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Full Length Research Paper

Screening of specific single-chain variable-fragment (scFv) antibody against human asialoglycoprotein receptor by capture phage enzyme-linked immunosorbent assay

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This study screened specific single-chain variable fragment (scFv) antibody against asialoglycoprotein receptor (ASGPR) by capture phage enzyme-linked immunosorbent assay (ELISA). The CRDH1/pET3b plasmid containing ASGPR gene was employed to amplify the CRDH1 gene of subunit H1 of ASGPR through PCR, which was then directionally cloned into prokaryotic expression vector pET-32c. The expression of CRDH1 was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) and the expression products underwent affinity purification through a Ni^{2+} chelating column. The humanized phage antibody library was screened and identified by capture phage ELISA. Then, the selected CRDH1 single chain antibody was sequenced, expressed, purified and identified by Western blot and compared with the antibody from indirect phage ELISA. The recombinant CRDH1 protein was a 35 kDa fusion protein which existed as an inclusion body. Affinity purification through a Ni^{2+} chelating column acquired the CRDH1 fusion protein. 44 colonies out of 60 colonies could specifically bind to CRDH1. Sequencing showed that 9 colonies had difference sequences and 9 colonies could express CRDH1 specific scFv. Furthermore, the purified scFv also specifically recognized rCRDH1. Capture phage ELISA effectively improved the GST fusion proteins, causing false positives and enhanced the positive rate. In addition, 9 rCRDH1 specific scFv antibodies were successfully obtained by capture phage ELISA.

Key words: Asialoglycoprotein receptor, enzyme linked immunosorbent assay, fusion protein, single-chain variable-fragment antibody.

INTRODUCTION

Asialoglycoprotein receptor (ASGPR) is an abundant hetero-oligomeric endocytic receptor that is predominantly expressed on the sinusoidal surface of the hepatocytes. The functional domain of ASGPR is a carbohydrate recognition domain with two homologous subunits: H1 subunit and H2 subunit. Study has shown that the H1 subunit plays an important role in the ligand recognition of ASGPR and endocytosis (Lee et al., 2009; Saunier et al., 2003). ASGPR has tissue specificity and is difficult to be saturated. Study revealed that ASGPR has potential roles in the targeted therapy of liver diseases mediated by targeting receptors, and in particular, adequate function and content of the ASGP receptor may provide protection against various toxin-mediated liver diseases (Dalton et al., 2009). In a previous study, 2 ASGRP specific single chain variable-fragment (scFv) antibodies were obtained through indirect enzyme linked immunosorbent assay (ELISA), but the false positive rate was as high as 60%

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(Cao et al., 2005). The CRDH1/pET3b plasmid containing ASGPR gene was employed to amplify the CRDH1 gene of subunit H1 of ASGPR through PCR, which was then directionally cloned into prokaryotic expression vector pET-32c. This study was aimed to screen the ASGRP specific scFv antibody by capture phage ELISA.

MATERIALS AND METHODS

Bacterial strains, plasmid and antibody library

DH5α, BL21 (DE3) and pET-32c strains were stored in the department. The pET3-CRDH1 plasmid containing CRDH1 cDNA was kindly provided by Prof. Martin Spiess of the University of Basel, Switzerland. A library of phage-displayed human single-chain Fv antibodies (Tomlinson I) was purchased from MRC, UK. The *Escherichia coli* strains TG1 and HB2151 and the helper phage KM13 were kindly provided by Dr. Zhu J. of the Department of Pathogen Biology, Nanjing Medical University.

Reagents

Isopropyl β-D-1-thiogalactopyranoside (IPTG), low-molecular-weight standard proteins, DNA polymerase, restriction endonucleases (Xho1 and NcoI), T4DNA polymerase, T4DNA ligase, plasmid DNA purification kit, PCR product purification kit, SDS-PAGE low molecular weight standard proteins, DNA marker, Coomassie brilliant blue R-250, diaminobenzidine (DAB) (Sino-American Biotechnology Co., Ltd), Ni^{2+} chelating HiTrap HP column, HiTrap Desalting column, HRP-anti-M13 (Amersham Biosciences Co., Ltd.), rabbit anti-thioredoxin polyclonal antibody, thioredoxin monoclonal antibody 2C9, Protein A-HRP, goat anti-rabbit IgG-HRP (Sigma, USA) and polystyrene ELISA plate (GongDong medical plastic factory) were used in the study. The primers for sequencing were synthesized in the Shanghai Yingjun Biotechnology Co., Ltd.

