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Fully human recombinant antibodies against EphA2 from a multi-tumor patient immune library suitable for tumor-targeted therapy

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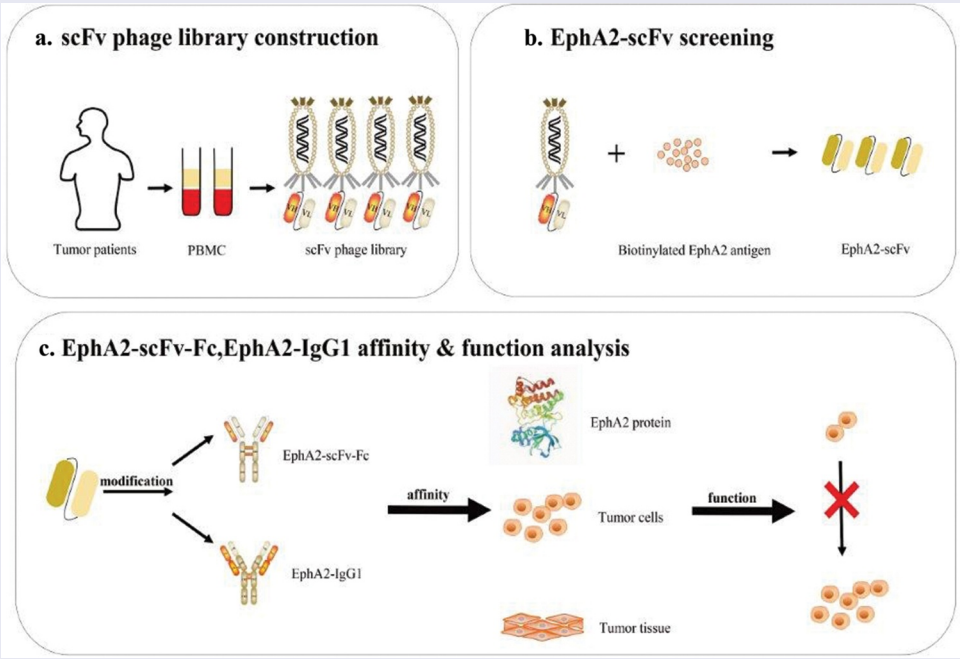
ABSTRACT

Enhanced EphA2 expression is observed in a variety of epithelial-derived malignancies and is an important target for anti-tumor therapy. Currently, Therapeutic monoclonal antibodies against immune checkpoints have shown good efficacy for tumor treatment. In this study, we constructed an immune single-chain fragment variable (scFv) library using peripheral blood mononuclear cells (PBMCs) from 200 patients with a variety of malignant tumors. High affinity scFvs against EphA2 can be easily screened from the immune library using phage display technology. Anti-EphA2 scFvs can be modified into any form of recombinant antibody, including scFv-Fc and full-length IgG1 antibodies, and the recombinant antibody affinity was improved following modification. Among the modified anti-EphA2 antibodies the affinity of 77-IgG1 was significantly increased, reaching a pmol affinity level (10^{-12}). We further demonstrated the binding activity of recombinant antibodies to the EphA2 protein, tumor cells, and tumor tissues using macromolecular interaction techniques, flow cytometry and immunohistochemistry. Most importantly, both the constructed scFvs-Fc, as well as the IgG1 antibodies against EphA2 were able to inhibit the growth of tumor cells to some extent. These results suggest that the immune libraries from patients with malignant tumors are more likely to screen for antibodies with high affinity and therapeutic effect. The constructed fully human scFv immune library has broad application prospects for specific antibody screening. The screened scFv-Fc and IgG1 antibodies against EphA2 can be used for the further study of tumor immunotherapy.



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1. Introduction

Erythropoietin-producing hepatocellular (Eph) receptors represent one of the largest subsets of the receptor tyrosine kinase (RTK) family, and are divided into two main categories (A and B) based on their extracellular structural domains. A total of 14 Eph receptors and eight ephrin ligands have been identified to date [1]. These molecules play an important role in tumor formation and progression by influencing various cell-related signaling pathways [2–4]. Erythropoietin-producing hepatocellular A2 (EphA2) is an important member of the Eph receptor family. In the human genome, the EphA2 gene is localized to chromosome 1p36.1, an oncogenic locus [5]. Unlike other Eph receptors, EphA2 is widely expressed in a variety of tissues or cell lines of epithelial origin in humans, and it is expressed at extremely low levels under normal conditions [1,6,7]. Evidence from numerous clinical studies indicates that EphA2 is highly expressed in a variety of epithelium-originated malignant tumors, including hepatocellular carcinoma [8], non-small cell lung cancer [9], prostate cancer [10], breast cancer [11], colorectal cancer [12], gastric cancer [13], cervical cancer [14], and cutaneous melanoma [15]. The importance of the expression pattern, localization, and function of EphA2 in multiple types of malignancies makes it an attractive therapeutic target, and it is also an important target for the delivery of drugs, toxins, and imaging agents to tumor tissues [16–18].

Monoclonal antibodies (MAbs) and new antibody structural domain-based molecules constitute the major therapeutic approach in clinical research for the treatment of cancer, as well as viral and inflammatory diseases [19]. Therapeutic antibodies against immune checkpoints have shown good efficacy for the treatment of tumors [20–22]. MAbs can be used as a vehicle for targeted chemotherapy or radionuclide therapy. They can also be used for immunomodulation and enhancing the anti-cancer immune response [20]. However, the selection of antibody carriers is the primary challenge associated with the development of radiopharmaceuticals based on targeted antibodies. The affinity, immunogenicity, clearance, and half-life of antibody carriers are often the main factors limiting the therapeutic effect of radiopharmaceuticals. In general, antibody fragments with a medium size and clearance (diabodies, minibodies, and scFv-Fcs with molecular weights of 50–110 kDa) may be more suitable as nuclear marker carriers [23–26]. Nevertheless, as an immunomodulator, full-length IgG1 antibodies are generally used, and most antibody-type anti-cancer drugs approved on the market belong to human IgG1 antibodies. This antibody activates Fcγ receptors present on natural killer cells, macrophages, and neutrophils, killing antibody-bound tumor cells through multiple mechanisms, including antibody dependent cell-mediated cytotoxicity (ADCC), complement dependent cytotoxicity (CDC), and antibody-dependent cellular phagocytosis (ADCP) [27]. As an antibody drug, fully human-derived antibodies, which are entirely encoded by human genes, are less immunogenic and more clinically effective, representing the primary research direction associated with antibody drug research. Screening for fully human antibodies with high affinity and the ability to inhibit tumor growth is key to the development of antibody drugs.

The theory of using the patient's own immune mechanism to target and kill tumor cells is long-standing [28,29]. Tumor cells almost always continuously express mutated proteins or antigens, which are 'exogenous' substances, unlike natural protein molecules, which can stimulate the immune system to mount an immune response [30,31]. Tumor patients may have antibodies with a high affinity for antigens that are highly expressed on the tumor, which function to inhibit tumor growth. Screening of high-affinity antibodies from tumor patients is more effective than traditional methods of screening antibodies from immunized mice or from nonimmune antibody libraries. In addition, it is easier to screen for antibodies with tumor therapeutic properties.

In this study, we hypothesized that therapeutic antibodies with a high affinity for specific targets can be screened from immune libraries derived from patients with multiple types of tumors. The present study aims to construct a large capacity fully human phage immune library and screen for high affinity antibodies based on the EphA2 target, and perform a preliminary exploration of the effect of the screened antibodies on tumor proliferation. First, mRNA was extracted from the PBMCs of 200 patients with multiple types of malignancies. This mRNA was used to construct a large capacity and diverse scFv immune library to screen for scFv

antibodies against EphA2 tumor targets. Furthermore, we constructed bivalent recombinant scFv-Fc and full-length IgG1 antibodies. Anti-EphA2 antibodies screened from the immune library of tumor patients retained their biological activity and high antigen affinity. Finally, we found that the screened scFv-Fc and IgG1 antibodies could inhibit tumor growth, which provides a foundation for subsequent antibody therapy for malignant tumors.

2. Materials and methods

2.1 Ethical statement

This study protocol was approved by the clinical trial ethics committee of the Affiliated Hospital of Southwest Medical University, China (ethics review number: KY2019276). Following the approval of the ethical committee, the patient volunteers signed the informed consent to collect the samples, and all methods were performed in accordance with the relevant guidelines and regulations. This study was compliant with the Declaration of Helsinki.

2.2. Cell lines and cell culture

The prostate cancer cell line, PC-3, hepatocellular carcinoma cell line, HepG2, lung adenocarcinoma cell line, A549, and 293 F eukaryotic cells were purchased from Institute of Cell Science, Chinese Academy of Sciences. PC-3, HepG2, and A549 were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Grand Island, USA) with 10% fetal bovine serum (Hyclon, Logan, Utah, USA) and 1% streptomycin (Beyotime, Shanghai, China), 293F eukaryotic cells were cultured as floating cells in HEK 293F complete medium (Sino Biological Inc, Beijing, China). All the cells were maintained in a humidified atmosphere at 37°C containing 5% CO_2.

2.3. Bioinformatics analysis based on the EphA2 gene

The Human Protein Atlas database is a comprehensive database that contains the tissue and cell distribution information of 24,000 human proteins, and provides the protein expression profiles for a wide range of human proteins [32]. The differential expression of the EphA2 gene at the mRNA level in different tumor cell lines was analyzed using the cell module in the online web server of the Human Protein Atlas database (http://www.proteinatlas.org). The gene name EphA2 was entered to investigate the level of gene expression in different tumor cell lines, and the results were expressed as bar charts.

To elucidate the relationship between EphA2 regulatory genes and tumor survival prognosis, we used the 'survival map' module of GEPIA2 [33] to obtain the overall survival (OS) and disease-free survival (DFS) significance map data for EphA2 in all TCGA tumors, using cutoff-high (50%) and cutoff-low (50%) values as expression thresholds to classify high and low expression cohorts. Hypothesis testing was performed using a log-rank test, and survival curves, which were obtained using the GEPIA2 'survival analysis' module.

