PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Preparation of single chain variable fragment of MG_7 mAb by phage display technology

Zhao-Cai Yu, Jie Ding, Yong-Zhan Nie, Dai-Ming Fan and Xue-Yong Zhang

Department of Gastroenterology, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, Shaanxi Province, China
Correspondence to Prof. Xue-Yong Zhang, Department of Gastroenterology, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, Shaanxi Province, China. xyzhang@fmmu.edu.cn
Tel: 0086-29-3374576
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Abstract

AIM **To develop the single chain variable fragment of MG_7 murine anti-human gastric cancer monoclonal antibody using the phage display technology for obtaining a tumor-targeting mediator.**

METHODS **mRNA was isolated from MG_7 producing murine hybridoma cell line and converted into cDNA. The variable fragments of heavy and light chain were amplified separately and assembled into ScFv with a specially constructed DNA linker by PCR. The ScFvs DNA was ligated into the phagmid vector pCANTAB5E and the ligated sample was transformed into competent *E. Coli* TG1. The transformed cells were infected with M13K07 helper phage to form MG_7 recombinant phage antibody library. The volume and recombinant rate of the library were evaluated by means of bacterial colony count and restriction analysis. After two rounds of panning with gastric cancer cell line KATOIII of highly expressing MG_7-binding antigen, the phage clones displaying ScFv of the antibody were selected by ELISA from the enriched phage clones. The antigen binding affinity of the positive clone was detected by competition ELISA. HB2151 *E.coli* was transfected with the positive phage clone demonstrated by competition ELISA for production of a soluble form of the MG_7 ScFv. ELISA assay was used to detect the antigen-binding affinity of the soluble MG_7 ScFv. Finally, the relative molecular mass of soluble MG_7 ScFv was measured by SDS-PAGE.**

RESULTS **The V-H, V-L and ScFv DNAs were about 340bp, 320bp and 750bp, respectively. The volume of the library was up to 2×10^6 and 8 of 11 random clones were recombinants. Two phage clones could strongly compete with the original MG_7 antibody for binding to the antigen expressed on KATOIII cells. Within 2 strong positive phage clones, the soluble MG_7 ScFv from one clone was found to have the binding activity with KATOIII cells. SDS-PAGE showed that the relative molecular weight of soluble MG_7 ScFv was 32.**

CONCLUSION **The MG_7 ScFv was successfully produced by phage antibody technology, which may be useful for broadening the scope of application of the antibody.**

Subject headings antibodies, neoplasms/ biosynthesis; antibodies, monoclonal; stomach neoplasms/ immunology; bacteriophages/genetics

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INTRODUCTION

In our previous studies, MG_7 hybridoma cell line had been successfully prepared by immunization of mouse with KATOIII gastric cancer cells and hybridization of the B cells from the spleen of the immunized mouse with the murine myeloma cell line SP 2/0. This hybridoma cell line generates a kind of monoclonal antibody against gastric cancer which can specifically recognize an ascertained gastric cancer associated antigen[1]. MG_7 antibody was confirmed to be of great value and good potency in the targeting gene therapy of gastric cancer due to the overexpression of its corresponding antigen in a large proportion of patients with gastric cancer. But owing to its murine origin, like many other similar antibodies, MG_7 antibody can elicit human anti-mouse immunoreaction and thus its use in clinical practice is restricted[2,3]. One of the efficient solutions to this problem is to remove the constant region of antibody which makes main contribution to the immunogenicity of the murine antibody to human being. It has been proved that antibody devoid of constant region still maintains its capacity of specific antigen-binding affinity[4-10]. Additionally, antibody without constant region, termed ScFv, is a small molecule and comprises 1/6 of its original antibody in molecular mass. Therefore, ScFv can more readily penetrate into the solid tumor *in vivo* and be easily cleared up from the normal tissue. In the early 90's, the emergence of recombinant phage library represented a great breakthrough in the antibody technology which provides an economical means to prepare the ScFv/Fab of any desired antibody[11-19]. In the present study, the MG_7 recombinant phage antibody derived from MG_7 hybridoma was constructed and screened to prepare the MG_7 ScFv which might help establish an efficient strategy of targeting gene therapy in gastric cancer.

