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• ESOPHAGEAL CANCER •

Production of a human single-chain variable fragment antibody against esophageal carcinoma

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Abstract

AIM: To construct a phage display library of human single-chain variable fragment (scFv) antibodies associated with esophageal cancer and to preliminarily screen a scFv antibody against esophageal cancer.

METHODS: Total RNA extracted from metastatic lymph nodes of esophageal cancer patients was used to construct a scFv gene library. Rescued by M13K07 helper phage, the scFv phage display library was constructed. esophageal cancer cell line Eca 109 and normal human esophageal epithelial cell line (NHEEC) were used for panning and subtractive panning of the scFv phage display library to obtain positive phage clones. Soluble scFv was expressed in *E.coli* HB2151 which was transfected with the positive phage clone, then purified by affinity chromatography. Relative molecular mass of soluble scFv was estimated by Western blotting, its bioactivity was detected by cell ELISA assay. Sequence of scFv was determined using the method of dideoxynucleotide sequencing.

RESULTS: The size of scFv gene library was approximately 9×10^6 clones. After four rounds of panning with Eca109 and three rounds of subtractive panning with NHEEC cells, 25 positive phage clones were obtained. Soluble scFv was found to have a molecular mass of 31 ku and was able to bind to Eca109 cells, but not to HeLa and NHEEC cells. Variable heavy (V_H) gene from one of the positive clones was shown to be derived from the γ chain subgroup IV of immunoglobulin, and variable light (V_L) gene from the κ chain subgroup I of immunoglobulin.

CONCLUSION: A human scFv phage display library can be constructed from the metastatic lymph nodes of esophageal cancer patients. A whole human scFv against esophageal cancer shows some bioactivity.

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INTRODUCTION

Esophageal cancer is one of the most common malignancies in China with a relatively high mortality rate. In recent years, antibody-mediated tumor immunoscintigraphy and immunotherapy have been used in the diagnostic and therapeutic approaches of cancers[1,2]. However, most antibodies are of murine origin, and repeated administration can induce human anti-mouse antibodies (HAMA). In addition, intact antibody is too large to penetrate into tumor masses, its application is limited. To overcome such deficiencies, many kinds of humanized antibodies including human-murine chimeric antibody and small molecular antibodies have been developed, but they are still of murine origin.

Recently, the emergence of genetically engineered antibodies and phage display libraries of human antibody fragments from immune or naïve donors has enabled the production of human antibody fragment targeting cancers[3]. In the present study, phage antibody library techniques were used to construct a human phage single-chain Fv antibody library from metastatic lymph nodes of esophageal cancer patients. To obtain a single chain Fv AD09, panning and subtractive panning were performed with human esophageal cancer cell line (Eca109) and normal human esophageal epithelial cell line (NHEEC) respectively. Soluble AD09 was expressed in *E.coli* HB2151 and purified by affinity chromatography using anti-E tag antibody, its bioactivity was then detected by cell ELISA assay.

MATERIALS AND METHODS

Cell culture

Human esophageal carcinoma cell line Eca109 (Cytology Institute of Chinese Medical Academy, Beijing) and HeLa cell line (Shanghai Cytology Institute, China) were cultured at 37 in RPMI1640 medium supplemented with 100 mL/L fetal calf serum (Hyclone, USA) in a humidified atmosphere of 50 mL/LCO_2. Normal human esophageal epithelial cell (NHEEC) line was a primary cell line from a 20-wk conception fetus cultured in RPMI1640 with 200 mL/L fetal calf serum.

Primer design

Primer sequences were created as previously described[4] with some modifications in PCR assembly part (Table 1B). We designed complementary coding sequences for a peptide linker at the 5'-end of J_H forward primers and the 3'-end of human V kappa (or lambda) back primers to optimize the diversity and efficiency of ligation. The primers were synthesized by Sunbiotech Company (Beijing, China) and the sequences are shown in Table 1. Sequences were given using the IUPAC nomenclature of mixed base (R = A or T, K = G or T, Y = C or T, S = G or C, H = A or C or T, N = A or C or G or T).

Library construction

Metastatic lymph nodes of 5 esophageal cancer patients were

Table 1 Oligonucleotide primers used for PCR of human immunoglobulin genes

A. Primary PCRs	
Human V_H back primers (sense)	
HuV_H1aBACK	5'-CAG GTG CAG CTG GTG CAG TCT GG-3'
HuV_H2aBACK	5'-CAG GTC AAC TTA AGG GAG TCT GG-3'
HuV_H3aBACK	5'-GAG GTG CAG CTG GTG GAG TCT GG-3'
HuV_H4aBACk	5'-CAG GTG CAG CTG CAG GAG TCG GG-3'
HuV_H5aBACK	5'-GAG GTG CAG CTG TTG CAG TCT GC-3'
HuV_H6aBACK	5'-CAG GTA CAG CTG CAG CAG TCA GG-3'
J_H forward primers (anti-sense)	
HuJ_H1-2FOR	5'-TGA GGA GAC GGT GAC CAG GGT GCC-3'
HuJ_H3FOR	5'-TGA AGA GAC GGT GAC CAT TGT CCC-3'
HuJ_H4-5FOR	5'-TGA GGA GAC GGT GAC CAG GGT TCC-3'
HuJ_H6FOR	5'-TGA GGA GAC GGT GAC CGT GGT CCC-3'
Human V kappa back primers (sense)	
HuV κ laBack	5'-GAC ATC CAG ATG ACC CAG TCT CC-3'
HuV κ 2aBack	5'-GAT GTT GTG ATG ACT CAG TCT CC-3'
HuV κ 3aBack	5'-GAA ATT GTG TTG ACG CAG TCT CC-3'
HuV κ 4aBack	5'-GAC ATC GTG ATG ACC CAG TCT CC-3'
HuV κ 5aBack	5'-GAA ACG ACA CTC ACG CAG TCT CC-3'
HuV κ 6aBack	5'-GAA ATT GTG CTG ACT CAG TCT CC-3'
Human J kappa forward primer (anti-sense)	
HuJ κ 1FOR	5'-ACG TTT GAT TTC CAC CTT GGT CCC-3'
HuJ κ 2FOR	5'-ACG TTT GAT CTC CAG CTT GGT CCC-3'
HuJ κ 3FOR	5'-ACG TTT GAT ATC CAC TTT GGT CCC-3'
HuJ κ 4FOR	5'-ACG TTT GAT CTC CAC CTT GGT CCC-3'
HuJ κ 5FOR	5'-ACG TTT AAT CTC CAG TCG TGT CCC-3'
Human V lambda back primers (sense)	
HuV λ 1BACK	5'-CAG TCT GTG TTG ACG CAG CCG CC-3'
HuV λ 2BACK	5'-CAG TCT GCC CTG ACT CAG CCT GC-3'
HuV λ 3aBACK	5'-TCC TAT GTG CTG ACT CAG CCA CC-3'
HuV λ 3bBACK	5'-TCT TCT GAG CTG ACT CAG GAC CC-3'
HuV λ 4BACK	5'-CAC GTT ATA CTG ACT CAA CCG CC-3'
HuV λ 5BACK	5'-CAG GCT GTG CTC ACT CAG CCG TC-3'
HuV λ 6BACK	5'-AAT TTT ATG CTG ACT CAG CCC CA-3'
Human J lambda forward primers (anti-sense)	
HuJ λ 1FOR	5'-ACC TAG GAC GGT GAC CTT GGT CCC-3'
HuJ λ 2-3FOR	5'-ACC TAG GAC GGT CAG CTT GGT CCC-3'
HuJ λ 4-5FOR	5'-ACC TAA AAC GGT GAG CTG GGT CCC-3'
B. PCR assembly	
Hu J_H-Linker primers	
HuJ_H1-2Linker	5'-AGAGCCACCTCCGCCTGAACCGCCTCCACCTGAGGAGACGGT GACCAGGGTGCC-3'
HuJ_H3Linker	5'-AGAGCCACCTCCGCCTGAACCGCCTCCACCTGAAGAGACGGT GACCATTGTCCC-3'
