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Optimal design of Ig 5' primers for construction of diverse phage antibody library established to select anti-HAb18GEF and anti-DOTA-Y Fabs for hepatoma pretargeting RIT

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1. ABSTRACT

Phage antibody library yields antibodies with higher affinity against different antigens, if diverse IgV gene repertoires can be amplified. As the currently available Fab primer sets cannot guarantee efficient amplification with high diversity, and because rare cloning sites can be found in certain Ig genes, here, we present an optimal set of Ig 5' primers, compatible with Fd 5' clone site replaced pComb3 vector, for diverse Fab phage display library construction. These novel Fab primes designed based on the newly classified IgV families, not only have best match and highest coverage for IgV family with minimized N-terminal amino acid changes, but also present good amplification diversity and efficiency of Ig gene from mice immunized with different forms of antigens (HAb18GEF, KLH-DOTA-Y, and HAb18G/pcDNA3). A high quality immune phage library with good diversity was constructed based on the mixed Ig repertoire, and five high affinity Fab antibodies were selected to specifically bind to HAb18GEF, DOTA-Y and an irrelevant antigen γ-sm, respectively. This novel Fab primers set can be applied to the construction of diverse phage antibody library and the anti-HAb18GEF and anti-DOTA-Y Fab antibodies lay a solid foundation for radioimmunotherapy of hepatoma.

2. INTRODUCTION

Hepatoma associated antigen, HAb18G, is a highly glycosylated transmembrane protein abundantly expressed in highly metastatic malignant tumors, especially in human hepatomas. By screening the hepatoma cDNA library with McAb HAb18 against HCC, the gene sequence of HAb18G was found to be identical to that of CD147, a member of the immunoglobulin superfamily, which has recently generated a great deal of interest in tumor invasion and metastasis (1-4). Previous studies demonstrated that HAb18G/CD147 promoted invasion of hepatoma cells by stimulating stromal cells to produce elevated levels of matrix metalloproteinase (MMPs) (5-7). Presence of HAb18G/CD147 on the surface of hepatoma cells makes it a promising target for radioimmunotherapy.

We previously used HAb18 $F(ab)_2$ labeled directly with $^{(131)}I$ for hepatoma targeting therapy in animal models and in liver cancer patients(8, 9). Despite efficient targeting with modest response rate, relatively slow pharmacokinetics and relatively high residence times of $^{(131)}I$ labeled HAb18 $F(ab)_2$ in the nontarget tissues were observed in hepatoma patients (unpublished data). These may be due to the medium affinity of McAb HAb18 and

the defect of conventional radioimmunotherapy with direct-targeting strategy. Since promising results have already been obtained with mAb-based pretargeting strategies in preclinical (10), preparation of high-affinity bispecific antibody composed of anti-HAb18GEF and anti-DOTA-Y would be an ideal approach for hepatoma pretargeting radioimmunotherapy.

Phage display technology allows for rapid isolation of specific McAbs with higher affinity from a large repertoire, and is now considered an attractive alternative to hybridoma technology especially in high-throughput generation of McAbs (11, 12). Fab and scFv antibody fragments were usually displayed on the surface of filamentous phages. However, scFvs are prone to dimer- and trimerisation which can hamper selection and characterization of specificity (13). Fab fragments, on the other hand, lack this tendency to multimerize, which facilitates screening assays to differentiate between antibody variants with differences in affinity or kinetics of binding. Most significantly, the advantages of the Fab format are (1) the retention of natural folding and binding characteristics; (2) that a wide range of available secondary antibodies may be used for detection and analysis; and (3) that few steps are used in PCR amplification and cloning (14).

To display Fab library on M13 phage, pComb3 phagemid vector system and its derivatives are often used to fused express of the heavy chain Fd gene to the N-terminus of minor coat protein gIII, and secreted express of the light chain gene into the periplasmic space where chain association efficiently forms an intact Fab (14). Since the library size, gene diversity and panning strategy are what greatly influence the pan-out of specific antibodies with higher affinity, the more diverse and larger the constructed libraries are, the more probable that rare but potent antibodies can be panned out (11). In most cases, the library size and gene diversity depend on (a) whether all the repertoire of Ig genes can be collected from murine or human sources, and (b) whether the collected Ig genes can be transformed into *Escherichia coli* with great efficiency. As high-efficient transformation can be realized by electroportion, designing extended primers to amplify all the Ig repertoire genes to the full is believed to be essential in the construction of high-quality libraries.

A great variety of primer sets had been designed for PCR-cloning of murine Ig genes. These primer sets can be classified as universal primers and degenerate primers. The former can not be used to uniformly amplify all the repertoire of Ig genes because of their lack of diversity (15-17), while the latter, though good for diversity, may diminish the amplification efficiency of some Ig genes, thus creating biased libraries (18, 19). Therefore, family-specific primers had been devised based on murine IgV genes classification. However, some of these family-specific primers were found to match inadequately for some minor gene families (18, 20- 22), some of them can not work properly in actual PCR process, and some of them result in truncated antibody genes if the restriction enzyme site resides frequently in certain region of Ig in some mice species (23-25). In addition, with the expansion of the updated kabat database, many new-submitted Ig sequences were not included in previously classification of IgV families (26). Therefore, designing of novel Fab primers set for the construction of diverse phage display libraries is needed firstly.

In this study, an original set of murine Fd 5' primers and Lκ 5' primers were firstly optimal designed based on the newly classified IgV families, and then used to improve the amplification diversity and efficiency of Ig gene from mice immunized with different forms of antigens. To demonstrate the general efficacy of our approach, a high quality immune phage library with good diversity were constructed, and several specific Fab antibodies with high affinity were selected to bind to HAb18GEF antigen, DOTA-Y hapten and an irrelevant antigen-γ-seminoprotein, respectively. These novel anti-HAb18GEF and anti-DOTA-Y Fab antibodies would lay a solid foundation for hepatoma pretargeting radioimmunotherapy in the next step, and these novel Fab primers set with limited degeneracy can be applied to the construction of diverse phage antibody library easily.

3. MATERIALS AND METHODS

3.1. Cells, vectors, bacterial strains, and reagents

Human hepatocellular carcinoma (HCC) cell lines FHCC-98 was established in our Lab (27), and HCC cell lines MHCC-97H (higher potential of metastasis) was kindly provided by Liver Cancer Institute of Zhongshan Hospital, Fudan University (Shanghai, China). Human normal liver cell line QZG were purchased from Type Culture Collection of Chinese Academy of Sciences (Shanghai, P.R.China). Phagemid vector pComb3 was kindly obtained from Carlos F. Barbas, III (The Scripps Research Institute, La Jolla, CA 92037, USA). pMD18-T vector, site-directed mutagenesis kit, and E. coli strain JM109 (F′ *traD36 proA*$^{+}$*B*$^{+}$ *lacI*^{q} *Δ(lacZ)M15/ Δ(lac-proAB) glnV44 e14*$^{-}$ *gyrA96 recA1 relA1 endA1 thi hsdR17*) were purchased from TaKaRa Biotechnology Ltd (Dalian, P.R. China). E. coli strain *XL-1 Blue* (*recA1,endA1,gyrA96,thi,hsdR17(rk*$^{-}$*,mk*$^{+}$*),supE44,relA1.λ*$^{-}$*,(lac), F′□proAB,lacI*^{q}*,lacZ□M15,Tn10(tet*^{r}*)*□) and Rosetta-gami B (F^{-} *ompT hsdS*$_B$($r_B^-m_B^-$) *gal dcm lacY1 aphC gor522*::Tn10 *trxB* pRARE^2(Cam^R,kan^R,Tet^R)) were purchased from Stratagene Corp.(La Jolla, CA USA) and EMD Biosciences Inc. (San Diego) , respectively. HAb18GEF antigen,γ-sm protein, BSA-DOTA-Y hapten conjugate, KLH-DOTA-Y hapten conjugate, HAb18G/pcDNA3 plasmid, and HAb18 Fab antibody were respectively constructed and purified as previously described (29, 8-32).

Trizol for total RNA isolation was purchased from Invitrogen Ltd. (Beijing, P.R. China). QIAquick Gel Extraction Kit, QIAquick PCR Purification Kits, MinElute Reaction Cleanup Kit and QIAprep Spin Miniprep Kit were purchased from Qiagen Ltd.(Shanghai, P.R. China). The restriction enzymes (*Sac* I, *Xba* I, *Spe* I, *Mfe* I, *Not* I), M-MLV Reverse Transcriptase, T4 DNA ligase, Vent$_R$ DNA polymerase, and M13K07 Helper Phage (kan^r) were

purchased from New England Biolabs Ltd.(Beijing, P.R. China). Peroxidase conjugated Rabbit anti-M13 McAb, Peroxidase Conjugated GOAT anti-mouse IgG Fab antibody, FITC Conjugated Goat anti-mouse IgG Fab antibody, Glycogen, PEG 8000 and IPTG were purchased from Sigma-Aldrich Inc. (Shanghai, P.R. China). Oligonucleotide primer synthesis and DNA sequencing was performed by ShengGong Ltd (Shanghai, P.R.China). Standard LB, SOB, SOC and 2YT medium were respectively used for liquid cultures. Standard LB, SOB agar plates were respectively were utilized as solid medium. All molecular biology procedures were performed according to conventional techniques as described by Sambrook and Russell (33) unless otherwise indicated.

3.2. Design of murine Fd 5' primers and Lκ 5' primers

The Kabat database (data updated to 2001-3-14) was downloaded from ftp.cbi.pku.edu.cn/pub/old/kabat.

For Fd 5' primers, the N-terminal amino acid and corresponding nucleotide acid sequences in FR1of known mouse Ig VH in the Kabat database were used. First, the frequency of each amino acid at positions 1 - 8 of FR1 was determined by using Kabatman software (34). Any amino acid at a frequency greater than 0.5% of known mouse Ig VH was selected and designated as "common" amino acid at that position. Next, all possible combinations of these "common" amino acids were searched for using Kabatman, and the number of hits from known mouse Ig VH with complete amino acid sequences at position 1-8 was recorded. Any "common" amino acid combination >0.5% was considered as a separate family. For each family, a list of all possible combinations of codon was made for positions 1 – 8, not including positions coding for 5' clone site. All these codon combinations were aligned in the Kabat database using Seqhunt II software (35). The Fd 5' primers were designed by three criteria: (a) the primers must match as much as possible to N-terminal nucleotide acid sequences of FR1 in Ig VH, and the total number of primers must not be too great, (b) the 5' cloning site residing in the primers must code for the "common" antibody residues at positions 1 - 8 and must be rarely found in murine IgV genes, (c) the 5' cloning site must be far away from the 3' end of the primers so that fewer N-terminal antibody residues will be altered.

For Lκ 5' primers, the N-terminal amino acid and corresponding nucleotide acid sequences in FR1of known mouse Ig Vκ in the Kabat database were used, and the similar searching and aligning approach were adopted to establish the new families classification of Lκ gene. The Lκ 5' primers were designed by same three criteria as described above.

For Fd 3' primers, IgG1, IgG2a (BALB/c), IgG3 and IgG2b primers, together with cloning site *Spe* I, described by M. Ostermeier (25) were used. For Lκ 3' primers, both the Kappa 3' primer and cloning site *Xba* I described by Kang (36) were used.

