



# Expression and Evaluation of HuscFv Antibody -PE40 Immunotoxin for Target Therapy of EGFR-Overexpressing Cancers

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**Background:** Epidermal growth factor receptor (EGFR) plays an important role in the progression and tumorigenesis of the various cancers. In this regards, anti-EGFR antibodies are valuable approved therapeutics for the EGFR over-expressing cancers. However, the occurrence of mutations in the *EGFR* and/or *KRAS* genes; a common phenomenon which is seen in many cancers, lead to the resistance to the EGFR-directed antibodies. EGFR based immunotoxins are capable of overcoming this limitation by directing the toxin moieties to the cancer cells resulting in cell death.

**Objectives:** In the present study, a novel immunotoxin consisting of the truncated *Pseudomonas* exotoxin A (PE-40) and anti-EGFR huscFv was developed and evaluated for the induction of cell death in EGFR positive A431 tumoral cells.

**Materials and Methods:** PE-40 fragment of the exotoxin A was amplified by using PCR and ligated to pET22b-huscFv. The reaction was confirmed by PCR and restriction digestion. The immunotoxin was expressed in *E. coli* BL21 (plysS) and then was purified by Ni-NTA affinity column. Subsequently, the toxicity of the purified immunotoxin was evaluated on EGFR over-expressing epidermoid carcinoma of skin, A431 cell line.

**Results:** PCR and restriction digestion experiments have verified the integrity of the immunotoxin construct. Purification by affinity column resulted in a highly purified recombinant immunotoxin. MTT assay revealed the growth inhibitory effect of the huscFv-PE40 immunotoxin on EGFR-over-expressing A431 cells with an IC<sub>50</sub> value of 250 ng.mL<sup>-1</sup>.

**Conclusion:** In conclusion, the results indicated that the immunotoxin developed in this study has a high toxicity on the EGFR-over-expressing tumor cells and could be considered as a promising candidate for the treatment of the EGFR positive cancers.

**Keywords:** Cancer target therapy, EGFR, HuscFv, Immunotoxin, *Pseudomonas* exotoxin A

## 1. Background

Immunotoxins are cytotoxic proteins that were emerged as a modern strategy for the cancer treatment (1). These proteins consist of the two moieties, a targeting moiety, and a toxic portion. Antibodies are among the common targeting moieties used in immunotoxin (IT)

preparations due to their specific bindings to the targets. Various toxic agents have been used in immunotoxins including chemical and biological toxins (1, 2).

Bacterial exotoxins derived from *Pseudomonas aeruginosa* (*P. aeruginosa*; PE) and *Corynebacterium* (*C.*) *diphtheriae* are the two most common toxins used

in immunotoxins are under clinical trials. Both of these exotoxins inhibit the elongation step of protein synthesis with effect on ADP-ribosylate elongation factor 2 (3). Pseudomonas exotoxin (PE) is composed of 613 amino acids and has three structural and functional domains. The N-terminal domain Ia is a cell binding domain. Domain II is translocation domain and mediates toxin translocation into the cells, and the domain III is the main catalytic part of the PE toxin. PE-40 is a derivative of the PE in which the cell binding domain has been deleted. In immunotoxins, an antibody replaces the cell binding domain of the toxin, and hence the immunotoxins only bind to the cells displaying targets for antibody moiety. PE immunotoxins enter into the cytosol with KDEL sequence at the C-terminal domain of the PE toxin (4-7).

Immunotoxins bind to the target cells via surface antigens on target cells such as EGFR (Epidermal growth factor receptor). EGFR is a member of receptor tyrosine kinase family of Erb that is over-expressed in several cancers. Upon ligand binding to EGFR, this receptor autophosphorylates and starts a cascade of signaling pathways that result in the cell proliferation (8). EGFR is over-expressed in many cancers such as ovary, pancreas, colorectal, brain, breast, kidney, lung, prostate, head and neck, and bladder (9, 10). Various strategies are used for targeting EGFR, including tyrosine kinase inhibitors via binding to the EGFR intracellular domain and inhibition of the ligand binding through interaction with the EGFR cell surface domain. At present several approved monoclonal antibodies (mAbs) are in use for colorectal and head and neck cancers. It's been shown that these antibodies bind to the extracellular domain of the EGFR and block EGFR (11-13). There are five mAbs against human epidermal growth factor receptor family (Trastuzumab, Pertuzumab, Cetuximab, Panitumumab, and Nimotuzumab) (14). Cetuximab (c225) is a chimeric anti-EGFR antibody that consists of the constant regions from human IgG and variable regions of the murine origin (15). This antibody recognizes EGFR with an affinity of two-times higher than its native ligand; EGF (16, 17). In spite of multiple advantages of the cetuximab, it has been shown to have several disadvantages such as immunogenicity due to its non-human origin and poor penetration into the solid tumors due to its large size (18, 19). There are various strategies that have been developed to overcome these limitations. Production of the small generation of the antibodies like single chain fragment variable (scFv) is used to overcome the size-related problems(8). Also, humanization of

