Expression and Purification of the Recombinant Human EGFR Extracellular Domain III

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Abstract

The epidermal growth factor receptor (EGFR) is a membrane surface protein having tyrosine kinase activity, which is ubiquitously expressed in human epidermal cells and stromal cells, and highly expressed in various human tumor cells. The extracellular domain of the EGFR protein contains a large number of epitopes, but the entire extracellular domain of EGFR is too large in molecular weight, and its extracellular domain III (EGFR-ECD$_3$) is a key site for the binding of EGF to EGFR. This study designed and constructed the pCMV-EGFR-ECD3 plasmid, which was transiently transfected into HEK293F cells, then expressed and purified by Ni-NTA affinity chromatography column, and biological activity identified by ELISA, CCK8 and phosphorylation experiments. Therefore, we have successfully obtained high purity, highly active EGFR-ECD$_3$ protein.

Keywords

EGFR-ECD$_3$, Expression and identification.

1. Introduction

Receptor tyrosine kinase (RTK) is an important cell surface receptor involved in signal transduction, which is a key component of the signaling pathway [1]. RTK plays a vital role in the physiological and biochemical processes of most cells, such as cell growth and differentiation, cell cycle, cell metabolism, etc. [2-4]. The epidermal growth factor receptor (EGFR or Her1 / ErbB1) gene is located on the short arm of chromosome 7, and the encoded EGFR is a transmembrane glycoprotein with a molecular weight of 170 kDa [5]. EGFR belongs to the receptor tyrosine kinase in normal epidermal cells. It plays an important role in growth and differentiation [6-8]. EGFR is divided into three parts, which are extracellular ligand binding region, transmembrane region and intracellular tyrosine kinase domain [9]. The extracellular ligand binding region consists of 621 amino acids, which is the extracellular appearance of EGFR. That part is where the binding sites of multiple ligands including Epidermal Growth Factor (EGF), Transforming Growth Factor-α (TGF-α) [10]. The transmembrane region is a hydrophobic region composed of 23 amino acids, which is highly conserved and can locate EGFR in the cell membrane [11]. The intracellular tyrosine kinase domain has a catalytic domain of tyrosine kinase activity, consisting of 542 amino acids, including the proximal membrane (JM) region, the tyrosine kinase (TK) region, and the C-terminal three subregions [12]. It is worth mentioning that the C-terminus consists of 229 amino acids and contains five autophosphorylation sequences, of which Tyr 1068, 1148, and 1173 are the major autophosphorylation sites. To be a site, it is closely related to the transmission of signals [13-14]. Overexpression and mutation of EGFR involves lung cancer, breast cancer, gastric cancer, colorectal cancer, head and neck cancer, pancreatic cancer and glioblastoma, which promote tumor cell growth, proliferation, angiogenesis, invasion, metastasis and death. The expression level of EGFR can be used as an indicator to judge the prognosis of various tumors [15-16]. At present, there are mainly chemical and biological drugs for this target, among which the chemical is a small molecule tyrosine kinase inhibitor, which can enter the cell and ATP competitively bind to the receptor intracellular tyrosine kinase domain, thereby preventing it

autophosphorylation [17]. The first generation of EGFR-TKI is a reversible tyrosine kinase inhibitor, but is susceptible to acquired resistance, and its molecular mechanism is mostly the EGFR gene exon20 T790M mutation [18-19]. The effect of the second-generation EGFR-TKI on overcoming T790M resistance is not satisfactory. The third-generation EGFR-TKI truly overcomes T790M resistance, but acquired C797S mutations cause resistance [20-23]. The fourth generation of EGFR-TKI is currently under investigation. The biopharmaceutical drug, monoclonal antibody, acts mainly in the extracellular region of EGFR, competitively inhibits ligand binding to EGFR, prevents EGFR activation and dimer formation, autophosphorylation, and downstream signal transduction, thereby inhibiting tumor cells proliferation, invasion and formation of new blood vessels [24]. It includes nimotuzumab, cetuximab, panitumumab and necitumumab. The monoclonal antibody has strong targeting, long half-life and long-term efficacy, but due to its difficult to penetrate solid tumor cells, large-scale production of monoclonal antibody is very high costly in the production process and the monoclonal antibody has certain immunogenicity, which makes its development limited [25-27]. Since the entire extracellular domain of EGFR has a large molecular weight (about 100 kDa), an expression plasmid is constructed to express a region that plays an important role in the binding activity of EGFR and EGF in the extracellular segment of EGFR (EGFR ECD3), and initially identified its biological activity. Highly pure and biological activity protein, laying the foundation for subsequent experimental Study.

