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Isolation and characterization of a novel human scFv inhibiting EGFR vIII expressing cancers

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ABSTRACT

EGFRvIII, a mutant form of epidermal growth factor receptor is highly expressed in glioblastoma, carcinoma of the breast, ovary, and lung but not in normal cells. This tumor specific antigen has emerged as a promising candidate for antibody based therapy of several cancers. The aim of the present study was isolation and characterization of a human single chain antibody against EGFRvIII as a promising target for cancer therapy. For this, a synthetic peptide corresponding to EGFRvIII protein was used for screening the naive human scFv phage library. Selection was performed using a novel screening strategy for enrichment of rare specific clones. After five rounds of screening, six positive scFv clones against EGFRvIII were selected using monoclonal phage ELISA, among them, a clone with an amber mutation in VH CDR2 coding sequence showed higher reactivity. The mutation was corrected through site directed mutagenesis and then scFv fragment was expressed after subcloning into the bacterial expression vector. Expression in BL21 pLysS resulted in a highly soluble scFv appeared in soluble fraction of *E. coli* lysate. Bioinformatic in silico analysis between scFv and EGFRvIII sequences confirmed specific binding of desired scFv to EGFRvIII in CDR regions. The specific reactivity of the purified scFv with native EGFRvIII was confirmed by cell based ELISA and western blot. In conclusion, human anti- EGFRvIII scFv isolated from a scFv phage library displayed high reactivity with EGFRvIII. The scFv isolated in this study can be the groundwork for developing more effective diagnostic and therapeutic agents against EGFRvIII expressing cancers.

1. Introduction

Epidermal growth factor receptor (EGFR) as a transmembrane tyrosine kinases receptor is a crucial regulator of normal cellular growth in epithelial tissues [1].

Dysregulated EGFR signaling as a result of overexpression or mutation is one of the main factors involved in epithelial malignancies [2]. EGF receptor variant III (EGFRvIII) with molecular mass of virtually 145 kDa is expressed mostly in glioblastoma, and in carcinoma of the ovary, breast, and lung whereas normal tissues lacking EGFRvIII [3,4]. This common mutant variant of EGFR is the result of deletion in exons 2–7 of the coding sequence and junction of exons 1 and 8 through a novel glycine residue [5,6]. This mutation creates a tumor specific immunogenic epitope and leads to unregulated growth, angiogenesis, survival, and invasion [7].

In recent years, many murine antibodies such as MR1, MR1-1, L8A4, and 3C10 have been developed against EGFRvIII; most of them have cross reactivity with EGFR wild type or have lower affinity to EGFRvIII. On the other hand, unfavorable HAMA responses induced by the murine origin of these antibodies limit their therapeutic applications [4,5,8].

Although, the presence of the Fc region in full length antibodies can be useful in antibody based therapies; the large size of these antibodies limits their efficiency for treatment of solid tumors, due

Abbreviations: Mabs, Monoclonal antibodies; Tomlinson I+J, The Human scFv phage libraries I+J; scFv, single-chain variable fragment; MPBS, Marvel milk powder in PBS; IPTG, β-D thiogalactopyranoside; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DAB, chromomeric substrate – 3, 3′diaminobenzidine; MW, molecular weight; PVDF, poly vinylidine difluoride membrane.

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to weak penetration into the solid tumors. So small fragments of antibodies like scFvs with higher tissue penetration rate are considered as suitable therapeutic or diagnostic reagents [9].

In contrast to the conventional methods used to generate antibodies, scFv phage libraries with high diversity of gene repertoires provide a rich source of scFvs to almost any antigen [10,11].

The present study aimed to isolate a novel human scFv against EGFRvIII using phage display technology as a potential candidate for treatment of EGFRvIII expressing cancers.

2. Material &methods

2.1. ScFv-Phage library, bacteria, and reagents

The Human scFv phage libraries I+J (Tomlinson I+J), HB2151and KM13 helper phage [12], (The Medical Research Council (MRC), Cambridge, UK) and, *E. coli* TG1were used for isolation of specific antibody clones and production of scFvs [12–14].

2.2. Synthetic peptide

The synthetic peptide LEEKKGNYVVTDHSGGK was selected from the N terminal region of EGFRvIII in which the first 13 residues is related to the tumor-specific deletion junction sequence and the SGG sequence served as a flexible spacer [5]. This peptide was synthesized with 97.2% purity (Biomatik, Life science).

2.3. Biopanning of scFv phage library

Biopanning process was performed with 50 μg/ml EGFRvIII peptide on a Maxisorp 96-well plate (Nunc Thermo Scientific Inc., Rochester, NY) in 50 mM carbonate buffer (pH 9.6). In beginning, the stock of libraries I & J were combined together then were amplified and titrated as described by the Medical Research Council (MRC) protocol [15]. During biopanning rounds, the concentration of EGFRvIII peptide was kept constant to avoid the loss of rare specific clones. Since, a significant part of antigen is often desorbed from the solid phase during blocking and washing processes, low antigen concentration can lead to reduction of phage yield [15,16]. Hence, in this study in order to increase the output of screening steps; we didn't reduce peptide concentration during biopanning rounds.

