Expression of a humanized single-chain variable fragment antibody targeting chronic myeloid leukemia cells in *Escherichia coli* and its characterization

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Abstract. Chronic myeloid leukemia (CML) is a malignant blood disease originating from hematopoietic stem cells. Drug resistance and tumor recurrence have become major problems for the treatment of this disease. Therefore, new therapeutic methods need to be developed. Antigens expressed on the surface of cancer cells are potential targets for antibody-mediated drug delivery. In our study, an anti-CML cell single-chain variable fragment (scFv) antibody has been produced and characterized because it is the first step towards the construction of a novel cancer-targeted agent for cancer diagnosis and treatment. Here, a 46 kDa antibody derivative was produced by genetic fusion of a humanized scFv antibody against a CML cell surface antigen with the 6xHis-tag, which can specifically bind to CML cells. The recombinant scFv against CML cells was expressed as a fusion protein containing the 6xHis-tag at its N-termini, and purified by Ni^{2+}-NTA column chromatography. The recombinant scFv, which was soluble, was expressed and produced in bacteria, and was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot assays. Finally, its cell-binding activity and immunoactivity were demonstrated by enzyme-linked immunosorbent assay (ELISA). Furthermore, flow cytometry analysis demonstrated that this scFv specifically targeted CML cells expressing the associated antigen (47.9 and 34.4%) other than non-expressing tumor cells (1.25%) *in vitro*. The results presented in this study illustrate that the humanized anti-CML cell scFv antibody may function as a novel therapeutic tool for CML.

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Introduction

Monoclonal antibodies (mAb) have been widely used in medicine, biology, immunology since Köhler and Milstein invented the hybridoma technology in 1975 (1). They have broad applications in diagnosing and treating a wide range of diseases due to their high specificity and affinity to the target cell surface or the intracellular antigen (2,3). However, as most mAbs derived from mice, the human anti-mouse antibody reaction (HAMA) can be induced by long-term administration (4). Moreover, intact mAbs are generally too large to penetrate tumor tissues, which limited their efficacy in clinical applications (5). With improvements in gene engineering technology, human origin antibodies, chimeric antibodies and single chain Fv are developed to progress murine origin mAbs (6-8).

Single-chain variable fragments (scFvs) are smaller fragments composed of antibody heavy (VH) and light chain variable (VL) regions with a flexible peptide linker containing 15-20 amino acids (9-11). scFvs are potentially very useful designer tools for the targeted delivery of drugs, toxins, or radionuclides to specific tumor tissues or cells (12). Compared with the larger Fab', F(ab')2, and IgG forms of the monoclonal antibodies from which they are derived, scFvs manifest lower retention times in non-target tissues, more rapid blood clearance, better tumor penetration, and decreased immunogenicity. Moreover, scFv antibodies can be produced in microbial expression systems in high yields, resulting in a fast and cost-effective production (13-16). These characteristics make scFvs potentially useful in cancer diagnosis and treatment as an effective carrier, making them attractive for therapeutic or diagnostic applications. Significant success has been achieved in this area (17-19). However, there is no scFv with specific targeting properties to chronic myeloid leukemia (CML) (20).

In this study, we have cloned a gene coding for the scFv domain from screened CML cell-specific mAb, then grafted the complementary determining regions (CDRs) of the mouse VH and VL genes to a human heavy chain and light chain

variable region genes with the highest homology, and assembled them with linker peptide $(Gly_4Ser)_3$ to form a humanized scFv fragment (VH-linker-VL). Then the T7-RNA polymerase based pET system was used to express the scFv protein in *Escherichia coli* (*E. coli*). scFv fragments were subsequently cloned into the pET-32a(+) vector, in which the genes of the humanized scFv were tagged with an N-terminal 6xHis-tag to allow easy purification. The expression, solubility, specificity, and antigen-binding activity of the humanized scFv have been characterized *in vitro*.