2. Materials and Methods

2.1 Reagents

1.*Kpn I, Xho I* FastDigest purchased from Fermentas.

2.DNA Marker was purchased from Takara.

3. T_4 DNA Ligase purchased from Thermo Scientific

4. Yeast Extract and Tryptone purchased from OXOID

4.Anti-his mouse antibody

5.Anti-EGFR rabbit antibody

6. Anti-pEGFR rabbit antibody

7.goat anti-mouse IgG-HRP antibody

8. goat anti-rabbit IgG-HRP antibody

2.2 Reagent setup

LB liquid medium was made by dissolving Yeast Extract 5 g, Peptone 10 g, NaCl 10 g and the pH adjusted to 7.0-7.4 in 1 L of deionized water, sterilization was then performed at 121°C for 20 min. PBS was made by dissolving KCl 0.2 g 、 $Na_2HPO_4 \cdot 12H_2O$ 3.58 g 、 KH_2PO_4 0.27 g and NaCl 8 g in 1 L of deionized water. 500 mM imidazole was made by dissolving Imidazole 35.0 g in 100 mL 10 x PBS (filtered membrane), then add 800 mL of ultrapure water and stir to fully dissolve. After add concentrated hydrochloric acid to adjust the pH to 7.4, add ultrapure water to make up to 1 L and store at 4 °C.

2.3 Cell lines and culture medium

Human non-small cell lung cancer cells A549 and human embryonic kidney cells HEK 293F were purchased from ATCC. A549 cell line was maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco). HEK 293F cell line was maintained in EX-CELL® 293 serum-free medium (Invitrogen). RPMI 1640 Medium (no phenol red) was purchased from Gibco. All cells were grown at 37°C in 5% CO_2.

2.4 Expression and purification of recombinant protein EGFR-ECD_3

The full length of the extracellular segment of EGFR (NCBI Sequence: NM_005228.4) was synthesized by Suzhou Hong Xun Biotechnology Co., Ltd. The EGFR-ECD_3 gene was amplified by PCR and cloned into pCMV plasmid (by our laboratory). The recombinant vector was transformed

to *E. coli* DH5α and the sequence of plasmid pCMV-EGFR-ECD$_3$ was confirmed by colony PCR and sequencing.

The plasmid pCMV-EGFR-ECD$_3$ was amplified in *E. coli* DH5α and purified using Endo Free Plasmid Maxi Kit E (Qiagen). HEK293F cells in the exponential growth phase were grown in medium (Invitrogen) until they reached a cell density of 1 x 10^6 cells/mL. Cells were transiently transfected with the transfection reagent, PEI, according to the manufacturer's instructions. Briefly, 400 μg of DNA and 1200 μg of PEI were diluted with basal medium RPIM1640, mixed and incubated for 5 minutes, then added dropwise to shake flasks containing 100 mL of cells, and the transfected cells were suspended in a shaker. These were left for 5 days (at 130rpm, 37°C, 5% CO_2), the blank control group without transfection was run concurrently under the same conditions. To evaluate the protein expression of the EGFR-ECD$_3$ proteins, a reducing SDS-PAGE and western blotting were performed on the samples. The collected cells were digested with 1% SDS at 100° C for 10 min. Protein concentration was determined by BCA to ensure the same quantity of protein was loaded for each sample. For western blotting, the proteins were transferred from the gels to a nitrocellulose membrane (GE Healthcare, UK), where residual protein binding sites were subsequently blocked with 5% milk. In order to detect EGFR-ECD$_3$ protein, the membrane was incubated with HRP anti-his mouse antibody (proteintech, china) at 4°C overnight, then developed with chemiluminescent substrate.

