Comparison of tear protein levels in breast cancer patients and healthy controls using a de novo proteomic approach

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Abstract. Noninvasive biomarkers are urgently needed for early detection of breast cancer since the risk of recurrence, morbidity and mortality are closely related to disease stage at the time of primary surgery. In the past decade, many proteomics-based approaches were developed that utilize the protein profiling of human body fluids or identification of putative biomarkers to obtain more knowledge on the effects of cancer emergence and progression. Herein, we report on an analysis of proteins in the tear fluid from breast carcinoma patients and healthy women using a de novo proteomic approach and 25 mixed samples from each group. This study included 25 patients with primary invasive breast carcinoma and 25 age-matched healthy controls. We performed a MALDI-TOF-TOF-driven semi-quantitative comparison of tear protein levels in cancer (CA) and control (CTRL) using a de novo approach in pooled samples. Over 150 proteins in the tear fluid of CTRL and CA were identified. Using an in-house-developed algorithm we found more than 20 proteins distinctly upregulated or downregulated in the CTRL and CA groups. We identified several proteins that had modified expression in breast cancer patients. These proteins are involved in host immune system pathways (e.g., C1Q1 or S100A8) and different metabolic cascades (ALDH3A or TPI). Further validation of the results in an independent population combined with individual protein profiling of participants is needed to confirm the specificity of our findings and may lead to a better understanding of the pathological mechanism of breast cancer.

Introduction

Breast cancer is still the leading cause of death in women worldwide (1). Although the detection rate of breast carcinoma has improved, many female patients die from metastatic relapse. Mammography is the best available method for detection of breast cancer after the age of 50; although, the detection rate of mammography is not as good in younger women due to their high density breast tissue (2). Early detection is beneficial in the fight against breast cancer. Currently, there are no clinical biomarkers available for early detection of breast cancer. Markers such as CA15.3 and CEA are useful, in combination with imaging and physical examination, for monitoring ongoing treatment in breast cancer patients with metastatic disease; although, they both lack the clinical specificity and sensitivity to be used routinely as a clinical diagnostic tool (3).

The development of high-throughput techniques in Proteomics expanded the search for new biomarkers and enabled the identification of proteins that may have a crucial role in emerging and progressing breast cancer. Proteome analysis of body fluids, such as sera, tear film, or urine, is a hot topic in Proteomics (4-6). Li et al found three differently regulated proteins in the sera of breast cancer patients and healthy subjects using surface-enhanced laser desorption/ionization time-of-flight based protein profiling in 2002 and Mathelin et al tried to validate these putative biomarkers, determining only two of them could be used for the discrimination of cancer patients (7,8) (reviewed in refs. 9,10). Some studies examined the nipple aspirate fluid of breast cancer patients and healthy patients (11). In 2005, Pawlik et al showed 17 distinctly regulated peptides; whereas, Li et al found different protein distribution patterns in the nipple aspirate fluid and ductal lavage with the use of SELDI-TOF mass spectrometry (12,13). Since then, many other protein profiling studies were published that used matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry with differently regulated proteins (14-16). The advantage of the MALDI-TOF-TOF MS is the subsequent identification of the proteins of interest. In a previous study, we reported data from MALDI-TOF-TOF-based profiling of the sera that could distinguish breast cancer patients from age-matched healthy controls, and we could classify cancer patients with a high sensitivity of 89% (17).
Another proteomics-based approach for the exploration of cancer-derived differences is the highly-precise microarray platform. This approach can serve, instead of the common ELISA test, as a validation tool for the biomarkers identified from prior MALDI-TOF-TOF-based explorations of the proteome. Here, the antibodies are fixed on a highly-optimized surface. In this manner, several protein levels can be measured simultaneously due to the small required volume (nl) of the reagents. After fixation of the antibodies, the surfaces can be incubated with body fluids containing the appropriate proteins. This high-throughput technique is also very common for the profiling of carcinoma tissue or body fluids of diseased patients due to its miniaturized size, accuracy, and automated handling (18-20) (reviewed in ref. 21). Several comparative studies of breast cancer and healthy sera have been published. Our study group reported the regulation of several proteins were significantly different in the sera of breast cancer patients (22). The discovery of different protein patterns in diseased cohorts and control samples and subsequent identification of these biomarkers is a promising method of obtaining knowledge about the effects of several diseases (6,23,24). A well-developed and clinically proven biomarker signature could lead to early detection of cancer, which can have great benefits for patients.

Most proteomic studies of breast carcinoma published so far concentrate on profiling the tissue or body fluids near the emergence spot. Little is known about the proteome changes of distant body fluids. Some research groups examined the protein profiles of alternative body fluids such as urine or saliva and several differently regulated proteins were reported (reviewed in refs. 25,26). Previously, we showed different protein distributions in the tear fluid of breast cancer patients and healthy controls in a SELDI-TOF-based profiling study (17,27). Another comparative MALDI-TOF-TOF-driven analysis of healthy dog's tear fluid and dogs diagnosed with cancer has been published (28). To our knowledge, no other comparative tear fluid proteomic studies for breast cancer have been reported. Tear fluid has unique properties as retrieval is minimally invasive and it does not contain as many highly-abundant proteins as serum.

Herein, we report a MALDI-TOF-TOF-driven semi-quantitative comparison of tear protein levels in cancer (CA) and control (CTRL) using a de novo approach in pooled samples. Using a signature of biomarkers significantly decreased or increased in groups of CA and CTRL could help to discriminate diseased women from the healthy population with high specificity and sensitivity and possibly lead to the establishment of a molecular diagnostic tool for breast cancer.

