Improvement of current flow cytometry and sandwich ELISA assays to detect cancer specific MUC1 in breast cancer

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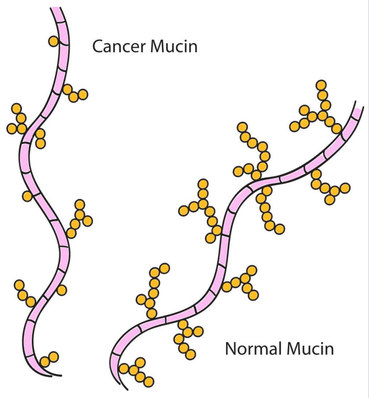
Background

The aim of the Microbiomed project is to develop a lab-on-a-chip system for the detection of breast cancer at an early stage. For this purpose cancer cell markers are being selected and a combination of these will be applied in different technical set-ups.

Mucin-1 (MUC1) is a well-known and extensively described cancer cell marker. This highly validated tumor specific antigen has therefore been chosen to evaluate the potential of the different new designs. Additionally, currently available techniques are being explored as a baseline.

MUC1 is a transmembrane glycoprotein that is expressed on epithelial cells. When expressed in healthy tissue it is heavily O-glycosylated, resulting in large chains of sugar molecules attached to the peptide backbone. On cancer cells MUC1 is expressed with a much more aberrant glycosylation pattern, with fewer sugar molecules attached to the peptide backbone. This characteristic is what makes MUC1 unique and ideal to be used as a tumor specific antigen. Antibodies have been developed in the past that recognize the aberrantly glycosylated version of MUC1 and that will not bind when any further glycosylation is present.

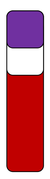


Research question: Is it possible to detect cancerous MUC1 using the current state-of-the-art techniques, flow cytometry – cell based and sandwich ELISA – protein based?

Methods

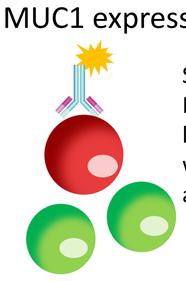
Flow cytometry – cell based

With the use of flow cytometry, an assay has been designed which combines two antibodies against MUC1 in order to generate a more sensitive and specific detection of cancer cells. These antibodies are 214D4, which binds to all MUC1 on both healthy and cancerous cells, and 5E5, which binds specifically to the MUC1 expressed on cancer cells.



Step 1: 5mL of whole blood added to 15mL of Ficoll and spun at 600 g for 20 minutes

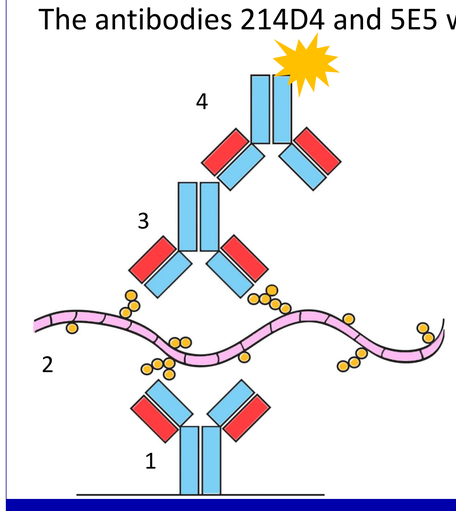
Step 2: Layer with peripheral blood mononuclear cells (PBMCs) isolated and red cell lysis performed



Step 3: PBMCs mixed with ZR75-1 breast cancer cells, stained with antibodies and analysed

Sandwich ELISA – protein based

The antibodies 214D4 and 5E5 were also used in the design of the sandwich ELISA.



1 Coating with 5E5, 3 µg/mL in 50mM Borate. Incubation overnight at 4°C.

2 Supernatant of breast cancer cell lines ZR75-1, MCF7, T47D or a blank media control. Incubation for 30 minutes at room temperature.

3 Addition of 214D4 labelled with biotin, 3 µg/mL in 1x PBS. Incubation for 30 minutes at room temperature.

4 Detection with streptavidin-HRP. Incubation for 30 minutes at room temperature.

All washes performed with 1x PBS supplemented with 0.1% Tween20
Development with TMB substrate and H_2SO_4 stop solution

Results

To what extent can we distinguish cancer cells from healthy cells with flow cytometry?

