Selection of anti-cancer-associated gene single-chain variable fragments derived from gastric cancer patients using ribosome display

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Abstract. The aim of this study was to construct a human single-chain variable fragment (scFv) gene library for gastric cancer, from which human anti-cancer-associated gene (CAGE) scFvs are selected. Human lymphocytes were isolated from the peripheral blood of 10 gastric cancer patients and whole human heavy and light chain genes were cloned by reverse transcription-polymerase chain reaction (RT-PCR). V_H and V_L were rearranged randomly by splicing by overlap extension PCR. The ribosome complexes were enriched against the recombinant CAGE protein conjugated to magnetic beads. scFv antibodies were evaluated by western blot analysis, and affinity constants in a solution of antigen-antibody complexes were determined by enzyme-linked immunosorbent assay. An scFv library was constructed using the peripheral blood lymphocytes. The expressed scFv proteins from the ternary ribosome complexes were analyzed by western blot analysis and the affinity [equilibrium dissociation constant (K_D)] of scFv for CAGE was determined to be $7.6x10^{-8}$ M. The ribosome display technique is efficient for selecting a fully human antibody fragment from a patient-derived gene pool.

Introduction

As a novel cancer/testis antigen, cancer-associated gene (CAGE) was originally isolated by serological analysis of a cDNA expression library (SEREX) with serum from a patient with gastric cancer (1). CAGE is known to be widely expressed among cancer cell lines and cancer tissues, yet restricted to testis tissue among normal tissues (2-4). Cancer/testis antigens have the notable property of being immunogenic in cancer patients and are considered promising targets for cancer vaccines due to their restricted expression pattern (5). Thus, anti-CAGE antibodies are applicable for payload delivery. Particularly in oncology, anti-CAGE antibodies may be combined with cytotoxic entities that have limited selectivity.

Traditionally, monoclonal antibodies (mAbs) are produced through rodent immunization using hybridoma technology. However, this approach is laborious and poses difficulties in generating antibodies against self-antigens. In recent years, *in vitro* display techniques, including phage and ribosome display, have become a platform technology for the design, selection and production of reagents for targeted therapies in cancer (6). Ribosome display has a number of advantages over phage display. A number of the limitations of phage display including the inability to select antibodies under conditions different from the cell environment, problems with the selection of proteins that are toxic, cells circumventing selection pressure and low transformation efficiency, may be circumvented using *in vitro* ribosome display (7). Ribosome display enables rapid and easy screening to isolate specific novel binders to target ligands. Over the past decade, ribosome display has been widely used for the selection of human antibodies for therapeutic intervention in various cancers (8-10). In the present study, we describe the construction of a single-chain variable fragment (scFv) antibody library and selection of a fully human antibody fragment from a gastric cancer patient-derived gene pool by *in vitro* ribosome display technology.

Materials and methods

Construction of the scFv library. Peripheral blood lymphocytes were isolated from 10 gastric cancer patients who initially did not receive chemotherapy. All patients provided a participant information statement and informed consent. This study was approved by the Human Ethics Committee of Nanchang University School of Medicine (Jiangxi, China). Total RNA was isolated individually from peripheral blood lymphocytes using an RNA purification kit for amplification of the scFv library. Primers were designed as previously described (11), with certain modifications for the construction of ribosome

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Table I. Oligodeoxynucleotide primers used for construction of the library.

