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Isolation of scFv antibody fragments against HER2 and CEA tumor antigens from combinatorial antibody libraries derived from cancer patients

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ABSTRACT

Tumor cells expressing HER-2/neu and CEA antigens are potentially ideal targets for antibody-targeted therapy. In this study, two large human combinatorial libraries have been generated from the lymph nodes of breast cancer patients that express HER2 and CEA antigens in their tumors. These ‘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. Libraries derived from these patients may contain a larger pool of anti-tumor antigen antibodies and are useful repertoire for isolating scFvs against any tumor markers. The results of this study showed that we were successful in obtaining human scFvs against HER-2/neu and CEA. For HER-2, cell-panning strategy was performed and resulted in two scFv binders that detected the complete HER-2 receptor on the cell membrane and internalized to the cells. Also, preliminary ELISA data showed that several anti-CEA scFv binders were isolated by panning.

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1. Introduction

Cancer therapy is among the many challenging areas in which monoclonal antibodies have primarily been applied [1]. The critical parameter in this area, is to generate antibodies or antibody fragments against tumor-specific antigens [1,2]. Extensive efforts have been made to identify tumor-specific antigens and quite a number of them, such as carcinoembryonic antigen CEA [3], epidermal growth factor receptor EGFR [4] and c-ErbB family of receptors [5], have already been considered valuable prognostic, diagnostic, and therapeutic biomarkers.

c-ErbB-2, also known as HER-2/neu, is a 185 kDa transmembrane glycoprotein belonging to the EGFR family. These

receptors have three domains: an intracellular tyrosine kinase, a hydrophobic transmembrane domain, and an extracellular domain (ECD), which is the ligand-binding portion of the receptor [6]. Overexpression of HER-2/neu in cancerous cells is detected in approximately 25–30% of breast cancers as well as in many other tumors such as ovarian, gastric, pancreatic, salivary gland and lung [7,8]. Tumor cells that express HER-2/neu are potentially ideal targets for antibody-targeted therapy since they are accessible cell surface receptors and are associated with malignant transformation and aggressive tumor behavior. Therefore, several anti-HER-2 mAbs have been generated that target the extracellular component of the HER-2 receptor, but application of numerous of these mAbs was limited in humans due to their non-human origin [5,9,10]. Herceptin is, indeed, a commercial recombinant humanized anti-HER-2 mAb that binds to targets and blocks intracellular signaling [11]. Some antibody fragments can trigger internalization of their receptors. These mAbs can be directly conjugated to agents such as toxins or drugs and be delivered into cytosol as more potential tools for therapy [12]. More recently, internalizing

Abbreviations: mAb, monoclonal Antibody; CEA, carcinoembryonic antigen; ECD, extracellular domain; scFv, single chain fragments; ctrl, control.

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anti-HER2 antibody fragments have been developed as a newer generation of anti Erb-B2 therapeutics [13–15].

CEA is a 180–200 kDa, epithelial cell glycoprotein and a member of a large immunoglobulin gene family [16]. The over expression of CEA in a wide variety of tumor types, such as colorectal, breast, and lung carcinoma [17–19], has made it an appropriate target antigen for antibody diagnosis and therapy [20]. Recently anti-CEA antibodies have been used in the radio-detection of several carcinomas such as colorectal carcinoma. Currently, these antibodies are being explored for use in radio-antibody therapy [3,21].

One of the major obstacles facing antibody therapy has been the issue of immunogenicity [22]. The emergence of powerful screening technologies such as phage display has allowed for the generation of fully human antibody fragments [23–25]. Lymph nodes of cancer patients serve as promising repertoire of tumor-specific antibodies and identification of anti-tumor antibodies from libraries derived from these sources has previously been reported [25,26]. The 25 kDa scFvs are the smallest Ab fragments with potential clinical advantages over whole mAb. Antitumor scFvs have greater tumor penetration, more rapid blood clearance, lower retention in non-target tissues, and less immunogenic response *in vivo* [27].

In this study, two large human combinatorial libraries from the lymph nodes of breast carcinoma patients were generated. It has been demonstrated that there is a humoral immune response to over-expressed tumor-related antigens such as CEA and HER-2/neu in patients with cancer [10,28–30]. Therefore, libraries derived from these patients may contain a larger pool of relevant antibodies which can be recovered by panning against specific tumor antigens.

scFv libraries have been panned against the two tumor antigens, namely, HER-2/neu and CEA. For HER-2, either cell panning strategy or panning against recombinant biotinylated ECD domain of the HER-2 was performed. Panning experiments on SKBR3 cell lines resulted in a number of scFv binders and their DNA sequences were determined. The results of this study showed the feasibility of isolation of specific scFv against tumor antigens and serve in paving the way towards isolation of human variable domains which can be useful tools for cancer diagnosis and therapy.

2. Materials and methods

2.1. Cell cultures

The human breast carcinoma cell lines MCF-7 (low expressing HER2/neu), SKBR3 (over expressing HER2/neu), BT549, and the human melanoma cell line A375 (all kindly provided by NICGEB, Trieste, Italy) were grown in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies Europe). HEK293 human kidney embryo cell line was transfected with human ECD-HER2 and cultured in DMEM containing 1 mg/ml G418 (Life Technologies). Medium was supplemented with 10% FCS, 50 units/ml penicillin, and 50 µg/ml streptomycin (Life Technologies).

2.1.1. Expression of biotinylated ECD-HER2/neu in HEK293 cell lines

The gene encoding the human HER2/neu-ECD domain (1985 bp in length) was amplified from cDNA which was synthesized on mRNA template extracted from SKBR3 cell lines. The PCR products were cloned into pcDNA3-SV5-BAP. HEK293 cell lines were co-transfected with the recombinant ECD vector and pcDNA3-BirA encoding the *E. coli* Biotin ligase gene, resulting in the production of biotinylated ECD domain of HER-2 in the transfected cell lines. Stable cell lines of HEK293 harboring the DNA encoding ECD-BAP-BirA were established. The culture supernatant was dialyzed extensively against phosphate buffer (5.8 mM KH₂PO₄, 40 mM Na₂PO₄, pH 8). The biotinylated ECD domain was finally purified by

SoftLink™ Soft Release Avidin Resin (Promega, USA) according to the manufacturer's protocol (Manuscript in preparation).

2.2. Construction of the phage display libraries

Briefly, a second *Sfi*I restriction enzyme site with a different compatible end and a histidine tag were introduced into the pHEN4 phagemid vector [31,32] which resulted in the construction of the pHEN7M phagemid.

