Selection of scFvs specific for the HepG2 cell line using ribosome display

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The aim of this study was to construct a ribosome display library of single chain variable fragments (scFvs) associated with hepatocarcinoma and screen such a library for hepatocarcinoma-binding scFvs. mRNA was isolated from the spleens of mice immunized with hepatocellular carcinoma cell line HepG2. Heavy and k chain genes (VH and k) were amplified separately by RT-PCR, and an anti-HepG2 VH/k chain ribosome display library was constructed by assembling VH and k into the VH/k chain with a specially constructed linker by SOE-PCR. The VH/k chain library was transcribed and translated *in vitro* using a rabbit reticulocyte lysate system. In order to isolate specific scFvs, recognizing HepG2 negative selection on a normal hepatocyte line WRL-68 was carried out before three rounds of positive selection on HepG2. After three rounds of panning, cell enzyme-linked immunosorbent assay (ELISA) showed that one of the scFvs had high affinity for the HepG2 cell and lower affinity for the WRL-68 cell. In this study, we successfully constructed a native ribosome display library. Such a library would prove useful for direct intact cell panning using ribosome display technology. The selected scFv had a potential value for hepatocarcinoma treatment.

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1. Introduction

Hepatocarcinoma, a form of cancer originating in the liver cells, is a challenging malignancy with high patient mortality rates (Melén *et al.* 2000). Finding a high-affinity antibody against hepatocarcinoma is an important way of detecting the development of hepatocarcinoma early and providing preoperative treatment.

Antibodies are the most widely used class of reagents for research, pharmaceutical, diagnostic and therapeutic applications (van Dijk and van de Winkel 2001; Taussig *et al.* 2007). Ribosome display technology offers an efficient and flexible route to the generation of recombinant antibodies (He and Taussig 1997; Hanes *et al.* 1998; He *et al*. 1999; Yau *et al.* 2003), and is a fully cell-free display method, which links nascent single-chain antibodies and their encoding mRNA as antibody–ribosome–mRNA (ARM) complexes. By interaction with an immobilized antigen, the mRNA is enriched for those antibodies that have the desired property (of binding the antigen) and can be enormously amplified by reverse transciptase-polymerase chain reaction (RT-PCR).

Screening antibodies or peptides directly on intact cells is an important method of isolating antibodies or peptides that bind to the cells (Zhu *et al.* 2008; Gao *et al.* 2003). This has the important advantage of requiring no prior knowledge of the cellular receptor. Furthermore, it leads to the eventual discovery of cell surface-specific antigens that may not

Keywords. Hepatocarcinoma; ribosome display; single-chain antibody

Abbreviations used: ARM, antibody–ribosome–mRNA; BSA, bovine serum albumin; CDR, complementarity-determining region; DMEM, Dulbecco modified Eagle high glucose medium; ELISA, enzyme-linked immunosorbent assay; FBS, foetal bovine serum; IPTG, isopropyl-b-D-thiogalactopyranoside; PBS, phosphate buffered saline; RT-PCR, reverse transcriptase-polymerase chain reaction; scFv, single-chain variable fragment; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; VH, variable region of heavy chain

have been considered as viable targets or have not yet been identified.

Probably due to the instability of the ARM complexes, ribosome display technology has not yet been used for intact cell panning. We applied ribosome display technology in this study to directly pan single-chain variable fragments (scFvs) on the hepatocellular carcinoma cell line HepG2, intending to obtain a specific novel scFv that bound to the cell surface.

2. Materials and methods

2.1 *Cell lines, BALB/c mice and bacterial strains*

The hepatocellular carcinoma cell line HepG2 and normal hepatocyte line WRL-68 were cryopreserved in our laboratory. HepG2 cells and WRL-68 cells were incubated in Dulbecco modified Eagle high glucose medium (DMEM) supplemented with 10% foetal bovine serum (FBS) at 37°C.

Female BALB/c mice (6 weeks old) were purchased from the Model Animal Research Center of Nanjing University.

Escherichia coli DH5*α* was used as the recipient of recombinant plasmids, and *E. coli* BL2l (DE3) was used as the host to produce scFvs.

2.2 *Immunization of mice*

Female BALB/c mice were given an intraperitoneal injection of HepG2 cells (1×10^6 cells/mouse, diluted in phosphate buffered saline [PBS]) once every 2 weeks to a total of 4 injections. Sera were collected for enzyme-linked immunosorbent assay (ELISA). The spleen was removed from the immunized mouse with the highest antibody titre.