Materials and methods

Materials. Restriction enzymes, T4 DNA ligase, dNTP and primerstar DNA polymerase were purchased from Takara Bio, Inc. (Dalian, China). Ni^{2+}-NTA agarose was purchased from Qiagen China Co., Ltd. (Shanghai, China). Anti-6xHis mouse monoclonal antibody was purchased from ZhongShan GoldenBridge Biotechnology Co., Ltd. (Beijing, China). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-mouse IgG were purchased from Beijing Huamei Biotechnology (Beijing, China). Primers were synthesized by Shanghai Sangon Biotechnology (Shanghai, China). All other chemical reagents were of analytical grade.

Bacteria culture and plasmids. *E. coli* DH5α and BL21 (DE3) were used as host strains for cloning and expression in *E. coli*. The *E. coli* expression vector pET-32a(+) (Novagen) was used for constructing plasmid vector. The strains containing expression vector pET-32a(+) were grown in Luria-Bertani (LB) broth (1% polypeptone, 1% NaCl, and 0.5% yeast extract, pH 7.0) or on LB agar supplemented with ampicillin (100 μg/ml). All strains were cultured at 37˚C with constant shaking (210 rpm).

Construction, expression, purification, and identification of scFv. Anti-CML cells scFv vector named pUCT vector was constructed previously and originated from pUC57 vector (Shanghai Sangon Biotechnology). Briefly, mRNA was extracted from $5x10^6$ hybridoma cells and cDNA was synthesized by reverse transcription reaction. VH and VL gene were separately amplified from the cDNA by polymerase chain reaction (PCR). Then, the obtained VH and VL gene were cloned into pMD19-T vector (Takara Bio, Inc.), and transformed into *E. coli* DH5α. The plasmids were identified by colony PCR and DNA sequencing. In http//www.imgt.org/IMGT_vquest/vquest, we blasted the sequences with human antibody sequences, and found that the murine VH had the highest homology with the human immunoglobulin heavy chain VI-2 gene (GenBank accession no. X62106) and the base pairing rate was 75.61%; the murine VL had the highest homology with the human immunoglobulin VKN light chain B1 gene (GenBank accession no. X12682) and its base pairing rate was 75.6%. By the DNA recombinant techniques, the CDR domains of the murine antibody VH and VL replaced the CDR domains of the corresponding variable region sequences, respectively. The linker DNA sequence, encoding a 15 residues long flexible peptide $(Gly_4Ser)_3$, were used to link the humanized variable region of heavy chain with the humanized variable region of the light chain, to form a humanized scFv fragment (VH-linker-VL). The recombinant fragment was constructed and sequenced in Shanghai Sangon Biotechnology.

Then the cDNA encoding the humanized scFv was amplified by PCR using primers P_1 and P_2. P_1 for *Eco*RI, 5'-CGGAATTCCAGGTGCAGCTGGTGCAGTCTG-3'; P_2 rev-*Hin*dIII, 5'-CCCAAGCTTCTATTATGATCTCCACCTT GGTCCCTCCGC-3' used to introduce the *Eco*RI and *Hin*dIII restriction sites (underlined) were synthesized in Shanghai Sangon Biotechnology. Then the products were digested with *Eco*RI and *Hin*dIII, and inserted into the *Eco*RI/*Hin*dIII sites of pET32a(+) vector. The resultant plasmid was designated as pET32a-scFv. Furthermore, the pET32a-scFv plasmid was confirmed by restriction enzyme digestions, colony PCR and DNA sequencing, and the clones with an insert orientation preserving the direction of transcription from the T7 promoter were selected.

Expression of scFv gene. The humanized scFv expressing bacteria were subsequently obtained by transformation of the constructed pET32a-scFv recombinant plasmid into the BL21 (DE3) expression strain. LB media (1 ml) was added to the cells and mixed gently. The cells were then incubated at 37˚C for 1 h at 210 rpm in a platform shaking incubator (Bioline Series 4600, Edwards Instrument Co., Australia) before an aliquot of 100 μl was spread onto an LB/amp plate and incubated at 37˚C for 12 h. The positive expression clones were subsequently screened out by colony PCR. The sequences amplified by PCR were confirmed by nucleotide sequencing.

