A novel diagnostic tool to detect early breast cancer

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Abstract

Breast cancer is the most common cancer in women worldwide. According to estimates, nearly 75.000 new cases occurred 2012 in Germany (Robert-Koch-Institute). For the improvement of the early detection of breast cancer the Interreg IV-A project MICROBIOMED (MICROtechnologies for BIOMEDical applications) was funded. The aim is to develop an antibody-based bioassay, which will later be integrated into a biochip system to achieve a simple and quick method for an early detection. In combination with the Epidermal Growth Factor Receptor (EGFR), which is well known to be overexpressed in 16-48% [1] of breast cancer, additional valuable markers will be analyzed.

The chosen biomarkers are expressed in various cancer types including lung, prostate, pancreatic and breast cancer. These markers are produced autocrinely and act as regulators to protect cells from apoptotic death and stimulate angiogenesis. As soon as these functions are dysregulated, antibodies might be used for their specific capturing. Thereby the growth of the cancerous cells could be inhibited. For the integration into the biochip, new antibodies against these antigens were generated by immunization of mice with recombinant protein. After the selection of monoclonal antibody producing hybridoma cells, the antibodies were purified via Protein G. Their binding specificity and functionality were analyzed in Enzyme-Linked Immunosorbent Assay (ELISA) and Western Blot experiments. Furthermore the antibodies will be tested regarding their suitability for the detection of the cancer-specific marker in patient material such as serum and tissue.

In cooperation with engineers from different institutes within the Euregio Meuse-Rhine a biochip system will be established, using the here developed antibodies. Possible measurements will essentially be based on label free detection methods.

In the here presented study, the production of two breast-cancer specific markers and the properties of the corresponding antibodies will be described.

Experimental Setup

The breast cancer specific serum markers were expressed in HEK293T cells and secreted into the cell supernatant mediated by their natural leader sequence. For the purification of the His-tagged proteins, a Ni-NTA Superflow Cartridge-Column (Qiagen, 5 ml) was used. The proteins were applied for ELISA and Western Blot experiments. For the immunization of mice with the here described breast cancer specific markers, GST-fusion proteins were generated. The small size of the markers required the GST-fusion to increase the immunogenicity. These proteins were expressed in *Escherichia coli* BL21 cells and purified via a Glutathione HiCap matrix (Qiagen). 6-8-week old BALB/c mice (female) were immunized with the GST-fusion proteins mixed with GERBU MM adjuvant (GERBU Biotechnik GmbH). 60 µg of the proteins were administered for priming and 30 µg for subsequent boosting. [2] For the generation of a hybridoma, Sp2/0-Ag14 myeloma cells were used. [3] The hybridoma cells were tested for monoclonality by limiting dilutions and sequence analysis. The produced IgG antibodies were purified via a protein G chromatography column.

Results

The two serum markers shown here belong to the same protein family and show a certain degree of sequence homology. In the following, the two serum markers will be described as protein 1 and protein 2 with their corresponding antibodies 1 and 2. The antibodies were characterized via different ELISA and Western Blot.

In initial experiments the optimal concentration of the antibodies for detection was determined via titration assays (figure 1). Therefore, protein 1 and protein 2 were coated on the ELISA plate. The corresponding antibodies were diluted from 2 µg to 156 pg. The highest absorption signal for antibody 1 (figure 1A) was reached with 40 ng. But also with 20 ng a 87% absorption signal could be obtained. For antibody 2 (figure 1B) the highest absorption signal was measured with 200 ng antibody but again, also an amount of 40 ng could be used.

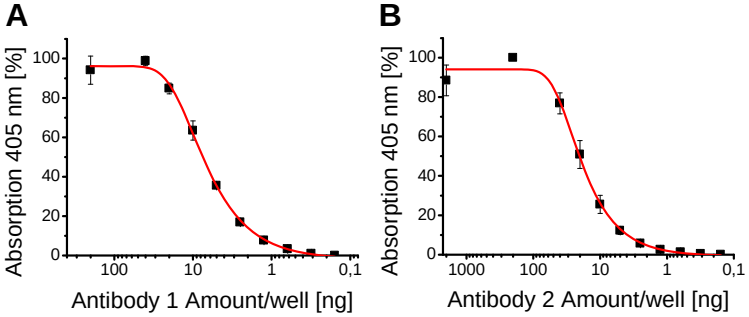

Figure 1: Titration experiments for the antibodies 1 and 2. The proteins 1 and 2 were coated with 50 ng per well. For the detection of the antibodies 1 and 2 a goat-anti-mouse antibody labeled with horseradish peroxidase (GaM PO) and ABTS were used. **A** titration antibody 1 **B** titration antibody 2. Data points are mean values of triplicate measurements from four independent experiments. Error bars represent the standard deviation of the mean values.

[1] Saxena, R. et al., *ErbB Family Receptor Inhibitors as Therapeutic Agents in Breast Cancer: Current Status and Future Clinical Perspective*. Med Res Rev. 2010 Oct 25.