Blood and tissue samples were collected from 15 consenting patients who were undergoing resection of breast cancer. Auxiliary lymph nodes were collected during surgical operation on patients. Sections of paraffin-embedded tumor tissues from each individual were subjected to immunohistochemical analysis of HER-2 tumor antigen [33]. Detection of CEA level in the sera of patients was done using the ELISA technique [34]. The lymph nodes of three patients, defined as positive for both tumor antigens, were selected and used for RNA extraction. Total RNA was extracted from 100 mg of frozen lymph nodes. First strand cDNA was then synthesized using oligo-dT primer (Fermentas, Russia). A total of 36 different and degenerate primers (MWG, Germany) were used for the amplification of VH and VL repertoire according to Barbas III et al. [35]. For amplification of the human VH 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 Vκ and Vλ gene repertoires, the same approach was used with 16 (of different Vκ gene family) and 27 (of different Vλ gene families respectively) separate PCR reactions for Vκ and Vλ, respectively. For PCR assembly of the scFv repertoires approximately 20 ng of each purified VH and VL PCR products were combined in a SOE-PCR [35] reaction mixture. Large scale PCR amplification for both types of scFvs with long and short linker were performed and purified. Phagemid vector DNA was also prepared in large scale. Digested vector and scFv fragments were ligated with LigaFast™ Rapid DNA Ligation System (Promega) for 1 h. Some 10 µg vector DNA, 12 µg scFv-SL, and 16 µg scFv-LL were used for ligation experiments separately. The ligated products were transformed into electro-competent *E. coli* TG1 cells using the Gene Pulser electroporation system (Biolab, Germany). The size of the libraries was estimated by counting the number of colonies in the sample plate. A colony-PCR assay was performed to assess the number of positive colonies with scFv inserts. The diversity of the constructed library was determined by two methods of restriction enzyme analysis and sequencing. Briefly, the PCR products from the colony-screening assay were purified and digested with the frequent-cutter restriction enzyme *Mva*I (Roche, Germany) at 37 °C for 1 h. Purified PCR products from 15 different clones were also sequenced (MWG, Germany). The alignment with germ line V genes was performed using Ig blast (NCBI database) and Kabat data base [36].

2.3. Selection of internalizing antibodies from phage libraries

The scFv phage-display libraries were prepared and used for selection against the SKBR3 cell line, naturally expressing high levels of HER-2 [37]. To start, 1×10^{13} rescued phage particles were blocked with 5% milk powder in PBS for 30 min. The blocked phages were then pre-incubated with 10^8 MCF-7 cells for 1 h at 4 °C with constant shaking. The depleted scFv phages were collected after centrifugation at 800 rpm for 5 min at 4 °C. SKBR3 cells plated in a 20-cm plate with 80–90% of confluency were incubated with depleted phage library in 20 ml of cold culture medium at 4 °C for 1.5 h. Cells were washed gently 6 times with 25 ml of PBS and then incubated with 30 ml of prewarmed culture medium at 37 °C for

45 min to allow internalization of surface-bound scFv-phage. The phages bound to the cell surface were eluted by 10-min washes with 20 ml glycine stripping buffer (50 mM glycine, pH 2.5, 500 mM NaCl) and cells were washed with 30 ml of PBS for neutralization. Cells were lysed by 100 mM triethylamine and then neutralized with 1.5 ml of 0.5 M Tris–HCl, pH 7.4. To re-infect the internalized phage into *E. coli*, 1.22 ml of eluted phage was added to 20 ml of exponentially growing *E. coli* strain TG1 (OD₆₀₀ ~ 0.3) and incubated at 37 °C for 30 min. TG1 infection was tittered by plating 10³ and 10⁴ dilution onto 100 mm TYE/amp/glu plates. The remaining bacteria were plated onto TYE/amp/glu plates and phages were then rescued for the next round of selection. A total of 4 rounds of panning were performed. To determine the number of individual selected clones, the amplified scFv products were used for DNA fingerprinting and sequence analyses as described previously.

2.4. Characterization of binders by enzyme-linked immunosorbent assay (ELISA)

Binding of individual scFv clones from the fourth round of panning was analyzed by scFv ELISA. Expression of soluble scFv was induced by isopropyl β-D-thiogalactopyranoside (IPTG) (Roche) from single colonies grown in 96-well microtiter plates as described [38]. Ninety-six-well microtiter plates (Falcon, Crop, USA) were coated overnight at 4 °C with 10 µg/ml of purified HER2-ECD. Plates were blocked with 4% skimmed milk powder and bacterial supernatant containing scFv was added directly to each well and incubated for 1 h at RT. Wells were washed three times with PBS, 0.1% Tween 20, and then with PBS. Plates were incubated with mouse anti-His antibody (DAKO, Glostrup, Denmark), diluted 1:3000, for 1 h and after washing detected by HRP-conjugated anti-mouse mAb (DAKO), diluted 1:2000, as well as ABTS (2,29-azinobis (3-ethylbenzthiazoline-6-sulfonic acid)) (Sigma, Germany) as substrate. The numbers of unique scFv sequences were estimated by PCR fingerprinting of the scFv genes with the restriction enzyme *MvaI* (Roche) as described previously.

2.5. Cell lysis, Western blot, and precipitation using scFv antibodies

SKBR3 cells were grown to 80–90% confluence on a 10 cm plate. Cells were removed in 2 mM EDTA-PBS and washed three times in PBS. Cell lysate was prepared using 0.5 ml of lysis buffer (0.4% (v/v) NP40, 10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM PMSF) and then cell debris were pelleted at 12,000 rpm for 15 min. Extracts were run on an SDS-PAGE and transferred onto nitrocellulose membranes. Blots were incubated with scFv (10 mg/ml in PBS, 0.05%, Tween 1% BSA) overnight at 4 °C. ScFvs were detected using mouse anti-His mAb (DAKO) and HRP-conjugated rabbit-antimouse antibody (DAKO). HER2/neu antigen was recognized with murine anti-HER2 mAb (Sino Biological Inc.).

For precipitation, the purified scFv from a 500 ml culture of *E. coli* TG1 was added to biotinylated HER2-ECD in MPBS (1% milk powder, PBS) at 4 °C for 1 h with shaking. Samples were incubated with 50 µl streptavidin magnetosphere (paramagnetic particles, Promega) and rotated at room temperature for 30 min. Beads were washed ten times with 1 ml 1% Tween 20/PBS and then ten times with PBS. The proteins were released by boiling in loading buffer and then centrifuged for 1 min at 1000 rpm to pellet the beads. The supernatant was fractionated by 7.5% SDS-PAGE and electro-blotted onto membranes. The scFv and HER-2 proteins were detected using anti-His and anti-SV5 mAbs (Invitrogen), respectively.

2.6. Soluble scFv expression and purification

pHEN7M HA-His harboring scFv fragments were transfected to *E. coli* non-suppressor strain HB2151. The cells were grown at 37 °C in 2xTY medium containing 100 µg/ml ampicillin and 1% glucose to

OD₆₀₀ ~ 0.5, then they were pelleted at 5000 rpm for 20 min and resuspended in a glucose-free medium. The scFv expression was induced by addition of IPTG to a final concentration of 1 mM in the cell culture and grown overnight at 28 °C. Cells were centrifuged again and resuspended in ice-cold 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, and 20% sucrose. Cells were incubated for 1 h on ice and then centrifuged at 12,000 rpm for 30 min at 4 °C. The periplasmic extract was dialyzed in PBS and soluble scFv was purified by immobilized metal affinity chromatography using a Ni-NTA column (Qiagen, GmbH, Hiden, Germany) and gel filtration as described [39]. The purified scFvs were analyzed by SDS-PAGE and Western blotting using mouse anti-His mAb (DAKO) and HRP-conjugated rabbit-anti-mouse antibody (DAKO).