Expression was induced by iso-propyl-β-D-thiogalactoside (IPTG) (Takara Bio, Inc.) at a final concentration of 1.0 mM at 25˚C for various time durations. In order to find the optimal induction conditions, a time course experiment was performed using log phase pET32a-scFv transformed BL21 (DE3) cells, and 1.0 mM IPTG was added to induce protein expression after OD 600 reached 0.4-0.6. Then 1 ml samples were taken at each time-points: 3, 4, 5 and 6 h after induction and lysed by sonication in ice water bath. The cell pellets collected from the protein expression cultures induced for 4 h were lysed with 1XHis binding buffer (20 mM Tris-HCl, 500 mM NaCl, 1 mM imidazole, pH 8.0) and frozen at -80˚C for 30 min. The cell pellets were then sonicated for 5 min and centrifuged at 16,000 x g for 30 min. The clear lysates, including the soluble protein remained in the supernatant and the insoluble protein found in the pellet, were applied to 10% SDS-PAGE for identification of the expression and localization of the protein.

Then 40 ml of overnight culture of pET32a-scFv transformed BL21 (DE3) was used to inoculate to 2 liters of fresh LB/amp medium for large-scale protein production. The cells were cultured at 37˚C for 4 h at 210 rpm in a shaking incubator until OD600 reached 0.4-0.6, and then 1.0 mM IPTG was added to induce protein expression. The culture was incubated for 4 h at 25˚C at 180 rpm before harvesting the cells by centrifugation at 13,000 rpm for 5 min at room temperature. The pellet was reserved at -80˚C.

Protein purification. The scFv protein was purified with nickel-nitrilotriacetic acid (Ni^{2+}-NTA) agarose resin column (Novagen). Frozen cell pellets, harvested from protein expression cultures were thawed for 15 min on ice and suspended with 5 ml of 1XHis binding buffer (20 mM Tris-HCl, 500 mM

NaCl, 1 mM imidazole, pH 8.0) per gram wet weight. The cells were lysed by sonification in ice water mixture for 20 min. The lysate was then centrifuged at 16,000 x g for 30 min at 4˚C to precipitate the cellular debris and the supernatant was filtered through 0.45 μm filters for affinity purification. Then the lysate-resin mixture was mixed gently and rested at 4˚C for 20 min in the column. After two gradient washing with wash buffer 1 (20 mM Tris-HCl, 500 mM NaCl, 10 mM imidazole, pH 8.0) and wash buffer 2 (20 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, pH 8.0), the protein was eluted with 10 ml of elution buffer 1 (20 mM Tris-HCl, 500 mM NaCl, 150 mM imidazole, pH 8.0) and with 10 ml of elution buffer 2 (20 mM Tris-HCl, 500 mM NaCl, 250 mM imidazole, pH 8.0). The eluted scFvs protein were dialyzed against 1X phosphate-buffered saline (PBS) (pH 8.5) at 4˚C to make the concentration of imidazole up to 0 mM and then the protein preparations were filtrated, sterilized and stored at -80˚C.

SDS-PAGE and western blotting. The purity of the purified 6xHis-scFv protein was examined by 10% SDS-PAGE with Coomassie blue G-250 staining. Meanwhile, a BCA protein assay was performed and equivalent amounts of each protein were identified by 10% SDS-PAGE, and then transferred to PVDF membrane and immunoblotted with anti-6xHis primary mouse antibody at a 1:1,000 dilution and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody at a 1:5,000 dilution. The blots were developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce), imaged and analyzed by the Bio-Rad Gel Imaging System.

Cell culture and protein treatment. Human erythroleukemia K562 cells and the basophilic leukemia KU812 cells, both of which were leukemia cells derived from the patients with CML at blast crisis, were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37˚C in a humidified atmosphere of 5% CO_2. Culture media changes were performed routinely, and the exponentially growing cells were used to conduct the following experiments. For the protein functional experiment, K562 cells or KU812 cells ($5x10^5$ cells/ml) were treated with scFv or bovine serum albumin (BSA) (as a negative control) at 37˚C for 6 h at various final concentrations ranging from 0 to 103 μg/ml in the complete medium.