2.3 *Construction of a VH/k chain library*

Total RNA was prepared from spleen cells using a Trizol reagent kit (Takara, Dalian, China). cDNA was synthesized with oligo (dT) primer using the PrimeScript™ 1st cDNA synthesis kit (Takara Bio, Dalian, China). DNA encoding the mouse variable region of heavy chain (VH) and k chain was obtained by PCR. Primers T7Ab/back (5′ GCAGCTAA TACGACTCACTATAGGAAGAACAGACCACCATG(C/G)AGGT(G/C)CA(G/C)CTCGAG(C/G)AGTCTGG 3′) (He and Taussig 2005) and VH/for (5′ TGAGGAGACGGTGAC CGTGGTCCCTTGGCCCC 3′) (He and Taussig 2005) were used for amplification of DNA encoding the VH; primers Vk/back (5′ GACATTGAGCTCACCCAGTCTCCA 3′) (Lee *et al.* 2005) and Ck/for (5′ GCTCTAGAACACTCA TTCCTGTTGGAGCT 3′) (Lee *et al.* 2005) were used for amplification of DNA encoding the k chain. The amplified VH and k were assembled using overlap extension PCR by a linker containing a sequence encoding $(Gly_4Ser)_3$ (5′ GTCACCGTCTCCTCAGGTGGTGGTGGTAGCGGC GGCGGCGGCTCTGGTGGTGGTGGATCCGACATTGA GCTCACCC 3′).

2.4 In vitro *transcription and translation*

Microtitre plates were coated overnight at 37°C with 1×10^5 HepG2 cells and WRL-68 cells. The coated plates were washed with PBS and blocked with sterilized 5% skim milk in PBSM (PBS with 5 mM $MgCl_2$) for 30 min at room temperature. After washing with PBS, the plates were blocked again with blocking buffer (1% BSA in PBSM) for 2 h. The plates were then washed thrice with PBSM and incubated on ice for at least 10 min.

TNT T7 Quick for PCR DNA kit (Promega, Madison, USA) was used for *in vitro* transcription and translation. The reaction volume was 25 μl and the reaction mixture contained 20 μl TNT Quick Master Mix, 0.02 mM methionine, 1 mM magnesium acetate, 10–100 ng VH/k PCR model. Sterile water treated with diethyl pyrocarbonate was added up to 25 μl. After 60 min of *in vitro* transcription and translation at 30°C, the mixture was immediately added to a tube containing ice-cold buffer (PBS containing 5 mM $MgCl_2$ and 1% BSA). This mixture was added to the prepared microtitre well coated with WRL-68 cells and incubated for pre-binding. After pre-binding, the supernatant was transferred to a well coated with HepG2 cells. The plate was incubated on ice for 1 h. After washing thrice with ice-cold PBSTM (PBS containing 5 mM $MgCl_2$ and 0.05% Tween-20) and washing twice with ice-cold PBSM, the retained ribosomal complexes were dissociated with 200 μl elution buffer (PBS containing 20 mM EDTA) for 10 min on ice. The mRNA was isolated from the eluted solution using EZ Spin Column RNA Purification kit (BBI, Ontario, Canada), as described by the manufacturer.

2.5 *RT-PCR*

Selected mRNA was reverse transcribed to cDNA using the PrimeScript™ 1st cDNA synthesis kit. The cDNA obtained was amplified by nested PCR in a 25 μl PCR mixture for 30 cycles (1 cycle is 50 s at 94°C, 50 s at 59°C, 2 min at 72°C) with T7Ab/back and D1 (5′ CGTGAGGGTGCTGCTCAT 3′) (He and Taussig 2005) instead of Ck/for using Taq DNA polymerase. We then used a different downstream primer for the subsequent cycle: D2 (5′ GGGGTAGAAGTTGTTCAAGAAG 3') (He and Taussig 2005). After the third panning cycle, VH/back (5′ (C/G)AGGT(G/C)C A(G/C)CTCGAG (C/G)AGTCTGG

3′) and Vk/for (5′ TGCAGCATCAGCCCGTTT 3′) were used for RT-PCR.

