ISSN: 1813-4890

Isolation and Analysis of Human Anti-CD133 Single-domain Antibodies

Wanyi Zhu[a], Xing Wei[b,*]

Department of Cell Biology, College of Life Science and Technology, Jinan University, Guangzhou Guangdong, 510632, China

[a]2240792710@qq.com, [b,*]2433461420@qq.com

Abstract

CD133 is a cell surface marker of cancer stem cells (CSCs), and its overexpression is related to the proliferation, migration and invasion of tumor cells. Human Single-domain antibodies (sdAbs) have the advantages of high tumor penetration, production in a variety of expression systems, no immunogenicity and easy genetic engineering. In this study, phage display technology was used to screen the human sdAbs phage library. After 4 rounds of library screening, the anti-CD133 sdAb phage library was effectively enriched. Then, 216 monoclonal phages were randomly taken from the fourth round of library screening and examined by monoclonal phage ELISA. A total of 14 anti-CD133 sdAb phages were identified by monoclonal phage ELISA and could specifically bind to CD133 antigen. This study can lay a necessary foundation for subsequent studies of these anti-CD133 sdAbs for their anti-tumor activity in vitro and in vivo.

Keywords

CD133; Single-domain Antibody; Phage Display; ELISA.

1. Introduction

Human CD133 is a member of the five-transmembrane glycoprotein prominin family and contains 865 amino acids with molecular weight 120 kDa [1]. CD133 has an extracellular N-terminal domain, two large glycosylated extracellular loops, two smaller intracellular loops, and an intracellular C-terminal domain [2]. Analysis of the CD133 amino acid sequence revealed a total of nine potential N-glycosylation sites including five in the first extracellular loop and four in the second extracellular loop [3]. CD133 is highly expressed in various types of cancers, such as breast cancer, liver cancer, colon cancer, prostate cancer, pancreatic cancer, lung cancer, and head and neck squamous cell carcinoma [4,5]. CD133 is widely used as a cell surface marker of cancer stem cells (CSCs), which has the characteristics of stem cells including strong self-proliferation, renewal and differentiation ability. CD133 can use its own amino acid residue phosphorylation to mediate various signaling pathways, and also mediates various signaling pathways by interacting with other related proteins, thereby affecting the growth and proliferation of cancer cells and tumor stem cells. CD133 expression contributes to the "stemness", tumorigenicity, epithelial-mesenchymal transition induction, invasion, and metastasis of tumor cells [6]. Several anti-CD133 monoclonal antibodies (such as C2E1 and AC133) with inhibitory effects on tumor cells bind to the second extracellular loop of human CD133 protein [7,8]. Therefore, we also selected a sequence (amino acids 507-716) in the CD133 second extracellular loop as the target antigen fragment.

sdAbs consist of four conserved framework regions (FR1-FR4) and three complementarity-determining regions (CDR1-CDR3). They have many advantages such as small molecular weight (15 kDa), strong affinity, low immunogenicity and easy production [9-11]. sdAbs can enhance tissue penetration and maintain their functional potential to specifically bind antigens,

ISSN: 1813-4890

and they can easily be modified to contain the Fc domain and and can be combined with drugs, radionuclides, photosensitizers and nanoparticles at specific positions. These properties make them particularly suitable for targeting tumors in vivo [12].

Phage display technology originated in 1985 and was discovered by George Smith [13]. Phage display technology allows rapid separation based on binding affinity to specific target molecules, and facilitates the identification of displayed peptides or proteins after the selection of phages with desired binding properties [14]. Antibody fragments are typically fused to the N-terminus of the pIII minor coat protein and displayed on the M13 phage. Most antibody libraries were cloned in phagemid vectors which contain the f1 origin of replication for single-stranded replication, the double-stranded origin of replication and the antibiotic resistance gene. *E. coli* was infected by phages, and phages require helper phages to provide phage genes required for phage assembly [15]. The antibody library screening process mainly includes "adsorption-elution-amplification". The anti-CD133 sdAb phages screened in this study laid a necessary foundation for their subsequent in vitro and in vivo anti-tumor activity studies.