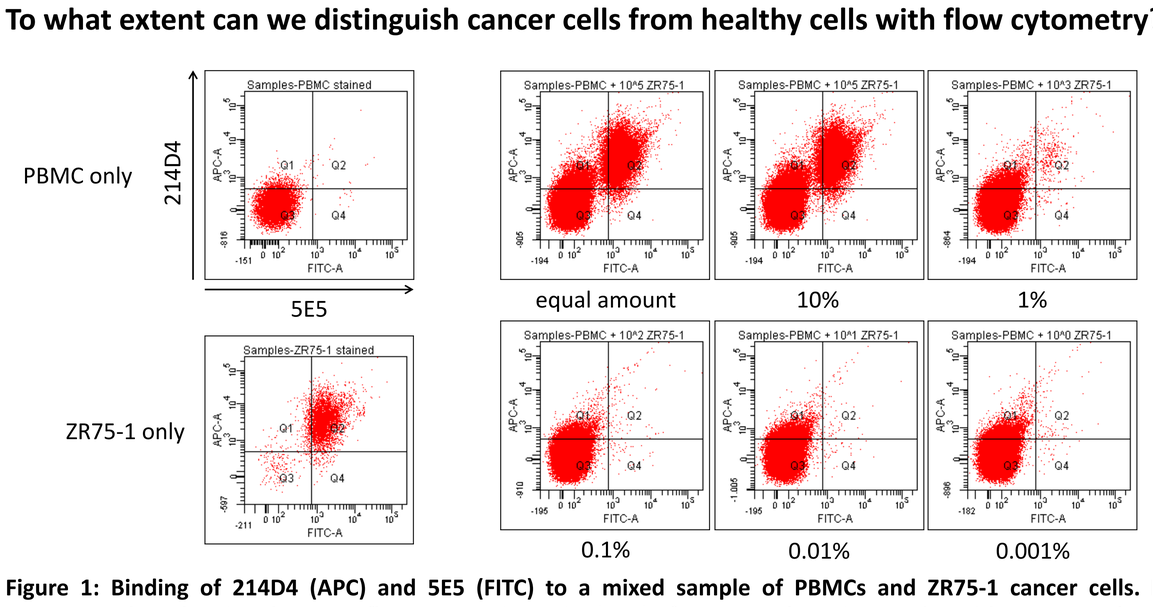
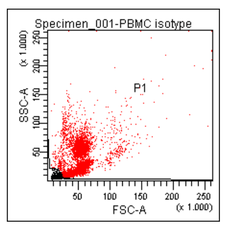


Figure 1: Binding of 214D4 (APC) and 5E5 (FITC) to a mixed sample of PBMCs and ZR75-1 cancer cells. Flow cytometry plots showing the mean fluorescence intensity (MFI) of 214D4 and 5E5 in samples with PBMCs and ZR75-1 cancer cells on their own and mixed in different ratios, always using 100.000 PBMCs per sample. Results are shown after gating out debris and quadrant gates are set in accordance with isotype controls.

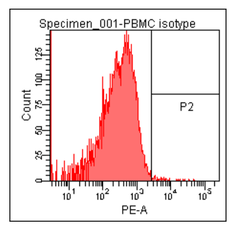
Separate population of ZR75-1 cancer cells measurable at 1% in total population of cells

Results continued

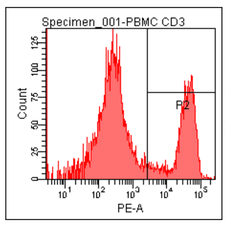
Are there additional markers to improve the sensitivity of the flow cytometry assay?



Step 1: Exclude debris



Step 2: Set gate on isotype control



Step 3: % positive cells determined

	PBMC MFI	% +	ZR75-1 MFI	% +
isotype	781	2	4229	2
CD3	10650	28	3460	1
CD4	11253	36	2919	0
CD8	6255	9	3231	1
CD14	7160	20	4303	1
CD16	66329	59	6776	1
CD19	537	2	3170	1
CD45	86434	99	2939	0
CD56	731	5	3418	1
CD66	4623	38	79378	49
CD80	246	1	3350	1

Figure 2: Screening of PBMC markers as possible negative markers for ZR75-1 cancer cells. Gating strategy for analysis show in 3 steps, including flow cytometry plots. The MFI and % of positive PBMCs and ZR75-1 cancer cells determined for a range of regularly used PBMC markers.

