Isolation and Evaluation of Specific Human Recombinant Antibodies from a Phage Display Library against HER3 Cancer Signaling Antigen

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Abstract

Background: The human epidermal growth factor receptor family comprises four homologous members: EGFR (ErbB1), ErbB2 (HER2), ErbB3 (HER3) and ErbB4 (HER4). This family plays an important role in the signaling pathway and cell proliferation. The heterodimerization of HER2 with HER3 leads to tumor cell proliferation. Monoclonal antibody to the human HER3 receptor blocks HER3 heterodimerization and inhibits the growth of breast cancer cells. Due to their human origin, small size, rapid penetration and high affinity properties, recombinant single chain antibodies (scFv) have been introduced as the most desired agents for cancer immunotherapy. In this study, we use a phage display system to select specific scFvs against HER3 for their use in cancer targeted therapy.

Methods: A phage antibody display library of scFv was panned against an immunodominant epitope of HER3. Phage rescue was performed on the library. The supernatant that contained the appropriate scFv (10^9 PFU/ml) was added to an immunotube which was coated with the peptide. Elution was done using log phase E. coli TG1. The clones were amplified by PCR and DNA fingerprinted to select the specific clones against the epitope. The specificity of the selected antibodies was tested in ELISA.

Results: The results represented two predominant patterns with the frequency of 25%. The other patterns showed the frequencies of 5%-10%. scFv1 and scFv2 demonstrated positive ELISA with absorbances of 0.63 and 0.46, respectively while the absorbances of wells without peptide were 0.19 and 0.11, respectively.

Conclusion: In this study two specific scFvs were selected against HER3 antigen in a successful panning process. Phage ELISA represented the specific binding of scFvs against HER3. The selected scFvs reacted only with the corresponding peptides. However, no reaction with the other peptides was detected. The selected anti-HER3 scFvs have suggested that these human high affinity and small antibodies that bind specifically to HER3 epitope can be considered in HER3 targeted approaches.

Keywords: Breast cancer, HER3, Specific scFvs, Antigen targeting

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Introduction

The rate of cancer incidences is increasing throughout the world, particularly in developing countries and breast cancer is the most common cancer among women worldwide. There are approximately one million new breast cancer cases each year comprising approximately 18% of female malignancies. It is also believed to be the most common cause of cancer-attributable deaths among women.2,3

The epidermal growth factor (EGFR) or ErbB family or HER family consists of four members: ErbB/HER1, ErbB/HER2, ErbB/HER3 and ErbB/HER4.4,5 ErbB family of type 1 receptor kinase (RTKs) and the remaining RTKs consist of a glycosylated extracellular ligand-binding domain and a hydrophobic transmembrane domain.5 HER2 over-expression occurs in about 25% to 30% of breast cancers. Homodimerization of HER2 and heterodimerization of this molecule with other HER2 members activates a network of signaling processes that lead to tumor cell proliferation, adhesion, angiogenesis, migration, and ability to decrease apoptosis.6,7

HER3 has been strongly implicated as a dimerization partner of HER2 in promoting cancer transformation by activating the PI3K pathway. Heterodimerization of HER2/HER3 is known as a strong oncogenic process in breast cancer that increases the risk of relapses and decreases overall chance of survival.8,9 HER3 is the only member of the HER family that has long been thought to be an inactive pseudokinase due to amino acid substitutions in the conserved kinase domain. Until recently, HER3 activity has been unknown, as it consists of an asparagine residue (N815) in place of the catalytic aspartic acid residue. Therefore, it has been broadly thought that HER3 depends on its combination with other active ErbB receptors for biological activity. However, it has been recently shown that HER3 does, in fact, exhibit residual kinase activity that may be crucial for ErbB signaling.10 Loss of HER3 in HER2-dependent cells results in decreasing the signaling through PI3K and cell proliferation, suggesting that HER2 may rely on HER3 to drive growth and survival of breast cancer cells.11 The high expression of ErbB3 in certain human cancers such as breast, ovarian, prostate and colorectal cancers suggests that it may play an important role in tumor development and can be taken into account as a therapeutic target.10-13 Monoclonal antibodies (MAbs) trastusumab (herceptin) and pertuzumab to HER2 are able to block HER2 homo and hetero dimerization14 and a monoclonal to HER3 (SGP1) has also been reported that binds to HER3 and inhibits HER2/HER3 heterodimerization15 but due to human anti-mouse antibody response (HAMA), these MAbs have limited clinical application.16,17 In addition, although humanized MAbs are less immunogenic, they still elicit an anti-idiotypic response because of non-human V region sequences.18,19

Advances in recombinant antibody technology during the past decade have facilitated the production of antibody fragments. These advances

Figure 1. PCR results of the library clones.
have introduced a large variety of engineered antibody molecules for research diagnosis and therapy. A scFv antibody contains a complete binding site and consists of variable regions of heavy (VH) and light (VL) chains joined by a flexible peptide linker. The unique properties of scFvs such as human origin, small size with excellent tumor penetration from the vasculature, high affinity, high specificity and ability for genetic manipulation have made these recombinant antibodies ideal components for cancer immunotherapy.