2. Materials and Methods

2.1. Materials and Reagents

E. coli TG1 was purchased from Novagen (EMD Millipore, Madison, WI, USA). Anti-M13-HRP, EpCAM, CD31 and NGF protein were purchased from Sino Biological Inc (Beijing, China). BSA (bovine serum albumin), TMB (3, 3′, 5, 5′-Tetramethylbenzidin), Kanamycin and Ampicillin were purchased from Beyotime Biotechnology (Shanghai, China). The 5 mL immune tubes and Nunc MaxiSorp 96-well plates were purchased from NUNC (Rochester, NY, USA).

2.2. Amplification of sdAb Phage Library

The sdAb phage library was purchased from Source BioScience (Nottingham, UK). The sdAb phage library was taken out from the -80°C refrigerator, transferred to 50 mL of 2×TY medium (containing ampicillin and glucose), and incubated at 37°C, 220 rpm for 2.5 h. Then a 5×10^{10} helper phage KM13 was added, and after 30 min of incubation at 37°C, the pellet was collected by centrifugation. The bacterial pellet was resuspended in 2×TY medium (containing ampicillin, kanamycin, and glucose), and cultured at 25°C and 220 rpm for 20 h. Then, the supernatant was collected by centrifugation, and 20% PEG/NaCl solution was added. After ice bathing for 1 h, the precipitate was collected by centrifugation. The precipitate was resuspended in PBS and centrifuged to collect the supernatant. The obtained phage library was diluted to a suitable gradient, infected TG1, and incubated at 37 °C for 30 min. The bacterial solution was spread on a TYE plate, and the library titer of the amplified sdAb phage was calculated according to the number of monoclonal phages.

2.3. Phage Library Screening

The nucleotide sequence of the human CD133 antigen fragment (amino acids 507-716) was obtained through NCBI, and was fused to the His-tag sequence. Suzhou Jinweizhi Biological Co., Ltd. inserted the nucleotide sequence of CD133 antigen into the prokaryotic expression plasmid pET-22b (+) by the restriction sites Nco I and Not I to construct the prokaryotic expression plasmid pET-22b-CD133-His. Soluble protein expression and purification were performed using the *E. coli* prokaryotic expression system.

CD133 antigen was added to an immune tube, and an equal volume of PBS was added to another tube. After incubation overnight at 4℃, tubes were washed with PBS and then blocked with 2% BSA at room temperature for 2 h. The blocking solution was discarded, tubes were washed with PBS, and 5×10^{12} pfu phages were added. All the solution was discarded, tubes were washed with PBST, and trypsin was added to elute the bound phages. The obtained phages were

ISSN: 1813-4890

infected with *E. coli* TG1, and the phage solution was spread on a culture plate after an appropriate dilution to calculate the phage titer. After centrifuging and resuspending the remaining bacterial solution, it was spread on another plate and cultivated. After overnight culture at 37℃, all the bacterial colonies on the petri dish were scraped and expanded to generate a phage library for the next round of screening. A total of 4 rounds of screening were conducted. Phage libraries obtained from the previous round of screening were used for the next round of screening. The CD133 antigen concentration was 100 μg/ml for the first round of library screening, 50 μg/ml for the second and third rounds and 25 μg/ml for the fourth round.

2.4. Polyclonal Phage ELISA

The enrichment degree of 4 rounds of phage library screening was verified by polyclonal phage ELISA. CD133 antigen (0.2 μg/well) was added to the 96-well immune plate, and blank control (PBS) and negative control antigens (CD31 and NGF) were included. After incubation overnight at 4°C, the plate was washed with PBS, and blocked with 2% BSA solution for 2 h at room temperature. The blocking solution was discarded, and the plate was washed with PBS. sdAbs phage library obtained after each round of screening was added and placed at room temperature for 1 hour. The solution was discarded, and the plate was washed with PBST. Anti-M13-HRP was added to each well and placed at room temperature for 1 h. The plate was washed with PBST, 100 μL TMB was added to each well, and the plate was kept in the dark at room temperature for 5 min. Then, sulfuric acid was added to each well and the OD450 nm absorbance was measured on a microplate reader (Bio-RAD, Hercules, CA, USA).