2.7. Flow cytometry analysis

Binding to whole cells was screened by fluorescence activated cell sorting (FACS) analysis using a FACS Calibur (Becton Dickinson) and the Cell Quest software. Experiments were performed on ice. Aliquots of 10⁵ cells were resuspended in 100 µl FACS buffer (PBS, 1% BSA) and incubated with 10¹² cfu/ml phage particles for 1 h. After two washes with FACS buffer, cells were incubated with murine anti-M13 mAb (DAKO). Cells were washed and incubated with FITS conjugated rabbit antimouse IgG (DAKO) for 30 min. After washing, cells were analyzed using a FacScan. FACS analysis also was performed with s-scFv (10 µg/ml in PBS/3% BSA). S-scFvs were incubated with 10⁵ cells in 100 µl of PBS/3% BSA. After washing, cells were incubated with murine anti-His mAb (DAKO) for 1 h and detected with FITS conjugated rabbit anti-mouse IgG for 30 min.

2.8. Fluorescence microscopy assay

Observation of scFv-phage internalization was obtained by confocal microscopy. Cells were grown to 60% confluency in 15-mm round cover slips and then media were changed. Final concentration of 10¹¹ cfu/ml phages or soluble scFv proteins (20 µg/ml) was added to cells and incubated at 37 °C for 1 h. The cover slips were washed 6 times with PBS and then 3 times for 10 min with glycine-stripping buffer to remove surface bound phages or scFv. Cells were fixed with PBS containing 4% paraformaldehyde for 5 min at room temperature, washed twice with PBS and permeabilized by incubating with 100% methanol for 10 min. Cells were blocked with PBS containing 1% BSA for 30 min. Intracellular phages or soluble scFvs were detected respectively with murine anti-M13 mAb (DAKO) or anti-His mAb (DAKO) (at RT for 1 h) followed by FITC-conjugated rabbit antimouse immunoglobulin (DAKO) (20 min at RT). The slides were mounted in ProLong mounting medium (Molecular Probes) and observed with a confocal Axiovert microscope (Zeiss).

2.9. Selection on CEA antigen

The library was panned on immunotubes (Maxisorp, Nunc) coated with 50 µg CEA antigen (ICNBiochemicals–Germany) and blocked with 2% (w/v) milk powder in phosphate buffered saline (MPBS). After blocking, 10¹² cfu phage particles suspended in 2 ml PBS containing 1% milk and 3% BSA were added and incubated for 2 h with rocking at room temperature. The tube was washed with PBS containing 0.1% Tween-20 (v/v) and bound phages were eluted with 1 ml of 100 mM triethylamine. The phage solution was neutralized by the addition of 0.5 ml of 1 M Tris–HCl, pH 7.4. Eluted phages were amplified by infection of fresh exponentially growing *E. coli* TG1 cells and then the phages were rescued. In total four rounds of selection were performed. Infections of the resultant colonies with M13K07 helper phage, expression of the library and phage rescue were repeated as described previously.

3. Results

3.1. Construction of two human scFv libraries

Pathological and medical records of 15 consenting patients in the hospital were studied. The presence of CEA and HER-2/neu in the patients' samples was confirmed by ELISA techniques and immunohistochemical studies, respectively. Lymph nodes from patients with metastatic tumors who had the highest score for the two tumor antigens were selected (data not shown). For construction of antibody library, pHEN7M phagemid was designed in which a second non-compatible *SfiI* restriction enzyme site and a histidine tag were inserted in the pHEN4 vector using the PCR method [32] (Fig. 1).

V_H , V_K , and V_L of immunoglobulin heavy and light chains were PCR-amplified using a combination of family-specific primers. In order to amplify the V_H encoding gene, 2 downstream primers and 2 sets of upstream primers with each set containing 6 different primers, were used. The first set was designed to produce scFv libraries with a short linker and the second set was designed for scFv with a long linker. The result of the assembly PCR of light- and heavy-chain variable fragments is a DNA fragment of about 750–800 bp in length that is shown in Fig. 2. After cloning in pHEN7M phagemid vector, the efficiency of cloning was estimated based on the number of positive and negative colonies as assayed by colony-PCR technique and digestion by *SfiI*. The results of titration confirmed library sizes of 5×10^9 and 8×10^8 for scFv-SL and scFv-LL, respectively. Digestion pattern of 20 randomly selected clones of both libraries with a frequent restriction cutter, *MvaI*, showed that they carried inserts of various sequences (Fig. 2). These results were confirmed by DNA sequencing. The heterogeneity of the libraries is measured based on the differences in the length and sequence of complementarity regions (CDRs) among individual clones with correct insert size. The heterogeneity percentage is estimated to be very high and sufficient for the next panning step.

The expression of scFv protein with molecular weight of ~30 kD was detected by anti His tag antibody using Western blotting (data not shown).

3.2. Selection of internalized scFv-phage

Phage was prepared from both human scFv phage libraries (with long and short linkers) for selection separately on SKBR3 cell line.

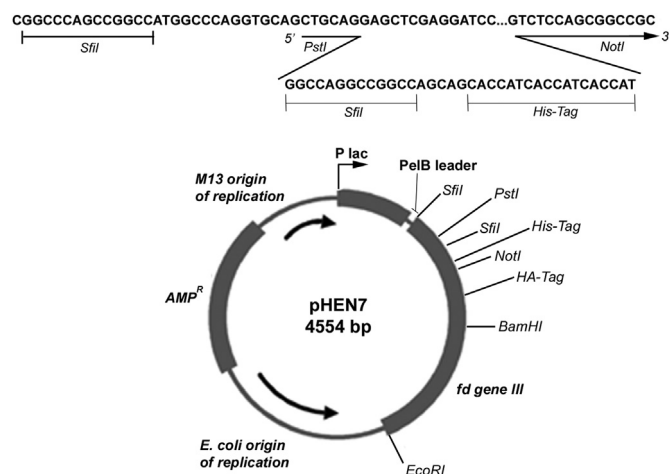


Fig. 1. The schemes of pHEN7M vector construction. A second *SfiI* restriction enzyme site with a different histidine tag compatible end was introduced into the pHEN4 phagemid vector by designing the upstream primer shown by the arrow.

