Constructing the Recombinant Antibodies Containing the Anti-EpCAM Human Single-domain Antibodies and Their Expression and Purification

Heng Zhang[1,a], Xing Wei[1,*]

[1]Department of Cell Biology, National Engineering Research Center of Genetic Medicine, Guangdong Provincial Key Laboratory of Bioengineering Medicine, College of Life Science and Technology, Jinan University, Guangzhou 510632, Guangdong, China.

[a]1252697861@qq.com, *2433461420@qq.com

Abstract

Previous studies showed that the abnormal overexpression of epithelial cell adhesion molecule (EpCAM) was closely related to the proliferation and metastasis of a variety of tumor cells. Two anti-EpCAM human single domain antibodies (sdAbs) pET22b-aEP3D4 and pET22b-aEP4G2 were previously isolated by screening an antibody library in a separate study by our laboratory and showed some in vitro and in vivo anti-tumor activity (unpublished data). In this study, these two sdAbs were used to constructed two bivalent recombinant antibody expression plasmids (pET22b-aEP3D4-aEP4G2, pcDNA3.1-aEP3D4-aEP3D4) to increase their anti-tumor effect. In addition, these two bivalent recombinant antibodies were combined with the anti-human serum albumin nanobody (anti-HSA nanobody) or human IgG1 Fc domain to construct two anti-EpCAM recombinant antibody expression plasmids (pET22b-aEP3D4-anti-HSA-aEP4G2, pcDNA3.1-aEP3D4-aEP3D4-Fc). Two recombinant antibody proteins were expressed by the prokaryotic expression system E. coli BL21 (DE3) (pET22b-aEP3D4-aEP4G2 and pET22b-aEP3D4-anti-HSA-aEP4G2).Two recombinant antibody proteins were expressed by the eukaryotic expression system 293f cells (pcDNA3.1-aEP3D4-aEP3D4 and pcDNA3.1-aEP3D4-aEP3D4-Fc). The expression of these four anti-EpCAM recombinant antibody proteins were detected by SDS-PAGE. This study lays the foundation for further research on the in vitro and in vivo functions of these recombinant antibodies for cancer therapy.

Keywords

EpCAM; Recombinant Antibody Proteins; Fc; Anti-HSA Nanobody; sdAb.

1. Introduction

The epithelial cell adhesion molecule (EpCAM) is also known as HEA125, TACSTD-1, 17-1A, ESA, CD326, MK-1, etc. It is a type I transmembrane glycoprotein normally expressed in the epithelial tissue and plasma cell basolateral membrane [1]. This molecule was first discovered as a tumor cell-specific antigen in the epithelial cell membrane during the process of immunizing mice with human colon cells in the 1970s [2,3]. Studies have shown that EpCAM is overexpressed in colorectal cancer, lung cancer, gastric cancer, prostate cancer, ovarian cancer, breast cancer and endometrial cancer [4-7]. Abnormal overexpression of EpCAM is associated with tumor cell proliferation, metastasis and poor prognosis [8-10]. Therefore, EpCAM can be used as a marker for tumor diagnosis and treatment [11].

Antibodies are a powerful weapon for targeted tumor therapy. Currently, more than 70 antibodies have been approved for clinical treatment of tumors [12]. Monoclonal antibodies have the advantages of less side effects and are good in the targeted cancer therapy. Monoclonal antibodies can specifically target tumor cell surface antigens, and can activate classic ADCC and CDC to kill tumor cells through the Fc segment [13]. However, due to their large molecular weight (~150 kDa), their penetration into tumor tissues is greatly restricted, which seriously affects the therapeutic effect [14].

Small-size single domain antibodies (sdAbs) can specifically bind to tumor cell surface antigens. Compared with monoclonal antibodies, they have the advantages of low immunogenicity, small molecular weight and easy penetration into tumor tissues [15-16]. However, the sdAb shortcomings include short half-life and quick concentration decrease from the blood circulation, which greatly affects the anti-tumor effect [17]. Therefore, these sdAb antibodies need to be changed through genetic engineering to improve their anti-tumor activity [18].

Two anti-EpCAM human sdAbs pET22b-aEP3D4 and pET22b-aEP4G2 were previously isolated by our laboratory from screening the antibody library in a saparate study (unpublished date). These two sdAbs were used to construct two bivalent recombinant protein expression plasmids (pET22b-aEP3D4 -aEP4G2, pcDNA3.1-aEP3D4-aEP3D4) and two anti-EpCAM recombinant antibody expression plasmids containing anti-human serum albumin nanobody (anti-HSA nanobody) or human IgG1 Fc. These recombinant antibodies were expressed by prokaryotic or eukaryotic expression systems and detected by SDS-PAGE. This study lays the foundation for further research on the in vitro and in vivo functions of these antibody proteins.