PBMCs highly positive for several markers, while ZR75-1 cancer cells are negative

A panel of different markers could be used, but CD45 can exclude most PBMCs suggesting an improvement of the sensitivity of our assay

Does our design of a sandwich ELISA with 214D4 and 5E5 improve the current standard used in the clinic, CA15.3?

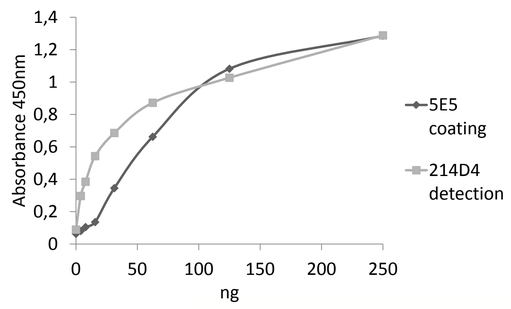


Figure 3: Titration of 5E5 coating and 214D4-biotin detection steps in the sandwich ELISA. The optimum amount of the antibodies 5E5 and 214D4 was determined in a titration assay, indicating the amount in ng that was added per well of a 96-well plate. For both antibodies a concentration at 150 ng/well resembling 3 µg/mL was found to be best to use in further assays.

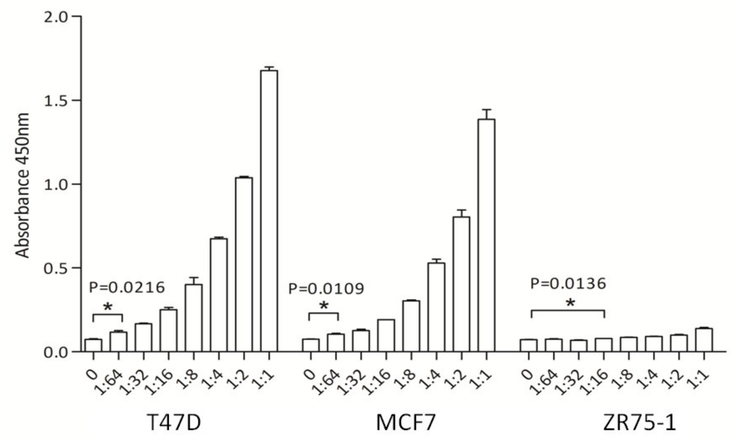


Figure 4: Results of the supernatants of the breast cancer cell lines T47D, MCF7, ZR75-1 measured in the sandwich ELISA designed for detection of cancerous MUC1 using 5E5 and 214D4. The presence of MUC1 was measurable at significant levels compared to the blank media control for T47D at 1:64 dilution, for MCF7 at 1:64 dilution and for ZR75-1 at 1:16 dilution. This is a significant increase compared to the results from the CA15.3 assay. These showed a detection limit at a 1:8 dilution for T47D, a 1:1 dilution for MCF7 and a 1:8 dilution for ZR75.1. The CA15.3 assay is the current standard in the clinic for MUC1 detection in serum of patients, but uses a different combination of MUC1 antibodies with apparent lower avidity.

The sandwich ELISA showed detectable MUC1 at a 1:64 dilution for T47D, 1:64 dilution for MCF7 and 1:16 dilution for ZR75-1. A significant increase compared to the current CA15.3 assay for detection of MUC1

Conclusions

Using flow cytometry, discrimination between cancer and non-cancer cells proved possible. ZR75-1 breast cancer cells were still detectable at ± 1% of the total number of cells when mixed with PBMCs

CD45 will be a good negative marker for ZR75-1 breast cancer cells in future experiments, increasing the sensitivity and specificity of the assay

Further improvements on the specificity of the flow cytometry assays are in progress by including antibodies against other breast cancer specific markers

With the sandwich ELISA a significant increase in sensitivity was observed compared to the CA15.3 assay used currently as the standard in clinical diagnostics.

Larger dilution series will be performed for the sandwich ELISA assays in order to further explore its possibilities

The assays described are essential for the Microbiomed project as standard of the currently available techniques and show great potential as an application in the clinic.

Project partners:

Funding: