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Guided Selection of Human Antibody Light Chains against TAG-72 Using a Phage Display Chain Shuffling Approach

Sang Jick Kim and Hyo Jeong Hong*

Therapeutic Antibody Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejon 305-333, Republic of Korea

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To enhance therapeutic potential of murine monoclonal antibody, humanization by CDR grafting is usually used to reduce immunogenic mouse residues. Most humanized antibodies still have mouse residues critical for antigen binding, but the mouse residues may evoke immune responses in humans. Previously, we constructed a new humanized version (AKA) of mouse CC49 antibody specific for tumor-associated glycoprotein, TAG-72. In this study, to select a completely human antibody light chain against TAG-72, guided selection strategy using phage display was used. The heavy chain variable region (VH) of AKA was used to guide the selection of a human TAG-72-specific light chain variable region (VL) from a human VL repertoire constructed from human PBL. Most of the selected VLs were identified to be originated from the members of the human germline VK1 family, whereas the VL of AKA is more homologous to the VK4 family. Competition binding assay of the selected Fabs with mouse CC49 suggested that the epitopes of the Fabs overlap with that of CC49. In addition, they showed better antigen-binding affinity compared to parental AKA. The selected human VLs may be used to guide the selection of human VHs to get completely human anti-TAG72 antibody.

Keywords: monoclonal antibody, phage display, TAG-72, chain shuffling, guided selection

The tumor-associated glycoprotein (TAG)-72 is expressed in the majority of human adenocarcinomas but is rarely expressed in most normal tissues, which makes it a potential target for the diagnosis and therapy of a variety of human cancers (Thor *et al.*, 1986, 1987). Mouse CC49 is a monoclonal antibody (mAb) with high affinity to TAG-72, and several clinical trials showed that radiolabeled mouse CC49 could successfully target such carcinomas (Divgi *et al.*, 1994, 1995a; Mulligan *et al.*, 1995). However, the administration of mouse CC49, like that of any other murine mAb, has led to the generation of human anti-mouse antibody (HAMA) immune responses in many patients (Divgi *et al.*, 1995b).

To overcome this problem, a humanized CC49 antibody (HuCC49) has been constructed by grafting the complementarity determining regions (CDRs) onto homologous human antibody, while retaining the framework (FR) residues that influence the CDR conformations and/or are involved in maintaining the structure of the the heavy (VH) and light (VL) chain variable domains (Kashmiri *et al.*, 1995). Subsequently, to reduce the immunogenicity of HuCC49, the mouse CDR and FR residues have been replaced individually with corresponding human residues, and the antigen-binding activity and potential immunogenicity of each variant have been analyzed (Iwahashi *et al.*, 1999; Tamura *et al.*, 2000; De Pascalis *et al.*, 2003; Gonzales *et al.*, 2003).

An alternative method, guided selection has been developed to convert murine antibodies into completely human antibodies with similar binding characteristics (Figini *et al.*, 1994; Jespers *et al.*, 1994; Figini *et al.*, 1998; Watzka *et al.*, 1998; Beiboer *et al.*, 2000). In this method, mouse VH and VL domains are sequentially or in parallel replaced by human VL and VL domains, respectively using phage selection to derive human antibody with best affinity. For example, original mouse VH domain is combined with VL repertoire derived human B cells, and the resulting human VL- shuffled antibody library is displayed on the phage surface followed by selection on the target antigen. The selected VLs are then used for construction of human VH-shuffled antibody library, followed by selection on the target antigen, which leads to the construction of completely human antibody with original specificity.

Previously, we constructed a new humanized version (AKA) of mouse CC49 to enhance its therapeutic potential (Yoon *et al.*, 2006). AKA still had several mouse residues to maintain the specificity and comparable affinity of mouse CC49. In this study, to generate a completely human antibody against TAG-72, guided selection strategy was used. The VH region of AKA was used to guide the selection of a human TAG-72-specific VL region from a human VL repertoire constructed from human PBL. The amino acid sequences of the selected VLs were determined, and TAG-72 binding characteristics of the Fabs with the VLs were compared with AKA and mouse CC49 using competition ELISA and BIAcore.

* To whom correspondence should be addressed.
(Tel) 82-42-860-4122; (Fax) 82-42-860-4597
(E-mail) hjhong@kribb.re.kr

Materials and Methods

Construction of human light chain-shuffled phage antibody library

About 100 ml of blood was collected from ten healthy donors, and PBLs were purified on a Ficoll gradient (Amersham Pharmacia Biotech., USA). Total RNA was extracted from the purified PBLs using RNAzol™ B (Tel-Test, Inc., USA), and first-strand cDNAs were synthesized using SUPERSCRIPT™ II (Life Technologies, USA) with 3'-primer specific for the constant region of human κ light chain. The cDNAs were then amplified by PCR using rTaq polymerase (TaKaRa, Japan) and 5'-family specific primers for human κ light chain variable region, as described previously (Burton *et al.*, 1991; Persson *et al.*, 1991) in combination with the 3'-primer. After gel-purifying 660 bp PCR products, the DNA was re-amplified with extension primers to increase the efficiency of restriction enzyme digestion and subsequent library construction. The final PCR products encoding the repertoire of light chains were digested with *Sac*I and *Xba*I (Boehringer Mannheim, USA) and ligated into the *Sac*I-*Xba*I sites of pC3Q-AKA (Yoon *et al.*, 2006), which was then electroporated into *E. coli* TG1 cells. The phages displaying human light chain-shuffled AKA Fab library were rescued by the infection of the transformed cells with VCSM13 helper phage (Stratagene, USA).

Biopanning

The wells of a microtiter plate (Nunc, USA) were coated with 1 μg of TAG-72-positive Bovine Submaxillary Mucin (BSM) (Type I-S, Sigma) or Porcine Submaxillary Mucin (PSM) (Sigma) at 4°C overnight. The wells were washed twice with PBS and blocked by 4% skim milk in PBS at 37°C for 2 h. After removing the blocking solution, 100 μl of the library phage (about 10^{11} colony forming units) in PBS with 2% skim milk was pre-incubated on the PSM-coated wells at 37°C for 30 min and then added to the BSM-coated wells. After incubation at 37°C for 2 h, the unbound phages were discarded and the wells were washed five times with 0.05% Tween 20 in PBS (PBST) and twice with distilled water. Bound phages were eluted with 0.1 M glycine-HCl (pH 2.2) and neutralized with 2 M Tris base. The eluted phages were amplified by infecting TG1 cells followed by their super-infection with the helper phage (Marks *et al.*, 1991). The amplified phages were then subjected to another round of panning. Four rounds of panning were carried out and the stringency of selection was increased with each round with an increase in the number of washes.

Screening for TAG72-specific clones

To screen and characterize anti-TAG72 clones, soluble Fab was prepared from randomly selected clones after panning. Briefly, the plasmid DNA isolated after the final round of panning was digested with *Spe*I and *Nhe*I (Boehringer Mannheim) to remove DNA segment containing gene III, recircularized by self ligation and then electroporated into *E. coli* TG1 cells. Sixty colonies were randomly selected, cultured in 5 ml SB with 100 μg/ml ampicillin, and induced for Fab expression using 1 mM Isopropyl β-D-thiogalactopyranoside. The culture supernatant of each clone was used for screening of the specificity for TAG72 by ELISA.

The clones showing positive signal in ELISA were subjected to DNA sequencing, and the nucleotide sequences determined were submitted to V-BASE (http://www.mrc-cpe.cam.ac.uk/DNAPLOT.php) for sequence analysis. Finally, five different clones (KCS1, KCS5, KCS8, KCS10, and KCS18) were selected, and their reactivity to TAG72 was analyzed using ELISA and BIAcore.

ELISA

Microtiter wells were coated with antigens or antibodies diluted in 50 mM sodium carbonate buffer (pH 9.6) at 4°C overnight, and blocked with 2% BSA or skim milk in PBS. The plates were washed four times in PBST between each step. All the incubations were carried out at 37°C for 1-2 h. Color was developed with 2',2'-Azino-Bis (3-Ethylbenz-thiazoline-6-s-Sulphonic Acid) diammonium salt (Sigma, USA), and the absorbance of developed color was measured at 405 nm using a microtiter reader (Emax, Molecular Devices, USA).

Kinetic analysis of Fab binding to TAG72

For the precise affinity ranking of the selected Fab clones, the culture supernatant of each clone was subjected to affinity chromatography on Protein G-Sepharose column (Amersham Pharmacia Biotech). The purity of assembled Fab was confirmed by 12% SDS-PAGE in non-reducing condition. BIAcore 3000 instrument (BIAcore AB, Sweden) was used to analyze kinetic properties of the purified Fab clones. BSM was immobilized to a 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC)/N-hydroxysuccinimide (NHS)-activated CM5 sensor chip (BIAcore AB). A similar surface was prepared using BSA as a control. The binding sensorgrams for five different concentrations of each Fab were obtained using a flow rate of 30 μl/min and analyzed using BIAevaluation software (version 4.1, BIAcore AB)