the antibodies by replacing murine framework parts of the antibodies with the human counterpart is used as an alternative solution for the problem associated with the immunogenicity(20). It has also been shown that the efficiency of anti-EGFR antibodies is affected by various mutations in *EGFR* and *K-ras* genes (20). This limitation could be overcome by immunotoxins that exerts direct toxicity to the target cells.

## 2. Objectives

The aim of this study was to develop huscFv-PE-40 immunotoxin and evaluate its toxicity on EGFR over-expressing tumoral cell line A431 as a model.

## 3. Material and Methods

### 3.1. Bacterial Strains, Plasmids, Culture

*P. aeruginosa* was obtained from DSMZ, GmbH, Germany. Nutrient agar medium and Luria-Bertani (LB) were obtained from Merck and Quelab, respectively. The *E. coli* BL21 (plys S) and pET-22b expression vector were purchased from Novagene, USA.

### 3.2. PCR Amplification of ExoA-PE40 Fragment

*Pseudomonas aeruginosa* was cultured on nutrient agar medium (15 g agar, 5 g peptone, and 3 g meat extract; the final pH was adjusted at 7.3) overnight at 37 °C. The total genomic DNA of *P. aeruginosa* was extracted with DNA extraction kit (Gene All, Korea). The PE-40 fragment of PE was amplified using primer set: PE-40-forward, 5'-TTCCAAGCTTACCCGTCATC GCCAGCC-3' and PE-40-reverse 5'-CTCG AGTTCTTCAGGTCCTCGCGCG-3' containing restriction sites for *Hind*III and *Xho*I on their 5' ends, respectively.

The Phusion® Master Mix kit with GC Buffer (Bio Lab, New England) was used for PCR amplification of this fragment. The thermo-cycler program was 98 °C for five min followed by 30 cycles of 98 °C for 30 s, 58 °C for 1 min, 72 °C for two min, and Finally 72 °C for 10 min.

### 3.3. Cloning of HuscFv- PE-40

Cloning of fusion gene was carried out by ligation. The recombinant vector PET22b-huscFv has previously been prepared in our laboratory (19). The PET22b-huscFv vector was digested with *Hind* III and *Xho*I and then was ligated to the PE-40PCR product digested with the same enzymes. Then, the recombinant huscFv-PE-40 was transformed into *E. coli* DH5α competent cells. The insert-positive clones were confirmed by PCR, restriction digestion, and sequencing.

### 3.4. Expression of Recombinant HuscFv- PE-40 Immunotoxin

For expression of huscFv-PE-40, the recombinant pET22b (+) plasmid was transformed into *E. coli* BL 21(plys S) strain. A 200 mL bacterial culture containing the recombinant pET22b (+) was prepared in LB media containing 100 mg.mL<sup>-1</sup> ampicillin in 37 °C. When the OD of the cell culture was reached to 0.7, isopropyl b-D-1-thiogalactopyranoside (IPTG) with a final concentration of 1 mM was added and the culture was continued for further four hours. The cell pellet was collected by centrifugation for 15 min, and the resultant 206 mg of the wet weight of cells was resuspended in 3 mL of the lysis buffer (100 mM NaCl, 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM PMSF, pH 8.0). The resuspended bacterial cell was disrupted by sonication for 30 times (30s pulse with 30s intervals) on ice. The cell lysate was centrifuged (12000 ×g, 30 min, 4 °C), and the resultant supernatant and pellet fractions were tested for the presence of huscFv-PE-40 using 10% SDS-PAGE followed by Coomassie blue staining.

