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Full Length Research Paper

Recombinant expression and purification of L2 domain of human epidermal growth factor receptor

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Epidermal growth factor receptor (EGFR) is one of the key molecules in cell growth and multiplication and plays an important role in some malignant processes. L2 domain of extra-cellular part of this receptor involved in ligand binding and its inhibition can prevent activation of related signaling pathways. The aim of the present study was cloning and expressing the fragment coding for L2 region of human EGFR for the production of recombinant L2 protein. The total RNA from A431 cells line was extracted and used for amplification of the sequence coding for L2 domain of EGFR by reverse transcriptase-polymerase chain reaction (RT-PCR) technique. The product was cloned into the PGEM-T vector and used for sequencing. In the next step, the insert was removed from the PGEM-T vector and subcloned into the PET22 expression vector. The expression construct was transformed into the *Escherichia coli* BL21 (DE3) and recombinant protein expression was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Analyzing the expression of produced recombinant protein showed that the recombinant L2 can be highly expressed by this expression system. This recombinant protein can be used for the production of specific mAb, screening for specific ligands and competitive inhibition of the EGFR.

Key words: Human epidermal growth factor receptor, domain L2, recombinant expression.

INTRODUCTION

Epidermal growth factor receptor (EGFR) belongs to a family of receptors also known as the type I receptor tyrosine kinases (Baselga, 2002). Activation of this receptor triggers multiple cellular events leading to cell proliferation, differentiation, migration, protection from apoptosis and induction of angiogenesis (Olayioye et al., 2000).

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Abbreviations: EGFR, Epidermal growth factor receptor; **RT-PCR,** reverse transcriptase-polymerase chain reaction; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis; **CR,** cysteine rich; **DEPC,** diethylpyrocarbonate; **BLAST,** basic alignment search tools; **LB,** Luria-Bertani; **IPTG,** isopropyl-D-thiogalactopyranoside.

Occurrence of mutation in the EGFR has been reported in several tumors (Salomon et al., 1995) and it's over expression having been shown in head and neck, lung and colon cancers. This over expression has been correlated with poor survival of patients (Brabender et al., 2001), whereas its inhibition was associated with inhibition of the xenograft of head and neck tumors (Carter et al., 1992; Chen et al., 1987; Drebin et al., 1985; Goldstein et al., 1995). These findings indicated the importance of EGFR in certain cancers and show that this molecule can be considered as a target for designing specific inhibitors (Li et al., 2008).

Three types of strategies have been developed for targeting of the EGFR according to the locations where the action takes place: at the intracellular tyrosine kinase domain, at the transmembrane lipophilic domain and at the N-terminal extracellular domain (Alroy and Yarden,

1997; Boonstra et al., 1995; Callaghan et al., 1993; Carpenter and Cohen, 1990; Ullrich et al., 1984). The extracellular part is composed of 621 amino acids containing four domains; two large homologous domains (L1 and L2) and two cysteine rich (CR) domains which referred to the CR1 and CR2 domains (Abe et al., 1998). The L1 and L2 subdomains are involved in the binding of ligands to the EGFR with high affinity (Ferguson, 2004). It has been shown that L2 domain plays a crucial role in ligand binding and activation of intracellular signaling pathway which initiates after binding of ligands to L2 domain. Therefore, targeting of the L2 domain through designing specific inhibitors or production of monoclonal antibodies can effectively be used for EGFR blockage. The current method for production of anti-EGFR antibodies or other inhibitors uses whole or extracellular part of EGFR which have been associated with low efficiency. The objective of this study was to clone and express L2 domain of human EGFR in *Escherichia coli* for the production of recombinant L2 protein.

MATERIALS AND METHODS

Cell culture and RNA isolation

In this study, the A431 cancer cells that express high levels of EGFR were used as a source of EGFR mRNA. The cell line was obtained from the American Type Culture Collection and cultured as described (Pourabbas et al., 2009). Briefly, the cells were cultured in tissue culture flasks in RPMI 1640 medium containing L-glutamine (2 mM) (Sigma, Germany) supplemented with 10% heat-inactivated fetal calf serum and penicillin/streptomycin.