HuJ_H4-5Linker	5'-AGAGCCACCTCCGCCTGAACCGCCTCCACCTGAGGAGACGGT GACCAGGGTTCC-3'
HuJ_H6Linker	5'-AGAGCCACCTCCGCCTGAACCGCCTCCACCTGAGGAGACGGT GACCGTGGTCCC-3'
Linker-Hu V κ primers	
Linker-HuV κ 2-3-6 BACK	5'-GTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGGAWRTTGTGHTGACKCAGTCTCC-3'
Linker-HuV κ 1-4 BACK	5'-gTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGGACATCSWGATGACCCAGTCTC C-3'
Linker- HuV κ 5 BACK	5'-GTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGGAAACGACACTCACG CAGTCTCC-3'
Linker-Hu V λ primers	
Linker-HuV λ 1-2BACK	5'-GTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGCAGTCTGYSYTGACKCAGCCKS C-3'
Linker-HuV λ 3BACK	5'-GTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGTCYTMTGWGCTGACTCAGSMMCC-3'
Linker-HuV λ 4-5BACK	5'-GTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGCASGYTRTRCTSACTCARCCGYC-3'
Linker-HuV λ 6BACK	5'-GTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGAATTTTATGCTGACTCAGCCC CA-3'
C. Reamplification with primers containing restriction sites	
Human V_H back (Sfi) primers (sense)	
HuV_H1aBACKSfi	5'-GTCCTCGCAACTGCGGCCCAGCCGGCCATGGCCCAGGTGCAGCTGGTGCAGTCTGG-3'
HuV_H2aBACKSfi	5'-GTCCTCGCAACTGCGGCCCAGCCGGCCATGGCCCAGGTCAACTTAAGGGAGTCTGG-3'
HuV_H3aBACKSfi	5'-GTCCTCGCAACTGCGGCCCAGCCGGCCATGGCCGAGGTGCAGCTGGTGGAGTCTGG-3'
HuV_H4aBACKSfi	5'-GTCCTCGCAACTGCGGCCCAGCCGGCCATGGCCCAGGTGCAGCTGCAGGAGTCGGG-3'
HuV_H5aBACKSfi	5'-GTCCTCGCAACTGCGGCCCAGCCGGCCATGGCCCAGGTGCAGCTGTTGCAGTCTGC-3'
HuV_H6aBACKSfi	5'-GTCCTCGCAACTGCGGCCCAGCCGGCCATGGCCCAGGTACAGCTGCAGCAGTCAGG-3'
Human J kappa forward (Not) primers (anti-sense)	
HuJ κ 1FORNot	5'-GAGTCATTCTCGACTTGCGGCCGCACGTTTGATTTCCACCTTGGTCCC-3'
HuJ κ 2FORNot	5'-GAGTCATTCTCGACTTGCGGCCGCACGTTTGATCTCCAGCTTGGTCCC-3'
HuJ κ 3FORNot	5'-GAGTCATTCTCGACTTGCGGCCGCACGTTTGATATCCACTTTGGTCCC-3'
HuJ κ 4FORNot	5'-GAGTCATTCTCGACTTGCGGCCGCACGTTTGATCTCCACCTTGGTCCC-3'
HuJ κ 5FORNot	5'-GAGTCATTCTCGACTTGCGGCCGCACGTTTAATCTCCAGTCGTGTCCC-3'
Human J lambda forward (Not) primers (anti-sense)	
HuJ λ 1FORNot	5'-GAGTCATTCTCGACTTGCGGCCGCACCTAGGACGGTGACCTTGGTCCC-3'
HuJ λ 2-3FORNot	5'-GAGTCATTCTCGACTTGCGGCCGCACCTAGGACGGTCAGCTTGGTCCC-3'
HuJ λ 4-5FORNot	5'-GAGTCATTCTCGACTTGCGGCCGCACCTAAAACGGTGAGCTGGGTCCC-3'