2. Materials and methods

2.1 Reagents and cell culture

The *E. coli* BL21 (DE3) and E. coli DH5α were purchased from Novagen (EMD Millipore, Madison, WI, USA). The Mini Plasmid Kit and Endo-Free Plasmid Maxi Kit were obtained from Omega Biotek Inc (Doraville, GA, USA). Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Sangon Biotech (Shanghai, China). Ni-NTA His Bind Resin was purchased from Sevensea Biotech (Shanghai, China). The 293f cell and KOP293 Transient Transfection Protein Expression System were purchased from Kairui Biotech (Zhuhai, China). The EpCAM and EGFR fragments were synthesized by Bootech (Shanghai, China).

Human 293f cells were cultured in KOP293 cell culture medium (Zhuhai, China) and incubated at 37°C and 5% CO 2 in a humidified suspension shaker incubator.

2.2 Construction of anti-EpCAM sdAb recombinant plasmids

In this study, two anti-EpCAM human sdAbs pET22b-aEP3D4 and pET22b-aEP4G2 were used to construct the recombinant antibody expression plasmids. The (G4S)3 linker was used to combine the two sdAbs. The two single sdAbs were combined with the anti-HSA nanobody or human IgG1 Fc fragment. Then, the recombinant genes were cloned into the prokaryotic expression plasmid pET22b (the restriction enzyme sites: Nco I and Not I) or eukaryotic expression plasmid pcDNA3.1 (the restriction enzyme sites: Xho I and Xba I), constructing the four anti-EpCAM recombinant antibodies expression plasmids (pET22b-aEP3D4-aEP4G2, pET22b-aEP3D4-anti-HSA-aEP4G2, pcDNA3.1-aEP3D4-aEP3D4 and pcDNA3.1-aEP3D4-aEP3D4-Fc). These anti-EpCAM recombinant antibody expression plasmids were transformed into *E. coli* BL21 (DE3) or transfected into 293f cells to express these anti-EpCAM recombinant antibody proteins. The construction of these recombinant plasmids was completed by Jinweizhi.

2.3 Expression and purification of the recombinant antibody proteins

Two prokaryotic expression plasmids (pET22b-aEP3D4-aEP4G2 and pET22b-aEP3D4-anti-HSA-aEP4G2) were expressed using *E. coli* BL21(DE3) (Novagen). The recombinant plasmids were transformed into *E. coli* BL21 (DE3) and cultured with shaking. The cultured bacterial solution was evenly spread on a LB plate containing ampicillin, which was incubated overnight at 37°C. Bacteria single clones were randomly picked, placed in 4 ml LB medium containing ampicillin and cultured overnight at 37°C and 220 rpm. The culture was added to 400 ml LB medium containing ampicillin and incubated at 37°C and 220 rpm until the OD600 reached 0.6. The 0.5 mM IPTG (Sangon Biotech) was added to induce protein expression. After the centrifugation, the pellet was resuspended in bacteria disruption buffer containing PMSF (Bio-Biotechnology). After sonication, the soluble protein was extracted by centrifugation. 293f cells are used for the expression of pcDNA3.1-aEP3D4-aEP3D4 and pcDNA3.1-aEP3D4-aEP3D4-Fc protein. The recombinant plasmids were transfected

into 293f cells according to the manufacturer's protocol, and cultured in a humid suspension shaking incubator at 37°C and 120 rpm for 5-6 days. The cell culture was centrifuged to collect the supernatant. The recombinant antibody proteins were purified using Ni-NTA His Bind Resin (Sevensea Biotech) and detected by SDS-PAGE.

