Homology modelling and bivalent single-chain Fv construction of anti-HepG2 single-chain immunoglobulin Fv fragments from a phage display library

MING NI[3], BING YU[*1], YU HUANG[2], ZHENJIE TANG[2], PING LEI[2], XIN SHEN[2], WEI XIN[2], HUIFEN ZHU[2] and GUANXIN SHEN[2]

[1]*Department of Pathogen Biology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China*

[2]*Department of Immunology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China*

[3]*Department of Infectious Diseases, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China*

Corresponding author (Email, andybing666@hotmail.com)

We prepared single-chain immunoglobulin Fv fragments (scFv) SLH10 specific for the HepG2 cell line after biopanning from a large human-naïve phage display library (Griffin. 1 Library). The three-dimensional (3D) structure of SLH10 was modelled by the Insight II molecule simulation software. The structure was refined using the molecular dynamics method. The structures with the least steric clashes and lowest energy were determined finally. The optimized structures of heavy (VH) and light (VL) variable chains of SLH10 scFv were obtained. Then SLH10 bivalent single-chain Fv (BsFv) was constructed that would be suitable for high-affinity targeting. SLH10 BsFv was generated by linking scFvs together and identified by sequencing. Its expression products were confirmed by western blot analysis. The relative molecular masses of scFv and BsFv were approximately 30 kDa and 60 kDa, respectively. Flow cytometry revealed that SLH10 BsFv bound the selected cell lines with greater signal intensity than the parental scFv. The improved antigen binding of SLH10 BsFv may be useful for immunodiagnostics or targeted gene therapy for liver cancer.

[Ni M, Yu B, Huang Y, Tang Z, Lei P, Shen X, Xin W, Zhu H and Shen G 2008 Homology modelling and bivalent single-chain Fv construction of anti-HepG2 single-chain immunoglobulin Fv fragments from a phage display library; *J. Biosci.* **33** 691–697]

1. Introduction

Single-chain immunoglobulin (Ig) Fv fragments (scFvs) are predominantly monomeric when the linker variable regions of the heavy (VH) and light (VL) chains are joined by polypeptide linkers (Adair 1992). scFvs maintain the binding specificity of the parental antibody; they are in small size and show improved properties for diagnostic and therapeutic applications (Presta 2003; Holliger and Hudson 2005 . scFvs have increased tumour penetration and faster clearance rates compared with the parent Ig (Beckman 2007; Carter 2001). They can also be used to create new forms of Fv modules of increased valency suited for *in vivo* imaging and therapy (Volkel *et al* 2001; Hudson *et al* 1999; Turner

Keywords. BsFv; homology modelling; scFv; three-dimensional (3D) structure

Abbreviations used: AMBER, assisted model building and energy; BSA, bovine serum albumin; BsFv, bivalent single-chain Fv; CDR, complementary determining region; CVFF, consistent valence force field; 3D, three-dimensional; FBS, foetal bovine serum; FITC, fluorescein isothiocyanate; FWR, framework region; HRP, horseradish peroxidase; Ig, immunoglobulin; IMAC, immobilized metal affinity chromatography; IPTG, isopropyl β-D-thiogalactoside; MPBS, milk phosphated-buffered saline; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PDB, Protein Data Bank; scFv, single-chain immunoglobulin Fv fragments; VH, heavy variable; VL, light variable

et al 1997). A simple method is to combine two scFvs to form a bivalent dimer (diabody) (Kortt *et al* 1997; Kortt *et al* 2001). A number of cancer-targeting scFv multimers have recently undergone pre-clinical evaluation for *in vivo* stability and efficacy, and have been shown to provide a significant increase in functional affinity (Peterson *et al* 2008). On the other hand, it is important to know more about the structural and functional role of the scFv residue. By computational approaches, combined sequence inspection and protein structural analysis can effectively provide relevant information about the functional role of the scFv residue. Knowledge at the atomic level of three-dimensional (3D) structure, which is, in turn, the main determinant of biological function, allows one to investigate or predict antibody properties. In this study, we prepared scFv SLH10 specific for the HepG2 cell line after biopanning from a large human-naïve phage display library (Griffin. 1 Library) (Yu *et al* 2005). We examined the functional characteristics of SLH10 through homology modelling of its 3D structure by the Insight II molecule simulation software (Cheng *et al* 2003; Saenz *et al* 2007). Then a bivalent single-chain Fv (BsFv) of SLH10 was constructed to increase the valency to suit high-affinity targeting. The improved antigen binding of BsFv may be applied to develop immunodiagnostics and immunotherapeutics for liver cancer.