collected for total RNA extraction (TRizol, Gibco BRL, UK). First-strand cDNA synthesis was performed in the presence of 40 U RNase inhibitor, 200 U Superscript II transcriptase (Gibco BRL, UK). The sample was finally treated with 2 U RNase H for 30 min at 37 °C and stored at -20 °C until use.

IgG-specific variable heavy (V_H) and light (V_L) chain gene fragments were amplified using Pyrobest PCR system (TarkaRa Biotechnology, Dalian, China) for 30 cycles (at 94 °C for 30 s, at 55 °C for 30 s and at 72 °C for 1 min), with each forward oligonucleotide primer and one of the back primers (Table 1A). The fragments were isolated from a 15 g/L agarose gel with the QIAex kit (QIAgen, Germany). Then fragments were used as templates for PCR amplification to extend a linker, V_H fragments used human J_H-linker primers and human V_H back primers, V_L fragments used linker-human Vκ primers (or linker-human Vλ primers) and human Jκ forward primers (or human Jλ forward primers), respectively.

The amplified V_H-linker and V_L-linker PCR products were combined in a SOE-PCR reaction mixture. First, approximately 100 ng each of V_H-linker and V_L-linker was assembled by PCR without primers in which the short regions of complementarities built into the ends of primers drove hybridization of various fragments. An initial denaturation step (at 94 °C for 5 min) was followed by five cycles (at 94 °C for 1 min, at 60 °C for 1 min and at 72 °C for 1.5 min) in the absence of primers. After the outer primers containing restriction sites (Table 1C) were added, 30 cycles (at 94 °C for 30 s, at 60 °C for 1 min and at 72 °C for 1.5 min) were performed. These were gel-purified, digested with *Sfi*I and *Not*I (TarkaRa Biotechnology, Dalian), cloned into the vector pCANTAB 5E (Amersham Pharmacia Biotech, Sweden) and transformed into electrocompetent *E.coli* TG1 (Amersham Pharmacia Biotech, Sweden). After electroporation, cells were plated on SOBAG medium (containing 20 g/L glucose and 100 mg/mL ampicillin) in 20 dishes and incubated overnight at 30 °C. The clones were scraped off the plates in 50 mL 2×YT medium with 100 mL/L glycerol and subsequently stored at -70 °C. Plasmid DNA was prepared from 10 randomly selected clones using Qiagen plasmid minikit (Qiagen, Germany). PCR and a *Sfi*I/*Not* I double digestion reaction were performed to identify the positive insert clones.

Rescue of phagemid libraries

Ampicillin-resistant colonies were scraped into 2×YT medium and superinfected by M13K07 (Amersham Pharmacia Biotech, Sweden) helper phage. After an overnight induction in 2×YTAK medium without glucose, the phage preparation was precipitated in 40 g/L PEG/0.5 mol/L NaCl and resuspended in 10 g/L PBS.

Panning and subtractive panning of phage antibody library

To screen the positive phage clones, live Eca109 cell line as the antigen was used for panning. Live NHEEC was used for subtractive panning. The panning procedure was carried out essentially as described previously[5]. After four rounds of panning and three rounds of subtractive panning, the unabsorbed phages were amplified.