2.5. Monoclonal Phage ELISA

Anti-CD133 sdAb phages were screened by the monoclonal phage ELISA. The phage library obtained in the fourth round of screening was plated. The bacteria colonies were randomly picked from the TYE plate and added to each well of 96 well-plate for culture. phages were derived from the infection of helper phage KM13. CD133 was the antigen, and PBS blank control group, CD31 and NGF were included as the negative controls. Other steps are the same as described in the polyclonal phage ELISA.

3. Results and Discussion

3.1. Amplification of sdAb Phage Library

It is necessary to amplify the sdAb phage library before antibody screening. The amplified phage library was infected with TG1 and incubated at 37°C for 30 min. The appropriate amount of bacterial culture was spread on TYE plate containing ampicillin and cultured at 37°C overnight. The library titer of the amplified sdAb phages was calculated according to the number of phages grown on the TYE plate, and the amplified sdAb phage library titer was 4.37×10^{13} pfu/mL.

3.2. Enrichment of Anti-CD133 sdAb Phages from Phage Libraries Using Phage Display Technology

To obtain the phages that specifically bind to the CD133 antigen, 4 rounds of sdAb phage library screening were performed. The coated antigen concentration was decreased for each subsequent round of screening. After the phage library was incubated with the CD133 antigen, the number of washes with PBST was increased to wash away unbound or poorly bound phages for each subsequent round of screening. As shown in Table 1, P indicates the phage library titer obtained by screening the phage library with CD133 antigen; N indicates the phage library titer obtained by screening the phage library with PBS. The P/N value of each round can reflect enrichment for specific anti-CD133 sdAb phages. In addition, because the amount of phage library added (Input phage) in each round of screening is 5×10^{12} pfu, the recovery rate of the

ISSN: 1813-4890

phage library by each round of screening can also reflect the enrichment. The P/N value gradually increased from 6.15 in the first round to 41.61 in the fourth round, and the recovery rate also increased from the first to the fourth round.

Table 1. Enrichment of anti-CD133 sdAbs after 4 rounds of library screening

Round	Antigen(ug/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}	3.97×10^{5}	6.46×10^{4}	7.94×10^{-8}	6.15
2	50	5×10^{12}	4.77×10^{7}	3.95×10^{6}	9.54×10^{-6}	12.08
3	50	5×10^{12}	4.85×10^{8}	1.88×10^{7}	9.70×10^{-5}	25.80
4	25	5×10^{12}	1.34×10^{9}	3.22×10^{7}	2.68×10^{-4}	41.61

Note: pfu indicates the phage forming unit; P indicates the phage library titer obtained by screening the phage library with CD133 antigen; N indicates the phage library titer obtained by screening the phage library with PBS.

3.3. The Enrichment of 4 Rounds of Library Screening was Verified by Polyclonal Phage ELISA

The enrichment of 4 rounds of library screening was verified by polyclonal phage ELISA. CD133 antigen was coated on 96-well plates, and PBS blank control, CD31 and NGF negative control antigens were included. The sdAb phage library obtained after each round of screening was used as a primary antibody for the next round of screening, and the anti-M13-HRP was used as a secondary antibody. As shown in Fig. 1, the enrichment of the anti-CD133 sdAb phages increased for each round of screening.