2.6 *Cloning and expression*

After three cycles of affinity selection, the selected scFv DNA was inserted into the pEASY-E1 expression T vector (Transgen, Beijing, China) and transformed into *E. coli* DH5*α*. To identify if the individual clone contained the scFv DNA with the correct orientation of the insert, colony PCR was carried out using the vector-specific upstream primer T7 Promoter (5′ TAATACGACTCACTATAGGGGA 3′) and scFv-specific downstream primer Vk/for. Plasmid DNA with the correct orientation of the insert was isolated and transformed into *E. coli* BL21 (DE3). The scFv proteins were induced and expressed from each clone. To purify recombinant scFv proteins, bacteria were centrifuged, re-suspended in binding buffer (8 M urea, 0.5 M NaCl, 5 mM imidazole, 20 mM Tris-HCl, pH 7.9) and then sonicated. The supernatants were applied to a His-bind resin column and bound antibodies were recovered according to the manufacturer's instructions. The eluent was dialysed against refolding buffer I (2 mM reduced glutathione and 0.2 mM oxidized glutathione) at 4°C overnight, and dialysis was then continued against refolding buffer II (20 mM Tris-HCl, 0.5 M NaCl, pH 7.9) at 4°C for 4 h. The buffer was changed and dialysis continued for another 4 h. Purified antibodies and bacteria were electrophoresed through a standard denaturing 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The purified scFvs were quantitated by the Bradford method (Bradford 1976).

2.7 *Cell ELISA for purified scFvs*

Microtitre plates were coated with HepG2 cells and WRL-68 cells as mentioned above. The purified scFvs were placed in each well and incubated for 1 h at 37°C. After washing five times with PBST, monoclonal antipolyhistidine (Promega, Madison, USA) was added and incubated for 1 h at 37°C. After washing five times with PBST, goat peroxidase-conjugated anti-mouse IgG (Calbiochem, Darmstadt, Germany) was added and developed by tetramethyl benzidine to measure the absorbance value at 450 nm.

3. Results

3.1 *Cloning and sequencing of VH and k chains*

VH and k chains were amplified by RT-PCR (figure 1). The amplified VH and k chains were cloned into the pMD18-T vector (Takara, Dalian, China) and sequenced. The lengths of VH and k were 399 bp and 647 bp, respectively. Comparison of VH and k with the IMGT/V-Quest database showed that VH and Vk belonged to the mouse Ig heavy family VH5 and light family Vk4/5, respectively.

3.2 *Construction, cloning and sequencing of VH/k*

VH and k were assembled into VH/k DNA fragments which were used for the preparation of mRNA (figure 2). VH/k DNA fragments were cloned into the pMD18-T vector and sequenced. The length of VH/k was 1093 bp. Comparison of VH/k with the IMGT/V-Quest database showed that VH/k belonged to the mouse Ig family VH1+Vk4/5.

The T7Ab/back primer contained a T7 promoter and ribosome-binding site, and the Ck/for primer had no stop codon. The Ck region of the k chain acted as a spacer that tethered the synthesized protein to the ribosome and supported the proper folding of scFv. An assembled VH/k chain DNA was used for the preparation of mRNA.

3.3 *Identification of feasibility by RT-PCR after panning*

After the first cycle of panning on intact cells, the eluted RNA was purified and amplified by RT-PCR (figure 3). However, during the panning process, it was possible that the HepG2 cells and WRL-68 cells had been lysed and released DNA or RNA, which might form a false scFv gene band. In addition, a false scFv gene band could also be formed if the VH/k chain library model had not been washed completely during the washing process. Thus, purified RNA that had not been reverse transcribed was used as a control. PBS was used as another control, which replaced the transcription and translation mixture during panning. The results showed that no false band had been formed.

3.4 *Cloning and expression of scFvs*

The scFv DNA selected after the third round was inserted into the expression vector pEASY-E1. After transformation in *E. coli* DH5*α*, 16/48 colonies contained the scFv DNA with the correct orientation of the insert, identified by colony PCR. Positive expression vectors were then isolated from *E. coli* DH5*α* and transformed into *E. coli* BL21 (DE3).

After induction, *E. coli* lysate of 16 tested clones was run on 15% SDS-PAGE, among which 5 tested clones expressed the scFv of approximately 31 kD and one clone expressed a truncated scFv of approximately 25 kD. We surmised that the non-expression or truncated expression of scFv was probably due to mutation or misfolding (Rothe *et al.* 2007).

One of the 5 clones was induced to identify the position of the expressed scFv proteins in *E. coli*. The figure indicated that the expressed proteins largely existed in inclusion form (figure 4). The expressed proteins were purified and each showed a single band on SDS-PAGE (figure 4).