3.3. Site-directed mutagenesis of pComb3 phagemid vector

The Fd 5' cloning site (*Xho* I) in pComb3 phagemid vector was substituted by newly designed 5' cloning site (*Mfe* I) by using site-directed mutagenesis kit as described by the manufacturer. Briefly, The small pre-mutant DNA fragment, double-digested by Spe I and Not I from pComb3 vector, was firstly cloned into pMD18T vector. The recombinant T-vector was PCR amplified by reverse-adjoining primers (A1: 5'-AGCAATTGCACCTGGGCCATGGCTGGT-3'; A2: 5'-GGTCGACGGTATCGATAAGC-3') to achieve site-directed mutagenesis. The gel-purified PCR products were blunting-kinated and self-ligated into cycle form. By double-digesting the resultant T vector with *Spe* I and *Not* I, small post-mutant fragment was ligated back to replace the original pre-mutant DNA fragment in pComb3 (denominated as pComb3Mf). The Fd and Lc cloning regions of pComb3Mf vector were sequenced respectively by sequencing primers (SFd: 5'-GGTGGCGGCCGCAAATTC-3', SLc: 5'-CTAAACTAGCTAGTCGCC-3').

3.4. Evaluation of murine Fd (Lκ) 5' primers and cloning of Ig gene

Both new designed Fd (or Lκ) 5' primers and literature-reported Fd (or Lκ) 5' primers (19, 20, 25, 36-40) were aligned to all murine Ig nucleotide acid sequences in the Kabat database, and the resultant sequence match percentages and family coverage percentages were compared respectively. The thermodynamics of the new designed primers were inspected using Primer Premier 5.00 software (www.PremierBiosoft.com).

To test the PCR amplification efficiency, three groups of BALB/c mice, one immunized by HAb18GEF protein (28), one immunized by HAb18G/pcDNA3 plasmid followed by mixed hepatoma cell boost (29), and the other immunized by KLH-DOTA-Y hapten conjugate (31), were sacrificed to isolate the spleen cells. Total RNA was extracted from 10^6 spleen cells using the TRIZOL technique. To prepare first-strand cDNA, total RNA was primed by oligo-dT with M-MLV reverse transcriptase. The new designed Fd (or Lκ) 5' primers and the traditional used Fd (or Lκ) 5' primers (36) were used separately for PCR amplifications with the same Fd (or Lκ) 3' primers. Each PCR amplification was conducted in 100 μL final volume containing: 2 μL cDNA, 40 pmol Fd (or Lκ) 5' and 3' primers respectively, 1 μL 10 mM dNTPs, 10 μL Vent polymerase buffer, 5.0 U $Vent_R$ DNA polymerase, and 2 mM $MgCl_2$. The cycling conditions were: initial melt at 94°C for 4 min followed by 30 cycles of a three-step program (94°C, 1 min; 50°C, 1 min; 72°C, 1 min). After a 10 min extension at 72°C, the PCR products were fractionated through 1.5% agarose gel and stained with ethidium bromide. The mixture of PCR-amplified Fd (or Lκ) gene products derived from three groups of spleen cells were purified on 1.5%, low melting temperature agarose gel by Qiaquick@ Gel Purification kit.

3.5. Construction and identification of bacterial-form Fab libraries

The PCR-amplified Lκ fragment and pComb3Mf plasmid were double-digested by *Sac* I and *Xba* I respectively, then purified using MinElute PCR Purification Kit and incubated (molecular ratio as 6:1) overnight at 16°C with T4 DNA ligase (3 U per ug). After

purified by QIAquick PCR Purification Kits and glycogen precipitation, the ligation product was resuspended in 10 μL ddH_2O and electrophorated into *E.coli* XL1-blue compentent cells using a Bio-Rad Gene Pulser (Bio-Rad Laboratories, CA) set at 2.5 kV and 200 Ohms. The transformed cells were mixed immediately with 1 mLfresh SOC medium, shaken for one hour at 37°C and plated on SOB-GAT (1% glucose; tetracycline, 40 μg/mL; ampicillin, 100 μg/mL) selective plates. The Lκ library plasmids were isolated from overnight-grown colonies by Qiaquick@Plasmid Extraction Kit. The gel purified Lκ library plasmid and the PCR-amplified Fd fragment were double-digested by *Mfe* I and *Spe* I respectively, ligated, electrophorated and plated on SOB-GAT selective plates as described above. All the overnight-grown colonies on the transformed plates were primary Fab antibody libraries in bacterial-form.

To evaluate of the quality of prepared libraries, 10 colonies randomly selected from the transformed plates were inoculated overnight in SOB-GAT medium and used for plasmid isolation. The Lκ (or Fd) gene recombinant percentage was determined by double digestion reaction with *Sac* I + *Xba* I (or *Mfe* I + *Spe* I). Cloned mouse Fd and Lκ gene insert in these plasmids were sequenced with automatic sequencing system. V gene diversity of the resultant Ig Lκ (or Fd) sequences was analyzed using the IMGT/V-QUEST online alignment program (http://imgt.cines.fr/textes/vquest/). The size of the prepared libraries was measured as ampicillin-resistant colony forming units (CFU) per mL.

3.6. Preparation and biopanning of phage display Fab libraries

All the colonies were then collected, mixed with glycerol, and stored at -70°C. To rescue phagemid particles from the library, 100 ml SOB-GAT medium was inoculated with 10^7 clones taken from the library stocks (approximately 100 μL) and grown with shaking (250 rpm/h) at 37°C. At OD600 of 0.5, helper phage M13K07 was added at a multiplicity of 50 and the infection process proceeded for 30 min at 37°C without shaking. After changing the culture medium to 2YT-AKT (ampicillin, 100 μg/mL; tetracycline, 40 μg/mL; kanamycin, 70 μg/mL), the cell incubation continued overnight at 30°C with shaking (200 rpm/h). Phage particles were precipitated by the addition of 4% (w/v) PEG 8000 and 3% (w/v) NaCl and incubated on ice for 30 min, followed by centrifugation (9000 rpm for 20 min at 4°C). Phage pellets were resuspended in 2 ml PBS (pH 7.3) and microcentrifuged for 3 min to remove the debris, and stored at 4°C. The titer of the prepared phage library was measured as ampicillin-resistant colony forming units using standard procedures (33).

100μL display Fab phage libraries with 10^{12} (PFU) titer in blocking solution (1%(w/v) BSA in PBS) were separately added to Microtiter wells coated respectively with 50 μg/mL HAb18GEF antigen, γ-sm protein, and BSA-DOTA-Y (in 50 m M NaHCO3 pH 9.6). After incubating for two hours at room temperature, the Microtiter wells were washed 10 times with PBST (PBS containing 0.05% Tween 20) and subsequently with PBS. The bound phage was eluted with 100 μL glycine-HCl (0.1 M, pH 2.2) for 10 min incubation at room temperature. Each eluate was neutralized with 2μL Tris (2M, pH 7.0) immediately and then incubated with 200μL of log phase XL1-blue cells at 37 °C for 30 min. All the infected cells were collected, propagated, and plated on SOB-GAT selective plates for the next panning round. The further panning process was repeated as described above except that the washes of bound phage with PBS/Tween and PBS in the two to four panning rounds were 20, 30 and 40 times, respectively. Following each round of panning, the percentage yield of phage was determined as (number of phage recovered/number of phage applied)×100 (41). Enrichment was calculated and compared with the percentage yield of the previous panning round.

3.7. Selection of different antigen-binding phage antibodies (PhAbs)

After the panning process, individual XL1-blue clones were picked, grown at 37 °C in 96-well plates, and rescued with M13KO7 helper phage as described earlier. The binding activity of the rescued PhAbs against different antigen was determined by phage ELISA. The amplified phage preparation was blocked with blocking solution (1%(w/v) BSA in PBS) at 4 °C for 1 h and then added to three 96-well microtiter plates coated respectively with different antigen (10 μg/mL HAb18GEF antigen, γ-sm protein, and BSA-DOTA-Y). After 90 min incubation at 37 °C, the plates were washed three times with PBST, and incubated with a HRP conjugated Rabbit anti-M13 McAb as the second antibody for 1 h. The plates were washed three times, TMB peroxidase substrate was added, and the absorbance was read at 450 nm using a microplate reader (Bio-Rad Laboratories, CA). The specificity of the positive clones was then further examined by phage ELISA using plates coated with a panel of six nonrelated antigens: BSA, ferritin, HBsAg, human IgG, and pepsin. The ELISA was performed as described above.

3.8. Soluble Fab expression, characterization and sequence analysis

To generate soluble Fab, plasmid DNA of the selected clones was prepared and transformed into *E. coli* Rosetta-gami B compentent cells. Single colony was grown in 40 ml LB (100 lg/ml ampicillin and 1% glucose) until OD600 =1.0 and then pelleted at room temperature for 10 min at 3000g. The cell pellet was resuspended in 40ml pre-warmed LB (100 lg/ml ampicillin and 1 mM IPTG) and cells were grown at 37 °C overnight. After incubation, cells were harvested by centrifugation and the cell pellet was resuspended in BugBuster Protein Extraction reagent (Novagen) using 1/10th of the original bacterial culture volume. The cell suspension was incubated on a shaking platform for 20 min at room temperature Insoluble cell debris was removed by centrifugation at 16,000g for 20 min at 4 °C and the supernatant containing soluble Fab was concentrated 10 times using Millipore Ultrafree-4 centrifugal filter units. Protein concentration was determined using the Bradford assay with the Bio-Rad Protein Assay Kit.

Concentrated samples were resuspended in equal volumes of Bio-Rad's Laemmli sample buffer with

(or without) β-mercaptoethanol, heated for 5 min at 95 °C, and loaded on a 12% SDS–polyacrylamide gel. ELISA assay of binding activity and specificity of the soluble Fab was the same as for the phage ELISA described above except that the second antibody was HRP-conjugated GOAT anti-mouse IgG Fab antibody. The apparent binding constants of the soluble Fabs were determined by inhibition ELISAs as described by Rath S (42). Briefly, dilutions of Fab preparations corresponding an ELISA OD450 reading of about 2.0 were chosen and mixed with increasing amounts of antigen (HAb18GEF or BSA-DOTA-Y orγ-seminoprotein) before transfer to corresponding antigen-coated ELISA plates, incubated for 2 hrs with rocking at room temperature and processed as for normal ELISAs. Apparent affinities were determined as the reciprocal of the antigen concentration required to inhibit 50% maximal binding. According to the results of inhibition ELISA, a range of different concentrations (1/1, 1/10 and 1/100) of soluble Fab preparations with higher apparent affinity was used to compete with another soluble Fab preparations for binding with the same antigen. This competitive inhibitory ELISA procedure helped to determine whether the isolated Fab antibodies recognize different epitopes of the same antigen. To further determine whether the selected anti-HAb18GEF (or anti-γ-seminoprotein) Fab antibody could specifically bind to human hepatoma cells (or prostate cancer cells), Immunofluorescence assays were carried out with FITC Conjugated Goat anti-mouse IgG Fab as the secondary antibody (1:800 dilution in 0.01% PBS-Evans blue) as previously described (29).

Gene sequencing of the selected clone was carried out on double-stranded DNA by the dideoxy chain-termination method. The variable region sequences analysis and corresponding germline gene assignment were performed using the IMGT/V-QUEST online alignment program.

