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Screening and Identification of Single Domain Antibodies Against CD44v6

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Abstract

CD44v6 antigen is overexpressed in many different cancers and is involved in the tumor formation, invasion, metastasis and development. Therefore, it is a good target for cancer therapy. Single domain antibody (sdAb) is a small antibody fragment that maintains the antigen binding function. At present, no anti-CD44v6 sdAbs have been approved for the treatment of cancers, and only a small number of anti-CD44v6 sdAbs are currently in the pre-clinical and clinical studies. In this study, a full human sdAb library was screened by phage display technology for the anti-CD44v6 sdAbs. After the four rounds of screening, the anti-CD44v6 sdAb phages were enriched. Some single phages were randomly picked from the fourth round of screening and tested by monoclonal phage ELISA. Four anti-CD44v6 sdAb phages were isolated and could specifically bind to CD44v6 antigen. These anti-Cd44v6 sdAbs can provide good candidates for the treatment of different cancers.

Keywords

Cancer; SdAb; Phage Display; ELISA.

1. Introduction

CD44 is a multifunctional transmembrane adhesion glycoprotein. The full-length CD44 gene consists of 20 exons and 19 introns [1]. Exons 1-5 and 16-20 are constant and encode the shortest isoform of CD44, which is known as CD44 standard isoform (CD44s). The intermediate 10 exons can be alternatively spliced and inserted between the exons 5 and 16 to form different variant isoforms (CD44v) [2]. The isoform of CD44v may contain a single exon, such as CD44v3 or CD44v6, or a combination of multiple different exons, such as CD44v6-v7 and CD44v8-v10 [3,4].

A large amount of evidence shows that both CD44s and CD44v are overexpressed in a variety of cancers and play different important roles in the occurrence and development of cancers [5]. CD44s is expressed in most cells of vertebrates, and its overexpression in cancer is mainly concentrated in solid tumors and hematologic malignancies [6]. Unlike CD44s, the expression of CD44v in normal cells is limited to a few normal cell types, such as embryonic cells, keratinocytes, monocytes and some hematopoietic cells [7]. However, CD44v is expressed in a variety of cancers, especially those in advanced cancer stages [8]. CD44v6 is one of the most studied CD44 variants, is over-expressed in most primary and metastatic head and neck squamous cell carcinoma (HNSCC) and is considered as a prognostic marker of HNSCC [9]. Currently, several monoclonal antibodies (mAbs) targeting CD44v6 have been developed for preclinical studies, such as 2F10, VFF4, VFF7, VFF18 (BIWA 1), U36, V6B3, HB-256 and Var 3.1. These mAbs can specifically bind to CD44v6 antigen and have been used in the diagnosis and treatment of different cancers in several clinical trials [10]. For example, the chimeric antibodies (chU36) and BIWA 4 were coupled to a radioactive label 99mTc for clinical phase I

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treatment of HNSCC [11,12]. In another clinical I study, the humanized mAbs BIWA 4 was coupled with a radioactive label 186Re for the treatment of head and neck cancer [13]. However, these clinical trials were discontinued because of severe skin toxicities with one fatal outcome attributed to mertansine conjugates [14].

Antibody-based cancer treatment strategy is one of the most successful and important strategies for the treatment of hematological malignant tumors and solid tumors. Single domain antibody (sdAb) is a small antibody fragment that maintains the antigen binding function [15]. At present, the sdAbs have been widely used in many aspects of life due to its small molecular weight (only 15 kDa), strong tissue penetration and low immunogenicity [16]. For example, because of its small size and high specificity, sdAbs are often coupled with various labels for disease diagnosis and detection [17]. In addition, sdAbs do not have Fc and can avoid being cleared and degraded by the body due to Fc-mediated immune responses. sdAbs are extremely popular carriers for mediating drug delivery in disease treatments [18].

Phage display is an in vitro method for selecting specific fusion peptides to be displayed on the phage surface [19]. The principle of this technique is to fuse the gene of foreign protein or peptide with the coat protein of phages, so that the target protein can be displayed on the surface of phages [20]. Then, phages with high affinity to specific antigen or ligand were isolated from a phage library by screening. Because of its low cost, and high screening efficiency, phage display is a powerful tool for isolating target specific ligands and has been widely used in antibody screening [21]. So far, at least 14 approved mAb drugs on the market have been obtained through phage display technology, and many other mAbs are in preclinical development or clinical trials [22].

