Abstract. Background: New biomarkers are needed to improve the early detection of breast cancer. This study describes the use of surface-enhanced laser desorption/ionisation time-of-flight mass spectroscopy (SELDI-TOF-MS) in serum and tear fluid. Materials and Methods: Blood and tear fluid of 10 women with breast cancer and 10 healthy age-matched women were screened for potential biomarkers. Blood samples and tear fluid were drawn prior to surgery. SELDI-TOF-MS were used for protein profiling with three different active surfaces of the protein chips. The data were analyzed by multivariate statistical techniques and artificial neural networks. Results: Complex protein and peptide patterns were found on all three surfaces. We identified the main proteins in tear fluid. Statistically significant differences in the protein pattern (p<0.001) were found between breast cancer patients and healthy controls. The diagnostic pattern differentiated cancer patients from controls with a specificity and sensitivity of approximately 90% in serum and tear fluid. Conclusion: Protein chip technology facilitates the discovery of new and better biomarkers in breast cancer. It is a promising approach to analyse a large number of patients with high sensitivity and specificity. Analysing tear fluid could show some advantages.

Recent trends in breast cancer incidence, survival and mortality rates provide evidence that both earlier detection and better treatment of breast cancer have contributed to the recent marked decline in breast cancer mortality (1). Unfortunately small lesions are frequently missed and may not be visible even by mammography, particularly in young women or women with dense breast tissue (2). The discovery of molecular markers that can potentially identify such lesions will provide a real opportunity to treat these neoplasms in time (3). But the lack of good serum tumor markers for breast cancer still creates many problems for its molecular diagnosis in early stages (4). Proteomics technologies offer the opportunity to discover novel biomarkers (5). Proteomic analysis of human body fluids has become one of the most promising approaches to the discovery of biomarkers for human diseases. Body fluid testing provides several key advantages including low invasiveness, minimum cost and easy sample collection and processing (6).

The potential of proteomics has been demonstrated in various medical areas (e.g. infectious disease (7), Alzheimer’s disease (8), cardiovascular disease (9), and oncology (10-14). In breast cancer, significant differences in protein expression in nipple aspirate fluid of healthy women and those with breast cancer were demonstrated (15). Serum protein profiles to classify breast cancer vs. normal benign breast diseases showed 90% sensitivity and 94% specificity (5). Other studies have demonstrated that saliva testing may be useful in breast cancer detection (16, 17).

In tears, proteins and peptides play an important role as antibacterial medium, for transport of water-insoluble molecules and as a protection for the cornea. The tear film is a very complex mixture of proteins, lipids, glycoproteins, neuropeptides and cytokines and is far from just being a simple reflection of blood plasma (31-34). Changes in tear protein expressions could be very important biomarkers to analyse disease states or for diagnosis. Biochemical analysis of tear-film composition might provide a useful approach to the search for disease-specific biomarkers and lead to the discovery of new diagnostic procedures and treatment options. Altered protein profiles in tear fluid were found in some ocular and systemic disease (e.g. dry eye, diabetes mellitus) (18-20). Contrary to tear fluid, removal of abundant proteins such as albumin or immunoglobulin is often desired.
prior to proteome analysis of serum. There has been concern regarding whether less abundant serum proteins are removed along with albumin or other proteins (6).

Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS; Ciphergen, Fremont, CA, USA) separates proteins on different solid-phase chromatographic surfaces (ProteinChip) and the proteins are subsequently ionized and detected by TOF-MS. ProteinChip technology is able to analyze protein profiles from a variety of complex biological materials, e.g. serum, tears, saliva, urine and cell lysates. This novel fast technology requires far less material compared to conventional methods and has higher reproducibility (21).

Using this technology, we performed a study comparing protein profiles in serum and tear fluid from a group of breast cancer patients and known healthy controls. To the Authors’ knowledge, no data on protein profiling of tear fluid in breast cancer patients have been reported to date.

Figure 1. SELDI-TOF mass spectra of tear protein profiles. SELDI-TOF-MS pattern of a healthy patient (1-3) and a patient with breast cancer (4-6). The intensity of proteins (U) is plotted versus the molecular mass in a range up to 6,000 Da analyzed with a low laser energy setting.
Figure 2. Detection of the two main tear proteins lysozyme (1) and lipocalin (2) analyzed on the three different array surfaces (H50, Q10, and CM10) at high laser energy.