Cloning, expression and purification of CRDH1

The primers for CRDH1 were designed according to its DNA sequence: P1: 5'-ATCCATGGGCTCAGAAAGGACCT-3', and P2: 5'-ATCTCGAGAAGGAGAGGTGGCTC-3'. The CRDH1/pET3b plasmid was used for amplification by PCR. The target fragment was directionally cloned into the prokaryotic expression plasmid pET-32c. The recombinant plasmid CRDH1/pET-32c was identified by restriction enzymes (NcoI and XhoI) digestion and subsequent DNA sequencing. A single colony of BL21 strain containing CRDH1/pET-32c was grown in LB broth followed by induction with 1 mmol/L IPTG. The CRDH1 expression was determined by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The inclusion body was processed in a conventional way and the fusion protein was purified through a Ni^{2+} affinity column.

Capture phage ELISA

Microplate wells were coated with 1 μg/ml of polyclonal Trx antibodies and then blocked with MPBS. Following the addition of non-denatured scFv-domain fusion proteins, 107 cfu monoclonal phages were applied to the microplate. The remaining steps were as described for indirect phage ELISA and measurements were carried out in triplicate. The Trx -specific monoclonal antibody G4 (provided by Michael Monecke, RWTH Aachen, Germany) was used as a positive control.

Screening by capture phage ELISA

The thioredoxin (Trx) polyclonal antibody was diluted with carbonate buffer (pH 9.6) into 1 μg/ml, and 100 μl of the solution was used to coat polystyrene ELISA plate at 4°C overnight. The plate was washed with PBS thrice followed by blocking with 2% nonfat milk in PBS (200 μl/well) at 25°C for 2 h. Then, 100 μl of rCRDH1 (5 μg/ml) were added to each well followed by incubation at 25°C for 2 h. The plate was washed with PBS thrice and 100 μl of human single-chain Fv antibody library (dilution: 1:10; 10^{10}CPU/ml) was added to each well followed by incubation at 25°C for 2 h. The plate was washed with 0.1% Tween 20 in PBS 10 times (20 times in the second, third and forth round). Elution was performed with 100 μl of 1 mg/ml trypsin and 50 μl of eluate were incubated with 350 μl of TG1 (A_{600} was about 0.4) at 37°C for 30 min followed by centri-fugation at 8 000 r/min for 2 min. Resuspension was performed with 50 μl of 2×TY medium and the suspension was spread on the TYE plate followed by incubation at 37°C overnight. Then, 2 ml of 2×TY medium were used to elute the colonies and 50 μl of eluate were inoculated in 50 ml of 2×TY medium, 10 ml of which was incubated with 10 μl of 5×10^{10} KM13 at 37°C for 30 min, followed by centri-fugation. Then, the bacteria were re-suspended in 50 ml of 2×TY medium followed by incubation at 30°C overnight under continuous shaking. Then, centrifugation was performed at 5000 r/min for 15 min, and 40 ml of supernatant were mixed with 10 ml of PEG/NaCl followed by incubation on the ice for 1 h and subsequent centrifugation at 5 000 r/min for 15 min. Then, the bacteria were suspended in 2 ml of PBS. The procedures above were repeated three times.

Identification of monoclonal phage by capture phage ELISA

After 4 rounds of screening, 50 μl of phage were used to prepare monoclonal supernatant. Then, 100 μl of supernatant were added to the polystyrene ELISA plate coated with Trx polyclonal antibody and rCRDH1 followed by incubation at 25°C for 1 h. The plate was washed with 0.1% PBST and then HRP-anti-M13 (1:5000) was added to each well followed by incubation at 25°C for 1 h. The plate was washed with 0.1% PBST three times and 50 μl of TMB were added to the plate followed by incubation for 5 min for color development. The reaction was terminated by adding 50 μl of 2 mol/L H_2SO_4 and the absorbance was determined at 450 nm (A_{450}). Then, ELISA was performed between the tag protein (from pET-32c blank vector after expression and purification) and the selected monoclonal phage. In addition, the cross-reaction between tag protein and mouse soluble antigen or *E. coli* antigen (membrane antigen + soluble antigen) was also detected.

