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Cloning and Expression of the Variable Regions of Anti-EGFR Monoclonal Antibody in *E. coli* for Production of a Single Chain Antibody

Farzaneh Jalalypour [1, 4], Safar Farajnia [2*], Fatemeh Mahmoudi [3], Behzad Baradaran [2], Davoud Farajzadeh [3], Leila Rahbarnia [4], Jafar Majidi [2]

[1]Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, I.R. IRAN
[2]Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, I.R. IRAN
[3]Department of Cellular and Molecular Biology, Azarbaijan Shahid Madani University, Tabriz, I.R. IRAN
[4]Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, I.R. IRAN

*Corresponding author: Safar Farajnia, Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, I.R. IRAN. Tel: +98-4113363234, Fax: +98-4113363231, E-mail: farajnias@tbzmed.ac.ir

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Background: Epidermal growth factor receptor (EGFR) overexpression is a characteristic of several malignancies and could be considered as an excellent target for designing specific inhibitors such as anti-EGFR monoclonal antibodies for cancer therapy. Drawbacks exerted by large sizes of full-length antibodies have lead to the development of single chain antibodies, which benefit from having smaller sizes and short circulation half-lives.
Objectives: The aim of this study was cloning, expression and purification of variable regions of anti-EGFR monoclonal antibody in *E. coli* for production of single chain antibodies.
Materials and Methods: The RNA, extracted from the C225 hybridoma cells, was reverse transcribed into cDNA and used for PCR amplification of genes encoding light and heavy chains from the variable regions. The PCR products were cloned and expressed in *E. coli* BL21 for production of a single chain antibody. The expressed protein was analyzed by SDS-PAGE and purified by Ni-NTA affinity chromatography. The reactivity of purified C225-scFv with EGFR-expressing A431 tumor cell line was tested by Western blotting and enzyme-linked immunosorbent assays.
Results: The results indicated that C225-scFv was highly expressed in *E. coli* and appeared as a protein with a mass of 27 kDa in the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the induced cell lysate. Reactivity analysis of the purified C225-scFv with A431 tumor cell line by western blotting and enzyme linked immunosorbent assay (ELISA) revealed high binding affinity of the recombinant C225-scFv to the target cells.
Conclusions: The results of this study indicated that C225-scFv is capable of binding to EGFR and could be considered as a useful tool for diagnosis and treatment of EGFR-overexpressing tumor cells.

Keywords: Single-Chain Antibody; EGFR; C225

1. Background

Epidermal growth factor receptor (EGFR) belongs to a family of transmembrane glycoprotein receptors with tyrosine kinase activity, which regulates various cell functions including growth, proliferation, differentiation, migration and angiogenesis via receptor-ligand binding. Previous studies have debated on the structure of the receptor and its various ligands (1, 2). Evidence from a variety of sources shows that amplification or mutation of the EGFR gene as well as overexpression of its protein is associated with various cancers. This overexpression is characteristic of many human tumors of epithelial origin, including head and neck, breast, lung, colorectal, prostate, kidney, pancreas, ovary, brain and bladder cancer and correlates with poor prognosis (3-5). Involvement of EGFR in initiation, development, and metastasis of cancers indicates that this molecule could be considered as an excellent object for target therapy and designing specific inhibitors (6). Unlike conventional therapeutics such as chemotherapy or radiation therapy, which are nonspecific and also influence nontumor tissues, EGFR target therapy can selectively affect cancer cells via specific binding to this receptor (7-9). These findings have led to the development of inhibitory monoclonal antibodies (mAb) against this receptor such as cetuximab

Implication for health policy/practice/research/medical education:
Over-expression of epidermal growth factor receptor (EGFR) is a characteristic of various malignancies and it is considered as a promising target for cancer therapy by development of specific inhibitors such as anti-EGFR monoclonal antibodies. Application of full length mAbs in cancer treatments is often limited by some obstacles due to their large size. In order to overcome this hurdle, smaller recombinant antibody (rAbs) fragments such as scFv have been developed. The small size of scFv fragments confers several advantages in immunotherapy applications. This paper describes the engineering process of the monoclonal antibody C225 into the scFv format and evaluation of its reactivity with cells expressing high levels of EGFR.