In this study we used amino acids 1330-1342 of the HER3 molecule and selected specific scFvs against this epitope. The specificity of the selected antibodies was evaluated in ELISA.

**Materials and Methods**

**Phage rescue**

A single chain Fv library was produced as previously described. E.coli bacteria that contained ligated phagemid (pCANTAB5 vector cloned with VH-Linker- VL) were grown overnight at 30°C on a 2TYG agar/ampicillin plate. The cells were scraped and inoculated into 2TYG broth and incubated with shaking at 37°C for 1 h. M13KO7 helper phage was then added and mixed with the culture and incubated at 37°C for 30 min followed by shaking at 37°C for 30 min. The culture was then centrifuged. The bacterial pellet was resuspended in 2TY broth that contained ampicillin kanamycin and incubated with shaking at 30°C overnight. The culture was centrifuged.

The supernatant was filtered using 0.2 µm filters (Orange, Belgium) to remove bacterial particles and subsequently stored at 4°C.

**Selection of specific single chain antibodies against HER3 epitope**

Specific scFvs were selected using the panning process. A total of 4 ml of the peptide with optimum concentration (10μg/ml) was coated on polystyrene immunotubes (Nunc, Denmark) at 4°C. The tube was then washed four times with PBS. Blocking buffer (2% skimmed milk in PBS) was added to the tubes which were then incubated at 37°C for 2 h. The tubes were washed with PBST and PBS, four times each. Diluted phage supernatant with blocking solution (1:1) was added to the tube and incubated at room temperature (RT) for 1 h. The tubes were washed with PBST and PBS. Log phase TG1 E. coli was then added to each tube and incubated at 37°C for 1 h. The tubes were centrifuged. The supernatant was removed and the pellets resuspended in 2TY broth medium, plated onto a 2TYG agar/ampicillin plate and incubated at 30°C overnight. Four rounds of panning were used to select specific antibodies against each peptide.

**PCR and fingerprinting of the library and selected clones**

The library clones and clones obtained after four rounds of panning were PCR amplified and Mva1 fingerprinted. Single colonies were picked up from the plates and incubated for 10 min at 94°C to lyse the cells and prepare the DNA.

![DNA fingerprinting of the library clones before panning. Different patterns represent the heterogeneity of the library.](image-url)
template. The inserts were amplified using a vector primer. Each PCR tube contained DNA template, primers (1 pmol), MgCl2 (2mM), dNTPs (2mM), buffer (1.5 mM), Taq polymerase (5U/µl) and distilled water to a final volume of 25µl and placed in a thermo cycler (Primus-96, Germany) with a thermal cycling program at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. After running the PCR products in the gel, we performed fingerprinting to check for diversity and frequency of the positive clones. Each PCR product was digested with a restriction enzyme (Fermentas, Germany) and the samples were run on a 2% agarose gel.

Phage ELISA

The peptide was coated on 96-well ELISA plates and placed overnight at 4°C. After washing three times with PBS, the wells were blocked using blocking solution (2% skimmed milk in PBS) and maintained at 37°C for 2 h. The wells were then washed with PBST and PBS. Afterward the phage rescue supernatant that contained the appropriate scFv diluted with blocking solution (1:1) was added to each well and allowed to remain at RT for 2 h. The plate was washed and anti-fd bacteriophage (Sigma Chemical Co., UK) was added to the wells. After 1.5 h, the wells were washed and HRP conjugated anti-rabbit antibody (1:300; Sigma Chemical Co, UK) was added to the well and incubated at RT for 1 h. After washing, the substrate and chromogen (H2O2 with ABTS in citrate buffer) was added and the plates were placed in the dark for 30 min. The absorbance was read at 405nm using an ELISA reader.

Results

PCR and DNA fingerprinting of the library and clones after panning

Figure 1 shows the PCR results of the library clones. The 950 base pair band was obtained for each clone which showed the presence of the VH-Linker-VL inserts. The DNA fingerprinting

![Figure 3. PCR results of the selected clones after panning.](image)

Figure 3. PCR results of the selected clones after panning.