Confirmation of scFv expressed by positive colonies

The positive phage colonies were used to infect *E. coli* HB2151. A single colony was inoculated in 100 μl of 2×TY medium followed by incubation at 37°C overnight under continuous shaking. Then, 2 μl of *E. coli* solution were inoculated in 200 μl of 2×TY medium followed by incubation at 37°C for 3 h (A600 was about 0.9) and 25 μl of 2×TY medium containing 9 mM ITPG were added followed by incubation at 30°C overnight under continuous shaking (250 r/min). Centrifugation was performed at 4500 r/min for 30 min and 50 μl of the supernatant were used for ELISA with rCRDH1. The secondary antibody was Protein A-HRP (1:5000).

DNA sequencing

Sequencing of positive phage colonies was performed in the

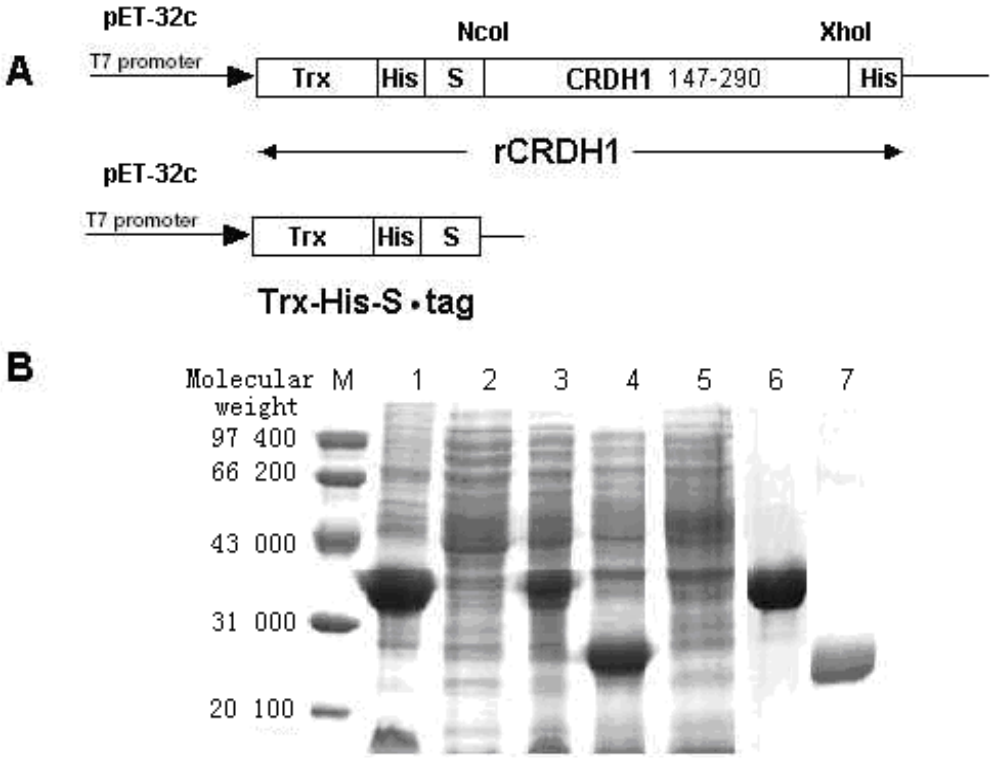

Figure 1. A diagram of the construction and 12% SDS-PAGE assay of the recombinant asialoglycoprotein receptor. A, CRDH1 was introduced to the prokaryotic expression vector pET-32c between the restriction sites of NocI and XhoI and the recombinant CRDH1 fused tags of Trx, His and S; B: Mw, Molecular weight; 1, lysate of vector pET-32c containing CRDH1; 2, supernatant of the lysate of vector pET-32c containing CRDH1; 3, sediment (inclusion body) of the lysate of vector pET-32c containing CRDH1; 4, lysate of vector pET-32c; 5, lysate of *E. coli* BL21; 6, Purified rCRDH1; 7, purified tag protein.

Shanghai Yingjun Biotechnology Co., Ltd. The primers were designed as follows: V_H: 5'-CGACCCGCCACCGCCGCTG-3' and V_K: 5'-CTATGCGGCCCCATTCA-3'.

