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Serum proteome profiling of metastatic breast cancer using recombinant antibody microarrays

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ABSTRACT

The driving force behind oncoproteomics is to identify biomarker signatures associated with a particular malignancy. Here, we have for the first time used large-scale recombinant scFv antibody microarrays in an attempt to classify metastatic breast cancer versus healthy controls, based on differential protein expression profiling of whole serum samples. Using this multiplexed and miniaturised assay set-up providing pM range sensitivities, breast cancer could be classified with a specificity and sensitivity of 85% based on 129 serum analytes. However, by adopting a condensed 11 analyte biomarker signature, composed of nine non-redundant serum proteins, we were able to distinguish cancer versus healthy serum proteomes with a 95% sensitivity and specificity, respectively. When a subgroup of patients, not receiving anti-inflammatory drugs, was analysed, a novel eight analyte biomarker signature with a further improved predictive power was indicated. In a longer perspective, antibody microarray analysis could provide a tool for the development of improved diagnostics and intensified biomarker discovery for breast cancer patients.

1. Introduction

Early and improved detection and diagnosis of breast cancer, which is the worldwide most common form (about 30%) of cancer in females, is essential.[1,2] The recent progress of proteomics has opened up novel avenues for cancer-related biomarker discovery.[3,4] However, adopting high-throughput proteomic approaches to multiplexed set-ups, providing a minimally invasive screening procedure, targeting non-fractionated biological fluids, such as blood, has proven to be challenging.[1,2,4] Antibody-based microarrays is a rapidly emerging affinity-proteomic technology that is likely to play an increasing role within oncoproteomics.[5] In recent years, the technology has made significant progress[6,7] (for review see Refs. 8–11) now allowing us to design miniaturised array platforms, capable of simultaneously profiling numerous low-abundant protein analytes in complex proteomes, such as plasma and serum, while consuming only minute amounts of sample.[6,12,13] Adopting antibody microarrays, translational proteomics is one immediate application where comparative protein expression profiling analysis of cancer versus normal proteomes could yield tentative predictive biomarker signatures.[12–18] From a clinical point of view, we also need increased possibilities to individually monitor disease progression and response to treatment, since no therapy has the same effect on a large number of patients with the same diagnosis.

To increase the diagnostic and predictive power in cancer, the critical value of using more than one biomarker has

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already been suggested,[2,19–21] which now drives the search for specific disease-associated biomarker signatures.[3,4] This is true, to an even higher degree, for heterogeneous disease, such as breast cancer, where a single biomarker is unlikely to give conclusive diagnostic information to successfully stratify all the different disease states known. This is illustrated by the fact that altered serum levels of a variety of (single) analytes, such as truncated forms of complement protein C3a,[22] cancer antigen (CA) 15-3,[3] carcinoembryonic antigen (CEA),[2] glycoproteins of the MUC family,[2] autoantibodies,[2] sialyl Lewis^x [21,23] and cytokines (e.g. IL-6, IL-8, IL-10)[24] have been observed in breast cancer patients, using traditional proteomic approaches. Still, these data are inconclusive and the specificity and sensitivity of these tentative single biomarkers are too low.

Recently, the first monoclonal antibody-based microarrays have been applied to analyse breast cancer cell lines, resulting in the identification of IL-8 as tentative key factor suggested in breast cancer invasion and progression,[25–27] IL-8/GRO as a HER2 (erB-2-)-induced cytokine signature,[28] as well as a five protein signature that may be associated with doxorubicin resistance.[29] Similarly, Hudelist and co-workers have used monoclonal antibody microarrays to detect a set of differentially expressed proteins in normal versus malignant breast tissue from one patient.[30]

In the present study, we have for the first time investigated the potential of large-scale recombinant scFv antibody microarrays to classify female, post-menopausal, age-matched metastatic breast cancer patients ($n = 20$) versus healthy controls ($n = 20$) based on differential serum protein profiling. The miniaturised set-up provided unique means to profile even pM range analytes, including mainly immunoregulatory proteins, in non-fractionated sera, while consuming only μL amounts of the clinical samples.[6,7,31] This proof-of-concept study showed that several differentially expressed serum proteins could be detected, and that this breast cancer-associated biomarker signature could be used to classify metastatic breast cancer patients.