Figure 3. Heat map of protein expression patterns in tears at each condition. In this graph, the intensity of protein expression is shown in colour values. The x-axis displays the corresponding molecular mass region and the chip surface and the y-axis the different groups (group 1 samples from controls, group 2 samples from cancer patients).
The aim of the study was to determine whether it was possible to protein profile tear and serum specimens using SELDI-TOF-MS and if these protein profiles were possibly altered in the presence of carcinoma of the breast.

**Patients and Methods**

*Patient characteristics.* This prospective study included 20 patients: 10 patients with breast cancer and 10 healthy patients matched for age. Informed consent was obtained from all patients participating in the study and the protocols were approved by the institutional Ethics Committee and conformed to the provisions of the Declaration of Helsinki. Blood samples and tear fluid were drawn prior to surgery. Patients’ blood was obtained 24-48 hours before surgery by peripheral venous puncture and was immediately centrifuged at 4000 xg for 5 min. The serum was frozen at –80°C until examination. Tear fluid was eluted from Schirmer strips. This basal secretry test (BST) to collect the tear fluid was also performed 24-48 hours before surgery and the Schirmer strips were stored at –80°C until use.

*Serum assay.* For preparation and purification of the serum samples, purification kits 100 MB-HIC 8 and 18, and MB-WCX (Bruker Daltonics Inc., Billerica, MA, USA) were used. The 100 MB-HIC 8 and 18 purification kits are based on super-paramagnetic microparticles with a highly porous surface functionalized with hydrophobic coatings.

Ten μl binding solution and 5 μl of the serum sample were mixed with 5 μl magnetic bead suspension by pipetting up and down five times. After 1 min, the beads were separated from the supernatant by a magnetic separator for 20 s. Washing with 100 μl wash solution was performed twice. To elute the peptides and proteins, 5 μl 50% acetonitrile were added and mixed thoroughly before transferring the elution to a fresh tube.

The MB-WCX kit is based on super-paramagnetic microparticles with negatively charged functional groups at their surface enabling cation exchange chromatography. Ten μl binding solution and 10 μl MB-WCX beads suspension were mixed by pipetting up and down before 5 μl serum sample were applied and mixed again. Incubation for 5 min was followed by collecting the supernatant. Washing with 100 μl wash solution, collecting the beads from the tube wall and removal of the supernatant was repeated twice. By adding 5 μl elution solution and pipetting up and down ten times, the beads were dissolved from the tube wall. After incubation for 2 min, the supernatants were transferred to a fresh tube and mixed with 5 μl stabilization solution.

*Tear fluid.* The Schirmer strips were eluted overnight in 500 μl 0.1% dodecylmaltoside and then for two hours in 300 μl 0.1% trifluoroacetic acid (TFA) resulting in two elutions, a TFA elution and a dodecyl elution. For automatic handling of all binding and washing steps with the TFA elution, a robotic laboratory automation station (Biomek 2000; Beckman Coulter, Fullerton, CA, USA) was used. Sodium acetate (pH 4) was used as a washing buffer. The station was extended by an integrated microplate mixer (Micromix 5; Diagnostic Products Company, Los Angeles, CA, USA), which held the ProteinChip Array BioProcessor (Ciphergen biosystems, Inc.). This BioProcessor was equipped with 12 ProteinChip Arrays, each having eight spots. With two BioProcessors, up to 192 wells with an approximate volume of 250 to 300 μl can be handled by the robotic station. The washing steps with the dodecyl eluate were performed without using the Biomek.

*ProteinChip analysis.* The two eluates from the tear fluid and the three eluates from the serum samples were assayed on three different chromatographic surfaces: a week cation exchange surface (CM10), a strong anion exchange surface (Q10) and a reversed-phase surface (HS0). All ProteinChip Arrays were pretreated according to the standard protocols of the manufacturer. Binding buffers were 5% acetonitrile/0.1% trifluoroacetic acid (HS0), 20 mM sodium acetate buffer (pH 5; CM10), and 50 mMTris (pH 8; Q10).