4. RESULTS

4.1 Design of murine Fd 5' primers and Lκ 5' primers

A collection of 2721 Hc and 1773 Lκ of murine Ig amino acid sequences were extracted from the Kabat databases. Among these, the FR1 regions of 1625 Hc and 1563 Lκ have complete N terminal sequence. The "common" amino acids at positions 1 – 8 were screened out (Table 1). From the hitting analysis, we identified 23 new VH families, which covered 79.8 % of all mouse Ig VH, and 25 new Vκ families, which covered 81.3 % of all mouse Ig Vκ in the Kabat database (Table 2). As most VH families had Gln-Leu at position 3-4 and most Vκ families had Asp-Ile at position 1-2, the best restriction enzyme that met our design criteria was Mfe I for Fd 5' clone site and Sac I for Lc 5' clone site. Virtually, Mfe I had fewer enzyme digest frequencies than Xho I to the known mouse IG gene (Table 3). Of all the codon possibilities derived from newly classified Ig VH families, only 61 combinations were found at all, which coded for 66.8% of all known mouse Ig VH gene. Of all the codon possibilities derived from newly classified Ig Kappa families, only 85 combinations were found at all, which coded for 80.2% of all known mouse Ig Vκ gene. The Fd and Lκ 5' primers were designed based on these matched codon combinations. To cover all known IgV gene as most as possible and to guarantee appropriate primer number simultaneously, the resultant primers list were respectively trimmed to 10 and 7 by bringing some limited degeneracy bases (Table 4).

4.2. Evaluation of murine Fd 5' primers and Lκ 5' primers

After the *Mfe* I cloning site, there was no mismatch in the last 11 bases of the new designed Fd 5' primers for >94.7 % and no more than one mismatch for >98.4% of all mouse Ig VH genes. After the *Sac* I cloning site, there was no mismatch in the last 17 bases of the new designed Lκ 5' primers for >94.3 % and no more than one mismatch for >99.2% of all mouse Ig Vκ genes. In comparison with previously designed Ig 5' primers, our primers are of limited degeneracy, and intend to cover most Ig genes and all Ig families in the Kabat database (Table 5). Furthermore, the new designed Fd 5' primers have 52.2% - 65.2% G+C content (average 56.8%) and 1 - 144 fold degeneracy (average 32.4 fold), the new designed Lκ 5' primers have 55.9% - 60.2% G+C content (average 57.3 %) and 2 - 144 fold degeneracy (average 58 fold).

Successful amplifications were observed in the two sets of reactions: the Lκ gene amplifications resulted in distinct products of predicted size (about 650 bp) whereas the Fd gene amplifications resulted in predicted-sized fragments with different content (Figure. 1, 2, 3). In comparison with traditional Ig 5' primers (36), better amplification quality was obtained with our new designed 5' primers, especially with H6&IgG1, H1&IgG3, H2&IgG3, H3&IgG3, H4&IgG3, L4&IgGK primers to amplify HAb18GEF-immunized spleen cells (Figure. 1), with H2&IgG2a, H1&IgG2b, H5&IgG2b, H3&IgG3, H5&IgG3 primers to amplify HAb18G/pcDNA3 plus hepatoma cells immunized spleen cells (Figure. 2), and with H1&IgG2a, H5&IgG2a, H3&IgG2b, H1&IgG3, H7&IgG3, H8&IgG3, L1&IgGK, L4&IgGK primers to amplify KLH-DOTA-Y immunized spleen cells (Figure. 3).

4.3. Identification of constructed Fab antibody libraries

Sequencing of pComb3Mf vector confirmed that Hc cloning site *Xho* I had been successfully replaced by *Mfe* I by Site-directed mutagenesis (data not shown here). Based on pComb3Mf phagemid vector and mixtures of PCR products amplified by new designed Fd and Lκ primers, a bacterial-form primary Fab libraries with 1×10^8 (CFU) library size had been constructed, which present 90% Lc recombinant percent and 70% Fd recombinant percent (Figure. 4). Sequencing results of 10 random-selected individual clones showed that these assembled genes all differ in sequence and derive from multiple murine Ig germline families. Most Fd 5' primers and Lc 5' primers were represented in these genes. Five of the Seven VH genes amplified by our primers belong to major VH gene families (VH1, VH2, VH3 and VH5), whereas the two others are related to minor families (VH12 and VH13). Similarly, major (Vκ1, Vκ4 and Vκ6) but also small-sized (Vκ7, Vκ8, Vκ12 and Vκ21) gene families are represented among the amplified Vκ genes (Table 6).

Table 1. Amino acid frequencies at 1-8 position of the FR1 region among known mouse Ig genes

1625 Mouse Heavy chains															
1		**2**		**3**		**4**		**5**		**6**		**7**		**8**	
E	49.3	**V**	83.5	**Q**	**59.8**	**L**	**88.7**	**Q**	51.1	**Q**	49.2	**S**	79.3	**G**	91.2
Q	29.0	---	12.2	**K**	**23.8**	---	9.8	**V**	26.3	**E**	43.4	**P**	11.6	**---**	6.5
D	7.1	**I**	3.3	---	11.9	**V**	0.6	**K**	7.2	**---**	6.5	**---**	6.5	**E**	1.1
---	12.8	M	0.4	**H**	1.5	M	0.4	**---**	6.5	D	0.3	**T**	2.2	V	0.4
G	1.0	A	0.3	**M**	1.0	P	0.3	**L**	5.5	R	0.2	F	0.2	D	0.3
R	0.2	F	0.1	**N**	0.7	F	0.1	**E**	2.2	V	0.2	L	0.1	R	0.2
H	0.1	G	0.1	**T**	0.6	Q	0.1	D	0.4	G	0.1	V	0.1	S	0.1
L	0.1			R	0.3	H	0.1	M	0.4	P	0.1			A	0.1
K	0.1			E	0.1			A	0.1	N	0.1				
P	0.1			P	0.1			R	0.1						
S	0.1			S	0.1			P	0.1						
T	0.1			I	0.1			I	0.1						
				Y	0.1										
1563 Mouse kappa light chains															
1		**2**		**3**		**4**		**5**		**6**		**7**		**8**	
D	**66.5**	**I**	**75.0**	**V**	69.1	**M**	54.6	**T**	93.8	**Q**	97.6	**S**	67.2	**P**	80.4
Q	19.1	**V**	17.7	**Q**	16.2	**L**	38.9	**S**	3.1	---	1.2	**T**	24.6	**T**	8.1
E	7.3	---	2.7	**L**	6.7	**I**	2.9	**I**	1.3	G	0.4	**A**	3.3	**A**	3.3
---	3.0	**N**	2.4	**K**	2.6	**V**	1.6	**N**	0.5	R	0.2	**D**	2.1	**H**	2.2
N	2.1	**L**	0.8	**E**	1.7	---	1.7	---	0.8	E	0.1	---	0.9	**E**	2.2
S	0.8	**T**	0.8	---	1.9	**Q**	0.1	A	0.3	D	0.1	**I**	0.7	**Q**	1.5
K	0.4	A	0.3	**M**	0.6	**P**	0.1	Q	0.1	A	0.1	F	0.4	---	0.9
G	0.2	M	0.1	**T**	0.6	**T**	0.1	V	0.1	H	0.1	E	0.1	**S**	0.7
		F	0.1	I	0.3					L	0.1	N	0.1	V	0.2
Y	0.1	D	0.1	A	0.2					K	0.1	L	0.1	M	0.1
T	0.1	Q	0.1	F	0.1					T	0.1	K	0.1	I	0.1
F	0.1			G	0.1					W	0.1	Y	0.1	G	0.1
P	0.1			D	0.1							P	0.1		
A	0.1											Q	0.1		
R	0.1											R	0.1		
												V	0.1		

Amino acids are shown as bold only if their frequency of occurrence is at least 0.5% of mouse known Ig V, and selected as 'common' amino acid at that position. Dot (---) represents a gap in this position.

Table 2. New classification of mouse Ig VH and Vκ families based on position 1-8 amino acid in FR1

Ig VH Family	**Amino acid sequence at position 1-8 of FR1**	**% of all mouse known Ig VH**	**Ig Vκ Family**	**Amino acid sequence at position 1-8 of FR1**	**% of all mouse known Ig Vκ**
1	EVQLQQSG	19.6	1	QIVLTQSP	16.5
2	EVKLVESG	12.7	2	DIVMTQSP	8.6
3	QVQLQQPG	9.6	3	DIVLTQSP	8.5
4	QVQLQQSG	8.4	4	DVVMTQTP	8.0
5	QVQLKESG	4.4	5	DIQMTQTT	6.5
6	EVKLLESG	3.3	6	DIQMTQSP	4.6
7	DVKLVESG	3.3	7	DVLMTQTP	4.2
8	QIQLVQSG	2.3	8	EIVLTQSP	3.5
9	EVQLQESG	2.3	9	DIVMTQAA	2.6
10	DVQLQESG	2.0	10	DIKMTQSP	2.4
11	EVKLEESG	1.8	11	DIVMSQSP	2.1
12	EVQLVESG	1.7	12	DIVMTQSH	2.1
13	QVQLKQSG	1.0	13	ENVLTQSP	1.9
14	EVKLVESE	1.0	14	DIVITQDE	1.7
15	EVMLVESG	0.9	15	DIELTQSP	1.5
16	EVQLLETG	0.9	16	DIVMTQSQ	1.2
17	QVKLQQSG	0.8	17	DVQITQSP	0.7
18	GVQLQQSG	0.7	18	NIVLTQSP	0.6
19	DVHLQESG	0.7	19	NIVMTQSP	0.6
20	DVQLVESG	0.7	20	DIQMTQSS	0.6
21	EVQLVETG	0.6	21	DVVVTQTP	0.6
22	QVTLKESG	0.6	22	QIVLSQSP	0.6
23	EIQLQQSG	0.5	23	DVQMIQSP	0.6
			24	SIVMTQTP	0.6
			25	ETTVTQSP	0.5
Total		79.8(1625)	Total		81.3(1563)

All 'common' amino acid combinations present at least 0.5% of mouse known Ig V are listed as new families, and two frequently-occurring amino acids among all families could code for a restriction site are shown as bold.

Table 3. Enzyme digest frequency analysis of mouse Ig V gene

Restriction enzyme	% of Mouse known IG gene		% of BALB/c known IG gene		% of Mouse germline IG gene	
	VH (2399)	VK (1307)	VH (690)	VK (431)	VH (145)	VK (94)
Xba	1.33	5.36	0.58	7.42	2.76	8.51
Sac	5.71	0.99	3.62	1.62	11.03	2.13
Spe	1.21	1.68	1.45	1.62	0	2.13
Xho	5.42	0.38	8.12	0.23	2.76	4.26
Mfe	0.75	1.98	0.72	1.39	0.69	6.38

Table 4. Designed Fd 5' Primers and Lκ 5' Primers based on the new families classification of mouse Ig V

Fd 5' Primer	Sequence(5'-3': *Mfe*site underlined)	% of all mouse Ig VH with exact match outside enzyme site	% of all mouse Ig VH with exact match for last 11 bases
H1	CANGTBCAATTGCARCARTCHGG	8.4	28.9
H2	GAVGTRCAATTGGTKGARTCTGG	17.9	19.2
H3	SRKRTSCAATTGGTGSAGTCTGG	15.2	16.4
H4	CAGGTSCAATTGCAGCAGCCTGG	8.3	10.9
H5	CARGTBCAATTGAARSAGTCWGG	7.4	7.9
H6	GADGTRCAATTGCAGGARTCRGG	4.8	5.4
H7	GARGTVCAATTGCTSGAGTCTGG	1.8	2.9
H8	GARGTGCAATTGGTGGARTCTGA	1.2	1.2
H9	GAAGTGCAATTGGAGGAGTCTGG	1.1	1.2
H10	GAGGTGCAATTGGTTGAGACTGG	0.7	0.7
Total	(1434)	66.8	94.7
Lκ 5' primer	**Sequence(5'-3': *Sac* site underlined)**	**% of all mouse Ig Vκ with exact match outside enzyme site**	**% of all mouse Ig Vκ with exact match for last 17 bases**
L1	CCAGTTCCGAGCTCGTKCTNACHCARTCTCC	—	29.3
L2	CCAGTTCCGAGCTCVWRRTGACHCARTCTCC	—	18.1
L3	CCAGTTCCGAGCTCGTYCTVACH CARTCTCC	—	16.9
L4	CCAGTTCCGAGCTCYWGATGACHCARACYMC	—	12.9
L5	CCAGTTCCGAGCTCGTRRTGACYCARACBCC	—	10.0
L6	CCAGTTCCGAGCTCGTGATGACMCAGTCTCA	—	3.9
L7	CCAGTTCCGAGCTCGTRATRACYCAGGMTGM	—	3.2
Total	(1113)	—	94.3