Table 1 – Patient demographics and clinical parameters

	Class	
	BC	N
Number of samples	20	20
Age		
Mean	60	57
Range	48–75	47–60
ER status		
ER+	15	–
ER–	3	–
n.d.	2	–
PgR status		
PgR+	7	–
PgR–	10	–
n.d.	3	
Clinical stage[a]		
I	2	–
II	8	–
III	5	–
IV	3	–
n.d.	2	–
Histological grade		
I	1	–
II	8	–
II–III	3	–
III	8	–
Histological type		
Ductal	12	–
Lobular	4	–
Mixed	2	–
n.d.	2	–
Drugs[b]		
Yes	14	14
No	6	6

ER = oestrogen receptor; PgR = progesterone receptor; n.d. = not determined.
a Refers to the stage at primary diagnosis.
b All BC patients were treated with a range of various agents (data not shown). Here, only those BC patients (and healthy controls) that had taken any anti-inflammatory drugs and/or hormones at the time when the serum samples were collected are defined.

2. Materials and methods

2.1. Samples

In total, 40 serum samples, supplied by the Department of Oncology, Lund University Hospital, Lund, Sweden, were included in this study. The serum samples were collected from female, post-menopausal patients, 20 of which suffered from metastatic breast cancer (denoted BC1 to BC20) and 20 healthy matched control subjects (denoted N1–N20). Patient demographics and information about additional clinical parameters, including oestrogen receptor (ER) status, progesterone receptor (PgR) status, clinical stage, histological type and histological grade, as well as information about intake of anti-inflammatory drugs/hormones, are shown in Table 1.

2.2. Labelling

The serum samples were labelled, using previously optimised labeling protocols for serum proteomes.[6,7,31] All serum samples were biotinylated, using EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL, USA). Fifty microliter serum aliquots were centrifuged at $16{,}000 \times g$ for 20 min at 4 °C and diluted 1:45 in phosphate buffer saline (PBS), resulting in a final protein concentration of about 2 mg/ml. Sulfo-NHS-biotin was then added to a final concentration of 10 mM and the samples were incubated on ice for 2 h, with careful vortexing every 20 min. Unreacted biotin was removed by dialysis against PBS for 72 h at 4 °C. Finally, the samples were aliquoted and stored at –20 °C prior to use.

2.3. Production and purification of scFv

129 human recombinant scFv antibody fragments against 60 different proteins mainly involved in immunoregulation (Table 2), were stringently selected from the n-CoDeR library,[32] and kindly provided by BioInvent International AB, Lund,

Table 2 – The different scFv specificities used for the antibody microarrays

Antigen (number of clones)	Antigen (number of clones)
IL-1a (3)	GLP-1 (1)
IL-1b (3)	GLP-1-R (1)
IL-1-ra (3)	C1q (1)
IL-2 (3)	C1s (1)
IL-3 (3)	C3 (2)
IL-4 (4)	C4 (1)
IL-5 (3)	C5 (2)
IL-6 (4)	Factor B (1)
IL-7 (2)	B (1)
IL-8 (3)	Properdin (1)
IL-9 (3)	C1 esterase inhibitor (1)
IL-10 (3)	CD40 ligand (1)
IL-11 (3)	PSA (1)
IL-12 (4)	Leptin (1)
IL-13 (3)	LDL (2)
IL-16 (3)	Integrin α-10 (1)
IL-18 (3)	Integrin α-11 (1)
TGF-β1 (3)	Procathepsin (1)
TNF-α (3)	Tyrosine-protein kinase BTK (1)
TNF-β (4)	Tyrosine-protein kinase JAK3 (1)
INF-α (3)	B-lactamase (1)
VEGF (4)	Lewis^x (2)
Angiomotin (2)	Lewis^y (1)
MCP-1 (3)	B cell lymphoma AG (1)
MCP-3 (3)	Sialyl Lewis^x (1)
MCP-4 (3)	MUC-1 (1)
Eotaxin (3)	Streptavidin (1) (control)
RANTES (3)	Digoxin (1) (control)
GM-CSF (3)	FITC (1) (control)
CD40 (4)	TAT (2) (control)

Sweden. Hence, some antigens were recognised by up to four different scFv clones. All scFv probes were produced in 100 ml *Escherichia coli* cultures and purified, from either expression supernatants or periplasmic preparations, using affinity chromatography on Ni-NTA agarose (Qiagen, Hilden, Germany). Bound molecules were eluted with 250 mM imidazole, extensively dialysed against PBS, and stored at 4 °C until further use. The protein concentration was determined by measuring the absorbance at 280 nm (average concentration 210 μg/ml, range 60–1090 μg/ml). The degree of purity and integrity of the scFv antibodies was evaluated by 10% SDS–PAGE (Invitrogen, Carlsbad, CA, USA).