![Figure 4. DNA fingerprinting of the selected clones after panning.](image)

Figure 4. DNA fingerprinting of the selected clones after panning. Two specific scFvs were selected: pattern 1, scFv1, (lanes 1,3,10,16 and 20) with a frequency of 25% and pattern 2, scFv2, (lanes 4,7,11,14 and 18), also with a frequency of 25%. The other patterns (lanes 2, 5, 6, 9, 12, 13, 15, 17 and 19) had frequencies of 5% and 10%.
of the library is shown in Figure 2. Various patterns confirmed the diversity and heterogeneity of the library. Figures 3 and 4 show PCR and fingerprinting of the selected clones after four rounds of panning, respectively. The 950 base pair band was obtained for all clones. As shown in Figure 4 DNA fingerprinting of the selected clones demonstrated pattern 1, scFv1, (lanes 1,3,10,16,20) with a frequency of 25% and pattern 2, scFv2, (lanes 4,7,11,14,18) with the frequency of 25%. The other patterns had frequencies of 10% and 5%. Dominant patterns 1 and 2 were selected for further evaluation.

**Phage ELISA results**

We performed phage ELISA to evaluate the specificity of the scFv antibody to the peptide of interest. The selected clones were phage rescued and used for phage ELISA. The wells without peptide, with unrelated peptide (prostate stem cell antigen peptide), with unrelated scFv (anti-HER2 scFv), and with M13KO7 (the helper phage) were used as controls. Absorbances at 405nm are shown in Figures 5 and 6. The ODs obtained from scFv1 and scFv2 reactions with the corresponding peptide were more than twice as much as the no-peptide wells, as negative controls.

**Discussion**

Although cancer chemotherapy is one of the major medical advances in the last few decades, the drugs used for this therapy have limited therapeutic effect. The resultant responses are often palliative and unpredictable. Unlike chemotherapy, targeted therapy is directed against cancer-specific molecules and, thus, has more limited nonspecific toxicity. In patients whose cancers express an over-abundance of the HER2 protein, trastuzumab is used to block the activity of this protein in cancerous cells which results in delaying cancer growth. Pertuzumab, another FDA approved MAb, has been used in combination with trastuzumab for the treatment of HER2 positive metastatic breast cancers, but some side effects, such as early cardiotoxicity, have been reported in these patients which limits the use of pertuzumab. The production of anti-HER3 MAbs is another attempt for blocking HER2 heterodimerization and inhibiting tumor growth. However, side effects such as allergic reactions and human anti-mouse antibody (HAMA) response show the necessity for an alternative treatment.

In this study we selected specific scFv antibodies against the HER3 epitope and evaluated
their specificity in ELISA for their use in HER3 blockage and breast cancer immunotherapy. We previously selected three scFvs against the HER2 epitope and evaluated their downstream signaling which resulted in inhibition of breast cancer cell growth, survival and metastasis. Since the central role of HER3 in the survival of HER2 positive cancer cells has been shown, anti-HER3 scFvs could be valuable agents in HER2 positive breast cancer immunotherapy and a combination of anti-HER2 and anti-HER3 scFvs might have more effective results in cell growth inhibition.

In this study we used amino acids 1330-1342 of the HER3 molecule (HSRLFPKANAQRT) and selected specific scFv1 and scFv2 against this epitope. This epitope has been reported as an immunodominant epitope of HER3 which plays an important role in heterodimerization with HER2. It has been used by some companies to produce effective antibodies against HER3. It forms heterodimers with other EGF receptor family members which show kinase activity. Heterodimerization leads to the activation of pathways which lead to cell proliferation or differentiation. According to reports, amplification of this gene and/or overexpression of its protein occurs in numerous cancers, including bladder, prostate and breast tumors.

The panning process that enriched the phage antibody and two specific scFvs at a frequency of 25% were selected. Fingerprinting of the selected clones of the library showed various patterns and confirmed the diversity and heterogeneity of these clones. The fingerprinting of 20 randomly selected clones after four rounds of panning represented five common patterns for each scFv. The phage-rescue supernatant of these clones were positive with ELISA at ODs of 0.63 (scFv1) and 0.46 (scFv2). The wells without peptides had ODs of 0.19 and 0.11, respectively. None of the other controls were positive. The ODs obtained for these controls could be lower if better washing steps were applied. The results of ELISA have confirmed the panning results and represented specific scFvs against the HER3 epitope. These specific scFvs offer the possibility of their role in the HER3 blocking and cancer targeted therapy since the prognostic significance of HER3 overexpression in invasive breast cancers has been reported. HER3 blocking not only interferes with HER2/HER3 dimerization which leads to the inhibition of HER2 positive breast cancer cell proliferation, but also these human antibodies can assist with inhibition of HER1/HER3 dimerization which provides a platform for increased growth signaling and is one of the trastuzumab resistance mechanisms. The selected libraries could also be helpful in the treatment of other cancers such as ovarian, prostate and colorectal cancers where HER1/HER3 dimerization contributes to cancer growth. Further research is needed to show the effects of the selected scFvs in vitro and in vivo.

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Conflict of Interest
No conflict of interest is declared.

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