MATERIALS AND METHODS

Detection of antigen-binding affinity of MG_7 antibody

MG_7 hybridoma cells and KATOIII cells were cultured with RPMI 1640 (purchased from Gibco) supplemented with heat-inactivated $100mL\cdot L^{-1}$ fetal bovine serum at 37℃ under $50mL\cdot L^{-1}$ CO_2. MG_7 hybridoma cells were harvested at log phase and stored at -70℃ with aliquot of 10^6 for RNA isolation. Supernatant was collected for detection of antigen-binding affinity of MG_7 antibody by ELISA. KATOIII cells in log phase were transferred into a 96 wellplate and immobilized on the wall by centrifiguation at $1\,000\times g$ for 10 min, finally

fixed by 0.25mL·L^{-1} glutaraldehyde. Supernatant of 0.2mL was applied to each well and incubated at 4℃ overnight, and 0.1mL HRP-labeled goat anti-mouse (HRP-GAM) Ig was added into each well. The absorbance value (*A*) at 492nm of reactant in each well was measured after incubation for 1 hour at 37℃ and staining with OPD.

Construction of MG_7 recombinant phage antibody library

According to the protocol of svtotal RNA isolation system and polyAT tract mRNA isolation system (purchased from Promega), mRNA was isolated from MG_7 hybridoma cells and quantified by gel electrophoresis for following reverse transcription reaction. Subsequently, reverse transcription reaction was performed with 0.3μg mRNA, 2U reverse transcripase (purchased from Promega) mixed together for incubation of 1 hour under 37℃. PCR was conducted with a mixture of 10μg product of reverse transcription, 2U *Taq* DNA polymerase and 2μL V H/V_L primers mix (purchased from Promega) in a total volume of 50μL. The procedure of PCR was arranged in the following order: 95℃×5min; 94℃ ×1min, 55℃×2min, 72℃×2min and 30 cycles; 72℃×10min. After precise quantification of PCR product by gel electrophoresis, 50ng of V_H and V_L product was respectively mixed with 50ng linker primer and 1μL *Taq* DNA polymerase to perform PCR (94℃×1min, 63℃×4min, 7 cycles). Subsequently,50ng RS primers (purchased from Promega) underwent another PCR (94℃×1min, 55℃×2min, 72℃×2min and 30 cycles; 72℃×10min). Two μL *Sfi* I and 0.5μg ScFv product were added into a sterile 0.5mL microtube and incubated at 50℃ for 4 hours. After being purified by PCR purification kit, 0.5μg *Sfi* I digested ScFv product mixed with 2μL *Not* I was incubated at 37℃ for 4 hours and purified again for later use. ScFv (150ng) and pCANTAB5E (250ng) mixed with 2μL T_4 DNA ligase was incubated at 16℃ for 16 hours. Ligated product was transformed into TG1 cell. Transformed product with aliquot of 100μL was placed onto SOBAG plates and incubated overnight at 37℃ to form bacterial clones.

Evaluation of volume and recombinant rate of phage antibody library

Colony count was adopted to exhibit the total number of clones formed on the SOBAG plates. Eleven clones were randomly singled out from the SOBAG plates and passaged into 5mL 2×YT-AG medium for an incubation of 12 hours at 37℃. Plasmid from each clone was respectively isolated and digested by *Eco* RI and *Hin* dIII. Gel electrophoresis was conducted with restriction digested product to examine the recombinant phagmid.

