Construction of human recombinant ScFv phage libraries from the advanced stages of breast carcinoma patients

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Abstract
Advances in the field of antibody engineering, and the emergence of powerful screening technology such as filamentous phage display allowed to generate fully human antibodies with high affinities against virtually any desired target from immune or even naIve human repertoires. As a result, the immunogenicity problems related to applications of non-human based recombinant antibodies as therapeutic reagents in human were bypassed. In this study, we constructed large human immunoglobulin libraries from the lymph nodes of breast carcinomas patients in two different formats of single-chain fragments of variable domains (scFv) of antibodies. The heterogeneity of the libraries were tested by restriction enzyme analysis and sequencing on DNA samples of randomly selected colonies. Functional expression of the selected scFv molecules in E. coli was demonstrated by Western blotting. Phage rescue and panning of these libraries against the candidate tumor antigens will lead to the identification of novel human scFvs for tumor detection and pave the way towards the generation of a fully human IgG with desired effector functions for possible future tumor therapy.

Keywords: *Human antibody library; single chain antibody; phage display; CEA; c-erbB2*

INTRODUCTION

There has been a rapid growth in the field of antibody engineering after it was shown that antibody fragments such as Fab (fragment for antigen binding) and Fv (variable fragment) could be expressed in the cytoplasm of *E. coli* (Boss *et al*., 1984; Riesenberg *et al*., 1991), or secreted as functional proteins into the periplasmic space and even into the culture medium (Better *et al*., 1988; Skerra and Pluckthun, 1988). Subsequently, the principles of the immune system for producing specific antibodies to a given antigen were mimicked by filamentous phage display technology by which large antibody libraries (10^8-10^{10}) established in *E. coli* could be directly screened for binding to candidate antigen(s) (Parmley and Smith, 1988; McCafferty *et al*., 1990; Hoogenboom *et al*., 1992; Breitling *et al*., 1991; Barabas *et al*., 1991). It is noteworthy to mention that immunoglobulin repertoires of mammals are quite heterogeneous in content and include many different variable region gene families. Consequently, crucial factors for construction of efficient antibody libraries are: designing the appropriate primer sets, optimizing the PCR conditions, applying proper immunization protocol, and last but not least using of an enriched source of immunized lymphocytes (Winter *et al*., 1994).

Filamentous phage display technology establishes a direct physical link between a gene, the protein it encodes, and the molecules recognized by the protein. Therefore, it is an efficient, high-throughput method by which very rare phage binders ($1/10^6$) can be selected from even a non-immune or naïve antibody libraries of sufficient size and diversity (Winter *et al*., 1994; Rader and Barbas III, 1997; Kipriyanov and Le Gall, 2004; Marks and Braddury, 2004; Bradbury and Marks, 2004; Hoet *et al*., 2005)

By applying this strategy, large immunoglobulin libraries from peripheral blood lymphocytes of healthy individuals (Griffiths *et al*., 1994) or patients (Rhyner *et al*., 2003) have been constructed and many human Fab or single-chain Fv (in which two variable domains of VH and VL are fused together by a short peptide linker) antibody fragments against human self-antigens such as Factor VIII, Calreticulin, Thyroglobulin (Griffiths *et al*., 1994) as well as tumor markers such as carcinoembrunoc antigen (CEA) (Osbourn *et al*.,

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1996; Ychou *et al.*, 1998), epidermal growth factor receptor (EGFR) (Mamot *et al*., 2003; Souriau *et al*., 2004)) ErbB/HER family (growth factor receptors) (Schier *et al*., 1995; Clark *et al*., 1997; Rothe *et al*., 2004) have been isolated. Moreover, construction of a desired immunoglobulin isotype (such as IgG, IgA, etc.), should it be necessary to trigger any effector functions, is now feasible by sub-cloning of the isolated, specific Fab or scFv into a mammalian expression vector harboring the constant regions cassette of antibodies (Morrison *et al*., 1984; Winter *et al*., 1994; Hoogenboom *et al*., 1998; Groner *et al*., 2004).

In this study, we tested the feasibility of generating large human libraries from the lymph nodes of breast carcinoma patients. It has been demonstrated that there is a humoral immune response to altered or over-expressed tumor-related antigen such as CEA and c-erbB2 (Her-2) in patients with realated cancers (Disis *et al*., 1994; Pupa *et al*., 1993). Therefore, libraries derived from these patients may contain a larger pool of relevant antibodies which can be recovered by phage panning against specific tumor antigens. Our study describe how such 'immune' libraries have been constructed in two different formats of scFv, differing in the length of the peptide linker connecting the two variable VH and VL domains. The shorter linker (7 amino acids in length) will allow the formation of diabodies (Holliger *et al*., 1993) which are divalent form of scFvs and therefore are mimicking their parental bivalent IgG in terms of antigen-binding units. The heterogeneity of the libraries has been confirmed by restriction enzyme analysis and sequencing data. The functional expression of the human scFvs in the peripalsmic space of *E. coli* was also shown by Western blotting technique.