2. Materials and methods

2.1 *Materials*

The structure of the SLH10 scFv was modelled using the Homology module of the Insight II software package (Biosym/MSI, San Diego, Accelrys Inc.2001). Vector pAB1 (provided by Dr R E Kontermann, Germany) was used for expression of recombinant antibody fragments (Volkel *et al* 2001). *Escherichia coli* strains TG1 and HB2151 (provided by Dr G Winter, UK) were used for expression of soluble BsFv. Goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) was provided by Kirkegaard Perry Laboratories (Gaithersburg, Maryland, USA). Goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) was obtained from Zhongshan Company (Beijing, China). FITC-conjugated anti-polyhistidine antibody was obtained from Covance Research Products (Princeton, NJ, USA). Ni-NTA columns were from Qiagen (Genetimes Technology, Inc. Shanghai, China) and flow cytometry reagents were from BD (Becton Dickinson Medical Devices, Shanghai, China).

2.2 *Methods*

2.2.1 *Cell culture:* The normal liver cell line L02, human hepatoma cell line SMMC-7721, cervical carcinoma cell line HeLa, breast cancer cell line MCF-7, human hepatocarcinoma cell line HepG2 were maintained in DMEM containing 10% foetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin and incubated at 37°C in a 5% CO_2 atmosphere.

2.2.2 *Molecular modelling:* The 3D structures of SLH10 were constructed by homology modelling based on Ig VL- and VH-domains with highly identical amino acid sequences and known structures. We searched the Protein Data Bank (PDB) for antibody sequences that had approximately 66% homology with SLH10. Two separate BLASTp searches were performed for VL and VH. The amino acid residue sequences of the VL and VH domains were compared with the primary sequences of all Ig deposited in the PDB using the BLAST program. The SLH10 scFv had a 69% exact match VH alignment to AB Ctm01 and 66% exact match VL alignment to Bence–Jones protein Cle protein. This close sequence homology allowed the construction of a 3D model of SLH10 using the software package Homology (Biosym, San Diego, CA, USA). Briefly, the two sequences were aligned, and coordinates from the alignment antibody were transferred to the SLH10 scFv, while coordinates for the linker were assigned by random search for the lowest energy conformation. The structure was then relaxed with the Discover program (InsightII, Biosym) by selective minimization of all atoms of the complementary determining region (CDR) loops and the side chains in the framework residues that were replaced. The backbone atoms of the framework were not minimized because of the close identity and conservation between the alignment antibody and SLH10. The structure refinement program Discover was used for energy minimization and simulation of annealing with the consistent valence force field (CVFF) and assisted model building and energy (AMBER) force fields. Based on these methods, the whole 3D structure was refined by the methods of molecular mechanics and dynamics (Heng and Othman 2006).

2.2.3 *Construction of BsFv:* Two pairs of polymerase chain reaction (PCR) primers were designed to introduce an interlinker G4S and the restriction endonuclease site for *Asc*I. Two fragments were amplified (SfiI-VH-VL-linker-*Asc*I and AscI-VH-VL-*Not*I) from the parental scFv SLH10 by PCR. We amplified the following SfiI-VH-VL-linker-*Asc*I fragments (P1, P2):

P1 VH *Sfi*I 5′-ATTAGGCCCAGCCGGCCATGGCCCAGGTGC-3′
P2 VL-linker
5′-ACTGGCGCGCCCTCCACTTCCACCTCCACCTAGGACGGTCAGCTTGGT-3′

We amplified the following AscI-VH-VL-*Not*I fragments (P3, P4):

P3 Linker-VH 5′-GGAGGGCGCGCCAGTATGGCCCA GGTGCAG-3′
P4 VL-*Not*I 5′-ATGTGCGGCCGCACCTAGGACGGT CAGCTT-3′

The target fragments were then digested with *Sfi*I and *Asc*I, or *Asc*I and *Not*I, and ligated into the expression vector pAB1, which had been digested with the same pair of restriction enzymes (figure 1). TG1 cells were transformed with the resulting phagmid pAB1–scFv using a standard chemical transformation protocol (Martineau *et al* 1998). Positive colonies that contained the insert were selected by PCR screening and restriction digestion. The two fragments were then cloned into the plasmid pAB1 to construct the expression vector pAB1–BsFv. In addition, the parental scFv was subcloned by *Sfi*I/*Not*I digestion to generate the expression vector pAB1–scFv.

2.2.4 *Sequencing of the BsFv construct:* Plasmid DNA was prepared from colonies expressing functional BsFv and sequenced by BoYa Shanghai Company (China). The sequencing results were analysed using the BLAST program in GenBank and IMGT/V-Quest software.

2.2.5 *Soluble expression and immunoblot analysis of BsFv:* A positive clone was transformed in non-suppressor *E. coli* (HB2151) for soluble expression of BsFv. After 8 h induction at 30°C with 2 mM isopropyl *β*-D-thiogalactoside (IPTG), soluble BsFv was harvested from the bacterial periplasm. The bacteria were pelleted by centrifugation for 30 min at 4000×*g* at 4°C. The bacterial pellets were resuspended in phosphate-buffered saline (PBS) and disrupted by three cycles of freeze–thawing. Insoluble material was removed by centrifugation for 20 min at

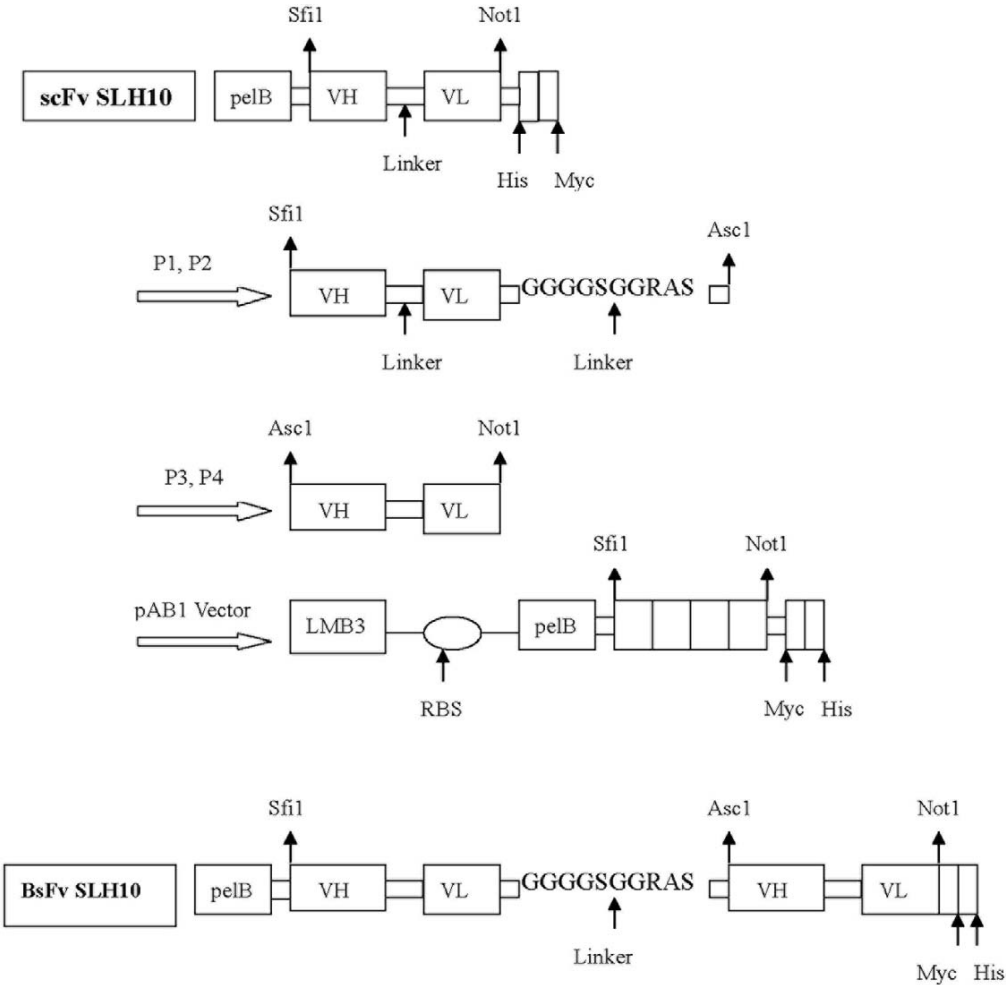


Figure 1. Schematic map of BsFv vector construction. Two pairs of PCR primers (P1, P2; P3, P4) were designed to introduce an interlinker G4S and the restriction endonuclease *Asc*I in the target gene. Two fragments (SfiI-VH-VL-linker-*Asc*I and AscI-VH-VL-*Not*I) were amplified from the parent scFv SLH10 by PCR. The two fragments were then cloned into the plasmid pAB1 to construct the expression vector pAB1–BsFv.

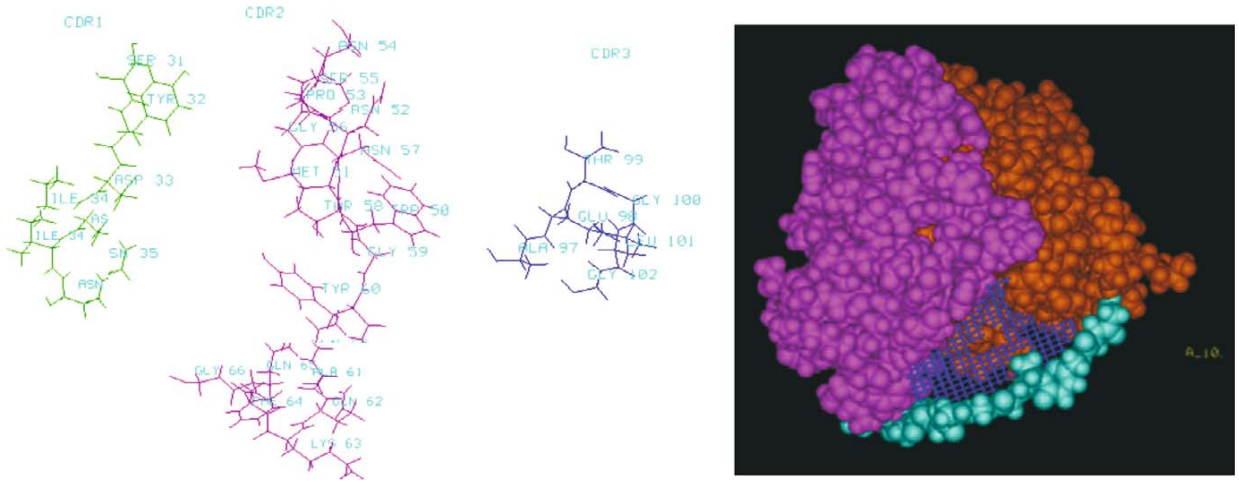
13 000 *g* and the BsFv- or scFv-containing supernatant filtered through a 0.22 μm membrane. Purified fragments were prepared using an immobilized metal affinity chromatography (IMAC) column, and the scFv fragments eluted with 200 mM imidazole in a final volume of 2 ml. The scFv and BsFv preparations were dialysed against PBS to remove the imidazole. We got purified scFv 600 μg and BsFv 400 μg from 1 litre bacterial culture, respectively. The samples were concentrated and stored at 4°C at a concentration of 1 mg/ml BsFv and scFv. The scFv and BsFv were resolved by 12% reducing SDS-PAGE and stained with Coomassie brilliant blue R-250. Proteins were resolved on 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked with 4% milk phosphate buffered solution (PBS), followed by incubation with the anti-his mouse antibody (1:2000, 4°C, 12 h) then the anti-mouse HRP conjugate (1:200, RT, 2 h) for detection of BsFv. The blot was developed using 3,3′-diaminobenzidine(DAB)/HRP.

2.2.6 *Evaluation of the binding activity of BsFv by flow cytometry:* Flow cytometry was used to determine the specificity of BsFv binding activity in L02, HepG2, HeLa, MCF-7 and SMMC7721 cell lines. 500 μl BsFv (1 mg/ml) and scFv were blocked with 6% bovine serum albumin (BSA) in PBS. The blocked BsFv was added to parallel plates containing 1×10^5 cells (1 h, 4°C, gentle agitation). The cells were washed twice and centrifuged. After three additional washes, the cells were analysed by flow cytometry. BsFv and scFv staining was detected using an FITC-conjugated anti-polyhistidine antibody. In each case, the intensities of the FITC and phycoerythrin (PE) staining of the cells were assessed.

3. Results and discussion

3.1 *3D models of SLH10*

The atomic coordinates of the antibody framework region (FWR) and CDR with a high degree of amino acid sequence homology to the SLH10 sequences were identified by a BLAST search of the RCSB PDB. For modelling of the SLH10 light chain, FWR1 to FWR4 and CDR1 to CDR3 were taken from the PDB entries 1LIL_A, and for the heavy chain the PDB entries 1AD9_H were selected. We investigated the structure–surface static electricity and hydrophobicity of scFv. Computer graphic modelling indicated that all sites of the VH and VL chains were correct in structure. Both the VH and VL regions were involved in the composition of the hydrophobic pocket. The linker was isolated from the VH and VL regions. The CDRs of the VH and VL regions surrounded the hydrophobic pocket (figure 2). Antigen-binding sites on the SLH10 VH and VL chains were identified by sequence homology with the CDRs of the corresponding protein structure. CDR1: SYDIN; CDR2: WMNPNSGNTGYAWKFWG; CDR3: AETGLG. We used the same method to identify the VL CDRs. In the 3D structure of SLH10, the VH and VL regions bind closely together by linkers through covalent binding. The linker chain extends out from between the VH and VL chains, but does not affect the formation of the scFv. The disulphide bonds contribute to the stability of the structure. The CDR3 region of SLH10 was surrounded by a pocket of seven hydrophobic amino acids – 101 (Leu), 102 (Gly), 219 (Thr), 220 (Val), 221 (Phe) and 222 (Gly). Furthermore, the hydrophobic pocket of the SLH10 chain contained 94 (Phe) and 221 (Tyr)(Zheng *et al*



(i) (ii)

Figure 2. 3D model of SLH10. (i) The complementary determining regions (CDRs) of the SLH10 VH chain ; (ii) the 3D model of the SLH10; the VL chain is purple, the VH chain is brown, the antigen-binding region is light blue and the linker is dark blue.

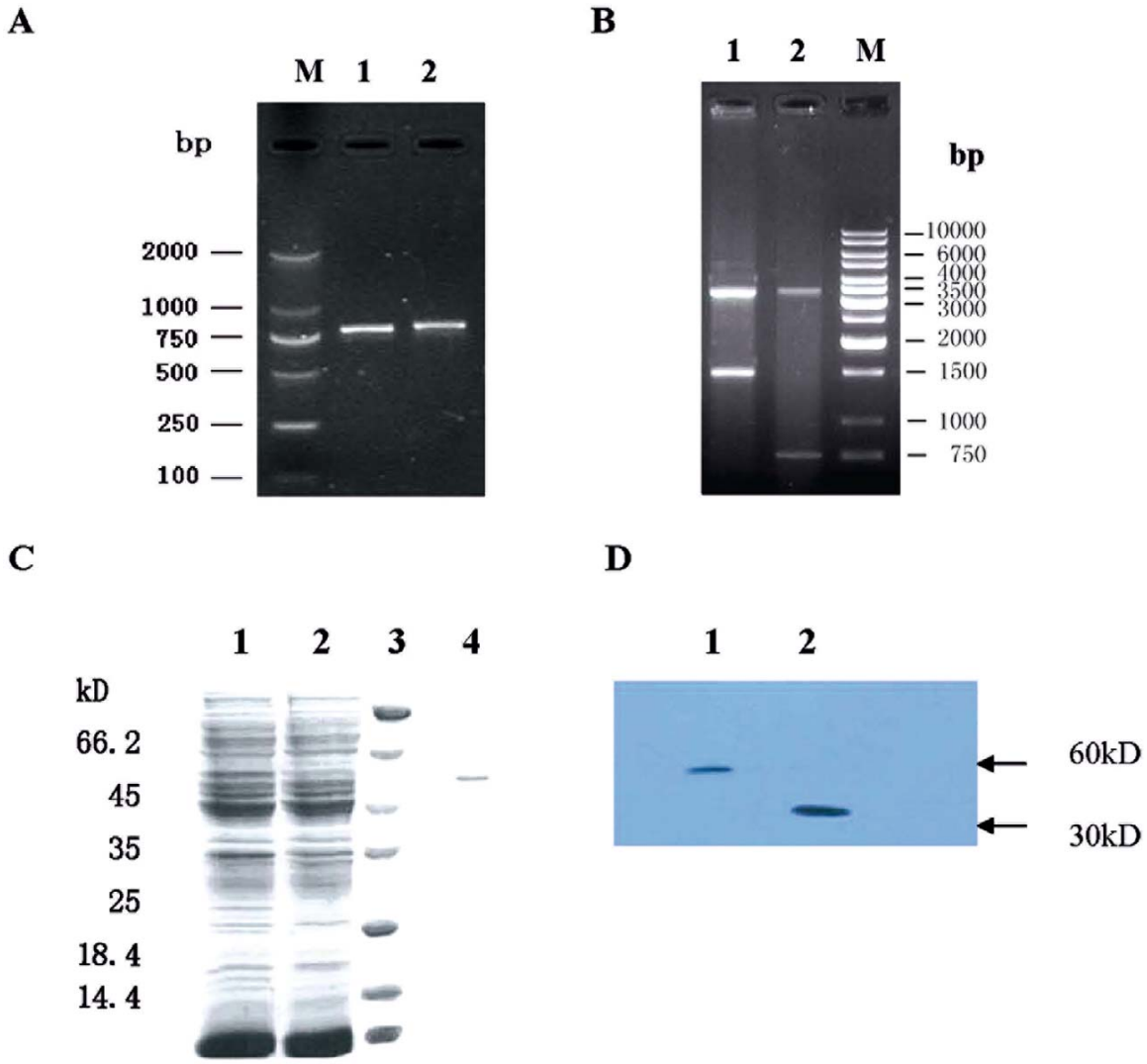


Figure 3. Construction and soluble expression of BsFv. (**A**) Electrophoresis of scFv fragments amplified by PCR. M, DNA marker; 1, PCR products of AscI-scFv-*Not*I fragments; 2, PCR products of *Sfi*I-scFv-linker-*Asc*I fragments. (**B**) Electrophoresis of *Not*I and *Sfi*I double digested products of BsFv and scFv. Lanes 1, 2: products of pAB1–BsFv and pAB1–ScFv digested with *Sfi*I/*Not*I. (**C**) SDS-PAGE of the purified products of BsFv. Lane 1: insoluble protein prepared from *E. coli* culture; Lane 2: soluble protein prepared from *E. coli* culture; Lane 3: molecular weight marker; Lane 4: purified BsFv expression products. (**D**) Immunoblot analysis of purified scFv and BsFv. Lanes 1, 2: The purified products of BsFv and scFv, respectively.

2005) amino acids that had benzene rings (Merienne *et al* 1997; Jorgensen *et al* 2002; Feng *et al* 2003;).

3.2 *Construction of BsFv*

The DNA encoding the VH and VL of SLH10 were amplified from the positive clone. Two pairs of PCR primers were designed to introduce an interlinker G4S and the restriction endonuclease site for *Asc*I. Two fragments were amplified (SfiI-VH-VL-linker-*Asc*I and AscI-VH-VL-*Not*I) from the parental scFv SLH10 by PCR (figure 3 A and B). The sequencing results and BLAST analysis showed that the SLH10 BsFv sequence was homologous to the scFv sequence. The scFv fragments were joined together by the linker GGGGSGGRAS. The scFv fragments were cloned successfully into the BsFv expression vector.

3.3 *Soluble expression and immunoblot analysis of BsFv*

SLH10 BsFv was expressed in its soluble form by induction of the infected HB2151 culture with 2 mM IPTG for 8 h

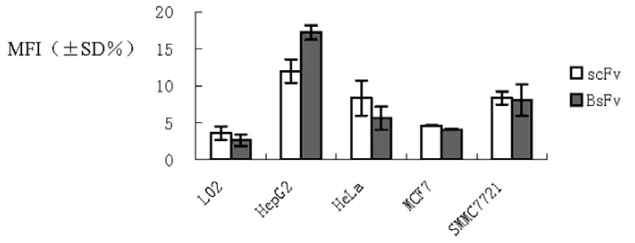


Figure 4. Binding activity of BsFv and scFv to different cell lines by flow cytometry. Results are expressed as the mean fluorescence intensity, which represents the level of scFv and BsFv binding to the total cells. MFI, mean fluorescence intensity.

at 30°C. After induction, a band with a molecular weight of approximately 60 kDa was detected in the periplasmic extract of infected HB2151 cells (figure 3 C and D).

3.4 *Evaluation of the binding activity of BsFv by flow cytometry*

Flow cytometry was used to detect the specificity of BsFv binding activity with the L02, HepG2, HeLa, MCF-7 and SMMC7721 cell lines. BsFv showed better binding properties with the HepG2 cell line than scFv (figure 4).