Standard abbreviations are used for mixed sites: N = (A, G, C, T), R = (A, G), Y = (C, T), B = (C, G, T), D = (A, G, T), H = (A, C, T), V = (A, C, G), K = (G, T), M = (A, C), S = (G, C), W = (A, T)

Table 5. Comparison of our designed murine Ig 5' primers with literature-reported Ig 5' primers

Literature	Fd primer number	% of Ig VH outside enzyme site	% of Ig VH after enzyme site	Cover number of Ig VH family	Lc primer number	% of Ig Vκ outside enzyme site	% of Ig Vκ after enzyme site	Cover number of Ig Vκ family
Ours	10	66.8	94.7	23	7*	—	94.3	25
Ostermeier[25]	9	35.9	70.2	18	7	—	32.8	10
Kang[36]	9	—	76.7	20	7	—	32.8	10
Hachiro[37]	5	—	25.1	9	5	—	25.0	11
Φrum[19]	25*	—	40.9	15	25*	—	38.9	13
Catherine[38]	10*	—	67.4	18	7*	—	44.9	20
Orlandi[39]	1*	14.6	31.9	10	1*	30.3	21.7	6
Jorge[20]	1*	—	46.3	16	1*	—	55.6	18
Zhongde[40]	2**	—	55	19	1**	—	46.8	15

Asterisk (*) represents low degeneracy primers and double asterisk (**) represents high degeneracy primers

Table 6. Gene diversity of random-selected colony from constructed Fab library

Clone	Isotype	V-D-J rearrangement of Heavy chain variable region	V-J rearrangement of Lignt chain variable region
1	ND/κ	ND	Vκ6S*01—Jκ2*03
2	IgG1/κ	VH13S1*01—DST4*01—JH3*01	Vκ12S41*02—Jκ1*01
3	IgG2b/κ	VH1S54*01—DSP2.13*01—JH4*03	Vκ4S78*01—Jκ2*02
4	ND/κ	ND	Vκ1S122*01—Jκ2*02
5	IgG1/ND	VH2S5*01—DSP2.8*01—JH4*01	Vκ8S30*01—Jκ1*02
6	IgG2a/κ	VH1S29*01—DSP2.7*01—JH3*01	Vκ6S17*01—Jκ4*01
7	ND/κ	ND	ND
8	IgG1/κ	VH3S1*02—DFL16.2*01—JH2*01	Vκ21S21*02—Jκ1*02
9	IgG1/κ	VH5S2*01—DSP2.12*01—JH2*01	Vκ1S117*02—Jκ2*02
10	IgG2a/κ	VH12S1*01—DSP2.2*01—JH3*01	Vκ7S33*01—Jκ2*03

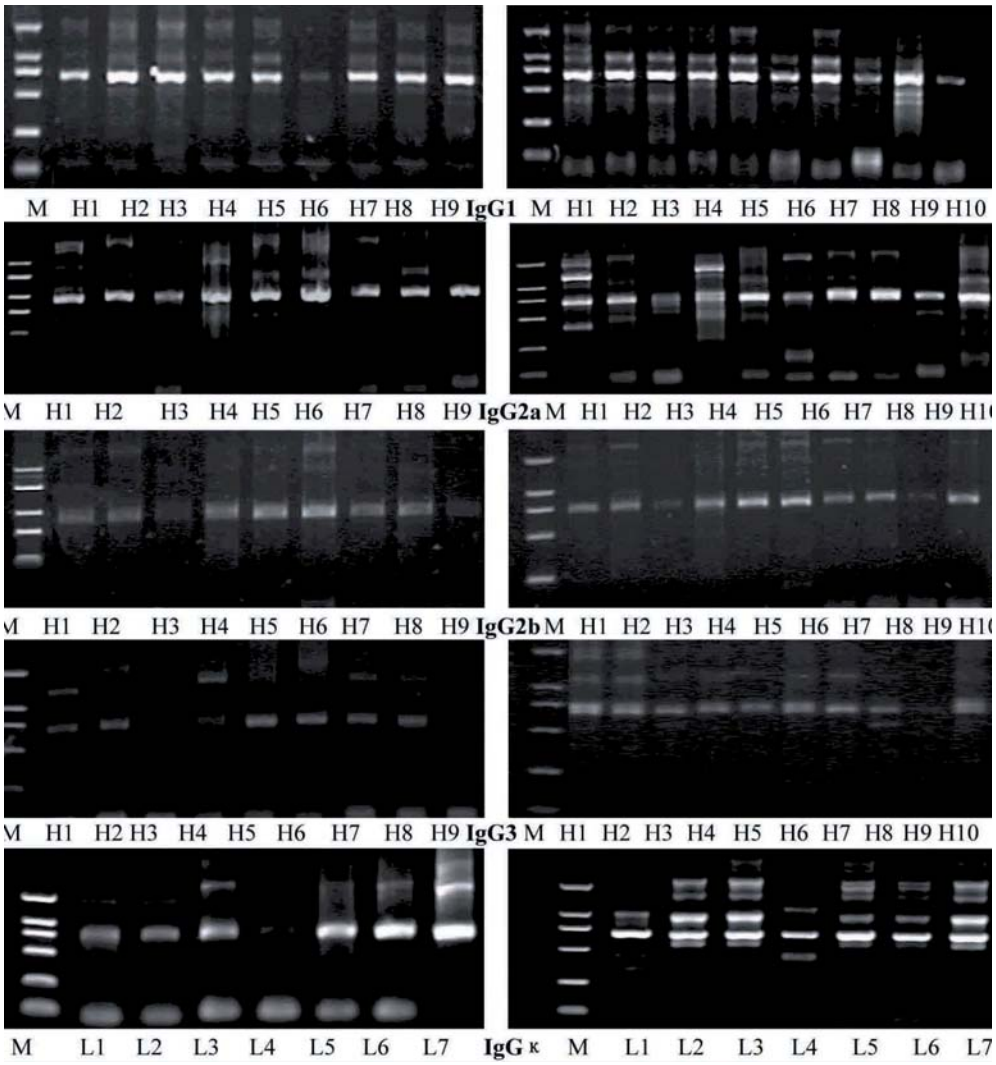

Figure 1. PCR amplification of Ig Fd and Lc gene from mice immunized by HAb18GEF. An aliquot of cDNA derived from the spleens of BALB/c mice immunized by HAb18GEF protein were amplified by PCR using different Ig 5' primers. New designed ten Fd 5' primer sets and seven Lc 5' primer sets listed in Table 4 were used respectively in the right column. Nine Fd 5' primer sets and seven Lc 5' primer sets described by Kang A. S. (36) were used respectively in the left column. The same Fd 3' primer sets (specific for IgG1, IgG2a (Balb/ c), IgG2b and IgG3) and Lc 3' primer (Ig Kappa) were used respectively in both columns. The first lane of each panel (M) contained molecular weight markers (prominent bands 2000, 1000, 750, 500, 250 and 100 bp). The number (X) below each lane corresponds to 5' primer used (HX or LX). Conditions for PCR were as described in Methods.

4.4. Biopanning of Fab phage libraries and selection of different antigen-specific PhAbs

After four rounds of panning on immobilized HAb18GEF, BSA-DOTA-Y andγ-seminoprotein respectively, the ratio of the number of recovered selected phages from the microtiter plates to the number of phages added increased from 38- to 220-fold (Figure. 5). These results showed successful enrichment on different antigen-specific clones, and also suggested the general diversity of our Fab library to some content. The panning procedure resulted in a step-wise enrichment for positive binders from 0 % (of 50 tested clones) in the pre-selected libraries to more than 30 % (of 90 tested clones) after four rounds of panning (data not shown here). Phage ELISA results showed that twelve clones with specific binding ability against HAb18GEF, five clones with specific binding ability against BSA-DOTA-Y, and two clones with specific binding ability against γ-seminoprotein had finally panned out (data not shown here). Interestingly, restriction enzyme digestion analysis showed that fourteen clones retain intact Fab gene insertion (four HAb18GEF-binding clones and one BSA-DOTA-Y -binding clones only retain Fd gene insertion). Partial DNA sequencing results further confirmed that only five clones contained intact Fab gene were unique, with different nucleotide and amino acid sequences (Table 7). Comparison with the sequences of the nearest germline V gene families indicated that the VH and Vk of the anti-HAb18GEF antibodies (clone H-15, H79, H-87) came from the mouse VH6, Vk6 and Vk12 families, respectively. The nearest germline V gene families of anti-