### 3.5. Protein Purification and Refolding From Inclusion Body

Ni-NTA affinity chromatography method (Qiagen, Chatsworth, CA, USA) was used for His-tagged fusion protein purification according to the manufacturer's instructions. Purification was performed under denaturing condition using 8 M urea for denaturation. The sonicated pellet (inclusion body) was washed three times with PBS + 1% Triton X-100 and was dissolved in 4-6 mL of 8 M urea lysis buffer and centrifuged for 10 min at 9000 ×g. The supernatant was then mixed with 2 mL resin, incubated at room temperature for 30 min and transferred to the column. The unwanted protein contamination was washed away from the column using wash buffer (20 mM sodium phosphate, 300 mM NaCl, 50 mM imidazol, pH 8.0). The protein of interest was then collected from the column by using elution buffer (20 mM sodium phosphate, 300 mM NaCl and 250 mM imidazole, pH 8.0).

For refolding, the stepwise dialysis method was used (21) and the output was checked by SDS-PAGE and Coomassie blue staining. The purity of the protein was analyzed by SDS-PAGE and protein concentration was assessed by Bradford's method.

### 3.6. Indirect ELISA for Antigen-Binding Activity of Anti-EGFRHuscFv- PE-40

The specificity and reactivity of the refolded huscFv-PE-40 were determined by indirect ELISA. The A431 cells were used as the EGFR-positive and CHO cells

were used as the EGFR negative control. Briefly, 10<sup>5</sup> cells.well<sup>-1</sup> were cultured in the culture plates overnight. Cells were fixed in 10% formalin-PBS (pH 7.4) for 15 min at room temperature (RT) and washed three times with 1% BSA-PBS. Then the plate was blocked by 3% BSA-PBS for 1 h at room temperature and subsequently was washed three times. Different concentrations of the refolded huscFv-PE-40 were added to the plate, incubated for 1.5 h and washed. Then, the plate was incubated with HRP-conjugated anti-human antibody (1:2000 dilution in 1% BSA-PBS) for 1.5h and subsequently was washed. Finally, the TMB substrate was added and the OD was read at 450 nm.

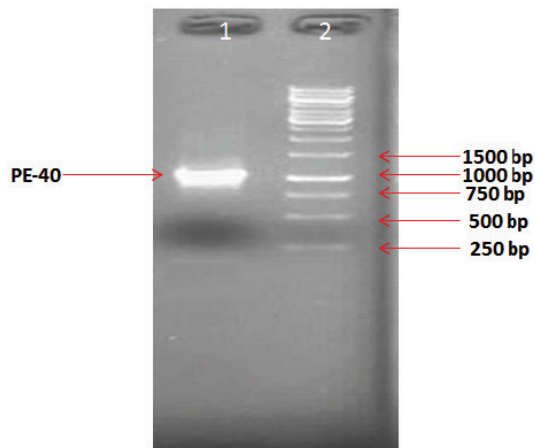
### 3.7. MTT Assay for Growth Inhibition Assessment

The ability of huscFv-PE-40 for inhibiting cancer cell lines was assessed on EGFR- over-expressing A431 cell line by MTT method. EGFR negative CHO cells were used as a negative control. The cell lines were coated in 96-well tissue culture plates (0.5 × 10<sup>5</sup> cells. well<sup>-1</sup>) for 24 h at 37 °C in a 5% CO<sub>2</sub> atmosphere. The plate was divided into 3 groups: group 1, including different concentrations of huscFv-PE-40 (ranging from 5 to 0.078 µg.mL<sup>-1</sup>); group 2, the antibody control group including various concentrations of huscFv and, group3, the negative control group including 200 µL of the growth medium alone. The plates were incubated for 72 h in a 5% CO<sub>2</sub> atmosphere at 37 °C and then the medium was replaced with 50 µL MTT solution (2.5 mg.mL<sup>-1</sup>) (Sigma , Germany) and 150 µL fresh growth medium. After 4h, the MTT solution was poured out and 150 µL DMSO and 25 µL Sorenson's buffer was added and incubated for 15 min. Finally, the absorbance was measured at the wavelength of 570 nm. All experiments were done in duplicate.