A, Primers for generating V_H-linker fragments.	
Upstream primer sequence (5'-3')	
HuV_H1b-7a/reverse	GAG(A/G)TGCAGCTGGTGCA(A/G)TCTGG
HuV_H1c/reverse	(G/C)AGGTCCAGCTGGT(A/G)CAGTCTGG
HuV_H2b/reverse	CAG(A/G)TCACCTTGAAGGAGTCTGG
HuV_H3b/reverse	(G/C)AGGTGCAGCTGGTGGAGTCTGG
HuV_H3c/reverse	GAGGTGCAGCTGGTGGAG(A/T)C(C/T)GG
HuV_H4b/reverse	CAGGTGCAGCTACAGCAGTGGGG
HuV_H4c/reverse	CAG(G/C)TGCAGCTGCAGGAGTC(G/C)GG
HuV_H5b/reverse	GA(A/G)GTGCAGCTGGTGCAGTCTGG
HuV_H6a/reverse	CAGGTACAGCTGCAGCAGTCAGG
Downstream primer sequence (5'-3')	
HuIgM linker/for	GGAGACGAGGGGGAAAAGGGTTGG
B, Primers for generating Vκ light chain.	
Upstream primer sequence (5'-3')	
Hu Vκ1b/reverse	CTTTTCCCCCTCGTCTCCGACATCCAG(A/T)TGACCCAGTCTCC
Hu Vκ2/reverse	CTTTTCCCCCTCGTCTCCGATGTTGTGATGACTCAGTCTCC
Hu Vκ3b/reverse	CTTTTCCCCCTCGTCTCCGAAATTGTG(A/T)TGAC(A/G)CAGTCTCC
Hu Vκ4b/reverse	CTTTTCCCCCTCGTCTCCGATATTGTGATGACCCACACTCC
Hu Vκ5/reverse	CTTTTCCCCCTCGTCTCCGAAACGACACTCACGCAGTCTCC
Hu Vκ6/reverse	CTTTTCCCCCTCGTCTCCGAAATTGTGCTGACTCAGTCTCC
Downstream primer sequence (5'-3')	
HuCκ/for	*GC*TCTAGA ACACTCTCCCCTGTTGAAGCT
C, Primers for generating V_λ fragment.	
Upstream primer sequence (5'-3')	
Hu V_λ1a/reverse	CTTTTCCCCCTCGTCTCCCAGTCTGTGCTGACTCAGCCACC
Hu V_λ1b/reverse	CTTTTCCCCCTCGTCTCCCAGTCTGTG(C/T)TGACGCAGCCCGCC
Hu V_λ1c/reverse	CTTTTCCCCCTCGTCTCCCAGTCTGTCGTGACGCAGCCGCC
Hu V_λ2/reverse	CTTTTCCCCCTCGTCTCCCA(A/G)TCTGCCCTGACTCAGCCT
Hu V_λ3a/reverse	CTTTTCCCCCTCGTCTCCTCCTATG(A/T)GCTGACTCAGCCACC
Hu V_λ3b/reverse	CTTTTCCCCCTCGTCTCCTCTTCTGAGCTGACTCAGGACCC
Hu V_λ4/reverse	CTTTTCCCCCTCGTCTCCCACGTTATACTGACTCAACCGCC
Hu V_λ5/reverse	CTTTTCCCCCTCGTCTCCCAGGCTGTGCTGACTCAGCCGTC
Hu V_λ6/reverse	CTTTTCCCCCTCGTCTCCAATTTTATGCTGACTCAGCCCCA
Hu V_λ7-8/reverse	CTTTTCCCCCTCGTCTCCCAG(A/G)TCGTGGTGAC(C/T)CAGGAGCC
Hu V_λ9/reverse	CTTTTCCCCCTCGTCTCCC(A/T)GCCTGTGCTGACTCAGCC(A/C)CC
Downstream primer sequence (5'-3')	
HuJ_λ/forward	GCAAGATGGTGCAGCCACACCTA(A/G)(A/G)ACGGTGAGCTTGGTC
D, Primers for generating full-length construct[a].	
Upstream primer sequence (5'-3')	
T7Ab/reverse	GCAGC *TAATACGACTCACTATAGGAACAGA* **CCACC**ATG (C/G)AGGT(G/C)CA(G/C)*CTCGAG*(C/G)AGTCTGG
Downstream primer sequence (5'-3')	
HuCκ/forward	GC*TCTAGA*ACACTCTCCCCTGTTGAAGCT

Table I. Continued.

E, Primers for generating constant region (Cκ) of κ light chain[b].	
Upstream primer sequence (5'-3')	
HuCκ/reverse	ACTGTGGCTGCACCATCTG
Downstream primer sequence (5'-3')	
HuCκ/forward	GC*TCTAGA*ACACTCTCCCCTGTTGAAGCT

[a]T7 promoter sequence is italicized. The Kozak sequence is indicated in bold. Initiation codon ATG is underlined. Underlined italics indicate restriction sites for cloning. [b]Underlined italics indicate a restriction site for cloning.

display libraries (Table I). First-strand cDNA was synthesized and V genes were amplified using suitable primers. Agarose gel electrophoresis revealed bands of the expected sizes. Splicing by splicing overlap extension polymerase chain reaction (PCR) led to a full-length human scFv repertoire linking the heavy and light chains. scFv antibody libraries in the format of V_H/κ and V_H/V_λ-Cκ were constructed for ribosome display.