The cell supernatant containing EGFR-ECD$_3$ was filtered with a 0.22 μm filter and purified by a Ni-NTA affinity chromatography column. The mixture of column was equilibrated with 1× PBS (pH 7.4). Then the targeted protein was eluted with 150 mM imidazole, and the purity was analyzed by SDS-PAGE and western blot.

2.5 Enzyme-linked immunosorbent assay

The EGF concentration was diluted to 0.5 μg/mL in a coating solution, then, added to a 96-well microtiter plate and coated at 4°C overnight. Plates were washed with PBST and blocked with 5 % BSA, then incubated at 37°C for 2 h. Plates were washed and with 0.5% BSA at an initial concentration of 10 μg/mL, then diluted twice before being added at 100 μL/well to the plate. The blank control was 0.5% BSA incubated at 37°C for 1 h. The plate was washed, then HRP anti-his tag mouse antibody added to plate, and incubated at 37°C for 1 h. The plate was washed, before TMB chromogenic solution was added to plate, incubated at 37°C for 10 min. 2.29% sulfuric acid was added to plate at 100 μL/well, and the OD was measured at 450 nm and 630 nm using a microplate reader.

2.6 CCK8 assay

The A549 cells in logarithmic growth phase were seeded in 96-well cell culture plates at 5×10^3 cells per well. The blank group contained medium only. Pre-culture was perfomed for 24 h (37°C, 5% CO_2), the medium discarded, and the cells washed twice with PBS. Cells were serum starved for 2 h in the appropriate serum-free medium. EGF was prepared with a different concentration gradient, and added to the plate; a control group with 0.4% FBS DMEM medium containing cells was used. The plate was incubated in an incubator for 48 h, and 10 μL of CCK8 solution was added to each well. The plate was incubated at 37ºC for 1 h. The absorbance at 450 nm to 630 nm was measured with a microplate reader, and the average value of 5 wells was taken, adjusting with the blank, and a proliferation inhibition curve was drawn. During this assay, the optimal concentration of EGF and different concentration gradients of EGFR-ECD$_3$, as determined by the A549 cell proliferation inhibition assay, were used in the test wells.

2.7 Phosphorylation experiment

Logarithmic growth phase A549 cells were seeded in 6-well cell culture plates at 4× 105 cells per well. The blank group contained medium only. The plates were pre-incubated in the incubator for 24 h (37°C, 5% CO2), then the appropriate protein added to the plates, and the sample taken after 48 hours. The protein lysate containing protease inhibitor was added, and the supernatant was collected by centrifugation. Protein concentration was determined by a BCA assay. Denaturation, gel loading,

electrophoresis, transfer, blocking, primary and secondary antibody incubation, and membrane development were performed as described previously.

2.8 Statistical analysis

The data from this paper were evaluated using analysis of deviation and variance.

3. Results

3.1 Expression and purification of recombinant protein EGFR-ECD$_3$

The EGFR- ECD$_3$ gene fragment was amplified from the full length of the extracellular domain of EGFR by PCR, and the EGFR- ECD$_3$ gene was inserted into the *Kpn1* restriction site, his tag and *Xho1* restriction sites were added to the end of the EGFR- ECD$_3$ gene. The pCMV vector (Fig.1A) was ligated, and the ligated product was transferred into *E. coli* DH5α competent state, identified by colony PCR (Fig. 1B) and sequencing, and the extracted recombinant plasmid was identified by double enzyme digestion. The results indicated that the recombinant plasmid pCMV-EGFR- ECD$_3$ was successfully constructed.

A large number of pCMV-EGFR-ECD$_3$ were extracted and transiently transfected into HEK293F cells. After three days of culture, the expression was confirmed. After five days of culture, the culture supernatant was collected. The EGFR-ECD$_3$ fusion protein was purified by Ni-NTA affinity chromatography column, 20 mM imidazole was equilibrated, and the target protein was eluted with 500 mM imidazole solution (Fig. 1C).

