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Generation and Characterization of a Recombinant Human Epidermal Growth Factor Receptor Extracellular Domain III Nanobody

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Abstract

Epidermal growth factor receptor (EGFR), a membrane surface protein with tyrosine kinase activity, is highly expressed in various human tumor cells. Despite breakthroughs being made in EGFR targeted therapeutics, nanobody (a single-domain antibody) is still considered a suitable candidate for development as EGFR inhibitors. Here, we previously produced and stored the recombinant EGFR extracellular domain III (EGFR-ECD3). In this study, nanobodies targeting EGFR-ECD3 were screened from a natural phage display nanobody library with 6.68×1011 CFU/Total. A positive clone, Nb_{H3}, which could specifically bind to EGFR-ECD3 with EC50 of 8.7619 μg/ml and negligibly cross-react with Her2 proteins in the same family, was selected. And NbH3 dose-dependently inhibited the proliferation of A549 cells induced by EGF and down-regulated the corresponding level of p-EGFR. The results suggested that the EGFR-targeted nanobody is promising for tumor treatments.

Keywords

EGFR, nanobody,· phage display library,·A549 cell,·proliferation inhibition.

1. Introduction

Receptor tyrosine kinases (RTKs) are widely distributed cell surface receptors and regulate nearly all kinds of various processes of life activities, like cell growth and differentiation, cell cycle regulation, and metabolism[1-3]. The epidermal growth factor receptor (EGFR or Her1 / ErbB1) is a transmembrane glycoprotein with a molecular weight of 170 kDa, and its gene is located on the short arm of chromosome 7. EGFR belongs to the receptor tyrosine kinase family in normal epidermal cells and possesses vital function in growth and differentiation[4-6]. The EGFR family contains four structurally similar receptors: EGFR (ErbB1, HER1), ErbB2 (HER2), ErbB3 (HER3), and ErbB4 (HER4). Overexpression and mutation of EGFR implicate in lung cancer, breast cancer, gastric cancer, colorectal cancer, head and neck cancer, pancreatic cancer, and glioblastoma, which stimulate tumor cell growth, cell proliferation, angiogenesis, cell invasion, and metastasis, etc. EGF/EGFR participates in the regulation of tumor cell metabolism and can be used to facilitate the prognosis of various tumors[7]. EGFR is divided into three regions: the extracellular ligand-binding region, the transmembrane region, and the intracellular kinase region. Presently, there are mainly chemical drugs and biopharmaceuticals that can act on EGF/EGFR. EGFR-TKI, a kind of epidermal growth factor receptor tyrosine kinase chemical inhibitor, enters cells through ion channels to competitively bind the receptor intracellular tyrosine kinase domain, to prevent autophosphorylation. The first generation of

ISSN: 1813-4890

EGFR-TKI is a reversible tyrosine kinase inhibitor but susceptible to acquired resistance, and its molecular mechanism is mostly through a mutation to the EGFR gene at exon20 T790M. The effect of the second-generation EGFR-TKI on overcoming T790M resistance was not satisfactory. The third-generation EGFR-TKI overcame T790M resistance but then acquired C797S mutations which also cause resistance and the fourth generation of EGFR-TKI is currently under investigation[8-12]. It still exists obstacles to chemical EGFR inhibitors development. The biopharmaceuticals, like monoclonal antibodies, mainly target the extracellular region of EGFR. Monoclonal antibodies, like nimotuzumab, cetuximab, panitumumab, and necitumumab, competitively inhibit ligand binding to EGFR, preventing receptor activation and downstream signal transduction, finally restaining tumor cells proliferation, invasion, and angiogenesis. But the defects of antibodies in difficult penetrating solid tumor cells, costly large-scale production, and specific immunogenicity make its development limited[13-17]. Nanobodies with small molecular weight, low immunogenicity, high expression and stability, possess the property superior to monoclonal antibodies, which gradually come into focus[18-21]. EGFR-ECD3 is the main binding domain of EGF targeting EGFR, EGFR-ECD3 nanobody is a potential choice. Here, this study reports a nanobody with high binding specificity and thermostability for EGFR-ECD3, screened by a large-capacity non-immune phage nanobody library.