Twenty microliters of the TFA elution and 20 μl of binding buffer were applied to each spot using the Biomek. The arrays were incubated on the DPC shaking platform for 1 hour and the solution in each well was removed by the Biomek. All wells were washed with sodium acetate (pH 4) followed by a wash step with distilled water for 5 min to remove buffer salts. After the wells were dry, the Biomek was used to apply 2 μl saturated sinapinic acid solution (an energy-absorbing molecule) in 50% acetonitrile and 0.5% TFA to each spot. After the spots were air dried, each spot was analyzed in a ProteinChip Reader. Each sample (2 μl per spot) was bound to each array surface in duplicate on separate arrays and BioProcessors.

The dodecyl eluate (2 μl per spot) was applied directly on the ProteinChips. The washing steps with sodium acetate (pH 4) and distilled water were carried out as mentioned above.

The three resulting eluates from the serum samples were also directly applied to the ProteinChips (2 μl eluate and 2 μl matrix per spot).

*Data acquisition and preprocessing.* ProteinChip Arrays were analyzed on a PBS-IIc ProteinChip Reader equipped with a ProteinChip Array AutoLoader using the ProteinChip Software version 3.2 (Ciphergen Biosystems, Inc.). The AutoLoader is able to analyze up to 24 ProteinChip Arrays (192 spots) at one time. Each array was read at two laser intensities: low intensity optimized for low molecular mass proteins and high intensity for high molecular mass proteins. The high-intensity protocol averaged 195 laser shots from each spot with a laser intensity of 190, a deflector setting of 3,000 Da, a detector sensitivity of 9, and a molecular mass detection range of 2,000 to 200,000 Da. For low-intensity measurements, the laser intensity was set at 180 and the deflector set at 1,500 Da. The raw data were transferred to the CiphergenExpress Data Manager Software version 2.1 (CE: Ciphergen Biosystems) for analysis.

*Data analysis.* The CE data manager software was used to normalize the spectra, to automatically detect peaks and to create the peak cluster lists. The peak intensities were normalized according to the total ion current. The cluster lists were exported as ASCII files to a statistical analysis program (Statistica, ver. 6.2; StatSoft, Tulsa, OK, USA). The cluster lists contained normalized peak intensity values for each sample within a group. Based on these normalized peak intensities, probabilities based on t-tests and multivariate discriminant analysis were calculated.

The separation between clinical groups for diagnostic purposes can generally be enhanced by using mult-marker panels of protein biomarkers rather than single biomarkers. The statistical software package (Statistica) was used to perform a multivariate discriminant analysis based on combinations of multiple marker peaks. This
discriminant analysis tests the zero hypothesis, namely that mean biomarker peaks of the different clinical groups derive from a multivariate normally distributed population, and also shows which of the various groups are statistically different. The discriminant analysis selected the eight most important protein biomarkers for best discrimination between the groups.

To assess the diagnostic power of this eight biomarker panel, a neural network was trained using these markers as input neurons. The traditional artificial neural network (ANN) model is the most widely used today and is a multiple-layer feed-forward network (MLFN) with the back-propagation training algorithm (22). In this study, the neural network module provided by the software (Statistica) was used. The network was trained by the eight biomarker peaks and the output neurons defined whether the protein pattern classified the patient as having breast cancer or not. To assess the performance of the network, the data set was randomly divided into two parts: the first half of the patients and controls was used as training set, the second as a test set. After completion of training, the success of the algorithm was tested using the test set, which comprised samples from patients and controls to which the neural network had not previously been exposed.

The software generates a receiver operating characteristic (ROC) curve by plotting sensitivity against 1 – specificity. A test that perfectly discriminates between two groups would yield a curve that coincided with the left and top sides of the plot. A global assessment of the performance of the test (sometimes called diagnostic accuracy) is given by the area under the ROC curve (AUC). A perfect marker with complete group separation would have an ROC AUC value of 1.

Results

Serum and tear samples were analyzed on three different ProteinChip Array chemistries as described. Figure 1 exemplarily shows the SELDI-TOF mass spectra of tear protein profiles at low laser energy. The figure also illustrates the complexity of tear protein profiles in the low molecular mass range and the variability from patient to patient.