MATERIALS AND METHODS

Construction of the phagemid display vector pHEN6: Briefly, a second *Sfi*I restriction enzyme site with a different compatible end and a histidine tag were introduced into the pHEN4 phagemid vector (Arbabi *et al*., 1997, Hoogenboom *et al.*, 1991) by designing the upstream primer PR3 (5´-GAA CTG CAG GGC CAG GCC GGC CAG CAC CAT CAC CAT CAC CAT GTC TCC AGC GGC CGC-3´) (*Pst*I cloning site underlined), PR4 (5´-GAA TGG ATC CTC ATT AAA GCC AGA-3´) (*BamH*I cloning site is underlined) as downstream primer. The multiple cloning sites and part of M13 gpIII encoding region in pHEN4 vector was amplified by Taq DNA polymerase (Roche, Germany) and the aforementioned primers. A PCR product of ~700 bp in length was purified by PCR purification Kit (Qiagen, Hilden, Germany), digested with *Pst*I *and BamH*I restriction enzymes (Roche, Germany) and ligated into pHEN4 vector which had been cut with the same restriction enzymes and purified from the gel (Roche Gel purification Kit). The nucleotide map of the new phagemid vector, pHEN6, was confirmed by restriction enzyme analysis and sequencing data (MWG, Germany).

Sample collection and patient details: Blood and tissue samples were collected from 15 patients who were undergoing resection of breast cancer at Institute of Cancer of Imam Khomeini Hospital in Tehran, Iran, at their consent. Auxilary lymph nodes were collected during surgical operation on patients and preserved immediately in liquid nitrogen. Clotted blood was centrifuged at 2000×g for 10 min and serum was stored in aliquots at -70°C before use.

Two different assays were performed for detection of HER-2 and CEA tumor antigens in samples collected from patients. Sections of paraffin-embedded tumor tissues from each individual were subjected to immunohistochemical analysis of HER-2 and CEA tumor antigen as described by Harlow *et al*. (1999). Detection of CEA level in the sera of patients was done using an ELISA (Margolis *et al*., 1994). The lymph nodes of three patients, defined as positive for both tumor antigens, were selected and used for RNA extraction.

Total RNA extraction and cDNA synthesis: 100 mg of lymph nodes was powdered in liquid nitrogen and homogenized in RNX-plus solution (Cinagen Inc. Iran). Total RNA was extracted according to manufacturer's instruction. The integrity of the RNA was analyzed by 1% (w/v) RNase-free agarose gel and its concentration was measured by spectophotometery. First strand cDNA was synthesized at 42°C for 1h using oligo-dT primer and M-MuLV reverse transcriptase (Fermentas, Russia) following the manufacturer's recommendations.

Repertoire amplification of human variable regions by PCR: A total of 36 different and degenerate primers were designed for the amplification of V_H and V_L repertoire according to Barbas III *et al*. (For complete list of the primers and their nucleotide sequences see Barbas III *et al.*, 2001).

Each reverse primer (downstream primer) of V_L set and forward primer (upstream primer) of V_H set has a complementary sequence tail that corresponds to the short linker sequence and these are used in assembly

PCR to make scFv DNA molecules. The 5´ end of the final V_L forward primer (VIII-1) and that of V_H reverse primer (VIII-2) contain two incompatible *Sfi*I restriction enzyme sites, respectively. For amplification of the human V_H gene repertoires 24 separate PCR reactions were set up by using 12 forward primers (6 primers with long linker and 6 primers with short linker) specific for the 5´-end of the VH regions of different gene families and 2 reverse primers specific for the CH1 domain of either IgG or IgM isotypes. For the amplification of V_κ and V_λ gene repertoires, the same approach was used with 16 (four forward primers and four reverse primers specific for the 5´-end and 3´-end of different V_κ gene family, respectively) and 27 (nine forward primers and three reverse primers specific for the 5´-end and 3´-end of different V_λ gene families) separate PCR reactions were performed.

PCR was performed in a volume of 50 ul with a final concentration of 25 pmol of primers, 200 µM of each dNTP, 1.5 mM $MgCl_2$, 1U Accueterm proof reading DNA polymerase (GeneCraft, Germany) and 0.5 µl of cDNA-RNA hybrid for 30 cycles (pre-denaturation step at 94°C for 3 min followed by 30s at 94°C, 30s at 54 to 56ºC and 45s at 72°C and final extension step at 72°C for 10 min.

The PCR products were purified on a 1% (w/v) agarose gel and isolated from the gel by DNA extraction kit (Roche, Germany). For PCR assembly of the scFv repertoires approximately 20 ng of each purified V_H and V_L PCR products were combined in a SOE-PCR reaction mixture. Assembly PCR was performed with an initial denaturation step (4 min at 94°C) followed by seven cycles of 30 s at 94°C, 1min at 56°C and 1.5 min at 72°C. Thereafter, primers RSC-F (VIII-1) and RSC-B (VIII-2) (10 pmol each) were added to each reaction mixture and cycled 30 times more at 94°C for 30s, 56°C for 30s and 72°C for 90s. The resulting scFv repertoires with an average length of 850 bp were purified on 1% agarose gel. The purified products of each amplification set of short linker ($V_{H\mu}$-V_κ, $V_{H\mu}$-V_λ, $V_{H\gamma}$-V_κ, $V_{H\gamma}$-V_λ) and of long linker ($V_{H\mu}$-V_κ, $V_{H\mu}$-V_λ, $V_{H\gamma}$-V_κ, $V_{H\gamma}$-V_λ) were separately used for subsequent cloning.