Materials and methods

Comparison of tear protein levels in pooled samples from CA and CTRL. This de novo study included 50 female subjects, 25 patients were diagnosed with primary invasive breast carcinoma and treated at the University Medical Center Mainz. At the time of diagnosis, none of the patients had developed distant metastases. Patients’ characteristics are summarized in Table I. The healthy control subjects were 25 age-matched women without any known malignancies who were treated at the University of Mainz medical center. All study members gave their informed consent for voluntary participation in this study. The protocols were approved by the institutional ethics committee in accordance with the ethical standard of Declaration of Helsinki (1964).

Sample retrieval. Tear fluid was obtained from all participants using a Schirmer Strip. After the samples were drawn, the strips were frozen immediately at -80°C to prevent protein degradation. Tear proteins were prepared under strict and identical conditions for all patients. Prior to the experiments, the wet strip part was cut into small pieces and incubated with n-Dodecyl-β-D-maltoside overnight at 4°C with constant shaking. The next day, the eluates were briefly centrifuged and transferred into fresh tubes. All samples were stored prior to analysis at -20°C.

Sample processing. For the comparison of protein levels in CTRL and CA, each of the 25 tear eluates were pooled together accordingly to the group and precipitated with three times the volume of acetone overnight at -80°C. The next day, tear proteins were centrifuged at 14000 x g and 4°C to prevent protein degradation. The supernatant was discarded and the proteins were resuspended in PBS. Protein concentrations were measured with the BCA Protein Assay kit (Thermo Scientific, Rockford IL, USA), according to the manufacturer's protocol.

1D SDS-PAGE and sample purification. Pooled tear proteins (60 µg) from CTRL and CA were separated by molecular weight using 1D SDS-PAGE (gels, buffers, and equipment all purchased from Invitrogen, Darmstadt, Germany). After gel electrophoresis, the lanes were stained overnight and then rinsed with double-distilled water. In the next step, the lanes were subdivided into 32 bands and the proteins were digested with endopeptidase trypsin according to the modified digestion

| Characteristic | Breast cancer patients n=25 (%) | Healthy controls n=25 |
|---------------|-------------------------------|----------------------|
| Mean age (distribution) | 58 (39-85) | 58 (39-85) |
| Tumor size | | |
| pT1 | 16 (64) |
| pT2 | 9 (36) |
| Nodal status | | |
| Negative | 18 (72) |
| Positive | 7 (28) |
| Grading | | |
| Well differentiated (G1) | 6 (24) |
| Moderately differentiated (G2) | 14 (56) |
| Poor/undifferentiated (G3) | 5 (20) |
| Distant metastases | | |
| M0 | 25 (100) |
| M1 | 0 (0) |
Results

In this study we conducted an explorative and comparative analysis of the tear proteome of breast carcinoma patients and age-matched healthy controls. We tried to minimize protein degradation and fluctuations of protein measurements to achieve a precise comparison of protein levels. One person performed the experimental steps for the preparation of tear samples for 1D SDS-PAGE until the transfer of digested fractions onto the sample plate for the robotic purification station. The peptide purification was performed automatically to avoid fluctuations due to the manual handling of samples. Likewise, the experimental steps from the precipitation of the tear eluates were also performed by the same person.

Semiquantitative comparison of protein levels in CA and CTRL. After destaining, a grid made of 32 bands was put under the gel for a more accurate comparison of the proteins. Each of the 32 bands from CTRL and CA were cut out and digested with trypsin overnight. Fig. 1 shows the samples after 1D SDS-PAGE separation and staining with Coomassie dye (Colloidal Blue Staining kit, Invitrogen). After digestion and automated fractionation, the peptides were measured in a MALDI-TOF/TOF mass spectrometer. Representative fractions from both groups are shown in Fig. 2. All spectra were normalized using Protein Pipeline Mainz software, which was developed in-house, and the appropriate tear proteins were identified with the MASCOT search tool.

Protein identification. After extensive comparison of the spectra obtained using the annotated proteins in the SWISSPROT Homo sapiens database under the given conditions and MOWSE score, we were able to identify over 150 proteins in the CTRL and CA. The complete merged list of identified proteins is summarized in Table II. To obtain an overview on the relevance and role of the identified proteins, we clustered the proteins in accordance to their molecular functions using the software Cytoscape 2.7.0, as shown in Fig. 3. The Cytoscape software often shows several overlapping molecular functions and distributions into several biological processes; therefore, we created an overview of one mapping possibility for a large number of the identified tear proteins.

Using the in-house-developed algorithm, we compared the protein levels in both groups. More than 20 proteins were distinctly upregulated or downregulated in the CTRL and CA groups and were involved in many biological processes such as metabolism (ALDH3A or TPI) or immune response (e.g., CIQ1 or SI00A8). Table III shows a detailed list of the increased or decreased proteins in the tear fluid of breast cancer patients. Of note, the findings include inflammation proteins or complement factors for pathologic processes such as cancer that have already been described (34-36). Moreover, several proteins show at least four-fold higher (Extracellular sulfatase Sulf-1, Cystatin SA, cst2; 5-AMP-activated protein kinase subunit gamma-3, prkag3; Triosophosphate isomerase, tpi1; Microtubule-associated tumor suppressor 1, mtus1; Transferrin receptor protein 1, trfc; and Putative lipocalin 1-like protein 1, lcnIII1) or lower levels (DNA damage-binding protein 1, ddb1; Protein S100-A9, s100a9; and GTP-binding protein Di-Ras2, diras2) in CA. An overview of the proteins differently regulated in the CA group was constructed according to their regulation using the STRING tool and is shown in Fig. 4 (32).