2.4. Production and processing of antibody microarrays

The production and handling of the antibody microarrays were performed according to a previously optimised set-up.[6,7,18,31] Briefly, the scFv microarrays were fabricated, using a non-contact printer (Biochip Arrayer1, Perkin Elmer Life & Analytical Sciences, Wellesley, MA, USA), which deposits approximately 330 pL/drop, using piezo technology. The scFv antibodies were arrayed by spotting 2 drops at each position and the first drop was allowed to dry out before the second drop was dispensed. The antibodies were spotted on to black polymer MaxiSorp microarray slides (NUNC A/S, Roskilde, Denmark), resulting on average 5 fmol scFv per spot (range 1.5–25 fmol). Eight replicates of each scFv-clone were arrayed to ensure adequate statistics. To assist the alignment of the grid during the subsequent quantification, a row containing Cy5 conjugated streptavidin (2 μg/ml) was spotted for every 10th row. In total, 160 antibodies and controls were printed per slide orientated in two columns with 8×80 spots per column. A hydrophobic pen (DakoCytomation Pen, DakoCytomation, Glostrup, Denmark) was used to draw a hydrophobic barrier around the arrays. The arrays were blocked with 500 μl 5% (w/v) fat-free milk powder (Semper AB, Sundbyberg, Sweden) in PBS overnight. All incubations were conducted in a humidity chamber at room temperature (RT). The arrays were then washed four times with 400 μl 0.05% Tween-20 in PBS (PBS-T), and incubated with 350 μl biotinylated serum diluted 1:10 (resulting in a total serum dilution of 1:450) in 1% (w/v) fat-free milk powder and 1% Tween in PBS (PBS-MT) for 1 h. Next, the arrays were washed four times with 400 μl PBS-T and incubated with 350 μl of 1 μg/ml Alexa-647 conjugated streptavidin diluted in PBS-MT for 1 h. Finally, the arrays were washed four times with 400 μl PBS-T, dried immediately under a stream of nitrogen gas and scanned with a confocal microarray scanner (ScanArray Express, Perkin Elmer Life & Analytical Sciences) at 5 μm resolution using six different scanner settings. The ScanArray Express software V3.0 (Perkin–Elmer Life & Analytical Sciences) was used to quantitate the intensity of each spot using the fixed circle method. The local background was subtracted and to compensate for possible local defects, the two highest and two lowest replicates were automatically excluded, and each data point represents the mean value of the remaining four replicates. For protein analytes displaying saturated signals, values from lower scanner settings were scaled and used instead.

2.5. Microarray data normalisation

Chip-to-chip normalisation of the dataset was performed, using a semi-global normalisation approach, conceptually similar to the normalisation method used for DNA microarrays. The coefficient of variation (CV) was first calculated for each analyte over all samples and ranked. The 15% of the analytes displaying the lowest CV-values over all samples were identified, corresponding to 21 analytes, and used to calculate a chip-to-chip normalisation factor. The normalisation factor N_i was calculated by the formula $N_i = S_i/\mu$, where S_i is the sum of the signal intensities of the 21 analytes for each sample, and μ is the average of S_i from all samples. Each dataset generated from one sample was divided with the normalisation factor N_i. Log2 values were calculated for the signal intensities for all analytes and ranked using a Wilcoxon test.

2.6. Microarray data analysis

The support vector machine (SVM) is a supervised learning method that we used to classify the samples as either healthy or breast cancer. The supervised classification was performed using a linear kernel, and the cost of constraints was set to 1, which is the default value in the *R* function SVM, and no attempt was performed to tune it. This absence of parameter tuning was chosen to avoid overfitting. The SVM was trained using a leave-one-out cross-validation procedure. Briefly, the training sets ($n = 40$) were generated in an iterative process

in which the samples were excluded one by one. The SVM was then asked to blindly classify the left out samples as either healthy or breast cancer, and to assign a SVM decision value, which is the signed distance to the hyperplane. No filtration on the data was done before training the SVM. Further, a receiver operating characteristics (ROC) curve, as constructed using the SVM decision values and the area under the curve, was found.

In those cases, where smaller cohorts of samples were compared, significantly up- or down-regulated analytes ($p < 0.05$) were identified using Wilcoxon test, log transformed and mean centered. The samples were then hierarchically clustered and visualised as a heat map, using Cluster and TreeView.[33]

3. Results

3.1. Evaluation of scFv microarrays

We analysed directly labelled, non-fractionated serum samples from metastatic breast cancer patients ($n = 20$) and healthy controls ($n = 20$), using a large-scale recombinant antibody microarray. The array was composed of 129 human recombinant scFv antibodies directed against 60 serum proteins, mainly of immunoregulatory nature (Table 2). A representative microarray image of a breast cancer serum is shown in Fig. 1A, demonstrating that dynamic signal intensities, homogenous and distinct spot morphologies, as well as high signal-to-noise ratios were obtained. The reproducibility of the set-up was validated by determining, the (i) intra-assay reproducibility, i.e. the spot-to-spot variation (Fig. 1B), and (ii) the inter-assay correlation, i.e. the reproducibility of duplicate experiments (same sample, but analysed on different arrays) (Fig. 1C). In agreement with previous results,[6] the reproducibility was found to be high, with an intra-assay reproducibility of 0.99 and an inter-assay reproducibility of 0.96. The specificity and sensitivity (pM range) of the microarray set-up for targeting complex proteomes has previously been validated.[6,7,18,31,32]

3.2. Classification of metastatic breast cancer

To evaluate the ability of the microarray set-up to classify metastatic breast cancer patients based on a simple blood test, we examined the serum protein expression profile generated by all 129 antibodies included on the chip (Fig. 2 and

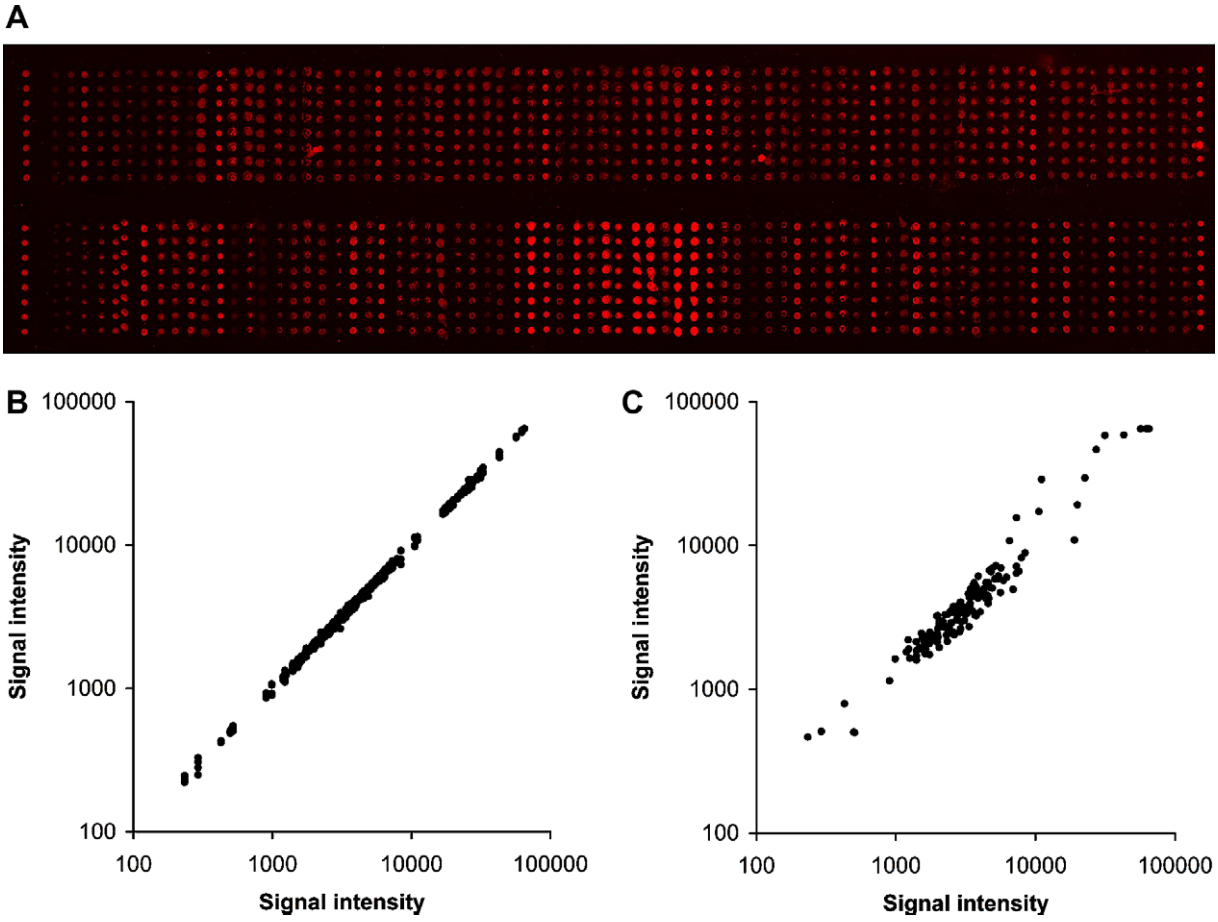

Fig. 1 – Evaluation of recombinant scFv antibody microarrays. (A) A scanned representative microarray image of a metastatic breast cancer sample containing 1280 data points. (B) Intra-assay reproducibility, i.e. spot-to-spot-variations. The correlation coefficient was found to be 0.99. (C) Inter-assay reproducibility, i.e. reproducibility of duplicate experiments. The correlation coefficient was found to be 0.96.