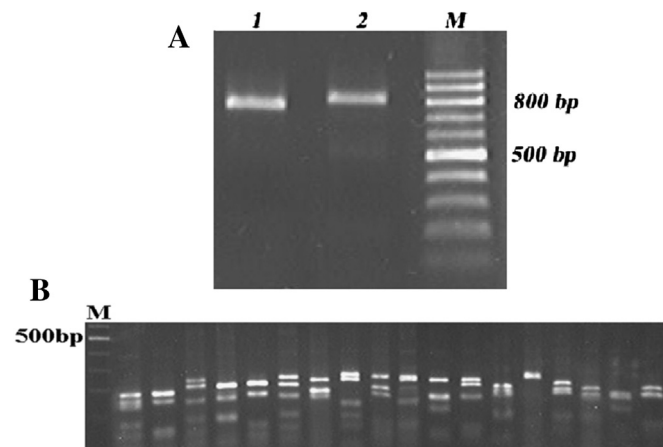


Fig. 2. A. Assembly of V_H and V_L into a scFv by an overlap extension PCR reaction. The results of the assembly PCR are DNA fragments of about 750–800 bp shown in lane 1 (for scFv with short linker) and 2 (for scFv with long linker). M is a molecular size marker of 100 bp ladder. B. Results of DNA fingerprinting on PCR-amplified scFv. The scFv DNA fragments were amplified from individual colonies, digested with *MvaI* restriction enzyme, and run on a 2% agarose gel. Different patterns of digestions with *MvaI* demonstrate that randomly selected clones are different from one another. M is a molecular size marker, 100 bp DNA ladder.

Libraries were first depleted of antibodies binding common antigens by adding phage to negative antigen cell line MCF7. Phages and SKBR3 cells were incubated at 37 °C for 1 h to allow endocytosis of surface-bound phages and then cells were washed extensively to remove weakly or nonspecifically bound phages. Cells were then stripped with a low pH glycine buffer to remove bound cell surface phages [12] and the internalized phages were released by cell lysate with a strong basic pH using TEA. The efficiency of selection was monitored by titrating the number of phages recovered from the cell lysate. A total of four rounds of selection were performed with SKBR3 cells whereas MCF7 cells were used for the depletion of non-specific phage antibodies in the library during each round of selection [15]. From the last selection round of the phage library with long linkers, the titer of internalized phages increase ~650 fold compared to the first round of panning. In contrast, no significant amplification was observed in the phage titers after four rounds of selection by phage antibodies rescued from the library with short linkers. Therefore, subsequent characterization of scFv clones was focused only on the scFv library with long linkers. After the fourth round of selection from this library, 30 distinct clones were picked up randomly and analyzed by PCR fingerprinting resulting in the identification of four different clones with unique CDRH3 sequences. These scFvs were named scFvHL-1, scFvHL-7, scFvHL-8 and scFvHL-10.

The success of selections was also monitored by flow cytometry. After each round of selection, polyclonal phages were prepared and analyzed for binding activity to cell lines expressing different quantities of HER2 (SKBR3 and MCF7). The fluorescent shift observed after the third round of selection on HER-2 positive cells was significantly greater than that observed after the first round of selection (Fig. 3). These results complemented the enrichment data from the panning experiment and strongly indicated the presence of HER-2 specific scFv-phage after four rounds of panning.

3.3. Isolation and characterization of monoclonal ECD-HER2 antibodies

After four rounds of panning, approximately 95 individual colonies were picked up and induced with IPTG to produce the soluble scFvs. An ELISA assay was performed on the periplasmic extracts

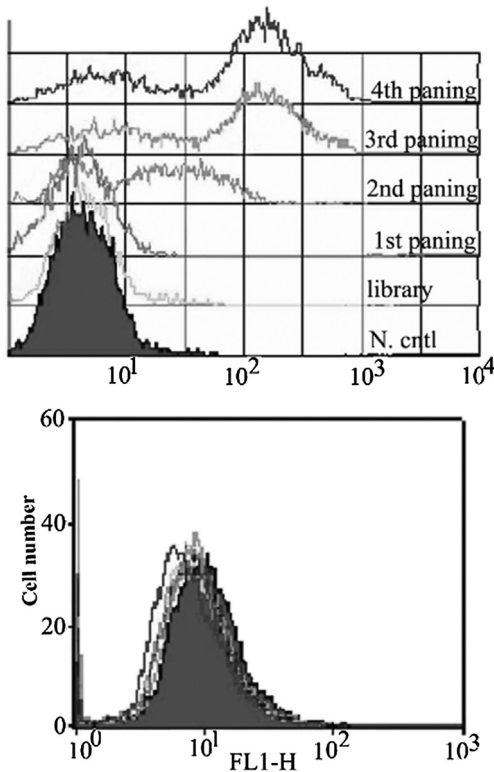


Fig. 3. FACS analysis of binding of polyclonal phages obtained from each round of selections to HER2/neu expressing (SKBR3) and non-expressing (MCF7) cells. At the upper panel, phage populations from the third and fourth rounds of selection showed significant fluorescent shift on SKBR3 cells. No difference in staining was observed on MCF7 cells compared to cell background (lower panel).

and showed the presence of 19 specific binders (approximately 20% of the clones) attached to the coated HER2-ECD. Two scFv clones with restriction patterns the same as scFvHL-8 and scFvHL-10 binders were identified by DNA fingerprinting and sequencing. The sequencing results of these clones are shown in Fig. 4.

Sequence analysis of these clones revealed that V_H of both scFvs belongs to the V3-9P gene family and show high homology, except in the CDR3 region, whereas there was no obvious homology among the V_L region of these two scFvs. V_L of scFvHL-10 and scFvHL-8 belonged to the IV3/8 and DPK1/018 gene families, respectively.

To obtain soluble scFv clones, individual phagemid vectors harboring the scFv genes fused to C-terminal hexa-histidine sequence were transformed in *E. coli* HB2151 and the cells were induced with IPTG. The expressed soluble proteins were extracted from periplasmic space [40] and purified by immobilized metal affinity chromatography (IMAC). Gel filtration and Western blotting analysis with an anti-His tag mAb indicated that all scFvs were monomeric and therefore, they have a single antigen-binding site. The results of Western blotting and SDS-PAGE are shown in Fig. 5A and B.

Four soluble scFvs were analyzed by ELISA for their ability to bind recombinant HER2-ECD. Again, only scFvHL-8 and scFvHL-10

showed high binding to HER2-ECD. Two other scFvs (scFvHL-7 and scFvHL-1) gave no significant binding activity above background (Fig. 5C).

To determine if soluble scFvs were specific for HER-2, the scFvs were assayed in a Western blot of the SKBR3 cell lysate. The two scFvHL-8 and scFvHL-10 were able to recognize a protein band of ~185 kDa on cell lysate which is the expected molecular weight for the complete HER-2 tumor antigen. A protein corresponding to the same size was detected by the murine anti-HER2 mAb. No bands were detected on SKBR3 cell lysates when a soluble anti-CEA scFv from our laboratory was used as a negative control (Fig. 5D).

In order to prove the binding of individual scFv protein to recombinant ECD domain expressed by the constructed cell lines, a precipitation assay was performed. The scFv proteins were incubated with culture supernatant of the HEK293 cell line expressing the biotinylated ECD domain of HER-2. Subsequently, streptavidin magnetic beads were used to precipitate and separate the bound proteins to ECD domain and, finally, the captured bead-protein complexes were boiled and run on an SDS-PAGE gel. The protein bands, scFv and HER2-ECD were detected in Western blot with anti-His antibody and anti-SV-5 antibody (epitop tag of HER2-ECD), respectively. As shown in Fig. 5E, only two scFvs (scFvHL-8 and 10) are able to bind the ECD domain. The precipitation and Western blotting results demonstrate that scFvHL-8 and scFvHL-10 specifically recognize the extracellular domain of HER-2.