3. Results

3.1 Construction of the four anti-EpCAM recombinant antibody plasmids

The two sdAbs (pET22b-aEP3D4 and pET22b-aEP4G2) were combined through a (G4S)3 linker, and an anti-HSA nanobody gene was added between the two sdAbs. The genes were cloned between Nco I and Not I sites of the pET-22b plasmid, and the two recombinant antibody expression plasmids (pET22b-aEP3D4-aEP4G2, pET22b-aEP3D4-anti-HSA-aEP4G2) were constructed. The same (G4S)3 linker was used to combine two pET22b-aEP3D4 to form the bivalent antibody. The human lgG1 Fc was added to the 3 'end of the bivalent antibody. The genes were inserted into Xho I and Xba I sites of the pcDNA3.1 plasmid, and the two recombinant antibody expression plasmids (pcDNA3.1-aEP3D4-aEP3D4 and pcDNA3.1-aEP3D4-aEP3D4-Fc) were constructed

3.2 Expression and purification of pET22b-aEP3D4-aEP4G2

The anti-EpCAM recombinant antibody expression plasmid pET22b-aEP3D4-aEP4G2 was transformed into *E. coli* BL21. IPTG was added to induce the soluble protein expression, and the recombinant antibody protein was purified by a Ni-NTA His Bind Resin. Nanodrop was used to detect the concentration of recombinant protein, and the yield of recombinant antibody protein was about 2.5mg/L. As shown in Fig. 1, the molecular weight of the recombinant antibody protein is about 30 kDa, and its position was marked by the arrow.

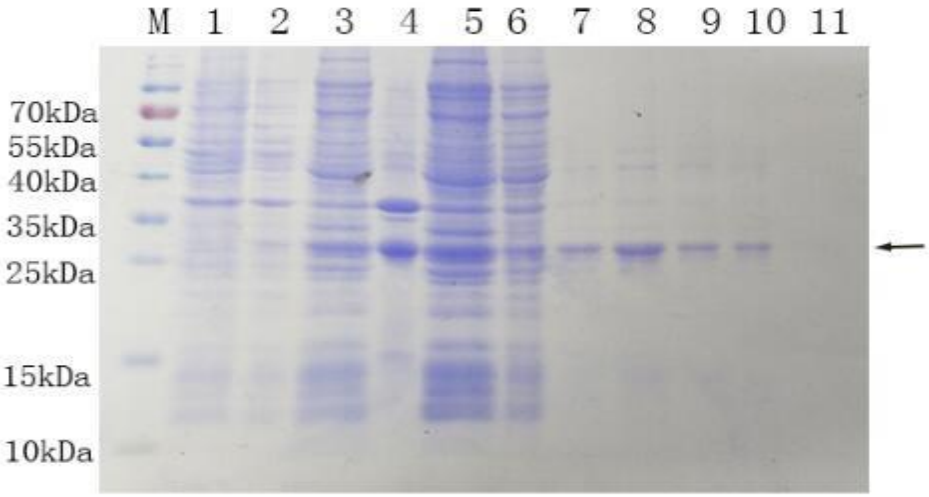

Fig. 1 SDS-PAGE was performed to detect the expression of pET22b-aEP3D4-aEP4G2. Lane M, protein marker; Lane 1, total proteins of the un-induced bacteria; Lane 2, total proteins of the induced bacteria; Lane 3, soluble supernatant after bacteria lysis; Lane 4, insoluble portion after bacteria lysis; Lane 5, flow-through fraction; Lane 6, wash buffer; Lane 7-11, proteins collected from the purification column. The position of the protein band was marked with the arrow.

3.3 Expression and purification of pET22b-aEP3D4-anti-HSA-aEP4G2

The anti-EpCAM recombinant antibody expression plasmid pET22b-aEP3D4-anti-HSA-aEP4G2 was transformed into *E. coli* BL21. IPTG was added to induce the soluble protein expression, and the recombinant antibody protein was purified by a Ni-NTA His Bind Resin. Nanodrop was used to detect the concentration of recombinant protein, and the yield of recombinant antibody protein was about 1mg/L. As shown in Fig. 2, the molecular weight of the recombinant antibody protein is about 50kDa, and its position was marked by the arrow.