Purification and western blot assay of rCRDH1 specific scFv

The *E. coli* HB2151 containing 1 ~ 9 phages were inoculated in 2×TY medium and the rCRDH1 expression was induced by 1.0 mM IPTG. The saturated ammonium sulfate solution was used to precipitate soluble scFv which was then dissolved in PBS. Dialysis and demineralization were performed with PBS followed by elution through Ni^{2+} column. The solution was subjected to 12% SDS-PAGE. Then, demineralization was performed with HiTrap desalting column in the AKTA prime high pressure liquid chromatography. The fusion protein was transferred into 1×PBS and its concentration was determined with a protein nucleic acid analyzer. The purity was measured by 12% SDS-PAGE. Then, 50 µl of CRDH1 and Trx-His-S·Tag recombinant protein were subjected to 12% SDS-PAGE and transferred onto nitrocellulose membrane which was then incubated with primary antibody (purified scFv; 1:10) and subsequently with HRP-anti-M13 and protein L-HRP (1:50). The primary antibody was replaced with Anti-His(C-term)-HRP as positive control and with *E. coli* HB2151 lysate as negative control. The substrate was 4-chloro-1-naphthol.

Data were expressed as means ± standard deviation ($\bar{x}$ ±s) and statistical analysis was performed with SPSS version 11.0 statistic software package. T test was applied for comparison between groups.

RESULTS

Prokaryotic expression and purification of CRDH1

The CRDH1 was introduced to the pET-32c between restriction sites of NotI and XhoI, and the expressed products were rCRDH1 fusing Trx, His and S tags but blank vectors directly expressed Trx-His-S·Tag after induction. After procession with ultrasound, the supernatant and sediment of the *E. coli* solution were subjected to SDS-PAGE. Results showed that the expressed rCRDH1 and Trx-His-S·Tag existed as inclusion body. After induction with 1 mmol/L IPTG, the BL21 containing CRDH1/pET-32c plasmid expressed a recombinant protein of about 35 kDa and the molecular weight of Trx-His-S·Tag was about 23 kDa (Figure 1). After affinity purification with Ni^{2+}

column, the purities of rCRDH1 and Trx-His-S·Tag were greater than 95% and the concentrations of rCRDH1 and