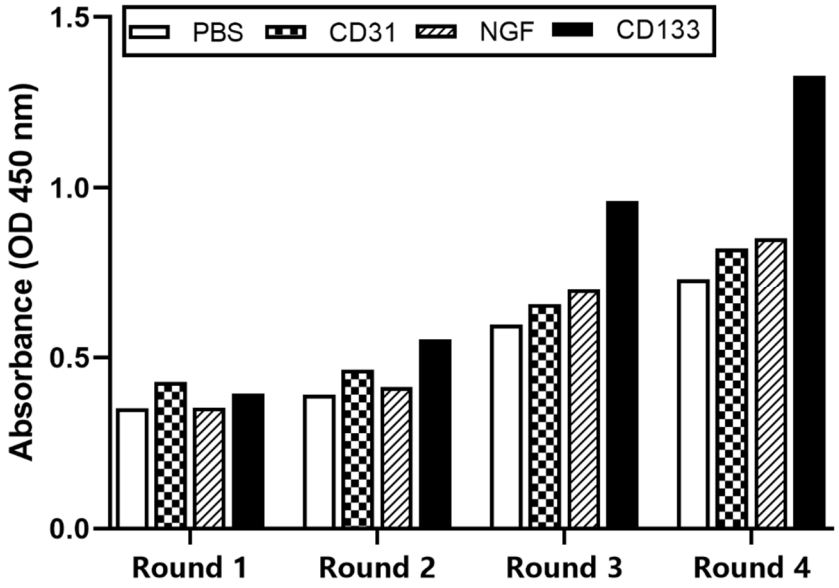

Fig 1. The enrichment of 4 rounds of library screening was verified by polyclonal phage ELISA. CD133 was used as antigen, and PBS was blank control, CD31 and NGF were negative controls. sdAb phage library obtained after each round of screening was used for the next round of screening as a primary antibody, and anti-M13-HRP was used as a secondary antibody. Polyclonal phage ELISA was performed to detect the enrichment of anti-CD133 sdAb phages. The higher the absorbance value represents the higher the enrichment of anti-CD133 sdAb phages

ISSN: 1813-4890

3.4. Screening of Anti-CD133 sdAb Phages by Monoclonal Phage ELISA

The sdAb phage library obtained in the fourth round of screening was infected with TG1, and monoclonal phages were randomly picked from the plates. The binding of phages to CD133 antigen was detected by monoclonal phage ELISA. PBS was the blank control, CD31 and NGF were the negative control antigens, and CD133 was the target antigen. aEP3D4 and aEP4D11 are the two anti-EpCAM sdAbs previously obtained in our laboratory, and were included as positive control antibodies. In this experiment, a total of 216 monoclonal phages were randomly picked from the fourth round of screening and tested by monoclonal phage ELISA. A total of 14 anti-CD133 sdAb phages were obtained. Fig. 1 shows the ELISA detection results of the representative 22 anti-CD133 phages.

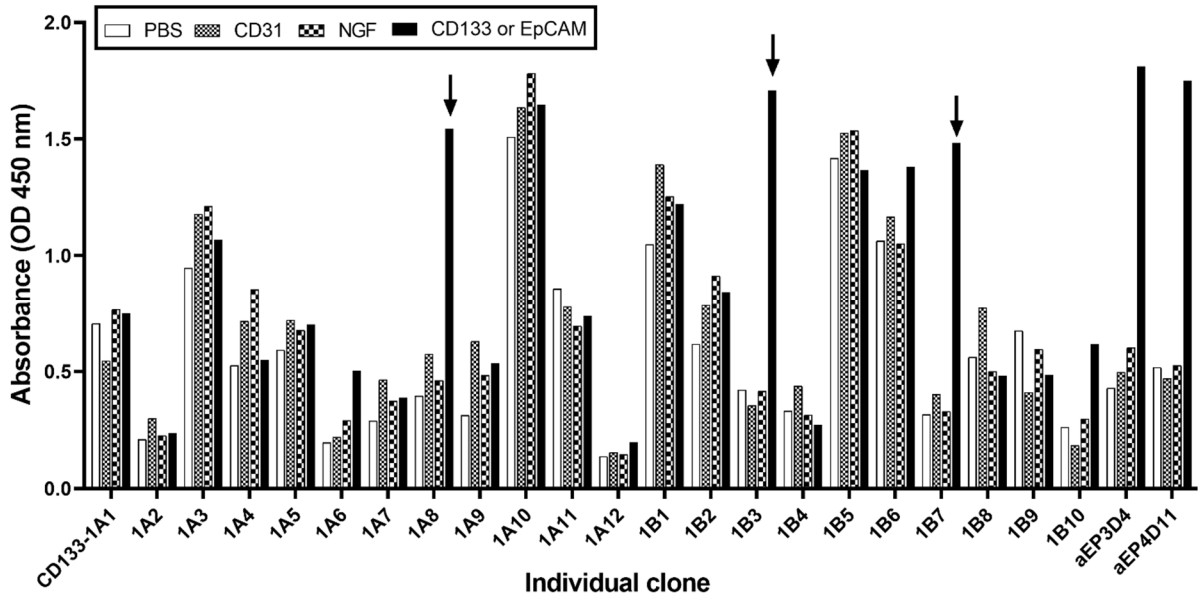

Fig 2. Anti-CD133 sdAb phages were tested by monoclonal phage ELISA. Monoclonal phages were randomly picked from the sdAb phage library obtained in round 4 and tested by monoclonal phage ELISA. It shows the ELISA results of only representative 22 monoclonal phages. aEP3D4 and aEP4D11are the two anti-EpCAM sdAbs previously obtained in our laboratory as positive control antibodies. PBS was the blank control, CD31 and NGF were the negative control antigens, and CD133 was the antigen. Arrows represent the positive phages that bind specifically to CD133 antigen