Figure 1. The stained tear samples from CTRL and CA. As an example, the composed grid is shown on the CTRL lane. The lane M shows the protein standard (SeeBlue® Plus2 Pre-Stained Standard Invitrogen, Darmstadt, Germany).
Discussion

Data from high-throughput proteomic technologies, such as SELDI-TOF MS, MALDI-TOF-TOF MS, and microarray platforms, have recently increased. These techniques allow simultaneous protein profiling and subsequent identification of proteins and their subunits (5,37,38). A huge number of proteome studies have been published for proteome comparison of cancer patients and controls. Likewise, different proteomic studies reported significant differences in protein levels in the body fluids of breast cancer patients and healthy subjects (38,39). In our study, we concentrated on the tear proteome for several reasons. First, the sample retrieval is minimally invasive for the participants and tear fluid is easy to obtain with a simple Schirmer test. Second, the tear proteome contains no high-abundant proteins, such as albumin and immunoglobulins that are found in serum; therefore, it is not necessary to perform additional depletion steps that may cause distortion of potentially important proteins. In addition, we find it very intriguing to explore the tear proteome for potential biomarkers of breast cancer as it is an uncommon approach.

Some of the differently regulated proteins in our de novo pooled experiment have been reported to be altered in the tear fluid of patients with ophthalmic disease. Zhou et al reported S100A8 and S100A9 are increased in patients with dry eyes and Grus et al reported an increase in protein S100A8 (34,40). Both proteins belong to the family of S100 calcium-binding proteins, whose members seem to be involved in pro-inflammatory pathways as previously reported by Nacken et al (35).
Table II. Proteins identified from tear proteomes of CA and CTRL.