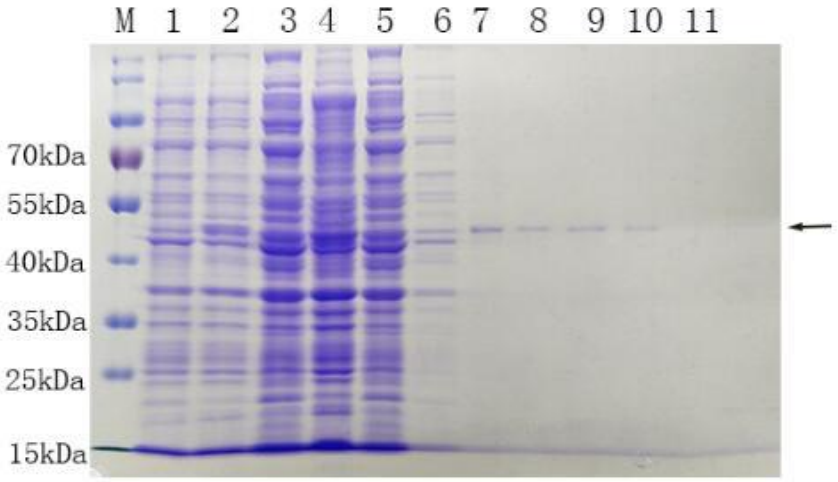

Fig. 2 SDS-PAGE was performed to detect the expression of pET22b-aEP3D4-anti-HSA-aEP4G2. Lane M, protein marker; Lane 1, total proteins of the un-induced bacteria; Lane 2, total proteins of the induced bacteria; Lane 3, soluble supernatant after bacteria lysis; Lane 4, insoluble portion after bacteria lysis; Lane 5, flow-through fraction; Lane 6, wash buffer; Lane 7-11, proteins collected from the purification column. The position of the protein band was marked with the arrow.

3.4 Expression and purification of pcDNA3.1-aEP3D4-aEP3D4

The anti-EpCAM recombinant antibody expression plasmid pcDNA3.1-aEP3D4-aEP3D4 was transfected into 293f cells and cultured in suspension to express the antibody protein, and the recombinant proteins were purified by a Ni-NTA His Bind Resin. Nanodrop was used to detect the concentration of recombinant protein, and the yield of recombinant antibody protein was about 10mg/L. As shown in Fig. 1, the molecular weight of the recombinant antibody protein is about 40kDa, and its position was marked by the arrow.

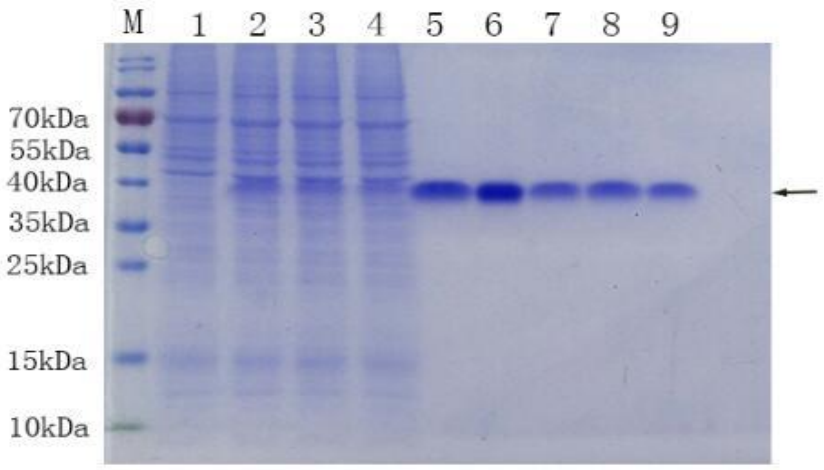

Fig. 3 SDS-PAGE was performed to detect the expression and purification of pcDNA3.1-aEP3D4-aEP3D4. Lane M: protein Marker; Lane 1: supernatant of the un-transfected cells; Lane 2: supernatant of the transfected cells; Lane 3: flow-through fraction; Lane 4: wash buffer; Lane 5-9: proteins collected from the purification column. The position of the protein band was marked with the arrow.

3.5 Expression and purification of pcDNA3.1-aEP3D4-aEP3D4-Fc

The anti-EpCAM recombinant antibody expression plasmid pcDNA3.1-aEP3D4-aEP3D4-Fc was transfected into 293f cells and cultured in suspension to express the antibody protein, and the

recombinant antibody proteins were purified by a Ni-NTA His Bind Resin. Nanodrop was used to detect the concentration of recombinant protein, and the yield of recombinant protein was about 10mg/L. As shown in Fig. 1, the molecular weight of the recombinant antibody protein is about 70kDa, and its position was marked by the arrow.