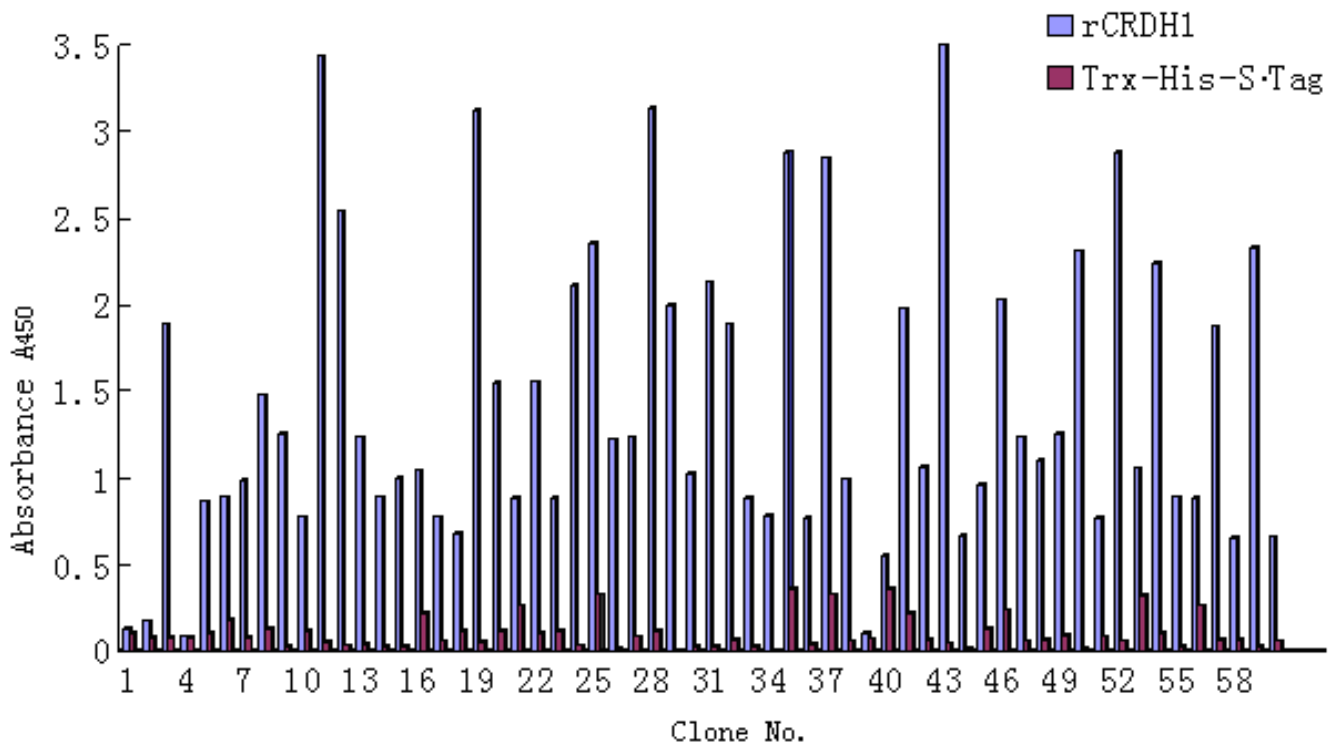

Figure 2. Screening of asialoglycoprotein receptor single chain antibody by capture phage ELISA.

Trx-His-S·Tag were about 2.0 and 4 mg/ml, respectively.

Screening of phage antibody and confirmation of positive phage colonies

After four rounds of screening, the absorbance in polyclonal phage ELISA did not increase any more, suggesting that the specific phage antibody was enriched. After screening, 60 monoclonal phage colonies were used for capture phage ELISA. Results showed that 45 colonies could bind to the rCRDH1 and only 1 colony had cross-reaction with recombinant protein tag (Figure 2). DNA sequencing revealed that different DNA sequence existed in 9 colonies. In indirect phage ELISA, the results showed that 24 out of 60 colonies had favorable binding activity with rCRDH1 and 14 colonies had cross-reaction with recombinant protein tag (Figure 3). DNA sequencing showed different DNA sequence in only 2 colonies but no cross reaction was observed between positive phages and mouse soluble antigen or *E. coli* antigen.

Sequencing of positive phage colonies

DNA sequencing showed that 9 out of 24 colonies had different light chain or heavy chain. This result demonstrated that the Tonlinson I had a big reservoir volume. Evidence showed the reservoir volume reached 10^7 (Holt et al., 2000).

Expression, purification and western blot assay of 9 soluble scFvs

The scFv expression of 9 positive phage colonies was induced by IPTG and ELISA showed that the supernatant specifically bound to the rCRDH1 (Figure 4). There were 6 His tags in the carboxyl terminal of scFv. One colony was subjected to purification through the Ni^{2+} affinity column and results showed a band of about 28 kDa which was consistent with the theoretical molecular weight of scFv (Figure 5A). The purified scFv specifically recognized rCRDH1 but did not react with Trx-His-S·Tag (Figure 5B).

False positive rate of capture phage ELISA

The indirect phage ELISA was used as the control. The ASGPR specific scFv was screened and confirmed. The results of indirect phage ELISA showed that 24 out of 60 monoclonal phage colonies had favorable binding activity with recombinant ASGPR antigen among which, 14 had cross reaction with recombinant protein tag. The false positive rate was as high as 58%. In the capture phage ELISA, there were 45 positive phage colonies in 60 monoclonal phage colonies and only 1 colony had cross