Indirect enzyme-linked immunosorbent assay. The binding activity of the recombinant protein scFv was determined by cell membrane-ELISA (CM-ELISA) using biological active membranes of tumor cells as described by Tur *et al* (21,22). In brief, 96-well plates were coated with 100 μl (~0.9 mg protein/ml) freshly prepared membrane fractions of the CML cell line K562 in 0.02 M bicarbonate buffer (pH 9.6) overnight at 4˚C. The plates were washed three times with PBS (pH 7.4) containing 0.2% (v/v) Tween-20 (TPBS) and blocked with 200 μl 10% fetal bovine serum in PBS. Then 0.01-103 μg/ml scFv or BSA diluted with 10% fetal bovine serum, and 0.05% Tween-20 (v/v) in PBS was added to the plate and incubated at 37˚C for 1 h. After washing with PBS containing 0.05% Tween-20, 100 μl of anti-6xHis mouse monoclonal antibody

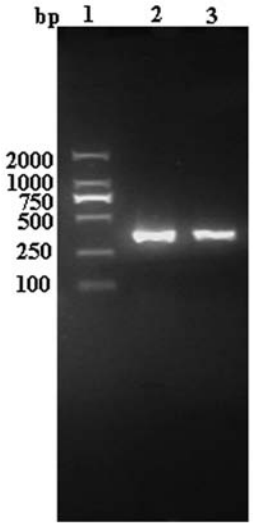

Figure 1. The amplification of VH and VL. Lane 1, DNA ladder (DL2000); lane 2, VH; lane 3, VL.

(ZhongShan GoldenBridge) in 1:1,000 was added to the plates and incubated for 1 h at 37˚C. After washing the plate three times, bound scFv protein was detected with HRP-conjugated goat anti-mouse IgG antibody. The assay was developed using an 3,3',5,5'-tetramethyl benzidine dihydrochloride (TMB) solution (KeHua Co., Shanghai, China) and the absorbance at 450 nm was measured by a microplate reader (Molecular Devices, Ismaning, Germany). The binding activity of scFv was determined by subtracting the absorbance of background binding from the value obtained with a scFv.

Flow cytometric analysis of scFv cancer-targeting ability in vitro. To identify the cancer-targeting ability of anti-CML cells scFv *in vitro*, flow cytometric analysis was performed on the CML cells (K562 and KU812 cells), along with the non-CML related cells (as a negative cell). All cells were respectively collected from culture flasks, washed and resuspended ($1x10^6$ cells/ml) in PBS. After blocking, cells were incubated with anti-CML scFv (100 μg/ml) or BSA (10 μg/ml) for 1 h at 37˚C. After three rounds of washing, anti-6xHis mouse monoclonal antibody was added for 1 h at 37˚C. After washing the plate, a FITC-conjugated goat anti-mouse IgG antibody for scFv was added to each sample and incubated for 30 min at 37˚C. The cells were then washed with PBS and analyzed with FACScalibur flow cytometer (Becton-Dickinson, Cowley, UK).

Results

Cloning of pET32a-scFv. The VH and VL were amplified from cDNA (Fig. 1). By the comparison analysis of three-dimensional conformation structure, the prepared humanized scFv fragment had a similar conformation with the known anti-hepatic cancer cell scFv fragment (GenBank accession no. AY686498), indicating that the humanized scFv fragment was constructed successfully (Fig. 2). Amplification products of approximately 732 bp were obtained using the specific primers for the scFv, which were subsequently cloned into pET32a(+) expression vector. The prokaryotic expression vector pET32a-scFv was successfully constructed as proven

