Development of a new diagnostic tool to detect early breast cancer in females

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Abstract

Breast cancer is the most common invasive cancer in women worldwide. According to estimates nearly 480,000 women died because of breast cancer in 2008.[1] Therefore it is increasingly important to develop a method, which identifies breast cancer at an early stage, so that a therapy can be induced very fast. The Interreg IV-A project MICROBIOMED (MICROtechnologies for BIOMEDical applications) was funded to achieve this goal by developing an *in vitro* diagnostic tool. At the end of the project an antibody-based assay will be presented, which is integrated into a biochip system to facilitate a simple and quick breast-cancer diagnosis. To reach this goal, the patients' blood will be analyzed and the final result will be available within minutes. The biomedical part of the project is the identification of different biomarkers, which are known to be (over)expressed in breast cancer and the production of the corresponding antibodies. By combining commonly used cancer markers e.g. HER2/neu, Epidermal Growth Factor Receptor (EGFR) and Muc1 with new markers, the system can be compared with existing diagnostic tools and we hope to increase the trust of medical doctors and patients into the new tool.

The first aim of this study was the development of a "proof-of-principle" for the biochip setup with the EGFR [2], which is overexpressed in 16-48% of breast cancers. Therefore a sandwich Enzyme-Linked Immunosorbent Assay (ELISA) with the natural ligand EGF and the single chain variable fragment 425 (scFv425) will be generated. The scFv425 binds to the EGFR but also inhibits the binding of EGF to the EGFR. Afterwards the setup will be adapted to the biochip and the specificity and sensitivity will be compared. In addition to this standard cancer marker, four secretory proteins were identified. Currently they are used to generate murine antibodies, which then can be utilized to setup further relevant diagnostic assays.

Aims / Objectives

Since the common diagnostic techniques like mammography, breast self examination, biopsy or magnetic resonance tomography (MRT) identify breast cancer at a relatively late stage, the early detection becomes more and more important. In the here described project a diagnostic tool will be developed using different biomarkers. These biomarkers are not exclusively specific for breast cancer but the combination of different markers will increase the sensitivity and specificity of the system. The first aim of this study was to develop a „proof-of-principle" for the experimental setup with the EGFRex, its natural ligand EGF and the scFv425 in an ELISA.

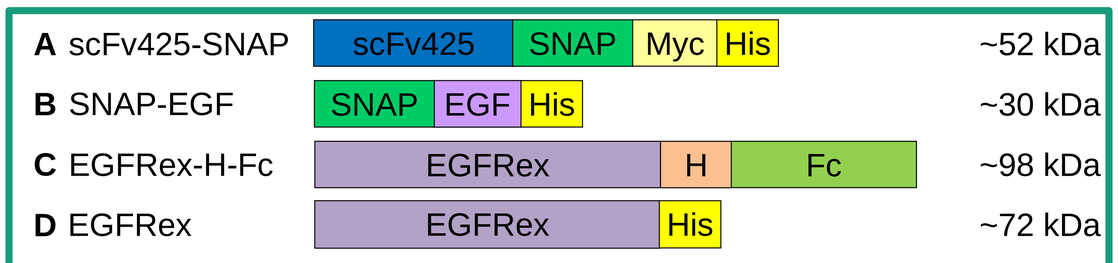

Fig. 1: Overview of different proteins used within the study. The different proteins shown within this scheme, were expressed in HEK293T cells and secreted into the cell culture supernatant by a murine Ig-K leader (A + B) or by its natural leader sequence (C + D). For the purification of the different His-tagged proteins, a Ni-NTA Superflow Cartridge-Column (Qiagen, 5 ml) was used, whereas the EGFRex-Hinge-Fc was purified using Protein A (Pall, 1 ml). The Myc-Tag could also be used for detection.

Experimental setup

The EGFR is well known to be overexpressed on breast cancer cells and other cancer cells. For the ELISA-experiment the proteins SNAP-EGF, in which EGF is the natural ligand and the scFv425-SNAP, in which the scFv425 binds to the EGFR but also inhibits the binding of EGF, will be used. To achieve a recombinant soluble EGFR, the extracellular domain of the receptor was cloned with and without the Fc part of a human immunoglobulin G 1 (IgG 1). All proteins were expressed in HEK293T cells and purified via IMAC or protein A (Fig. 1). To specifically label the proteins with different fluorophors, the SNAP-tag technology (NEB) was used.[3] The binding specificity of the proteins SNAP-EGF and scFv425-SNAP was tested in flow cytometry with EGFR-positive (A431, MDA-MB-231) and -negative cell lines (U937). Since the scFv425 inhibits the binding of EGF, the coating for the sandwich ELISA was performed with SNAP-EGF. Different setups were tested concerning the binding specificity of both recombinant EGFRex proteins and the cell line MDA-MB-231. For ELISA, scFv425-SNAP was labeled with Biotin and the detection was done using streptavidin-horseradish peroxidase (Strep-PO) and ABTS. For other ELISAs, the murine anti-SNAP antibody M2D11 (in house) and GaM-PO (Sigma) were used for detection.

[1] http://www.who.int/research/en/

[2] Adamczyk K.A., et al., *Characterization of soluble and exosomal forms of the EGFR released from pancreatic cancer cells*. Life Sci. 2011. 89(9-10):304-12.

[3] Kampmeier, F., et al., *Site-specific, covalent labeling of recombinant antibody fragments via fusion to an engineered version of 6-O-alkylguanine DNA alkyltransferase.* Bioconjug Chem, 2009. **20**(5): p. 1010-5.