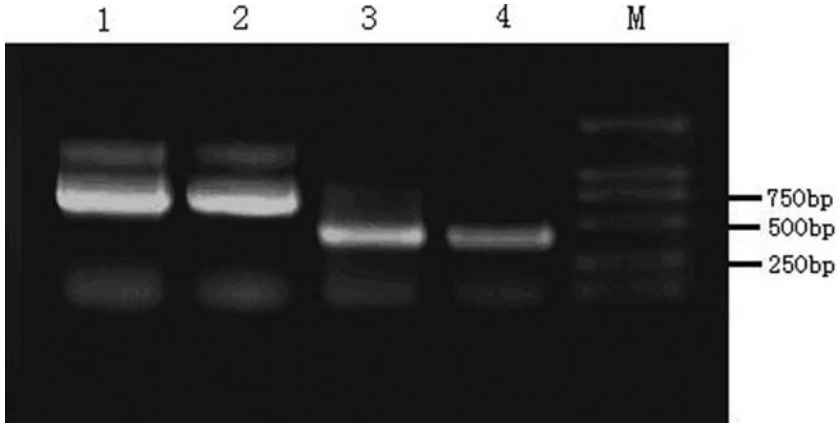

Figure 1. Agarose gel electrophoresis of the PCR product of VH and k. VH DNA and k chain DNA were separately amplified by RT-PCR from the total RNA of spleen cells. M, DNA marker DL 2000; 1 and 2, k fragment; 3 and 4, VH fragment

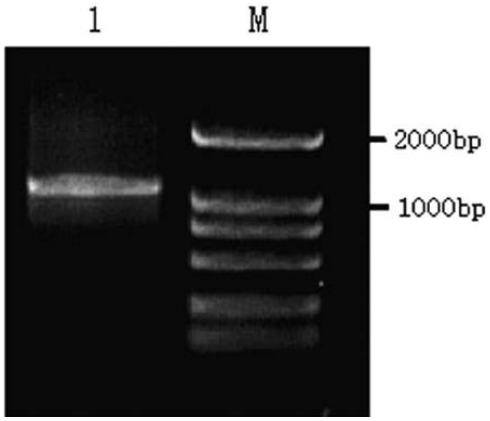

Figure 2. Agarose gel electrophoresis of the PCR product of VH/k. VH and k were assembled into VH/k DNA fragments by a linker containing $(Gly_4Ser)_3$. M, DNA marker DL 2000; 1, VH/k fragment

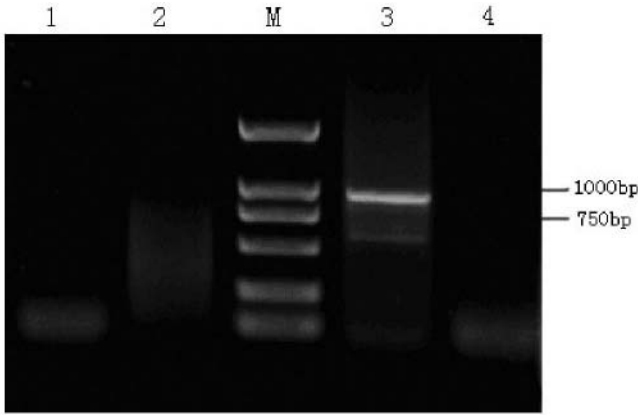

Figure 3. Agarose gel electrophoresis of PCR after the first cycle of panning. After selection, the eluted RNA was amplified by RT-PCR and comparisons were made to confirm whether the potential DNA or RNA was released from lysed cells or the VH/k chain library model had not been washed completely and would give false results. M, DNA marker DL 2000; 1, No model control; 2, Purified RNA as model; 3, Synthesized cDNA as model; 4, PBS panning control. This result indicates that no DNA or RNA released from the lysed cells was amplified and the VH/k chain library model had been washed completely.

3.5 *Characterization of purified scFvs*

The affinity of the 5 scFvs was tested by cell ELISA (figure 5). The result showed that all the purified scFvs of the 5 clones had binding activity to HepG2 cells. However, clone 3 showed the highest binding activity to HepG2 cells and relatively low binding activity to WRL-68 cells.

4. Discussion

Tumour-targeting therapy can be an efficacious way of curing a malignant tumour. In this study, we have described the selection of HepG2-specific scFvs by ribosome display. HepG2 cells were used to immunize mice and a DNA library of its VH/k antibody fragments was constructed using a PCR-based recombination method and spleen from immunized mice. Using this DNA library, we were able to show HepG2-specific selection and enrichment by ribosome display. After three rounds of selection, the selection pool was cloned into an expression vector and each clone was identified by ELISA. One scFv showed specific binding activity to HepG2 cells.