CAGGTGCAGC TGGTGCAGTC TGGGGCTGAG GTGAAGAAGC CTGGGGCCTC
AGTGAAGGTC TCCTGCAAGG CTTCTGGCTA CACGTTCACC AGCTACTGGA
VH-CDR1
TGCACTGGGT GCGACAGGCC CCTGGACAAG GGCTTGAGTG GATGGGATGG
ATTGATCCTT ACGATAGTGA AACTAACTAT GCACAGAAGT TTCAGGGCAG
VH-CDR2
GGTCACCATG ACCAGGGACA CGTCCATCAG CACAGCCTAC ATGGAGCTGA
GCAGGCTGAG ATCTGACGAC ACGGCCGTGT ATTACTGTGC AAGATATGGT
TACGACGGGA CGGGGTTTGC TTACTGGGGC CAAGGGACCA CGGTCACCGT
VH-CDR3
CTCCTCATCA GGAGGAGGCG GATCCGGAGG CGGTGGATCT GGAGGCGGTG
$(G4S)_3$-Linker
GATCCGACAT TGTGCTGACC CAGTCTCCAG CCTCCTTGGC CGTGTCTCCA
GGACAGAGG GCCACCATCA CCTGCAGAGC CAGTAAAAGT GTCAGTACAT
VL-CDR1
CTGGCTATAG TTATATTCAC TGGTATCAGC AGAAACCAGG ACAACCTCCTA
AACTCCTGAT TTACCTTGTA TCCAATAAAG ACACTGGGGT CCCAGCCAGG
VL-CDR2
TTCAGCGGCA GTGGGGTCGG GACCGATTTC ACCCTCACAA TTAATCCTGT
GGAAGCTAAT GATACTGCAA ATTATTACTG TCAGCACATT AGGGAGCTTA
VL-CDR3
CACGTTCGGC GGAGGGACCA AGGTGGAGAT CA

Figure 2. Nucleotide sequences of the humanized scFv.

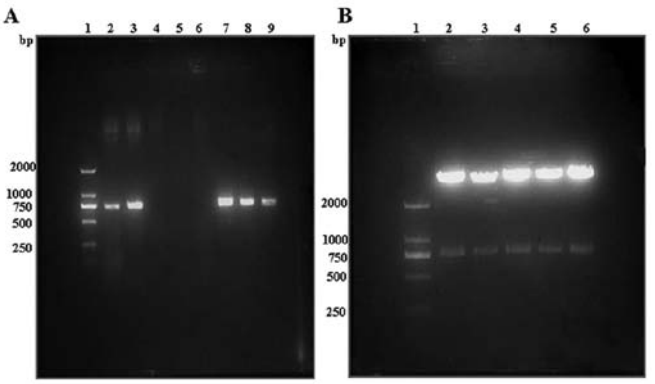

Figure 3. Cloning of the target pET32a-scFv. (A) Bacterial colony PCR for the detection of the DH5α clones. Lane 1, DNA ladder (DL2000); lanes 2-3, and 7-9, positive bacterial colonies for pET32a-scFv; lanes 4-6, the negative bacterial colony pET32a-scFv. (B) Double endonuclease digestion of the prokaryotic expression vector pET32a-scFv. Lane 1, DNA ladder (DL2000); lanes 2-6, double digestion with *Eco*RI/*Hin*dIII.