3.4. Cell binding and internalization of phage antibodies and scFvs

Following the characterization of scFv proteins, their ability to bind to SKBR3 cell lines was demonstrated in FACS assay. Flow cytometric analysis showed that all clones (scFvHL-1, 7, 8, 10) stained HER-2 expressing cells (SKBR3) whereas they failed to stain MCF7 cells. Furthermore, no fluorescence was detected with anti-CEA scFv-phage using SKBR3 cells. The results of this experiment are shown in Fig. 6A.

The ability of the phage antibody internalization was determined by fluorescence microscopy. The selected clones, both in the phage and in the soluble form, were tested. Cells grown on cover slips were incubated with scFv-phage and then extensively washed with PBS to remove nonspecific binding. Surface phages were removed with low-pH glycine and, after fixation and permeabilization of the cells, the intracellular phages were detected using an anti-M13 phage mAb and a FITC-conjugated second antibody. The staining of the cells was assessed directly by immunofluorescent microscopy. Intracellular phages scFvHL-8 and scFvHL-10 were visualized inside SKBR3 cells but no staining was seen in MCF7. Additionally, a control anti-CEA scFv-phage gave no intracellular staining (Fig. 6B).

For purified native scFvs, the experiment was repeated on cells by scFvs. Intracellular scFvs were detected using anti-His tag mAb followed by anti-mouse FITC labeled antibody. Identical results were observed with the soluble scFvs. The results, shown in Fig. 6B, demonstrated that all scFvs or their phages are able to bind to the SKBR3 cell surfaces as well as enter the cells.

For further characterization of non-HER2 binding antibodies, binding of scFvHL-1 and scFvHL-7 phage antibodies was assessed with a panel of cell lines (including A735 human melanoma, BT549

FR-L1	CDR-L1	FR-L2	CDR-L2	FR-L3	CDR-L3	FR-L4
scFvHL-8: EIVMTQSPSSLSASVGRVTITC	QASQDISNYLN	WYQQKPGKAPKLLIY	DASNLET	GVPSRFSGSGSGTDFTITSSLPEDIATYYC	QYYDNPLPT	FGGGTRLEIK
scFvHL-10: SYELTQPPSVSVAPGKTARITC	GGNLESKSVH	WYQQKPGQAPVYVY	GDIDRPS	GIPERFSGSKSGNTAITLITRVEAGDEADYHC	QVWDSDHWV	FGGGTKLTVL
FR-H1	CDR-H1	FR-H2	CDR-H2	FR-H3	CDR-H3	FR-H4
scFvHL-8: QVQLVESGGGLVQPGRSRLRSCAAS	GTFTDDYAMH	WVRQAPGKGLEWVSG	ISWNSGSGIYADSVKG	RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR	GGAFIPAFDI	WGQGTITVTVSS
scFvHL-10: EVQLLETGGGLVQPGRSRLRSCAAS	GTFTDDYAMH	WVRQAPGKGLEWVSG	ISWNSGSGIYADSVKG	RFTISRDNAKNSLYLQMNSLRAEDTALYYCAK	DMVNSGWYSPFFDI	WGQGTITVTVSS

Fig. 4. Amino acid sequence of Framework (FR) and complementary determining regions (CDR) of V_H and V_L from scFvHL-8 and scFvHL-10 clones.