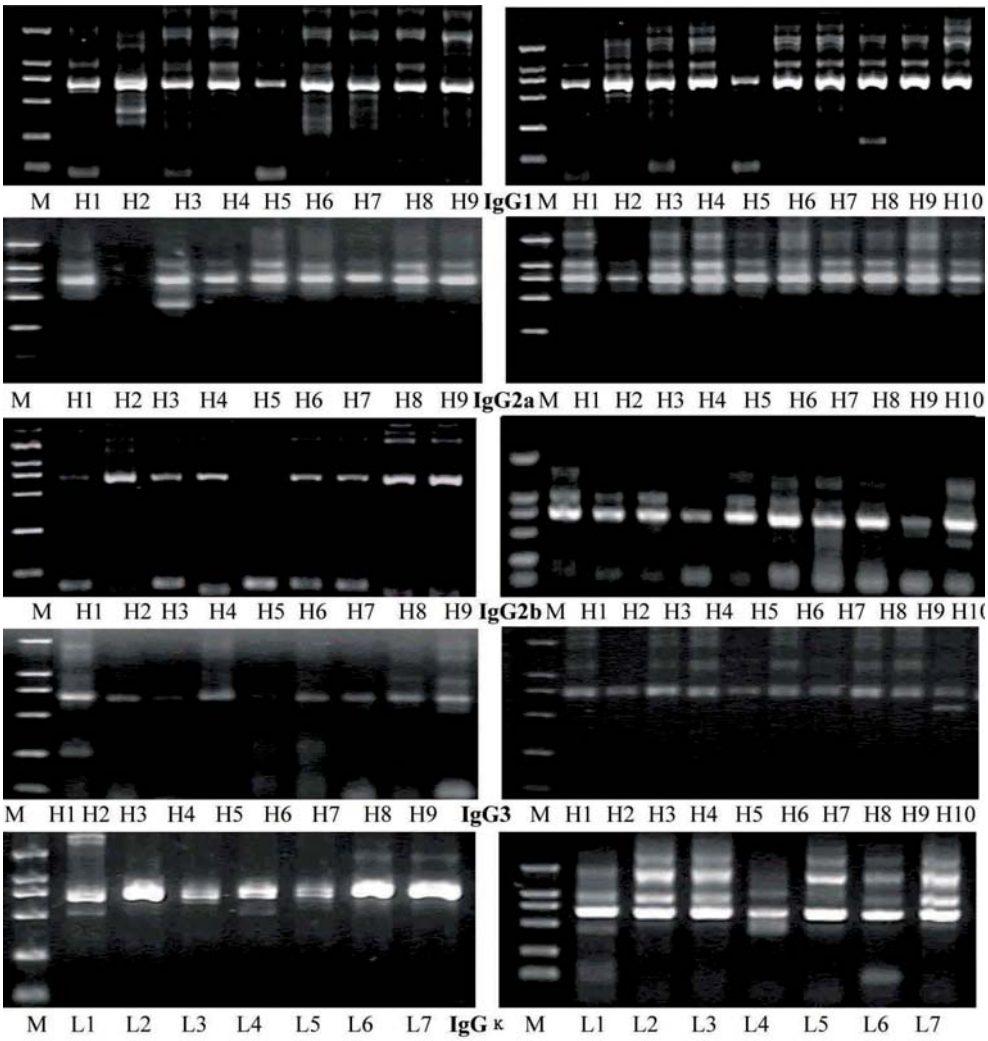

Figure 2. PCR amplification of Ig Fd and Lc gene from mice immunized by HAb18G/pcDNA3 followed hepatoma cells boost. An aliquot of cDNA derived from the spleens of BALB/c mice immunized by HAb18G/pcDNA3 followed mixed hepatoma cells boost were amplified by PCR using different Ig 5' primers. New designed ten Fd 5' primer sets and seven Lc 5' primer sets listed in Table 4 were used respectively in the right column. Nine Fd 5' primer sets and seven Lc 5' primer sets described by Kang A. S. (36) were used respectively in the left column. The same Fd 3' primer sets (specific for IgG1, IgG2a (Balb/ c), IgG2b and IgG3) and Lc 3' primer (Ig Kappa) were used respectively in both columns. The first lane of each panel (M) contained molecular weight markers (prominent bands 2000, 1000, 750, 500, 250 and 100 bp). The number (X) below each lane corresponds to 5' primer used (HX or LX). Conditions for PCR were as described in Methods.

-seminoprotein (clone R-41) antibody were VH2 and Vk6, respectively. In contrast, VH5 and Vk1 germline families were used in anti-DOTA-Y antibody (clone D-73).

4.5. Soluble Fab expression and characterization

Soluble Fab fragments of the three anti-HAb18GEF antibody clones, one anti-γ-seminoprotein antibody clone, and one anti-DOTA-Y antibody clone were successfully expressed in E.Coli Rosetta-gami B with visualized bands of 45 Ku (assembled Fab) and 25-Ku (free light chain or heavy chain Fd fragment) (Figure. 6). Scanning analysis by Smartview software indicated that the quantities of expressed Fab were about 34.3 % (H-87), 20.5 % (H-15), 29.6 % (H-79), 30.1 % (R-41), 32.8 % (D-73) of concentrated bacterial total soluble protein, respectively. Indirect ELISA with different antigens further showed that these soluble Fabs had highly reactivity and specificity to corresponding antigen (Figure. 7 A-E). Inhibition ELISA curves demonstrated that these five soluble Fab antibodies had high affinities to corresponding antigen. The value of apparent binding constants (Ka) for clone H-87, H-79, H-15, R-41 and D-73 were 2.25×10^{9} M^{-1}, 1.21×10^{9} M^{-1}, 7.89×10^{8} M^{-1}, 5.50×10^{8} M^{-1} and 2.95×10^{8} M^{-1}, respectively (Figure. 8). When the HAb18GEF antigen was first reacted with the H-79 Fab, the reaction of the H-15 Fab with antigen was markedly reduced in competitive inhibitory ELISA. The inhibitory effect was concentration dependent. On the other hand, the H-87 Fab did not interfere with the H-79 or H-15 or HAb18 Fab antibody-antigen reaction, and the H-79 Fab did not interfere with HAb18 Fab reaction

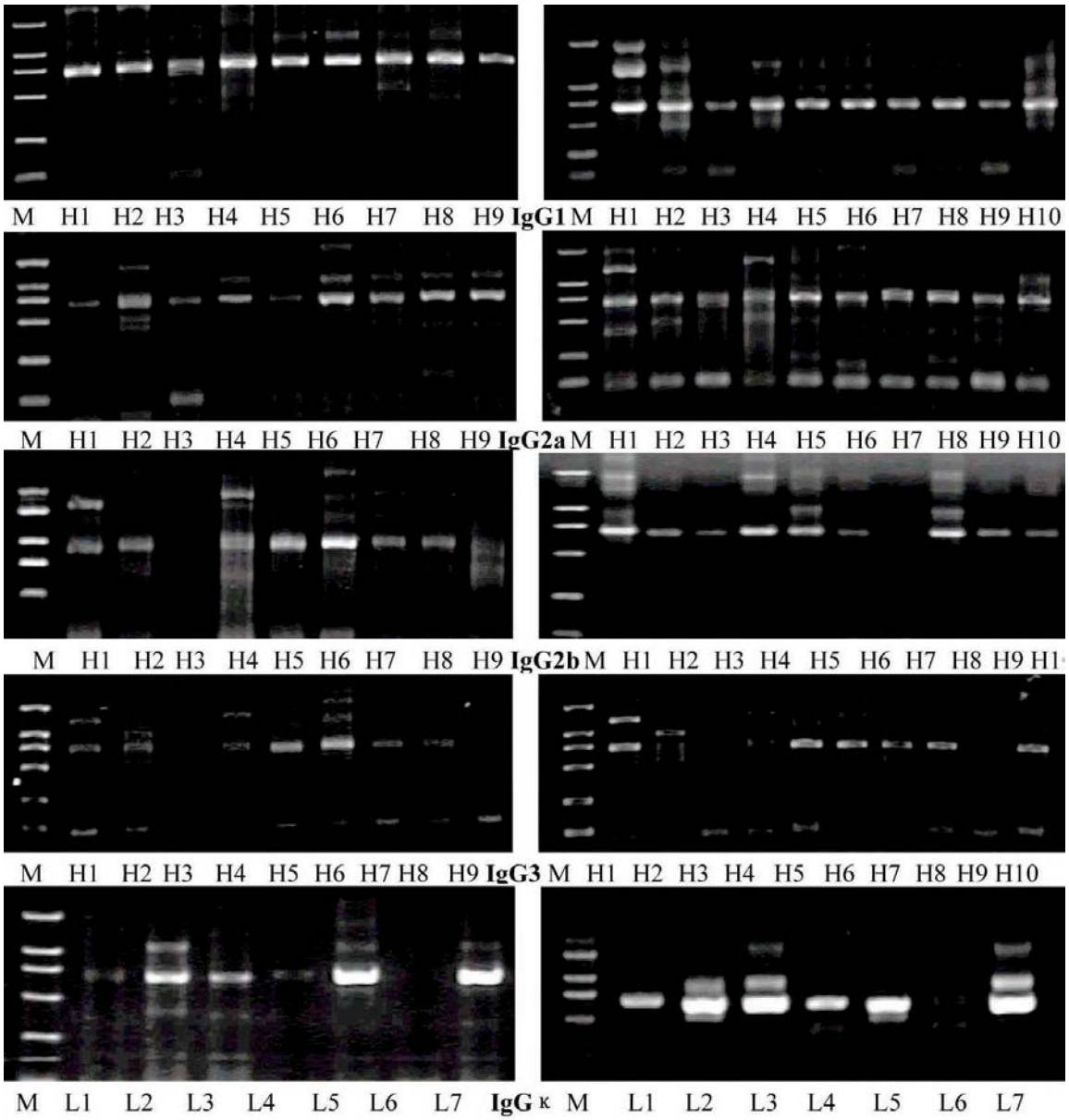

Figure 3. PCR amplification of Ig Fd and Lc gene from mice immunized by KLH-DOTA-Y conjugate. An aliquot of cDNA derived from the spleens of BALB/c mice immunized by KLH-DOTA-Y hapten conjugate were amplified by PCR using different Ig 5' primers. New designed ten Fd 5' primer sets and seven Lc 5' primer sets listed in Table 4 were used respectively in the right column. Nine Fd 5' primer sets and seven Lc 5' primer sets described by Kang A. S. (36) were used respectively in the left column. The same Fd 3' primer sets (specific for IgG1, IgG2a (Balb/ c), IgG2b and IgG3) and Lc 3' primer (Ig Kappa) were used respectively in both columns. The first lane of each panel (M) contained molecular weight markers (prominent bands 2000, 1000, 750, 500, 250 and 100 bp). The number (X) below each lane corresponds to 5' primer used (HX or LX). Conditions for PCR were as described in Methods.

(Figure. 7 F). These suggested that the H-79 and H-15 Fab antibody recognize the same epitope on HAb18GEF antigen that differed with H-87 or HAb18 Fab antibody recognition. Strongly positive Immunofluorescence staining reactions of anti-HAb18GEF Fabs (H-87, H-79 and H-15) were indicated on binding to human hepatoma cell FHCC-98 and high-metastasis hepatoma cell MHCC-97H. Whereas, normal human liver cell QZG showed a weak reaction, and human prostate cancer cell LnCap and PC3 showed a negative reaction (Figure. 9).

5. DISCUSSION

Bispecific mAbs-based 2-step pretargeting approach first requires the production of mAbs with dual specificity: high affinity for the tumor-associated antigen

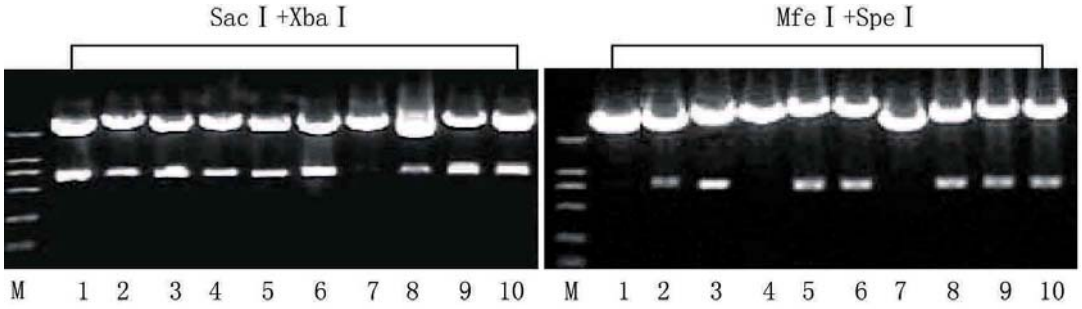

Figure 4. Restriction enzyme digestion analysis of gene recombinant percentage of Fab libraries. Fd and lc gene recombinant percentage of 10 clones selected randomly from the constructed Fab libraries on SOB-GAT plate were determined respectively by double digestion reaction with Mfe I + Spe I and Sac I + Xba I.