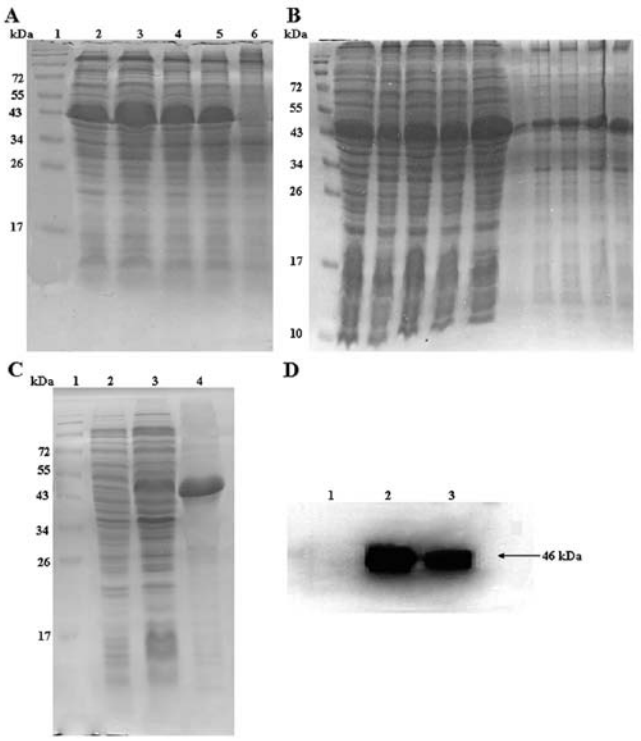

Figure 4. Expression and purification of the scFv protein in *E. coli* containing pET32a-scFv. (A) Time course analysis of scFv protein expression 3-6 h after IPTG induction by SDS-PAGE. Samples of bacterial culture were taken every hour starting at 3 h and up to 6 h after IPTG induction. The cell pellets from each time point were suspended in 1X SDS protein loading buffer, boiled for 5 min at 100˚C, centrifuged at 10,000 x g for 10 min and the supernatant was collected for 10% SDS-PAGE analysis. Lane 1, molecular weight standards; lanes 2-5, hourly induction samples (3-6 h); lane 6, uninduced culture. (B) Solubility analysis of the scFv protein by 10% SDS-PAGE. The cell pellets collected from the protein expression cultures induced for 4 h were lysed, sonicated and centrifuged. The clear lysate was analyzed by SDS-PAGE as the soluble fraction and the remaining cell pellet as the insoluble fraction. Lane 1, molecular weight standard; lanes 2-6, soluble fraction; lanes 7-11, cell pellet. (C) Purification of the his-tagged scFv recombinant protein by Ni^{2+}-NTA metal affinity chromatography. Lane 1, molecular weight standard; lane 2, uninduced bacterial lysate; lane 3, IPTG induced culture; lane 4, purified protein. (D) Detection of scFv by western blotting using anti-6xHis antibody. Lane 1, uninduced bacterial lysate; lane 2, IPTG induced culture; lane 3, purified protein.

by bacterial colony PCR (Fig. 3A), restriction enzyme digestions (Fig. 3B), and complete sequencing (Fig. 2).

Expression and purification of the scFv recombinant protein. The prokaryotic expression vector pET32a-scFv was transformed into the *E. coli* BL21 (DE3) expression host strain for protein overexpression. A small-scale time course experiment was performed to determine the kinetics of protein expression in the bacterial culture. It was determined that the cells should be harvested at 4 h after IPTG induction, as the maximum amount of the correct 46 kDa size soluble scFv protein which contained the His-tag protein was produced at this time point (Fig. 4A). The solubility of the expressed protein at 4 h was further investigated in detail. Cell pellets were abruptly frozen on dry ice and underwent a freeze thaw cycle. The cells were then sonicated (Branson Sonifier 250, Branson, Danbury, CT, USA) and the sample was clarified by centrifugation to separate the clear lysate containing the soluble protein from the cell debris pellet. Most of the target protein was detected in the clear lysate (Fig. 4B). Therefore, the recombinant 6xHis-scFv protein was expressed in a soluble form in the bacterial cells. The scFv protein was expressed as a recombinant fusion protein with His-tag, consisting of scFv and other for His-tag and its upstream sequence, which was consistent with the theoretically predicted value (46 kDa). The expression and purification was identified by 10% SDS-PAGE gel and Coomassie brilliant blue staining (Fig. 4C). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed that the purity of the prepared scFv was >90% and the concentration was 7.8 mg/ml, which demonstrated its potential usefulness in clinical application. Moreover, the expression and purification of the recombinant protein was validated by immunoblotting (Fig. 4D) using anti-6xHis antibody.

Cell membrane binding activity of the recombinant scFv antibodies. The anti-CML cells scFv, prepared by immunizing BALB/c mice with CML patient cells, can be used for

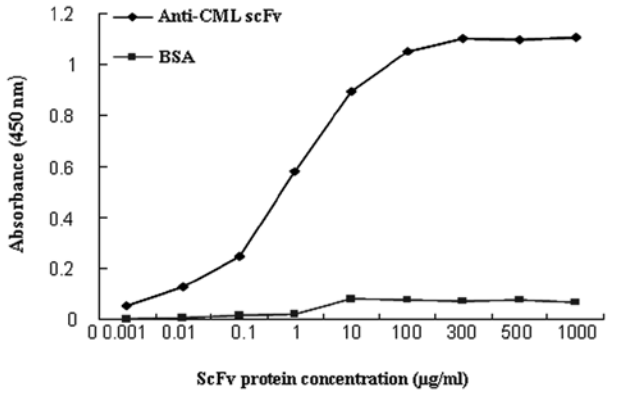

Figure 5. Analysis of the cell membrane binding activity of purified anti-CML cells scFv by CM-ELISA. A 96-well microtiter plate was coated with the freshly extracted cell membrane fractions and probed with various concentrations of anti-CML cells scFv. Anti-CML cells scFv was purified from *E. coli* cultures and its binding activity to antigen (CML cell surface antigen) was analyzed by ELISA. BSA was used as a negative control.