| Protein          | Description                      | Organism species (OS) | Gene name (GN) |
|------------------|----------------------------------|-----------------------|----------------|
| TRFL_HUMAN       | Lactotransferrin                 | Homo sapiens          | LTF            |
| LCN1_HUMAN       | Lipocalin-1                      | Homo sapiens          | LCN1           |
| ALBU_HUMAN       | Serum albumin                    | Homo sapiens          | ALB            |
| IGKC_HUMAN       | Ig κ chain C region              | Homo sapiens          | IGKC           |
| SG2A1_HUMAN      | Mammaglobin-B                    | Homo sapiens          | SCGB2A1        |
| LYSC_HUMAN       | Lysozyme C                       | Homo sapiens          | LYZ            |
| PIP_HUMAN        | Prolactin-inducible protein      | Homo sapiens          | PIP            |
| DMBT1_HUMAN      | Deleted in malignant brain tumors 1 protein | Homo sapiens          | DMBT1          |
| IGHA1_HUMAN      | Ig α-1 chain C region            | Homo sapiens          | IGHA1          |
| IGHA2_HUMAN      | Ig α-2 chain C region            | Homo sapiens          | IGHA2          |
| GSTP1_HUMAN      | Glutathione S-transferase P      | Homo sapiens          | GSTP1          |
| ZA2G_HUMAN       | Zinc-α-2-glycoprotein            | Homo sapiens          | AZGP1          |
| ACTB_HUMAN       | Actin, cytoplasmic 1             | Homo sapiens          | ACTB           |
| CYTN_HUMAN       | Cystatin-SN                      | Homo sapiens          | CST1           |
| LC1L1_HUMAN      | Putative lipocalin 1-like protein 1 | Homo sapiens          | LCN1L1         |
| PROL4_HUMAN      | Proline-rich protein 4           | Homo sapiens          | PRR4           |
| CYTS_HUMAN       | Cystatin-S                       | Homo sapiens          | CST4           |
| ACTBL_HUMAN      | β-actin-like protein 2           | Homo sapiens          | ACTBL2         |
| POTEE_HUMAN      | POTE ankyrin domain family member E | Homo sapiens          | POTEE          |
| POTEF_HUMAN      | POTE ankyrin domain family member F | Homo sapiens          | POTEF          |
| ACTC_HUMAN       | Actin, α cardiac muscle 1        | Homo sapiens          | ACTC1          |
| LAC2_HUMAN       | Ig λ-2 chain C regions           | Homo sapiens          | IGLC2          |
| SG1D1_HUMAN      | Secretoglobin family 1D member 1 | Homo sapiens          | SCGB1D1        |
| S10A9_HUMAN      | Protein S100-A9                  | Homo sapiens          | S100A9         |
| K1C9_HUMAN       | Keratin, type I cytoskeletal 9   | Homo sapiens          | KRT9           |
| TMC8_HUMAN       | Transmembrane channel-like protein 8 | Homo sapiens          | TMC8           |
| K2C1_HUMAN       | Keratin, type II cytoskeletal 1  | Homo sapiens          | KRT1           |
| LAC1_HUMAN       | Ig λ-1 chain C regions           | Homo sapiens          | IGLC1          |
| CYTT_HUMAN       | Cystatin-SA                      | Homo sapiens          | CST2           |
| PIGR_HUMAN       | Polymeric immunoglobulin receptor | Homo sapiens          | PIGR           |
| S10A8_HUMAN      | Protein S100-A8                  | Homo sapiens          | S100A8         |
| APOA1_HUMAN      | Apolipoprotein A-I               | Homo sapiens          | APOA1          |
| PROL1_HUMAN      | Proline-rich protein 1           | Homo sapiens          | PROL1          |
| HSPB1_HUMAN      | Heat shock protein β-1           | Homo sapiens          | HSPB1          |
| LACRT_HUMAN      | Extracellular glycoprotein lacritin | Homo sapiens          | LACRT          |
| ABCA3_HUMAN      | ATP-binding cassette sub-family A member 3 | Homo sapiens          | ABCA3          |
| IGJ_HUMAN        | Immunoglobulin J chain           | Homo sapiens          | IGJ            |
| ANXA2_HUMAN      | Annexin A2                      | Homo sapiens          | ANXA2          |
| SSH2_HUMAN       | Protein phosphatase Slingshot homolog 2 | Homo sapiens          | SSH2           |
| KV301_HUMAN      | Ig κ chain V-III region B6       | Homo sapiens          |                |
| KV307_HUMAN      | Ig κ chain V-III region GOL      | Homo sapiens          |                |
| TPIS_HUMAN       | Triosephosphate isomerase        | Homo sapiens          | TPI1           |
| LEG3_HUMAN       | Galectin-3                       | Homo sapiens          | LGALS3         |
| NGL_A_HUMAN      | Neutrophil gelatinase-associated lipocalin | Homo sapiens          | LCN2           |
| POP1_HUMAN       | Ribonucleases P/MRP protein subunit POP1 | Homo sapiens          | POP1           |
| ZC3H1_HUMAN      | Zinc finger C3H1 domain-containing protein | Homo sapiens          | ZFC3H1         |
| CLIC1_HUMAN      | Chloride intracellular channel protein 1 | Homo sapiens          | CLIC1          |
| LIME1_HUMAN      | Lck-interacting transmembrane adapter 1 | Homo sapiens          | LIME1          |
| HV307_HUMAN      | Ig heavy chain V-III region CAM   | Homo sapiens          |                |
| GNL3_HUMAN       | Guanine nucleotide-binding protein-like 3 | Homo sapiens          | GNL3           |
| POTEI_HUMAN      | POTE ankyrin domain family member I | Homo sapiens          | POT1           |
| Protein            | Description                                      | Organism species (OS) | Gene name (GN) |
|--------------------|--------------------------------------------------|-----------------------|----------------|
| ENOA_HUMAN         | α-enolase                                        | Homo sapiens          | ENO1           |
| PRDX1_HUMAN        | Peroxiredoxin-1                                 | Homo sapiens          | PRDX1          |
| MECP2_HUMAN        | Methyl-CpG-binding protein 2                     | Homo sapiens          | MECP2          |
| K2C78_HUMAN        | Keratin, type II cytoskeletal 78                 | Homo sapiens          | KRT78          |
| ZG16B_HUMAN        | Zymogen granule protein 16 homolog B             | Homo sapiens          | ZG16B          |
| YM012_HUMAN        | Uncharacterized protein DKFZp434B061             | Homo sapiens          |                |
| YV021_HUMAN        | Uncharacterized protein LOC284861                | Homo sapiens          |                |
| ILEU_HUMAN         | Leukocyte elastase inhibitor                     | Homo sapiens          | SERPINB1       |
| ANXA1_HUMAN        | Annexin A1                                       | Homo sapiens          | ANXA1          |
| POTEJ_HUMAN        | POTE ankyrin domain family member J              | Homo sapiens          | POTEJ          |
| PLSL_HUMAN         | Plastin-2                                        | Homo sapiens          | LCP1           |
| NCOA5_HUMAN        | Nuclear receptor coactivator 5, protein existence (PE), 1; sequence version (SV), 2 | Homo sapiens | NCOA5          |
| B2MG_HUMAN         | β-2-microglobulin                                | Homo sapiens          | B2M            |
| KLH34_HUMAN        | Kelch-like protein 34                            | Homo sapiens          | KLHL34         |
| ANX13_HUMAN        | Annexin A13                                      | Homo sapiens          | ANXA13         |
| MDHC_HUMAN         | Malate dehydrogenase, cytoplasmic                | Homo sapiens          | MDH1           |
| AIFM2_HUMAN        | Apoptosis-inducing factor 2                      | Homo sapiens          | AIFM2          |
| STAG3_HUMAN        | Cohesin subunit SA-3                            | Homo sapiens          | STAG3          |
| SMCA4_HUMAN        | Transcription activator BRG1                     | Homo sapiens          | SMARCA4        |
| DDB1_HUMAN         | DNA damage-binding protein 1                     | Homo sapiens          | DDB1           |
| RM18_HUMAN         | 39S ribosomal protein L18, mitochondrial         | Homo sapiens          | MRPL18         |
| KRIT1_HUMAN        | Krev interaction trapped protein 1              | Homo sapiens          | KRIT1          |
| PERT_HUMAN         | Thyroid peroxidase                               | Homo sapiens          | TPO            |
| HPT_HUMAN          | Haptoglobin                                      | Homo sapiens          | HP             |
| F184A_HUMAN        | Protein Fam184A                                  | Homo sapiens          | FAM184A        |
| AAKG2_HUMAN        | 5'-AMP-activated protein kinase subunit γ-2      | Homo sapiens          | PRKAG2         |
| AAKG3_HUMAN        | 5'-AMP-activated protein kinase subunit γ-3      | Homo sapiens          | PRKAG3         |
| EIF2A_HUMAN        | Eukaryotic translation initiation factor 2A      | Homo sapiens          | EIF2A          |
| RGP2A_HUMAN        | Ras GTPase-activating protein subunit α-2        | Homo sapiens          | RALGAPA2       |
| TUT4_HUMAN         | Terminal uridylyltransferase 4                   | Homo sapiens          | ZCCHC11        |
| ATP4A_HUMAN        | Potassium-transporting ATPase α chain 1          | Homo sapiens          | ATP4A          |
| YJO17_HUMAN        | Putative uncharacterized protein LOC439951       | Homo sapiens          |                |
| AINX_HUMAN         | α-internexin                                     | Homo sapiens          | INA            |
| TTBK2_HUMAN        | Tau-tubulin kinase 2                             | Homo sapiens          | TTBK2          |
| SPTN2_HUMAN        | Spectrin β chain, brain 2                       | Homo sapiens          | SPTBN2         |
| MDGA1_HUMAN        | MAM domain-containing glycosylphosphatidylinositol anchor protein 1 | Homo sapiens | MDGA1          |
| FREM3_HUMAN        | FRAS1-related extracellular matrix protein 3     | Homo sapiens          | FREM3          |
| PDE4C_HUMAN        | cAMP-specific 3',5'-cyclic phosphodiesterase 4C  | Homo sapiens          | PDE4C          |
| SULF1_HUMAN        | Extracellular sulfatase Sulf-1                   | Homo sapiens          | SULF1          |
| LRC4C_HUMAN        | Leucine-rich repeat-containing protein 4C        | Homo sapiens          | LRRC4C         |
| S10A4_HUMAN        | Protein S100-A4                                  | Homo sapiens          | S100A4         |
| LRFN6_HUMAN        | Leucine-rich repeat and fibronectin type-III domain-containing protein 6 | Homo sapiens | ELFN2          |
| IGHG3_HUMAN        | Ig γ-3 chain C region                            | Homo sapiens          | IGHG3          |
| IGHG2_HUMAN        | Ig γ-2 chain C region                            | Homo sapiens          | IGHG2          |
| ELOA1_HUMAN        | Transcription elongation factor B polypeptide 3  | Homo sapiens          | TCEB3          |
| DLG3_HUMAN         | Disks large homolog 3                            | Homo sapiens          | DLG3           |
| PDZD7_HUMAN        | PDZ domain-containing protein 7                  | Homo sapiens          | PDZD7          |
| HV315_HUMAN        | Ig heavy chain V-III region WAS                  | Homo sapiens          |                |
| HV304_HUMAN        | Ig heavy chain V-III region TIL                  | Homo sapiens          |                |
Table II. Continued.