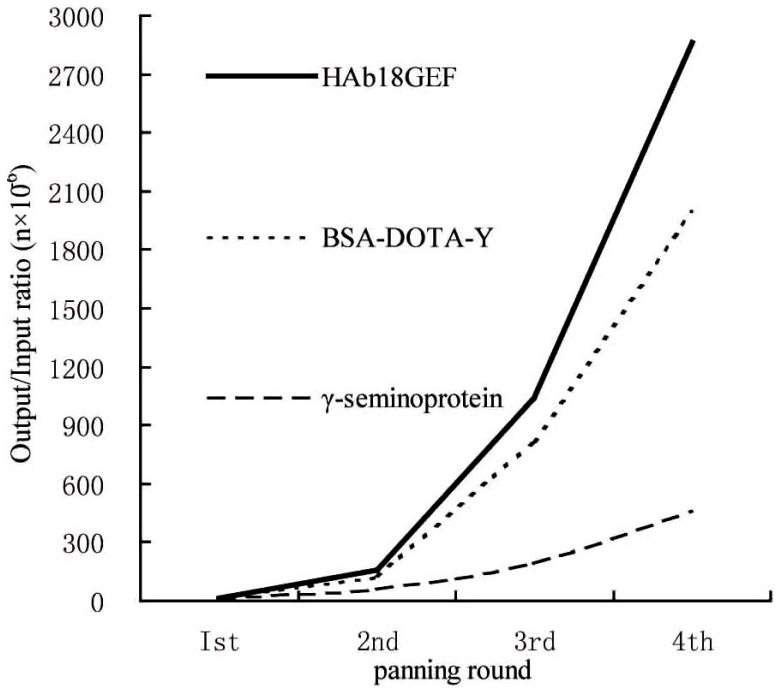

Figure 5. Selection of diverse phage-Fab libraries by panning on 96-well microtiter plates. Selection on various antigens was performed by panning on antigen-coated 96-well microtiter plates with (A) HAb18GEF, (B) BSA-DOTA-Y, (C) γ-seminoprotein; The ratio was calculated as follows: (output phage titer)/(input phage titer). The second, third and fourth panning input phage preparations were amplified from the last rounds of the output phages.

on the one hand and hight affinity for the radiolabeled hapten on the other (10). As reconstruction on HAb18 Fab for affinity enhancement is laborious and dicey, using phage antibody library technology to rapid isolate of new anti-HAb18G Fab anibodies with higher affinity is a feasible choice. Immune phage antibody library is usually constructed to enrich Ag-specific Abs, some of which will have been affinity-matured by the immune system. This method not only permits to yield Abs with higher affinity than obtained from hybridoma technology but also select out rare but potent Abs against different antigens, if diverse IgV gene repertoires could be amplified and cloned (14). In immunoglobulin repertoire library cloning, the homology between a particular primer sequence and that of its target template, and the diversity capacity of a primer pool are the two most important parameters that determine the cloning efficiency and the diversity of a resulting repertoire library. In this report we present an optimized set of Fab primers, compatible with Fd 5' clone site replaced pComb3 vector, for diverse Fab phage display library construction.

Fab primers are generally designed with 5' primers directed to conserved region of FR1 or signal sequences (15, 16, 18, 20-22, 38, 39). Initially, we considered the signal sequences as the targets for Fab 5' primer designing so the complete sequence of the IgV would be determined. However, we abandoned this approach for two reasons. First, mouse signal sequences,

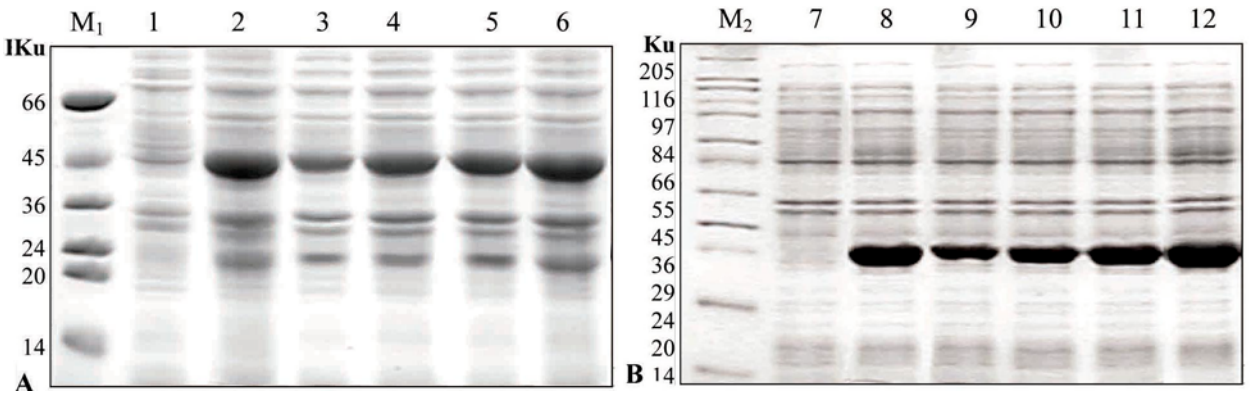

Figure 6. SDS-PAGE analysis of the expressed products of the anti-HAb18GEF, anti-γ-seminoprotein and anti-DOTA-Y antibody clones. Equal amounts (twenty micrograms) of concentrated cell lysate was separated by non-reducing (A) and reducing (B) 12% SDS–PAGE respectively. 1, 7: Extract of uninduced pComb3Mf/ Rosetta-gami B; 2, 8: Extract of induced H-87 clone; 3, 9: Extract of induced H-15 clone; 4, 10: Extract of induced H-79 clone; 5, 11: Extract of induced R-41 clone; 6, 12: Extract of induced D-73 clone;

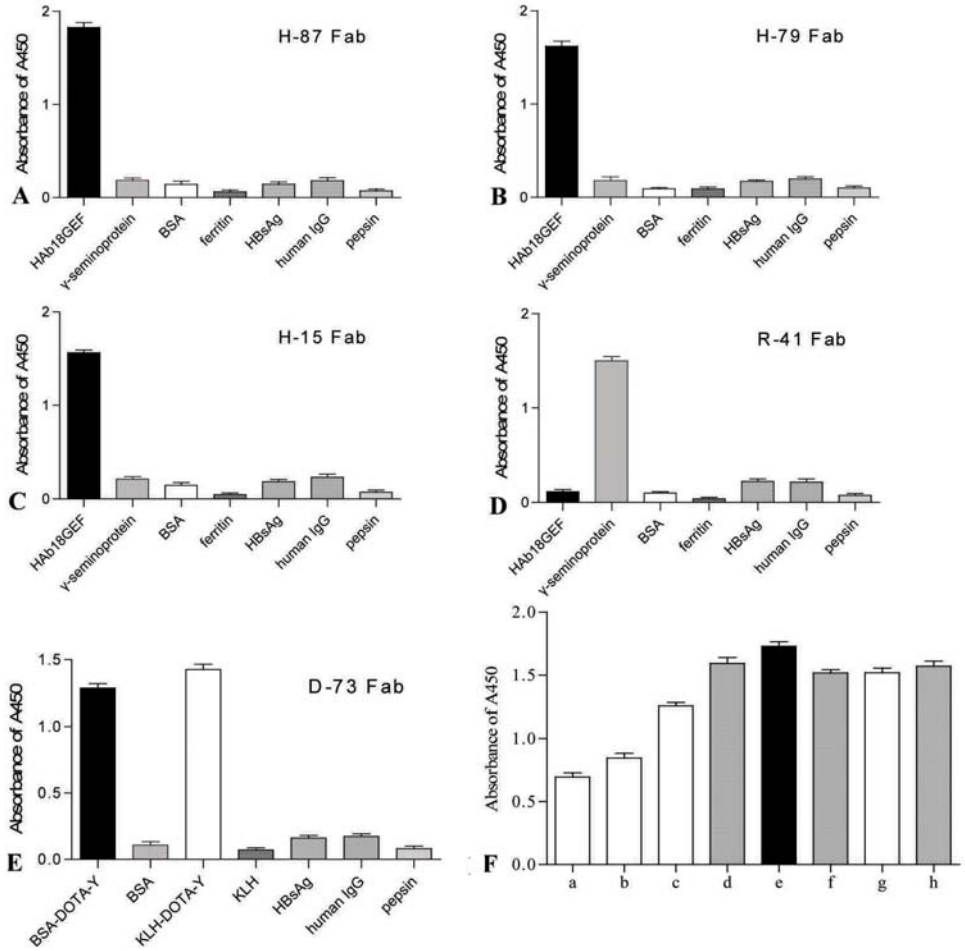

Figure 7. Specificity of anti-HAb18GEF soluble Fab (H-87, H-79 and H-15), anti-γ-seminoprotein soluble Fab (R-41) and anti-DOTA-Y Fab (D-73). Binding specificity was determined by indirect ELISA (A, B, C, D, E) to a variety of proteins including HAb18GEF, γ-seminoprotein, BSA-DOTA-Y, KLH-DOTA-Y, BSA, KLH, Ferritin, HBsAg, Pepsin and huma IgG; Epitope specificity of anti-HAb18GEF Fabs was determined by competing inhibitory ELISA (F). H-79 Fab at different concentrations (a 1/1, b 1/10, c 1/100) competed with H-15 Fab for binding with HAb18GEF, whereas H-87 Fab (d) did not change the original reaction (e). In addition, either H-87 Fab (f) or H-79 Fab (g) did not compete with HAb18 Fab (h) for binding with HAb18GEF.

Table 7. Amino acids sequence of anti-HAb18GEF, anti-DOTA-Y and anti-γ-seminoprotein Fab antibodies by IMGT/V-QUEST

Clone	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
	VL						
H-15	ELVMTQSPKSDSM SVSERVTLSCKLS	EIVGTY	VSWRQQKP EQSPKLLI	YGAS	NRYTGVPDRFTG SKSATDFTLTISQV QAEDLADYAC	GQMYSYPFT	FGSGTKLE IK
H-79	ELVMTQSPKSMSM SVPERVTLSCKLS	ENVGRY	VSWYQWK PEQSPKELI	YGAS	NRYTGVPDRVTG SGSATDFTLTIASV QAEDLADYHC	GQSYSYDFT	FGSGTKLE IK
H-87	ELQMTQSPASTSAS VGELVTIQCGAS	ENIYGR	LNWYQRKW GKSPQGLI	YGAT	NLADGASSRFSGS GSGRQYSPKISSLH PDNVATYYC	QTVLSFPYT	FGGGTKLE IK
D-73	ELVMTQAALTTSP GEGVTLTCRKS	SGAVTLNNY	ANWVQEKP DTLFTGLI	GGTN	ERAPGVPARFTGS LIGDKDALTITGAQ TEDEAYYFC	ALWFSDHVV	FGGGTELT VLG
R-41	ELVMTQTPSPLLL SASDRVTITCRGS	QSVSRD	VAWYQQKP TQSPRLLK	YYAS	SRYTGVPDIFTGS GYGTDFTFTISTV QAEDIAVYFC	QQDYSWYT	FGAGTKLE IK
	VH						
H-15	QLEESGGGLVWP GGSHKLSCVAS	GFRFSNYW	VHWVRQSPE TGLEWVME	IRLKSINQAT	HYARSVKGRFTIS RDDSKSSVYLQM NELRTEDSGIYYC	TSYDVEY	WGQGTLIT VSAAK
H-79	QLEESGGGLVDP GGAWKLSCVAS	GFTFSNLW	MNWVRQSP YKGLEWVA	EIALKSNQYAT	HYAESVKNRFTIS RDDSKPSVYLQMT NLRTEDTGIYYC	TSYDSEY	WGQGTLVT VSI
H-87	QLLESGGGLVSPG GSMKLLCVAS	GFTFSDAW	MDWVRQSP EKGLEWVA	EIRAKMNNHAP	YYSESVKGRFTISR DDSKSIEYLQMN NTLRHTGIYLLC	QGYSGYPL	GPRDSVHC LC
D-73	QLQQSGSGSGLVK PGGSHTLSCADS	GFTFTNHS	MSWVRQPP EKTLEWVAT	ISGGYTYT	YFPNSFQGRFTISSD VAKNTLYLQMSSL RAEDTSMYFC	TRHGLYRYAFGY	WGQGTLVT VSCA
R-41	QLKESGPGLVAP SQNVKISCTVS	GFSLTSYG	VHWVRQPP GHGLEWIGV	IWTGRDT	TYNSALMSRLSIS KDNSSSQVFLKM NSLQTDDSAIVYC	GRGGLITSFVFDY	WGQGTTVT VSS

when examined by the approach in Table 1, are less conserved than the FRI regions. Second, when building an antibody gene library for phage display, mouse signal sequences will be unnecessarily incorporated into the gene and may interfere with antibody function. Since murine IgV genes had been classified into 15 VH and 18 Vκ gene families, some family-specific primers have been devised for the amplification of diverse Fab libraries (24, 43, 44). However, it was observed that although 11.6% of mouse Ig VH in the Kabat database had proline at position 7 (Table 1), none of the fifteen known mouse Ig VH gene families coded for proline at this position. This observation pointed out a danger on designing primers solely on previous classified Ig families. So, we chose to define our own "families" based on N-terminal amino acid sequence homology of IgV FR1 in the updated Kabat database (26), and then new classification of 23 Hc families and 25 Lc families emerged. These newly classified families could cover almost all known mouse Ig genes in the updated Kabat database (Table 2). Despite remaining 5.3% of Ig VH and 5.7% of Ig VL in the updated Kabat database not being covered, these were rare sequences of "common" amino acid combination or contained at least one uncommon amino acid at positions 1 – 8. Thus, our primers designed to amplify these newly classified families would have a reasonable chance of amplifying these "uncommon" sequences as well.

