The Construction and Analysis of the Affinity-matured Library of the Anti-EpCAM Single Domain Antibodies

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Abstract

Single domain antibody (sdAb) is a new type of low molecular weight antibody for broad clinical applications. EpCAM is over-expressed in many cancer cells and is a good target for cancer therapy. The anti-EpCAM sdAb aEP3D4 was previously cloned by our laboratory in a separate study. In this study, error-prone PCR was performed to introduce random mutations in aEP3D4 to increase its affinity. PCR product was ligated into phagemid pComb3XSS, and the ligated phagemid was transformed into E. coli DH5α to create an affinity-matured library containing 3.58×10^7 cfu (colony-forming unit). Five rounds of screening the library were conducted to enrich affinity-matured sdAbs, and the P/N ratio of each round of screening indicated that the enrichment of EpCAM-specific sdAbs increased along with each round. In addition, polyclonal phage ELISA was performed to verify the screening result. The result showed that the specificity of the affinity-matured library to EpCAM also increased along with each round, which was consistent with the P/N values. This affinity-matured library will be used for the isolation of the high affinity anti-EpCAM sdAbs, which may become good cancer therapeutics.

Keywords

Affinity Maturation; Error-prone PCR; Phage Display; ELISA; sdAb.

1. Introduction

Single domain antibody (sdAb) is a low molecular weight antibody, and it contains only a variable region of antibody heavy chain and is the smallest complete antigen binding fragment [1]. Human sdAb has no immunogenicity, high solubility and good thermostability, which provides the broad commercial applications [2]. Since the requirement of high antibody affinity in clinical application for better therapeutic effect, sdAbs need to be affinity-matured in vitro [3]. In antibody engineering, the most common modification is the antibody affinity, which is closely related to the efficacy, dosage and side effects of antibody drugs [4-5]. In the process of sdAb affinity maturation, error-prone PCR is usually used to introduce random mutations in wild-type sdAbs to create an in vitro antibody library with high diversity [4]. Phage display, yeast display and ribosome display are often used for affinity maturation of antibodies in vitro, and the affinity of various antibodies was significantly increased [6-7].

Phage display technology was first developed in 1985 and has been widely used in the antibody discovery field [8]. Five years later, McCafferty described phage displayed antibodies for the first time, and the gene encoding the whole antibody binding domain was linked to phage gene III, which helped to display sufficient antibodies on the outer surface of phage to screen phages that could recognize specific antigen [9]. Compared with traditional antibody screening methods, phage display technology has many advantages. Phage display technology could screen antibodies in vitro, thus avoiding immune response, and accurately control antigen conformation and concentration [6, 10-11]. It could screen for specific antibodies using recombinant proteins, cells, organs and even the whole organism as substrates [12-13]. In addition, it also has the advantages of large library capacity,

simple operation and low cost [14-15]. Many antibodies screened by phage display have been approved for marketing or are in clinical research. Belimumab was approved in 2011 [16], and Avelumab targeting PD-L1 is currently in phase III clinical study [17].

At present, phage display has become a powerful platform for sdAb screening and a useful tool to improve antibody characteristics [11]. After introducing random mutations into the wild-type sdAb gene, the mutant sdAbs with matured affinity were further isolated by phage display [18]. The studies showed that the sdAb affinity was increased by 8-1300 times through random mutations and phage display [19-22]. The anti-EpCAM sdAb aEP3D4 was previously cloned by our laboratory in a separate study (unpublished data). In this study, we performed in vitro affinity maturation of human sdAb aEP3D4 targeting EpCAM, and preliminarily analyzed the affinity-matured library. This study can lay a necessary foundation for the isolation of the high affinity sdAbs for clinical application.

2. Materials and Methods

2.1 Materials and reagents

GeneMorph II Random Mutagenesis Kit was purchased from Agilent Technologies (Santa Clara, CA, USA), and phagemid vector pComb3xss was obtained from MiaoLingBio (Wuhan, China). The E. coil DH5α and TG1 were purchased from Novagen (Madison, WI, USA). The immune tube and 96-well plate were purchased from NUNC (Rochester, NY, USA). The EpCAM, CXCR4 and EGFR fragments were synthesized by Bootech (Shanghai, China). The BSA (Bovine Serum Albumin) and PEG 6000 were purchased from Sigma Aldrich (St. Louis, MO, USA). Anti-M13-HRP was purchased from Sino Biological (Beijing, China). TMB (3, 3′, 5, 5′-Tetramethylbenzidin) was from Beyotime (Shanghai, China).