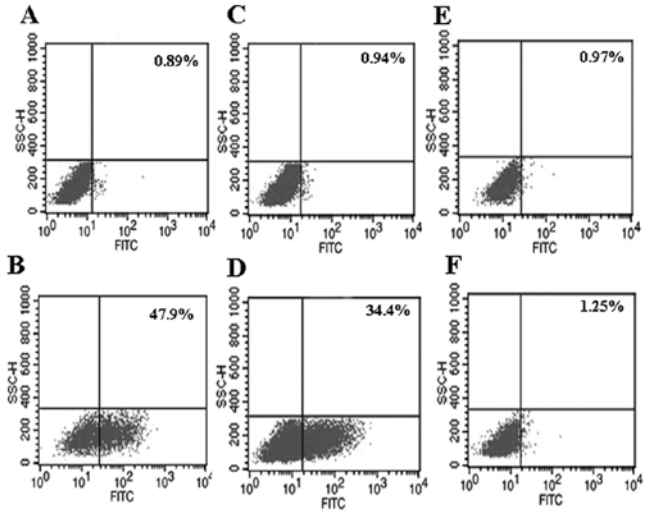

Figure 6. Flow cytometric analysis for cancer-targeting activity of anti-CML cells scFv *in vitro*. K562, KU812 were incubated with anti-CML cells scFv or BSA and then incubated with mouse anti-6xHis monoclonal antibody, and accordingly stained with FITC-conjugated anti-mouse IgG for scFv and analyzed. HEK293 cells as a negative control. The fluorescence on cells stained with anti-CML cells scFv or BSA was shown. The binding rates of K562 and KU812 cells with scFv were (A and B) 47.9%, (C and D) 34.4%, respectively, while that of HEK293 cell was (E and F) only 1.25%. (A, C and E) The cells were treated with BSA and (B, D and F) were treated with anti-CML cells scFv.

membrane targeting of fusion proteins. To identify whether the scFv recognizes and has high binding activity to the CML cell membrane components, CM-ELISA was performed. The ELISA plate was coated with freshly prepared membrane fractions of the CML cell line K562 and detected by incubation with anti-6xHis mouse monoclonal antibody and HRP-conjugated goat anti-mouse IgG antibody. The results of CM-ELISA, revealed that the anti-CML cells scFv showed high binding activity to the cell membrane of K562 compared with the control (Fig. 5).

Cancer-targeting ability of anti-CML scFv in vitro. To identify the different expression rate of the targeting antigen on K562, KU812 and the non-CML related cells, flow cytometric analysis was conducted. Anti-CML cells scFv interacted with 47.9% of K562 cells (Fig. 6A and B) and 34.4% of KU812 cells (Fig. 6C and D) versus 1.25% of the non-CML related cells (Fig. 6E and F), which demonstrated that the targeting antigen was highly expressed on CML cells (K562 and KU812 cells) and there was hardly any expression on the non-CML related cells.

Discussion

In the recent years, recombinant antibody fragments against tumor cell surface antigens have been produced successfully for tumor diagnostic and therapeutic applications (23). With the advances of gene recombinant technology and other biotechniques, antibody preparing, screening and the cost-effectiveness of large-scale antibody production have acquired great progress in this field. The products originated from scFvs could allow us to avoid the major limitations previously observed when administering murine intact mAbs to cancer patients. A number of scFv-based biological products are continuously entering clinical trials but many aspects remain to be elucidated and procedure improvements are needed before the scFvs could be routinely used for diagnostic and therapeutic applications. Among the most critical issues, careful monitoring of toxicity, improvement of the scFv capacity of penetrating tumors and deeper characterization of immunoregulatory pathways and molecular mechanisms of growth regulation are also needed for further study. Also, the potential of scFvs as diagnostic reagents has not been fully explored.