Since most Fab primers have cloning sites within the IgV or own high degeneracy, a certain number of N-terminal gene sequences may be deleted by restriction enzyme or be altered by high degeneracy (39, 45). Those undesired N-terminal changes could significantly affect antigen binding activity in some cases, because crystal structures show that the N-terminal of VH is near the CDR3 and amino acid at position two of VL is part of the predicted canonical structure of CDR1 (46-49). In addition, considering different restriction enzymes have different internal digestion frequency in IgV (such as all BALB/c mice have higher frequency internal XhoI site in IgG2a heavy chains (25)), we feel that designing of limited degenerate primers with new cloning site to move the primer annealing position as close as possible to the 5' end is the best approach to amplify more diverse Ig gene repertoire. Virtually, the best cloning sites we chose for Hc and Lc was Mfe I and Sac I, respectively. These two 5' clone site also own lower internal digestion frequency (Table 3). Compared with other Fab primers, our primers own highest match percentage for mouse Ig genes and own limited degeneracy (Table 5). Satisfied amplification effect was observed in the two sets of PCR amplification reactions, independent of the IgG subtype and immunogen kinds (Figure. 1). This not only demonstrated the high efficiency of our new primers but also confirmed it's universal applicability for mouse Ig gene clonging. From the constructed Fab library, we have detected at least 6 VH genes of 15 IGHV families and at least 7 Vk genes of 19 IGKV families identified by Lefranc M.P (50). Those amplified Ig sequences covered the majority of the mouse Ig families and presented a good diverse family distribution (Table 6). Ig families that we have not encountered may not be involved in the immune response to the immunizing

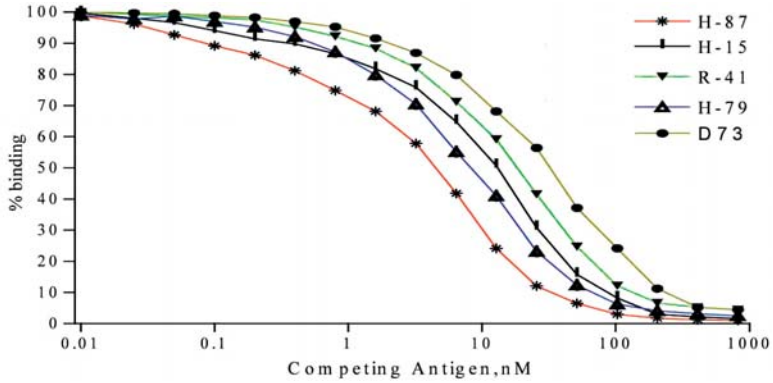

Figure 8. Binding characteristics of expressed Fab antibodies as measured by inhibition ELISA assays. The cell lysate of expressed clones H-15, H-79, H-87 and R-41 were collected respectively, and the apparent binding constants were tested by inhibition ELISAs as described in Materials and Methods. The binding percentage was determined relative to the reactivity of the same number of soluble Fabs with immobilized HAb18GEF (BSA-DOTA-Y orγ-seminoprotein) but without competing antigen. The inhibition curves and apparent affinities were calculated by program GraphPad Prism 4..

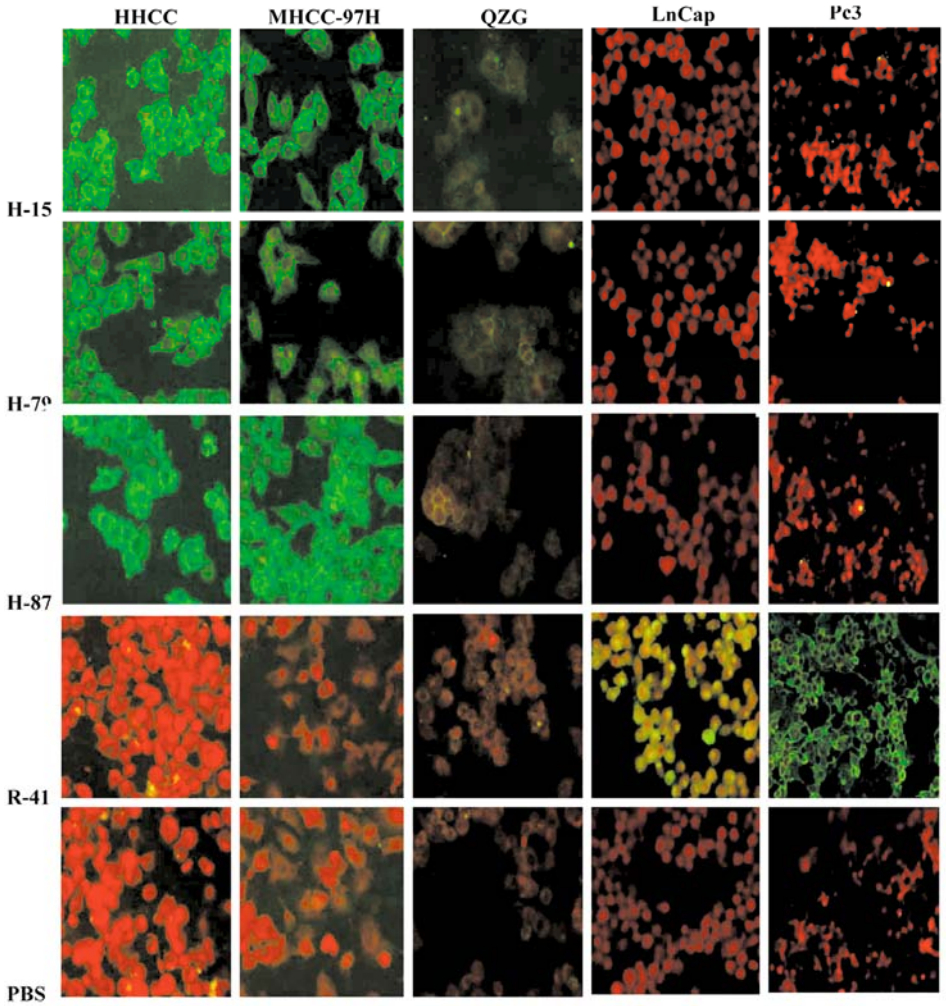

Figure 9. Immunofluorescent staining of different cells with expressed Fab antibodies. Soluble anti-HAb18GEF Fabs H-15 (first row), H79 (second row), H87 (third row) and anti-γ-seminoprotein Fab R-41 (fourth row) were used separately as the primary antibody (1:200 dilution, PBS as the control) to bind HHCC cells (first column), MHCC-97H cells (second column), QZG cells (third column), LnCap cells (fourth column) and PC3 cells (fifth column) respectively. FITC-labeled goat anti-mouse IgG (H+L) (1:1000 dilution) was used as the secondary antibody to light up the stained cells (×400).

antigen, or it may simply be that we have not sampled enough clones to observe them.

To test the diversity of our Fab antibody library, HAb18GEF antigen, BSA-DOTA-Y hapten conjugate and an irrelevant antigen γ-seminoprotein were used for biopanning simultaneously. Five specific Fab antibodies with high affinity had successfully been selected in a cycle of panning, and some specific clones containing incomplete antibody gene insertion were also panned out (this possibly owing to high stringency of selective pressure during multiple panning rounds). These not only demonstrated the high quality of our Fab library but also confirmed it's gene diversity avilable for other antigen panning. Moreover, the selected anti-HAb18GEF Fabs (H-87, 2.25×10^{9} M^{-1}; H-79, 1.21×10^{9} M^{-1}) not only own higher affinity than HAb18 Fab (2.95×10^{8} M^{-1}) but also recognize different epitopes on HAb18GEF (Figure. 7, 8). Since the MMPs secretion level of stromal cells was only determined by the local motif of extracellular fragement of HAb18G/CD147 (4, 51, 52), these different epotopes recognized anti-HAb18GEF Fabs might present better anti-metastasis characteristic by more efficient blocking effect in the future. Moreover, it should be noted that selected anti-HAb18GEF Fabs could efficiently bind to Human hepatocellular carcinoma cell FHCC-98 and high metastasis hepatocellular carcinoma cell MHCC-97H in Immunofluorescence staining.

Taken together, the anti-HAb18GEF and anti-DOTA-Y Fab antibodies we obtained in the present study could be used to compose of new-type pretargeting durg against human hepatoma. Along with chemeric reconstruction in the next step, these high affinity Fab antibodies will play important role on specifically enhancing the radiation dose compared with directly labeled HAb18 mAbs. Moreover, the newly designed primers are feasible to construct diverse Fab antibody libraries, and may also supply an alternative system on studying V gene selection in B-cell development for any response antigen.