Antibody-ribosome-mRNA (ARM) ribosome display. The eukaryotic ribosome display was performed as described previously (12), with certain modifications. The PCR libraries were expressed in a coupled rabbit reticulocyte lysate system to generate ARM complexes. The reaction was performed in a volume of 50 µl containing 40 µl TNT T7 Quick for PCR DNA, up to 5 µg PCR DNA, 1 µl 1 mM methionine, 0.5 µl 100 mM magnesium acetate and 3.5 µl ddH_2O. The mixture was incubated in a siliconized tube at 30˚C for 60 min. Forty units RNase-free DNase I were added to the mixture together with 6 µl 10X DNase I digestion buffer and ddH_2O, yielding a final volume of 60 µl. The mixture was then incubated at 30˚C for an additional 15 min. Cold (4˚C) phosphate-buffered saline (PBS; 60 µl) was added prior to antigen selection. Antigen-linked Dynabeads (2 µg) were then added and the mixture was incubated at 4˚C for 2 h with gentle agitation to select a CAGE-specific antibody fragment. The beads were collected magnetically and then washed three times with cold washing buffer (PBS, 1% Tween-20, and 5 mM magnesium acetate), followed by washing twice with cold ddH_2O. The beads were collected by magnetic particle clutch and resuspended in 10 µl nuclease-free ddH_2O for reverse transcription (RT)-PCR to recover antigen-selected mRNA. The beads were stored at 4 or -20˚C.

The selected mRNA was reverse transcribed to cDNA using a PrimeScript® One Step RT-PCR Kit (ver. 2) for 30 min at 50˚C for the reverse transcription and then for 2 min at 94˚C for deactivation of the reverse transcriptase. The obtained cDNA was amplified in a 50 µl PCR mixture for 30 cycles (30 sec at 94˚C, 30 sec at 55˚C and 1 min at 72˚C) with T7A1 (5'-GCAGCTAATACGACTCACTATAGAACA GACCACCATG-3') and Hu1 (5'-GCTCAGCGTCAGGGT GCTGCT-3'). For nested PCR, different downstream primers for subsequent cycles were used to enrich production: Hu2 (5'-CTCTCCTGGGAGTTACC-3') in the second cycle and Hu3 (5'-GAAGACAGATGGTGCAGC-3') in the third cycle. Purified products were applied to the subsequent selection round.

Cloning and expression. The DNA generated using the above-described steps was digested with *Xho*I and *Xba*I and ligated into the vector pBluescript SK^+ for periplasmic expression of the antibody fragment. *Escherichia coli (E. coli)* BL21 containing the expression plasmid was grown in 20 ml 2X tryptone-yeast extract (TY; 16 g tryptone; 10 g yeast extract, 5 g NaCl) medium containing 100 µg/ml ampicillin and 0.5% glucose at 37˚C overnight. Following centrifugation at 300 x g for 10 min, the bacterial pellet was induced by resuspension in 50 ml fresh 2X TY containing 100 µg/ml ampicillin and 0.5 mM isopropylthio-β-galactoside (IPTG) and then grown at room temperature (20-25˚C) for 5-7 h. The bacteria were then harvested by centrifugation at 300 x g for 10 min and the pellet was either stored at -20˚C or used immediately. The pellet was first washed with 1 ml 20% (w/v) sucrose, 0.3 M Tris-HCl (pH 8.0) and 1 mM ethylenediaminetetraacetic acid (EDTA) to prepare a periplasmic extract. Following centrifugation, the bacteria were subjected to osmotic shock by rapidly mixing with 1 ml ice-cold 0.5 mM $MgCl_2$ and leaving on ice for 10 min with periodic agitation. The supernatant periplasmic fraction containing the soluble scFv was recovered by centrifugation at 13,000 x g for 10 min at room temperature. Recombinant protein was further purified by affinity chromatography using Ni-NTA spin kits. Following binding of the protein to Ni-NTA agarose, the column was washed with increasing concentrations of imidazole. The expressed protein was eluted with 200 mM imidazole and dialyzed overnight in PBS at 4˚C.