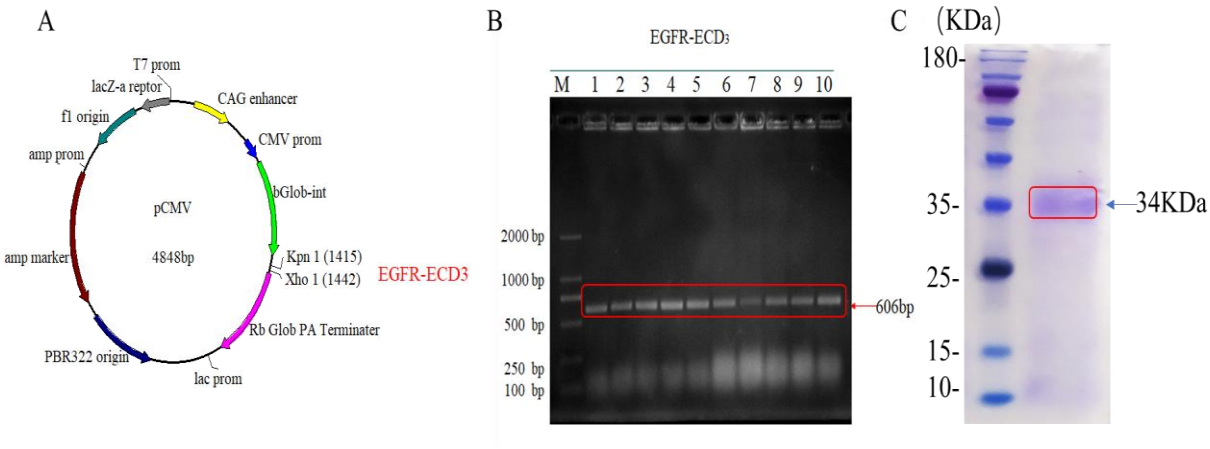

Fig. 1. Construction, expression and purification of the antigen EGFR-ECD$_3$

A: pCMV vector map, EGFR- ECD$_3$ is inserted between *Kpn I* and *Xho I*.

B: 10 monoclonal clones for pCMV-EGFR- ECD$_3$ plasmid for colony PCR verification.

C: EGFR- ECD$_3$ purified staining map.

3.2 Identification of EGFR-ECD$_3$ protein affinity by ELISA

Biological activity is an important criterion for screening antibodies as antigens. EGFR- ECD$_3$ must have the ability to bind to EGF. The 96-well plate was coated with 50 ng of EGF per well, and the diluted EGFR- ECD$_3$ was added. The relative amount of EGFR-ECD$_3$ binding to EGF was determined by the magnitude of the absorbance. As the concentration of EGFR-ECD$_3$ increased, its absorbance increased, indicating that the amount of EGFR-ECD$_3$ bound to the plate gradually increased. To fit the curve, the EC_{50} of EGF binding to EGFR-ECD$_3$ was 1.163 μg/mL and the minimum saturation concentration was 2.5 μg/mL (Fig. 2). The experiments have therefore shown that the EGFR-ECD$_3$ protein obtained herein has a biological activity that strongly binds to EGF.

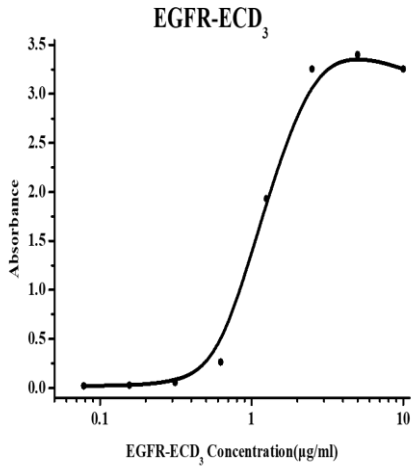

Fig. 2. EGFR-ECD3 protein affinity identification

3.3 EGFR-ECD$_3$ effectively inhibited A549 cells proliferation induced by EGF

EGFR- ECD$_3$ effectively inhibited tumor cell proliferation induced by EGF. The effect of EGFR-ECD$_3$ on proliferation of A549 cells induced by EGF was examined using a CCK8 assay. EGFR-ECD$_3$ has a significant anti-proliferative effect on A549 cells induced by EGF, with an inhibition rate as high as 65%, and a dose-dependent effect is observed (Fig. 3A-B).