Likewise, the use of mAbs has greatly improved the treatment of patients with lymphoma and leukemia. Rituximab (anti-CD20) was the first mAb developed for the treatment of B-cell lymphomas and has significant activity in chronic lymphocytic leukemia (CLL) (24). In recent years, many antibodies are developed for hematologic tumors, for example alemtuzumab (anti-CD52) for untreated or refractory CLL patients (25); gemtuzumab ozogamicin (GO, anti-CD33) for acute myeloid leukemia and myelodysplastic syndromes (26); and ofatumumab (Hu-Max-CD20) (27). Many mAbs developed against all these antigens have been further developed in the scFv format to improve therapeutic performance in patients (28,29).

There is a high incidence of CML. The most effective treatment for CML is allogeneic hematopoietic stem cell transplantation, but age, physical condition and other factors limit the application of this method, and graft-versus-host disease, the incidence of opportunistic infections and other complications may lead to death. Although BCR/ABL tyrosine kinase inhibitors can specifically inhibit BCR/ABL protein-positive leukemia cell proliferation and induce apoptosis, patients still may have the possibility of relapse or resistance, leading to irreversible malignant progression. Therefore, exploring other more effective treatments is urgently needed for the treatment of CML. In a previous study, we immunized BALB/c mice with CML patient cells to prepare a monoclonal antibodies library against CML cell surface antigens (unpublished data). From these prepared monoclonal antibodies, we screened one of them specifically against CML patient cells to prepare our anti-CML cells scFv. In this study, we have cloned,

overexpressed, purified, retrieved, and identified the prepared humanized anti-CML cells scFv in *E. coli*.

However, expression of foreign proteins at high levels in *E. coli* often results in the formation of inclusion bodies (30), which are composed of insoluble aggregates of the expressed protein. Generally, procedures such as washing, denaturation, purification, refolding and concentration are needed in order to obtain an effective target recombinant protein (31). The prepared scFv protein expressed mostly in the soluble form which suggested that the *E. coli* protein expression system can effectively be used for producing humanized scFv. Besides, the T7 promoter system of pET32a(+) has the advantage of high-level expression, which was an important factor to have scFv expression at high levels. Here, we have selected an anti-CML cell scFv based on its ability binding to the CML cells *in vitro*. After sequencing, it was demonstrated that it is composed of the CDRs of the mouse antibody and the FRs of the human antibody with the highest homology. Although CDR grafting could make antibodies less immunogenic and could reduce affinity compared to the parent murine antibody (32), we have found that the selected scFv has high specificity and affinity to CML cells in this study. This scFv maintains its antigen-binding activity as shown in flow cytometric and ELISA analyses. Moreover, anti-CML cells scFv has cancer-targeting activity to CML cells *in vitro*, as it can specifically bind to human erythroleukemia K562 cells and the basophilic leukemia KU812 cells but not to non-CML related cell line. In our further studies, some other remodeling methods of antibodies, such as resurfacing, could be considered to increase the affinity of scFv.

Furthermore, as shown in flow cytometric analyses, anti-CML cell scFv is capable of targeting CML cells, while there is no binding to non-CML tumor cells. The *in vitro* binding of anti-CML cell scFv to CML cell lines shows promise for targeting CML cells *in vivo*. Further studies are underway to test the cancer-targeting potentials of anti-CML cells scFv *in vivo*. Moreover, they can be used to develop therapies based on targeting delivery strategies. For example, the scFv can be fused with drugs or drug-loaded nanoparticles, engineered toxin molecules, or appropriate radionuclides to obtain a targeted cell killing function (33,34). These targeted agents could offer an opportunity to determine if *in vivo* eliminating of tumor-initiating cells results in improved therapeutic effects such as suppression of recurrence in theory.

In conclusion, we have characterized a highly efficient model for the generation of humanized scFv with potential clinical diagnostic and therapeutic applications. We constructed a humanized scFv from a selected anti-CML patient cells monoclonal antibody library. Our results suggest that this constructed scFv has high specificity and affinity to CML cells and can specifically bind to CML cells. In future, anti-CML scFv can be conjugated with drugs or drug-loaded nanoparticles, engineered toxin molecules, or appropriate radionuclides to impart a targeted cell killing function for CML diagnosis and therapeutics.

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