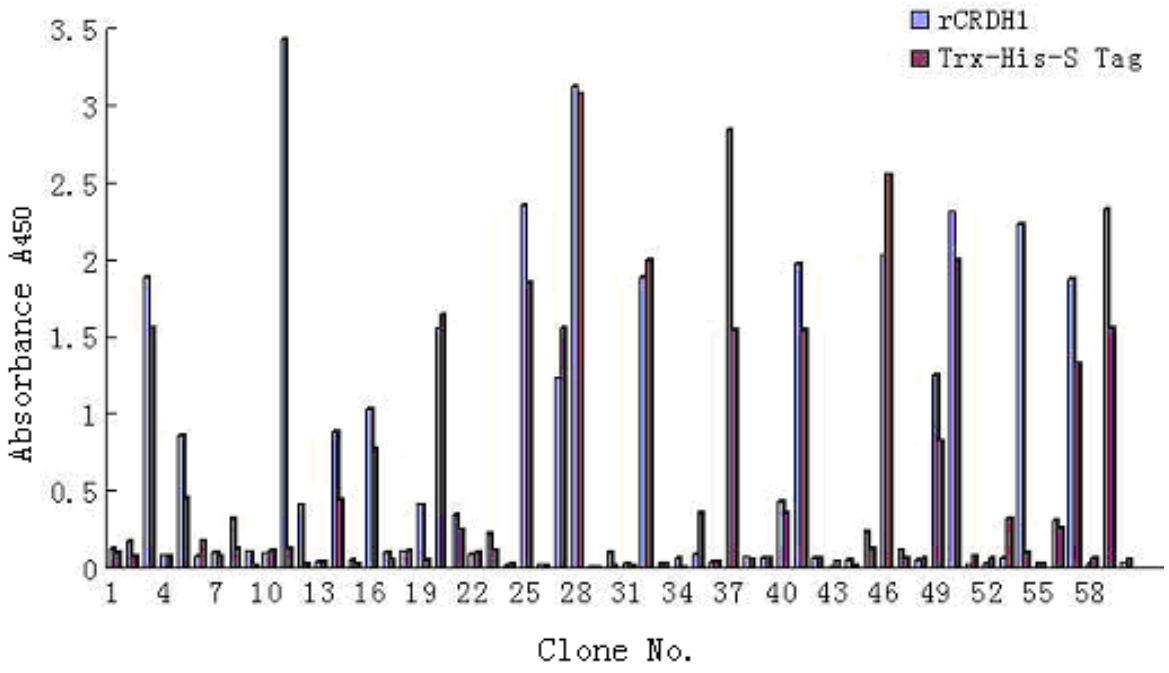

Figure 3. Screening of asialoglycoprotein receptor single chain antibody by indirect phage ELISA.