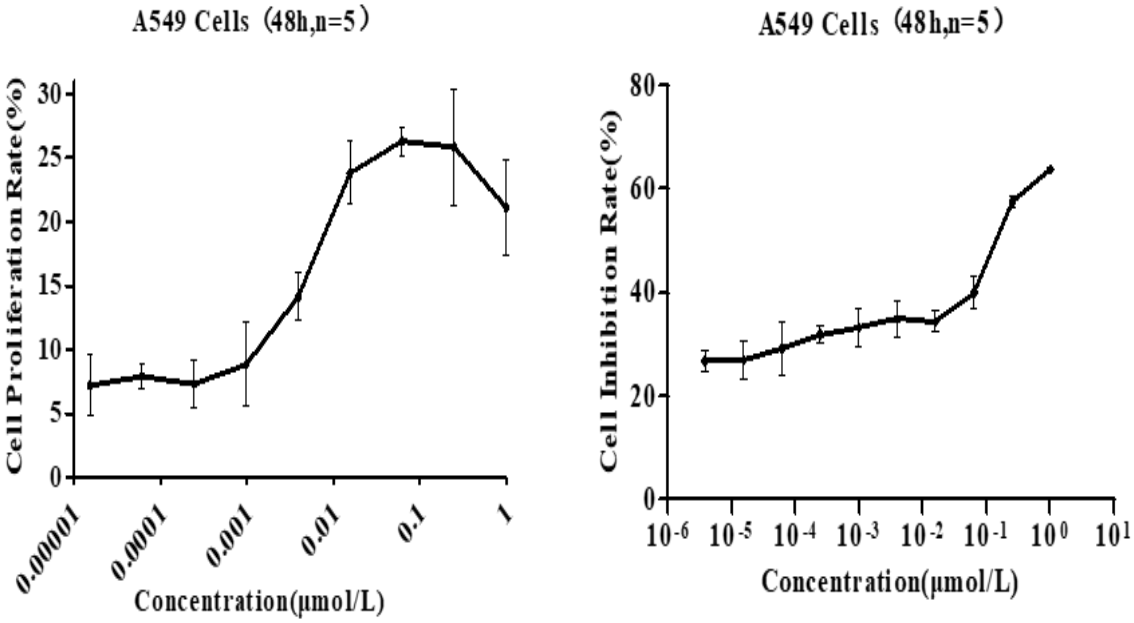

Fig. 3 EGFR-ECD3 inhibits the proliferation of A549 cells

A: EGF can promote A549 cell proliferation.

B: EGFR-ECD$_3$ inhibition of A549 cell proliferation caused by EGF.

3.4 EGFR-ECD$_3$ inhibits phosphorylation of EGFR on the surface of A549 cells

A Western Blot assay was used to determine the effect of EGFR-ECD$_3$ on EGF-induced endogenous EGFR phosphorylation in A549 cells. When the concentration of EGF in A549 cells is 0.0625 μmol/L, the phosphorylation of EGFR protein on the cell surface was significantly increased. When both EGF and EGFR-ECD$_3$ concentrations are used 0.0625 μmol/L, the phosphorylation level of EGFR protein on the cell surface was significantly inhibited. Therefore, all of the above experiments indicated that the EGFR-ECD$_3$ protein obtained in these experiments has specific biological activity (Fig. 4A-D).