Panning and enrichment of MG_7 recombinant phage antibody

The initial recombinant phage antibody library was incubated for 1 hour at 37℃ with shaking at 250r·min^{-1}, and helper phage M13KO7 was added and incubated for another hour at 37℃ with shaking at 250 r·min^{-1}[20]. The culturing product was spinned at 1000×*g* for 10 min to precipiate the cells. Then the entire sample was gently resuspended in 10 mL 2× YT-AK medium. After an overnight incubation at 37℃ with shaking at 250r·min^{-1}, the culturing product was spinned at 1 000×*g* for 20 min and the supernatant which contained the recombinant phages was collected. Then, 2mL PEG/NaCl was added and placed on ice for 45 min for precipitation of recombinant phage clones. It was spinned at 10000×*g* for 20 min at 4℃ and the pellet was resuspended in 16mL 2×YT medium diluted with 14 mL blocking buffer containing 0.1g·L^{-1} sodium azide and incubated at room temperature for 15 min. Twenty mL of the diluted recombinant phage was then added to the flask which was coated with KATOIII cells and well blocked. The flask was incubated for 2 hours at 37℃, washed 10 times with PBS plus another 10 times with PBS containing 1mL·L^{-1} Tween20. Ten mL log-phase TG1 cells were added to the flask and incubated with shaking at 37℃ for 1 hour for reinfection. After two rounds of panning, reinfected TG1 cells with bound phages directly in the panning flask were plated for colony isolation.

Screening for MG_7 recombinant phages

Recombinant phages were rescued from individual clones and screened for MG_7 binding by ELISA. Microtiter wells were coated with KATOIII cells. Bound phages were detected by incubation with a 1:5000 dilution of conjugate (Pharmacia Biotech). And the detection was achieved by addition of TMB substrate. Clones reacted to KATOIII cells were referred to as positives.

Competitive test of positive selected MG_7 recombinant phages

Microtiter wells were coated with KATOIII cells as mentioned above. The supernatant of the selected positive MG_7 recombinant phages was applied into each well (100mL·well^{-1}) and then incubated for 1 hour at 37℃. After disposing of the supernatant, MG_7 antibody (100mg·L^{-1}, 50μL·well^{-1}) was added and incubated for 1 hour at 37℃. PBST was used to wash 5 times, and HRP-GAM Ig (1:1000 diluted, 50μL·well^{-1}) was added and developed by TMB to measure the absorbance value at 450nm. The inhibiting ratio of selected positive MG_7 recombinant phages with MG_7 antibody for binding of KATOIII cells was calculated by the following formula: Inhibiting ratio = 1- (value of sample/value of control)×100%

Detection of antigen-binding affinity of the soluble MG_7 ScFv

The positive phages were transfected into *E.coli* HB2151 cells for the production of a soluble form of the MG_7 ScFv. Five mL culturing product of transfected *E.coli* HB2151 cells with overnight induction of 1mmol·L^{-1} isopropyl β-D-thiogalactopyanoside (IPTG) was centrifugated at 1000×*g* to collect the sediment and the supernatant (containing extracellular soluble ScFvs). The sediment was given osmotic shock to prepare periplasmic extracts. Microtiter wells were coated with KATOIII cells, and ELISA test was made twice to detect the antigen-binding affinity of soluble MG_7 ScFv.

Measurement of the relative molecular weight of soluble MG_7 ScFv

Periplasmic extracts from transfected *E.coli* HB2151 cells induced by IPTG was adopted to measure the relative molecular weight of soluble MG_7 ScFv by SDS-PAGE.

RESULTS

Antigen-binding affinity of MG_7 antibody

The ELISA showed that the A_{492} absorbance of reactant with presence of MG_7 antibody was up to 0.65 (0.208 in control).

Amplification of V_H, V_L and ScFv gene

On electrophoresis, V_H product formed a band at 350bp and V_L at 320bp, and ScFv was successfully spliced together to form a fragment of 750bp, as shown in Figure 1.