Construction of human scFv antibody libraries: Large scale phagemid DNA of pHEN6 was prepared and digested with *Sfi*I restriction enzyme (Roche, Germany). Purified scFv DNA fragments were also digested with *Sfi*I. Digested vector and scFv fragments were purified with DNA purification kit (Roche, Germany). Ligation reaction was performed in a volume of 100 µl containing 15 µg cut pHEN6, 3 µg scFv fragments, 10 µl 10X ligation buffer and 200 units of T4 DNA ligase (Roche, Germany) and incubated overnight at 16°C. The ligated products were purified and transformed into electrocompetent *E. coli* TG1 cells using the Gene Pulser electroporation system (Biolab, Germany). After electroporation, transformed cells were grown in SOC medium at 37°C for 1h. A small aliquot of transformed cells was diluted and plated on LB-agar containing 100 mg/ml ampicillin and incubated overnight at 37°C. The size of the libraries was estimated by counting the number colonies in sample plate. A colony-PCR assay was performed to determine the number of positive colonies with scFv inserts. Briefly, single colonies were used as template in a PCR experiment as described above using the universal reverse primer (URP) (upstream) in vector and the reverse (downstream) primer of scFv (VIII-2).

DNA fingerprinting and sequencing of positive clones: The diversity of the constructed library was determined by two methods of restriction enzyme analysis and sequencing. Briefly, the resulted PCR products from colony-screening assay product was purified and digested with the frequent-cutter restriction enzyme *Mva*I (Roche, Germany) at 37°C for 1h. Digested materials were run on a 2% agarose gels and analyzed. Purified PCR products from 15 different clones were also sequenced (MWG, Germany).

Expression of scFv fragments: Selected positive clones from the libraries were grown overnight at 37ºC. A 1:100 dilution of the pre-cultures of individual clones were incubated in 2xTY medium containing 100 µg/ml ampicillin-0.1% glucose at 37°C in a shaker incubator. The bacteria were grown to an OD_{600} = 0.7-0.8, and scFv expression induced by the addition of IPTG to a final concentration of 1mM. The bacterial cultures were incubated at 37ºC for 4 hours. Subsequently, the cells were pelleted and cell lysate were prepared. Analysis of expressed proteins was done by electrophoresis on 12% SDS-PAGE. Also, the same protein samples were electro-transferred onto nitrocellulose membrane (Roche, Germany) for immunoblotting experiment. Briefly, after blocking with 2.5% nonfat dry milk for 1h at room temperature, the membrane was incubated with primary anti-His tag mAb (1:3000 dilution) (Sigma Company) for 1h at 37ºC, washed three times with PBST, and incubated for another 1h at 37ºC with secondary HRP-conjugated anti-mouse antibody (1:2000 dilution) (DAKO). Finally, the membrane was washed three times with PBST and stained with DAB substrate solution. Chromogenic reaction was stopped by rinsing the membrane twice with water.

RESULTS

Serological studies: Pathological and medical records of 15 patients in hospital were studied. The presence of selected tumor markers, namely, carcinoembryonic antigen, an oncofetal antigen, and Her-2/neu (c-erb-B2), a member of protein kinase receptor family, in patients' samples were confirmed by immunohistochemical (Fig. 1) and ELISA techniques (data not shown). Lymph nodes from patients with metastatic tumors who had the highest score for the two tumor antigens, namely, CEA and HER-2, were selected (data not shown). The total RNA of the axillary lymph node cells was extracted and oligo-T primed complementary DNA (cDNA) was synthesized. The integrity of RNA was shown by agarose gel and its concentration and purity was determined by spectophotometry.

Construction of phagemid: For construction of antibody library, pHEN6 phagemid was designed in which a second non-compatible *Sfi*I restriction enzyme site and a Histidine tag were inserted into pHEN4 vector (Arbabi *et al.*, 1997). Application of the new phagemid vector simplifies the cloning procedures for library construction by using a single restriction enzyme (*Sfi*I) and the purification steps of recombinant protein by using IMAC.

Amplification of V_H and V_L gene repertoire of immunoglobulins: V_H, V_κ and V_λ of immunoglobulin heavy and light chains were PCR-amplified using synthesized cDNA repertoire. For amplification of V_H encoding gene, two downstream primers (complementary to 3´-end of VH region) and 2 sets of upstream primers (complementary to the 5´-end of VH region) with each set containing 6 different primers were used. The first set was designed to produce scFv library with a short linker and the second set was designed for scFv library with long linker. For amplification of V_κ fragments, 4 upstream primers and 4 downstream primers, and for V_γ fragments, 9 sense primers and 3 anti-sense primers were used. PCR products of the correct size ~400 bp in length for the V_H and ~350 bp in length for the V_κ and V_γ were obtained, respectively (Fig. 2A, B).

Assembly of VH and VL into scFv: The resulting V_H and V_L fragments were used for assembly of scFv molecules. In an overlap extension reaction by PCR, equimolar quantities of light- and heavy-chain variable fragments were used. After seven cycles of PCR, RSC-F (complementary to the 5´-end of VL region) and RSC-B (complementary to the 3´-end of VH region) primers with tailed restriction enzyme were added to amplify single chain fragments of variable regions of antibodies. The result of the assembly PCR is a DNA fragment of about 750-800 bp in length and is shown in Figure 3.