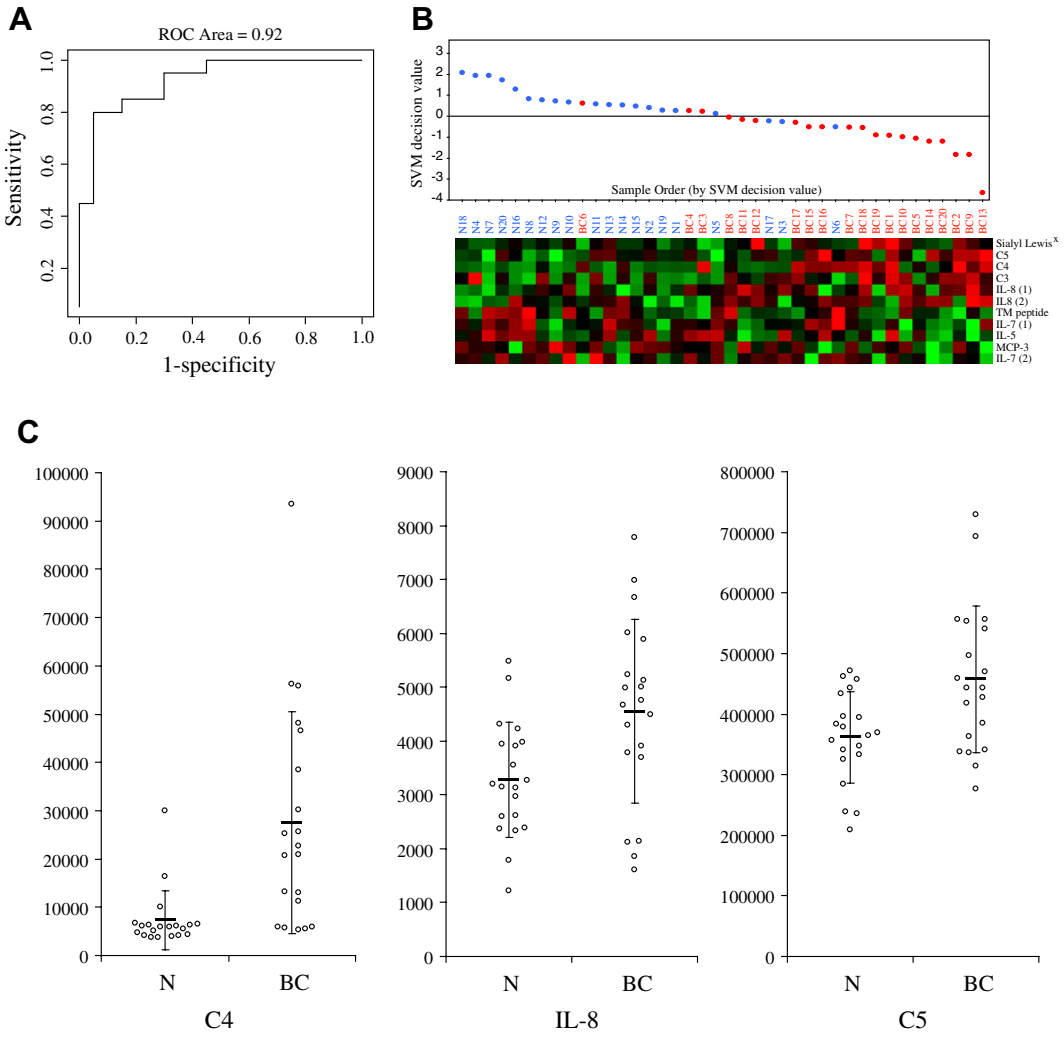

Fig. 2 – Classification of metastatic breast cancer patients by serum protein profiling, using recombinant scFv antibody microarrays. (A) A receiver operator characteristics (ROC) curve obtained for metastatic breast cancer patients (*n* = 20) versus healthy controls (*n* = 20) based on all 129 analytes, using a leave-one-out cross validation approach with a Support Vector Machine (SVM). (B) Classification of the serum samples, using the SVM prediction values based on all 129 analytes. A heat map where the 11 highest ranked, i.e. significantly differentially expressed, analytes, corresponding to nine non-redundant serum analytes, are hierarchically clustered is shown. (C) The signal intensities observed for the top 3 differentially expressed analytes, C4, IL-8 and C5. The mean values are indicated.

Table 2). Consequently, we ran a leave-one-out cross validation, with a Support Vector Machine (SVM), and collected the decision values for each sample. In Fig. 2A, a receiver operating characteristics (ROC) curve was constructed, using the decision values produced by SVM. The results showed that the metastatic breast cancer patients versus healthy controls could be discriminated, displaying an area under the curve of 0.92.

The decision value is the output of the predictor, and samples with a prediction value below a threshold are predicted to be breast cancer. The threshold parameterises the trade-off between sensitivity and specificity and is often set to zero. The 20 metastatic breast cancer samples obtained decision values in the interval of –3.64 to 0.26, and the healthy controls in the interval from –0.51 to 2.11 (Fig. 2B). Thus, with a threshold value of zero, the sensitivity and specificity was 85% in our dataset. Notably, in the training and testing of the SVM, no filtration of data was performed, i.e. data from all analytes measured were included in the analysis.

Furthermore, the 11 highest ranked, i.e. significantly differentially expressed, analytes corresponding to nine non-redundant serum analytes, are shown in Fig. 2B, suggesting a breast cancer-associated serum biomarker signature. When including only these highest ranking analytes in the analysis, the ROC area under the curve was increased to 0.97. This predictor signature allowed us to classify the metastatic breast cancer patients versus healthy controls displaying a sensitivity and specificity of 95%, respectively (data not shown).

The tentative signature of the 11 top differentially expressed serum analytes contained both analytes previously associated with breast cancer, e.g. sialyl Lewis^x,[21,23] C3,[22] C4[34] and IL-8,[24–27] as well as markers previously not observed in the disease, e.g. IL-5 and IL-7. The signal intensities observed for the top three differentially expressed analytes,

including C4, IL-8 and C5, are shown in Fig. 2C. The results showed that the signal intensities in serum from breast cancer patients had increased 1.6 times (IL-8 and C5) and 3.6 times (C4). Of note, the observed differences in signal intensities can be interpreted in terms of relative changes of the amount of each individual analyte present. However, they do not necessarily reflect the magnitude of absolute changes for one analyte compared to another, due to the inherent limitations associated with direct labeling of different analytes in complex proteomes.[35]

3.3. *Effect of anti-inflammatory drugs and/or hormones*

Screening serum samples of metastatic breast cancer patients, as well as other forms of cancers,[12] will reflect not only directly cancer-related affects, but also indirect systemic responses due to, e.g. inflammatory-associated events. In addition, intake of various drugs may also affect the protein expression signatures observed. In an attempt to address this issue, we examined the expression profiles of only those metastatic breast cancer patients ($n = 6$) and healthy controls ($n = 6$) that had not taken any anti-inflammatory drugs and/or hormones at the time when the serum samples were collected. The differentially expressed serum analytes ($p < 0.05$) in these two restricted cohorts of samples were identified, using a Wilcoxon test, and visualised as a heat map (Fig. 3A) based on unsupervised hierarchical clustering. The results showed that eight serum proteins were identified that completely distinguished between metastatic breast cancer patients and healthy controls. Notably, only two of the analytes, C4 and IL-7, overlapped with the first signature, composed of nine non-redundant analytes, which was generated by analysing all samples.

3.4. *Sub-classification of metastatic breast cancer patients*

In an attempt to further stratify the metastatic breast cancer patients, we compared the known clinical parameters (Table 1), including ER status, PgR status, clinical stage, tumour volume, and histological features, with the observed serum protein expression profiles. No correlation could, however, be observed with respect to PgR status or clinical stages. Similarly, no correlation could be observed with respect to ER status, tumour volume, and histological features, although these analyses were hampered by the small sets of sample groups available. Of note, five serum proteins distinguishing between the most advanced clinical stages of the metastatic breast cancer patients, stages III and IV ($n = 8$) versus healthy controls ($n = 6$), excluding those controls that had taken any inflammatory drugs and/or hormones, could be observed (Fig. 3B). Except for procathepsin, 4 of these 5 analytes overlapped with the observed signature distinguishing between non-treated (anti-inflammatory drugs and/or hormones) breast cancer patients and healthy controls.