2. Materials and Methods

2.1. Materials and Reagent

A naive phage nanobody library was supplied by our laboratory. HRP anti-his mouse antibody, goat anti-rabbit IgG-HRP antibody, anti-human EGFR rabbit antibody, anti-pEGFR rabbit antibody, the large-capacity natural phage nanobody library, VCSM13 helper phage, E. coli strain WK6, and E. coli strain TG1 were in our laboratory.

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. TBS was made by dissolving Tris-base 2.423 g and NaCl 8.006 g in 1 L of deionized water.

2.3. Cell Culture

Human non-small cell lung cancer cells A549 (ATCC® CCL-185™) were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). A549 cell line was maintained in DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and grown at 37°C in 5% CO_2.

2.4. Screening, Expression, and Purification of Nanobodies Targeting EGFR-ECD_3

(1) Amplification of the natural phage nanobody library. Took 100 ml phage nanobody library (6.68×1011 CFU/Total) and added to E. coli TG1 solution in the logarithmic growth phase, and the mixture was allowed to rest for 30 min and then supplemented with 2×YT/Amp-Glu medium for shaking culture. The helper phage was added and shaken, and kept at room temperature for 30 min, before being centrifuged, and the supernatant discarded. Then resuspended in 2×YT/Amp-Kan medium and shaken overnight. The overnight culture was centrifuged, the supernatant was added to a volume of PEG/NaCl solution and mixed in an ice bath for 30 min, before being centrifuged again. The phage pellet was resuspended in sterile PBS, centrifuged, and was filtered through a 0.22 μm filter into a sterile EP tube. Freshly prepared phage library preparations were used for panning experiments.

ISSN: 1813-4890

(2) Panning of the natural phage nanobody library. EGFR-ECD3 was diluted with the coating solution to the appropriate concentration, added to a 96-well microtiter plate, and coated at 4 °C overnight. The next day, after blocking, 100 μL of the phage amplified library was added to each well and incubated for 1 h at room temperature, and then washed separately 5, 10, 15 times, increasing in increments. Freshly prepared 100 mM triethylamine solution was added and allowed to stand at room temperature for 10 min. The liquid was transferred to a sterile EP tube and an equal volume of 1 M Tris-HCl was added. It constituted a round of phage library after panning. And then repeated the "adsorption-elution-amplification" panning three times. The phage eluted after the third panning was infected with E. coli TG1, and 96 single colonies were randomly picked. After incubation for 1 h in 2YT medium, helper phage VCSM13 (1011 CFU/ml) was added and incubated for 30min at room temperature. Performed Centrifugation and the sediment was resuspended in 2 × YT/Amp-Kan liquid medium and cultured overnight. The supernatant comprises cloned recombinant phage and could be detected by ELISA.

(3) Enrichment verification and screening of EGFR-ECD3 nanobody in the natural phage nanobody library. EGFR-ECD3 was coated at 4°C overnight, and the blank coat was used as a control. After the next day of blocking, the ELISA plate wells coated with the antigen and the corresponding negative control wells were added and incubated at 37°C for 1 h, then the anti-M13-HRP antibody was added and incubated at 37°C for 1 h. 100 μL TMB solution was added per well and incubated at 37°C for 10 min. Finally, 2.29% sulfuric acid was added to stop the reaction. OD450 was measured with a microplate reader. The plate was washed after each round of incubation. The positive clone corresponding to the largest OD value was inserted into a 5 mL LB/Amp medium, cultured to a suitable concentration, and then extracted with a small amount of plasmid to be sent for detection, and sequence analysis to obtain the EGFR-ECD3 nanobody sequence. The pMECS-NbH3 plasmid stock was electrotransferred into E. coli WK6. The transformed bacteria were sequenced, cultured in LB medium containing ampicillin resistance, and when they reached the logarithmic growth phase, IPTG was added at 28°C overnight. The culture was centrifuged, and the pellet was resuspended in PBS. After centrifugation, the cells were resuspended in an appropriate volume of TES and shaken at 200 rpm for 6 h at 4°C. Another volume of TES was added and kept at 4°C, with shaking at 200 rpm overnight. The sample was centrifuged at maximum speed and the supernatant and precipitate were run on SDS-PAGE.

(4) Expression and purification of nanobodies. To purify the target protein, 1 L of the transformed bacterial solution was cultured and induced with IPTG at 28°C, and then extracted as described above. The clarified supernatant was filtered through a 0.22 μm filter, purified using Ni-NTA affinity chromatography column before the column mixture was equilibrated with PBS (pH 7.4). The target protein was then eluted with 200 mM imidazole and the purity was analyzed by SDS-PAGE and western blot.