Cell ELISA assay with phage

To detect the scFv-phage recombinant antibody, cell ELISA was performed. Eca109 cells (5×10^4) as antigens were grown in 96-well plates at 37 °C for 24 h, then fixed with 25 g/L glutaradehyde and blocked with 10 g/L BSA. This was followed by incubation with scFv-phage at 37 °C for 2 h. After washed three times with PBS, HRP/anti-M13 monoclonal conjugate (1:5 000) (Amersham Pharmacia Biotech, Sweden) was added into wells with scFv-phage and they were incubated for 1 h at 37 °C. After washed again as above, 1×ABTS substrate solution was added, and incubated in darkness for 30 min and the reactions were read at 405 nm. PBS was used as negative control. The absorbance reading for the positive was 0.2 or above at least three times higher than that for the negative control.

Expression and purification of soluble scFv

To produce soluble scFvs, strongly positive recombinant phage clones were used to infect log-phage *E.coli*.HB2151 (Amersham Pharmacia Biotech, Sweden). Expression of soluble scFv was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mmol/L and the cultures grown overnight at 30 °C. The induced culture was centrifuged at 1 500 r/min for 20 min. Cell pellets were resuspended in 2% of culture volume ice-cold 1×TES. Subsequently, 3% of culture volume ice-cold 0.2×TES was added, the mixture was incubated on ice for 30 min to induce a mild osmotic shock. The contents were centrifuged at 12 000 g for 10 min. The supernatant, containing the soluble antibodies from the periplasm, was transferred to the clean tubes and stored at -20 °C.

Soluble scFvs from periplasm were purified by affinity chromatography. Anti-E tag antibody (Amersham Pharmacia Biotech, Sweden) was covalently coupled to a protein G column (Amersham Pharmacia Biotech, Sweden) and soluble scFvs were selected by binding to anti-E tag antibody. After washed with 20 mmol/L phosphate buffer, pH 7.0, +0.5 g/L NaN3, scFvs were eluted from the column with 0.1 mol/L glycine-HCl, pH 3.0, and neutralized immediately with 1 mol/L Tris/HCl, pH 8.2, +0.5 g/L NaN3. Column fractions were assayed and positive fractions were pooled and stored at -70 °C.The expressed soluble scFv proteins were analyzed by 120 g/L sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and confirmed by Western blotting. Purity and concentration of proteins were determined with Bradford assay.

Cell ELISA assay for activity of soluble scFv

To detect the activity of soluble scFv, HRP/anti-E tag antibody (Amersham Pharmacia Biotech, Sweden) was used. Eca109 cells, HeLa cells and NHEEC cells (5×10^4) were used as antigens. The cell ELISA assay procedure was performed as described above.

Sequencing

Plasmid DNA was prepared from recombinant clones using the Qiagen plasmid minikit (Qiagen, Germany). Nucleic acid sequencing was carried out on the ABI PRISM 377 DNA sequencer by the method of dideoxynucleotide sequencing. DNA and deduced amino acid sequence were compared with NCBI database.

RESULTS

Library construction and panning

The presence of V_H and V_L gene fragments obtained by RT-PCR was confirmed by electrophoresis, with their sizes being approximately 350 bp (Figure 1 A). The scFv genewas assembled successfully. Its size was about 750 bp (Figure 1B).

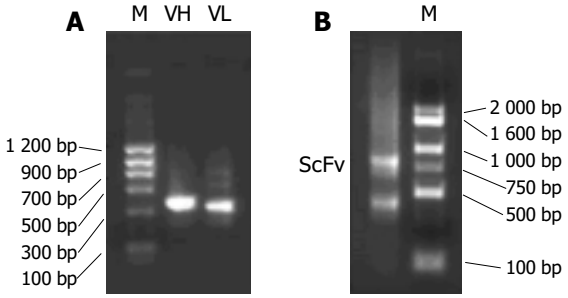

Figure 1 V_H and V_L and scFv fragments in 20 g/L agarose gel electrophoresis with staining of ethdium bromide (EB). (A) V_H and V_L fragments, (B) scFv fragment, M: DGL 2 000 marker.