Production of anti-TAG72 IgGs

The VL sequences in the Fab expression plasmids were amplified by PCR and combined with the leader sequences of immunoglobulin (Ig) light chain by recombinant PCR. The VL with leader sequences was subcloned into the *Hind*III-*Bsi*WI site of an AKA expression cassette, pdCMV-dhfr-AKA containing the human γ1 and κ constant regions, as described previously (Yoon *et al.*, 2006). The resulting expression plasmid was introduced into COS7 cells using lipofectamin (Life Technologies) according to the protocol suggested by the supplier. After two day cultivation of the transfected cells, the supernatant was harvested and the concentration of antibody in the culture supernatant was determined by sandwich ELISA. Then the binding activity of whole IgG to TAG72 was measured by indirect ELISA.

Results and Discussion

Construction and panning of human light chain-shuffled Fab library

Previously, we obtained a humanized antibody, AKA by grafting parts of the CDRs of original murine antibody, CC49 onto homologous human immunoglobulin (Ig) germ-

line segments. However, it still has mouse amino acid residues from CC49 which can evoke HAMA response in patients. To completely remove these mouse residues, in this study light chain shuffling was employed. A human light chain-shuffled Fab library was constructed by cloning the kappa light chain repertoire from human PBL into the phagemid vector containing AKA Fab, pC3Q-AKA. The resulting Fab library had diverse human kappa light chains combined with Fd region of AKA Fab, and consisted of $\sim 2 \times 10^7$ clones.

The library was subjected to panning against TAG-72-positive BSM four times. Each round of panning included subtraction panning against TAG-72-negative PSM to remove non-specific binders. Antigen-binding activity of the pooled phages from each round of the panning was analyzed by ELISA, which showed successful enrichment of BSM-specific binders (Fig. 1).

Selection and characterization of TAG72-specific clones

From the final round of panning, 60 clones were randomly selected, subjected to soluble Fab expression, and tested for their reactivity to BSM by ELISA. Among them, 17 clones were highly positive for antigen binding and were identified as five unique clones (KCS1, KCS5, KCS8, KCS10, and KCS18) by DNA sequence analysis. All five have unique light chain CDR3 (LCDR3) sequences which differ from that of AKA (Table 1). Sequence alignment using V-base revealed the closest human germline VK gene and somatically hypermutated nucleotides of each clone. Among the five clones, most VK sequences belong to germline VK1 family, while only one VK sequence (KCS5) was homologous to DPK24, which is only one germline VK belonging to germline VK4 family. In our previous study, DPK24 was used as the human VK template for the generation of humanized VK of AKA since its sequence was highly homologous to that of CC49 VK (Yoon *et al.*, 2006). This study first shows that not only VK4 sequence but also VK1 family sequences can be used to create the antibodies specific to TAG-72.

For precise characterization of the selected clones, soluble Fabs were expressed and purified as described in the Materials and Methods. The purity and integrity of the purified Fabs were confirmed by SDS-PAGE analysis, which showed more than 95% purity of assembled Fabs (Fig. 2). The purified Fabs were used for further studies.

Characterization of binding kinetics and affinity was performed by surface plasmon resonance (SPR) measurements of association of Fab with immobilized BSM. Figure 3 shows representative sensorgrams for binding of 1 μM Fab to im-

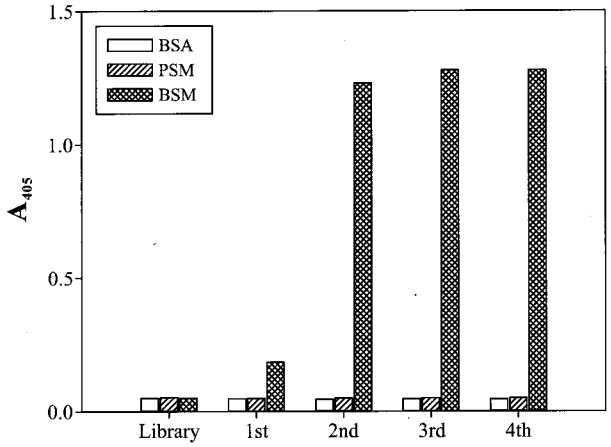

Fig. 1. Phage ELISA showing the enrichment of TAG-72-specific Fab-phages during panning cycles. After each round of panning, the output phage were amplified and applied to microtiter wells coated with BSA, TAG-72 negative PSM or TAG-72 positive BSM. Each microtiter well was coated with 200 ng of the antigen. Bound phages were detected by horseradish peroxidase (HRP)-conjugated anti-M13 antibody.