2.5. Identification of Biological Activity of Nb_{H3} By ELISA Assay

EGFR-ECD3 (at 100 ng/well) was coated overnight at 4°C and the plate was washed with 0.1% PBST and added the blocking solution, the next day. NbH3 was diluted and added to the plate with a concentration of 10 μg/mL for 1 h at 37°C. Then washed with PBST, and added mouse anti-HA antibody for 1 h at 37°C. The plate was washed again with PBST and then added HRP goat anti-mouse antibody for 1 h 37°C. Washed with PBST again, then added TMB for 10 min at 37°C and terminated with dilute sulfuric acid. OD450-630 was measured using a microplate reader and the absorbance reflected the nanobody affinity. Pretreatment of NbH3 was performed at different temperatures (25°C, 37°C, 60°C, 90°C) and at different times (0 min, 10 min, 30 min, 60 min, 120 min, and 180 min). The NbH3 treated under different conditions was subjected to an antigen-antibody reaction, and the stability was measured by the change of the absorbance value, the steps were the same as described for the detection affinity.

ISSN: 1813-4890

2.6. Identification of Biological Activity of Nb_{H3} by CCK8 and Western Blot

The A549 cells in the logarithmic growth phase were seeded in 96-well cell culture plates at 5×103 cells per well. The blank group contained medium only. Pre-culture was performed for 24 h (37°C, 5% CO2), and the cells were 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 for 1 h at 37ºC. OD450-63 was measured with a microplate reader and drew the proliferation inhibition curve. The optimal concentration of EGF and different concentration gradients of NbH3 were determined by the A549 cell proliferation inhibition assay.

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 was added to the plates for 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 and prepared for western blot.

2.7. Identification Nb_{H3} Localization in A549 Cells

A549 cells in the logarithmic growth phase were trypsinized to obtain a single-cell suspension. 1×106 cells/EP tube were centrifuged at 1000 rpm for 5 min. The supernatant was discarded and the pellet was washed twice with 1×PBS, before being treated with the nanobody. The anti-EGFR monoclonal antibody was used as a positive control and a PBS group as a negative control. These were incubated at 37℃ for 1 h, then washed three times with PBS to remove the unbound antibody. FITC-conjugated mouse anti-his antibody and goat anti-rabbit IgG (FITC) were separately added and incubated at 37℃ for 1 h. Data were analyzed using a flow cytometer.

A549 cells in the logarithmic growth phase were inoculated into a 24-well plate at 8×104 cells/well per well, and pre-incubated in a constant temperature incubator (37℃, 5% CO2) for 24 h, then added to 4 % poly formaldehyde for fixation. Then washed with PBS and blocked with 2% BSA. Sequentially incubated with antibodies and imaged using a fluorescence microscope.

2.8. Statistical Analysis

Statistical significance was determined by Student's t-test (two-tailed). All statistical analysis and curve fitting was performed with OriginPro (2019b), and p values were reported (*$p<0.05$; **$p<0.01$).

3. Results

3.1. Nb_{H3} Targeting EGFR-ECD3

3.1.1. Screening Nanobodies Targeting EGFR-ECD3

Nanobodies targeting EGFR-ECD3 were screened from a large-capacity native phage nanobody display library. After three rounds of bio-panning, the phage nanobody library was significantly enriched (Fig. 1A-B). After screening, a positive clone NbH3 (Nanobody H3) was obtained. The cloned phage antibody consists of 122 amino acid residues with a molecular weight of approximately 13 kDa and an isoelectric point (pI) of 7.82. And the phage antibody has four framework regions (FR) and three complementary determining regions (CDRs).

3.1.2. Expression and Purification of Nb_{H3}

NbH3 was next used in the functional experiment, where it was first transformed into E. coli WK6 and overexpressed. The expression of the target protein was induced by 1 mM Isopropyl-β-D-Thiogalactopyranoside (IPTG) at 28℃. Because of its expression in the periplasmic space,

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the recombinant protein is necessary to be extracted by osmotic shock. The results of the SDS-PAGE showed that NbH3 was expressed as a soluble protein at 28℃. Since his tag was present in the pMECS vector, the target protein was purified by affinity chromatography using a Ni-NTA column. The protein was eluted from the Ni-NTA column with 200 mM imidazole to a purity of more than 95% (Fig. 1C). Approximately 5 mg of highly purified NbH3 was recovered from 1 L of recombinant E. coli WK6 culture.