and panitumumab, directed towards the extracellular domain, and a large number of low-molecular-weight tyrosine kinase inhibitors (TKIs) which act intracellularly by blocking the ATP binding site of EGFR (8). The extracellular domain of EGFR is involved in ligand binding, and its targeting by monoclonal antibodies intercepts the activation of relevant signaling pathways, resulting in suppression of tumor growth (5, 7). In brief EGF, as the most important ligand of EGFR, binds to the epitopes on domain I and III and blockage of one site can inhibit ligand binding. Accordingly, cetuximab, a chimeric mAb is able to block EGF binding to its receptor by covering domain III of EGFR (10).

2. Objectives

Since full-length mAbs are large molecules, some obstacles often limit their application in cancer treatments. Whole antibodies diffuse poorly into solid tumors, retain in blood circulation for a long time and subsequently accumulate in the liver (11). To overcome this obstacle, smaller recombinant antibody (rAbs) fragments such as scFv, Fab, dsFvs and single-domain antibodies have been developed (12). One of the most common rmAbs is single chain fragment variable (scFv), with a molecular weight of 25-26 kDa. It contains the complete antigen-binding site and comprises the variable regions of heavy (VH) and light (VL) chains, which are commonly joined via a flexible peptide linker (12-15). Several different linkers have been examined for bridging the variable domains of scFv, varying in length and amino acid composition (16). Among these, the flexible Gly- and Ser-rich sequences (e.g. (Gly4Ser)3) (12, 17) have been the most commonly used linker. This linker seems to be an optimal linker that can easily be expressed in its functional form in *E. coli* and allows scFv to fold into the correct conformation (16, 18). These scFvs with short linkers do not have the ability to fold correctly, and it has been shown that scFvs with linkers shorter than 12 resides cannot assemble into a functional Fv domain (15, 19). Furhtermore, scFvs are constructed in both orientations of VL-linker-VH and VH-linker-VL; however, the most extensively used orientation is VH-linker-VL (12, 20). The scFv antibodies are nonglicosylated fragments which can be expressed efficiently in a bacterial expression system. The small size of scFv fragments also confers several advantages in immunotherapy and medical applications. It can be used in cancer treatment, gene therapy, drug delivery and radioimmunodetection by conjugation to different kinds of reagents including drugs, toxins, radionuclides, nanoparticles, viruses, biosensors and liposomes (12, 15, 21, 22). Besides, the novel modified scFvs that are derived from chimeric and humanized antibodies are more suitable in medical applications due to minimizing the immunogenicity of murine antibodies in humans (17, 23, 24). In this paper, we described the engineering of C225 monoclonal antibody into a scFv, and evaluated its reactivity with cells expressing high levels of EGFR.

3. Materials and Methods

3.1. Cell Culture and RNA Isolation

In this study, C225 hybridoma cells expressing murine anti-human EGFR were used as sources of mRNA, coding anti-human EGFR mAb. Briefly, the cells were cultured in RPMI containing inactivated fetal bovine serum (FBS), penicillin and streptomycin. The cells were harvested and the total RNA was isolated from the cells in the log phase using TRIzol reagent. The cells were homogenized in TRIzol solution, extracted with chloroform, precipitated with isopropanol and dissolved in diethylpyrocarbonate (DEPC)-treated distilled water, after washing with 75% ethanol and drying.