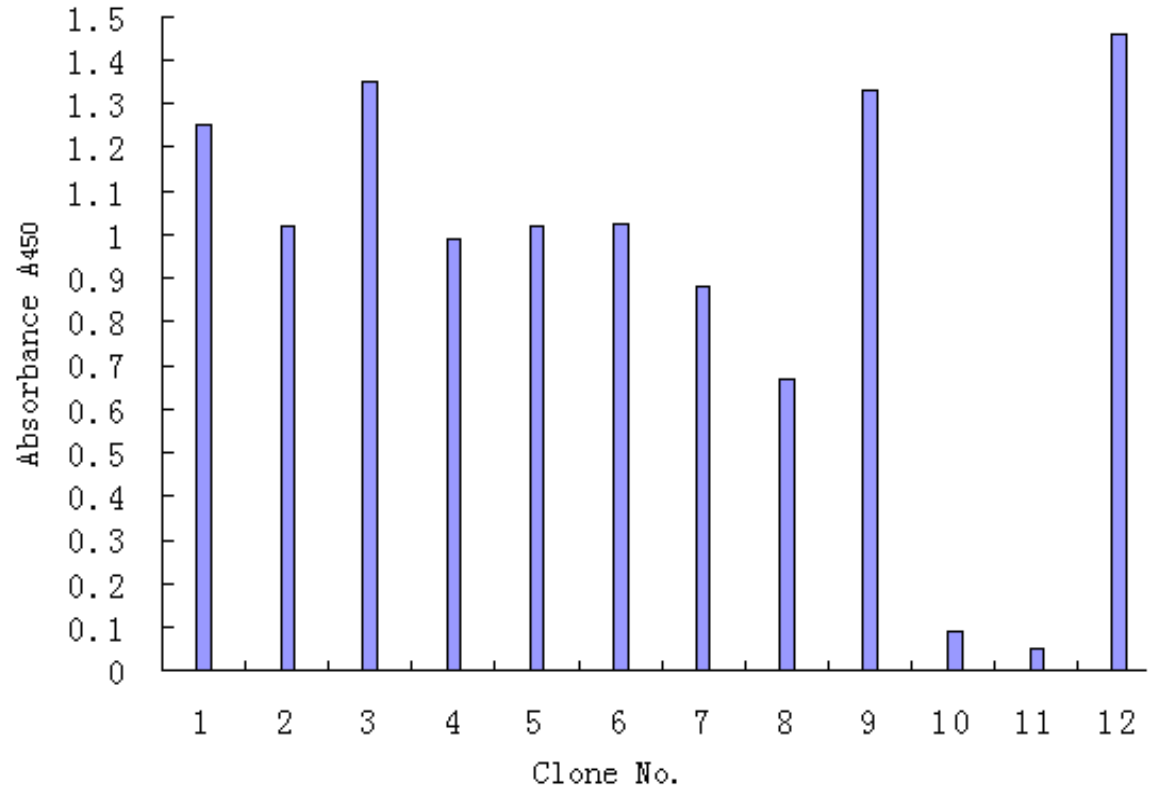

Figure 4. Soluble expression and confirmation of asialoglycoprotein receptor single chain antibody. 1 μg/ml rabbit anti-Trx polyclonal antibody was used to coat ELISA plate which then was incubated with rCRDH1 overnight. Then, 100 μl of the supernatant was used for cloning and expression of phage by capture ELISA (1 to 9, phage colonies with different sequences; 10, vector pIT2; 11, supernatant from negative phages; 12, Trx specific monoclonal antibody as positive control)

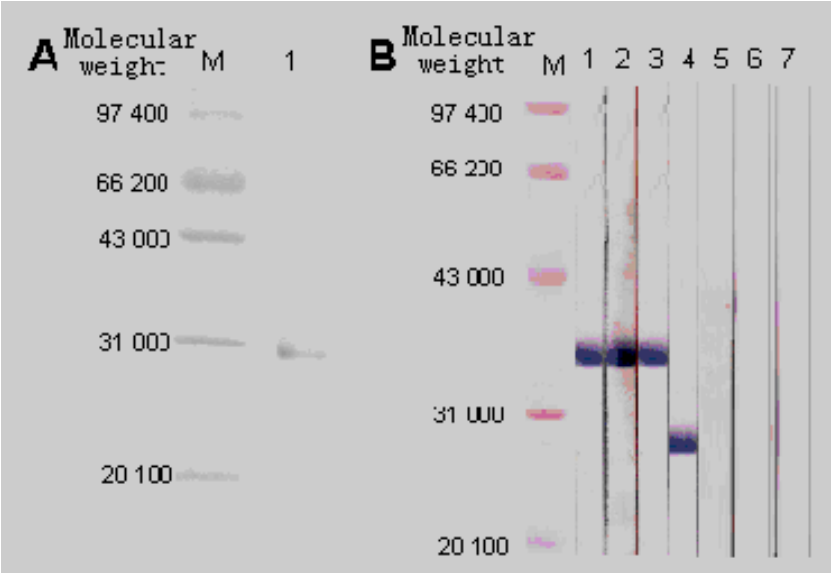

Figure 5. 12% SDS-PAGE assay of scFv after purification. M, molecular weight of proteins; A1, purified scFv; B1, recognition of rCRDH1 by Anti-his(C-term)-HRP; B2, recognition of rCRDH1 by scFv; B3, recognition of rCRDH1 by phages; B4, recognition of Trx-His-s tag by Anti-His(C-term)-HRP; B5, recognition of Trx-His-s tag by Anti-ASGPR scFv; B6, LYSATE of *E. coli* HB2151; B7, PBS.

reaction with recombinant protein tag. The false positive rate was only 2%.

DISCUSSION

Phage display is a highly valuable technique for antibody selection which is related to this study. In brief, a diverse group of antibody genes are inserted into a phagemid vector or the phage genome so that when the protein is expressed, it becomes anchored on the surface of the phage by fusion to a coat protein. A diverse library of recombinant antibodies is generated in this way and can then be exposed or panned on the antigen of interest. Phage display has been extensively used to study protein-protein interactions, receptor- and antibody-binding sites, and immune responses, to modify protein properties, and to select antibodies against a wide range of different antigens. Phage display technology is a new powerful tool for making antibodies outside the immune system, thus avoiding the use of experimental animals, and phage display antibody library technique has achieved rapid progress which contributes to its high efficiency in screening antibody (Pansri et al., 2009).

Screening of antibody library with antigen can be performed in several ways. For example, the antigens are directly (direct phage ELISA) and indirectly (capture phage ELISA) coated on the ELISA plate or plastic tube, cross-linked on the chromatography media. In addition, screening can also be conducted in liquid phase with biotin-streptavidin-poly beads (Bradbury and Marks, 2004). Currently, evidence has shown that the phages are mainly screened with immobilized pure antigens. In addition, recombinant proteins can also be applied as immobilized antigens for screening. Zhang et al. (2001) applied immobilized antigens to screen the NSM specific scFv, and results showed that the scFv from 68 phage colonies had cross reaction with GST. Similarly, in a previous study, proteins fused with 109aa Trx•Tag™ were employed to screen Tonlinson I antibody library, but the false positive rate was as high as 60% (Cao et al., 2005). This study showed that capture phage ELISA isolated clones should be evaluated by capture phage ELISA to exclude the false positives not identified by indirect phage ELISA.