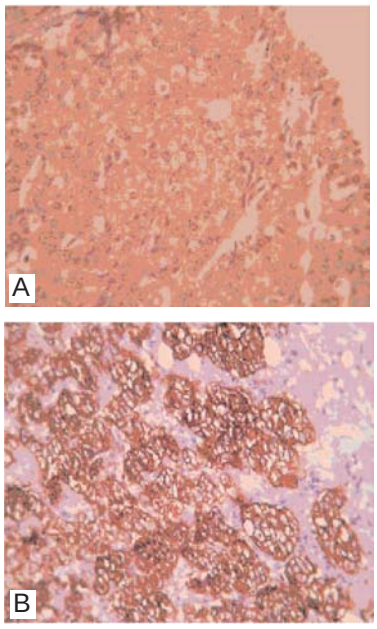

Figure 1. The results of immunohistochemistry assay using tissue sections of both normal and cancerous tissues: (A) normal tissue, (B) breast carcinoma tissue showing overexpression of c-erbB2 tumor antigens.

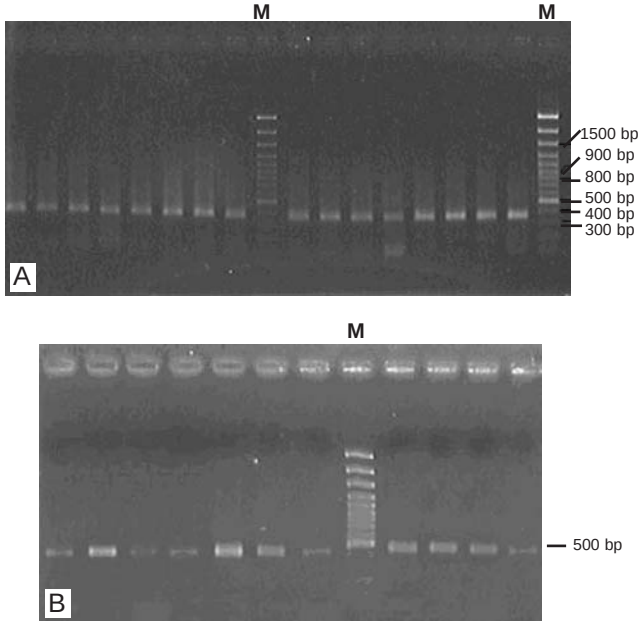

Figure 2. Amplification of variable regions (VH and VL fragments) of immunoglobulin gene repertoire by PCR. Different PCR products have been obtained using the synthesized cDNA as templates and different PCR primer sets: (A) V_L PCR products of ~350 bp by 8 V_K primer pairs, and (B) V_H PCR products of ~400 bp by 12 V_H primer pairs. M is a 100 bp DNA ladder.

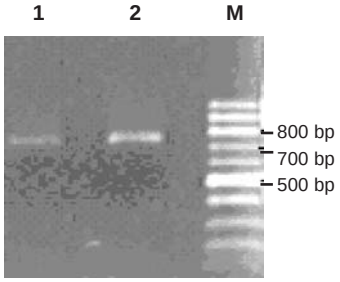

Figure 3. Assembly of V_H and V_L into a scFv fragment by an overlap extension PCR reaction using RSC-B and RSC-F primers. The results of the assembly PCR are DNA fragments of about 750-800 bp shown in lane 1 (for scFv with short linker) and 2 (for scFv with long linker). M is a molecular size marker of 100 bp ladder.

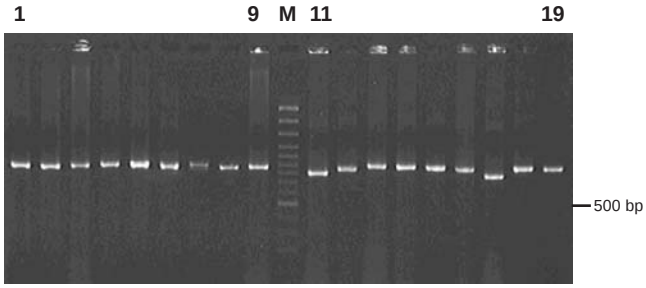

Figure 4. Results of colony-PCR assay on a 2% agarose gel. Eighteen randomly selected colonies were directly used as templates in PCR reactions. Amplification of scFv inserts in pHEN6 vector was performed by using a universal reverse primer in the vector (as upstream primer) and the RSC-B primer which resulted in DNA bands of approximately 900bp in lengths (lane 1-9 and lane 11-19). M is the same molecular size marker as in Figure 2.

Construction of antibody fragments library: Large scale PCR amplification for both type of scFvs with long and short linker were performed and gel purified. Phagemid vector DNA was also prepared in large scale. The digestion conditions with *Sfi*I were optimized in order to increase the efficiency of ligation reaction. Small-scale ligation to assess the optimal ratio of the vector and inserts was performed. The ligated material was transformed and the efficiency of cloning was estimated based on the number of positive and negative colonies as assayed by colony-PCR technique. The results showed that a ratio of 1 vector to 3 insert gives the highest number of colonies. For colony- PCR, a universal upstream primer in the vector and the gene-specific RSC-B primer were used and fragments of about 900 bp in length were amplified (Fig. 4). The extra length of PCR fragment (about 100 bp) is related to the distance of universal primer to the first cloning site of scFv fragment in pHEN6 vector. Large scale ligation and transformation under the optimized conditions were performed. To end this, some 25 µg vector DNA and 15 µg scFv-SL and 14 µg scFv-LL were used for ligation experiment. The ligated materials were purified by *n*-butanol precipitation method, 0.6 µg DNA was used per each electroporation cuvette and 10^7 colonies were obtained per one mililer of transformed bacteria. Subsequently, all of the transformed bacteria were mixed, grown for 2h in the presence of 2% glucose and titrated to estimate the final size of the libraries. The results of titration confirmed library sizes of 5×10^9 and 8×10^8 for scFv-SL and scFv-LL, respectively.