| Protein          | Description                                           | Organism species (OS) | Gene name (GN) |
|------------------|-------------------------------------------------------|-----------------------|----------------|
| WBS23_HUMAN      | Williams-Beuren syndrome chromosomal region 23 protein| Homo sapiens          | WBSCR23        |
| PKHH3_HUMAN      | Pleckstrin homology domain-containing family H member 3| Homo sapiens          | PLEKHH3        |
| DMXL2_HUMAN      | DmX-like protein 2                                     | Homo sapiens          | DMXL2          |
| CBR3_HUMAN       | Carbonyl reductase [NADPH] 3                           | Homo sapiens          | CBR3           |
| CE164_HUMAN      | Centrosomal protein of 164 kDa                        | Homo sapiens          | CEP164         |
| USPL1_HUMAN      | Ubiquitin-specific peptidase-like protein 1            | Homo sapiens          | USPL1          |
| TRFE_HUMAN       | Serotransferrin                                        | Homo sapiens          | TF             |
| MPPA_HUMAN       | Mitochondrial-processing peptidase subunit α           | Homo sapiens          | PMPCA          |
| CAPI1_HUMAN      | Calcium-binding protein 1                              | Homo sapiens          | CAPI1          |
| TFR1_HUMAN       | Transferrin receptor protein 1                         | Homo sapiens          | TFR1           |
| ZNF446_HUMAN     | Zinc finger protein 446                                | Homo sapiens          | ZNF446         |
| MTC2_HUMAN       | Bifunctional methylenetetrahydrofolate dehydrogenase/  | Homo sapiens          | MTHFD2         |
|                  | cyclohydrolase, mitochondrial                          |                       |                |
| CT151_HUMAN      | Uncharacterized protein C20orf151                      | Homo sapiens          | C20orf151      |
| LIP2_HUMAN       | Liprin-β-2                                             | Homo sapiens          | LIP2           |
| ZSWM5_HUMAN      | Zinc finger SWIM domain-containing protein 5           | Homo sapiens          | ZSWM5          |
| WDR60_HUMAN      | WD repeat-containing protein 60                        | Homo sapiens          | WDR60          |
| C1QC_HUMAN       | Complement C1q subcomponent subunit C                  | Homo sapiens          | C1QC           |
| CNOT1_HUMAN      | CCR4-NOT transcription complex subunit 1               | Homo sapiens          | CNOT1          |
| CDK13_HUMAN      | Cyclin-dependent kinase 13                             | Homo sapiens          | CDK13          |
| GLE1_HUMAN       | Nucleoporin GLE1                                       | Homo sapiens          | GLE1           |
| RFI4_HUMAN       | Rab11 family-interacting protein 4                     | Homo sapiens          | RAB11FIP4      |
| AL3A1_HUMAN      | Aldehyde dehydrogenase, dimeric NADP-preferring        | Homo sapiens          | ALDH3A1        |
| FRMD7_HUMAN      | FERM domain-containing protein 7                        | Homo sapiens          | FRMD7          |
| SEM4C_HUMAN      | Semaphorin-4C                                          | Homo sapiens          | SEMA4C         |
| PRTG_HUMAN       | Protagenin                                             | Homo sapiens          | PRTG           |
| PTPRR_HUMAN      | Receptor-type tyrosine-protein phosphatase R            | Homo sapiens          | PTPRR          |
| HV305_HUMAN      | Ig heavy chain V-III region BRO                         | Homo sapiens          |                |
| TGS1_HUMAN       | Trimethylguanosine synthase                             | Homo sapiens          | TGS1           |
| LRRK2_HUMAN      | Leucine-rich repeat serine/threonine-protein kinase 2  | Homo sapiens          | LRRK2          |
| BMP2_HUMAN       | Bone morphogenetic protein receptor type-2             | Homo sapiens          | BMP2           |
| F178A_HUMAN      | Protein FAM178A                                        | Homo sapiens          | FAM178A        |
| MOV10_HUMAN      | Putative helicase MOV-10                               | Homo sapiens          | MOV10          |
| K0556_HUMAN      | Uncharacterized protein KIAA0556                       | Homo sapiens          | KIAA0556       |
| KAT2A_HUMAN      | Histone acetyltransferase KAT2A                        | Homo sapiens          | KAT2A          |
| EAP1_HUMAN       | Enhanced at puberty protein 1                          | Homo sapiens          | EAP1           |
| CA175_HUMAN      | Uncharacterized protein C1orf175                       | Homo sapiens          | C1orf175       |
| ENOG_HUMAN       | γ-enolase                                              | Homo sapiens          | ENO2           |
| ENOB_HUMAN       | β-enolase                                              | Homo sapiens          | ENO3           |
| LOX5_HUMAN       | Arachidonate 5-lipoxygenase                            | Homo sapiens          | ALOX5          |
| MTMR4_HUMAN      | Myotubularin-related protein 4                         | Homo sapiens          | MTMR4          |
| YQ050_HUMAN      | Putative uncharacterized protein FLJ45831              | Homo sapiens          |                |
| TRI75_HUMAN      | Tripartite motif-containing protein 75                  | Homo sapiens          | TRIM75         |
| LRIG3_HUMAN      | Leucine-rich repeats and immunoglobulin-like domains protein 3 | Homo sapiens          | LRIG3          |
| DSCL1_HUMAN      | Down syndrome cell adhesion molecule-like protein 1    | Homo sapiens          | DSCAML1        |
| CD20_HUMAN       | B-lymphocyte antigen CD20                              | Homo sapiens          | MS4A1          |
| IGHG4_HUMAN      | Ig γ-4 chain C region                                  | Homo sapiens          | IGHG4          |
| MIDA_HUMAN       | Protein midA homolog, mitochondrial                    | Homo sapiens          | C2orf56        |
| SI1L3_HUMAN      | Signal-induced proliferation-associated 1-like protein 3| Homo sapiens          | SIPAIL3        |
| TLE2_HUMAN       | Transducin-like enhancer protein 2                      | Homo sapiens          | TLE2           |
| KLIH17_HUMAN     | Kelch-like protein 17                                   | Homo sapiens          | KLIH17          |
| COTAI_HUMAN      | Collagen α-1(VII) chain                                | Homo sapiens          | COL7A1         |
| MRGRD_HUMAN      | Mas-related G-protein coupled receptor member D        | Homo sapiens          | MRGRPD         |
| MCF2L_HUMAN      | Guanine nucleotide exchange factor DBS                 | Homo sapiens          | MCF2L          |
| MTUS1_HUMAN      | Microtubule-associated tumor suppressor 1              | Homo sapiens          | MTUS1          |
of the proteins may be of high interest, e.g., Mitochondrial
tumor suppressor 1, MTUS1 and DNA damage binding
protein, DDB1. MTUS1 regulates the cell cycle by acting as a
tumor suppressor and DDB1 is involved in nucleotide excision
repair. In addition, many of the differently regulated proteins
are involved in metabolic processes, e.g., TPI or MDH1 in
glycolysis and the citric acid cycle, which are both increased in
the tear fluid of cancer patients. However, higher levels of auto-
antibodies against TPI1 have been reported in the sera of breast
cancer patients (36). In our previous studies, we found several
alterations in protein expression in the sera and tear fluid of
breast cancer patients (22,41). Further analysis of the SELDI-
TOF-based tear proteome profiling identified the protein
S100A4 to be increased in the tears of breast cancer patients
(data not shown). This result was confirmed in this study. The
protein S100A4 was also previously found to be upregulated in
patients with dry eye syndrome (40). Noteworthy, we observed
several alterations in the level of proteins involved in immune
response, such as complement factor C1Q1 or fragments of
immunoglobulins (Table II). Also, several complement factors