3.2. cDNA Synthesis and Polymerase Chain Reaction Amplification of Variable Regions of Light and Heavy Chains

Six microliters of total RNA was used for first strand cDNA synthesis of the first strand (Fermentas, Lithuania) in a reverse transcriptase (RT) reaction, containing 1 μL of oligo-dT primer, 4 μL of 5X buffer, 1 μL of Riboblock (Fermentas), 2 μL of dNTPs and 1 μL of murine leukemia virus (M-MLV) reverse transcriptase. The mixture was kept at 23°C for 10 minutes, followed by incubation at 42°C for 60 minutes. The enzyme was finally inactivated by heating at 95°C for 5 minutes. For amplification of VH and VL gene segments by PCR, 1 μL of the prepared cDNA was subjected to polymerase chain reaction (PCR) with VH-specific primers of VH-F and VH-R, and VL-specific primers of VL-F and VL-R, respectively. Primers were designed by the Gene Runner software using the sequence reported in the patent related to cetuximab (WO 135259A2). Sequences of primers used for amplification of light chain variable regions were, VL-F: 5'-ATAAGCTTGGTGGAGGCGGTTCAGGCGGAGGTGG CTCTGGCGGTGGCGGATCAGACATCTTGCTGACTCAGTCTCC-3', introducing a HindIII restriction site and containing the common linker coding sequence (Gly4Ser) 3 (bold letters); and VL-R: 5'- TACTCGAGTTTCAGCTCCAGCTTGGTCC-3', introducing an *XhoI* restriction site. Sequences of VH specific primers were, VH-F: 5'- TACATATGCAGGTGCAGCTGAAGCAGTC-3', introducing an NdeI restriction site; and VH-R: 5'- ATAAGCTTTGC AGAGACAGTGACCAGAGTC-3', introducing a HindIII restriction site to bind to the VL and VH coding sites. PCR amplification was carried out in a final volume of 25 μL, containing PCR buffer (1X), 2 mM $MgSO_4$, 0.2 mM of each deoxynucleoside triphosphate (dATP, dCTP, dTTP and GTP), 0.4 μL of each primer and 2.5 U of Pfu DNA polymerase. Each PCR cycle consisted of denaturation at 94°C for 30 seconds, annealing at 60°C for 1 minute, extension at 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. Thirty-two cycles were performed. The reaction mixture was further incubated for 25 minutes with Taq DNA polymerase to achieve 3' end tailing with single a base. PCR products

were visualized on 1% agarose gel under UV light, after staining with ethidium bromide.

3.3. Cloning and Sequencing of VL and VH Fragments

The PCR products were purified using a PCR purification kit (Qiagen, Germany) according to the manufacturer's instructions. T-A cloning was performed using the pGEAM-Teasy cloning kit (Promega, USA) and yielded plasmid clones of PCR2.1-VL and PCR2.1-VH. These products were separately transformed into *E. coli* DH5a competent cells, and the positive clones were submitted for sequencing. The yielded nucleotides and the predicted amino acid sequences were compared with the data provided by the basic alignment search tool (BLAST).

3.4. Construction of the scFv Expressing Vector

The VL gene segment was excised with *Hind*III - XhoI restriction enzymes and subcloned into the pET22b expression vector (Novagen, USA), in frame with a carboxy-terminal 6-histidine tag, yielding a plasmid subclone of pET22b-VL. Subsequently, the VH gene segment, released by HindIII-NdeI digestion was subcloned into the recombinant plasmid pET22b-VL in the VH-linker-VL orientation. This structure was named the pET22b-Anti-EGFR scFv construct. Finally, the resulting expression vector was confirmed by PCR and DNA sequencing.

3.5. Expression of Recombinant C225-scFv in E. coli

The pET22b-Anti-EGFR scFv construct was transformed into E. coli BL21, grown at 37°C overnight with vigorous shaking in Luria-Bertani (LB) medium, containing 100 μg/mL ampicillin and 1% glycerol. Protein expression was induced by addition of 0.3 mM isopropyl-D-thiogalactopyranoside (IPTG), and the cells were harvested 1, 3, 6 and 24 hours later. Expression of the recombinant protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 15% gel (25). For expression optimization, *E. coli* BL21 cells, transformed with the expression construct, were grown in LB broth with different inducer concentrations for 1, 3, 6 and 24 hours, and the protein expression was analyzed by SDS-PAGE.

3.6. Purification of the Recombinant C225-scFv

After culturing for 3 hours, the cells were pelleted and resuspended in 4 mL of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 1 Mm phenylmethylsulfonyl fluoride (PMSF), then sonicated for 30 seconds with a thirty-second pause, for 15 times at 80W on ice. The lysate was centrifuged and the supernatant was collected and saved for purification using Ni-NTA affinity chromatography.