Immunoblot analysis. The purified periplasmic extracts from selected anti-CAGE-producing clones were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% polyacrylamide gel. Following SDS-PAGE, the gel was transferred to a nitrocellulose membrane [2% (w/v) skimmed milk in PBS]. The transblotted membrane was blocked for 1 h at room temperature with blocking solution and then incubated for 1 h at room temperature with peroxidase-conjugated mouse anti-His tag (1/1000 dilution with blocking solution). 4-Chloro-1-naphthol was used as a peroxidase substrate to visualize the immunoreactivity. Protein concentration was determined using the BCA protein assay kit and purity was assessed with SDS-PAGE analysis.

Determination of scFv titer and affinity constant. The equilibrium dissociation constant (K_D) value of scFv antibodies was

determined in the solution phase by enzyme-linked immunosorbent assay (ELISA) (13,14). A 96-well plate was first coated with 5 μg/ml CAGE protein and then serially diluted scFv protein in 2% skimmed milk was added at 37˚C for 1 h. The bound antibodies were detected with normal ELISA. The concentration of scFv antibodies presenting 50% of the maximum antigen-binding activity was used in competitive ELISA, which was performed on immobilized CAGE as described above, with the exception that CAGE at various concentrations was mixed with a constant amount of antibody in 100 mM PBS (pH 7.4) supplemented with 10 mg/ml bovine serum albumin (BSA). The antibody concentration was derived using ELISA as mentioned above. After overnight incubation at room temperature, 50 μl each mixture was transferred into the well, coated with CAGE and incubated for 1 h at 37˚C. After washing, the bound scFv antibodies were detected as above in triplicate and their affinity was calculated using the Scatchard analysis equation.

Sequencing analysis. Plasmid DNA from anti-CAGE-producing clones was isolated from *E. coli* BL21. The scFv DNA was sequenced using the dideoxy method with the pBluescript SK$^+$ sequence primer set in an ABI Prism automated sequencing machine system.

Results

Antibody library construction. In this study, a large non-immune ribosome display antibody library was established for routine isolation of a high-affinity human scFv antibody to the CAGE antigen. Purified lymphocytes from blood samples were obtained from 10 gastric cancer patients for amplification of the scFv library. Following total RNA isolation, first-strand cDNA synthesis and amplification of V genes with their corresponding primers were performed. Agarose gel electrophoresis revealed bands of the expected sizes (Fig. 1). Splicing by overlap extension PCR was then used to generate full-length scFv gene templates, i.e., V_H/κ and V_H/V_λ-Cκ, which were detected by gel electrophoresis (Fig. 2). Although variable regions in the light and heavy chains of the scFv genes were different, their upstream primer, downstream primer and linker were the same. Thus, these scFvs had an identical sequential pattern graph. DNA sequencing of randomly selected clones confirmed in-frame cloned V genes of human origin derived from various V-gene families (data not shown).

ARM ribosome display. The scFv library was expressed *in vitro* in the rabbit reticulocyte coupled transcription/translation system to generate ternary RNA-ribosome-scFv complexes. The ribosome complexes were enriched by the binding of scFv antibody to recombinant CAGE protein-conjugated Dynabeads. Following RT-PCR recovery of the selected RNA in the complexes, the small DNA band was detected following three rounds of subtractive panning, as shown by the results of agarose gel electrophoresis (Fig. 3). For nested PCR, we used the downstream primer Hu2 in the second cycle and Hu3 in the third cycle in order that the recovered DNA became progressively shorter in each cycle. The shortening only affected the constant domain of the κ-light chain.