After the scFv gene repertoires were transformed into *E. coli* TG1 cells, approximately 9×10^6 ampicillin-resistant clones grew. PCR and *Sfi*I/*Not*I double digestion reactions showed the positive insert ratio was about 95% (19/20). Rescued by M13K07 helper phage, the recombinant phage antibody library (about 9×10^{11}cfu/mL) was constructed.

Four rounds of panning with Eca109 cells resulted in a 130-fold enrichment of tumor cell binding scFv-phage. After three rounds of subtractive panning with NHEEC cells, individual phage-displayed scFv fragments were tested for reactivity with Eca109 cells by cell ELISA. Of the 95 clones screened, 25 were positive. The highest A_{405} nm value was found in AD09 clone.

Expression and purification of soluble scFv

The soluble scFv was stably expressed in *E. coli* HB2151 transfected with AD09 positive phage clone. In pCANTAB 5E, the pel B signal peptide upstream from the scFv directed the expression to periplasmic compartment. The periplasmic extract of AD09 was run through anti-E tag antibody affinity chromatography column and soluble AD09 was eluted from the column as a single peak (data not shown). The expressed and purified AD09 was loaded on 120 g/L SDS-PAGE and analyzed by Western blot. This protein migrated with a molecular mass approximately 31 ku (Figure 2). The overall yield of soluble AD09 in *E. coli* flask culture was more than 0.55 mg/L. The purity was about 90%.

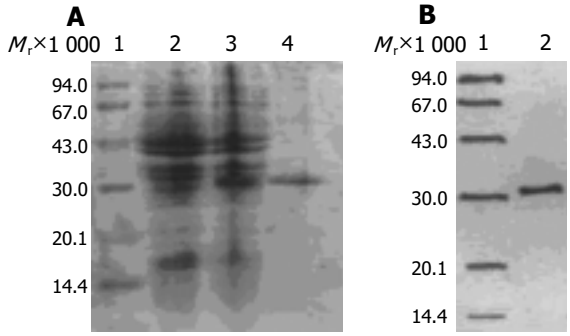

Figure 2 Expression and purification of AD09. A: SDS-PAGE. Lane 1: Markers (Amersham Pharmacia Biotech, Sweden). Lane 2: Product of *E.coli* HB2151 without scFv gene. Lane 3: Expression of AD09 in *E.coli* HB2151 with induction of IPTG. Lane 4: Purified AD09 protein. B: Western blot. Lane 1: Markers. Lane 2: Purified AD09 protein.

Determination of immunoreactivity of soluble scFv

The immunoreactivity of purified soluble AD09 was determined by ELISA. The result revealed that AD09 was highly specific and could bind to Eca109 cells, but not to HeLa and NHEE cells (Table 2).

Table 2 Immunore activity of soluble scFv determined by ELISA

Sample	A_{405nm} (mean±SD)		
	Eca109	HeLa	NHEE
Soluble scFv	0.78±0.12	0.21±0.09[d]	0.23±0.07[b]
PBS	0.14±0.04	0.13±0.08	0.15±0.02

[d]P<0.001, [b]P<0.01 *vs* Eca109 group.

Sequence analysis

Sequencing of six randomly selected antibodies from the positive clones was performed with an ABI PRISM 377 DNA sequencer using pCANTAB5 sequencing primer set (Amersham Pharmacia Biotech, Sweden). The sequence f AD09 clone is shown in Figure 3. Compared using BLAST, both V_H and V_L had sequence similarities to the variable fragments of some known human antibodies. Alignment with the V_H and V_L sequences of Ig, blast analysis of immunoglobulin sequences showed that V_H was the γ chain subgroup IV of human immunoglobulin and V_L was the κ chain subgroup I of human immunoglobulin.