3.7. Western Blotting

A431 is a tumor cell line, naturally expressing high levels of EGFR. For analyzing the activity of C225-scFv antibody expressed in *E. coli* BL21, A431 cells were subjected to SDS-PAGE, followed by western blotting. The proteins were transferred to PVDF filters, blocked by 5% skim milk, and incubated with purified C225-scFv antibody. After washing, the reaction was developed with an HRP-conjugated antimouse IgG. The reaction signals were detected using the 3, 3'-diaminobenzidine (DAB) substrate.

3.8. Reactivity Assessment of the Recombinant C225-scFv Against A431 Cells by Enzyme-Linked Immunosorbent Assay

The reactivity and specificity of the recombinant C225-scFv was examined by enzyme-linked immunosorbent assay (ELISA), using A431 cells. The cells were sonicated and coated on ELISA plates (SPL, Seoul, Korea)) overnight at 4°C. After being washed with PBS and blocked by 3% BSA, the purified C225-scFv was added and incubated for 1 hour at room temperature. The wells were then washed and detected by 3, 3', 5, 5'-Tetramethylbenzidine (TMB) substrate after incubation with goat anti mouse secondary antibody. The reaction was stopped by addition of 1 M H_2SO_4 and the absorbance was measured at 450 nm by an ELISA reader.

4. Results

4.1. Amplification, Cloning and Sequencing of VL and VH Fragments

The VL and VH genes were amplified by RT-PCR as described, and visualized on agarose gel, revealing specified bands of about 366 bp for the VH gene and 378 bp for the VL gene (Figure 1). These fragments were separately cloned into the PCR 2.1 vector and prepared for sequencing. DNA sequencing of the VH and VL genes indicated that these genes possessed the intended sequence.

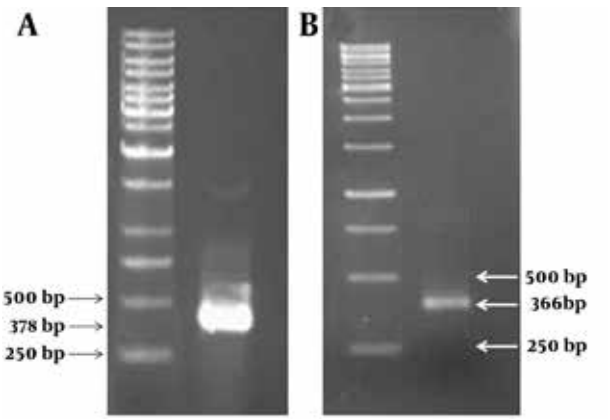

Figure 1. Agarose Gel Electrophoresis of PCR Amplification of VL (A) and VH (B) Coding Sequences From C225 Hybridoma Cells.

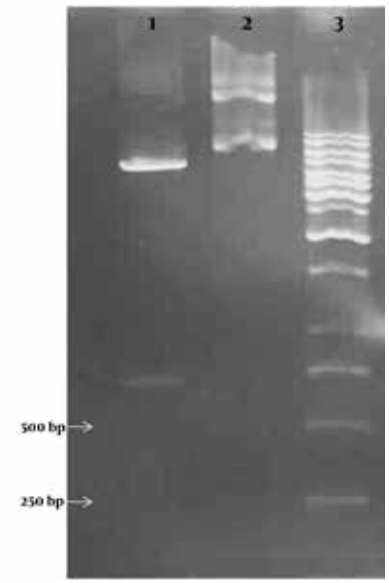

Figure 2. Confirmation of Cloning of scFv by Restriction Digestion. Lane 1, Undigested pET22b-anti-EGFR scFv; Lane 2, pET22b-anti-EGFR scFv digested with XhoI and NdeI; Lane 3, 1 kb ladder.