In tears, SELDI-TOF-MS succeeded in identifying the main tear proteins, lysozyme and lipocalin, for the three different chip surfaces as shown in Figure 2. Furthermore, Figure 3 shows a heat map of tear eluate, where the protein expressions are shown as pseudocolors for the molecular mass region between 3,000 and 70,000 Da (x-axis) and relative peak intensity (y-axis) for the different groups (CRTL=1 and CA=2) for the various conditions. The heat map provides a more comprehensive overview of the complex protein patterns found on the different array surfaces.

In conclusion, the results shown in Figures 1 to 3 provide evidence that SELDI-TOF-MS ProteinChip Array analysis is very useful for the analyses of tear and serum protein profiles.

The other objective of this study was to search for proteins or peptides that are differentially expressed in healthy women and those with breast cancer. Cluster lists were generated for each condition (low- and high-energy laser settings) and for each surface (CM10, H50, and Q10). For each of these cluster lists, a multivariate discriminant analysis was performed which defined a total of 8 peaks with significant differences between the tear ($p<0.0080$) and serum ($p<0.0023$) protein profiles of control and cancer samples. Figures 4 and 5 show the different protein profiles in serum and tears of control and cancer samples, respectively. Using this biomarker panel for input, an artificial neural network was trained with a training data set. The performance of the trained net was assessed using a test data set. Figures 6 and 7 show the ROC curves of sera and tears with an AUC of 0.95 and 0.90, and a specificity and sensitivity of approximately 90% for each in sera and tears, respectively.

Discussion

The findings of this study appear to satisfy its objectives: it was possible to protein profile tear and serum specimens using mass spectrometry and these protein profiles were somewhat altered in the presence of carcinoma of the breast.

The results of the pilot study suggest that protein profiling using SELDI technology and other mass spectrometry-based approaches could be applied to tear fluid and/or serum biomarker discovery with regard to breast cancer. Based on the evidence provided in the Results section, the investigation suggests that tear fluid did not require special sample preparation as serum samples did.

The technology primarily used to date, namely two-dimensional (2-D) gel electrophoresis, allows analysis and quantification of proteins and the establishment of a protein map. However, this technology is very time consuming and is limited by problems in reproducibility, which is particularly important when it is used in clinical routine with many samples. Furthermore, the sensitivity of 2-D electrophoresis limits the analysis to proteins larger than 8 to 10 kDa (20). In contrast, the SELDI-TOF-MS technology allows rapid sample analysis because very small sample volumes can be directly applied to the ProteinChip Array surfaces and the process can easily be automated for high-throughput analysis (23).

To identify tears and serum biomarkers in this study, comparisons were made between the breast cancer individuals and the healthy controls. We found statistically significant biomarker clusters which discriminated between breast cancer patients and healthy controls in both tear and serum samples. At the present time, we do not know the identity of these ion signals. After validating the biomarker profiles in a larger group of patients, we will identify the proteins in a second step.

For breast cancer, early diagnosis is much more significant than any treatment (24). The main diagnostic measures such as self-examination, clinical examination and mammography do little for patients whose tumors are small and current serum tumor markers such as CA 15-3 and CEA do not have enough sensitivity or specificity for these patients (4).
Figure 4. Protein profiles of serum samples from controls (group 1) and cancer patients (group 2).

Figure 5. Protein profiles of tear samples from controls (group 1) and cancer patients (group 2).
Figure 6. ROC curves of serum samples.

Figure 7. ROC curves of tear samples.
As in other tumor entities, in breast cancer some authors tried to find protein profiles to discriminate between breast cancer patients and healthy controls (5, 6). Serum samples, nipple aspirate and saliva have been used as diagnostic fluid. Besides serum samples, we chose tear fluid because it is easily and noninvasively obtained and the lack of highly abundant proteins obviates the need for purification of the samples. Comparable to the published data (25-30), we found significantly different protein profiles in breast cancer patients and in healthy controls, both in tear fluid and serum. The specificity and the sensitivity were both over 90%.

The results of this study suggest that SELDI-TOF-MS may be a useful tool in the development of the detection of tear and serum biomarkers in breast cancer.

References


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