2. Materials and Methods

2.1. Materials and Reagents

TMB (3, 3', 5, 5'-Tetramethylbenzidine) was purchased from Beyotime (Shanghai, China). Anti-M13-HRP was from Sino Biological (Beijing, China). BSA (Bovine Serum Albumin) was from Newprobe (Beijing, China). The Plasmid Maxi Preparation Kit was purchased from Omega Biotech (Doraville, GA, USA). PEG 6000, Tryptone and Yeast extract were from Sigma Aldrich (St. Lous, MO, USA). E. coil BL21(DE3) and E. coil DH5α were from Novagen (Madison, WI, USA). The recombinant human EGFR extracellular protein and recombinant human CD28 extracellular protein were purchased from Sino Biological (Beijing, China).

2.2. Amplification of the sdAb Phage Library

The sdAb phage library used in this experiment was purchased from Source BioScience (Nottingham, UK). To amplify the sdAb phage library, 100 μ L of the original sdAb phage library (contains about $3x10^9$ phage clones per milliliter) was inoculated to 50 ml 2 × TY culture medium and cultured at 37°C and 220 rpm until the OD600 to 0.5. About $5×10^{10}$ colony-forming unit (cfu) KM13 helper phages were added, incubated for 30 min in a water bath at 37 °C and then cultured at 220 rpm for two hours. The infected E. coli TG1 was collected by centrifuging at 3300g for half an hour, the supernatant was discarded, and then the pellet was resuspended again in 50 ml 2 × TY medium (containing 100 ug/ml ampicillin, 50 μg/ml kanamycin and 1% glucose) and cultured at 30°C overnight. The next day, phages were collected by PEG/NaCl precipitation after centrifugation at 3300 g for half an hour. The amplified sdAb phage library was filtered and sterilized by 0.45 um filter and stored at - 80℃.

2.3. Screening of CD44v6-specific sdAbs from the sdAb Phage Library

The CD44v6 antigen was chosen as the target for the selection of specific sdAbs from the sdAb phage library. Briefly, the nucleotide sequence of human CD44v6 was obtained in genebank and

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was synthesized and inserted between Nco I and Not I digestion sites of pET-22b expression plasmid after being optimized by GENEWIZ (Guangzhou, China). His tag was added at the 3' end of CD44v6 to facilitate purification and detection. The expression plasmid containing CD44v6 antigen was transformed into E. coli BL21 for the soluble expression and purification by Ni-NTA column.

Plate panning was used to select the specific anti-CD44v6 sdAbs. Briefly, the human CD44v6-His antigen (400 ug) in phosphate buffered saline (PBS) was coated on immune tube (NUNC) at 4°C overnight, and after washing with PBS for 3 times, 2% bovine serum albumin (BSA) in PBS was added and incubated for 2-2.5 hours. Each tube was washed with PBS and then incubated with 5×10^{12} cfu displayed phages (Input phages) with 2% BSA in PBS for 2 hours. Unbound or weakly bound phages were removed by washing with PBST for 10 times for the first round of screening and for 20 times for the 2-4 rounds of screening. The specific phages were then eluted with triethylamine (1mg/ml) for 5-10 minutes. The eluted phages were named output phages and used to infect TG1 cells at 37°C for half an hour, which were amplified by the help of KM13 helper phages for next round of panning. this process represented one round of bio-panning, and 2-4 rounds of library screening were carried out by coating lower amount of CD44v6 antigen ranging from 200 μg to 100 ug.

2.4. Polyclonal Phage ELISA

After four rounds of screening, the CD44v6-specific phages were enriched gradually. The enrichment degree of four rounds of library screening was detected by polyclonal phage ELISA. Each well of 96-well plates was coated with PBS control, EGFR and CD28 fragment as unrelated protein controls or the CD44v6 antigen at 4°C overnight, and the coating concentration was 0.2 ug per well. Then, each well of 96-well plates was blocked with 2% BSA in PBS for 2-2.5 hours. After washing with PBST, 10^{12} cfu eluted phages from each round of screening were added to the wells and the plates were incubated at room temperature for 1 hour. Then, 100 μL of diluted anti-M13-HRP secondary antibody was added into each well and incubated for 1 hour at room temperature. After adding tetramethylbenzidine (TMB), reaction was stopped by the addition of diluted H_2SO_4, and the optical density (OD) value was measured with ELISA reader (Bio-RAD, Hercules, CA, USA) at 450 nm.