Total RNA was extracted from A431 cancer cells using RNAzol solution according to the manufacturer's instructions. Briefly, 10^{-6} cells were homogenized in 1 ml of RNAzol solution and mixed with 200 µl chloroform. The mixture was centrifuged in 12000 rpm for 15 min and RNA from aqueous phase was precipitated by equal volume of isopropanol. The sediment was dissolved in diethylpyrocarbonate (DEPC) treated distilled water after washing with 75% ethanol and drying.

cDNA synthesis and PCR amplification of L2 domain

6 µl of total RNA was entered in a reverse transcriptase (RT) reaction containing 1 µl oligo-dT primer, 4 µl of 5X buffer, 1 µl Ribobloc, 2 µl dNTPs and 1 µl of MMLV reverse transcriptase. The reaction was performed at 42°C for 60 min. 1 µl of prepared cDNA was subjected to polymerase chain reaction (PCR) amplification with primer pair L2-F: 5'-TACATATGCGCAAAGTGTGTAACGG AATAG-3' and L2-R 5'-TGCTCGAGCAAGTTCTTGTTTCCTGAA ATTATC-3' which was complementary to 5' and 3' ends of L2 domain of human EGFR gene, respectively. PCR amplification was carried out in a final volume of 25 µl containing PCR buffer (1X), 2 mM $MgCl_2$, a 0.2 mM concentration of each 2'-deoxynucleoside triphosphate (dATP, dCTP, dTTP and dGTP), a 0.4 □M concentration of each primer and 2.5 U of *Pfu* DNA polymerase (Fermentas Inc.). PCR conditions were: primary denaturation at 94°C for 4 min followed by 30 cycles of denaturation at 94°C for1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 7min. The reaction further incubated for 20 min with Taq DNA polymerase for 3' end tailing with single A base.

Cloning and sequencing of L2 domain gene

The PCR product purified with a PCR purification kit (Qiagen) according to the manufacturer's instructions and used for T-A cloning using pGEM-T easy cloning kit (Promega), yielded the plasmid clone pGEM-L2. The reaction was transformed into an *E. coli*, DH5a competent cells and a positive clone was submitted for sequencing. Nucleotide and predicted amino acid sequences were compared to data available by the basic alignment search tools (BLAST) search method.

Recombinant expression of L2 domain in *E. coli*

The coding region for L2 domain was isolated by digestion of pGEM-L2 clones with *NdeI-XhoI* restriction enzymes and subcloned into the pET 22 b expression vector (Novagen) in frame with a carboxy-terminal six histidine tag, yielding the plasmid subclone pET22b-L2. The construct was transformed into *E. coli* Bl21 strain, cultured at 37°C in Luria-Bertani (LB) medium containing 100 µg ml^{-1} ampicillin to an absorbance at 600 nm (A_{600}) of 0.7. Protein expression was induced by adding isopropyl--D-thiogalacto-pyranoside (IPTG) to a final concentration of 1 mM and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Purification of recombinant L2 was carried out as described (Kafshnochi et al, 2010). Briefly, 500 ml LB media containing 100 µg ml^{-1} ampicillin was inoculated with 5 ml of an overnight culture of recombinant bacteria and incubated at 37°C with vigorous shaking (150 rpm). Culture were induced at mid-log phase by adding 0.125 mM IPTG and growth was continued for 6 h. Bacteria was harvested (10000 x g, for 10 min), resuspended in 10 ml lysis buffer A (50 mM NaH_2PO_4, pH-8, 300 mM NaCl, 10 mM imidazole, 1 mg/ml lysozyme) and disrupted by sonication (five 30 s pulses interrupted with cooling on ice). Soluble and insoluble fraction was separated by centrifugation at 12000 x g for 10 min at 4°C and recombinant protein was found to be mainly in the insoluble fraction as inclusion body.

SDS-PAGE analysis

SDS-PAGE was performed using the Laemmli buffer system. Prior to electrophoresis, the samples were heated at 100°C for 10 min in dissociating buffer containing 2% SDS and 5% 2-mercaptoethanol. Protein markers used were phosphorylase (97.4 kDa), bovine serum albumin 66.3 kDa), ovalbumin (45.0 kDa), lactate dehydrigenae 35.0 kDa), RE Bsp981 (25.0), beta-lactoglobolin (18.4) and lysozyme (14.4 kDa).

Expression optimization

E. coli BL21 cells transformed with pET 22b-L2 were grown in liquid medium at different inducer concentrations and induction times to check their ability to higher level of expression. After 1, 3, 6, 12 and 24 h induction, 1 ml of bacterial culture was centrifuged and the protein expression was checked by SDS-PAGE.

RESULTS

Amplification, cloning and sequencing of L2 domain of human EGFR

The amplification of L2 domain of human EGFR by RT-PCR resulted in the production of a specified band of

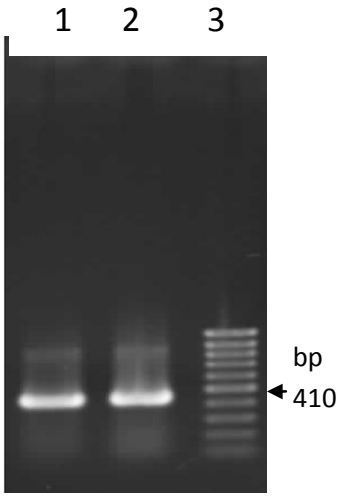

Figure 1. RT- PCR amplification of human EGFR, L2 domain coding region.