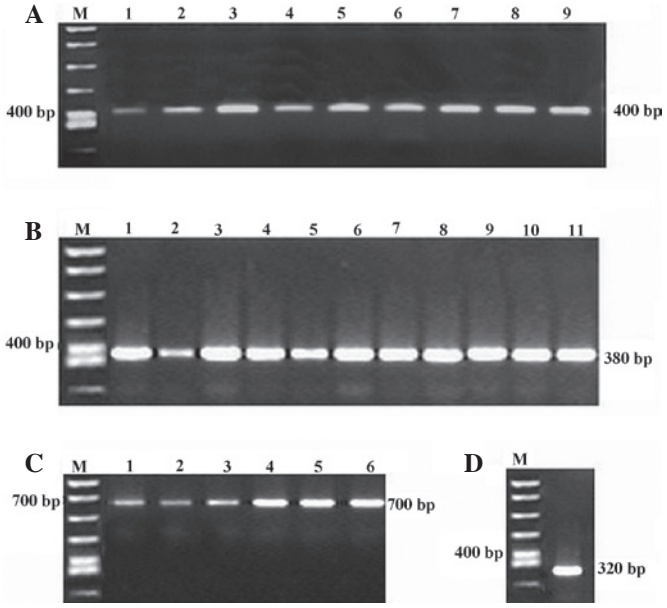

Figure 1. Electrophoretogram of the production of V_H linker, V_λ, Vκ and Cκ. (A) Polymerase chain reaction (PCR) products of the V_H repertoire analyzed by electrophoresis on 1.5% agarose gel. (B) PCR products of the V_λ repertoire analyzed by electrophoresis on 1.5% agarose gel. (C) PCR products of the Vκ repertoire analyzed by electrophoresis on 1.5% agarose gel. (D) PCR products of the Cκ repertoire analyzed by electrophoresis on 1.5% agarose gel.

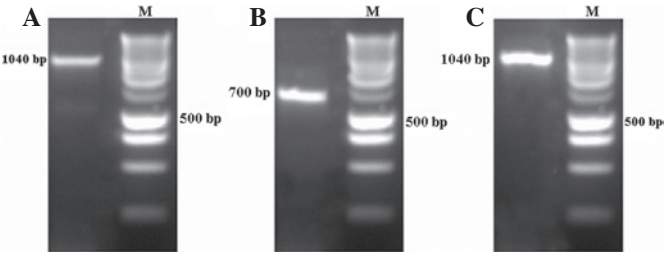

Figure 2. Electrophoretogram of the production of single-chain variable fragment (scFv). (A) Polymerase chain reaction (PCR) products of the assembled V_H/κ DNA library analyzed by electrophoresis on 1.5% agarose gel. (B) PCR products of the assembled V_λ-Cκ DNA library analyzed by electrophoresis on 1.5% agarose gel. (C) PCR products of the assembled V_H/V_λ-Cκ DNA library analyzed by electrophoresis on 1.5% agarose gel.

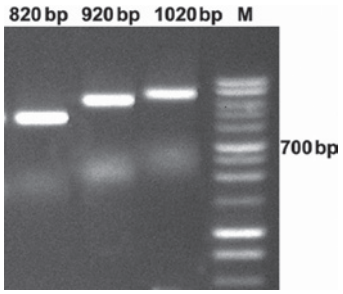

Figure 3. Selection of human V_H/κ or V_H/V_λ-Cκ fragments against cancer-associated gene (CAGE) over 3 cycles of antibody-ribosome-mRNA (ARM) display. Agarose gel analysis (1.5%) of DNA recovered by reverse transcription-polymerase chain reaction (RT-PCR) after each ARM display cycle.

Western blot analysis. After three rounds of panning, the CAGE-specific scFv antibody genes were digested and ligated into the expression vector. The expressed scFv antibodies were

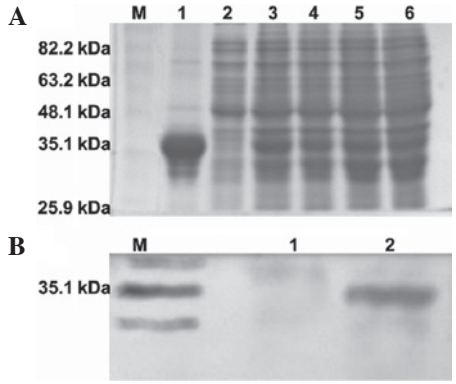

Figure 4. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of *E. coli* BL21 expression of anti-cancer-associated gene (CAGE) single-chain variable fragment (scFv) gene. M, marker of protein; lane 1, purified anti-CAGE scFv; lane 2, lysates of uninduced total bacteria; lanes 3-6, crude lysates of bacteria with different levels of isopropylthio-β-galactoside (IPTG) induction. (B) Western blot analysis of CAGE scFv-expressed protein. Lane 1, uninduced total bacterial proteins; lane 2, purified anti-CAGE scFv.