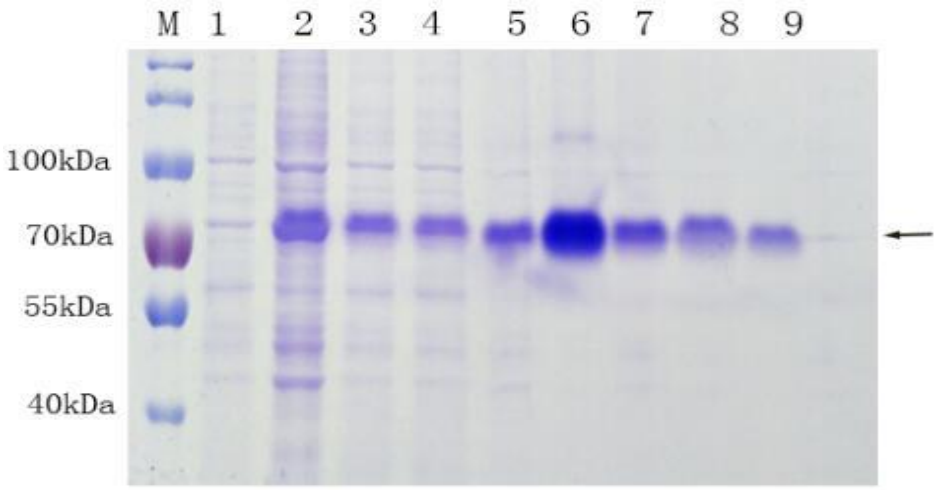

Fig. 4 SDS-PAGE was performed to detect the expression and purification of pcDNA3.1-aEP3D4-aEP3D4-Fc. Lane M: protein Marker; Lane 1: supernatant of the un-transfected cells; Lane 2: supernatant of the transfected cells; Lane 3: flow-through fraction; Lane 4: wash buffer; Lane 5-9: proteins collected from the purification column. The position of the protein band was marked with the arrow.

4. Discussion

There is no doubt that the use of antibodies for tumor therapy has achieved great success. In the decades since antibodies were first used in tumor treatment [18], the various methods were used to construct a variety of modified and optimized antibodies including use of the antibody Fc domain, toxins or nanoparticles and the bispecific antibodies (bsAbs).

The recombinant antibody including the antibody Fc domain is produced by combining the antibody Fc domain with another protein [19]. For example, Tang et al. isolated the SD1 human antibody domain and combined it with human Fc to produce the recombinant protein (SD1-hFc). In vitro cell experiments showed that SD1-hFc could produce strong ADCC and CDC activity against tumor cells, and in addition, it could inhibit the growth of xenograft tumors in nude mice [20]. Solitomab (MT110) is a CD3/EpCAM bispecific single-chain fusion antibody [21], which induced target cell lysis by activating cytolytic T cells [22]. Solitomab showed good anti-tumor activity in preclinical EpCAM-positive ovarian tumor xenograft models. In addition, clinical data showed that it also had an excellent anti-tumor activity on tumor cells in the ascites of patients with gynecological carcinosarcoma [23]. In order to improve the penetration of solid tumors, Teesalu T et al. fused EGFR-targeting sdAb with iRGD, which showed good anti-tumor activity in human tumor xenograft models, which could significantly reduce tumor volume [24]. In addition, anti-EGFRsdAb-iRGD combined with paclitaxel-loaded silk fibroin nanoparticles produced significant anti-tumor activity in cells expressing EGFR in vivo and in vitro [25]. Mischa R. Müller et al. [26] isolated high-affinity anti-HSA VNAR antibodies by immunizing sharks, and in vivo pharmacokinetic experiments proved that binding to human serum albumin (HSA) prolonged the serum half-life of VNAR antibodies.

In this study, two sdAbs were used to construct two bivalent recombinant antibody expression plasmids (pET22b-aEP3D4-aEP4G2, pcDNA3.1-aEP3D4-aEP3D4). In addition, these two sdAbs were combined with anti-human serum albumin nanobody (anti-HSA nanobody) or human IgG1 Fc fragment to construct two anti-EpCAM recombinant antibody expression plasmids. These recombinant antibody proteins were expressed by prokaryotic or eukaryotic expression systems. This

research lays the foundation for further research on the in vivo and in vitro functions of these antibody proteins.

5. Conclusions

In this study, the four anti-EpCAM recombinant antibody expression plasmids pET22b-aEP3D4-aEP4G2, pET22b-aEP3D4-anti-HSA-aEP4G2, pcDNA3.1-aEP3D4-aEP3D4 and pcDNA3.1-aEP3D4-aEP3D4-Fc were constructed. These four anti-EpCAM recombinant antibody proteins were expressed and purified separately through prokaryotic or eukaryotic expression systems. This study lays the foundation for further research on the in vitro and in vivo functions of these antibody proteins.

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