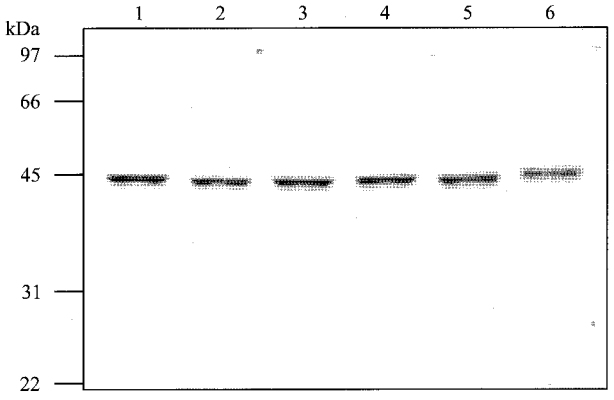

Fig. 2. SDS-PAGE (12%, Non-reducing) of purified Fabs. Lane 1, AKA; 2, KCS10; 3, KCS18; 4, KCS8; 5, KCS1; 6, KCS5. Molecular weight markers are indicated in kilodalton on the left.

Table 1. Sequence analysis of human VL genes in the selected TAG-72-specific Fab clones

Clone	Human VL family	Closest human VL germline gene	Differences from germline (Nucleotides, Amino acids)[a]	LCDR3
AKA	VK4	DPK24		QQ**YYSY**PLT
KCS1	VK1	HK137	(9, 6)	QQ**YNYY**PIT
KCS5	VK4	DPK24	(11, 8)	QQ**HYRA**PIT
KCS8	VK1	L12a, DPK9	(16, 6)	QQ**VKYY**PLT
KCS10	VK1	L12a	(10, 5)	QQ**LNSY**PIT
KCS18	VK1	L12a, DPK9	(17, 6)	QQ**TYIA**PIT

[a] Nucleotide and amino acid differences in V-gene segment, excluding CDR3. The nucleotide sequences were analyzed using V-BASE (http://www.mrc-cpe.cam.ac.uk/DNAPLOT.php).

Table 2. Binding parameters of the selected Fabs with the shuffled human light chains. Association (k_{on}) and dissociation (k_{off}) rate constants were determined using surface plasmon resonance. TAG-72 (+) Bovine submaxilary mucin (BSM) was immobilized on the sensor chip. The dissociation constant (K_D) was calculated from k_{off}/k_{on}

Clone	$k_{on}/10^4$ ($M^{-1}s^{-1}$)	$k_{off}/10^{-3}$ (s^{-1})	K_D (nM)
AKA	1.3	13.3	1,020
KCS1	1.01	4.33	429
KCS5	1.35	5.06	375
KCS8	1.68	6.33	377
KCS10	0.48	5.14	1,045
KCS18	2.21	8.41	381

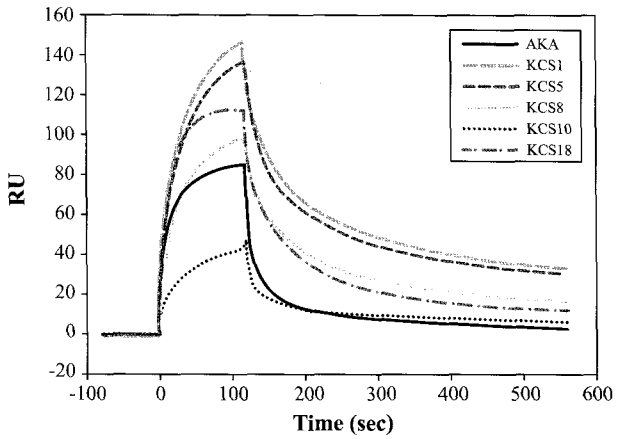

Fig. 3. Biacore sensorgrams for the binding of the Fabs (1 μM) to immobilized BSM on a sensor chip.