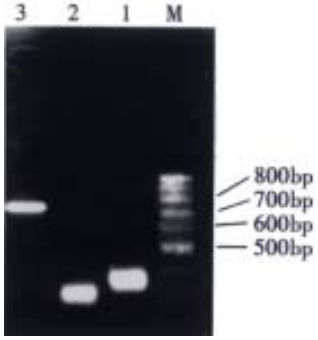

Figure 1 RT-PCR of V_H, V_L and ScFv fragment of MG_7 antibody. 1: V_H; 2: V_L; 3: ScFv; M: 100bp ladder

Volume of MG_7 phage antibody library

Colony counts showed that MG_7 phage antibody library consisted of 2×10^6 clones.

Recombinant rate of MG_7 recombinant phage antibody library

Eight of 11 random clones were found to release a 2.1 kb DNA fragment and confirmed to be recombinant phagmid by restriction analysis and gel electophoresis (Figure 2). The recombinant rate was 72.7%.

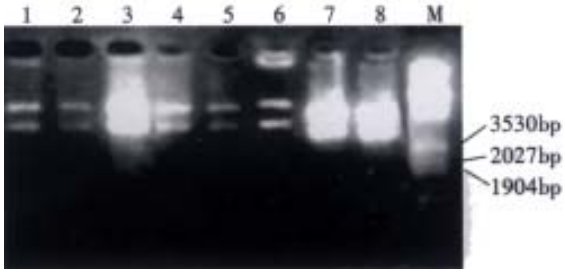

Figure 2 Enzymatic analysis of MG_7 recombinant phage antibody library with *Eco* RI and *Hin* dIII. 1-8: Recombinant clones from library; M: λ / *Eco* RI and *Hin* d III

Screening of MG_7 positive recombinant phages

Using ELISA assay, we yielded six strains of positive clones which had a good reaction with KATOIII cells (Table 1).

Table 1 ELISA results of screening from enriched phage displayed antibody library

Screening	Number of positive clones (A- value)						Neg. ctrl
	1	2	3	4	5	6	
First round	0.495	0.508	0.488	0.805	0.845	0.580	0.157
Second round	0.543	0.606	0.560	0.840	0.796	0.758	0.185

Results of competitive ELISA

Two strong positive clones were found to inhibit the binding of MG_7 antibody and KATOIII cells with the inhibiting ratio of 26.1% and 30%, respectively.

Antigen-binding affinity of soluble MG_7 ScFv

By means of ELISA assay, one of the strong positive clones exhibited the capacity of binding with KATOIII cells (Table 2).

Table 2 ELISA results of the soluble MG_7 ScFv for binding with KATOIII cells

ELISA	Number of strong positive clones (*A* value)		Neg. ctrl
	1	2	
First round	0.776	0.287	0.201
Second round	0.802	0.346	0.223

The relative molecular weight of soluble MG_7 ScFv

From Figure 3, an extra band on the lane of sample was visualized at M_r 32, as compared with the negative control. The relative molecular weight of soluble MG_7 ScFv was 31.

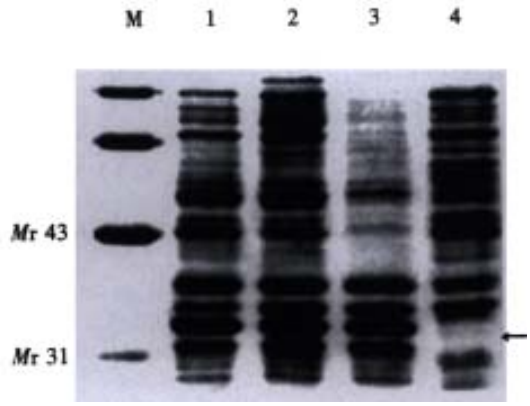

Figure 3 Measurement of the relative molecular weight of soluble MG_7 ScFv. 1-3: Periplasmic extracts; 4: Neg. ctrl; M: Low molecular mass protein marker

DISCUSSION

The phage antibody technique is one of the most remarkable achievements in antibody technology. With this technique, the repertoire of V_H and V_L genes are amplified and joined together by PCR and finally inserted into phagmid[20]. After transformation into *E.coli*, phage with the fusion of exogenous ScFv and pIII protein exposed on the surface was released from *E.coli* with the aid of the helper phage M13KO7. This technique uniforms the phenotype of ScFv to its genotype. By immunosorbance of the immobilized antigen, phage with functional ScFv can be bound and enriched. Subsequently, the desired phage harboring functional ScFv gene can be selected from the enriched phage antibody library by ELISA. The resultant ScFv can be solubly expressed in *E. coli* HB2151. The primary structural information of ScFv of antibody is accessible by DNA sequence of phagmid from the bound phage. Therefore, phage antibody has become an optimal measure to develop the ScFv of desired antibody[21-26].