In this study, gene-engineering technique was applied to prepare the CRD (ASGPR H1 subunit) recombinant antigens which were used as coating antigens. Then, direct phage ELISA was performed to screen rCRDH1 specific scFv, achieving the false positive rate of 60%. Furthermore, capture phage ELISA was conducted in which rabbit anti-Trx polyclonal antibody was coated and the rCRDH1 captured by the antibody was used as antigen followed by screening of the library of phage-displayed human single-chain Fv antibodies. Results showed that the false positive rate was only 2%. The low false positive rate may be explained thus: (1) although the recombinant protein underwent purification with GST and 6×His tags, the antigen was not completely pure. Preparation of immobilized pure antigens through coating ELISA plate may generate non-specific adsorption and (2) the Trx fused tag was derived from *E. coli* and its

molecular weight was about 23 kDa. In the screening of human single-chain Fv antibody library, this protein has potent antigenicity. The anti-Trx polyclonal antibody can block certain epitopes of Trx which enhances the specificity of CRDH1. In addition, CRDH1 can bind to the ELISA plate and non-specific bands are removed by PBS which also increases the positive rate.

Recently, the advantages of capture phage ELISA have been fully used to detect phenotypic variants that are not easily detected by routine analytical methods. For example, due to high specificity of capture phage ELISA, Rozand and Feng, (2009) conducted a specificity analysis for the detection of *E. coli* O157:H7 and phage ligand assay showed no cross-reactivity to the other *E. coli* serotypes. Mi et al. (2006) successfully screened carbohydrate-specific phage antibodies against recombinant human erythropoietin (rhuEPO).

Therefore, in the screening of phage antibody library with recombinant proteins as antigens, the capture phage ELISA is preferable to avoid the false positive caused by fusion tag.

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REFERENCES

Bradbury AR, Marks JD (2004). Antibodies from phage antibody libraries. J. Immunol. Methods, 290(1-2): 29-49.

Cao LM, Si J, Zhu YC, Wang WY, Zhao XR, Ye Q (2005). Expression and purification of the human asialoglycoprotein receptor H1 subunit and selection of anti-CRDH1 scFv. Chin. J. Microbiol. Immunol. 25(9): 756-61.

Dalton SR, Lee SM, King RN, Nanji AA, Kharbanda KK, Casey CA, McVicker BL (2009). Carbon tetrachloride-induced liver damage in asialoglycoprotein receptor-deficient mice. Biochem. Pharmacol. 77(7): 1283-1290.

Holt LJ, Bussow K, Walter G, Tomlinson IM (2000). By-passing selection: direct screening for antibody-antigen interactions using protein arrays. Nucleic Acids Res. 28(15): E72.

Lee SM, Casey CA, McVicker BL (2009). Impact of asialoglycoprotein receptor deficiency on the development of liver injury. World J. Gastroenterol. 15(10): 1194-1200.

Mi J, Ding X, Zhao M, Chang W, Guo Z (2006). Screening of carbohydrate-specific phage antibodies against recombinant human erythropoietin (rhuEPO) using a phage display antibody library: preliminary study. J. Immunoassay. Immunochem. 27(2): 115-128.

Pansri P, Jaruseranee N, Rangnoi K, Kristensen P, Yamabhai M (2009). A compact phage display human scFv library for selection of antibodies to a wide variety of antigens. BMC. Biotechnol. 9: p. 6.

Rozand C, Feng PC (2009). Specificity analysis of a novel phage-derived ligand in an enzyme-linked fluorescent assay for the detection of *Escherichia coli* O157:H7. J. Food. Prot. 72(5): 1078-1081.

Saunier B, Triyatni M, Ulianich L, Maruvada P, Yen P, Kohn LD (2003). Role of the asialoglycoprotein receptor in binding and entry of hepatitis C virus structural proteins in cultured human hepatocytes. J. Virol. 77(1): 546-559.

Zhang MY, Schillberg S, Zimmermann S, Liao YC, Breuer G, Fischer R (2001). GST fusion proteins cause false positives during selection of viral movement protein specific single chain antibodies. J. Virol. Methods, 91(2): 139-147.