Heterogeneity analysis: *Mva*I restriction analyses of 18 randomly selected clones of both libraries showed that they carried inserts of various sequences since the pattern of digestions were different among individual colonies (Fig. 5). DNA samples from 15 of these colonies (10 clones from short-linker library and 5 clones from long linker library) were sequenced and the sequencing data confirmed the results of restriction enzyme analyses (Table 1). The hetergeneity of the libraries is measured based on the differences in the length and sequence of complementarity regions (CDRs) among individual positive clones. According to the obtained sequencing data, there were no two clones with the same nucleotide sequences and, therefore, the heterogeneity percentage is estimated to be very high. Although further sequencing data will complete heterogeneity percentage of the libraries, our limited data proved that the generated libraries are of sufficient heterogeneity for the next screening step.

Expression of scFvs: *E. coli* TG1 cells harboring recombinant phagemid pHEN6-scFv were grown at 30°C and induced for the expression of recombinant

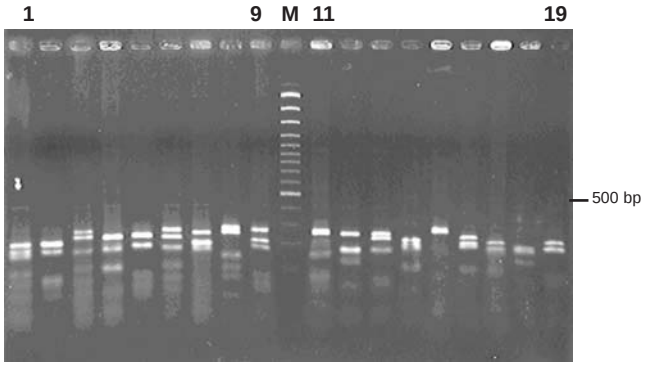

Figure 5. Results of DNA fingerprinting on PCR-amplified scFv fragments. The scFv fragments were amplified from individual colonies as in Figure 4. The PCR products were digested with *Mva*I restriction enzyme (lane 1-9 and 11) and run on a 2% agarose gel. Different patterns of digestions with *Mva*I demonstrate that randomly selected clones are different from each other. M is a molecular size marker as in Figure 2.

Table 1. Sequencing results (for simplicity only the nucleotide sequences of CDR regions were shown). Sequence alignment of the CDR regions from ten different scFv clones with short hinges (the first ten sequences) and five scFv clones with long hinges (the last five sequences). The sequences start with CDRs in the VL domains and ends with CDRs in the VH domains. The CDR regions were defined according to Kabat *et al.* (2001). Dashes indicate either empty space generated either due to the sequence alignments or base ambiguities. There are clear differences in the CDR lengths and nucleotide sequences among individual clones.

CDR1 (light chain)

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C G G G C A A G T C A G A G - - - - - - - - T A T A A G A A A C T A T C T T A G T - -   HuscFv01.seq
A C C C G C A G C A G T G G C A G - C A T T G C C A G C A A C T A T G T G C A G - -   HuscFv07.seq
C G G G C A A G T C A G A G - - - - - - - - T A T A A G A A A C T A T C T T A G T - -   HuscFv05.seq
A G G G C C A G T C A G A G - - - - - - - - T G T T A G C A G C A A C T T A G C C - -   HuscFv06.seq
A C C C G C A G C A G T G G C A G - C A T T G C C A G C C A C T A T G T G C A G - -   HuscFv12.seq
A G G G C A A G T C A G G G - - - - - - - - C A T T A G A A A T G A T T T A G G C - -   HuscFv09.seq
A C C G G C A G C G G T G G C A G - C A T T G C C G C C A A C T A T G T A C A C - -   HuscFv10.seq
A G G G C C A G T C A G A G - - - - - - - - T G T T A G C A G C A A C T T A G C C - -   HuscFv11.seq
T C G G G C A A T - A G G G - - - - - - - - C A T T A T A A A T G A T T T A T G C - -   HuscFv15.seq
T T C C A C A A T T G G A G C T G T C A C C A G T G G T T A C T A T - T A A A C - -   HuscFv14.seq
- - - C G G G C A A G T C A G A G C A T T A G C A G C T A T T - T A A - - - A T - -   HuscFv04.seq
A - - - G G G C C A G T C A G A G T G T T A G T A G C A A C T - T A G C C - - - - -   HuscFv02.seq
A C C C G C A G C A G T G G C A G C A T T G C C A G C C A C T - A T G T G C A G - -   HuscFv03.seq
G C T T C C A C A A T T G G A G C T G T C A C C A G T G G T T A C T A T C T A A A C   HuscFv08.seq
C - - - G G G C A A G T C A G G G C A T T A G C A G T T C T T - T A G C C - - - - -   HuscFv13.seq
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CDR2 (light chain)

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G C - T G C G T C C A G T T T G C A A A G T   HuscFv01.seq
G A G G A T G A C C A A - A G A T C G T C T   HuscFv07.seq
G C - T G C G T C C A G T T T G C A A A G T   HuscFv05.seq
G G - T G C A T C C A G C A G G G C C A C T   HuscFv06.seq
G A G G A T G T C C A A - A G A C C G T C T   HuscFv12.seq
G A - T G C A T C C A C T T - G C A A A G T   HuscFv09.seq
G A G G A T A A C C A A - A G A C C C T C T   HuscFv10.seq
G G - T G C A T C T A T C A G G G C C A C T   HuscFv11.seq
G A - T G C A T C C A C T T T G C A A A G T   HuscFv15.seq
G A A T A T G A C A A C - A A A C A T T C C   HuscFv14.seq
G C T G C A T C C A G T T T G C A A A G T -   HuscFv04.seq
G G T G C A T C C A C C A G G G C C A C T -   HuscFv02.seq
G A G G A T G T C C A A A G A C C G T C T -   HuscFv03.seq
A A T A T C G A C A A C A A A C A T T C C -   HuscFv08.seq
G A T G C C T C C A G T T T G G A A G T T -   HuscFv13.seq
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CDR3 (light chain)