Table III. Proteins increased or decreased at least 2-fold in CA.

A, Increased proteins in CA with fold increase

| Protein ID                                  | Fold increase | Number of compared peptides |
|---------------------------------------------|---------------|----------------------------|
| Extracellular sulfatase Sulf-1              | 44            | 1                          |
| Cystatin-SA                                 | 9             | 2                          |
| 5-AMP-activated protein kinase subunit γ-3  | 6             | 1                          |
| Triosephosphate isomerase                   | 5.5           | 5                          |
| Microtubule-associated tumor suppressor 1   | 4.7           | 13                         |
| Transferrin receptor protein 1              | 4.5           | 6                          |
| Keratin, type I cytoskeletal 9              | 4.4           | 17                         |
| Putative lipocalin 1-like protein 1         | 4.1           | 1                          |
| Malate dehydrogenase, cytoplasmic           | 4             | 5                          |
| Ig α2 chain C region                        | 3.2           | 2                          |
| Ig heavy chain V-III region BRO             | 3.2           | 6                          |
| Protein S100-A4                             | 3.2           | 1                          |
| Keratin, type II cytoskeletal 1             | 3.1           | 36                         |
| Pericentrin                                 | 2.8           | 49                         |
| Ig heavy chain V-III region WEA             | 2.7           | 2                          |
| Complement C1q subcomponent subunit C       | 2.6           | 1                          |

B, Decreased proteins in CA with fold increase

| Protein ID                                    | Fold decrease | Number of compared peptides |
|-----------------------------------------------|---------------|----------------------------|
| Aldehyde dehydrogenase, dimeric NADP-preferring| 2.1           | 6                          |
| Immunoglobulin J chain                        | 2.4           | 14                         |
| Ig γ-3 chain C region                         | 2.4           | 12                         |
| POTE ankyrin domain family member F           | 2.5           | 6                          |
| Protein S100-A8                               | 2.5           | 18                         |
| Uncharacterized protein C20orf151             | 2.9           | 9                          |
| Ig γ-4 chain C region                         | 3             | 1                          |
| WD repeat-containing protein 60               | 3             | 3                          |
| DNA damage-binding protein 1                  | 3.3           | 3                          |
| Protein S100-A9                               | 3.3           | 11                         |
| GTP-binding protein Di-Ras2                   | 10            | 1                          |