2.4. Total RNA extraction from PBMCs and PCR amplification of light and heavy chain genes

The following experiments were performed as previously described [34–37]. PBMCs were isolated using the lymphocyte isolate (Ficoll solution) from the peripheral blood of 200 tumor patients (the patient information is summarized in Table 1) to construct a scFv immune library. The total lymphocyte RNA of the PBMCs was extracted using TRIzol reagent (TaKaRa, Dalian, China), and purified RNA was used as a template to synthesize first-strand cDNA by reverse transcription with Oligo (dT) used as a primer. Polymerase chain reaction (PCR) was performed to amplify the coding regions of heavy chain variable domain (VH), light chain variable domain (VL, including Vλ, and Vκ) antibody fragments by designing primer sequence combinations based on the light and heavy strand framework regions of human antibody sequences (see Qing Yuan et al. for the primer sequences and details [38]). The following PCR conditions were used: 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, for a total of 30 cycles. The purified VH or VL DNA of different patients were mixed to achieve subsequent VH and VL splicing.

2.5. Construction of a fully human EphA2-scFv phage library

The following experiments were performed as previously described [39,40]. Briefly, the scFv fragment

Table 1. Tumor patients information summary form.

Tumor type	Total cases	Gender ratio		Age range	
		Male	Female	≥ 60 years	< 60 years
Bronchial or lung tumors Cervical cancer Ovarian cancer Nasopharyngeal carcinoma Esophageal cancer Colorectal cancer Stomach cancer Breast cancer Pancreatic cancer Lymphoma Liver cancer	200	45.5%	54.5%	38%	62%

contained *Sfi I* and *Not I* restriction sites and was amplified by overlapping PCR using purified VH and VL as templates. The classical $(Gly_4Ser)_3$ sequence was used as a linker between the heavy chain variable region and the light chain variable region. The first round overlapping extension PCR reaction conditions were as follows: without primers, 94°C for 1 min, 68°C for 1 min, for 20 cycles. The product of the first round of PCR was used as a template for the second round of PCR, with the upstream primer VHFvF and the downstream primer VλFvR or VκFvR. The PCR reaction conditions: 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, for 30 cycles. The PCR products were recovered by 1.5% agarose gel electrophoresis and stored at −20°C.

The following primer sequences were used in the present study:

V_HFvF: 5′-ATCGACGCTACTGCGGCCCAGCC GGCCCAGGT-3′;

VλFvR: 5′-ACGGCTGCGTCAGAGTGCGGC CGCACGTTT-3′; and

VκFvR: 5′-ACGGCTGCGTCAGAGTGCGGC CGCACC-3′.

The scFv library gene was created by mixing VH-Linker-Vκ and VH-Linker-Vλ. The scFv library gene and pCANTAB5E vector plasmids were digested with *Sfi I* and *Not I* restriction enzymes, respectively, and ligated at 16°C overnight using T4 DNA ligase. The ligation products were then transformed into the TG1 competent cells, and the transformation products were cultured in Luria-Bertani (LB) solid medium containing 100 μg/mL ampicillin and 2.0% glucose (LBAG) overnight at 37°C. The next morning, all of the transformed bacteria were scraped from the culture plate with LBAG medium, and the scFv bacterial library was preserved with a final concentration of 20% glycerol at −80°C. To assess the diversity of the scFv library, a total of 21 single colonies from the library were randomly selected for PCR identification, using the method as previously described [41–43]. The PCR products were digested with *BstN I* to form a DNA fingerprint.

2.6. Phage amplification

The phage amplification experiment was performed as previously described [44,45]. A volume of 150 μL library bacterial solution was stored at −80°C and inoculated into 40 mL LBAG liquid medium, incubated at 37°C, shaken at 250 rpm, and cultured to bring the OD_{600} value of the bacterial solution to approximately 0.2. The samples were centrifuged for 10 min at 4,500 rpm at room temperature. The bacterial sediment was collected and resuspended in 40 mL LBA liquid medium (containing 100 μg/mL ampicillin without glucose). The addition of a final concentration of 3×10^9 PFU/mL M13KO7 helper phage in LBA liquid medium, and infected for 15 min at 37°C. The samples were placed on a horizontal shaker at 37°C and 200 rpm, and shaken for 2 h. Next, kanamycin was added to a final concentration of 20 μg/mL and incubated overnight at 32°C. The samples were centrifuged at 8000 rpm at 4°C for 20 min. The supernatant was collected and 1/5 of the final volume of polyethylene glycol-sodium chloride (20% PEG, 2.5 M NaCl) was added to resuspend the bacteriophage precipitate and centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was discarded and the precipitate was resuspended with 1 × PBS buffer.

2.7. EphA2-scFv affinity screening

Affinity screening of scFv was carried out using a liquid phase screening method [46–48]. The purified EphA2 protein was biotinylated according to the EZ-Link® SulfoNHS-LC-Biotinylation (Thermo Science, Rockford, USA) manual. The amplified library phage was incubated with blocking buffer (containing 3% fetal bovine serum albumin [BSA]) for 1 h at room temperature. 2 μg of biotinylated EphA2 protein was added to the blocked library phage and incubated at room temperature for 2 h. Bound antibody antigen complexes were captured with streptavidin-coated Dynabeads M-280 (Invitrogen, Grand Island, USA) and washed 5–10 times with 1 × PBST (1 × PBS containing 0.05% Tween-20). The phages were eluted with 0.1 M Glycine-HCl (pH = 2.2) and neutralized with 1 M Tris to pH 7.0 after elution. TG1 in the logarithmic growth phase was infected with the eluted and neutralized phage, spread onto LBAG plates, and the colonies were harvested for the next round of screening.

Further amplification was performed as described above. In the second and third rounds of affinity screening, 1 μg of biotinylated EphA2 protein was used. At the same time, multiple monoclonal colonies were randomly selected from three rounds of enrichment affinity screening of the phage library for PCR amplification [41]. The amplification products were digested in *BstNI* to form a DNA fingerprint profile, which was used to assess the diversity of the scFv phage library following enrichment affinity screening.

2.8. ELISA for the detection of scFv-binding affinity

2.8.1. Phage ELISA

After three rounds of screening, colonies were randomly selected from the scFv phage library, and each scFv-phage was amplified according to the phage amplification protocol. The expressed phage scFv from each clone was then detected by phage ELISA, and affinity analysis of the scFvs was conducted by ELISA as described previously [49]. Briefly, the purified EphA2 protein was diluted to 2 μg/mL with coating buffer (0.1 M $NaHCO_3/Na_2CO_3$) and coated overnight at 4°C. The coating buffer was discarded and washed three times with washing buffer (0.05% Tween-20 in 1 × PBS) for 5 min per wash, and the wells were blocked with ELISA blocking buffer (containing 5% skimmed milk powder) at 37°C for 1 h. The wells were washed and phage supernatant with blocking buffer was added to the wells and incubated at 37°C for 1 h. After washing, Horseradish peroxidase (HRP)-conjugated anti-M13 antibodies (anti-M13-HRP, 1:5000 dilution, Abcam, Cambridge, UK) were used to bind to the EphA2-scFv phage. Tetramethylbenzidine liquid substrate (TMB) was incubated for 30 min protected from light and the reaction was terminated by the addition of 50 μL 2 M H_2SO_4 and read at OD_{450}.

2.8.2. Phage cell ELISA

For the phage cell ELISA, the experimental method was performed as previously described [50]. The PC-3, HepG2, and A549 cells (1.0×10^5 cells/mL) were seeded into 96-well plates and cultured in RPMI-1640 medium with 10% fetal bovine serum for 24 h – 48 h and until the cells reached a cell density of 80% – 90% in each well of the 96-well plate. The medium was discarded and the plate was washed twice with 1 × PBS. After the plates had dried, 80 μL of fixative (0.25% glutaraldehyde solution prepared from 1 × PBS) was added to each well and fixed for 10 min. To block the latter wells, phage scFv and anti-M13-HRP secondary antibodies were incubated together. Color development is shown in the phage ELISA scheme.

2.9. Homologous recombination and expression of the EphA2 bivalent recombinant antibody scFv-Fc and full-length IgG1 antibody

The screened EphA2-scFv with good binding activity and specificity was ligated with a pcDNA3.1/SP-Fc (human IgG1-Fc) recombinant vector constructed in our laboratory and recombined to form a bivalent EphA2-scFv-Fc in accordance with the experimental methods in [51]. The full-length IgG1 antibody was constructed as previously described [52]. VH and VL were amplified by PCR using EphA2-scFv as template, and VH was homologously recombined into the pcDNA3.1/SP-CH vector constructed in our laboratory. VL was homologously recombined into the pcDNA3.1/SP-CL vector constructed in our laboratory. All recombinant product was transformed into *E.coli* TOP10-competent cells and gene sequencing was

performed to verify whether scFv and VH and VL were homologously recombinant with the recombinant vector. The methods used for fusion protein expression and purification were performed as previously described [53,54]. The homologous recombinant pcDNA3.1/SP-scFv-Fc plasmids were extracted using an Endotoxin-free Plasmid Extraction Kit (Tiangen, Beijing, China) and transfected with PEI to express the EphA2-scFv-Fc fusion protein in 293 F eukaryotic cells. The pcDNA3.1/SP-VH-CH and pcDNA3.1/SP-VL-CL plasmids were transiently cotransfected in equal proportions into 293 F cells and the EphA2-IgG1 protein was expressed in 293 F eukaryotic cells. On day 7 of expression, the supernatant was collected by centrifugation at 8,000 rpm for 10 min and the antibody was purified from the supernatant using a protein A affinity chromatography column (GE).

2.10. Macromolecular interaction technique for the detection of antigen-antibody affinity

We measured the binding affinity of bivalent recombinant antibody scFv-Fc and full-length IgG1 antibody to the EphA2 protein by biolayer interferometry (BLI). The BLI experiments were performed as previously described [49,55]. Biotinylated EphA2 protein (200 μL; Sino Biological Inc, Beijing, China) was diluted in 1 × PBST (10 μg/mL) and coupled to a SA biosensor (Pall ForteBio, Fremont, CA, USA). Subsequently, 200 μL purified recombinant scFv-Fc or full length IgG1 antibody against EphA2 (0 μg/mL, 5 μg/mL, 10 μg/mL, and 20 μg/mL) were used to assess antigen-antibody interactions. Equilibrium dissociation constant (KD) values were calculated by systematically analyzing antigen-antibody binding and dissociation. The interaction between antigen (EphA2) and antibody (scFv-Fc or full length antibody IgG1) was analyzed by Octet RED96 (Pall ForteBio, Fremont, CA, USA). The acquired data were analyzed using custom ForteBio software Data Acquisition 9.0.