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C A C C A G A C T T A C A - - T G A C T T C G - T A C A C T   HuscFv01.seq
C G G T C T T A T G A T A C C - A A C A T T A T A G T G - -   HuscFv07.seq
C A C C A G A C T T A C A - - T G A C T T C G - T A C A C T   HuscFv05.seq
C A G C A G C G T A A C A T C T G G C C - - - G C T C A C T   HuscFv06.seq
C A G T C T T A T C A T A A G - A C T A C T G T G G T C - -   HuscFv12.seq
C T A C A G C A T G G T A - C T T A C G T C G - - - - - - -   HuscFv09.seq
C A G T C T T A T G A T A A G - T A C A A T C T T G T A - -   HuscFv10.seq
C A G C A G T A T G G T A G C T C A C C T C G A T A C A C T   HuscFv11.seq
C T A C A G C A T G G T A - C T T A C G T C G - - - - - - -   HuscFv15.seq
C T G C T C T A C G G T C G T - G G T G C T T G G G T G - -   HuscFv14.seq
C A A C A G - - - - - - A G C T A C A G T A C C C C A A C T   HuscFv04.seq
C A G C A G T A T A A T A A C T G G C C T C C T T A C A G T   HuscFv02.seq
C A G T C T T A T C A T A A - - - G A C T A C T G T G G T C   HuscFv03.seq
C T G C T C T A C - - - G G T C G T G G T G C T T G G G T G   HuscFv08.seq
C A A C A G - - - - - - - - - - G G A T T C - - - A C - - T   HuscFv13.seq
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CDR1 (heavy chain)

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A G T A G C T A T G C C A T G A G C   HuscFv01.seq
A G T G A T A G G T A C T G G A G T   HuscFv07.seq
A G T A G C T A T G C C A T G A G C   HuscFv05.seq
A C C A G T T A T G A T A T C A A C   HuscFv06.seq
A C C G A C T A C T A T A T G C A C   HuscFv12.seq
A C C G A C T A C T A T A T A C A C   HuscFv09.seq
A C C A A C T A T G G T A T C A G C   HuscFv10.seq
A G C A G C T A T G G T A T C A G T   HuscFv11.seq
A C C G A C T A C T A T A T A C A C   HuscFv15.seq
A C C A G T T A T G A T A T C A A C   HuscFv14.seq
A G C A G T C G T A A T G G A G A C   HuscFv04.seq
A C C A G T T A T G A T A T C A A C   HuscFv02.seq
A C C G A C T A C T A T A T G C A C   HuscFv03.seq
A C C A G T T A T G A T A T C A A C   HuscFv08.seq
A C C A G T T A T G A T A T C A A C   HuscFv13.seq
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                                        CDR2 (heavy chain)
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G C T A T T A G T G G T A G T G G T G G T A G C A C A T A C T A C G C A G A C T C C G G A   HuscFv01.seq
G A A A T C A A - - - T C A T A G T G G A A G G A C C A A T T A C A A C C C G T C T C C A   HuscFv07.seq
G C T A T T A G T G G T A G T G G T G G T A G C A C A T A C T A C G C A G A C T C C G G A   HuscFv05.seq
T G G A T G A A C C C T A A C A G T G G T A A C A C A G G C C A T G C A C A G A A G T C C   HuscFv06.seq
C G G A T C C A C C C T A A C A A T A T T G G C A C A A A C T T T G C A C A G A A G T T C   HuscFv12.seq
C G G A T C A A C C C T A A C A G T G G T G G C A C A A A C T A T G C A C A G A A G T T C   HuscFv09.seq
T G G A T C A G C G C T T A C A A T G G T G A C A C A A A C C A T G C A C A G A A G G C C   HuscFv10.seq
A G G A T C A T C C C T A T C G T T G G T G T A G C A A A G T A C G C A C A G A A G T C C   HuscFv11.seq
C G G A T C A A C C C T A A C A G T G G T G G C A C A A A C T A T G C A C A G A A G T T C   HuscFv15.seq
T G G A T G A A C C C T A G C A G T G G T A A C A G A G G C C A T G C A C A G A A G T C C   HuscFv14.seq
C A C A T C T A T T C T A A T A C T G G G G A C A C G T A C T A C A A C C C G T C C C C A   HuscFv04.seq
T G G A T G A A C C C T A A C A G T G G T A A C A C A G G C T A T G C A C A G A A G T C C   HuscFv02.seq
C G G A T C C A C C C T A A C A A T A T T G G C A C A A A C T T T G C A C A G A A G T T C   HuscFv03.seq
T G G A T G A A C C C T A A C A C T G G T A A C A C A A A C T A G T T G G C G T C T G G T   HuscFv08.seq
T G G A T G A A C C C T A A C A G T G G T A A C A C A G G C T A T G C A C A G A A G T C C   HuscFv13.seq

                                        CDR3 (heavy chain)
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- - - - - - - - C A G T G G C G G T G G C G G C T C C A C T C - - T T T G A C C T C         HuscFv01.seq
G G A T T G G G G T G C T G T T C G G C G C A C C T A T T A C G G T C T G G A C G T C       HuscFv07.seq
- - - - - - - - C A G T G G C G G T G G C G G C T C C A C T C - - T T T G A C C T C       HuscFv05.seq
G - - T C - C C T G - T A G C A G - - - C A G C T G - G T A A - - T - G A C T A C T       HuscFv06.seq
G T - - - - - - - - - - - - - - - - - C - - - C A T A C C G - - - - - - A C A G C       HuscFv12.seq
G - - C G A C G T G G C A G T A G T G G C G A T A G T G C T A - - A C T T T G A T T       HuscFv09.seq
G A G G G G G G T A G T G - - - G G A G C - - - T A T T T T G - - - - - - A C T A C       HuscFv10.seq
G - - C T A C T T G - T A G T A G G A G C - - - T A T T T T G - - - - - - A C T A C       HuscFv11.seq
A - - C - - - - - T G G C A C T A - T G G C A T A G C T G C T A - - A C T T T G A C T       HuscFv15.seq
A G G - - - - - - - G C C A T C G G A G G C - - - - - - - - - T G G T T C G - A C C C C       HuscFv14.seq
G G G G G A T A T T G T T G G A G T T C C A G C T G C C C A T A C G G C C G A G T C
A A T A C C T T T G A C T T C                                                           HuscFv04.seq
- G A G G A T G G T - T C - G G G G A G A A C C T T - - A C G G T A T G G A C G T C       HuscFv02.seq
- G A G A - - - G T - C C A - - - - - - - - - - - - - - - - T A C C - - - G A C A G C       HuscFv03.seq
G A - - C T T C T G A G T A C C - - T T C A T C A - A A G G C T G G G T A C C G A C       HuscFv08.seq
- G A G G A T G G T - T C G G G G - - - A G A A C C T T A C G G T A T G G A C G T C       HuscFv13.seq
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antibodies by IPTG. The clones were randomly selected from the library. The expressed proteins are transported to the periplamic space of *E. coli* with the help of *pelB* signal peptide. The presence of the expressed antibody fragments in *E. coli* extracts were confirmed by Western blotting technique. A histidine tag is located at the C-terminal of scFv proteins and a mouse monoclonal anti-His was used for their detection. The Western blotting results demonstrated the presence of scFv protein bands with molecular weight of approximately 30 kD for the selected clones (Fig. 6).

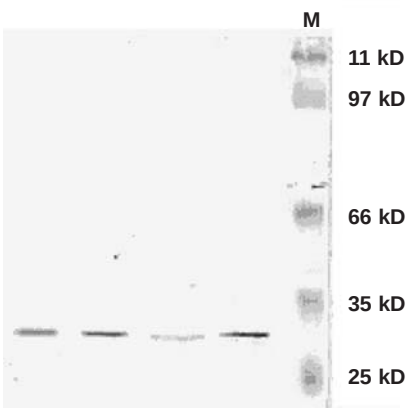

Figure 6. Immunoblot analysis of scFv proteins expressed in 4 different clones randomly selected from the library. Proteins, extracted from periplasmic space of *E. coli* cells, were electrophoresed on a 12% SDS-PAGE and transferred onto a nitrocellulose membrane. The scFv protein bands of approximately 30 kD in size were detected using anti-His-tag mAb and a secondary mAb-HRP conjugate. All the samples correspond to 200 µl of bacterial culture. A protein molecular weight marker was run next to the protein samples and shown on the right side of the blot.

DISCUSSION

Antibody engineering and phage display have revolutionized the applications of immunoglobulin in various fields of medical sciences including diagnosis and therapy (Souriau and Hudson, 2003; Funaro *et al*., 2000). With approval of 19 monoclonal and engineered antibodies for therapy by the FDA (Stockwin and Holmes, 2003; Groner 2004) and more than a 1000 of them in different phases of clinical trials, there is no doubt that the future demand for therapeutic antibodies will be of great importance. Today, more than 30 percent of all biopharmaceutical products are antibodies or antibody-based reagents (Souriau and Hudson, 2003).

Cancer diagnosis and therapy are among the challenging areas in which mAbs have primarily been applied and indeed, the first series of FDA-approved drugs of such kinds were tumor-specific mAbs, and currently more than 50% of all the FDA-approved monoclonal and engineered antibodies are used only

for cancer treatment (Souriau and Hudson, 2003; Stockwin and Holmes, 2003; Groner, 2004). The ideal 'magic bullets' for cancer treatment are those antibodies or their fragments that specifically detect cancerous cells in the earliest stage of their growth and/or destroy them before spreading throughout the human body. In this regard, the critical parameter is to generate antibodies or antibody fragments against tumor-specific markers (antigens) in order to either reduce the background (for diagnostic applications) or diminish the undesired side effects during different phases of tumor treatment (Stockwin and Holmes, 2003; Russeva and Adams, 2004; Groner *et al*., 2004; Funaro *et al*., 2000).