## 4. Results

### 4.1. Construction and Expression of the Recombinant Immunotoxin

PCR amplification of the sequence coding for PE-40 of *Pseudomonas aeruginosa* with the specific primer resulted in a PCR product of about 1017 bp (**Fig. 1**). The PCR product was cloned into the *Hind*III- *Xho*I site of the pET22b-huscFv resulted in a pET22b-huscFv-PE40 expression construct that was confirmed by the restriction digestion (**Fig. 2**) and PCR (**Fig. 3**). Several expression strains of the *E. coli* were checked for the expression of recombinant protein among which *E. coli* BL21 (plys S) cells showed a higher expression than the other strains. *E. coli* BL21 (plys S) cells containing pET22b-huscFv-PE-40 were cultured in LB media and the expression of the recombinant huscFv-PE-40 was

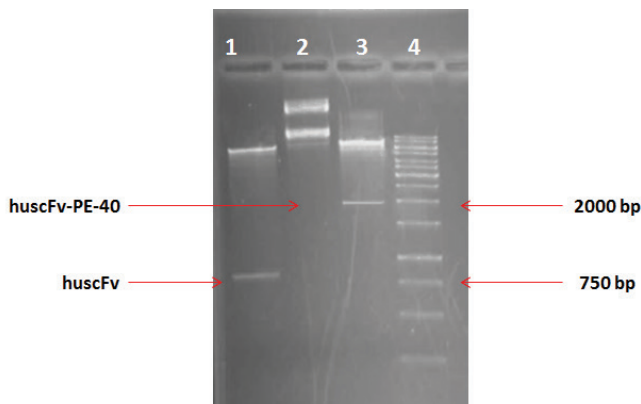


**Figure 1.** PCR amplification of the PE-40 sequence from *Pseudomonas aeruginosa*.

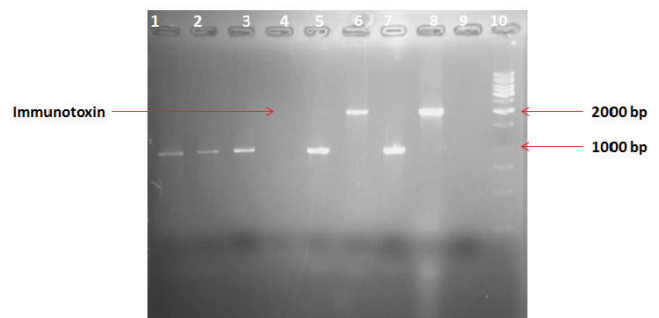
induced by 1mM IPTG at 37 °C for different time course (i.e., hours). The results indicated that the highest level of recombinant IT was expressed at 4h. SDS-PAGE analysis revealed that huscFv-PE-40 expressed as a sharp band of about 66 kDa. The expression analysis of the recombinant IT revealed that most of the protein product was expressed as the inclusion body and appeared in pellet fraction (**Fig. 4**).

**4.2. Purification and Refolding of the Recombinant IT**

For purification and refolding of the huscFv-PE-40, the inclusion bodies were solubilized in 8M urea and purified by affinity chromatography using Ni-NTA resin. This purification method leads to a protein with a purity of about 98% as was appeared in SDS- PAGE



**Figure 2.** Double digestion of the recombinant plasmid pET22b-huscFv-PE-40. Lane 1; pET22b- huscFv-PE-40 double digested with the NdeI-HindIII. Lane 2; Undigested pET22b -huscFv-PE-40. Lane 3; pET22b- huscFv-PE-40 double digested with the NdeI-XhoI. Lane 4; DNA size marker.

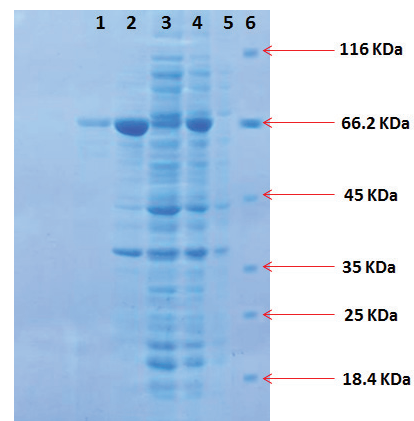


**Figure 3.** Confirmation of the huscFv-PE-40 construction by PCR. Lane 1-3, 5, and 7; indicate huscFv, Lane 6 and 8; immunotoxin (huscFv-PE-40), Lane 9; Negative control, Lane 10; DNA size marker.

analysis of the purified protein (**Fig. 4**). The stepwise dialysis method was used to obtain recombinant IT with correct folding. The Bradford protein assay showed the quantity of the refolded recombinant IT 10 µg.mL<sup>-1</sup>.

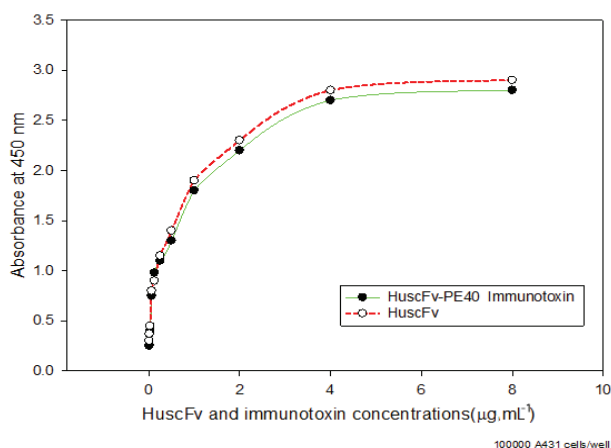
**4.3. ELISA Binding Assay Result**

Analysis of the reactivity of the huscFv-PE-40 on A431 carcinoma cells by ELISA showed that refolded anti-EGFR huscFv-PE-40 is able to recognize and bind to the EGFR on A431 cells surface. The maximum OD in ELISA was obtained at 5 µg.mL<sup>-1</sup> concentration of the refolded huscFv-PE-40 (**Fig. 5**). The 50% binding activity of the recombinant IT and huscFv were calculated as 0.504 and 0.470 µg.mL<sup>-1</sup>, respectively.



**Figure 4.** SDS-PAGE analysis of the expression and purification of the recombinant huscFv-PE-40 immunotoxin in the *E. coli* BL21 (plys S). Lane 1 indicates the purified and refolded huscFv-PE-40 immunotoxin. Lane 2 corresponds to the insoluble fraction. Lane 3 shows the soluble fraction. Lane 4 indicates cell lysates after 4 h induction with 1 mM IPTG. Lane 5 correlates with the cell lysates before induction. Lane 6, protein size marker.

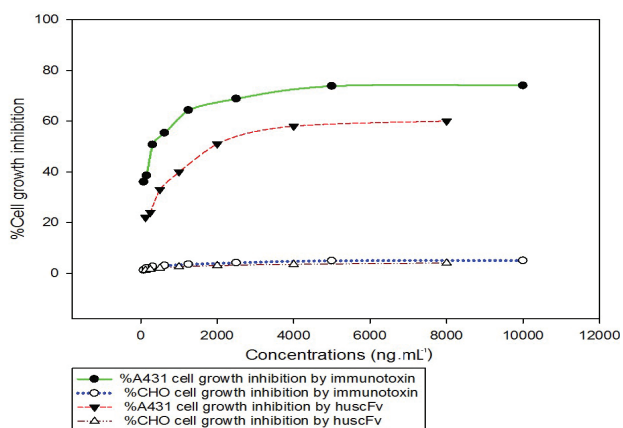




**Figure 5.** The results of cell-ELISA for huscFv-PE-40 immunotoxin and huscFv antibody. The immunotoxin and huscFv antibody showed a similar activity on the EGFR-over-expressing A431 cell line.

#### 4.4. MTT Assay

The cytotoxicity and cell growth inhibitory effect of the huscFv-PE-40 was assessed by MTT assay. Results of the MTT assay demonstrated that 74, 69, 64, 58, 55, 39, and 36 % cell growth inhibition activity could be achieved when amounts of 5, 2.5, 1.25, 0.625, 0.312, 0.156, and 0.078  $\mu\text{g.mL}^{-1}$  of the recombinant IT are applied. The IC<sub>50</sub> values of this immunotoxins and huscFv were 250 ng and 2000  $\text{ng.mL}^{-1}$ , respectively. The immunotoxin showed no reactivity and growth inhibitory effect on EGFR-negative CHO cell line (**Fig. 6**).



**Figure 6.** The cell growth inhibition assay of the huscFv-PE-40 immunotoxin and huscFv antibody by applying MTT test. HuscFv-PE-40 and huscFv antibody showed the inhibitory effect on the A431 cells (EGFR-over-expressing cell line), while, they had not such a significant inhibitory effect on the EGFR-negative CHO cells. The immunotoxin showed a significantly higher growth inhibitory effect on the A431 cells compared to the huscFv antibody.

## 5. Discussion

The severe side effects of the conventional cancer therapies encouraged the scientists to look for new the innovate strategies. Immunotoxins are the novel class of the therapeutics in which the therapeutic agents directly target the cancer cells with no or minimal effect on the normal cells (22). The success of this method is highly dependent on the specificity of the biomarker on the target cancer cells. These markers are proteins that are expressed solely or over-expressed in tumor cells and are involved in the cancer cell multiplication and metastasis (23, 24).

EGFR is a transmembrane protein that plays an important role in the proliferation and metastasis of the several cancers. This property has proposed this molecule as a promising target for the use of cancer targeting therapy. Considering the specificity of the antibodies, anti-EGFR antibodies have been attempted for cancer treatment. In the present study, a humanized fragment antibody was used for development of the immunotoxin for targeting EGFR over-expressing tumoral cells.

The immunogenicity and penetration potency are two important limitations in the immunotoxins therapy. Humanized or human versions of small antibody fragments such as scFv have been suggested as a solution for these limitations (22). Zhou *et al.* (2012) was used an anti-EGFR EGF scFv for treating non-small cell lung cancer (NSCLC) (23). In our study, an anti-EGFR huscFv antibody fused to the modified PE toxin (PE-40) by a peptide linker was used for IT preparation. Safdari *et al.* (2014) have produced a humanized version of anti-EGFRvIII antibody. They have reported that the affinity of the humanized scFv (humMR1) was 22-times higher than that of parental scFv (25). Akbari *et al.* (2016) have reported that humanization of the anti-EGFR scFv is associated with a decreased immunogenicity while the affinity of the huscFv was 2.5 times higher than murine counterpart (20). In agreement with the above reports, our results also indicated that fusion of PE40 to the anti-EGFR huscFv did not reduce the affinity of the antibody moiety.

In the present study, the correct folding and anti-tumor activity of the immunotoxin was confirmed by ELISA and MTT assay. The results of ELISA showed that the refolded immunotoxin recognizes A431 cells with high affinity ( $\text{OD}_{50\%} = 0.504 \mu\text{g.mL}^{-1}$ ). Our results were in agreements with several other previous studies. Alderson and colleagues (2009) have developed an anti-CD22 immunotoxin which has high affinity to its target (26). Chandramohan *et al.* (2014) reported that anti-EGFRwt and EGFRvIII recognize their target

with high affinity (27). In this study, the results of MTT showed that our immunotoxin has a high capability to inhibit EGFR positive tumor cells with an IC<sub>50</sub> around 250 ng.mL<sup>-1</sup> compared to the huscFv with IC<sub>50</sub> around 2 µg.mL<sup>-1</sup>. These data revealed that the immunotoxin was more effective than huscFv in the inhibition of the EGFR-over-expressing A431 cell line. This finding is not surprising because PE based on immunotoxins induce cell death by inhibition of the protein synthesis via ADP-ribosylation of the EF2. Our results were inconsistent with the several previous studies. Lv *et al.* (2015) produced an anti-EpCAM immunotoxin and reported that scFv2A9-PE immunotoxin with an IC<sub>50</sub> of 50 pM show growth inhibition on EpCAM positive cells (22). In 2013, Chandramohan *et al.* have reported that D2C7-(scdsFv)-PE38KDEL immunotoxins show IC<sub>50</sub> of 0.18 to 2.5 ng.mL<sup>-1</sup> on cells expressing EGFRwt (27). Krietman *et al.* (2011) have also reported that anti-CD22 immunotoxin has a high growth inhibition effect on the CD22 positive cells with IC<sub>50</sub> of 2-30 ng.mL<sup>-1</sup> (28).

In conclusion, the results of this study showed that huscFv-PE-40 immunotoxin developed in this study have the binding affinity and proliferation inhibitory effect on EGFR-over-expressing A431 cell line. This immunotoxin could be considered as a potential alternative tool for the treatment of the cancers that over-express EGFR marker.

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