.

3.2. Screen for Geneticin[®] resistant transformants

In this step of screening for Geneticin[®] resistant we have confirmed the observed level of Geneticin[®] resistance. There were only a few Geneticin[®] resistant colonies, and they were of different sizes, but the colony morphology was the same. The number of the resistant colonies were in inverse proportion with the Geneticin concentration as it shown in figure 2, we may see a very few colonies resistant to 3.0 mg/ml Geneticin[®]. "Jackpot" clones resistant to these high levels of Geneticin[®] are very rare.

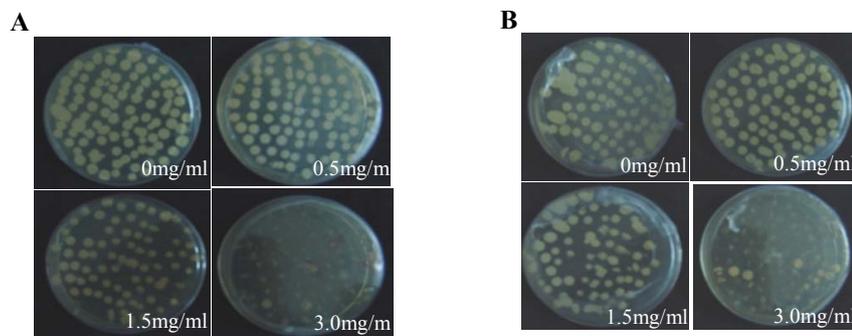


Figure 2. Screen for Geneticin[®] resistant transformants. **A**, transformants containing *hEGFfr-PEII-scFv* construct; **B**, control (transformants containing the parent plasmid) using different concentrations of

3.3. Recombination and integration in *P.pastoris*

The figure below shows the result of a typical PCR analysis of *P.pastoris* integrants (**Fig 3**). Genomic DNA was isolated from *Pichia* recombinants and from appropriate controls. Ten microliters samples from each PCR were run on a 0.8% agarose gel. In the lanes which contain the *Pichia* recombinants we see clearly two bands. One is corresponding to the size of our gene of interest cloned into pPIC9K (1191 bp + 492 bp = 1683bp), the other to the wild-type AOX1 gene (approximately 2.2 kb). From this result we determined the Mut phenotype of the transformants. Because we see clearly two bands we can conclude that the integrants are Mut⁺.

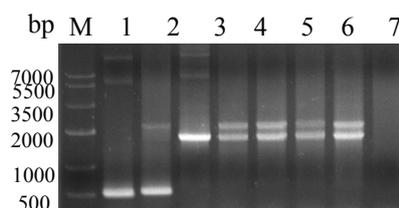


Figure 3. PCR analysis of *Pichia* integrants. M, D7000 marker. Lane 1 shows the 492 bp PCR product made from pPIC9K by priming with the 5' and 3' AOX1 primers. Lane 2 shows the 492 bp product and the wild-type AOX1 gene (2.2 kb) from GS115/pPIC9K. Lane 3 shows the expected size of our gene of interest cloned into pPIC9K (1191 bp + 492 bp = 1683bp). Analyzing the *Pichia* recombinants in lanes 4-8, it can be seen that lanes 4-7 contain insert. Lane 8, the His⁺ transformant, does not contain the gene of interest.

4. Discussion

Antibody engineering and advances in bacterial and yeast expression systems have enabled production of soluble and active heterologous proteins. However, the functional expression yields of these heterologous proteins vary widely. In this work, in the first step the *His*Tag-hEGFfr-PEII-scFv* fusion gene was PCR amplified from plasmid pET-28a(+)-hEGFfr-PEII-scFv, this gene contains 1191 bp. The gene was inserted into the pPIC9K plasmid between the *Avr* II and *Sna*B I sites. The recombinant plasmid was linearised and transformed into *P.pastoris* cells by electroporation. The linearised plasmids, recombinant plasmid and the empty plasmid, were integrated into the yeast genomic DNA to form stable transformants (Fig 1). In the second step, we needed as many His⁺ transformants as we can conveniently generate. Recall that statistically 1-10% of the His⁺ transformants will have more than one insert. This means that if the frequency of multicopy inserts is 1%, it will have to screen 1000 His⁺ transformants to get 10 Geneticin[®] hyper-resistant colonies to test. This may require 1-5 plates containing His⁺ transformants. It is not unusual to screen thousands of colonies. In this work we found that the number of the resistant colonies were in inverse proportion with the Geneticin concentration as it shown in figure 2, we may see a very few colonies resistant to 3.0 mg/ml Geneticin[®]. In a GS115 his4 strain a gene replacement (omega insertion) event arises from a double crossover event between the AOX1 promoter and 3' AOX1 regions of the vector and genome. This results in the complete removal of the AOX1 coding region (i.e. gene insertion). The resulting phenotype is His⁺ Mut⁺. His⁺ transformants can be readily and easily screened for their Mut phenotype, with Mut⁺ serving as a phenotypic indicator of integration via gene insertion at the AOX1 locus. The net result of this type of gene insertion is a loss of the AOX1 locus (Mut⁺) and the gain of an expression cassette containing plasmid AOX1, the gene of interest, and HIS4 (Fig 3).

5. Conclusion

Pichia pastoris expression system provides today an excellent system for recombinant proteins production. There is two ways for this production, the intracellular production way and the secretion into the medium using a secretion factor. The results described in this work elucidates the cloning procedure and results of the hEGFfr-PEII-scFv into *Pichia pastoris* cells. The fusion gene derived from the pET-28a(+) recombinant plasmid. This fusion gene was successfully into a *P.pastoris* expression vector pPIC9K fused with α -factor, a secretion signal for protein secretion.

6. Acknowledgements

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7. References

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