2.2 Generation of affinity-matured library by error-prone PCR

The construction of affinity-matured library was performed with the anti-EpCAM sdAb aEP3D4. Random mutations were introduced by the error-prone PCR, which was performed using a GeneMorph II Random Mutagenesis kit. According to the protocol of the kit, one ng plasmid DNA of the aEP3D4 was used as the PCR template, and 30 cycles were conducted to introduce mutations. The reaction product was purified, digested by SfiI, and then ligated into the phagemid pComb3xss at a molar ratio of 10:1. To construct the affinity-matured library, the ligated phagemid was then transformed into E.coli DH5α through electroporation, and the electro transformation was carried out twenty times to enlarge the library size. Then, 10 μl of transformed bacteria was diluted and cultured on TYE plate which containing 100 μg/ml ampicillin, and the growing colonies were counted to calculate the size of the bacterial library. The remaining bacteria were cultured in a 2×TY medium containing 100 μg/ml ampicillin for 2 hours at 37 ℃ and then stored at -80 ℃ with 15% glycerol.

The bacteria stock was culture-enlarged at 37 ℃ in 2×TY culture medium containing 100 μg/ml ampicillin and 1% glucose. The KM13 helper phages were added and incubated at 37 ℃ for 30 min to rescue the phage library. After incubation, the precipitate was collected by centrifugation at 3200 g for 10 min, and then cultured in 2×TY culture medium containing 100 μg/ml ampicillin, 50 μg/ml kanamycin and 0.1% glucose for 20 h at 25 ℃. After centrifugation at 3200 g for 10 min, the supernatant was collected, and the phage library was precipitated with PEG/NaCl. The phage library was resuspended in PBS and the size of the phage library was determined by calculating the growing colonies.

2.3 Phage library screening

The EpCAM antigen fragment with a concentration of 100 μg/ml was added to the NUNC immune tube for antigen coating. Meanwhile, PBS buffer was added to another immune tube as the negative control. After overnight coating at 4 ℃, the immune tubes were washing three times with PBS, and then blocked with 2% BSA solution at room temperature for 2 h. Discarded the blocking solution, washed the immune tubes with PBS three times, added the phage library (5×10^{12} PFU), and incubated at room temperature for 2 h. Discarded the phage solution and washed the immune tubes for 10 times with PBST, and the antigen binding phages were eluted by trypsin solution (1 mg/ml).

The eluted phages obtained above were used to infect E. coil TG1, and 10 μl of infected bacteria was diluted and cultured on TYE plate containing 100 μg/ml ampicillin for calculating the titer of eluted phages. After centrifugation and resuspension, the remaining 990 μl of bacterial solution was cultured on TYE plate containing 100 μg/ml ampicillin and 1% glucose. After overnight culture at 37 ℃, all colonies growing on the plate were scraped off and enlarged to produce phage library for the next round of screening. A total of 5 rounds of screening were carried out, the concentration of antigen coated in the 2nd to 5th round of screening was 50, 50, 25, 25 μg/ml, respectively. The phage library added to the immune tube for incubation was obtained from the previous round of screening. In addition, the washing times of the immune tube with PBST in each round increased to 20 times.

2.4 Polyclonal phage ELISA

To verify the results of five rounds of screening, the phage libraries obtained from five rounds of screening were used as primary antibodies for polyclonal phage ELISA. Firstly, 100 μl of EpCAM fragment solution with concentration of 2 μg/ml was added to NUNC 96-well immune plate for antigen coating, and PBS, CXCR4 and EGFR fragments were used as blank and unrelated antigen controls, respectively. After the immune plate was coated overnight and washed with PBS for three times, it was blocked with 2% BSA solution at room temperature for 2 h. Discarded the blocking solution, washed the immune plate with PBS for three times, added the phage libraries obtained from five rounds of screening and incubated at room temperature for 1 h. Washed the immune plate with PBST for three times, and then added 100 μL anti-M13-HRP as the secondary antibody, and incubated at room temperature for 1 h. Washed the immune plate with PBST for three times, added 100 μl TMB solution to each well at the same time, incubated at room temperature in dark for 5 min to develop color, added 50 μl 1M H_2SO_4 to each well to terminate the reaction, and then measured the absorbance at 450 nm on the microplate reader (Bio-RAD, Hercules, CA, USA).