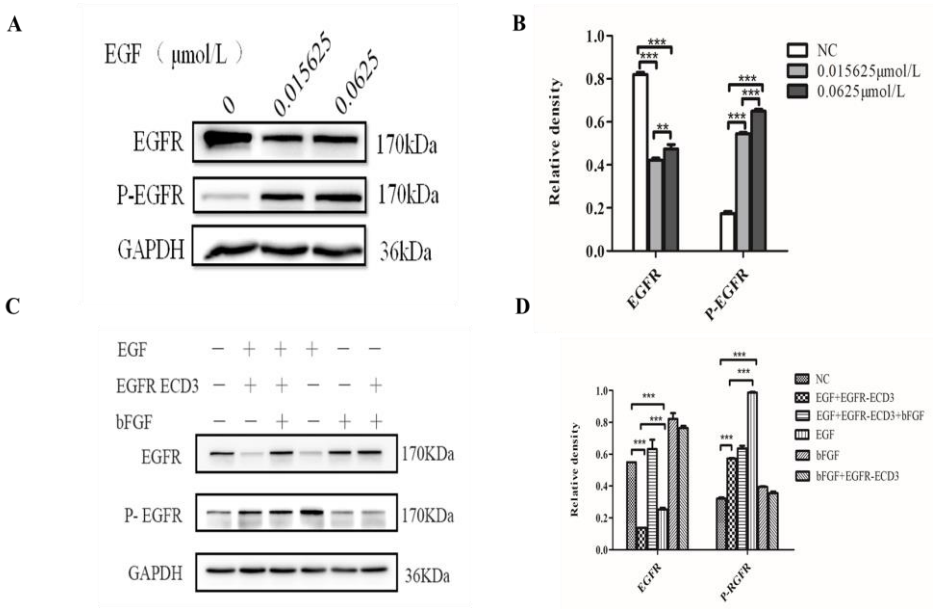

Fig.4. EGFR-ECD$_3$ inhibits p-EGFR protein on the surface of A549 cells induced by EGF

A: EGF can promote phosphorylation of EGFR protein on the surface of A549 cells.

B: Image J analysis of A.

C: EGFR-ECD$_3$ inhibits p-EGFR protein on the surface of A549 cells induced by EGF.

D: Image J analysis of C.

4. Discussion

The epidermal growth factor receptor EGFR is highly expressed in various tumors, such as lung cancer, colon cancer, head and neck cancer, and the like. For a long time, China's EGFR-targeted drug market has been monopolized by foreign imported drugs. In 2008, China's first functional monoclonal antibody drug-nimotuzumab used to treat malignant tumors [28]. Approved to go public, breaking the foreign monopoly for the first time. EGFR has long been a hot research target.

In this study, a large number of high-purity, high-activity EGFR-ECD$_3$ proteins were prepared, which was the first step in obtaining a highly bioactive anti-EGFR- ECD$_3$ antibody by screening a large-volume natural phage library. pCMV is a eukaryotic vector that expresses EGFR-ECD$_3$ to the culture supernatant. Currently, researchers have expressed EGFR-ECD protein in *E. coli* BL21. However, proteins expressed by *E. coli* BL21 exist as inclusion bodies that need to be purified and renatured to become bioactive soluble proteins. Therefore, in this study we used the HEK293F eukaryotic expression system. The pCMV-EGFR-ECD$_3$ transfected HEK 293F cells were detected by Western Blotting and the results showed that EGFR-ECD$_3$ was successfully expressed in the supernatant. Proteins were purified using protein A Ni-NTA affinity chromatography. Protein purity was verified by SDS-PAGE.

At present, EGFR monoclonal antibodies have some side effects. But nanobody have good stability and high affinity, and overcome the shortcomings of monoclonal antibodies. It also has low molecular weight, low immunogenicity and strong tissue penetration. With these advantages, it has great application prospects in disease diagnosis and treatment, and has great potential to become a tumor means. We can use HEK293F cells to express EGFR-ECD$_3$ and then screen for specific nanobodies against EGFR-ECD$_3$ using phage display technology.

Acknowledgements

The research was supported by Guangdong Provincial Science and Technology Program (2015A020211016).

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