An scFv antibody SLH10 was identified which could bind extensively to the HepG2 cell line by biopanning from a large, naive phage display library. Its DNA sequence was submitted to GenBank (accession nos: AY686499) (Yu *et al* 2005). In this study, the 3D structure of SLH10 was modelled using the Insight II molecule simulation software. The reasonable scores of SLH10 homology modelling after optimization of molecular dynamics were identified by Profile-3D as 113.219917 (Bose *et al* 2003; Li *et al* 2007; Zheng *et al* 2005). This resulted in a more protruding and open hydrophobic pocket in SLH10, which leads to easier antigen binding. The structure of SLH10 scFv modelling was stable, thereby providing a basis for analysing the function of scFv.

scFvs have relatively low avidity due to their single antigen-binding site (Thomas *et al* 1996). Although the scFv itself has a small molecular weight that would be suitable for penetrating tumour cells, its stability is poor. Through an intermediate connecting peptide (interlinker), two scFvs can be constructed into a bivalent antibody which may be folded with high binding properties (Bera *et al* 1998; Muller *et al* 2007). Our results show that the SLH10 BsFv expression vector was constructed successfully and expressed active protein molecules. Compared with scFv, BsFv showed higher cell-binding activity. BsFv has two binding sites that promote the avidity of antibody binding (Wang *et al* 2007). The sequence and length of the linkers are important for bivalent antibody-mediated cell effects. The most commonly used binding linker sequence is GGGGS. However, the linkers should not be too short, since they should maintain favourable antibody formation with a space that is conducive to stability and conformation (Jain *et al* 2007; Todorovska *et al* 2001). Therefore, in this study, we designed a linker that had a length of 10 amino acids with a sequence of GGGGSGGRAS. The bivalent format should prove useful for many antibody phage display applications and for the display of other proteins that require dimerization or increased avidity. BsFv showed better binding properties with the HepG2 cell line than scFv due to its bivalent binding. We expect that the improved binding properties of BsFv will improve diagnostic and therapeutic applications such as tumour imaging or targeted cancer therapy.

Acknowledgements

We thank Dr Zhou Chun and colleagues for helpful discussions, support and critical review. This project was financially supported by the Key Program of the National "863" High-Tech Development Plan of China (863 Program, No.2006AA02Z158) and the New Faculty Member Program of the Chinese Department of Education (No. 20070487111).

References

Adair J R 1992 Engineering antibodies for therapy; *Immunol. Rev.* **130** 5–40

Beckman R A, Weiner L M and Davis H M 2007 Antibody constructs in cancer therapy: protein engineering strategies to improve exposure in solid tumors; *Cancer* **109** 170–179

Bera T K, Onda M and Pastan I 1998 A bivalent disulfide-stabilized Fv with improved antigen binding to erbB2; *J. Mol. Biol.* **281** 475–483

Bose B, Chugh D A, Kala M, Acharya S K, Khanna N and Sinha S 2003 Characterization and molecular modeling of a highly stable anti-hepatitis B surface antigen scFv; *Mol. Immunol.* **40** 617–631

Carter P 2001 Improving the efficacy of antibody-based cancer therapies; *Nat. Rev. Cancer* **1** 118–129

Cheng L S, Liu A P, Yang J H, Dong Y Q, Li L W, Wang J, Wang C C and Liu J 2003 Construction, expression and characterization of the engineered antibody against tumor surface antigen, p185(c-erbB-2); *Cell Res.* **13** 35–48

Feng J, Xie Z, Guo N and Shen B 2003 Design and assembly of anti-CD16 ScFv antibody with two different linker peptides; *J. Immunol. Methods* **282** 33–43

Heng C K and Othman R Y 2006 Bioinformatics in molecular immunology laboratories demonstrated modeling an anti-CMV scFv antibody; *Bioinformation* **1** 118–120

Holliger P and Hudson P J 2005 Engineered antibody fragments and the rise of single domains; *Nat. Biotechnol*. **23** 1126–1136

Hudson P J and Kortt A A 1999 High avidity scFv multimers; diabodies and triabodies; *J. Immunol. Methods* **231** 177–189

Jain M, Kamal N and Batra S K 2007 Engineering antibodies for clinical applications; *Trends Biotechnol.* **25** 307–316