Figure 4. Using the software, STRING, we constructed an overview of the
proteins that were at least 2-fold differently regulated in CTRL and CA. The
appropriate gene names are abbreviated. The arrows show the increase (red
arrow) or decrease (blue arrow) of the proteins in CA. Some of the known
interactions of the proteins are shown with connection lines. The thickness
of the lines shows how strong the interactions are.
have been reported to be differentially regulated in the sera of cancer patients (42,43). Although, some of the results were controversial and may have resulted from different storage and handling conditions (44). Thus, members of the complement system may have additional roles. Markiewski et al reported tumor growth was promoted by C5a in their experiments with a cervical cancer mouse model (45,46).

To our knowledge, little is known about protein expression in the tear fluid of breast cancer patients. Only a very small number of tear proteome studies concerning proteome changes during breast cancer or cancer in general have been published. Further subsequent analyses and validation of our results in a tear protein study with an independent population and a higher number of participants will follow that also includes individual profiling. The findings from this study are intriguing as they may deepen the understanding of the impact of cancer and several cancer-driven pathways. Our study demonstrates that different biological processes are altered not only in prominent and broadly investigated body fluids such as serum and plasma, but also in discrete fluids such as tears that are located far away from the cancer site. As we already mentioned, several proteins have been reported to be modified in various types of body fluids, such as nipple aspirate fluid or urine. Our pilot study adds to these findings and shows again the complexity and multiple impacts of breast cancer while emerging and developing in the host, affecting biological processes and signal cascades. Moreover, we propose that a biomarker panel consisting of different proteins could accurately discriminate cancer patients from healthy controls. Therefore, it is important to examine the protein levels in an independent study population using individual protein profiling to validate our results. Further de novo approaches and validation of our results could lead to a better understanding of the pathological mechanism of breast cancer.

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References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D: Global cancer statistics. CA Cancer J Clin 61: 69-90, 2011.
2. Brekelmans CT, Seynaeve C, Bartels CC, et al: Effectiveness of breast cancer surveillance in BRCAl/2 gene mutation carriers and women with high familial risk. J Clin Oncol 19: 924-930, 2001.
3. Harris L, Fritsche H, Mennel R, et al: American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. J Clin Oncol 25: 5287-5312, 2007.
4. Tyers M and Mann M: From genomics to proteomics. Nature 422: 193-197, 2003.
5. Elrick MM, Walgren JL, Mitchell MD and Thompson DC: Proteomics: recent applications and new technologies. Basic Clin Pharmacol Toxicol 98: 432-441, 2004.
6. Banks RE, Dunn MJ, Hochstrasser DF, et al: Proteomics: new perspectives, new biomedical opportunities. Lancet 356: 1749-1756, 2000.
7. Li J, Zhang Z, Rosenzweig J, Wang YY and Chan DW: Proteomics and bioinformatics approaches for identification of serum biomarkers to detect breast cancer. Clin Chem 48: 1296-1304, 2002.
8. Mathelin C, Cromer A, Wendling C, Tomasetto C and Rio MC: Serum biomarkers for detection of breast cancers: A prospective study. Breast Cancer Res Treat 96: 83-90, 2006.
9. Baskin Y and Yigitbasi T: Clinical proteomics of breast cancer. Curr Genomics 11: 528-536, 2010.
10. Huijbers A, Velstra B, Dekker TJ, et al: Proteomic serum biomarkers and their potential application in cancer screening programs. Int J Mol Sci 11: 4175-4193, 2010.
11. Pawelczak CP, Trock B, Pemenen M, et al: Proteomic patterns of nipple aspirate fluids obtained by SELDI-TOF: potential for new biomarkers to aid in the diagnosis of breast cancer. Dis Markers 17: 301-307, 2001.
12. Li J, Zhao J, Xu Y, et al: Identification of biomarkers for breast cancer in nipple aspirate and ductal lavage fluid. Clin Cancer Res 11: 8312-8320, 2005.
13. Pawlik TM, Fritsche H, Coombes KR, et al: Significant differences in nipple aspirate fluid protein expression between healthy women and those with breast cancer demonstrated by time-of-flight mass spectrometry. Breast Cancer Res Treat 89: 149-157, 2005.
14. de Noo ME, Deelder A, van der Werff M, Ozalp A, Mertens B and Tollenaar R: MALDI-TOF serum protein profiling for the detection of breast cancer. Onkol Logie 29: 501-506, 2006.
15. Villanueva J, Shaffer DR, Philip J, et al: Differential exoprotease activities confer tumor-specific serum peptide patterns. J Clin Invest 116: 271-284, 2006.
16. Engwegen JY, Gast MC, Schellens JH and Beijnen JH: Clinical proteomics: searching for better tumour markers with SELDI-TOF mass spectrometry. Trends Pharmacol Sci 27: 49-59, 2006.
17. Lebrecht A, Boehm D, Schmidt M, Koelbl H and Grus FH: Surface-enhanced laser desorption/ionisation time-of-flight mass spectrometry to detect breast cancer markers in tears and serum. Cancer Genomics Proteomics 6: 75-83, 2009.
18. Hudson ME, Pozdnyakova I, Haines K, Mor G and Snyder M: Identification of differentially expressed proteins in ovarian cancer using high-density protein microarrays. Proc Natl Acad Sci USA 104: 17494-17499, 2007.
19. Hodgkinson VC, ElFadl D, Drew PJ, Lind MJ and Cawkwell L: Repeatedly identified differentially expressed proteins (RIDEPs) from antibody microarray proteomic analysis. J Proteomics 74: 698-703, 2011.
20. Shi W, Meng Z, Chen Z, Luo J and Liu L: Proteome analysis of human pancreatic cancer cell lines with highly liver metastatic potential by antibody microarray. Mol Cell Biochem 347: 117-125, 2011.
21. Yang L, Guo S, Li Y, Zhou S and Tao S: Protein microarrays for systems biology. Acta Biochim Biophys Sin (Shanghai) 43: 161-171, 2011.
22. Bohn D, Keller K, Boehm N, et al: Antibody microarray analysis of the serum proteome in primary breast cancer patients. Cancer Biol Ther 12: 772-779 2011.
23. Bertucci F, Birnbbaum D and Goenvalles A: Proteomics of breast cancer: principles and potential clinical applications. Mol Cell Proteomics 5: 1772-1786, 2006.
24. Diamandis EP: Analysis of serum proteomic patterns for early cancer diagnosis: drawing attention to potential problems. J Nati Cancer Inst 96: 353-356, 2004.
25. Downes MR, Byrne JC, Dunn MJ, Fitzpatrick JM, Watson RW and Cunningham SR: Application of proteomic strategies to the identification of urinary biomarkers for prostate cancer: a review. Biomarkers 11: 406-416, 2006.
26. Radpour R, Barekati Z, Kohler C, Holzgreve W and Zhong XY: New trends in molecular biomarker discovery for breast cancer. Curr Opin Text Mol Biomarkers Oncol 13: 565-571, 2008.
27. Lebrecht A, Boehm D, Schmidt M, Koelbl H, Schwartz RL and Grus FH: Diagnosis of breast cancer by tear proteomic pattern. Cancer Genomics Proteomics 6: 177-182, 2009.
28. de Freitas Campos C, Cole N, Van Dyk D, et al: Proteomic analysis of dog tears for potential cancer markers. Res Vet Sci 85: 349-352, 2008.
29. Shevchenko A, Loboda A, Ens W, Schraven B, Stading KG and Shevchenko A: Archived polycrylamide gels as a resource for proteome characterization by mass spectrometry. Electrophoresis 22: 1194-1203, 2001.
30. Perkins DN, Pappin DJ, Creasy DM and Cottrell JS: Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 20: 3561-3567, 1999.
31. UniProt Consortium: Ongoing and future developments at the Universal Protein Resource. Nucleic Acids Res 39: D214-D219, 2011.
32. Szklarczyk D, Franceschini A, Kuhn M, et al: The STRING database in 2011: functional interaction networks of proteins, globally integrated and scored. Nucleic Acids Res 39: D561-D568, 2011.

33. Cline MS, Smoot M, Cerami E, et al: Integration of biological networks and gene expression data using Cytoscape. Nat Protoc 2: 2366-2382, 2007.

34. Grus FH, Podust VN, Bruns K, et al: SELDI-TOF-MS ProteinChip array profiling of tears from patients with dry eye. Invest Ophthalmol Vis Sci 46: 863-876, 2005.

35. Nacken W, Roth J, Sorg C and Kerkhoff C: S100A9/S100A8: Myeloid representatives of the S100 protein family as prominent players in innate immunity. Microsc Res Tech 60: 569-580, 2003.

36. Tamesa MS, Karamitsu Y, Fujimoto M, et al: Detection of auto-antibodies against cyclophilin A and triosephosphate isomerase in sera from breast cancer patients by proteomic analysis. Electrophoresis 30: 2168-2181, 2009.

37. El Yazidi-Belkoura I, Adriaenssens E, Vercoutter-Edouart AS, Lemoine J, Nurcombe V and Hondermarck H: Proteomics of breast cancer: outcomes and prospects. Technol Cancer Res Treat 1: 287-296, 2002.

38. Gast MC, van Gils CH, Wessels LF, et al: Influence of sample storage duration on serum protein profiles assessed by surface-enhanced laser desorption/ionisation time-of-flight mass spectrometry (SELDI-TOF MS). Clin Chem Lab Med 47: 694-705, 2009.

39. Shin BK, Wang H and Hanash S: Proteomics approaches to uncover the repertoire of circulating biomarkers for breast cancer. J Mammary Gland Biol Neoplasia 7: 407-413, 2002.

40. Zhou P, Lowery M, J AD and Kuiken T: Towards improved myoelectric prosthesis control: high density surface EMG recording after targeted muscle reinnervation. Conf Proc IEEE Eng Med Biol Soc 4: 4064-4067, 2005.

41. Böhm D, Keller K, Wehrwein N, et al: Serum proteome profiling of primary breast cancer indicates a specific biomarker profile. Oncol Rep 26: 1051-1056, 2011.

42. Oner F, Savas I and Numanoglu N: Immunoglobulins and complement components in patients with lung cancer. Tuberk Toraks 52: 19-23, 2004.

43. Liu W, Liu B, Xin L, et al: Down-regulated expression of complement factor I: a potential suppressive protein for gastric cancer identified by serum proteome analysis. Clin Chim Acta 377: 119-126, 2007.

44. Gast MC, van Gils CH, Wessels LF, et al: Influence of sample storage duration on serum protein profiles assessed by surface-enhanced laser desorption/ionisation time-of-flight mass spectrometry (SELDI-TOF MS). Clin Chem Lab Med 47: 694-705, 2009.

45. Markiewski MM, DeAngelis RA, Benencia F, et al: Modulation of the antitumor immune response by complement. Nat Immunol 9: 1225-1235, 2008.

46. Markiewski MM and Lambiris JD: Is complement good or bad for cancer patients? A new perspective on an old dilemma. Trends Immunol 30: 286-292, 2009.