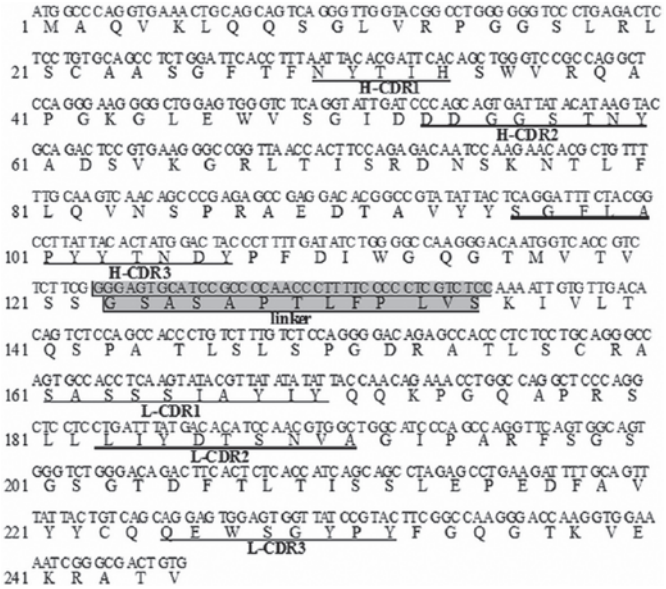

Figure 5. Nucleotide and derived amino acid sequences of the single-chain variable fragment (scFv) gene.

collected from the periplasm of the bacteria and purified by affinity chromatography using Ni-NTA spin kits. The amount of the best-expressed scFv anti-CAGE proteins expressed in *E. coli* BL21 was ~30% of the total bacterial proteins. SDS-PAGE results for the full-length fusion proteins are shown in Fig. 4A. The expression of scFv was confirmed by western blot analysis using anti-His tag antibodies (Fig. 4B). The selected scFv antibody presented a single band measuring ~35 kDa in size.

Dissociation constant of CAGE-specific scFvs and sequence analysis. According to the Scatchard analysis equation $[A_0/(A_0 - A) = K_D/A_g + 1$, where A_0 is the absorbance when the antibody was incubated without any antigen, A is the absorbance corresponding to free antibody following incubation with antigen and A_g is the free antigen concentration that is equal to the antigen obtained for experimentation considering a pseudo-first-order reaction], the K_D of scFv for CAGE was 7.6×10^{-8} M. Fig. 5 shows the RT-PCR product sequence obtained following the third screening cycle and its deduced amino acid sequence. Complementarity determining regions (CDRs) are marked as per the Kabat definition.

Discussion

Gastric cancer is one of the most common malignancies in the digestive system. Chemotherapy remains the mainstay of treatment for advanced gastric cancer; however, its efficacy is modest. An understanding of the molecular biological mechanisms underlying the formation, progression and metastasis of advanced gastric cancer has enabled the use of this new approach to treat this disease in clinical practice. In recent years, molecular-targeted therapies have emerged as a novel approach to the treatment of gastric cancer (15). mAb-based cancer immunotherapy has attracted significant research interest. Immunoconjugate agents are obtained by coupling radioactive materials, chemotherapeutic agents and biotoxins with mAbs (16-18). The coupled agents produce antitumor effects by directing mAbs to the cell surface that expresses the corresponding antigen. The coupled agents also reduce the damage to normal tissues.