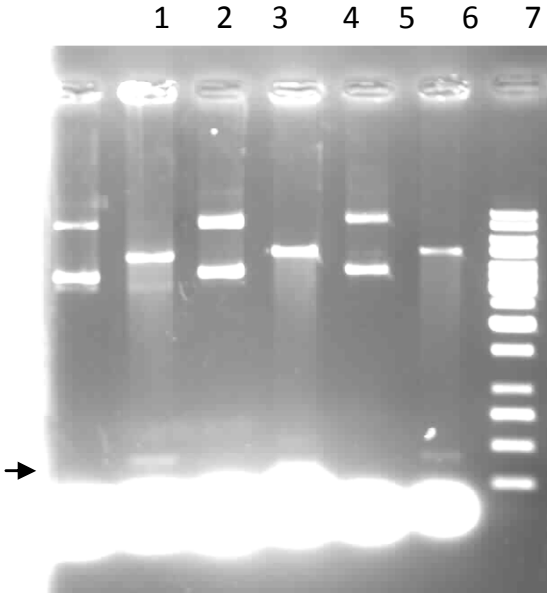

Figure 2. Subcloning of L2 fragment in pET 22 b expression vector. Lanes 1, 3 and 5 represent 3 pET22b-L2 clones before restriction digestion and lanes 2, 4 and 6, represent same clones after digestion with NdeI-Xho I. Lane 7 shows 1 Kb DNA ladder.

about 410 bp (Figure 1). The PCR product was ligated to pGEM-Teasy vector and cloning was verified by PCR and restriction digestion analysis (Figure 2). Sequencing and multiple alignment of nucleotide and predicted amino acid sequence of cloned gene by BLAST program confirmed the identity of clone as showed 100% homology with L2 domain of human EGFR.

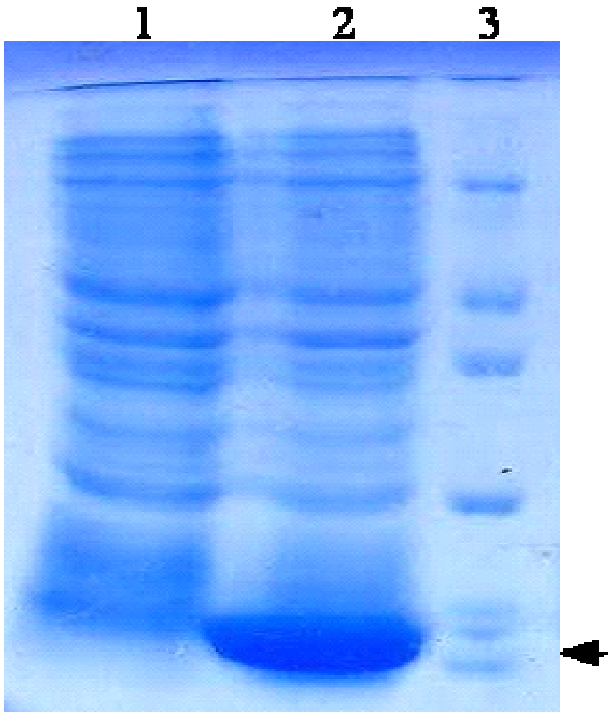

Figure 3. Recombinant expression of L2 coding region in *E. coli* BL21. Lane 1, before induction; lane 2, after induction and lane 3, molecular weight size marker.

Recombinant expression and purification of L2 domain

For high-level expression of L2 protein in *E. coli*, the L2 insert was removed from pGEM-T vector and subcloned into the pET 22 b expression vector (Figure 3). The nucleotide sequence of this construct was confirmed by sequence analysis. Induction with IPTG of *E. coli* BL21 transformed with expression cassette pET 22 b-L2 resulted in a high level expression of recombinant protein as appeared in SDS-PAGE analysis of lysate of induced bacteria. Figure 4 shows Commassie blue-stained SDS-PAGE gels of *E. coli* culture before and after induction. Recombinant L2 protein was expressed as a 16 - 18 KDa protein in high concentration.

Optimization of recombinant protein expression

The optimal IPTG concentration for production of recombinant L2 was 0.125 mM (Figure 4). Thus, the best conditions for the maximum production of recombinant L2 by *E. coli BL*21 were: incubation of the recombinant cells at 37°C in the presence of 0.125 mM concentration of IPTG and 6 h culture time after induction.