6. ACKNOWLEDGEMENT

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7. REFERENCES

1 Chen ZN, Mi L, Xing JL: Gene cloning of CD147/HAb18G and study of its function in human hepatoma cells. *J Tumor Marker Oncol* 16(4): 225-228 (2001)

2 Chen ZN: Significance and application of anti-malignant hepatoma MAb HAb18 in radioimmunal diagnosis of human hepatocellular carcinoma. *Zhonghua Zhong Liu Za Zhi* 14(1): 9-12 (1992)

3 Chen,ZN, Yang,Z, Mi,L: Structure and function analysis of hepatoma associated factor HAb18G.. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi* 15(1):34 (1999)

4 Toole B.P.: Emmprin (CD147), A cell surface regulator of matrix metalloproteinase production and function. *Curr Top Dev Biol* 54:371-89 (2003)

5 Huang Y, Jiang J, Dou K, Chen Z: HAb18G/CD147 enhances the secretion of matrix metalloproteinases (MMP) via cGMP/NO-sensitive capacitative calcium entry (CCE) and accordingly attenuates adhesion ability of fibroblasts. *Eur J Cell Biol* 84(1):59-73 (2005)

6 Jiang JL, Chan HC, Zhou Q, Yu MK, Yao XY, Lam SY, Zhu H, Ho LS, Leung KM, Chen ZN: HAb18G/CD147-mediated calcium mobilization and hepatoma metastasis require both C-terminal and N-terminal domains. *Cell Mol Life Sci* 61(16):2083-91 (2004)

7 Jiang JL, Zhou Q, Yu MK, Ho LS, Chen ZN, Chan HC: The involvement of HAb18G/CD147 in regulation of store-operated calcium entry and metastasis of human hepatoma cells. *J Biol Chem* 276(50):46870-7 (2001)

8 Lou C, Chen ZN, Bian HJ, Li J, Zhou SB: Pharmacokinetics of radioimmunotherapeutic agent of direct labeling mAb 188Re-HAb18. *World J Gastroenterol* 8(1):69-73 (2002)

9 Qiu K, Wang BC, Chen ZN, Fang P, Liu CG, Wan WX, Liu YF: 99mTc-labeled HAb18 McAb Fab fragment for radioimmunoimaging in nude mice bearing human hepatocellular carcinoma. *World J Gastroenterol* 4(2):117-120 (1998)

10 Boerman O.C., van Schaijk F.G., Oyen W.J., Corstens F.H.: Pretargeted radioimmunotherapy of cancer: progress step by step. *J Nucl Med* 44(3):400-11 (2003)

11 Kretzschmar T., Von Ruden T.: Antibody discovery: phage display. *Curr Opin Biotechnol* 13: 598-602 (2002)

12 Hust M., Dubel S.: Mating antibody phage display with proteomics. *Trends Biotechnol* 22(1):8-14 (2004)

13 Bird R.E., Walker B.W.: Single chain antibody variable regions. *Trends Biotechnol* 9: 132-137 (1991)

14 O'Brien, Philippa M. and Aitken, Robert: Antibody Phage Display: Methods and Protocols, *Methods in Molecular Biology*, 178: Humana Press, DOI: 10.1226/0896037118 (2001)

15 LeBoeuf,R.D., Galin,F.S., Hollinger,S.K., Peiper,S.C., Blalock, J.E.: Cloning and sequencing of immunoglobulin variable-region genes using degenerate oligodeoxyribonucleotides and polymerase chain reaction. *Gene* 82: 371-377 (1989)

16 Chaudhary,V.K., Batra,J.K., Gallo,M.G., Willingham,M.C., FitzGerald,D.J., Pastan,I.: A rapid method of cloning functional variable region antibody genes in *Escherichia coli* as single chain immunotoxins. *Proc Natl Acad Sci USA* 87: 1066-1070 (1990)

17 Dattamajumbar,A.K., Jacobson,D.P., Hood,L.E., Osman,G.E.: Rapid cloning of any rearranged mouse immunoglobulin variable genes. *Immunogenetics* 43: 141-151 (1996)

18 Jones ST, Bendig MM.: Rapid PCR-cloning of full-length mouse immunoglobulin variable regions. *Biotechnology (N Y)* 9: 579 (1991)

19 Ørum,H., Anderson,P.S., Øster,A., Johansen,L.K., Riise,E., Bjornvad,M., Svendsen I, Engberg J.: Efficient method for constructing comprehensive murine Fab antibody libraries displayed on phage. *Nucleic Acids Res* 21: 4491-4498 (1993)

20 Gavilondo-Cowley J.V., Coloma M.J., Vazquez J., Ayala M., Macias A., Fry K.E., Larrick J.W.: Specific amplification of rearranged immunoglobulin variable region genes from mouse hybridoma cells. *Hybridoma* 9: 407-417 (1990)
21 Coloma M.J., Larrick J.W., Ayala M., Gavilondo-Cowley J.V.: Primer design for the cloning of immunoglobulin heavy-chain leader-variable regions from mouse hybridoma cells using the PCR. *Biotechniques* 11: 152-154 (1991)
22 Coloma M.J., Hastings A., Wims L.A., Morrison S.L.: Novel vectors for the expression of antibody molecules using variable regions generated by polymerase chain reaction. *J Immunol Methods* 152: 89-104 (1992)
23 Nicholls, P.J., Johnson, V.G., Blanford, M.D., Andrew, S.M.: An improved method for generating single-chain antibodies from hybridomas. *J Immunol Methods* 165: 81-91 (1993)
24 Seijen A.M., Seijen H.G., Bos N.A.: Systematic design of mouse Vh gene family-specific oligonucleotides. *J Immunol Methods* 254: 161-168 (2001)
25 Ostermeier M., Benkovic S.J.: A two-phagemid system for the creation of non-phage displayed antibody libraries approaching one trillion members. *J Immunol Methods* 237: 175-186 (2000)
26 Johnson G., Wu T.T.: Kabat database and its applications: 30 years after the first variability plot. *Nucleic Acids Res* 28: 214-218 (2000)
27 Lou CY, Feng YM, Qian AR, Li Y, Tang H, Shang P, Chen ZN: Establishment and characterization of human hepatocellular carcinoma cell line FHCC-98, *World J Gastroenterol* 10: 1462-5 (2004)
28 Zhang SH, Xing JL, Yao XY, Chen ZN: Non-fused expression of HAb18GEF by reducing stability of translational initiation region in mRNA. *Sheng Wu Gong Cheng Xue Bao* 20: 175-80 (2004)
29 Zhang SH, Xing JL, Yang XM, Song F, Chen ZN: Generation of high titer anti-sera against the native form of hepatoma associated antigen HAb18G/CD147 by the DNA-cell boost method. *J Tumor Markers Oncol* 18: 246-248 (2003)
30 Zhang SH, Yang XM, Xing JL, Chen ZN: Comparison of polyclonal anti-sera against extracellular domain of hepatoma associated antigen HAb18G/CD147 prepared by different immunization schemes. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi* 21: 65-8 (2005)
31 Song F, Xing JL, Zhang SH, Yang XM, Chen ZN: Construction and characterization of anti-DOTA-Y phage antibody library. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi* 19: 476-9 (2003)
32 Hao XK, Reng WT, Liang GD: Production of anti-sera againstγ-seminoprotein purified from prostate cancer patients. *Zhong Hua Jian Yan Yi Xue Za Zhi* 12: 212-214 (1989)
33 Sambrook J., Russell D.W.: *Molecular cloning: a laboratory manual* 3rd edn. Cold Spring Harbor Press, Cold Spring Harbor, NY (2001)
34 Martin, A.C.R.: Accessing the Kabat antibody sequence database by computer. *Proteins* 25: 130-133 (1996)
35 Johnson G., Wu T.T., Kabat E.A.: SEQHUNT: a program to screen aligned nucleotide and amino acid sequences. *Methods Mol Biol* 51: 1-15 (1995)
36 Kang, A.S., Burton, D.R., Lerner, R.A.: Combinatorial immunoglobulin libraries in phage lambda. *Methods: Companion Methods Enzymo*l 2: 111-118 (1991)
37 Yamanaka H.I., Kirii Y., Ohmoto H.: An improved phage display antibody cloning system using newly designed PCR primers optimized for Pfu DNA polymerase. *J Biochem (Tokyo)* 117: 1218-1227 (1995)
38 Kettleborough C.A., Saldanha J., Ansell K.H., Bendig M.M.: Optimization of primers for cloning libraries of mouse immunoglobulin genes using the polymerase chain reaction. *Eur J Immunol* 23, 206-211 (1993)
39 Orlandi R., Gussow D.H., Jones P.T., Winter G.: Cloning immunoglobulin variable domains for expression by the polymerase chain reaction. *Proc Natl Acad Sci USA* 86: 3833-3837 (1989)
40 Wang Z., Raifu M., Howard M., Smith L., Hansen D., Goldsby R., Ratner D.: Universal PCR amplification of mouse immunoglobulin gene variable regions: the design of degenerate primers and an assessment of the effect of DNA polymerase 3' to 5' exonuclease activity. *J Immunol Methods* 233: 167-177 (2000)
41 Barbas C.F., Lerner R.A.: Combinatorial immunoglobulin libraries on the surface of phage (Phabs): rapid selection of antigen-specific Fabs. *Methods: Companion Methods Enzymol* 2: 119-124 (1991)
42 Rath S., Stanley C.M., Steward M.W.: An inhibition enzyme immunoassay for estimating relative antibody affinity and affinity heterogeneity. *J Immunol Methods* 106: 245-249 (1988)
43 Zhou H., Fisher R.J., Papas T.S.: Optimization of primer sequences for mouse scFv repertoire display library construction. *Nucleic Acids Res* 22: 888-889 (1994)
44 Chardes T., Villard S., Ferrieres G., Piechaczyk M., Cerutti M., Devauchelle G., Pau B.: Efficient amplification and direct sequencing of mouse variable regions from any immunoglobulin gene family. *FEBS Lett* 452: 386-394 (1999)
45 Sastry L., Alting-Mees M., Huse W.D., Short J.M., Sorge J.S., Hay B.N., Janda K.D., Benkovic S.J., Lerner R.A.: Cloning of the immunological repertoire in *Escherichia coli* for generation of monoclonal catalytic antibodies: construction of a heavy chain variable region-specific cDNA library. *Proc Natl Acad Sci USA* 86: 5728-5732 (1989)
46 Chothia C., Lesk A.M., Tramontano A., Levitt M., Smith-Gill S.J., Air G., Sheriff S, Padlan E.A., Davies D., Tulip W.R.: Conformations of immunoglobulin hypervariable regions. *Nature* 342: 877-883 (1989)
47 Bernstein F.C., Koetzle T.F., Williams G.J, Meyer E.J., Brice M.D., Rodgers J.R., Kennard O., Shimanouchi T., Tasumi M.: The Protein Data Bank: a computer-based archival file for macromolecular structures. *J Mol Biol* 112: 535-542 (1977)
48 Panka D.J., Mudgett-Hunter M., Parks D.R., Peterson L.L., Herzenberg L.A., Haber E., Margolies M.N.: Variable region framework differences result in decreased or increased affinity of variant anti-digoxin antibodies. *Proc Natl Acad Sci USA* 85: 3080-3084 (1988)
49 Chien N.C., Roberts V.A., Giusti A.M., Scharff M.D., Getzoff E.D.: Significant structural and functional change of an antigen-binding site by a distant amino acid substitution: proposal of a structural mechanism. *Proc Natl Acad Sci USA* 86: 5532-5536 (1989)

50 Lefranc M.P: IMGT-ONTOLOGY and IMGT databases, tools and Web resources for immunogenetics and immunoinformatics. *Mol Immunol* 40: 647-660 (2004)
51 Guo H, Zucker S, Gordon MK, Toole BP, Biswas C: Stimulation of matrix metalloproteinase production by recombinant extracellular matrix metalloproteinase inducer from transfected Chinese hamster ovary cells. *J Biol Chem* 272(1): 24-7 (1997)
52 Biswas C, Zhang Y, DeCastro R, Guo H, Nakamura T, Kataoka H, Nabeshima K: The human tumor cell-derived collagenase stimulatory factor (renamed EMMPRIN) is a member of the immunoglobulin superfamily. *Cancer Res* 55(2): 434-9 (1995)

Abbreviations: bp, basepairs; CFU, colony forming units; PFU, plaque forming unit; Fab, fragment antigen binding; PCR, polymerase chain reaction; VH, heavy chain variable domain; Vκ, Kapa light chain variable domain; CDR, complementarity determining region; FR, framework region; IgV, immunoglobulin variable region; Hc, heavy chain; Lc, light chain; HAb18GEF, hepatoma associated antigen HAb18G extracellular fragement; DOTA-Y, dodecane-tertraacetic acid-yttrium. BSA, Bovine serum albumin; KLH, keyhole limpet haemocyanin; γ-sm, gamma-seminoprotein; PEG, polyethylene glycol; ELISA, enzyme-linked immunosorbent assay; IPTG, isopropyl b-dthiogalactopyranoside

Key Words: Phage antibody library, Primer, Fab, Diversity, Radioimmunotherapy

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