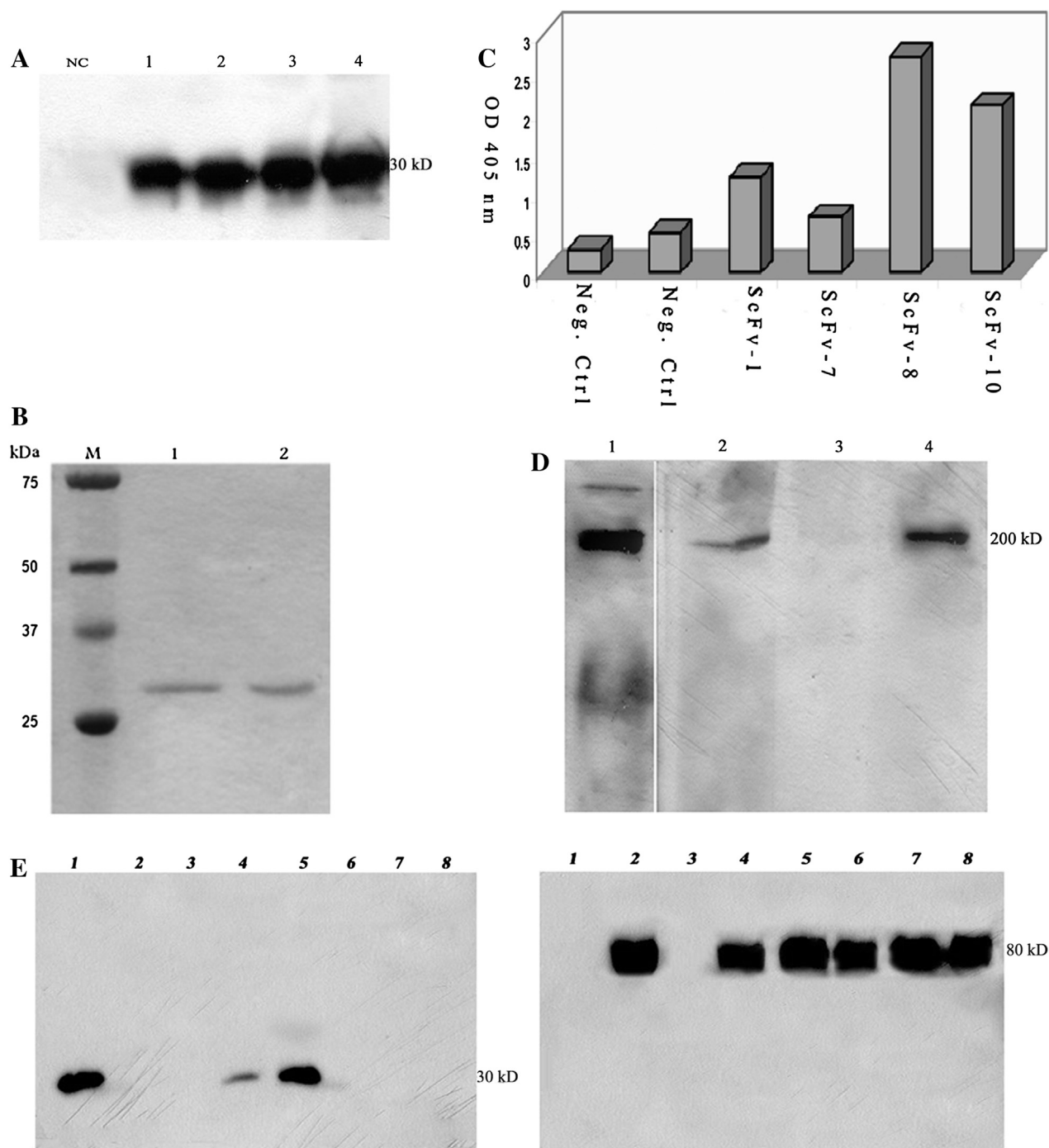


Fig. 5. Characterization of selected binders. A. Immunoblotting experiment was performed on the expressed scFv protein in HB2151 *E. coli* strain. The recombinant scFvHL-1, 7, 8 and 10 were detected in lanes 1–4 respectively by anti-His tag and anti-mouse-HRP antibodies. The presence of a single band of about 30 kDa demonstrates the presence of monomer scFv molecules in bacterial periplasmic extracts. B. SDS-PAGE of purified scFvHL-8 (lane 1) and scFvHL-10 (lane 2) stained with Coomassie. Purified scFvs are observed at ~30 kDa according to the protein marker (M). C. The ELISA results from incubating soluble scFvs extracted from clones scFvHL-1, 7, 8 and 10 with recombinant HER2-ECD domains pre-coated in ELISA wells. Only scFv clones 8 and 10 showed significant binding to the recombinant ECD domain. D. Western-blot analysis of cell lysates using scFvs. SKBR3 cells were lysed, run on an SDS-PAGE, and blotted onto a nitrocellulose membrane. As primary antibodies, purified scFvHL-8 (line1), scFvHL-10 (line2), anti CEA scFv (line 3), and anti HER2 mAb (lane 4), as secondary antibody, anti-His, and then HRP conjugated antibodies were used. ScFvHL-8 and 10 recognized a protein of approximately 185 kDa on cell lysate. E. Immunoblotting experiment performed on the culture supernatant from cell line stably expressing the biotinylated HER2-ECD domain. Soluble scFvs from clones scFvHL-1, 7, 8, and 10 were incubated with culture supernatants and the bound proteins were captured by magnetic streptavidine beads. The same proteins were blotted in both membranes in the same order and developed by anti-His tag antibody (tag of scFv) (left panel) and anti SV-5 antibody tag (tag of ECD) (right panel). Lane 1 (negative control) is scFv protein without beads, lane 2 (positive control) is HER2-ECD complexed with beads, lane 3 (negative control) is scFv protein incubated with beads, lanes 4–7 are scFv proteins obtained from clones 10, 8, 1, 7 respectively, and lane 8 is anti CEA scFv incubated with HER2-ECD. As shown, only scFvHL-8 and 10 proteins are able to bind the HER2-ECD domains.

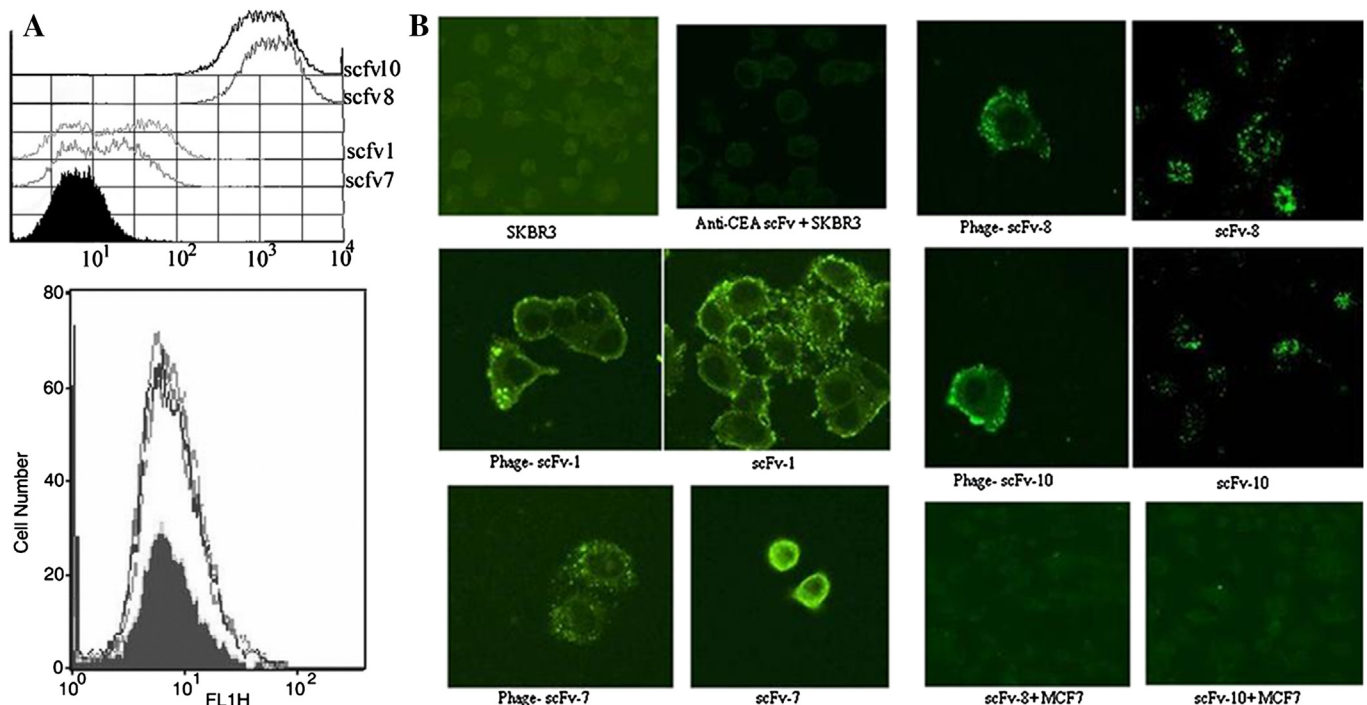


Fig. 6. Internalization of the recombinant scFvs. A. FACS assay to demonstrate the binding of individual scFv proteins to the SKBR3 cell lines. In the upper panel, all clones showed a shift upwards compared to the cell background (black histogram), however, scFvHL-8 and 10 stained SKBR3 cells more intensely. The same binding assay on the MCF-7 cell line (negative control) is shown in the bottom part. B. The results of fluorescent microscopy experiment to demonstrate the ability of scFv clones or their phage particles in binding to SKBR3 cells and entering into the cells. Cells were fixed and permeabilized, intracellular antibodies were detected by anti-His or anti-M13 antibodies as primary antibodies and FITC-conjugated secondary antibody. As negative control, MCF-7 cells were used which did not show any color development.

breast tumor, HEK293, and SP20 mouse myeloma cell lines) by flow cytometry. The two phage antibodies significantly stained SKBR3 and BT549 cells which are both breast cancer cell lines. On the other hand, they did not stain human melanoma and normal kidney cell lines or SP20 (Fig. 7).

3.5. Selection of CEA antibodies

The phage scFv at 10^{12} pfu (input) was subjected to immunotubes coated with purified CEA antigen. After a 2 h incubation period, the immunotubes were extensively washed to remove nonspecific binders, and the bound phages (output) were calculated after each panning. Phage scFv to CEA were specifically enriched after four rounds of panning and individual colonies were

picked up and grown for phage preparation. The result of phage–ELISA is shown in Fig. 8. ScFv clones 4, 5 and 7 showed significant binding to the CEA tumor antigen. The nucleotide sequences of these clones demonstrated that they have different CDR lengths and amino acid combinations, and that they belong to different V_H and V_L gene families (data not shown). Further analysis of these scFv binders, including cell assay, is suggested for future studies.