It is well known that the immune system will be triggered and activated in response to the presence of certain antigens in patients with some kinds of diseases, such as tumor, infective diseases and autoimmune diseases[27-32]. The immune system will produce abundant B lymphocyte clones which can yield and secrete antibody directed against the disease associated antigens in these patients. Therefore, the B lymphocyte

population isolated from PBMC of these patients can be used as an ideal material source for construction of the recombinant phage antibody library[29-31]. Additionally, the B lymphocyte isolated from PBMC of immunized animals with given antigen is an alternative material source[20]. Besides the B lymphocyte population from patients or immunized animals, many kinds of antibody-producing hybridomas are also suitable as a kind of material source for construction of the recombinant phage antibody library[6,19]. Owing to the unraveling of biological functions over many antigen recognized by antibody from hybridomas, hybridomas are more favorable as material source for construction of recombinant phage antibody library.

In order to understand the quality of MG_7 hybridoma as a material source for construction of MG_7 recombinant phage antibody library, we detected the antigen-binding activity of MG_7 antibody in present study. ELISA assay showed that the *A* 492nm value of reactant with presence of MG_7 antibody was 0.65 which was over twice higher than that with absence of MG_7 antibody (0.208 only). It demonstrated that MG_7 hybridoma could secrete functional antibody and could be used as the source of mRNA to amplify the V_H and V_L genes of MG_7 antibody. Colony count and restriction analysis were conducted for evaluating the volume and quality of MG_7 recombinant antibody library. The large volume of MG_7 recombinant antibody library (2×10^6) and high recombinant rate (72.7%) confirmed that MG_7 phage antibody library comprised sufficient repertoire of recombinant clones for further research. ELISA assay and SDS-PAGE showed that the soluble MG_7 ScFv had antigen-binding activity and was M_r 31. Taken together, we have successfully constructed the MG_7 recombinant phage antibody library and prepared the phage-displayed/soluble MG_7 ScFv.

Gastric cancer is a highly prevalent neoplasm and is the first killer among various malignancies. In advanced cases, many current therapeutic approaches, including surgery combined with chemotherapy, appear to be palliative. These therapeutic approaches can not be targeted to and completely annihilate individual tumor cells, which leads to the failure of preventing metastasis and recurrence of many tumors. Besides, some kinds of therapeutic approaches, such as chemotherapy, can cause damage to both the tumor cells and normal tissue cells. Thus, introduction of a new way of targeting therapy for tumor is desperately needed to overcome these obstacles with the conventional approaches, such as surgery and chemotherapy[33-44]. Targeting therapy for tumors in the last decade has become a highlight in the field of tumor therapy. This therapy mediated by antibody still remains as a promising curative modality among the ways of tumor therapy and attracts worldwide attention[45-50].

Developing ScFv of the MG_7 is of great significance in both early diagnosis and treatment of gastric cancer. For instance, MG_7 ScFv fused with avidin can be used as a reagent in immuno-PCR for early diagnosis of gastric cancer. Additionally, a new immunotoxin with curative effect on gastric cancer can be developed by fusing the MG_7 ScFv and A subunit of ricin. MG_7 ScFv can direct the A subunit of ricin to MG_7 positive gastric cancer cells. Thus, the construction of MG_7 phage antibody library and subsequent preparation of MG_7 ScFv may be a step forward in seeking an efficient way for targeting therapy for gastric cancer.

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