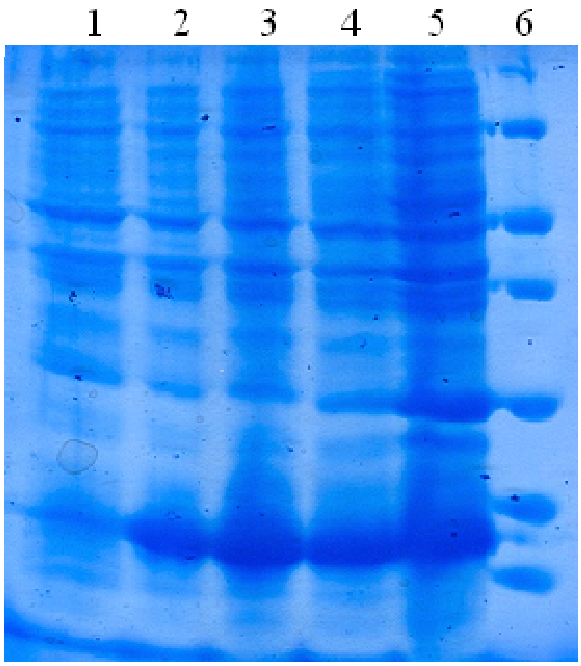

Figure 4. Effect of different IPTG concentration on the level of recombinant L2 expression. Lane 1, lysate of *E. coli* BL21 containing pET22-L2 before induction; lanes 2 - 5, the same expression after induction with IPTG concentration of 0.0625, 0.125, 0.25, 0.5, respectively.

DISCUSSION

Conventional methods used for treatment of cancer affect non-selectively, both normal and cancerous cells and leads to severe side effects. Recent studies in oncology focused on molecular events and key molecules that are involved in cell growth, differentiation and migration (Roskoski, 2004; Woodburn, 1999). The objective of such studies is the development of therapeutics that selectively target molecules expressed differentially on malignant cells without affecting normal cells. Growth factors and their receptors are among the molecules that are involved in cell multiplication processes and seem as good targets for interventional therapies. EGFR is a receptor tyrosine kinase that expresses in a significantly higher level in several types of cancer cells and its activation results in cell proliferation, differentiation, cell adhesion, migration and angiogenesis. Ultrastructural studies have shown that L2 domain from extracellular part of EGFR is involved in ligand binding and receptor activation (Li et al., 2008). It has being shown that monoclonal antibodies such as Cetuximab that binds to L2 domain of EGFR can block and inhibits receptor activation. These findings indicated that l2 domain could be considered as a target for the development of specific inhibitors. Furthermore, soluble L2 can be used for competitive inhibition of EGFR by collecting ligands accessible for cell surface EGFR. Thus, the L2 domain is an important molecule in researches related to EGFR and production of recombinant L2 would be useful for further applications in cancer research.

For the production of recombinant L2, the A431 cell was used as a source of EGFR mRNA. The A431 cell is a human epithelial carcinoma cell line that expresses EGFR in very high concentration (Rizzino et al., 1988). The RNA extracted from these cells was used for cDNA synthesis and RT-PCR amplification of L2 coding region by standard method (Schmidt, 2004). The T-A cloning method was used for cloning of the PCR product. It has being shown that this method is a rapid and efficient method for cloning of PCR product for further use in reactions such as sequencing, expression and any other studies. Sequencing of the cloned gene showed 100% nucleotide homology with domain L2 of EGFR and confirmed the identity of the clone. Recombinant expression of L2 fragment was carried out by *E. coli* BL21 and pET 22 b expression vector which carry T7 promoter. SDS PAGE analysis of induced cells revealed that this system is capable of producing a high level of recombinant L2.

Different expression systems have been developed for recombinant expression of proteins, including *E. coli* prokaryotic system, yeast expression system, insect and mammalian cell expression systems. Each of these systems has their own advantage and disadvantages (Sambrook and Russell, 2001). It has being shown that *E. coli* system is a very rapid, inexpensive and efficient system for production of recombinant proteins (Li et al., 2008). But this system does not contain posttranslational modification mechanisms such as, glycosylation, phosphorylation, acetylation and carboxylation. Thus, this system cannot be used for the production of recombinant proteins; thus, posttranslational modification is necessary for their correct structure/function. In case of L2 domain, sequence analysis showed that this domain does not have any site for such posttranslational modification. So, *E. coli* could be a suitable expression system for the production of recombinant L2. Findings of our study indicated that L2 domain are stably expressed in *E. coli* and can be purified for further use in applications such as production of monoclonal antibodies, screening for specific ligands and studies on competitive inhibition of EGFR.

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