2.11. Flow cytometry

To examine the specific binding of the selected scFv-Fc and IgG1 antibodies to the EphA2 receptor on the surface of tumor cells, flow cytometry was performed as previously described [56,57]. The purified bivalent recombinant antibody, scFv-Fc; and full-length antibody, IgG1, were added to PC-3 and A549 cells (2×10^5 in 1 × PBS) as primary antibodies with final concentrations set at 1.25 μg/mL, 5 μg/mL, and 20 μg/mL for an incubation at 4°C for 1 h. The final concentration in the blank group was 1.25 μg/mL. The cells were washed twice with 1 × PBS followed by the edition of an anti-Human-IgG fluorescent antibody (Jackson ImmunoResearch. West Grove, PA, USA) at a dilution of 1:500 and incubated for 1 h at 4°C, protected from light. The cells were washed twice and the cell suspension was collected in 1 × PBS and assayed by flow cytometry (NovoCyte 2070 R, ACEA, San Diego, CA, USA).

2.12. Immunohistochemistry

Immunohistochemistry was performed as previously described [58]. Adenocarcinoma and adjacent normal lung tissues were fixed, dehydrated, paraffin-embedded, and sectioned. The dewaxed sections were placed in hydrogen peroxide (containing 3% methanol) for 10 min at room temperature and washed with 1 × PBS. The tissue sections were immersed in 0.01 M citrate buffer solution (pH 6.0) and heated to boiling. Following cooling, the sections were washed with 1 × PBS. Blocking solution (goat serum) was added dropwise to block for 20 min at room temperature. The purified bivalent recombinant antibody, EphA2-scFv-Fc, and the full-length antibody, EphA2-IgG1 was separately added dropwise as a primary antibody. Moreover, the human IgG1 (Shanghai-yuanye Biotechnology, Shanghai, China) was used as an isotypic control and incubated overnight at 4°C. Goat-anti-human-IgG-HRP (Abcam, Cambridge, USA) was added dropwise as a secondary antibody and incubated for 90 min at 37°C and washed with 1 × PBS at the end of the incubation. A DAB chromogenic reagent kit (Zhongshan Jinqiao, Beijing, China) was used to stain at room temperature. After hematoxylin counterstaining and dehydration, the sections were sealed with neutral gum.

2.13. Determination of cellular proliferation using a CCK8 assay

The ability of the screened bivalent recombinant scFv-Fc and full-length IgG1 antibodies to inhibit the proliferation of tumor cells was determined by performing a CCK8 assay as previously described [59]. A549 cells were evenly seeded into 96-well plates at a density of 6×10^4 cells/mL at 100 μL per well. The experimental wells, blank control wells, and negative control wells were established according to the CCK8 reagent instructions. The 96-well plates were incubated at 37°C for 24 h in an incubator containing 5% CO_2. After discarding the medium, bivalent recombinant EphA2-scFv-Fc and full-length EphA2-IgG1 antibodies diluted with complete medium were added to the experimental wells at 100 μL each, with antibody concentrations set at 2 μg/mL, 10 μg/mL, and 50 μg/mL. The wells were incubated at 37°C in an incubator containing 5% CO2. Next, 10 μL of CCK8 reagent (APE×BIO, Houston, USA) was added to each well at 24 h, 48 h, and 72 h post-incubation. The absorbance at 450 nm was measured using an enzyme marker after a 1 h incubation.

2.14. Statistical analysis

The statistical analysis was performed using Prism 8 (GraphPad Software). Data were presented as the mean ± SD. Significance was calculated using a *t*-test for comparison between two groups. A value of $P > 0.05$ was deemed to be not statistically significant (ns); * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$.

3. Results

In the current study, we used phage display technology to screen anti-EphA2-scFv from the constructed fully human immune library. This extensive ELISA screening ensured the affinity of the screened scFv. This affinity was further improved when scFv was further modified into scFv-Fc and full-length IgG1 antibodies. Notably, our data revealed that the constructed full-length antibody, 77-IgG1 exhibited affinity at the pmol level (10^{-12}). Moreover, the flow cytometry and immunohistochemistry results showed that both the screened scFv-Fc and IgG1 antibodies were able to effectively bind to the EphA2 receptor expressed on the surface of tumor cells and tissue. Importantly, the results of a CCK8 assay showed that the screened anti-EphA2 antibodies could inhibit the proliferation of tumor cells to a certain extent. Taken together, these results suggest that the constructed fully human scFv immune library has broad applications for specific antibody screening. Furthermore, the screened scFv-Fc and IgG1 antibodies against EphA2 could be used for the further study of tumor immunotherapy.

3.1 EphA2 significantly affects tumor survival and prognosis

The level of EphA2 gene expression in each cell line was analyzed using the Human Protein Atlas database (http://www.proteinatlas.org). Figure 1(a) showed that EphA2 was highly expressed in multiple tumors, including male reproductive tumors (PC-3), lung cancer cell lines (A549), and hepatobiliary tumors (HepG2). The tumor cases were divided into high and low expression groups according to the level of EphA2 expression and TCGA dataset was used to investigate the correlation between EphA2 expression and the prognosis of patients with different tumors. As shown in Figure 1(b), high EphA2 expression was associated with a poor prognosis in glioblastoma multiforme (GBM, P = 0.034), brain lower grade glioma (LGG, P = 3.8e-07), lung adenocarcinoma (LUAD, P = 0.032), and pancreatic adenocarcinoma (PAAD, P = 0.0074) tumors in TCGA projects. Data from the disease-free survival (DFS) analysis (Figure 1(c)) showed that high EphA2 expression was associated with a poor prognosis in LGG (P = 0.0035) and PAAD (P = 0.011) in TCGA data. In addition, low expression of the EphA2 gene was associated with a poor overall survival (OS) in kidney renal clear cell carcinoma (KIRC, P = 0.049), thyroid carcinoma (THCA, P = 0.029) and poor DFS in KIRC (P = 0.015). The above data suggested that the level of EphA2 gene expression significantly affected the prognosis of tumor patients and EphA2 was an important target for tumor therapy.

3.2 Construction of a human scFv phage library

PBMCs were isolated from the peripheral blood of 200 tumor patients and mRNA was extracted to synthesize first-strand cDNA. The synthesized cDNA appeared as a 0.1 kb-8 kb on the

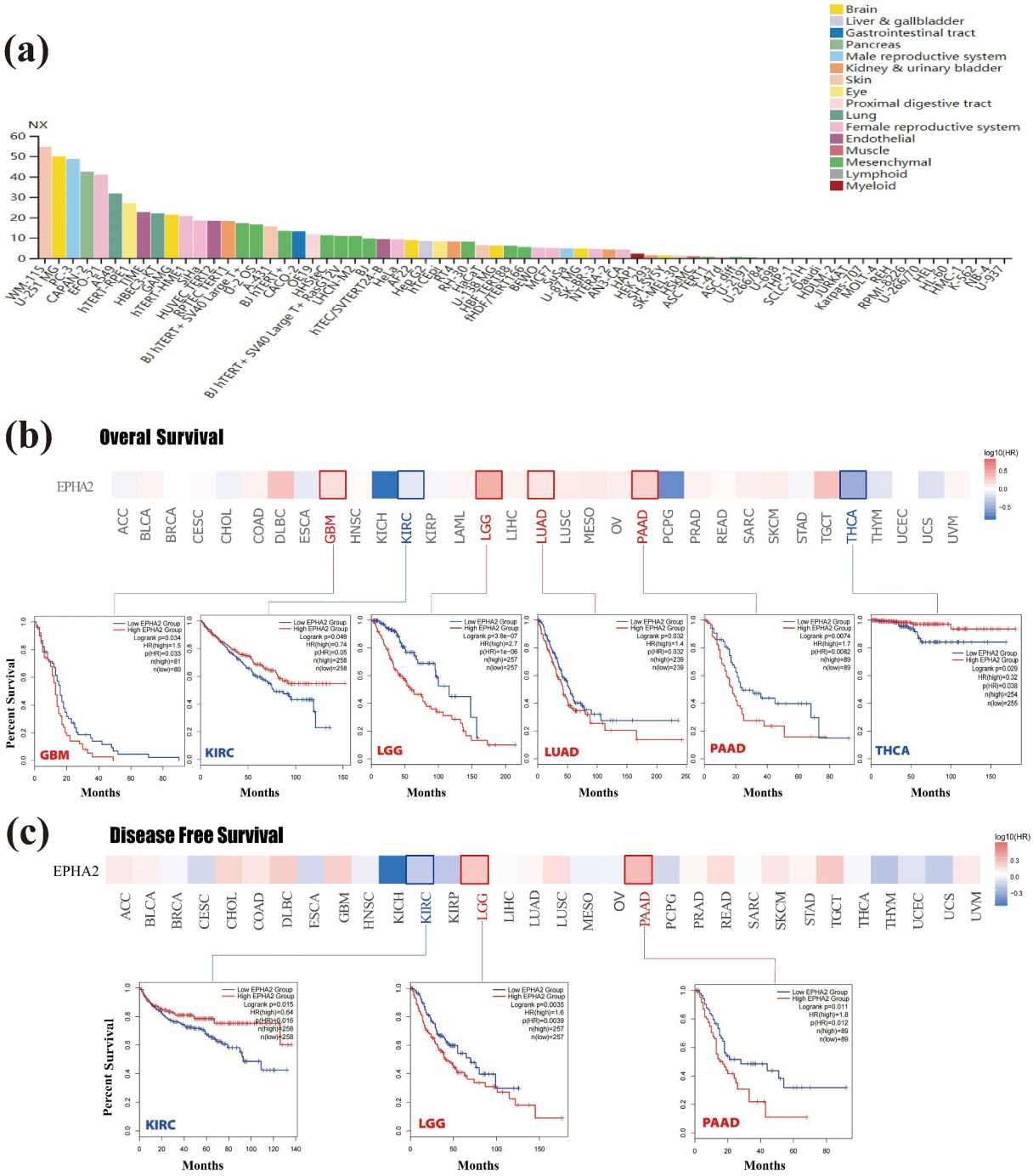


Figure 1. Expression of the EphA2 gene in tumor cells and the relationship between EphA2 gene expression and survival prognosis of TCGA tumors. (a) We used the cell panel of the Human Protein Atlas database (http://www.proteinatlas.org) to analyze the level of EphA2 gene expression in each cell line. In male genital tumors, hepatobiliary tumors, and lung cancer tumor cell lines, PC-3, HepG2, and A549 cell lines, the gene expression of EphA2 was more prominent. (b-c) We used the GEPIA2 tool to analyze the relationship between EphA2 gene expression and overall survival (1b) and disease-free survival (1 c) of the different tumors in TCGA database. Survival plots and Kaplan-Meier curves are presented.