4. Conclusion

In this study, human CD133 was used as the antigen, and phage display technology was performed to screen the human sdAb phage library. After four rounds of library screening, monoclonal phages were randomly taken from the fourth round of library screening. Positive monoclonal phages that specifically bind to CD133 were screened by monoclonal phage ELISA. This study can lay a necessary foundation for subsequent studies of anti-CD133 sdAbs on anti-tumor activity in vitro and in vivo.

Acknowledgments

The authors are grateful for the help from members of our lab. The authors declare no conflict of interest.

ISSN: 1813-4890

References

[1] Shmelkov S V, ST Clair R, Lyden D, et al. AC133/CD133/Prominin-1[J]. Int J Biochem Cell Biol, 2005, 37(4): 715-719.

[2] Thamm K, Graupner S, Werner C, et al. Monoclonal Antibodies 13A4 and AC133 Do Not Recognize the Canine Ortholog of Mouse and Human Stem Cell Antigen Prominin-1 (CD133)[J]. PLoS One, 2016, 11(10): e0164079.

[3] Akbari M, Shomali N, Faraji A, et al. CD133: An emerging prognostic factor and therapeutic target in colorectal cancer[J]. Cell Biol Int, 2020, 44(2): 368-380.

[4] Ferrandina G, Petrillo M, Bonanno G, et al. Targeting CD133 antigen in cancer[J]. Expert Opin Ther Targets, 2009, 13(7): 823-837.

[5] Mehra N, Penning M, Maas J, et al. Progenitor marker CD133 mRNA is elevated in peripheral blood of cancer patients with bone metastases[J]. Clin Cancer Res, 2006, 12(16): 4859-4866.

[6] Nomura A, Banerjee S, Chugh R, et al. CD133 initiates tumors, induces epithelial-mesenchymal transition and increases metastasis in pancreatic cancer[J]. Oncotarget, 2015, 6(10): 8313-8322.

[7] Chen H, Luo Z, Sun W, et al. Low glucose promotes CD133mAb-elicited cell death via inhibition of autophagy in hepatocarcinoma cells[J]. Cancer Lett, 2013, 336(1): 204-212.

[8] Wang D, Guo Y, Li Y, et al. Detection of CD133 expression in U87 glioblastoma cells using a novel anti-CD133 monoclonal antibody[J]. Oncol Lett, 2015, 9(6): 2603-2608.

[9] Muyldermans S. Nanobodies: natural Single-domain antibodies[J]. Annu Rev Biochem, 2013, 82: 775-797.

[10] Siontorou C G. Nanobodies as novel agents for disease diagnosis and therapy[J]. Int J Nanomedicine, 2013, 8: 4215-4227.

[11] Arezumand R, Behdani M, Mahdian R, et al. Camel Heavy Chain Polyclonal Antibody Raised Against Recombinant Murine Placental Growth Factor Expressed in Escherichia coli[J]. Monoclon Antib Immunodiagn Immunother, 2015, 34(2): 126-130.

[12] Bannas P, Hambach J, Koch-nolte F. Nanobodies and Nanobody-Based Human Heavy Chain Antibodies As Antitumor Therapeutics[J]. Front Immunol, 2017, 8: 1603.

[13] Smith G P. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface[J]. Science, 1985, 228(4705): 1315-1317.

[14] Nixon A E, Sexton D J, Ladner R C. Drugs derived from phage display: from candidate identification to clinical practice[J]. MAbs, 2014, 6(1): 73-85.

[15] Shim H. Antibody Phage Display[J]. Adv Exp Med Biol, 2017, 1053: 21-34.