Extensive efforts have been made to identify tumor-specific antigens and quite a number of them such as CEA (Chhieng *et al*., 2003), EGFR (Mamot *et al*., 2003) and c-erbB family of receptors (Roskoski, 2004) which include four distinct groups of c-erbB1-B4 (also called HER1-4) have already been considered as valuable prognostic, diagnostic and therapeutic biomarkers. Trastuzumab (Herceptin) is, indeed, a commercial recombinant humanized anti-HER-2 mAb that binds the extracellular domain of the receptor and blocks intracellular signaling. Patients with breast cancer have been effectively treated when this mAb is used in combination with chemotherapy (Slamon *et al*., 2001, Vogel *et al*., 2002, Hudson *et al.,* 2001).

In the present study, two important principles have been taken into account: a) the existence of a specific antibody response against breast cancer antigens, a fact which has also been proven in recent studies (Croce *et al*., 1995; Disis *et al*., 1994); b) generation of fully human isotype-specific antibodies by phage display technology and other engineering techniques (Griffiths *et al*., 1994; Marks *et al*., 2004).

Investigations with different solid tumors document tumor specific antibodies in blood serum as well as alterations in the cellular compositions of regional lymph nodes (Lores-Vazquez *et al*., 1996). Moreover, lymph node tissue of cancer patients was determined to be the most appropriate source of for the generation of rAb libraries (Yip *et al*., 1997). This is most likely due to the fact that reactive lymph nodes of patients with malignancies contain more memory B-cells and antigen-activated cells than other source of immunocompetent cells such as peripheral blood mononuclear cells. The results of immunohistochemistry and ELISA in this study confirmed the immunoreactivity of both tumor antigens in tissue and serum of patients (data not shown). Therefore, lymph nodes from our cancer patients are likely a rich and a promising source of immunoglobulin V-gene repertoire for the generation of specifically binding recombinant antibodies. Libraries generated from these cancer patients might preferentially lead to the isolation of antibodies with higher affinity and specificity.

By using the human immunoglobulin databases (Chothia *et al*., 1992; Tomlinson *et al*., 1995, Williams *et al*., 1996) and published primer sequences (Barbas *et al*., 2001, Caros F *et al*., 2000), we designed 36 primers to completely cover all the human variable domain heavy and light families. It is necessary to mention that there are seven VH families, seven D_H, six JH families, six VLκ families, five Jκ families, ten V_λ lambda families and four J_λ families in human and each families has more than 30% differences at their DNA sequences (Tomlinson, *et al*., 1992; Bentley *et al*., 1980; Daley *et al*., 1992). In addition, linker DNA sequences of either long linker with 18 flexible amino acids or short linker with 7 amino acids have been built into the 3´-end of the VL and 5´-end of the VH primers. The short linker DNA sequences at the end of VH and VL fragments anneal to each other during the assembly PCR and, thereafter, extended by polymerases to generate the single chain antibody (scFv) fragment. It has been shown that scFvs with short DNA linkers (generally below 10 amin acids) tend to intermingle upon expression in *E. coli* and form dimmers, called diabodies. These bivalent molecules mimick IgG molecules in terms of binding capacity and due to their smaller size have greater tumor penetration (Holliger *et al*., 1993).

Distinct VH and VL bands from 67 individual PCR reactions were obtained under optimized conditions, and the corresponding amplified VH and VL (of both κ and λ types) fragments were combined. Subsequently, these fragments were used in an assembly PCR under standardized conditions and repertoires of scFv fragments with either short or long linker were resulted. These repertoires were cloned into pHEN6 phagemid vector and libraries with average sizes of approximately 10^8 individual colonies were generated. Considering that the V genes have been amplified from a rather immune source of lymphocytes, size of the constructed libraries are large enough to select binders of high affinity and specificity against the candidate tumor antigens.

In addition to the size, the quality of immunoglobulin libraries is also determined by the heterogeneity factor which is calculated as percentage of randomly selected clones with different amino acid sequences in their CDR regions. We applied two strategies to demonstrate the heterogeneity of our libraries. First, PCR products of a colony-PCR assay were digested with a frequent restrictions cutter (4-cut-

ter) and the digestion patterns were compared on a 2% agarose gel. As shown in Figure 5, all 18 clones seem to have different digestion patterns. Second, some 15 DNA samples from the above-mentioned clones were sequenced. The nucleotide sequences of 10 scFv DNA fragments with short linker and five scFv DNA fragments with long linker showed quite distinct and different CDR sequences. Nucleotide and amino acid sequence comparison of all 15 scFv clones with the published human VL and VH nucleotide sequences (Chothia *et al*., 1992; Tomlinson *et al*., 1995; Williams and Winter, 1993) showed that different human VL (V_κ and J_κ, or V_λ or J_λ) and VH (V_H, D_H and J_H) families have been used to generate the final VH and VL gene fragments. These data, altogether, demonstrate that the constructed libraries are of sufficient heterogeneities and can be used for the following phages rescue and screening steps to fish scFv binders against our candidate tumor antigens.

Finally, in order to prove that our libraries can be efficiently expressed in *E. coli*, a few clones from the libraries were randomly selected and induced to express the recombinant scFv proteins. The presence protein bands of approximately 30 kD in the blotting experiments confirm the effectiveness of expression of the libraries in *E. coli*.

In conclusion, we have amplified the immunoglobulin repertoires from a well-established B lymphocyte source that is likely to be very promising for the selection of specifically binding recombinant antibodies to our pre-defined tumor antigens. We, therefore, constructed scFv libraries of sufficient size and diversities and showed their expression in bacterial host. Screening of these libraries by displaying the recombinant scFvs on the surface of filamentous phages (M13) and panning against the candidate tumor antigens to select potential binders are the following steps of the present study.

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