However, for increasing the screening stringency, incubation time of the pool phages with antigen were decreased while washing numbers were increased between screening rounds (Table 1). After washing EGFRvIII- immobilized plate with PBS and blocking with 3% Bovine Serum Albumin (BSA) for 2 h, 10^{12-13} pfu phages diluted with blocking solution were added. The plate was incubated on a platform shaker (Heidolph Titramax 1000) with speed of 150 rpm for 30 min at room temperature (RT) and then stood for further 30 min at RT. Afterward, plate was washed with PBS containing Tween 20 (PBST), the bound phages were eluted by treatment with 100 μl/well of trypsin-PBS (100 μl of 10 mg/ml trypsin stock solution in10 ml PBS) and incubating for 15 min at RT on a platform shaker (150 rpm). Details related to the phage incubation period and washing number is presented in Table 1. All the titration, amplification and purification processes of phages were carried out as described elsewhere [15].

Totally, five rounds of biopanning were carried out to select EGFRvIII −specific phage clones. In round 4, before starting screening, an invert biopanning against BSA was performed to deplete sub library from specific clones to BSA then, the depleted library from BSA binders was added to the EGFRvIII- immobilized wells [17].

To perform invert biopanning, in a 96-well Maxisorp plate, 16 wells were coated with 3%BSA, and incubated overnight at 4 °C. After washing plate with PBS, the eluted phages from 3th round (diluted in PBS) were added to BSA-immobilized wells and incubated for 1hr at RT. The depleted library from BSA binders was then added to the EGFRvIII- immobilized wells and incubated for 1hr at RT. After washing with PBST, the bound phages were eluted by trypsin as detailed above [17].

2.4. Specificity analysis of selected phages by polyclonal phage ELISA

To examine the specificity of phages selected from each round of panning, a polyclonal phage ELISA was performed against EGFRvIII peptide. For this purpose, EGFRvIII peptide with concentration of 25 μg/ml and BSA were coated into the plates separately overnight at 4 °C. After blocking with 3% BSA for 2 h and washing with PBST, the eluted phages (1:10 dilution in1% BSA–PBS) were added to plates and incubated for 1hr. After washing, the plates were incubated with anti-M13- HRP (1:2000 dilutions in 1% BSA–PBS) for 1hr and the reaction was developed with TMB substrate. The optical density (OD) was read at 450 nm.

2.5. Selection of scFvs clones against EGFRvIII peptide by monoclonal phage ELISA

To identify specific scFv clones against EGFRvIII, single colonies from the fifth round of panning were selected randomly, and their reactivity with EGFRvIII peptide was determined using monoclonal phage ELISA. For this purpose, individual colonies were inoculated into 2xTY medium (1.6% [w/v] tryptone, 1% [w/v] yeast extract, and 0.5% [w/v] sodium chloride) containing100 μg/ml ampicillin and 4% glucose in a 96 well plate at 37 °C for 2 h [18,19]. After 2 h incubation, 10^9 helper phages were added to each well and incubated for 1hr at 37 °C without shaking. Then plate was spined in 3000 g for 10 min, aspirated off the supernatants and the bacteria pellets were resuspended in 2xTY containing 100 μg/ml ampicillin and 50 μg/ml kanamycin. Cultures were continued overnight at 30 °C with shaking (250 rpm) and culture supernatants (1:2 dilution in1% BSA–PBS) were used in phage ELISA. So that, EGFRvIII peptide with concentration of 25 μg/ml was coated into the ELISA plates overnight at 4 °C. After blocking with 3% BSA for 2 h and washing with PBST, the eluted phages (1:2 dilution in 1% BSA–PBS) were added to the plates and incubated for 1hr. After washing, the plates were incubated with anti-M13- HRP (1:2000 dilutions in 1% BSA–PBS) for 1hr and the reaction was developed with TMB substrate. The optical density (OD) was read at 450 nm.

2.6. PCR amplification and sequence analysis

The presence of both of VH and VL fragments in selected phage clones was examined using PCR amplification. For this, plasmid DNA were extracted from positive phages clones and PCR amplification was performed using pIT2-vector specific primers: Forward: 5′-CAGGAAACAGCTATGAC-3′, Reverse: 5′-CTATGCGGCCCCATTCA-3′in a 25 μl reaction for 31 cycles (94 °C for 30s, 58 °C for 30s, 72 °C for 45s) after an initial denaturation at 94 °C for 4 min. Products were analyzed on%1 (w/v) agarose gel. The positive clones with confirmed size were sequenced using pIT2-vector specific primers and sequences were then analyzed with V-BASE (http://vbase.mrc-cpe.cam. ac.uk/) and Ig BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi) using the Kabat numbering system to identify the complementarity determining regions(CDRs) in heavy chain (VH) and light chain (VL) variable regions [20].