4. Discussion

Phage display library is a powerful technique that can be used to identify tumor specific antibodies by panning against live tumor cells [41,42]. In this study, scFv format of antibody was selected for library construction since relatively simple genetic manipulation is

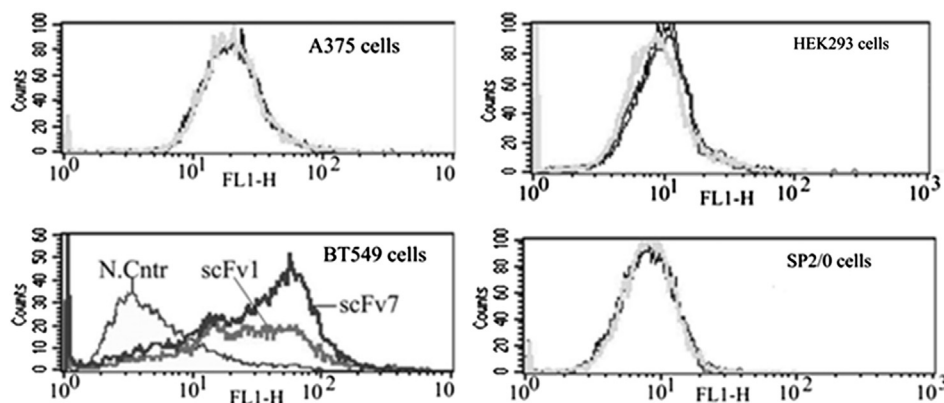


Fig. 7. Flow cytometric analysis of scFvHL-1 and scFvHL-7 phage antibody binding to various cell lines. (A375) Human melanoma cells, (BT549) human breast carcinoma cells, (HEK293) human kidney embryo cells, and (SP2/0) mouse myeloma cells. Control = M13K07 helper phage. Both scFvHL-1 and scFvHL-7 bind to BT549 cell line.

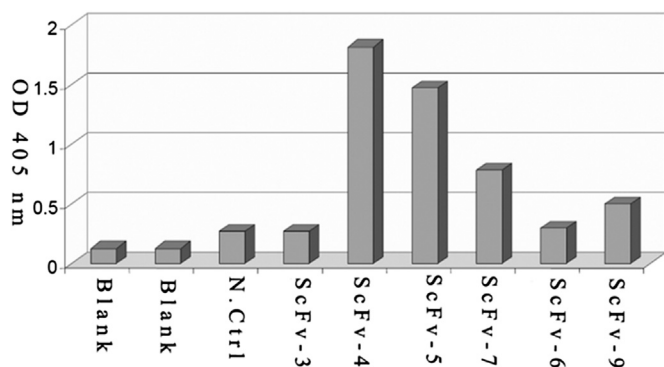


Fig. 8. The Phage-ELISA results after four rounds of panning against CEA antigen. Only three clones (scFvHL-4, 5, and 7) showed significant binding to CEA.

required to assemble two variable domains. There is also a higher level of scFv in *E. coli* that lead to a more efficient display of these molecules on the surface of filamentous phages [43].

For library construction, metastatic lymph nodes of breast cancer patients, that overexpress HER-2/neu (i.e. an Immunohistochemical score of 3+) and CEA antigens, were used to maximize the chance of isolating the V_H and V_L genes of in vivo-matured antibodies. Overexpression of self-proteins can enhance immunogenicity in cancer patients [44]. Expression of HER-2/neu and CEA antigens is up-regulated in breast cancer cells and humoral immune response was detected against both of these antigens in patients [30,45,46]. Indeed, more than half of these patients have anti-CEA antibody circulating in their sera [30]. The level of HER-2/neu-specific antibodies correlates with the level of HER-2/neu overexpression and is also associated with disease progression as reflected by lymph node and metastatic status [44,46,47]. Previous studies have demonstrated that HER-2/neu-specific humoral responses are significantly more prevalent in patients with +3 protein overexpression in their tumors [44]. Moreover, in patients with breast cancer, B lymphocytes in metastatic peritumor lymph nodes are most likely pre-immunized by tumor-associated proteins as previously demonstrated for melanoma antigens [48,49]. Therefore, it was determined that the lymph node tissue of cancer patients is the most appropriate source for the generation of mAb libraries [50].

For the construction of the immunoglobulin V-genes library, a new phagemid vector (pHEN7M) was designed which simplified the cloning strategy for library construction and increased the diversity of scFv genes by using a single restriction enzyme (*Sfi*I). In addition, it allowed for the detection of recombinant antibodies by anti-His antibodies and purification of recombinant scFvs by affinity chromatography.

Functionality of the vector was evaluated by making a small model library from peripheral blood lymphocytes (PBL). The V_H genes originating from both IgM and IgG were amplified to maximize retrieval of the wide possible naïve and immune V-gene repertoire and increase the library diversity. Distinct V_H and V_L bands from 67 individual PCR reactions with 36 primers were obtained. These repertoires of scFv with both short and long linkers were cloned separately into pHEN7M phagemid vector and two library sizes of approximately 10^9 individual colonies were generated. Considering that the V-genes have been amplified from a rather immune source of lymphocytes, the constructed library sizes are large enough to select binders against the candidate tumor antigens. The quality of immunoglobulin libraries is also determined by the heterogeneity factor which is calculated as the percentage of randomly selected individual colonies with different amino acid sequences in their CDR regions. Nucleotide and amino

acid sequence comparison of scFv clones with the published human V_L and V_H nucleotide sequences [51] showed that different human V_L ($V_{L\kappa}$ and $J_{L\kappa}$, or $V_{L\lambda}$ or $J_{L\lambda}$) and V_H ($V_{H\mu}$, $D_{H\mu}$ and $J_{H\mu}$) families have been used to generate the final V_H and V_L domain sequences.

The aim of this study was the generation of specific antibodies against HER2 and CEA, however, constructed libraries are also valuable sources of antibodies against any potential related tumor antigen. The effective factors to finding interested binders in an antibody library are library size, diversity, and the source of immunoglobulin genes. As mentioned previously, the considerable size and variability of this library makes it a useful repertoire for isolating scFvs against other related tumor antigens. In addition, immune responses to tumor-related antigens occur quite frequently in patients affected by breast cancer [47,52], thus in comparison with unaffected individuals, the immunoglobulin repertoires of these patients provide a strong source of antitumor profiles. In contrast to other libraries, mAb libraries generated from a combination of antibody repertoires of various patients should potentially enable the recognition of a more complete coverage of antigenic determinants on the tumor cell surface [24,25]. This ability makes these scFv combinatorial libraries attractive candidates for the isolation of targeted cancer therapeutic agents.