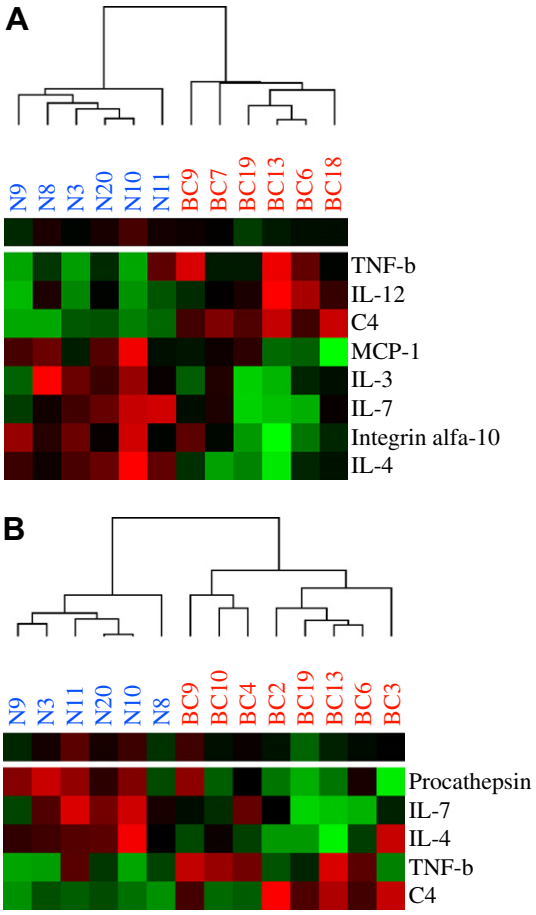

Fig. 3 – Effect of drugs and clinical parameters on the classification of metastatic breast cancer patients. (A) Classification of metastatic breast cancer patients ($n = 6$) and healthy controls ($n = 6$) not taking any drugs (anti-inflammatory drugs and/or hormones) at the time when the serum samples were collected. Differentially expressed analytes ($p < 0.05$) were identified using Wilcoxon test, and visualised in a heat map by unsupervised hierarchical clustering. (B) Classification of metastatic breast cancer patients, stages III and IV ($n = 8$) and healthy controls ($n = 6$). Differentially expressed analytes ($p < 0.05$) were identified, using Wilcoxon test, and visualised in a heat map by unsupervised hierarchical clustering.

4. Discussion

Novel cancer biomarker signatures for early and improved detection and diagnostics, that in the long run also could be used to predict tumour relapses, monitor treatment, and stratify patients based on non-invasive set-ups are critical, since more than 11 million people are diagnosed with cancer every year.[1–5] In this study, we have shown that large-scale recombinant scFv antibody microarrays could provide an unique, miniaturised mean to perform classification of metastatic breast cancer, by multiplexed serum protein profiling of a blood sample. The results showed that the cancer patients could be classified with high sensitivity and specificity.

In comparison, antibody-based microarrays have previously been used to profile, e.g. bladder cancer,[13] colon cancer,[17] lung cancer,[36] liver cancer,[37] ovarian cancer,[38] pancreatic cancer,[12], prostate cancer[15,16] and squamous cell carcinoma[14] (for review see Refs. 5,10,39). Albeit successful, outlining the potential of the technology within oncoproteomics,[5,10,39] the ability of the biomarker signatures to distin-