Jorgensen T O, Solem ST, Espelid S, Warr G W, Brandsdal B O and Smalas A 2002 The antibody site in Atlantic salmon; phage display and modeling of scFv with anti-hapten binding ability; *Dev. Comp. Immunol.* **26** 201–206

Kortt A A, Dolezal O, Power B E and Hudson P J 2001 Dimeric and trimeric antibodies: high avidity scFvs for cancer targeting; *Biomol. Eng.* **18** 95–108

Kortt A A, Lah M, Oddie G W, Gruen C L, Webster R G and Hudson P J 1997 Single-chain Fv fragments of anti-neuraminidase antibody NC10 containing five- and ten-residue linkers form dimers and with zero-residue linker a trimer; *Protein Eng.* **10** 423–433

Lawrence L J, Kortt A A, Iliades P, Tulloch P A and Hudson PJ 1998 Orientation of antigen binding sites in dimeric and trimeric single chain Fv antibody fragments; *FEBS Lett.* **425** 479–484

Li B, Wang H, Zhang D, Qian W, Hou S, Shi S, Zhao L, Kou G, Cao Z, Dai J and Guo Y 2007 Construction and characterization of a high-affinity humanized SM5-1 monoclonal antibody; *Biochem. Biophys. Res. Commun.* **357** 951–956

Martineau P, Jones P and Winter G. 1998 Expression of an antibody fragment at high levels in the bacterial cytoplasm; *J. Mol. Biol.* **280** 117–127

Merienne K, Germain N, Zinn-Justin S, Boulain J C, Ducancel F and Menez A 1997 The functional architecture of an acetylcholine receptor-mimicking antibody; *J. Biol. Chem*. **272** 23775–23783

Muller D and Kontermann R E 2007 Recombinant bispecific antibodies for cellular cancer immunotherapy; *Curr. Opin. Mol. Ther.* **9** 319–326

Peterson E C, Laurenzana E M, Atchley W T, Hendrickson H P and Owens S M 2008 Development and preclinical testing of a high-affinity single-chain antibody against (+)-methamphetamine; *J. Pharmacol. Exp. Ther.* **325** 124–133

Presta L 2003 Antibody engineering for therapeutics; *Curr. Opin. Struct. Biol*. **13** 519–525

Saenz H, Lareo L, Poutou R A, Sosa A C and Barrera L A 2007 Computational prediction of the tertiary structure of the human iduronate 2-sulfate sulfatase; *Biomedica* **27** 7–20

Thomas T C, Rollins S A, Rother R P, Giannoni M A, Hartman S L, Elliott E A, Nye S H, Matis L A, Squinto S P and Evans M J 1996 Inhibition of complement activity by humanized anti-C5 antibody and single-chain Fv; *Mol. Immunol.* **33** 1389–1401

Todorovska A, Roovers R C, Dolezal O, Kortt A A, Hoogenboom H R and Hudson P J 2001 Design and application of diabodies, triabodies and tetrabodies for cancer targeting; *J. Immunol. Methods* **248** 47–66

Turner D J, Ritter M A and George A J 1997 Importance of the linker in expression of single-chain Fv antibody fragments: optimisation of peptide sequence using phage display technology; *J. Immunol. Methods* **205** 43–54

Volkel T, Korn T, Bach M, Muller R and Kontermann R E 2001 Optimized linker sequences for the expression of monomeric and dimeric bispecific single-chain diabodies; *Protein Eng.* **14** 815–823

Wang W W, Das D, McQuarrie S A and Suresh M R 2007 Design of a bifunctional fusion protein for ovarian cancer drug delivery: single-chain anti-CA125 core–streptavidin fusion protein; *Eur. J. Pharm. Biopharm.* **65** 398–405

Yu B, Ni M, Li W H, Lei P, Zhu H F and Shen G X 2005 Human scFv antibody fragments specific for hepatocellular carcinoma selected from a phage display library; *World J. Gastroenterol.* **11** 3985–3989

Zheng Q C, Li Z S, Sun M, Zhang Y and Sun C C 2005 Homology modeling and substrate binding study of Nudix hydrolase Ndx1 from *Thermos thermophilus* HB8; *Biochem. Biophys. Res. Commun.* **333** 881–887

MS received 10 April 2008; accepted 2 September 2008

*e*Publication: 20 October 2008

Corresponding editor: Dipankar Chatterji