In order to obtain phage antibodies which are able to bind to HER-2 receptors and can be endocytosed by the cell, the subtractive cell panning strategy was selected. Direct panning of the antibody library on living cells allows isolation of antibodies that recognize the native antigen in a physiologic context [41,42,53]. In this study, the isolation of internalizing antibodies was carried out since scFvs isolated from cell surface selection of phage libraries have, typically, not shown antitumor activity [54]. In contrast, intrinsic antitumor activity has been observed with antibodies that trigger internalization [55]. Additionally, this strategy can identify novel tumor antigens that may be used for clinical applications [53,56].

As mentioned previously, two libraries with two different linkers were constructed in this study. The linker DNA sequences were either long linker with 18 flexible amino acids (GGSSRSSSGGGGSGGGG) or short linker with 7 amino acids (GGSSRSS) [35]. It has been shown that scFvs with short DNA linkers (generally below 10 amino acids) tend to intermingle upon expression in *E. coli* and form diabodies [57] that can crosslink receptors and induce internalizing more efficiently than monomer scFv [58]. However, the scFv library with short linkers did not show specific enrichment in the phage population after the fourth round of panning and, consequently, no binders were identified. On the basis of previous investigations, it was determined that increased enrichment ratios are essential for selection of antibody libraries on heterogeneous antigens on the cell surface [56]. A possible explanation for that could be that a functional Fv domain could not be folded by a scFv molecule with a linker of 7 residues and instead, it led to the formation of a diabody [57]. Unfortunately, a diabody configuration form is not stable and several factors such as concentration of scFv molecules in solution, buffer components, pH and ionic strength are important for dimer generation [59,60].

Successful selection of four rounds of panning of scFv library with long linker on SKBR3 cell lines was demonstrated by flow cytometric analysis as well as by titration of the internalized phages. Four different clones were isolated that showed high expression in *E. coli* and had unique sequences with CDR3 length of 9–14 amino acids. Binding of these purified phages or their soluble scFv proteins to the SKBR3 cell were demonstrated by FACS analysis and no binding to a negative cell line was observed. The results of fluorescent microscopy studies demonstrated that all four scFvs, namely, scFvHL-1, 7, 8 and 10, were able to bind and enter into the SKBR3 cells. Further analysis of the binding properties of the

antibodies showed that scFv-8 and scFv-10 were able to specifically recognize the ECD domain of the HER-2 absorbed to ELISA plates and in solution. In addition, binding of both scFv-8 and scFv-10 to complete HER-2 receptors was confirmed on the cell lysates by Western blotting. Finally, sequencing data obtained from the heavy and light DNA fragments of scFvHL-8 and scFvHL-10 showed that their CDR regions, particularly CDR3 region, have significantly different sequences suggesting that they may recognize different epitopes with differential affinity.

These results confirmed that our approach for identification of the antibodies' ability to enter the tumor cells has been successful. Monomeric scFv has been shown to induce receptor-mediated endocytosis either by mimicking the natural ligand binding or by spontaneous generation of divalent antibodies [53,56,61] that crosslink receptors. Such antibodies that trigger HER2/neu internalization can have desirable biological function such as blocking the HER2/neu signaling, and receptor down regulation. In addition, they may inhibit tumor cell growth and induce apoptosis [15,62]. Internalizing anti HER-2 antibodies remove HER-2 from the cell surface which leads to the prevention of HER-2 dimerization with other surface HER proteins, and induces down-regulation of the HER-2 signaling pathway [63]. Furthermore, it seems that internalizing anti HER-2 antibodies act by homodimerization of receptors. Since the HER-2 homodimers are at the degradation pathway, these antibodies reduce signaling activity that resulted from HER-2 hetero or homodimers by degradation of HER-2 and decrease the level of HER-2 at the cell surface [63,64]. These types of antibody binders are advantageous for drug delivery and some innovative therapeutic agents such as antisense technology or nanoparticles [65]. A great number of antibodies against HER2-ECD have been identified [5,9], however, most of them have had nonhuman origin. Although Herceptin, a humanized variant of anti-HER-2 murine antibody, has been used for treatment of advanced breast cancer, it can be internalized after long periods of incubation with cells [66].

To date, two groups have identified human scFvs against ECD-HER2 that are able to internalize cells [15,56,58]. Among these two groups, Erbicin has been considered an immunoagent with effective properties and human origin [15,67]. To our knowledge, the anti-HER2 antibody fragments developed in this experiment, scFvHL-8 and scFvHL-10, are the first reported that have been isolated from a large human library immunized *in vivo* against this antigen. As an immune antibody library efficiently enriched for a diverse set of matured human mAbs, scFvHL-8 and scFvHL-10 can be potentially used as anti-tumor agents with mature properties. Furthermore, in comparison with Erbicin that has been internalized into the HER2-overexpressing SKBR3 cells within 16 h of incubation at 37 °C [15], both scFvHL-8 and scFvHL-10 were rapidly internalized within 1 h with the same conditions as demonstrated by confocal microscopy.

Western blot and flow cytometric analysis on some cell lines revealed that clones scFvHL-1 and 7 bind specifically to two different breast cancer cell lines (BT549 and SKBR3), suggesting that they might bind alternative endocytosing receptors commonly expressed by breast tumor cells and therefore, likely recognize novel cell surface antigens. These internalizing scFvs may be bound to active receptors or transporter molecules that are important in tumor survival. Identification of the antigens bound by these antibodies will be addressed in the future. Since these two scFvs were isolated from subtractive panning of Ab library on breast cancer cell line MCF7, it is probable that a much larger number of tumor-specific antibodies remain to be identified by screening breast tumor sections.

In the case of CEA, a number of monoclonal antibodies have been developed to date [3,20,68]. Due to low expression in normal tissues,

CEA has been accepted as a suitable target for antibody-based localization and therapy strategies by radioimmunodetection and radioimmunotherapy [21]. ScFvs are advantageous over whole antibodies for these purposes due to its rapid clearance from the bloodstream [27]. Anti-scFv antibodies, like MFE-23 scFv [3] and T84.66 mAb [68], that are used in clinics, have murine origins. Three different anti-CEA binders isolated here are completely human antibodies with different CDR sequences. Although further investigations are necessary for the characterization of these antibody fragments, to our knowledge, they are the first anti-CEA antibodies isolated from an immune human scFv library.

5. Conclusions

The immunoglobulin repertoires were amplified from a well-established B lymphocyte source that shows high selectivity of specifically binding recombinant antibodies to the predefined tumor antigens. To construct large effective scFv antibody libraries, we exploited the *in vivo* occurrence of maturation humoral immune response to cancer antigens. By this method, repertoire of anti-tumor antibody genes was enriched. These libraries are large enough to provide useful sources for isolation of recombinant scFv antibodies against a broader array of target antigens (cancer or non cancer antigens). Furthermore, they yield a panel of human tumor specific antibodies, could be useful for mapping the tumor cell surface epitope space, and can be used to probe neoplastic variations on the surface of breast cancer cells.

The results obtained so far demonstrated that we were successful in obtaining human scFvs against our candidate antigens and further studies on the obtained antibodies may open new windows of opportunities for the detection of HER-2 and/or CEA-tumor cells in patients and pave the way for improved future tumor diagnosis and treatment (as a 'naked' antibody or as a delivery molecule). However, further studies are needed to determine the specific interaction of these antibodies with their tumor targets.

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