[2] Püttmann, C., Kohlberg, K. et al., *A monoclonal antibody for the detection of SNAP/CLIP-tagged proteins*. Immunological Letters. 2013, 150, 69– 74

[3] Köhler, G., Milstein, C., *Continuous cultures of fused cells secreting antibody of predefined specificity*. Nature .1975, 256, 495–7.

[4[Branch, D.W., Brozik, S.M., *Low-level detection of Bacillus anthracis simulant using Love-wave biosensors on 36°YX $LiTaO_3$*. Biosensors and Bioelectronics. 2004, 19, 849-859

Results continued

In further experiments the detection limit of the antibodies was determined. Therefore the corresponding proteins were coated in a dilution series.

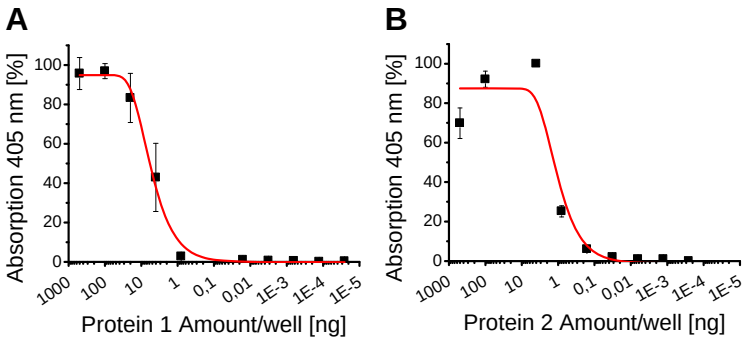

Figure 2: Detection limit of antibodies 1 and 2. The proteins were coated in a range from 500 ng to 0,256 pg. The antibodies were used in a concentration of 0,4 ng/µl. The detection was performed with GaM PO and ABTS. **A** detection limit of antibody 1 **B** detection limit of antibody 2. Data points are mean values of triplicate measurements from four independent experiments. Error bars represent the standard deviation of the mean values.

Based on the fitting curve, the detection limit of antibody 1 could be determined at about 1 ng of coated antigen (figure 2A). The detection limit for antibody 2 is lower, approximately 0,16 ng antigen (figure 2B).

The cross-reactivity of antibodies 1 and 2 with other proteins was also tested in an ELISA. Therefore different proteins were coated on the plates.

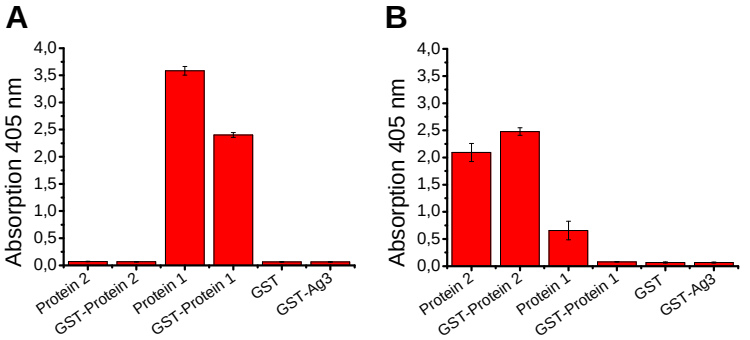

Figure 3: Cross-reactivity of the antibodies with different proteins. The proteins were coated with 50 ng per well, the GST-fusions in an equimolar range. The detection was performed with GaM PO and ABTS. The additional GST proteins were used as negative controls. **A** detection with antibody 1 **B** detection with antibody 2. Antibodies 1 and 2 were used in a concentration of 0,4 ng/µl. Data points are mean values of triplicate measurements from four independent experiments. Error bars represent the standard deviation of the mean values.

As expected, both antibodies recognize their corresponding protein, as well as the corresponding GST-fusion (figure 3). Protein 1 is also detected by antibody 2 but the binding is much weaker (figure 3B). A possible cross-reactivity of the antibody can be explained by the sequence homology of the two proteins.

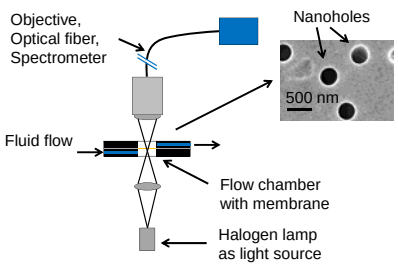

Figure 4: Schematic structure of the SPR apparatus. The experimental setup is based on a microscope with an attached spectrometer.

In cooperation with the engineers from the Institute of Materials in Electrical Engineering 1, initial experiments were performed via surface plasmon resonance (SPR) (figure 4). Therefore a thin gold layer was clamped into a flow chamber allowing fluids to pass through the layer and adhere. First, the gold surface was coated with Protein A [4] to improve the adsorption of the antibody. Second, after the coupling of antibody 2, antigen 2 was captured out of the pouring liquid. The specific binding of the protein and antibody could be measured in the shift of the wavelength.

Conclusions

- Successful production of antibodies directed against two breast cancer specific serum markers
- Characterization of generated antibodies in ELISA and Western Blot
- Antibodies recognize the conformational epitopes of the antigens but not the linearized epitopes
- First experiments in a biochip setup using SPR

Project partners:

Funding:

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