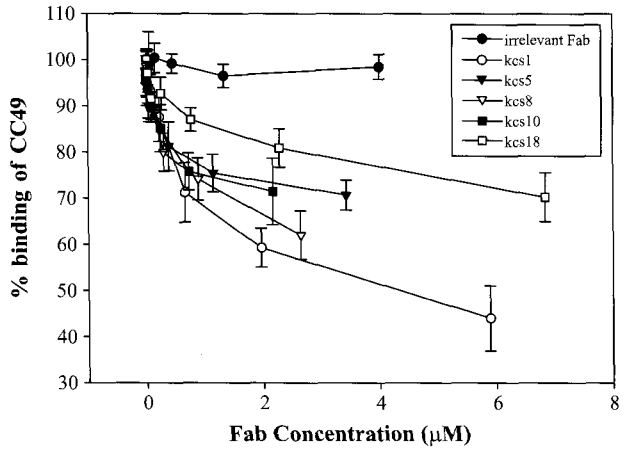

Fig. 4. Competitive binding assays with murine CC49. CC49 antibody was incubated with BSM (200 ng/well) coated in the microtiter wells in the presence of dilutions of the selected Fabs and irrelevant Fab specific to HAV. Bound murine CC49 antibody was detected by HRP-conjugated goat anti-mouse IgG (Fc-specific) antibody (Pierce). 100% binding corresponds to the value of absorbance obtained in the absence of competing antibody. Data points represent Means±Standard Deviation of triplicates.

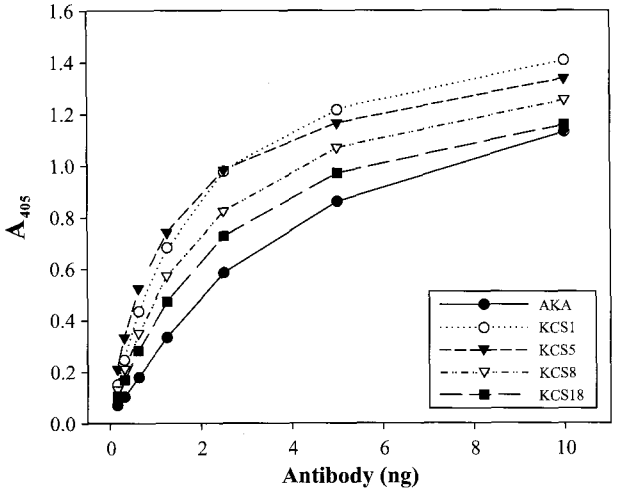

Fig. 5. ELISA with whole IgG form. COS7-expressed IgGs were tested for their binding ability to BSM by incubating with BSM (200 ng/well) coated in the microtiter wells. Bound IgG was detected by HRP-conjugated goat anti-human IgG (Fc-specific) antibody (Pierce).

mobilized BSM. Direct comparison of the sensorgrams indicated that most of the selected Fabs had better binding affinity compared to AKA Fab. For precise comparison of binding affinity, kinetic rate constants of each Fab were estimated from sensorgrams obtained at five different concentrations of Fab by using BIAevaluation program. Table 2 shows the estimated value of association rate constant (k_{on}), dissociation rate constant (k_{off}), and dissociation constant (K_D) for each Fab. Four Fabs (KCS1, KCS5, KCS8, and KCS18) showed higher binding affinities (K_D) compared with that of AKA Fab. The affinity (375 nM) of KCS5 Fab, the best one, was approximately three-fold higher than that (1,020 nM) of AKA Fab. Considering that the affinity of whole IgG form of AKA is 10.5 nM (Yoon *et al.*, 2006), the affinity of antibody fragment for TAG-72 is approximately 100-fold lower than that of whole IgG. This is consistent with the previous report that the K_A of mouse CC49 ScFv was 1.4×10^6/M, as measured by SPR (Pavlinkova *et al.*, 2000).

A competition binding assay with CC49 was performed to test whether the selected Fabs maintain original epitope specificity of CC49. As shown in Fig. 4, the binding of CC49 to immobilized BSM was inhibited in a dose-dependent manner by increasing concentrations of the selected Fabs but not by an irrelevant Fab, anti-Hepatitis A virus Fab HA6 (Kim *et al.*, 2004). Hence, the selected Fabs with the replaced human kappa light chain were suggested to bind to the same epitope recognized by original CC49 antibody.

Finally, the four Fabs with higher affinities compared to AKA Fab were converted to whole IgG (γ1, κ) with human heavy and κ light chain constant regions. The whole IgG was transiently expressed in COS7 cells and subjected to indirect ELISA to compare the relative binding activities of IgGs (Fig. 5). Like in Fab form, the IgGs with the human kappa light chains showed better antigen-binding activity

than IgG form of AKA.

In summary, using guided selection strategy, the human light chains specific for TAG-72 were isolated from human light chain repertoire constructed from human PBL. Most of the selected human light chains were identified to be originated from the members of the human germline VK1 family, whereas the VL of AKA or murine CC49 is more homologous to the VK4 family. The Fabs with the selected human light chains maintained similar specificity of CC49 antibody. In addition, they showed better TAG-72-binding affinities than the parental AKA. In a consecutive step, the selected human VL region will be applied to select a human TAG-72-specific VH region from a human VH repertoire to get a completely human anti-TAG-72 antibody.

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