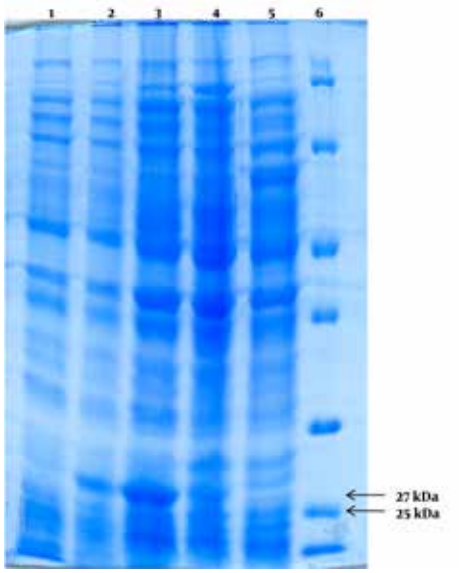

Figure 4. SDS-Page Analysis of Anti-EGFR scFv Expression in *E. coli*. Lane 1, before induction; Lane 2, 1 hour after induction; Lane 3, 3 hours after induction; lane 4, 6 hours after induction; lane 5, 24 hours after induction; lane 6, size marker.

ATGCAGGTGCAGCTGAAGCAGTCAGGACCTGGCCTAGTGCAGCCCTCACAGAGCCTGTCCA
TCACCTGCACAGTCTCTGGTTTCTCATTAACTAACTATGGTGTACACTGGGTTCGCCAGTCTCC
AGGAAAGGGTCTGGAGTGGCTGGGAGTGATATGGAGTGGTGGAAACACAGACTATAATACA
CCTTTCACATCCAGACTGAGCATCAACAAGGACAATTCCAAGAGCCAAGTTTTCTTTAAAAT
GAACAGTCTGCAATCTAATGACACAGCCATATATTACTGTGCCAGAGCCCTCACCTACTATGAT
TACGAGTTTGCTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCAAAGCTTGGTGGAG
GCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCAGACATCTTGCTGACTCAGTCTCC
AGTCATCCTGTCTGTGAGTCCAGGAGAAAGAGTCAGTTTCTCCTGCAGGGCCAGTCAGAGT
ATTGGCACAAACATACACTGGTATCAGCAAAGAACAAATGGTTCTCCAAGGCTTCTCATAAA
GTATGCTTCTGAGTCTATCTCTGGGATCCCTTCCAGGTTTAGTGGCAGTGGATCAGGGACAG
ATTTTACTCTTAGCATCAACAGTGTGGAGTCTGAAGATATTGCAGATTATTACTGTCAACAAAA
TAATAACTGGCCAACCACGTTCGGTGCTGGGACCAAGCTGGAGCTGAAATAA

MQVQLKQSGPGLVQPSQSLSITCTVSGFSLTNYGVHWVRQSPGKGLEWLGVIWSGGNTDY
NTPFTSRLSINKDNSKSQVFFKMNSLQSNDTAIYYCARALTYYDYEFAYWGQGTLVTVSAKLG
GGGSGGGGSGGGGSDILLTQSPVILSVSPGERVSFSCRASQSIGTNIHWYQQRTNGSPRLLIK
YASESISGIPSRFSGSGSGTDFTLSINSVESEDIADYYCQQNNNWPTTFGAGTKLELK

Figure 3. The Nucleotide and Amino Acid Sequences of C225 scFv.

4.2. Construction of the scFv Expression Vector

Insertion of the VL gene into the pET22b vector created the light-chain intermediate plasmid (pET22b-VL). Subsequently, VH was subcloned into this intermediate plasmid (pET22b-anti-EGFR scFv). We used PCR and restriction enzyme digestion methods to prove the cloning of these fragments into the expression vector and construction of scFv (Figure 2). The results showed that the total gene of scFv against human EGFR was about 750 bp, consisting of a 366 bp VH gene, a 378 bp VL gene, and a 45 bp linker encoding the (Gly4Ser)3 gene, which corresponded with the expected molecular sizes of the fragments. The nucleotide sequence of this construct was confirmed by sequence analysis (Figure 3). The resulting vector was transformed into the *E. coli* BL21 strain.