gel, and the size, intensity, and yield were satisfactory (data not shown). The size of the amplified VH, Vλ, and Vκ gene bands was approximately 400 bp (Figure 2(a-c)). The purified amplification products of VH, Vλ, and Vκ from different patients were mixed in equal amounts, respectively. The VH-linker gene library was amplified by adding the *SfiI*

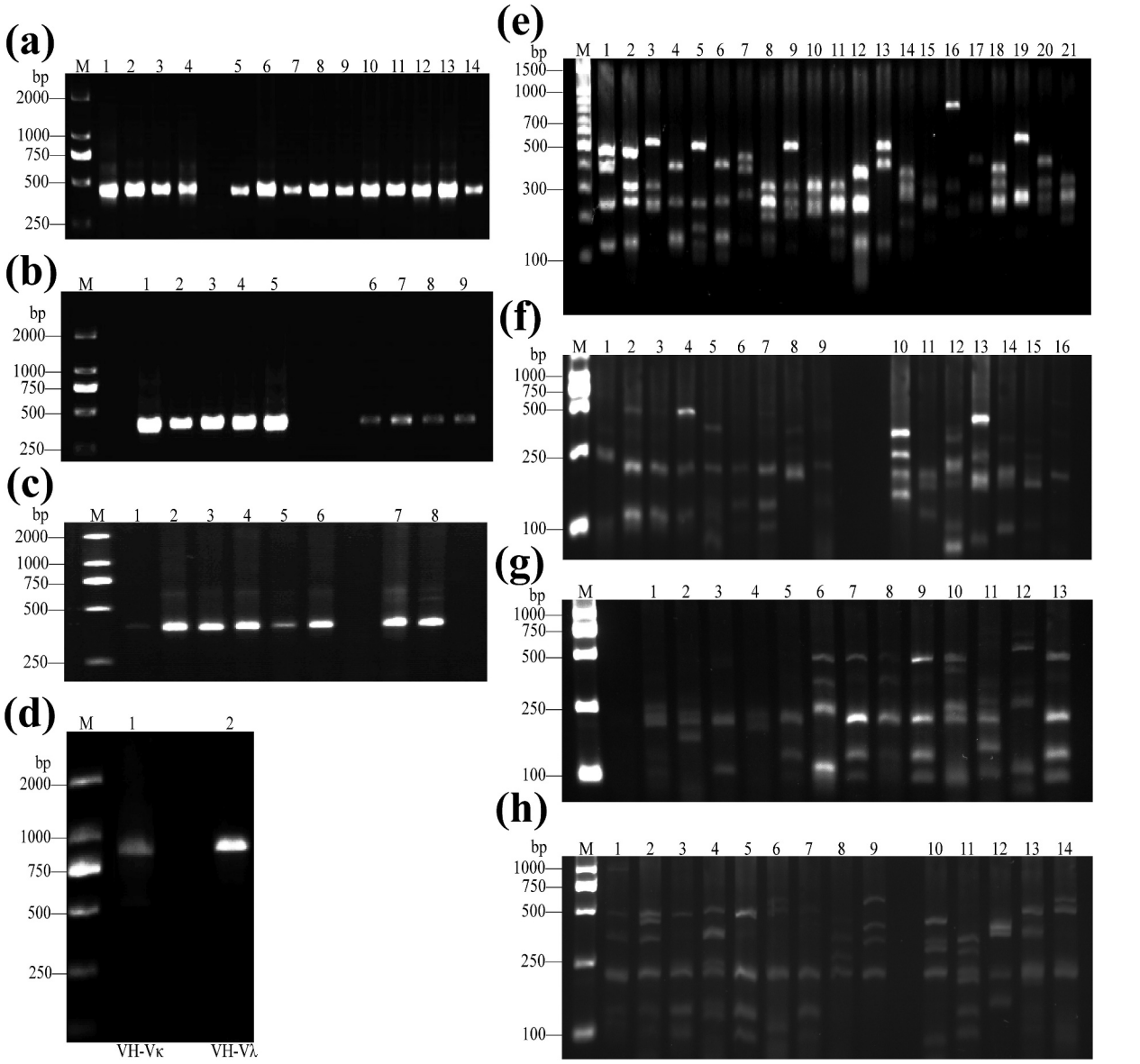


Figure 2. Construction of scFv and analysis of the diversity of phage immune libraries. (a) M is a DL 2,000 bp DNA marker, 1–14 are the amplified VH gene bands. (b) M is DL 2000 bp DNA marker, 1–9 are the amplified Vκ gene bands. (c) M is DL 2,000 bp DNA marker, 1–8 are the amplified Vλ gene bands. (d) scFv to the VH-linker-VL approximately 800 bp in size were obtained by assembling VH-linker-Vκ and VH-linker-Vλ by overlapping extension PCR. M is the DL 2000 bp DNA marker, 1 is the amplified VH-Vκ gene band, and 2 is the amplified VH-Vλ gene band. (e) DNA fingerprints formed by random digestion of scFv from the phage library by restriction enzyme *BstN I*. M is a DL 1,500 bp DNA marker, 1–21 are sample numbers of different monoclonal colonies from the phage library. (f) DNA fingerprinting formed by the random digestion of scFv by restriction enzyme *BstN I* after the first round of enrichment affinity sieve. M is a DL 2,000 bp DNA marker, 1–16 are sample numbers of different monoclonal colonies. (g) DNA fingerprinting formed by the random digestion of scFv by the restriction enzyme, *BstN I*, after a second round of enrichment affinity sieve. M is a DL 2,000 bp DNA marker, 1–13 are sample numbers of different monoclonal colonies. (h) DNA fingerprinting formed by the random digestion of scFv by restriction enzyme *BstN I* after a third round of enrichment affinity sieve. M is a DL 2,000 bp DNA marker, 1–14 are sample numbers of different monoclonal colonies.

site to the upstream primer and the linker sequence to the downstream primer. The Vλ-linker and Vκ-linker gene libraries were amplified by adding a linker sequence to the upstream primer and a *Not I* site to the downstream primer, respectively. The VH-linker-VL gene library (approximately 800 bp) was obtained by overlap extension PCR (Figure 2(d)) and a fully human scFv DNA library was successfully constructed.

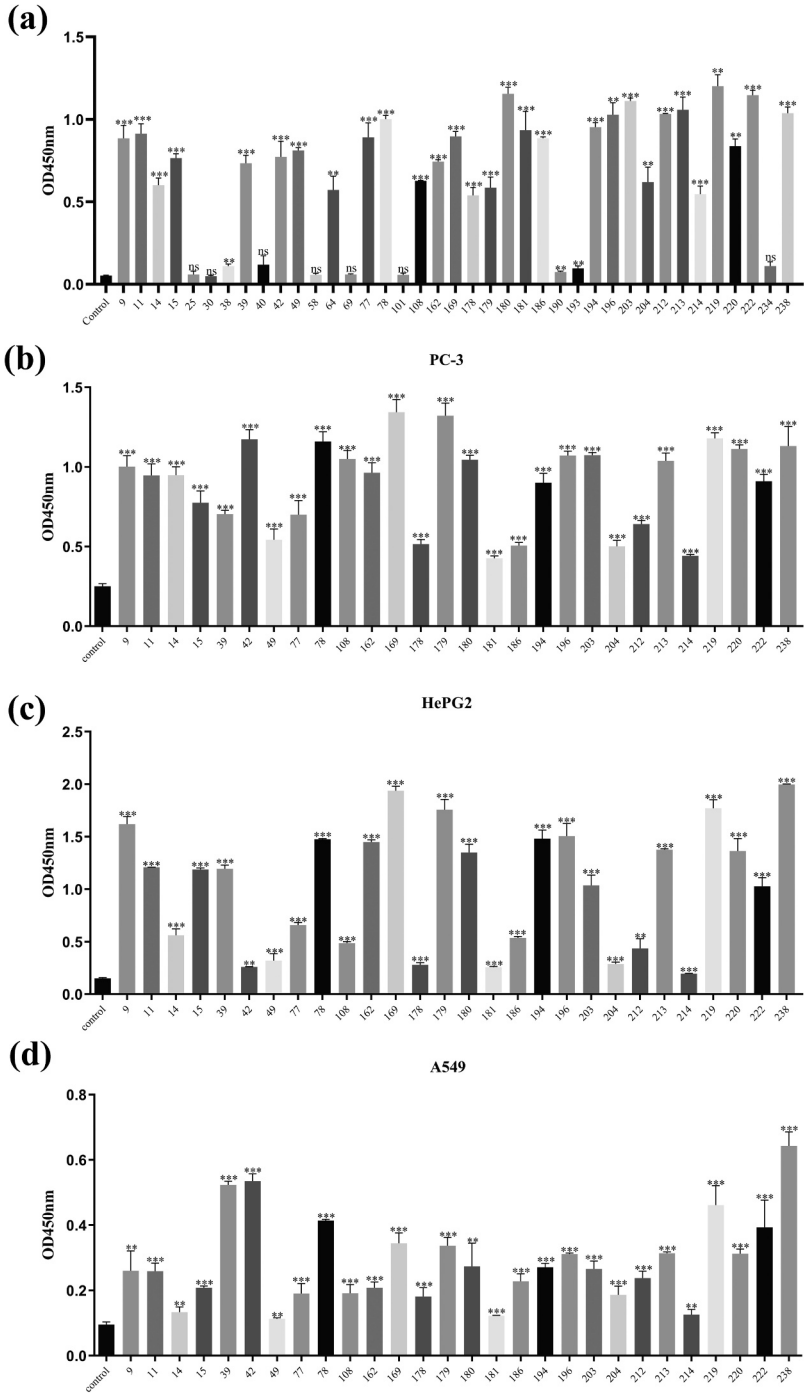


Figure 3. Screening for high affinity scFvs. (a) A phage ELISA identified the binding activity of 39 scFv antibodies from the initial screen to the target antigen EphA2. The results at OD_{450} were used to determine whether the initial screen of phages consistently expressed scFvs. (b) A phage cell ELISA identified the binding of 28 strains of scFv to the EphA2 receptor on PC-3 cells and OD_{450} values were read. (c) A phage cell ELISA identified the binding of 28 strains of scFv to the EphA2 receptor on HepG2 cells, and OD_{450} values were read. (d) A phage cell ELISA identified the binding of 28 strains of scFv to the EphA2 receptor on A549 cells, and the OD_{450} values were read. (n ≥ 3 of per experiment group; ns, not statistically significant; * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$. A *t*-test for group design was used for statistical analysis).

DNA from the scFv library was ligated to the phage vector, pCANTAB5E, and transformed into *E.coli* TG1. A total of 21 monoclonal colonies were randomly selected, and the scFv insert was identified by PCR. The results showed that all monoclonal colonies inserted the full-length scFv gene, the DNA of

scFv was digested with the restriction enzyme, *BstNI*. DNA fingerprinting demonstrated that each scFv was distinct (Figure 2(e)), the constructed human scFv phage library was diverse, and the gene sequences in the antibody library were enriched.

3.3 Screening and affinity determination of EphA2-scFv

The biotinylated EphA2 protein was added to the amplified scFv phage library using a liquid phase screen, and the bound phage scFv and antigen was captured with streptavidin-coated Dynabeads M-280 (Invitrogen). After three rounds of enrichment screening, scFv clones were enriched against target antigens. After each round of affinity screening, *E.coli* TG1 were infected with the screened phages and multiple monoclonal colonies were randomly selected to digest the inserted scFv DNA using the restriction enzyme, *BstN I*. DNA fingerprinting showed that after three rounds of enrichment affinity screening, the diversity of scFv against the EphA2 target antigen gradually decreased, the specificity gradually increased (Figure 2(f-h)).

After three rounds of affinity screening, 240 monoclonal colonies were randomly selected for expression. The binding activity of the phage scFvs and target antigen, EphA2, was detected using an ELISA after three rounds of enrichment affinity screening, and the strains were preserved based on the results obtained at OD_{450}. The positive scFv preserved strains were subjected to another ELISA to test whether the preserved strains consistently expressed scFv (Figure 3(a)) to screen for scFv with high binding activity to the target antigen, EphA2. The results showed approximately 71% positive ELISA reactions and the OD of the positive reactions was twice as high as the negative values.

Since EphA2 is highly expressed on PC-3, A549, and HepG2 tumor cells, the binding activity of the screened anti-EphA2 scFvs to the three tumor cell surface antigens were detected. According to the phage ELISA results, the scFvs that were highly bound to the target antigen, EphA2, after multiple screening was used as the primary antibody, and anti-M13-HRP as the secondary antibody for cellular ELISA. As shown in Figure 3(b-d), most of the screened anti-EphA2 scFvs were capable of binding to the three tumor cells. The binding activity of the screened anti-EphA2 scFv to the tumor cell surface antigen with higher values at OD_{450} than the negative control wells.

3.4 Homologous recombination and expression of EphA2-scFv-Fc and EphA2-IgG1

Based on the results of the repeated ELISAs, cell ELISA, and gene sequencing, we further detected the antigen and antibody affinity of the positive scFvs with different sequences by macromolecular interaction technology. Finally, three scFvs with an affinity of 10^{-8} (Data not shown) were identified for subsequent recombinant antibody construction. For the construction of scFv-Fv antibodies, scFv was homologously recombined into a pcDNA3.1 vector carrying human IgG1 Fc. Moreover, a full-length EphA2-IgG1 antibody was constructed using EphA2-scFv as a template. EphA2-scFv-Fc and EphA2-IgG1 were successfully constructed, as verified by gene sequencing (Figure 4(b-e)). The transient cotransfection of the constructed expression vector into 293F eukaryotic cells and the expression supernatant was collected on day 7 of expression (Figure 4(f)). The collected expression supernatant was purified using protein A resin, and the expression supernatant was verified by SDS-PAGE and the results showed that the target protein was well-purified, and with the molecular size of bivalent EphA2-scFv-Fc being about 100 kDa, and that of bivalent EphA2-IgG1 being about 150 kDa (Figure 4(a, d)).

3.5 Affinity identification of EphA2 bivalent recombinant antibody scFv-Fc and EphA2 full-length antibody IgG1

3.5.1. Bivalent recombinant antibody EphA2-scFv-Fc was constructed into full-length antibody EphA2-IgG1, which improved their antigen affinities

This improvement in affinity arises from changes in both the Kd and Ka rates. The affinity dissociation constant (KD, KD = Kd/Ka) values were calculated by systematically analyzing the binding and dissociation of antigenic antibodies. By comparing the KD values of the three bivalent recombinant antibodies scFv-Fc and

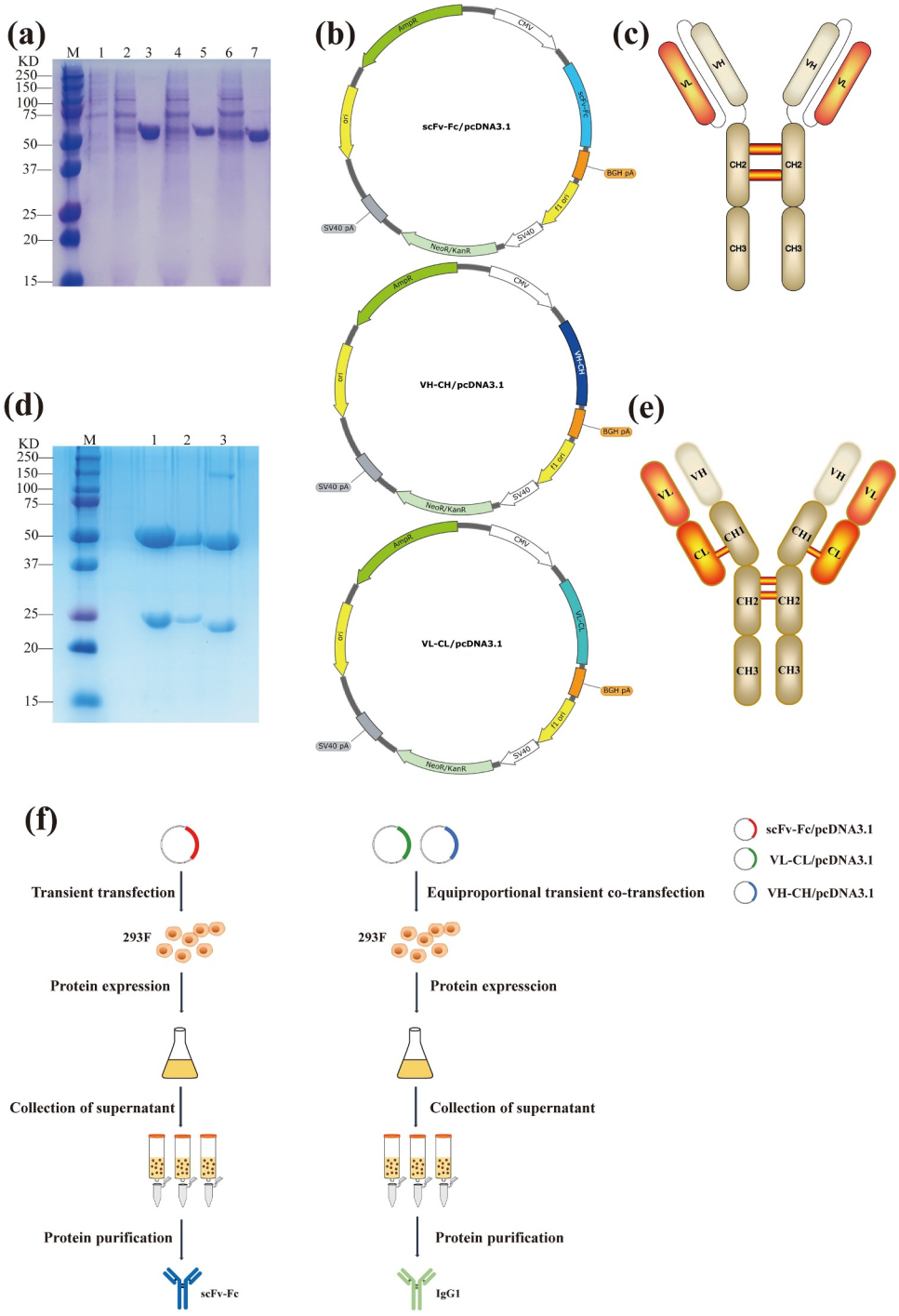
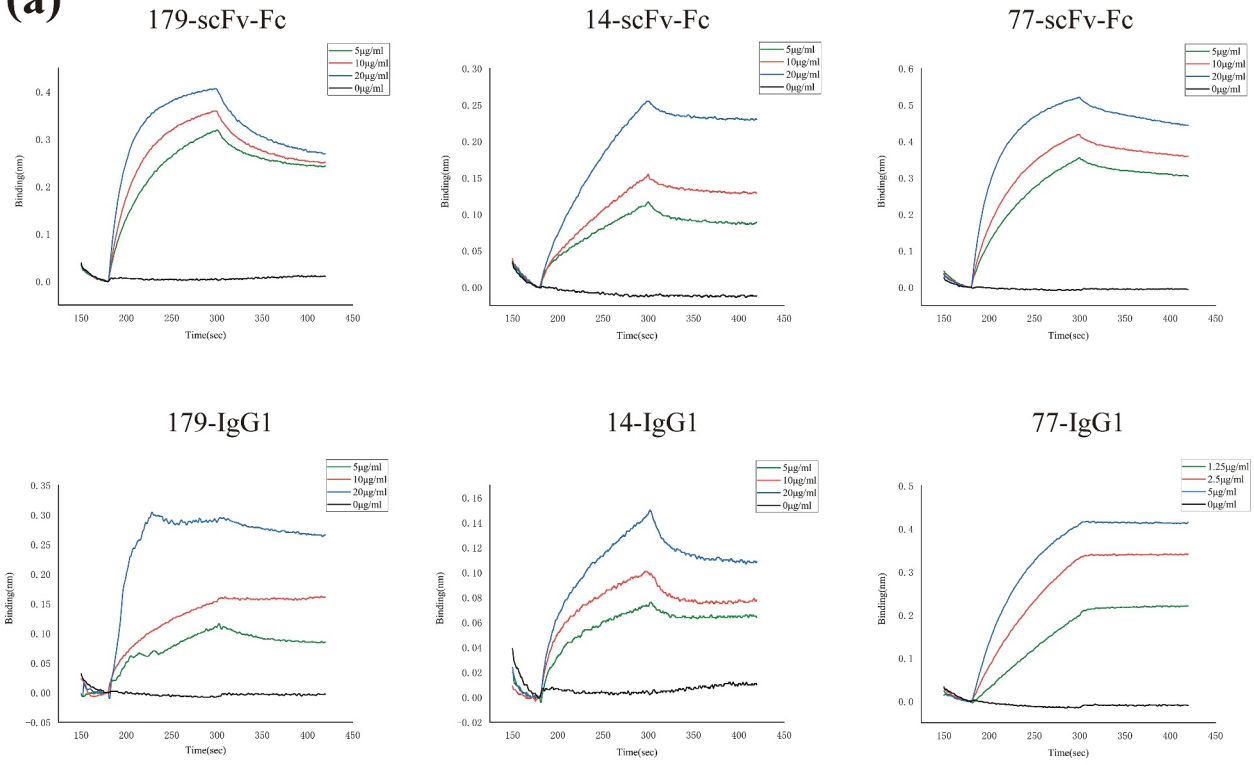