2.5. Monoclonal Phage ELISA

To screen specific monoclonal phages against CD44v6, 100 ul of E. coli TG1 in the exponential growth phase (OD600 = 0.5) was infected with the phages eluted from the 4th round of screening and cultured on TYE plate (containing 100 μg/mL ampicillin and 1% glucose). The next day, individual clones were picked randomly and cultured with shaking overnight in 1.5 mL tubes containing 200 ul of 2×TY (1% glucos and 100 μg/mL ampicillin). $1x10^8$ cfu KM13 helper phages were then added to aids in phages amplification and replication, and then the infected E. coli TG1 was collected by centrifuging at 3300g for half an hour. The precipitate was re-suspended in 200 μl 2 × TY medium (containing 50 μg/ml kanamycins, 100 μg/ml Ampicillin and 0.1% glucose) and shacked at 220 rpm for 20 hours. Phage clones were collected by PEG/NaCl precipitation and centrifugated at 3200g for 10-20 minutes.

For monoclonal phage ELISA, A 96-well plate was coated overnight with CD44v6 antigen (0.2 ug/well) and blocked with 2% BSA in PBS. Two unrelated antigens (EGFR and CD28) were included to help to screen the specific anti-CD44v6 monoclonal phages. 100 ul of the selected phage clones were added in each well of a 96-well plate and incubated for 1 hour. After washing with PBS, 100 μL of diluted anti-M13-HRP was added into each well and incubated for 1 hour. After adding tetramethylbenzidine (TMB), reaction was stopped by the addition of diluted sulfuric acid, and the optical density (OD) value was measured by an automated microplate reader (Bio-RAD, Hercules, CA, USA).

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3. Results and Discussion

3.1. Screening sdAb Phages Against CD44v6

The titer of the original sdAb phage library was about $3x10^9$ pfu (plaque forming unit) /ml and needs to be amplified before screening the sdAb phage library. The titer of the amplified sdAb phage library was 4.2×10^{13} pfu/ml.

CD44v6 antigen was coated on the plastic surface of NUNC 96 well plates and incubated with phages from the library. Phages which unbound or weakly bound to CD44v6 antigen were removed by washing with PBST, and phages specifically bound to CD44v6 antigen were eluted with trypsin and amplified again by infecting E. coli TG1. PBS blank control was included to verify the results of each round of screening. As shown in Table 1, the enrichment ratio (P/N) increased significantly from 3.88 after the first round of selection to 51.81 after the fourth round of selection.

Table 1. Enrichment of anti-CD44v6 sdAb phages from screening the phage library

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}	4.42×10^5	$1.14x\ 10^5$	8.84×10^{-8}	3.88
2	50	5×10^{12}	2.83×10^7	2.51×10^6	5.66×10^{-6}	11.27
3	50	5×10^{12}	3.21×10^8	$8.56x\ 10^6$	6.42×10^{-5}	37.50
4	25	5×10^{12}	1.43×10^9	2.76×10^7	2.86×10^{-4}	51.81

P represents the titer of the sdAb phage library eluted when CD44v6 antigen was coated on the tube. N represents the titer of the sdAb phage library eluted when PBS as a control was added to the tube.

3.2. The Four Rounds of Library Screening were Detected by Polyclonal Phage ELISA

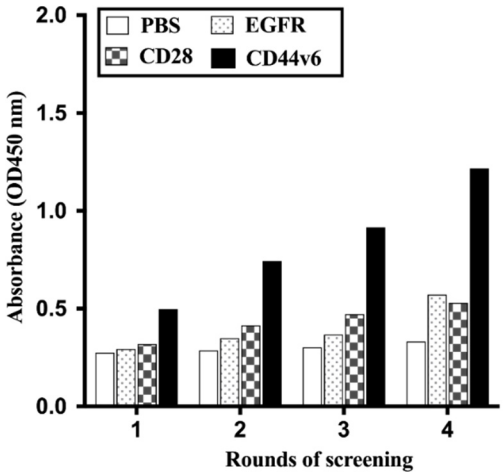

Fig 1. The anti-CD44v6 sdAbs enriched by four rounds of library screening was detected by polyclonal phage ELISA. PBS, EGFR, and CD28 were negative controls. The primary antibody was the sdAb phages after each round of screening. The secondary antibody was M13-HRP

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After four rounds of screening, the enrichment of anti-CD44v6 phages was detected by polyclonal phage ELISA. As shown in Fig.1, the absorbance of OD450 nm after each round of screening increased compared with the previous round of screening when the wells were coated with CD44v6 antigen, and the absorbance did not increase when the wells were coated with PBS and two negative control antigens (EGFR and CD28), indicating that specific anti-CD44v6 sdAb phages were enriched after the four rounds of selection.