guish between different carcinomas or between cancer and inflammation has been difficult to achieve, except in a few cases.[13,16,18,40] To a great extent, this reflects the performance of the array set-ups, e.g. functionality, sensitivity and range of antibody specificities[5,10,39], as well as the fact that the serum signature most likely will mirror both directly cancer-associated affects as well as indirect systemic affects. In the present study, the former issue has been minimised by adopting a proven recombinant scFv antibody microarray technology platform[6,7], enabling us to target high- as well as low-abundant immunoregulatory proteins in non-fractionated serum proteomes, thus providing several key advantages.[31] We found that the metastatic breast cancer patients could be classified with a specificity and sensitivity of 85% and a ROC = 0.92, using all 129 analytes, corresponding to 60 non-redundant serum analytes. To make this tentative signature more manageable, we also chose to present a condensed signature, composed of the 11 most significantly differentially expressed analytes, which corresponded to nine non-redundant biomarkers. Although additional screening will be required to validate this signature, our data showed that it was capable of classifying breast cancer with 95% specificity and sensitivity and an ROC = 0.97. Of note, this 11 marker signature had only 1 of 14 (IL-5) analytes in common with one signature identifying *Helicobacter pylori* infected stomach tissue,[18] and 4 of 35 (C3, C5, IL5- and IL-7) in common with systemic lupus erythematosus, an autoimmune disorder with a significant inflammatory component (Wingren et al., manuscript in press.). This indicated that the breast cancer signature was not related to general inflammation. In addition, the breast cancer signature was also different from that observed for, e.g. bladder cancer,[13] lung cancer,[40] pancreatic cancer[12] (Ingvarsson et al., submitted) and prostate cancer.[16] In the case of gastric adenoma carcinoma,[18] 7 of 28 (not C4 and Il-5) biomarkers overlapped, indicating a similarity to this much larger signature, although it should be noted that the tissue extracts and not serum samples were analysed in that particular study.

The strength of the 11 analytes' breast cancer signature was further highlighted by the fact that the breast cancer patients could be adequately classified, although they were individually treated with a wide range of therapeutic agents that might influence their serum signatures differently. In this context, it was of interest to note that an additional biomarker signature with a higher predictive power was indicated, when only those patients who had not taken anti-inflammatory drugs and/or hormones were profiled. Although larger sample cohorts need to be analysed to validate these results, this second signature overlapped less with the gastric adenoma carcinoma signature.[18]

Amongst the nine non-redundant analyte signatures, five were up-regulated (sialyl Lewis^x, C3, C4, C5 and IL-8) and 4 down-regulated (TM peptide, IL-5, IL-7 and MCP-3). In agreement, increased levels of sialyl Lewis^x, a molecule of importance for the interaction between tumour cells and endothelium, have previously been observed for breast cancer patients.[21,23] Similarly, increased levels of truncated forms of C3a, originating from C3, have also been confirmed.[22] C3 is a versatile complement protein, supporting the activation of all three pathways of complement activation, and that has been suggested to function in immune surveillance against tumours, although the mechanisms for the latter remains unknown.[22,41] The role of the complement system was further highlighted by the increased serum levels of both C4 and C5, supported by early work of Lamoureux and co-workers.[34] In addition, the up-regulation of IL-8, suggested to be involved in breast cancer invasion and progression, has also been observed in several studies.[24–28,42] Furthermore, IL8 is expressed by both breast carcinoma and stroma cells and has been implicated in tumour angiogenesis.[43] The novel findings that serum levels of IL-5, IL-7 and MCP-3 were down-regulated could, for example, reflect a lowered tumour immune surveillance by eosinophiles (IL-5),[44] impaired maintenance of T cell memory (IL-7)[45] and a reduced attraction of leucocyte subsets, which potentially recognise and destroy tumour cells (MCP-3).[46] In contrast, IL-7 was shown to mediate tumour growth *in vitro*[47] and levels of IL7 expression in tumour tissue also correlated with tumour aggressiveness in breast cancer patients.[48]

The panels of eight biomarkers observed when profiling a focused cohort of breast cancer, where patients taking any anti-inflammatory drugs and/hormones had been excluded, showed an up-regulation of typical TH1 cytokines, e.g. TNF-β and IL-12, accompanied by a down-regulation of TH2 cytokines, e.g. IL-4, indicating a TH1 skewing of the immune system. Increased levels of IL-12 and an induced TH1 response have previously also been observed in breast cancer tissue.[24,42,49]

In the previous work, a relationship between a single clinical parameter, such as ER status, and the expression levels of, e.g. IL-8, has been observed.[24–27] With the possible exception for the combined cohort of the most advanced forms of breast cancer, stages III and IV, we did not detect any such relationship, although these particular analyses were impaired by too small cohorts.

Taken together, in this first proof-of-principle study, we have been able to classify metastatic breast cancer with a high specificity and sensitivity, based on a blood-test, using the novel approach of recombinant antibody microarray analysis. The signature, consisting of nine non-redundant serum proteins, now needs to be validated in a larger cohort of breast cancer patients. The work has, however, clearly outlined the potential of affinity proteomics for biomarker discovery for breast cancer that in the long run could provide us with the required tools to perform, e.g. improved early diagnosis, prediction of relapses and monitoring of therapy.

Conflict of interest statement

None declared.

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