Figure 4. Expression and purification of bivalent recombinant scFv-Fc and full-length IgG1 antibodies. (a) SDS-PAGE results of the EphA2-scFv-Fc recombinant protein. M is the marker, 1 is the blank control; 2, 4, and 6 are the pre-purification bands for recombinant protein 179-scFv-Fc, 14-scFv-Fc, and 77-scFv-Fc; 3, 5, and 7 are the purified protein bands for recombinant protein 179-scFv-Fc, 14-scFv-Fc, and 77-scFv-Fc. (b) The structure of the recombinant vector for scFv-Fc and IgG1. (c) The scFv-Fc antibody structural diagram. (d) SDS-PAGE results of the EphA2-IgG1 recombinant protein. M is the marker; 1–3 are the purified protein bands for recombinant proteins 179-IgG1, 14-IgG1, and 77-IgG1. (e) IgG1 antibody structural diagram. (f) Expression and purification flow chart of scFv-Fc and IgG1.

the three full-length antibodies IgG1, we found that after 179-scFv-Fc was constructed as 179-IgG1, the KD value changed from 1.859e-8 to 1.081e-9, with a 10-fold increase in affinity. Similarly, after the construction of 14-scFv-Fc into 14-IgG1, the same effect was observed, with a 10-fold increase in affinity. Notably,

(a)



(b)

Structural forms	Antibody number	KD(M)	Ka(1/Ms)	Kd(1/s)
scFv-Fc	179	1.859e-8	2.585e5	4.805e-3
	14	9.706e-8	1.625e4	1.577e-3
	77	7.652e-9	1.957e5	1.497e-3
IgG1	179	1.081e-9	8.176e5	8.838e-4
	14	2.148e-8	1.764e5	3.79e-3
	77	<1e-12	2.403e5	<1e-7

Figure 5. There was a significant increase in antibody affinity after a conversion of the bivalent recombinant scFv-Fc antibody to a full-length IgG1 antibody. (a) scFv-Fc and IgG1 dose-response curves for binding with the biotinylated EphA2 protein. (b) Data regarding the macromolecular interactions of scFv-Fc and IgG1 with biotinylated EphA2 proteins. KD means equilibrium dissociation constant; Ka means combination constant; Kd means dissociation constant (the data are representative of one of three independent experiments).

after 77-scFv-Fc was constructed as 77-IgG1 with a KD value of less than 1e-12 and a 1000-fold increase in affinity at the pmol level (< 10^{-12}) (Figure 5(a,b)). The above results indicate that the bivalent recombinant scFvs-Fc antibody was constructed as a full-length IgG1 antibody, and the affinity of the antibodies was further enhanced.

3.5.2. Binding activity of the bivalent recombinant scFv-Fc antibody and full-length IgG1 antibody to tumor cell surface antigens

We previously detected the binding affinity between recombinant antibodies (scFv-Fc and full-length antibody IgG1) and the soluble antigen EphA2 using the macromolecular interaction technique.

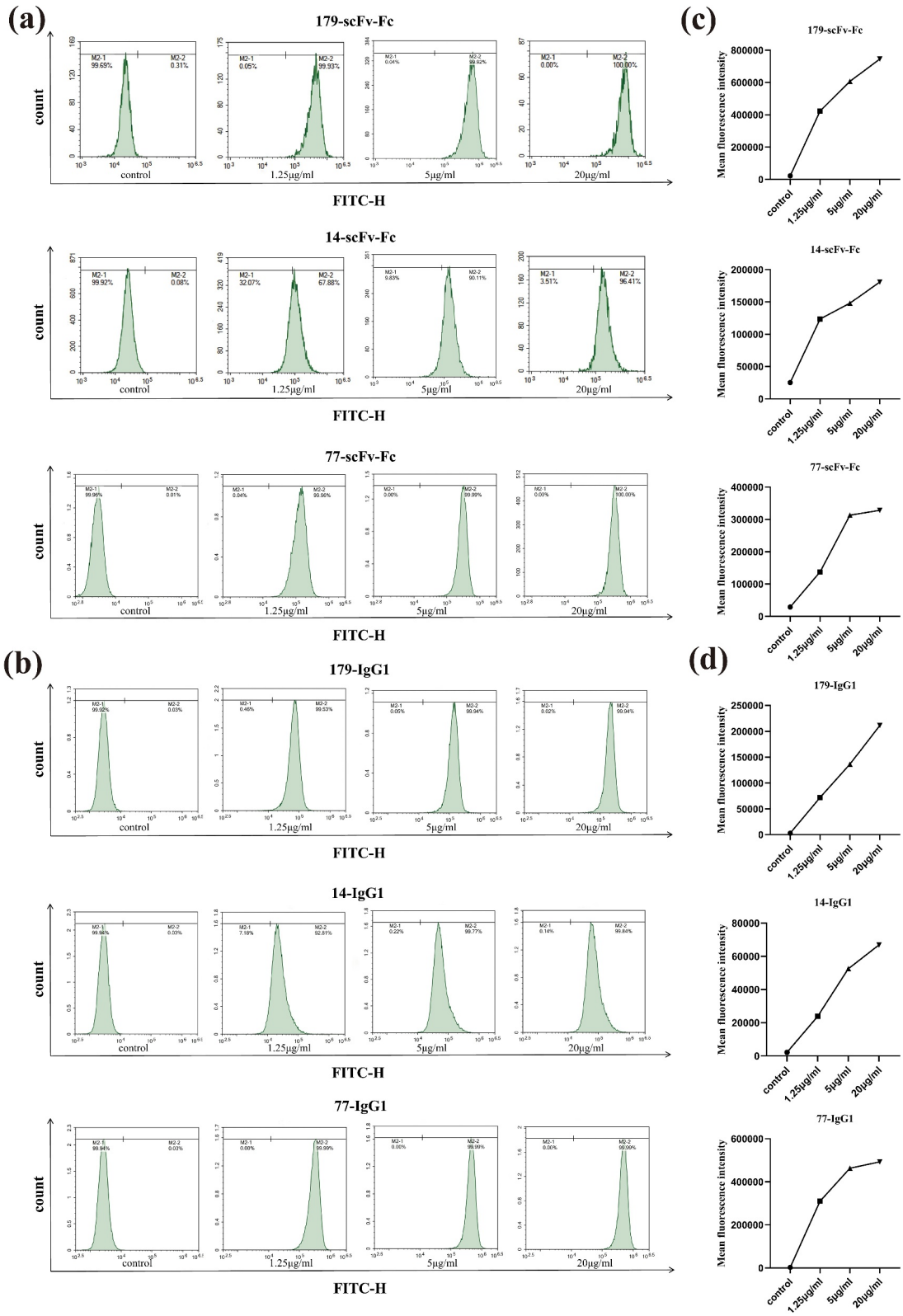


Figure 6. Bivalent recombinant scFv-Fc and full-length IgG1 antibodies bind with high affinity to the EphA2 receptor on the surface of tumor cells. FSC-sorted PC-3 and A549 tumor cells were SSC-labeled. Positive-bound target cells were then circled with controls. (a) The binding of scFv-Fc to EphA2 receptors on PC-3 cells at different concentrations. (b) The binding of IgG1 to EphA2 receptors on PC-3 cells at different concentrations. (c) Changes in the mean fluorescence intensity of scFv-Fc when bound to EphA2 receptors on PC-3 cells at different concentrations. (d) Changes in the mean fluorescence intensity of IgG1 when bound to EphA2 receptors on PC-3 cells at different concentrations. (e) The binding of scFv-Fc to EphA2 receptors on A549 cells at different concentrations. (f) The binding of IgG1 to the EphA2 receptors on A549 cells at different concentrations. (g) Changes in the mean fluorescence intensity of scFv-Fc when bound to EphA2 receptors on A549 cells at different concentrations. (h) Changes in the mean fluorescence intensity of IgG1 when bound to EphA2 receptors present on A549 cells at different concentrations (the data are representative of one of three independent experiments).