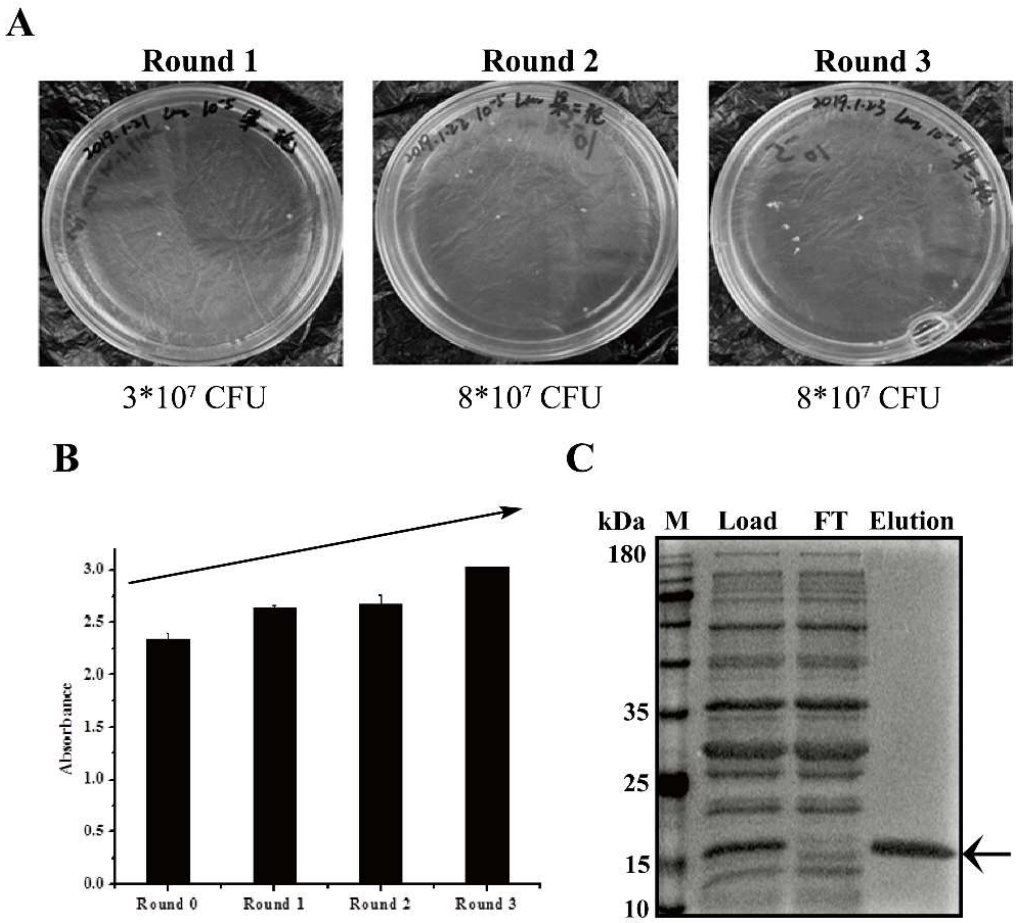

Fig. 1. NbH3 was screened by a large-capacity natural phage library. (A) and (B) show after three rounds of panning, the library was significantly enriched; (C) shows SDS-PAGE analysis of purified NbH3.

3.2. Specificity and Stability of Nb_{H3}

3.2.1. Nb_{H3} has a Biological Activity that Effectively Binds to EGFR-ECD3

The affinity was determined by a non-competitive enzyme-linked immunosorbent assay. 50 ng/well of antigen was coated in a 96-well plate, and the diluted NbH3 was added to the plate. The absorbance was measured and the "S" curve was fitted to obtain half absorbance. The value corresponds to the NbH3 concentration (EC50) is 8.7619μg/mL (Fig. 2A).

3.2.2. Nb_{H3} Specific Binds to EGFR-ECD3

Three proteins, HER2, EGF, and bFGF, were used as control antigens, PBS was used as a blank control, and HRP anti-his tag antibody was used as a positive control. The binding specificity of NbH3 was detected by ELISA. The absorbance value directly reflects the specific-binding ability of NbH3 to EGFR-ECD3 (Fig. 2B). NbH3 has a strong specific binding ability to EGFR-ECD3, and has the weak binding capability to the other three control antigens, and is determined to be an anti- EGFR-ECD3 specific nanobody.

ISSN: 1813-4890

3.2.3. Nb_{H3} with High Thermal Stability

The positive control HRP anti-his tag antibody and NbH3 were placed at 25°C, 37°C, 60°C, 90°C for 10 min, 30 min, 60 min, 120 min, and 180 min, respectively. Samples treated at different temperatures and times were collected, and their binding activity to EGFR-ECD3 was detected by ELISA, to determine the thermostability of NbH3. The relative activities of the HRP anti-his tag antibody and NbH3 were maintained above 90% after treatment at 25°C compared to untreated samples; and the control antibody was 90% at 37°C, the NbH3 decreased but remained above 85%. After treatment at 60°C, the relative activity of the control antibody, decreasing significantly with time, was close to 40% after 180 min, whereas NbH3 remained above 80%. NbH3 was significantly better performing compared to the control antibody at 60°C; after 90°C treatment, the relative activity of the control antibody decreased significantly and had down to 0% when treated for 10 min (Fig. 2C). The relative activity was still greater than the monoclonal antibody after 180 min of NbH3 treatment, indicating that NbH3 is more stable than the HRP anti-his tag antibody at 90°C (Fig. 2D).

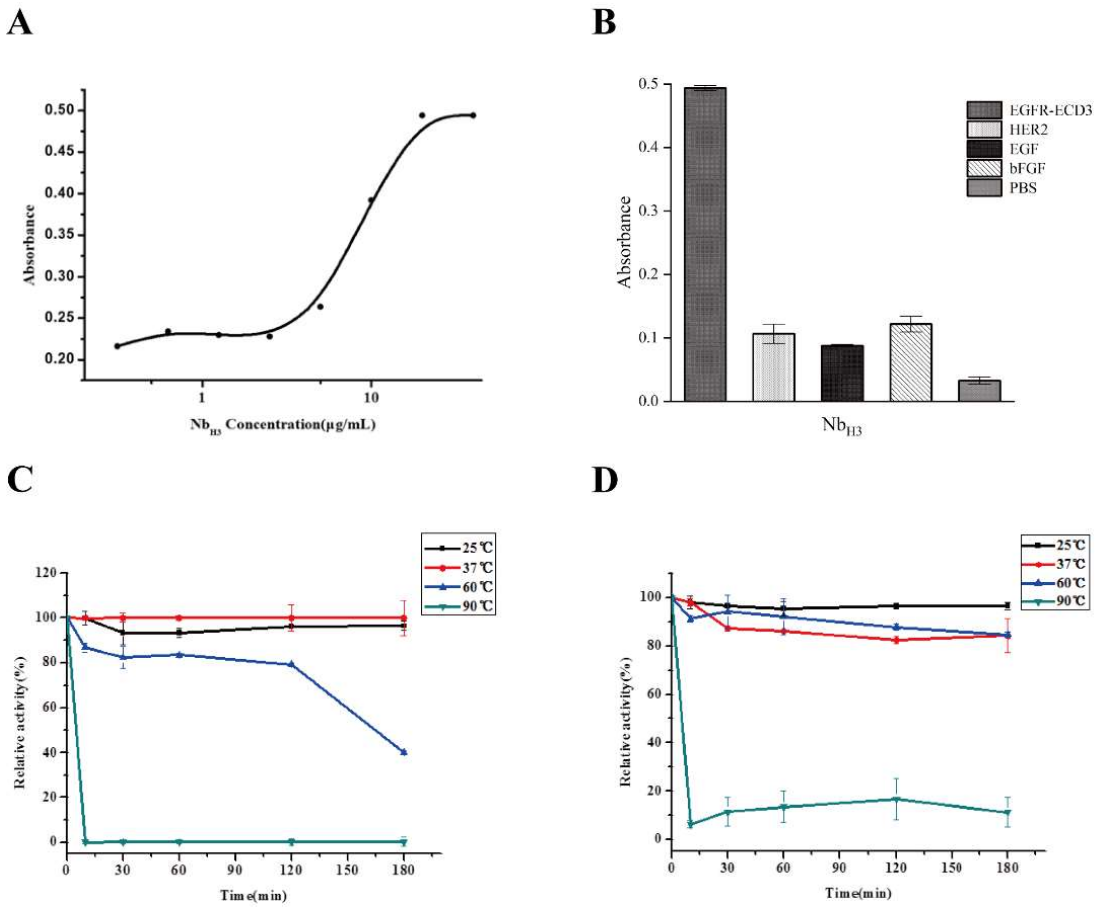

Fig. 2. Analyze NbH3 affinity, specificity, and stability by ELISA assay. (A) shows affinity analysis; (B) shows specificity analysis; (C) and (D) show the thermostability of HRP anti-his tag antibody and NbH3, respectively.

3.3. Nb_{H3} Inhibits A549 Cell Proliferation and the Phosphorylation of EGFR Protein Expression Induced by EGF

The effect of NbH3 on the proliferation of A549 cells induced by EGF was examined using the CCK8 assay. NbH3 has a certain anti-proliferative effect on A549 cells stimulated by EGF, the inhibition rate is 25%, and a dose-dependent effect is observed (Fig. 3A).

A Western Blot assay was used to detect the effect of NbH3 on the phosphorylation of endogenous EGFR in A549 cells facilitated by EGF. When only EGF was applied to A549 cells,

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the phosphorylation of EGFR protein on the cell surface was significantly increased. While A549 cells were treated with the same concentration of NbH3 in the condition of EGF, the phosphorylation of EGFR protein on the cell surface was prominent inhibited (Fig. 3B).

3.4. Nb_{H3} Specifically Binds to the A549 Cell Surface

Flow cytometry was analyzed that NbH3 binds to the EGFR protein on the surface of A549 cells, and its binding ability is stronger than that of anti-EGFR antibody (Fig. 3C-D). Correspondingly, the immunofluorescence assay demonstrated the binding sites of NbH3 were on the A549 cells membrane. (Fig. 3E).

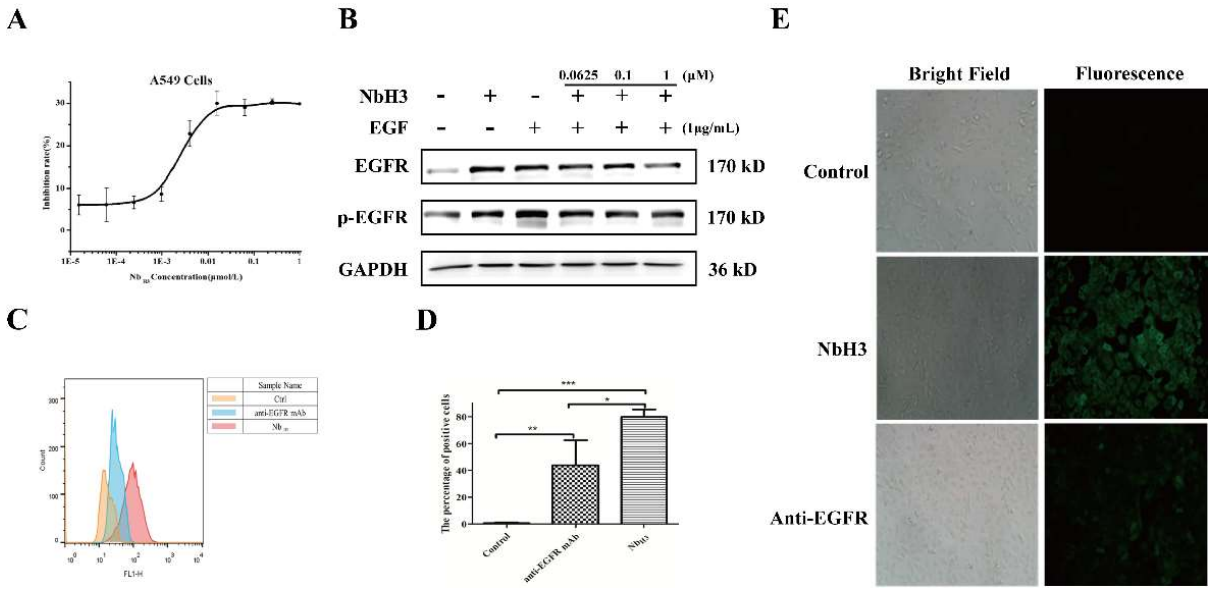

Fig. 3. NbH3 effects in vitro and NbH3 location on A549 cells. (A) shows NbH3 can inhibit A549 cell proliferation in a concentration-dependent manner; (B) shows NbH3 can inhibition of phosphorylation of A549 cell surface EGFR protein induced by EGF; (C) and (D) show NbH3 bind to the EGFR protein on the surface of A549 cells analyzed by flow cytometry; (E) shows NbH3 localized on A549 cells membrane by immunofluorescence detection

4. Discussion

EGFR protein has been found in a variety of malignancies, making it a target for research and development. At present, for targeting EGFR, the most popular and successful new drug development is tyrosine kinase inhibitors against the intracellular domain of EGFR and monoclonal antibodies against the extracellular domain[22,23]. The former is limited by the recurring of drug resistance after its drug acts on the target; the latter still exists some inevitable shortcomings and limitations with costly production, low stability, and harsh transportation and storage conditions, etc. These unresolved challenges have caused a bottleneck in the development of traditional antibodies. A single domain antibody, containing only one heavy chain antibody variable region, that is, a nanobody, stands out because of its small molecular weight, high stability, and high-temperature resistance[24]. Here, we aimed to develop a candidate material for EGFR-ECD3 nanobody that blocks the activity of the EGF/EGFR pathway, laying the foundation for subsequent drug development.

EGFR extracellular region is the binding epitope of EGF. To reduce the non-specificity of NbH3 to EGFR, we considered the EGFR-ECD3 as the binding site and added his tag for the convenience of recombinant protein purification. EGFR has glycosylation sites in the natural conformation. To harvest the EGFR-ECD3 recombinant protein with approximately the same modification as the native EGFR, the eukaryotic expression vector pCMV was selected in this

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study. And mammalian cells, like human embryonic kidney cells (HEK 293F), which took on the property of high expression, easy transfection, natural glycosylation modification, correct folding of proteins, and post-translational modification, were as the host for the expression of EGFR- ECD3 fusion protein.

Current preparation techniques for antibody libraries include phage display techniques, yeast display techniques, and ribosome and mRNA display techniques, the most widely used of which is phage display technology[25]. Screening antibodies using phage display technology is not only time- and labor-saving, but also high-throughput. In this study, we chose a large-capacity natural phage nanobody library with a short screening period and the application to multiple types of antigens. The phagemid pMECS carrying the anti-EGFR-ECD3 nanobody sequence, a high expression vector, can be directly utilized for transforming the expression host E. coli WK6. NbH3 can be expressed in the periplasmic with the IPTG low-temperature induction, and be extracted through an osmotic shock method, and final can be harvested with the purity (>95%) and the yield (about 5 mg/L).

The affinity and thermostability of NbH3 were investigated employing an ELISA. NbH3 inhibits A549 cells proliferation and phosphorylation promoted by EGF, detected by CCK8 and phosphorylation assays. And, the most authoritative method of affinity measurement is to measure the interaction between biological macromolecules by surface plasmon resonance (SPR) technology, and the antigen-antibody binding and dissociation constants can be analyzed. But the conditions are limited, this study used a non-competitive enzyme-linked immunosorbent assay to measure its affinity. NbH3 can competitively restain the binding of EGF to EGFR-ECD3 to a certain extent, but it is only a preliminary experiment, and it is not possible to determine the relationship between their binding epitopes. Therefore, their crystal structures and respective binding sites can be further analyzed, both of which could determine the relationship and characteristics. NbH3 can identify the EGFR extracellular domain, but more activity assays are necessary to verify the function and safety.

5. Conclusion

In this study, human EGFR extracellular domain III (EGFR-ECD3) was used as a target, phage display technology was to screen nanobodies specific for EGFR-ECD3 in a large-capacity phage nanobody library, and finally, the biological properties of EGFR-ECD3 were identified. And provide materials and shreds of evidence for the identification of drug candidates with potential for use as targeted anti-Cancer therapeutics.

6. Author contributions

S.X. designed this study; XL.L. conducted the experiments and drafted the manuscript; ZL.Z., MZ.L assisted in assays.

7. Conflicts of interest

The author declares there are no conflicts of interest.

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