Ribosome display technology has some potential advantages over *in vivo* display. These include an increase in library, ease of affinity maturation, speed of display, selection recovery of genotype and potential for synthesis of molecules toxic to *in vivo* expression systems (Irving *et al.* 2001). In spite of the advantages of ribosome display technology, to the best of our knowledge, no report has been published about

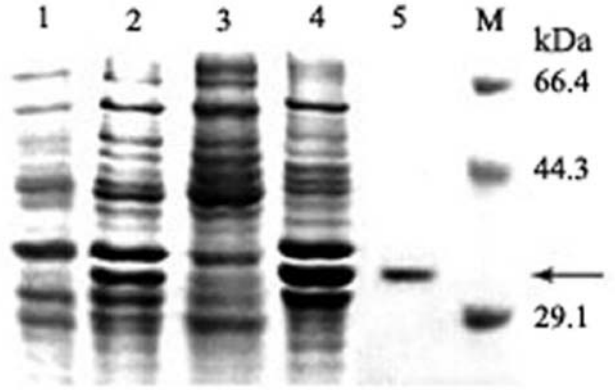

Figure 4. SDS-PAGE analysis of expressed and purified proteins. The position of the expressed scFv proteins in *Escherichia coli* was identified by SDS-PAGE. M, Protein molecular weight marker (low); 1, Non-induced clone; 2, Induced whole-cell extract; 3, Induced cell supernatant; 4, Induced cell inclusion; 5, The purified scFv proteins with Ni affinity chromatography.

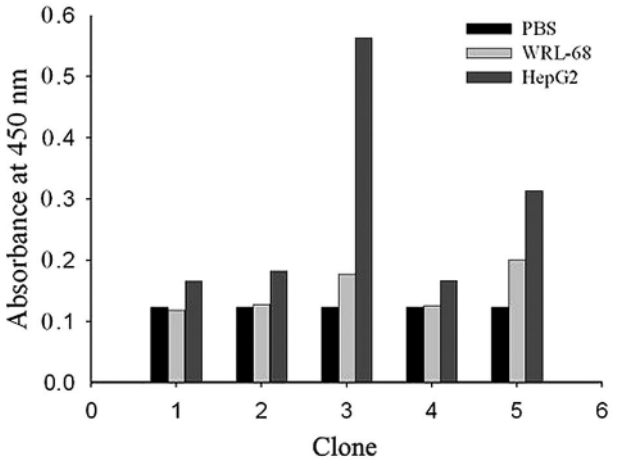

Figure 5. Characterization of individual selected clones from round 3. The purified scFvs from each clone were tested against WRL-68 cells and HepG2 cells by cell ELISA.

cell-based panning using ribosome display. This is probably due to the fragility of ARM complexes to potential DNase, RNase or protease released by cell lysates. After each of the three panning cycles, the correct band on agrose gel by RT-PCR demonstrated that ribosome display technology could be used for intact cell panning. In addition, we also confirmed that the potential DNA or RNA released from lysed cells was not amplified by PCR after each panning cycle.

We used an internal primer for the RT-PCR. It has been reported that the results are much better with an internal primer than with a 3′ primer (He and Taussig 2005; Lee *et al.* 2005). After three cycles of panning, the scFv repertoire was cloned into the pEASY-E1 expression T vector and transformed into *E. coli* DH5*α*. After 12 h at 37°C, more than 200 clones would be expected to develop. While *E. coli* BL21 (DE3) was directly transformed with the ligation mix, it yielded no clones. This demonstrated that *E. coli* BL21 (DE3) had relatively lower transformation efficiency for the pEASY-E1 expression vector than *E. coli* DH5*α*. Thus, in our study, the ligation mix of the scFv repertoire and pEASY-E1 expression T vector was first transformed into *E. coli* DH5*α*, and the positive clones identified by colony PCR were isolated and transformed into *E. coli* BL21 (DE3).

The scFv that we have constructed here is the smallest antigen-binding antibody fragment consisting of VH and k joined together by a flexible peptide linker $(Gly_4Ser)_3$. The binding affinity of the scFv molecules resides at the surface presenting the variable heavy and variable light domains, each of which contributes three complementarity-determining regions (CDRs). The binding face therefore comprises six CDRs. The scFv format was chosen because of its probable advantage for the treatment of solid cancers (Verhaar *et al.* 1995). Because of their small size, these proteins penetrate faster and more deeply into tissues and clear more rapidly from the blood than whole immunoglobulin G or antigen-binding fragments. Also, the lack of constant regions mitigates retention by Fc receptors found in most tissues and organs, which further reduces their possible side-effects.

In conclusion, this study describes, possibly for the first time, the selection of antibodies against complete HepG2 cells from a native scFv gene library using ribosome display technology. The scFvs generated after three cycles of panning preferably bound to HepG2 cells rather than to WRL-68 cells. One of the selected scFvs had significant potential value for the targeted treatment of liver cancer. However, further studies are needed to investigate the binding specificity of the selected scFv to human hepatoma tissues and the application of the selected scFv in clinical oncology.

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