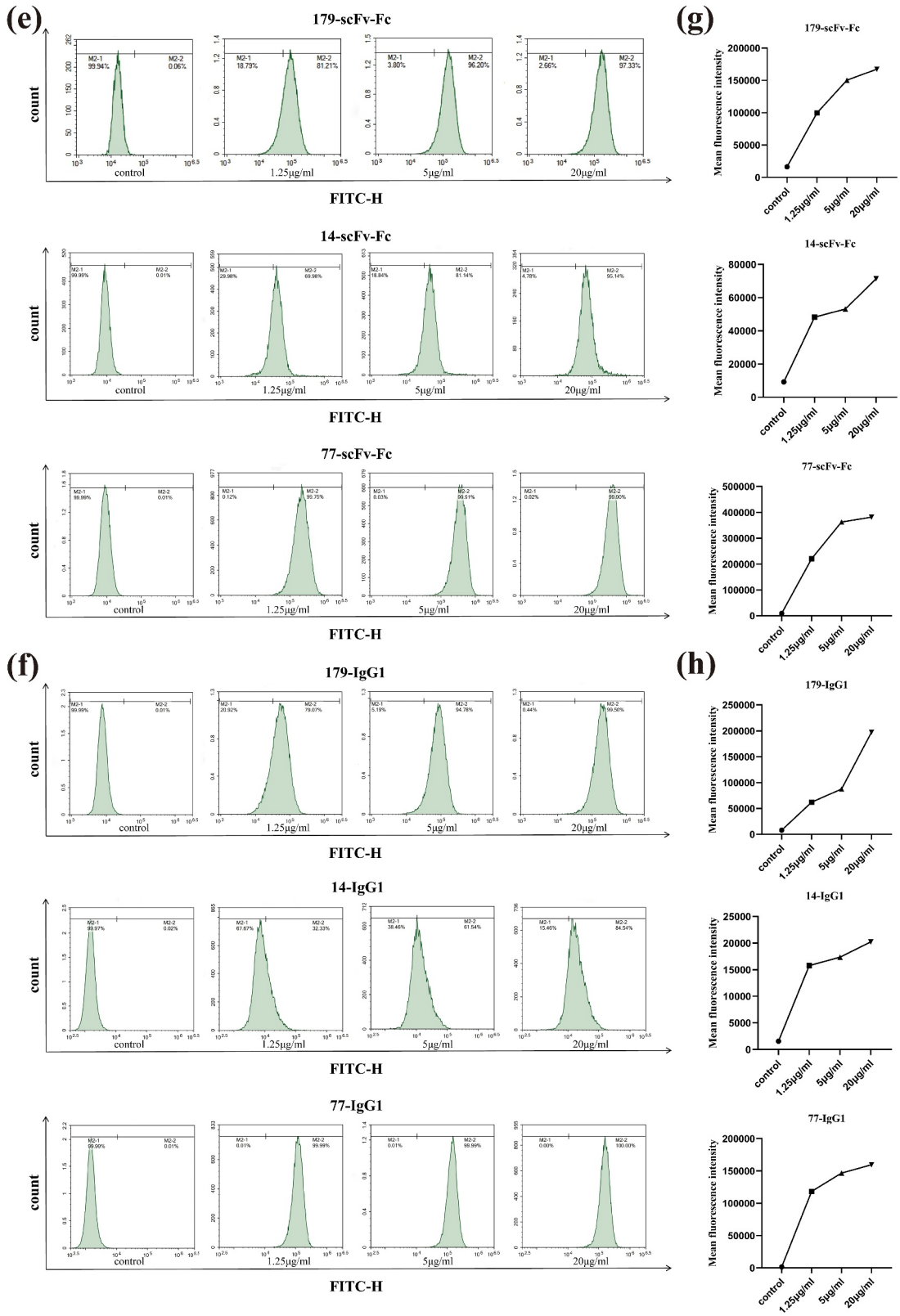


Figure 6. Continued.

Here, we further detected the binding activities between the recombinant antibodies and the cell lines (PC-3 and A549) with a high expression of EphA2 by flow cytometry. The binding with PC-3

cells revealed that (Figure 6(a,b)): the binding rates of 179-scFv-Fc and 77-scFv-Fc at 1.25 μg/ mL and above were greater than 99; the binding rates of 14-scFv-Fc at 5 μg/ mL and above were greater than 90%; the binding rates of 179-IgG1 and 77-IgG1 at 1.25 μg/ mL and above were greater than 99%; and the binding rates of 14-IgG1 at 1.25 μg/ mL and above were greater than 90%. Binding with A549 cells showed that (Figure 6(e,f)): the binding rates of 77-scFv-Fc at 1.25 μg/mL and higher were more than 99%, 179-scFv-Fc at 1.25 μg/ mL and higher were more than 81%, and 14-scFv-Fc at 5 μg/mL and higher were higher than 81%; the binding rates of 77-IgG1 at 1.25 μg/mL and higher were more than 99%, 179-IgG1 at 5 μg/ mL and higher were more than 94%, and 14-IgG1 at 5 μg/mL and higher were higher than 61%. The above results indicated that all three of the bivalent recombinant antibodies and full-length antibodies that we constructed could effectively bind to EphA2 receptors on PC-3 and A549 cells. The mean fluorescence intensity was positively correlated with the antibody concentration (Figure 6(c, d, g, h)

3.5.3. Binding of bivalent recombinant antibody scFv-Fc and full-length IgG1 antibody to tumor tissue surface antigens

Immunohistochemical results showed that compared with adjacent normal lung tissue, all three purified bivalent recombinant scFv-Fc and purified full-length IgG1 antibodies could effectively bind to the EphA2 antigen on the surface of adenocarcinoma tissues with good binding activity. In contrast, the isotype control groups did not bind to adenocarcinoma tissues surface receptors (Figure 7(a,b); magnification: 40 ×; scale bar: 40 μm).

3.6 Bivalent recombinant scFv-Fc and full-length IgG1 antibodies inhibit tumor cell proliferation

To assess the inhibitory effect of the bivalent recombinant scFv-Fc and full-length IgG1 antibodies in human lung adenocarcinoma A549 cells, we used a CCK8 assay to detect the inhibitory effect of different concentrations of the bivalent recombinant scFv-Fc and the full-length IgG1 antibodies on tumor cells. These results reveal that the bivalent recombinant scFv-Fc and the full-length IgG1 antibodies exhibited time- and dose-dependent inhibition of A549 tumor cells (Figure 8). The magnitude of the OD at 450 nm for A549 target cells decreased with an increasing antibody concentration and with increasing co-culture time. Compared to the negative control group, the proliferation of A549 cells was inhibited by three bivalent recombinant scFv-Fc antibodies and three full-length IgG1 antibodies at concentrations of 10 μg/mL and 50 μg/mL after 72 h co-culture (both $P < 0.05$), and the inhibition was more pronounced at 50 μg/mL (both $P < 0.05$). The maximum inhibition was 40% at a concentration of 50 μg/mL for the bivalent recombinant antibody scFv-Fc and 34% at a concentration of 50 μg/mL for the full-length antibody IgG1. The above results indicate that the constructed bivalent recombinant scFv-Fc antibody and the full-length IgG1 antibody have some inhibitory effect on the proliferation of tumor cells.

4. Discussion

In this study, 200 peripheral blood PBMC samples from patients with different malignant tumors were used to construct a large and diverse scFv library. The scFvs with affinity 10^{-8} could be easily screened from the library. The affinity was further improved when scFv was further modified into scFv-Fc and full-length IgG1 antibodies. The affinity of the full-length antibody 77-IgG1 reached 10^{-12} (pmol). The constructed scFv-Fc and full-length IgG1 antibodies could specifically bind to EphA2 expressed on tumor cells and tissues and had the ability to inhibit tumor cell growth, which can provide the basis for the subsequent immunotherapy of malignant tumors.

The antibodies screened from different types of phage antibody libraries exhibit different affinities. Antibody libraries can generally be classified into immune and nonimmune libraries, depending on the source of the library [60]. Immune libraries are constructed from antibody V genes isolated from B cells (*IgG*) of patients or immunized animals and are commonly used in medical research to obtain antibodies against a specific target antigen. In contrast, nonimmune libraries are constructed from antibody V genes in B cells (*IgM*) from unimmunized donors and cannot specifically bind to antigens [61]. It has been shown that antibodies screened from nonimmune libraries lack *in vivo*

rearrangements, and thus the antigen binding affinity is low [62]. In addition, the high background of antibody screening in nonimmune libraries and the low abundance of antibodies against specific target antigens require a larger library capacity to screen for higher affinity antibodies, which greatly increases the difficulty associated with screening. Unlike nonimmune libraries, immune libraries contain a large number of antibodies against specific target antigens and their screening background is greatly reduced. In addition, antibodies screened from the immune library have undergone an *in vivo* affinity maturation process in the host and have a high affinity [63,64]. The acquisition of high affinity antibodies is a complex process influenced by multiple factors, and the source of the phage antibody library is only one factor that affects the level of affinity. Notably, the *in vitro* screening of antibody affinity is also a key influencing factor. In this study, phage display technology was used to enrich and select the scFv library, and scFvs with a high affinity were easily screened from the scFv library after three rounds of affinity screening.

Different types of antibodies can be used for different purposes [65]. IgG-like antibodies are known to be the most abundant immunoglobulins in the blood and are also the main form of therapeutic antibodies. The molecular weight of full-length IgG is approximately 150 kDa and its blood clearance half-life (T1/2) in humans is 21 days [66]. Most therapeutic antibodies that are currently available are developed in the IgG framework; the majority of which belong to the IgG1 subtype [67,68]. IgG1 has a 60% abundance distribution in the serum and primarily binds protein antigens, is highly stable and resistant to aggregation, and has a greater ability to activate antibody-dependent cytotoxicity and complement-dependent cytotoxicity [69,70]. In contrast, another antibody fragment based on the IgG form, scFv-Fc, is similar to full-length IgG in terms of tumor uptake [66] and has a better tumor targeting profile. In addition, scFv-Fc has a smaller molecular weight than full-length IgG, while retaining the ease of penetration of the monovalent antibody scFv into tumors [60]; however, compared to the monovalent binding of scFv, scFv-Fc is a recombinant bivalent molecule that has significantly increased affinity, reduced aggregation tendency, and an increased serum half-life [71]. In addition, the added Fc segment can restore the immunological functionality of the Fc region. Therefore, this bivalent recombinant antibody can serve as a good radionuclide carrier in radioimmunotherapy, and can also serve as a therapeutic antibody, which can obtain a better therapeutic effect. In the present study, scFv screened from a library of fully human-derived phage single-chain antibodies was further modified and transformed into scFv-Fc and full-length IgG1 antibodies. Following the modification, the affinity of the antibodies was further improved with the highest affinity reaching the pmol level (10^{-12}), which indicated that the construction of immune libraries from a wide range of tumors was successful and the screened antibodies were affinity matured with high affinity in the patients. Three bivalent recombinant antibodies and three full-length antibodies were screened for their ability to inhibit tumor growth, suggesting that functional antibodies with tumor therapeutic properties could be more easily screened from a library of antibodies from tumor patients.

In the 35 years since the first monoclonal antibody drug was approved by the US FDA in 1986, 100 antibody drugs have subsequently been launched. Despite the large number of approved antibody drugs, their associated targets remain highly limited, and the development of antibody drugs based on tumor targets has become the key to antibody research [72]. EphA2 is an important oncogenic protein and an emerging drug target [18,73,74]. In addition, EphA2-based targeted therapies have appeared in clinical trials at various stages in a variety of malignancies [75–77]. Among these, antibodies are preferred for EphA2-targeted therapeutics due to their high specificity, affinity, stability, and multiple mechanisms of action with cell surface proteins [78]. A range of antibodies against the EphA2 receptor have been investigated in preclinical studies. A monoclonal antibody targeting EphA2 was produced by Kelly Carles-Kinch et al., who further demonstrated that this EphA2 monoclonal antibody had a significant inhibitory effect on the growth of malignant tumor cells, as well as distant metastasis by inducing the

autophosphorylation and degradation of the EphA2 protein [79]. In another study, EphA2 antibodies also exhibited strong therapeutic potential. Three anti-EphA2 monoclonal antibodies were prepared by Atsushi Sakamoto et al. In studies of melanoma cell lines, EphA2 monoclonal antibodies were found to significantly inhibit the cellular migration, invasion, and other cell metastatic behaviors. Moreover, when researchers coupled EphA2 monoclonal antibodies to immunotoxins, it was found that the coupled EphA2 monoclonal antibodies were strongly cytotoxic, could inhibit growth, and held therapeutic promise for the treatment of melanoma, an aggressive tumor [80]. The above studies showed that antibody research based on the EphA2 target was extremely important in improving tumor treatment modalities and enhancing the effectiveness of tumor therapy; however, to date, there have been limited studies on radioactive immunotherapy using antibodies as a carrier for the Epha2 target. We screened three strains of human Epha2 bivalent recombinant scFv-Fc and full-length IgG1 antibodies with high affinity through phage display technology, providing a basis for tumor immunotherapy drugs. In the future, we will continue to explore the targeted killing of tumor cells by anti-EphA2 antibodies in vivo to obtain a good immunotherapeutic effect.