Results

Previous ELISA-experiments showed that 50 ng of EGFRex and EGFRex-H-Fc demonstrate reliable results using the scFv425 for detection. In the sandwich ELISA EGFRex can be detected with a dilution of 1:584 (app. 125 ng) of scFv425-SNAP-Biotin. The binding of scFv425-SNAP-Biotin to the EGFRex-H-Fc is more efficient and the detection limit is a dilution of 1:4672 (15,6 ng, Fig. 2).

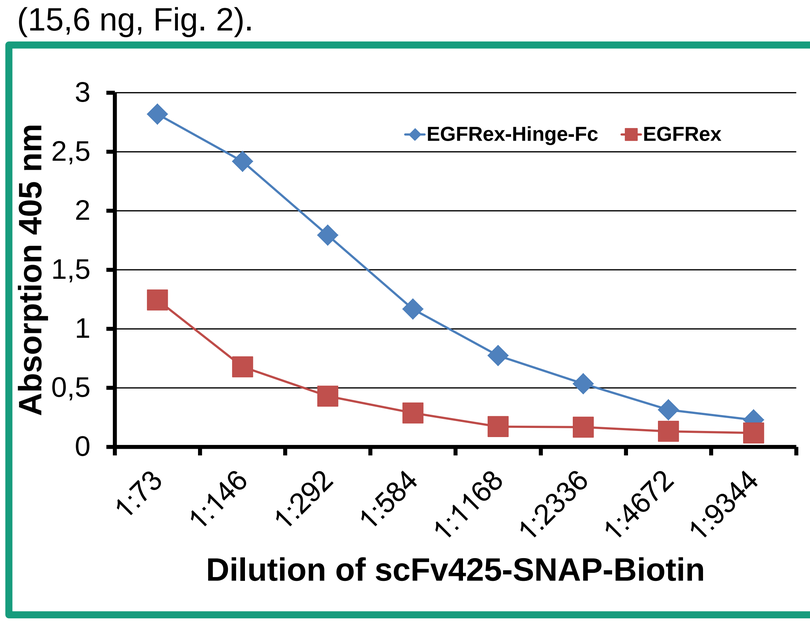

Fig. 2: Sandwich ELISA with recombinant proteins. SNAP-EGF was coated with a concentration of 100 ng, EGFRex-H-Fc and EGFRex were used in a concentration of 50 ng to saturate all binding sites (tested in ELISA). scFv425 was labeled with Biotin to perform the detection with Strep-PO and ABTS.

For the sandwich ELISA, two EGFR-positive cell lines MDA-MB-231 and A431 were tested in advance in flow cytometry to determine the amount of scFv425-SNAP, SNAP-EGF and EGF, which lead to a saturation of all EGFR binding sites (Fig. 3). A total amount of 20-25 µg (scFv425-SNAP: 250 ng/µl, SNAP-EGF: 200 ng/µl, EGF: 160 ng/µl) of all proteins was necessary for MDA-MB-231 cells. In contrast to this, only 10 µg (scFv425-SNAP: 180 ng/µl, SNAP-EGF: 100 ng/µl) protein is necessary to saturate all binding sites on A431 cells.

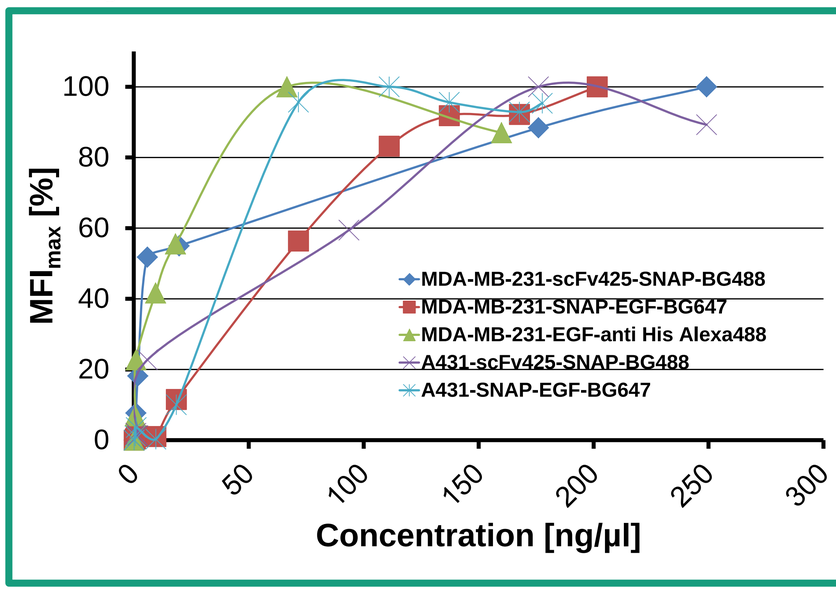

Fig. 3: Determination of EGFR saturation. The saturation of the EGFR on the surface of A431 and MDA-MB-231 cells was measured using flow cytometry. scFv425-SNAP was labeled with BG488 and SNAP-EGF with BG647. EGF was detected using an anti His Alexa488.

Conclusions:

- Successful ELISA-experiments with recombinant EGFRex proteins
- Saturation of EGFR binding sites with different recombinant proteins
- Establishment of a sandwich ELISA as a „proof-of-principle" for the later biochip system

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