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ATG CCA GCC GGC CAT GGC CGA GGT GCA GCT GGT GCA GTC TGC CCA GGA CTG
 M  P  A  G  H  G  R  G  A  A  G  A  V  C  P  G  L
GTG AAG CCT TCG GAG ACC CTG TCC CTC ACC TGC ACT GTC TCT GGT GGC TCC
 V  K  P  S  E  T  L  S  L  T  C  T  V  S  G  G  S
ATC AGT AGT TAC TAC TGG AGC TGG ATC CGG CAG CCC CCA GGG AAG GGA CTG
 I  S  S  Y  Y  W  S  W  I  R  Q  P  P  G  K  G  L
          CDR1
GAG TGG ATT GGG TAT ATC TAT TAC AGT GGG AGC ACC AAC TAC AAC CCC TCC
 E  W  I  G  Y  I  Y  Y  S  G  S  T  N  Y  N  P  S
                                   CDR2
CTC AAG AGT CGA GTC ACC ATA TCA GTA GAC ACG TCC AAG AAC CAG TTC TCC
 L  K  S  R  V  T  I  S  V  D  T  S  K  N  Q  F  S

CTG AAG CTG AGC TCT GTG ACC GCT GCG GAC ACG GCC GTG TAT TAC TGT GCG
 L  K  L  S  S  V  T  A  A  D  T  A  V  Y  Y  C  A
AGA GAG CGT GCC GAG ATG GCT ACA ATC GGG GGT GCT TTT GAT ATC TGG GGC
 R  E  R  A  E  M  A  T  I  G  G  A  F  D  I  W  G
                   CDR3
CAA GGG ACC ACG GTC ACC GTC TCC TCA GGT GGA GGC GGT TCA GGC GGA GGT
 Q  G  T  T  V  T  V  S  S  G  G  G  G  S  G  G  G
                                                Linker
GGC TCT GGC GGT GGC GGA TCG GAC ATC CTG ATG ACC CAG TCT CCA TCC TCC
 G  S  G  G  G  G  S  D  I  L  M  T  Q  S  P  S  S

CTG TCT GCA TCT GTA GGA GAC AGA GTC ACC ATC ACT TGC CGG GCA AGT CAG
 L  S  A  S  V  G  D  R  V  T  I  T  C  R  A  S  Q

AGC ATT AGC AGC TAT TTA AAT TGG TAT CAG CAG AAA CCA GGG AAA GCC CCT
 S  I  S  S  Y  L  N  W  Y  Q  Q  K  P  G  K  A  P
CDR1
AAG CTC CTG ATC TAT GCT GCA TCC AGT TTG CAA AGT GGG GTC CCA TCA AGG
 K  L  L  I  Y  A  A  S  S  L  Q  S  G  V  P  S  R
                CDR2
TTC AGT GGC AGT GGA TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC AGT CTG
 F  S  G  S  G  S  G  T  D  F  T  L  T  I  S  S  L
CAA CCT GAA GAT TTT GCA ACT TAC TAC TGT CAA CAG AGT TAC AGT ACC CTG
 Q  P  E  D  F  A  T  Y  Y  C  Q  Q  S  Y  S  T  L
                                   CDR3
TAC ACT TTT GGC CAG GGG ACC A AG CTG GAT ATC AAA CGT GCG GCC GCA
 Y  T  F  G  Q  G  T  K  L  D  I  K  R  A  A  A
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Figure 3 Sequence of AD09 gene.

DISCUSSION

The discovery of hybridoma technology by Kohler and Milstein in 1975[6] has heralded a new era in antibody research and clinical development. However, until recently, there were few antibody-based products suitable for clinical trial. This delay can be largely explained by the fact that murine antibodies could trigger a human anti-mouse antibody response[7-9]. Human monoclonal antibodies seldom triggered a harmful immune response and have been used in cancer immunotherapy[10]. However, there are many difficulties in making human monoclonal antibodies by hybridoma technology. Phage display antibody library technique provides a powerful tool to produce human antibody. In this study, we selected a human single chain antibody against esophageal cancer from a phage displayed antibody library. It confirmed that this technique was feasible.