5. Conclusion

Overall, it was demonstrated that therapeutic antibodies with high affinity for specific targets can be screened from immune libraries derived from

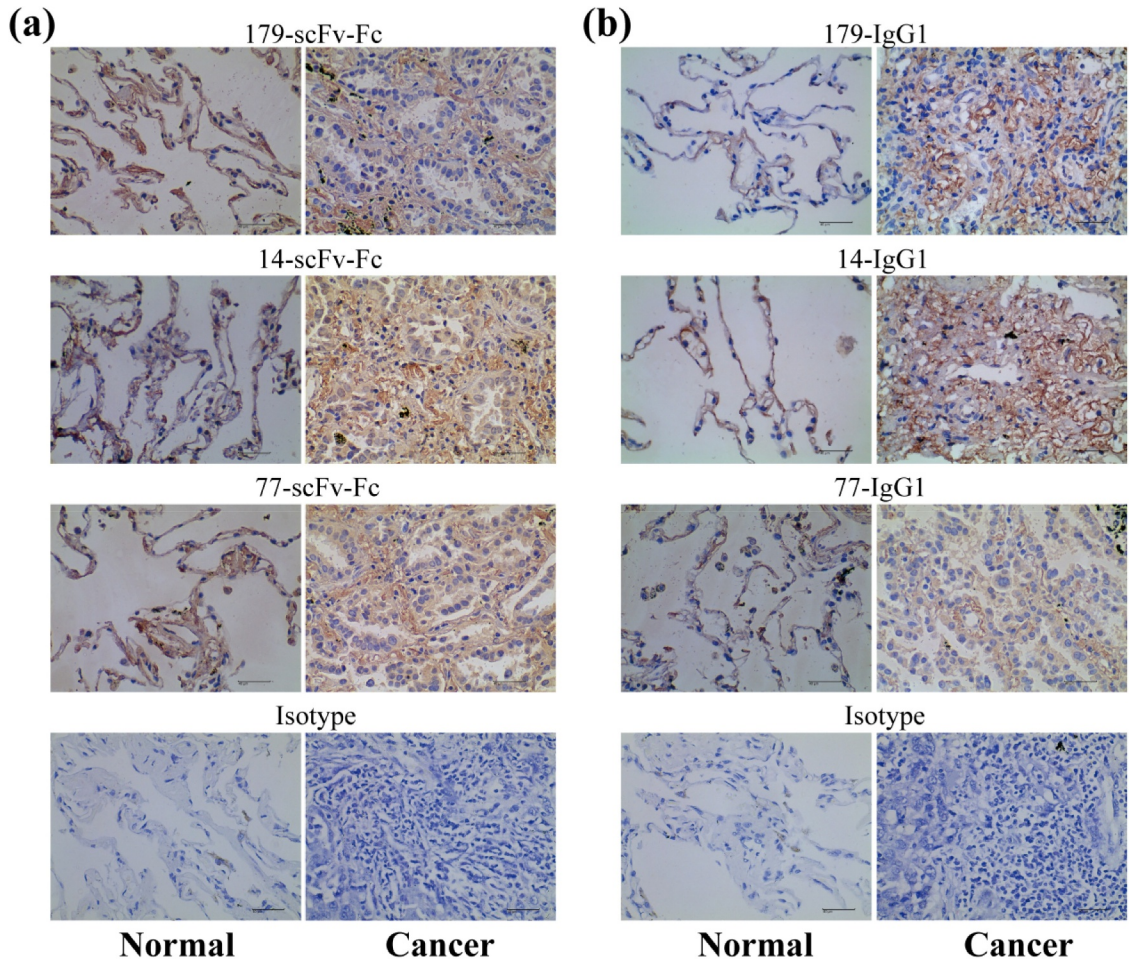


Figure 7. The bivalent recombinant scFv-Fc antibody and full-length IgG1 antibody bind effectively to EphA2 receptors on the surface of tumor tissues. (a) The binding of scFv-Fc to adenocarcinoma tissue and adjacent normal lung tissue. The isotype controls are background staining controls. (b) The binding of IgG1 to adenocarcinoma tissue and adjacent normal lung tissue. The isotype controls are background staining controls (magnification: 40×; scale bar: 40 μm).

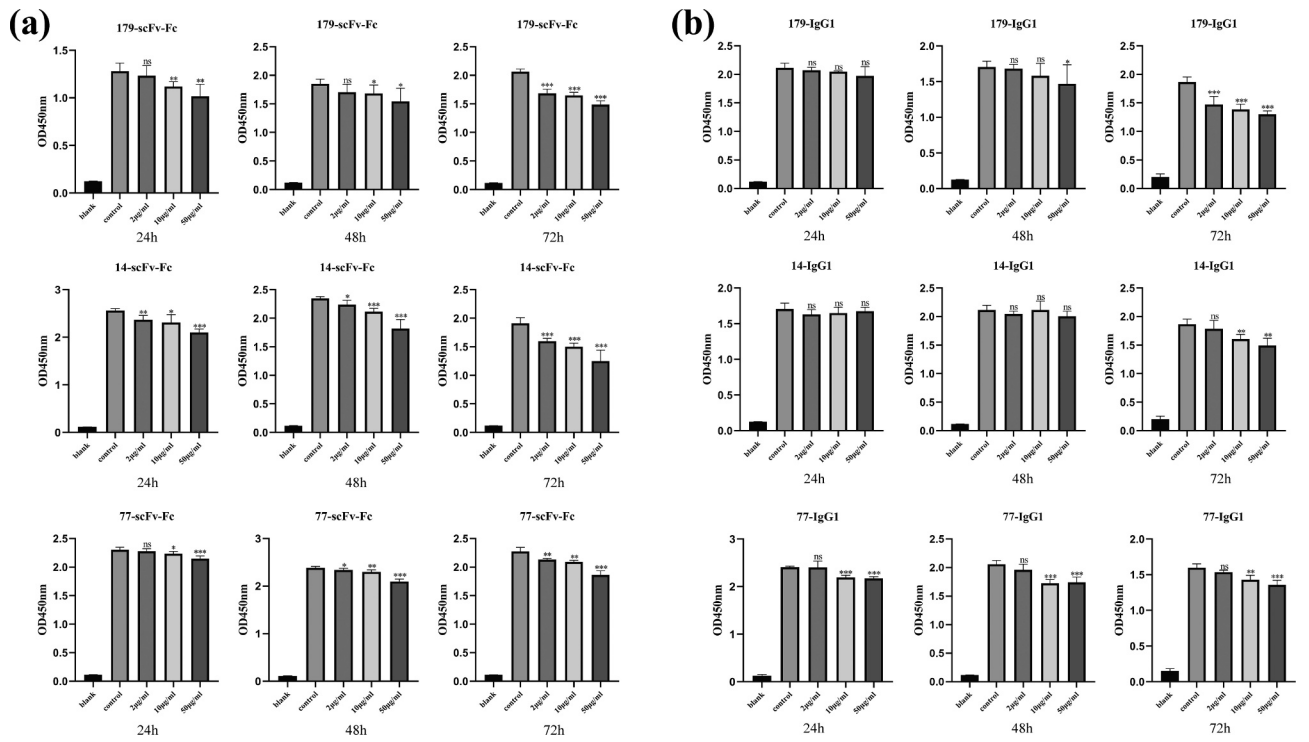


Figure 8. Both the bivalent recombinant scFv-Fc antibody and full-length IgG1 antibody inhibited the growth of tumor cells to some extent. (a) Inhibition of tumor cell growth by scFv-Fc at different concentrations and various durations of action. (b) Inhibition of tumor cell growth by IgG1 at different concentrations and durations of action (n ≥ 3 per experimental group; ns, not statistically significant; * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$. A *t*-test for group design was used for statistical analysis.

patients with multiple types of tumors. The research results suggest that screened scFv-Fc and IgG1 antibodies against EphA2 could be used for the further study of tumor immunotherapy; however, further *in vivo* and *in vitro* experiments, including clinical trials, should be conducted to validate our results.

Highlights

- High affinity scFvs against EphA2 can be easily screened from the tumor immune library.
- Affinities were further improved after modification of anti-EphA2 scFvs into scFv-Fc or IgG1.
- scFv-Fc and IgG1 against EphA2 were able to inhibit the growth of tumor cells to some extent.

Abbreviations

The full name; Eph: Erythropoietin-producing hepatocellular; RTK:Receptor tyrosine kinase; EphA2: Erythropoietin-producing hepatocellular A2; MAb: Monoclonal antibodies; scFv: Single-chain fragment variable; ADCC: Antibody dependent cell-mediated cytotoxicity; CDC: Complement dependent cytotoxicity; ADPC: Antibody dependent phagocytosis; PBMCs: Peripheral blood mononuclear cells; OS: Overall survival; DFS: Disease-free survival; VHHeavy chain variable domain: VL: Light chain variable domain; Vλ: λ light chain; Vκ: κ light chain; LB: Luria-Bertani; BSA: Bovine serum albumin; HRP: Horseradish peroxidase; Anti-M13-HRP: Horseradish peroxidase-conjugated anti-M13 antibodies; TMB: Tetramethylbenzidine liquid substrate; RPMI: Roswell Park Memorial Institute; Fc: Crystallizable fragment; GBM: Glioblastoma multiforme; LGG: Brain lower grade glioma; LUAD: Lung adenocarcinoma; PAAD: Pancreatic adenocarcinoma; KIRC: Kidney renal clear cell carcinoma; THCA: Thyroid carcinoma; BLI: Biolayer interferometry

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Author contributions

Yaqi Yang and Siji Nian designed the study, Yaqi Yang completed the cell experiments and wrote the article, Siji Nian completed the data analysis, and processed the images. Lin Li, Xue Wen, Qin Liu,

Bo Zhang, and Yu Lan completed the antibody expression, as well as the purification. All authors contributed to drafting of the manuscript and approved the final version.

Disclosure statement

The authors declare that the submitted work was not carried out in the presence of any personal, professional, or financial relationships that could potentially be constructed as a conflict of interest.

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