At present, therapeutic mAbs are one of the most rapidly growing components of the biopharmaceutical industry. Investigators have created large numbers of mouse mAbs to treat or diagnose human diseases. However, the availability of mouse mAbs has been greatly limited by their high immunogenicity in humans and rapid clearance due to the human anti-mouse antibody reactions that occur in patients (19). Thus, mouse mAbs have exhibited significantly limited and inefficient effector functions in clinical trials. To reduce the immunogenicity of mouse mAbs, an attempt has been made to establish methods to generate human-derived mAbs. Several methods for generating human mAbs have been established, including the phage and ribosome display methods, as well as transchromosome mice technology, in which human Ig genes are expressed (20). Ribosome display is considered to be one of the most promising biotechnologies among the next generation of display technologies (21). It is a technique used to perform *in vitro* protein evolution to create proteins that bind to a desired ligand (22). The process results in translated proteins associated with their mRNA progenitor, which is used as a complex to bind to an immobilized ligand in a selection step. scFv-ribosome-mRNA complexes bind to their specific antigens and non-specific complexes are removed by extensive washing. Eluted mRNAs from the remaining complexes are reverse transcribed to cDNA and reamplified by PCR. The outcome is a nucleotide sequence that may be used to create tightly binding proteins. In addition, ribosome display has advantages in selection from larger libraries and is capable of affinity maturation without any need for additional ligation, transformation or construction of a second library (23).

Together with the progression of genome medical science, an increasing number of target molecules in various diseases have been revealed. Cancer testis antigens are a unique class of tumor antigens expressed in a variety of cancer tissues but silent in normal tissues, with the exception of the testis. Due

to their restricted gene expression in the testis and various malignancies, cancer testis antigens are potential defined targets for antigen-based vaccination and antigen-directed immunotherapy for the regulation of cancer growth (24). CAGE is a typical cancer/testis antigen that is overexpressed in gastric cancer tissues, whereas its expression in normal tissues is limited to the testis (25). Studies have shown that anti-CAGE IgG antibodies are present in the sera of patients with various cancers (26). Therefore, CAGE may be an ideal target for delivering cytotoxic agents to tumors.

In this study, we used ribosome display to select a fully human anti-CAGE scFv. Small antibody fragments, including Fab and scFv, exhibit applicable pharmacokinetics for tissue penetration and full binding specificity as the antigen-binding surface is unaltered (27). A single-chain antibody library in the format of V_H/κ and V_H/V_λ-Cκ was constructed using a PCR-based recombination method. Using this DNA library, we performed CAGE-specific scFv selection and enrichment by ribosome display. After three rounds of selection, anti-CAGE scFv was cloned into an expression vector.

In vitro display techniques have certain potential advantages over *in vivo* display methods. These include ease of generating larger libraries, fewer biases than would be caused by cell expression and more facile application of round-by-round mutagenesis (28). In this study, we performed ribosome display, an *in vitro* display method, using an *in vitro* transcription and translation process. As the library size of a ribosome display is not limited by transformation, anti-CAGE scFv is more easily obtained from DNA libraries using ribosome display than by *in vivo* display methods. We used a rabbit reticulocyte lysate system since such a system has a lower level of RNase activity compared with the *E. coli* ribosome display system, rendering the selection conditions less complex. Moreover, eukaryotic conditions may improve the translation and/or folding efficiency of certain proteins. Therefore, our study is valuable as an example of antibody selection from an antibody library by ribosome display using a eukaryotic translation system.

Although we observed antigen-specific gene recovery by RT-PCR and monitored the enrichment rate during the selection procedure, our results suggest that three rounds are not sufficient for complete selection. One reason may be that stringent selection conditions are essential for the enrichment of specific binders (29). Low temperature and RNase-free conditions are required to maintain intact scFv-ribosome-RNA complexes. With the aim of developing more efficient selection in ribosome display using eukaryotic translation, we are currently studying the factors involved in maintaining ARM complexes. To the best of our knowledge, this is the first time that this technique has been used successfully for the direct generation of human scFv against CAGE. As the scFv originates from humans, it is expected to be less immunogenic.

In summary, a new human anti-CAGE scFv was isolated using *in vitro* ribosome display technology. The technological challenge of the ribosome display methodology is reflected in the screening process. Nevertheless, it successfully isolates a fully human antibody construct with specificity to a clinically relevant target antigen. It may provide the basis for the development of anti-scFv-drug conjugates for the proliferation and metastasis of gastric cancer cells.

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