Since scFv could penetrate faster and deeper in solid tumors, scFv format of monoclonal antibody was selected. ScFv is a small antigen-binding antibody fragment consisting of V_H and V_L joined together by a flexible peptide linker. The main advantage of scFv over intact whole IgG or Fabs was its small size (one sixth of intact IgG), making it penetrate a solid tumor mass more rapidly and evenly[11,12].In addition, the lack of constant regions decreased retention by Fc receptors found in most tissues and organs, which further reduced the side effects[13,14].These characteristics rendered scFv an ideal vector for delivery of

agents such as radionuclide, enzyme, drugs or toxin *in vivo*[14,15].

Normally, peripheral blood lymphocytes were the main source for the construction of antibody libraries[16]. However, it was inconvenient to isolate at least 200 mL of peripheral blood to obtain 10^7 B-lymphocytes for constructing a large antibody library[3]. There are thousands of B cells in metastatic peritumor lymph nodes which may be preimmunized by tumor-associated antigens in esophageal cancer patients. The preimmunized B cells are sufficient to construct a library, and can be directly used for screening recombinant antibodies, since the heavy and light chain genes have been rearranged and ligated to specific targets of tumor antigens. Therefore, it has become a better source of B cells for human antibody library construction[17].

Recombinant antibodies in phage antibody library could be best captured with purified tumor antigens[18]. Unfortunately, esophageal cancer associated specific antigens have not yet been identified. Human esophageal cancer cell line, Eca109 could express human esophageal cancer-associated specific antigens[19]. In addition, live cells could facilitate the identification of antibodies better than fixed cells[5,20] since the antibodies bound to native rather than denatured antigens. Moreover, using live cancer cells to screen phage antibody library was a feasible method[20,21]. So we used live Eca109 as antigens to screen the phage antibody library, and live NHEEC cell line was used for subtractive panning to obviate the cross-reaction with normal human esophageal epithelial cells. This was done to facilitate subsequent expression cloning of corresponding antigens, as well as to enhance the therapeutic potential of the antibodies obtained.

Generation of large repertoires of scFv genes is a crucial step during phage antibody display. To construct a large scFv gene repertoire, a number of methods were used. (1) Multiple primers covering most of the immunoglobulin heavy and light chain variable genes were used. (2) To optimize the diversity and efficiency of ligation, linker sequences were designed in PCR assembly primers. These made the linker easily synthesized and increased the diversity of scFv genes. (3) Electroporation transformation was used to obtain an efficiency of 10^9cfu/ug for pUC18 and 3×10^7 cfu/ug for ligation products. (4) The quality of mRNA appeared essential in the PCR amplification step and in subsequent construction of the library. To preserve intact mRNA, DEPC and RNase inhibitor were used during total RNA extraction and cDNA synthesis.

To further identify the bioactivity of scFv, soluble scFv protein was expressed in *E.coli* HB2151 induced by IPTG. SDS-PAGE and Western blot showed that the molecular mass was about 31 ku, which was consistent with the theoretically predicted product. The soluble expression level of scFv in *E.coli* HB2151 was still low, but it was sufficient for bioactivity detection. Cell ELISA assays showed that the soluble scFv had esophageal cancer associated antigen-binding activity. Whether this scFv shows affinity and specificity for tissue *in vivo* remains to be determined. Finally, DNA sequencing determined that scFv had common characteristics shared by other known scFvs[3].

Our study demonstrated that specific human antibodies against tumor-associated antigens could be selected from a phage library constructed from the metastatic lymph nodes of esophageal cancer patients. The approach did not depend on immunization procedures. Since the antibody is entirely of human origin, it is expected to be much less immunogenic than murine antibodies and more efficient in targeting tumor cell surface. It may also be used as research reagents or a starting point for the development of therapeutic antibodies.

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