3.3. Screening Anti-CD44v6 sdAb Phages by Monoclonal Phage ELISA

To obtain positive monoclonal phages against CD44v6, some individual phage clones were picked randomly from the phage library obtained from the fourth round of screening and checked by monoclonal phage ELISA. To select the anti-CD44v6 monoclonal phages with good specificity, PBS and two unrelated antigens (EGFR and CD28) were used. aEP3D4 and aEP4D11 against EpCAM are the two sdAb phages as positive controls which were previously screened by our laboratory. Fig. 2 shows the results of ELISA with 34 monoclonal phages, and the arrows represent the four positive monoclonal phages with specific binding to CD44v6, and include CD44-1A5, CD44-1B2, CD44-1B7, CD44-1C10.

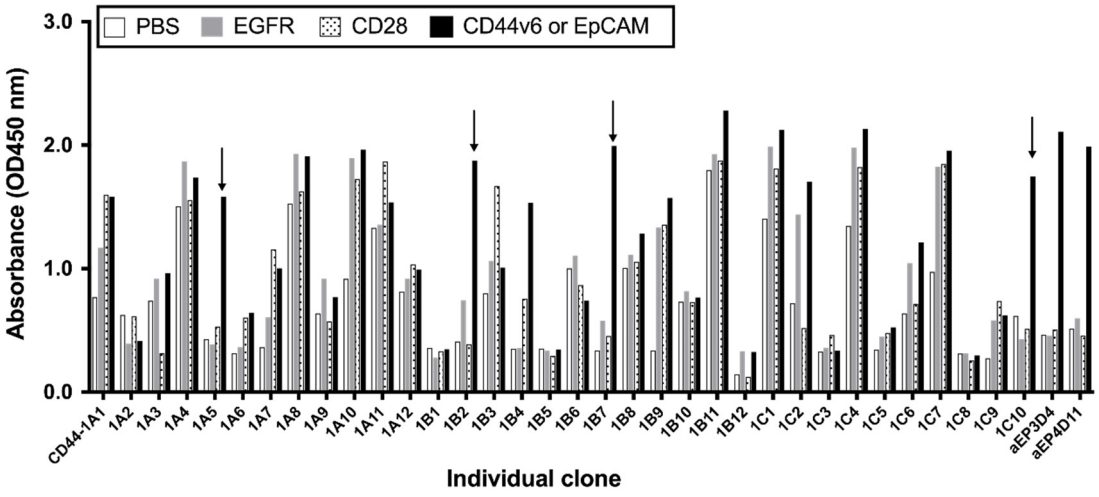

Fig 2. The positive monoclonal phages binding to CD44v6 antigen were screened by monoclonal phage ELISA. Monoclonal phages were randomly picked from the phage library enriched after the fourth round of library screening, and their binding ability to CD44v6 antigen was detected by monoclonal phage ELISA. The arrows represent the four positive monoclonal phages with specific binding to CD44v6. PBS, EGFR, and CD28 were negative controls, and aEP3D4 and aEP4D11 were two positive control sdAbs against EpCAM

4. Conclusion

The incidence rates of cancers are increasing year by year and the death rates continue to rise, sdAb is a promising new approach for cancer thrapy because of its small molecular weight (only 15 kDa), strong tissue penetration and low immunogenicity.

In this study, CD44v6 was used as an antigen to screen the sdAb phage library. After four rounds of screening, sdAb phages with specific binding to CD44v6 antigen were effectively enriched and confirmed by polyclonal phage ELISA. Then, individual phage clones were picked randomly from the phage library obtained from the fourth round of screening, and the specificity of each selected monoclonal phage binding to CD44v6 antigen was detected by monoclonal phage ELISA. The four monoclonal phages with good specificity against CD44v6 antigen were finally obtained. This study has laid a foundation for the use of these anti-CD44v6 sdAbs for the treatment of different cancers.

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