4.3. Expression of Recombinant C225-scFv

E. coli BL21 was transformed with the recombinant plasmid, pET22b-Anti-EGFR scFv, and induced as described above. The results indicated that C225-scFvs are highly expressed in *E. coli*, which appeared as a protein with a mass of 27 kDa in the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the induced cells (Figure 4). This corresponded with the expected molecular size of the scFv.

4.4. Optimization of the Recombinant Protein Expression

Analysis of the recombinant protein expression in different conditions revealed that the best condition for maximum production of C225-scFv by *E. coli* BL21 was cul ture of the recombinant cells at 37°C in the presence of 0.1 mM IPTG for 3 hours.

4.5. Protein Purification and Western Blot Analysis

After disturbing the *E. coli* BL21 cells by sonication, the protein was successfully collected from the supernatant and purified by Ni-NTA affinity chromatography. Analysis by SDS-PAGE showed a purity of > 90% by a single purification step. Evaluation of the reactivity to the A431 cell line by ELISA, resulted in an OD of >3 with 10 μg/mL of purified scFv.

Analysis by western blotting revealed specific binding of purified C225-scFv with A431 cells, verifying the activity of the recombinant single chain antibody (Figure 5).

Figure 5. Western Blot Analysis of Reactivity of Anti-EGFR scFv With the A431 Cell Lysate. Lane 1, The A431 cell lysate incubated with anti-EGFR scFv; lane 2, The A431 cell lysate incubated with secondary antibody; lane 3, Size marker.

5. Discussion

EGFR is the prototypic member of the class I (ErbB, HER) family of RTKs, which includes EGFR, HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4), and mediates multiple cellular functions, including cell proliferation, migration, apoptosis and differentiation. EGFR overexpression occurs in several human solid tumors, which promotes oncogene activation and alters the signaling pathways of the cells (8, 26-28). Therefore, EGFR could be used for targeted therapy of various malignancies. EGFR targeting by monoclonal antibodies is one of the most important strategies for treatment of EGFR-positive cancers, and several anti-EGFR monoclonal antibodies are currently in clinical use or in different phases of preclinical evaluations (29). Furthermore, mAb C225 is an FDA-approved chimeric anti-EGFR antibody, used in treatment of head, neck and colon cancers, and has recently been approved for treatment of lung cancer (30). Although this antibody has shown promising results in cancer therapy, its usage is limited by some impediments related to its large size (11). Moreover, scFvs are the smallest units of the immunoglobulin molecule, composed of VH and VL regions packed into a single molecule (approximately 27 kDa) via a short flexible link (33, 34); scFvs are expected to be less immunogenic than full length antibodies, while retaining their specificity to their own targets (35). These scFvs can also be produced on large scales at low cost in *E. coli* (36). Considering the various advantageous of scFv antibodies, this study was devoted to the development and evaluation of an anti-EGFR scFv originated from C225 mAb. Expression of this scFv in *E. coli* resulted in high amounts of recombinant C225-scFv that was purified by a single step Ni-NTA affinity chromatography. This result was consistent with previous reports on expression of scFv in the *E. coli* expression system (14, 37). Analysis of reactivity of recombinant C225-scFv against A431 cells by the ELISA test revealed that scFv bound to EGFR on A431 tumor cell line with high affinity. Western blotting showed that C225-scFv recognized a protein with a mass of 140 kDa on A431 cells that was equivalent to the molecular weight of EGFR. This finding was in line with some previous studies reporting similar affinity and specificity for scFvs to their parental full-length counterparts (14, 38, 39).

In conclusion, the results of this study indicated that C225-scFv, produced in this study are capable of binding to EGFR and thus, could be used for the diagnosis and treatment of EGFR overexpressing tumor cells.

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Authors' Contributions

Farzaneh Jalalypour performed the experiments and wrote the manuscript. Safar Farajnia designed and supervised the study and analyzed the data. Fatemeh Mahmoudi, Davoud Farajzadeh and Behzad Baradaran and Leila Rahbarnia provided consultation.

Financial Disclosure

The authors declared that they had no competing interests to disclose.

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