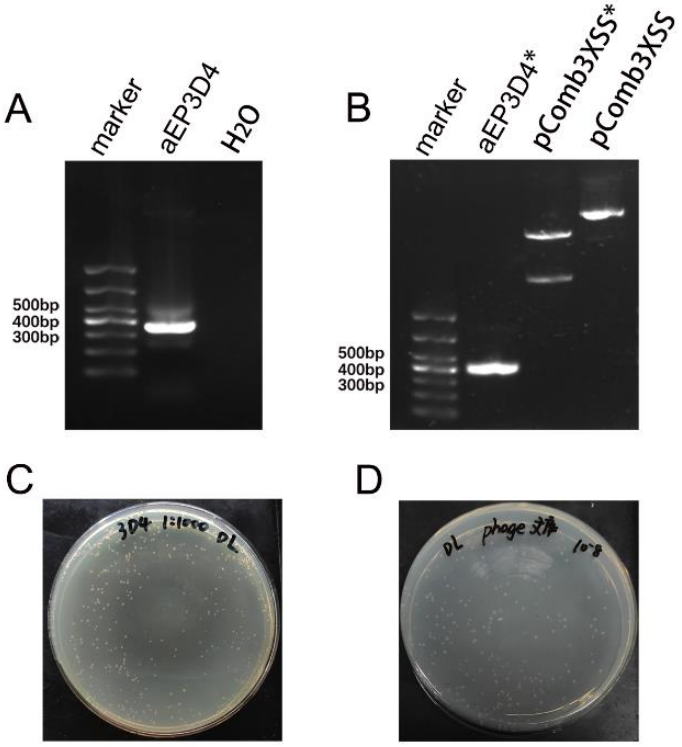

Fig. 1 Construction of affinity-matured library by error-prone PCR. (A) The gene of wild-type sdAb aEP3D4 was used as the template for error-prone PCR, and ddH_2O as blank control. The PCR products were verified by electrophoresis. (B) The PCR product of aEP3D4 and phagemid pComb3XSS were digested overnight and verified by electrophoresis, *indicates that they were digested. (C) After electroporation, 10 μl of bacterial solution was diluted and cultured on TYE plate to determine the size of bacterial library. (D) The phage library was diluted and used to infect TG1 to calculate the titer of phage library.

3. Results and discussion

3.1 Construction of affinity-matured library by random mutation

The wild-type human sdAb aEP3D4 was amplified by error-prone PCR and then the PCR product was ligated with phagemid pComb3xss to construct the affinity-matured library. One ng of aEP3D4 gene was used as the template for error-prone PCR, and ddH_2O as the blank control. As shown in Fig.1A, the band of aEP3D4 was about 400 bp, and there was no band in the blank control. The PCR product of aEP3D4 and phagemid pComb3XSS were digested by SfiI at 50℃ overnight, and the digested products were identified by electrophoresis. As shown in Fig.1B, the complete pComb3XSS was 4900bp, the digested pComb3XSS contained 3300bp and 1600bp. The digested PCR product was ligated into the digested pComb3XSS overnight at 16℃. The ligated product was transformed into E.coli DH5α by electroporation to prepare the bacterial library. The size of the library obtained by one electroporation was about 1.79×10^6 cfu as showed in Fig.1C. The electroporation was performed 20 times, and the obtained bacterial libraries were mixed. The total library size was about 3.58×10^7 cfu. The bacterial library was culture-enlarged, and the phage library was rescued by adding the helper phage KM13. As shown in Fig.1D, the titer of phage library was 1.43×10^{13} pfu/ml.

3.2 Enrichment of the EpCAM-specific sdAbs from the affinity-matured phage library

The phage display screening was performed to enrich affinity-matured sdAbs from phage library. The EpCAM fragment was coated on the immune tube, and phage library was added for incubation, the non-specific phages were removed by continuous washing with PBST, and the EpCAM specific phages were eluted with trypsin. In order to obtain sdAbs with higher affinity, the concentration of coated antigen was decreasing gradually, and the washing times of PBST after phage incubation were increased. In addition, the PBS was included as a blank control. The titer of the phage library eluted in each round was determined, and the phage titer was expressed by P for the EpCAM group and N for the blank control group. The P/N ratio of each round of screening shows the enrichment of antigen-specific phages. As shown in Table 1, the P/N ratio of each round was increasing gradually, which indicated that the enrichment of EpCAM specific sdAbs was increasing. After five rounds of screening, the P / N value increased from 5.67 in the first round to 48.92 in the fifth round of screening, and the recovery rate of phage library was also increased.

Table 1. The results of five rounds of phage library screening

Round	Antigen (μg/ml)	Input phage (pfu)	Output phage (pfu) (P)	Output phage of negative control (pfu) (N)	Recovery Rate (P/Input phage)	P/N
1	100	5×10^{12}	3.22×10^{5}	5.68×10^{4}	6.44×10^{-8}	5.67
2	50	5×10^{12}	4.51×10^{7}	4.06×10^{6}	9.02×10^{-6}	11.11
3	50	5×10^{12}	4.72×10^{8}	1.98×10^{7}	9.44×10^{-5}	23.84
4	25	5×10^{12}	8.68×10^{8}	2.69×10^{7}	1.74×10^{-4}	32.27
5	25	5×10^{12}	5.43×10^{9}	1.11×10^{8}	1.09×10^{-3}	48.92

Note: pfu is the plaque forming unit; P represents the titer of phage library eluted in the antigen group; N represents the titer of phage library eluted in PBS group.

3.3 The verification of screening results by polyclonal phage ELISA

After five rounds of screening, the specificity of the obtained affinity-matured phage libraries was verified by polyclonal phage ELISA. EpCAM fragment was coated on 96-well immune plate, and PBS, CXCR4 and EGFR were coated as blank and unrelated antigen controls. The phage libraries obtained from five rounds of screening were used as the primary antibody to verify their specificity for EpCAM. As shown in Fig. 2, the absorbance at 450 nm of each round of screening was increased obviously, indicating that the specificity to EpCAM of the library obtained from each round of screening was gradually increasing. The result was consistent with the P/N values.

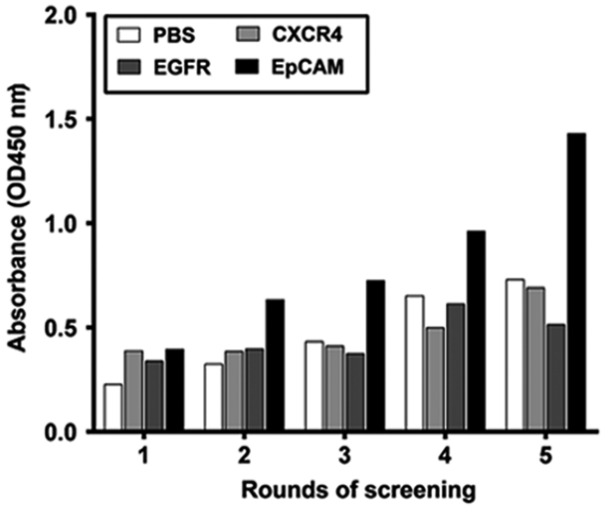

Fig. 2 The specificity verification of the affinity-matured phage library by polyclonal phage ELISA. PBS was used as blank control, and CXCR4 and EGFR as unrelated antigen controls. The phage libraries obtained from five rounds of screening were used as the primary antibody to verify their specificity for EpCAM. The higher absorbance at 450 nm indicated higher antigen specificity.

4. Conclusion

sdAb is a low molecular weight antibody with the broad commercial applications. Due to the high affinity requirements of antibody drugs in clinical application, we performed affinity maturation by error-prone PCR to introduce random mutations in wild-type sdAb aEP3D4 to get better therapeutic effect. The mutated sdAbs were ligated into phagemid pComb3XSS to create an affinity-matured library. Then, five rounds of screening and polyclonal phage ELISA were carried out to enrich and verify the EpCAM specific sdAbs. Our study will lay a necessary foundation for further sdAb application.

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