A Technical Triade for Proteomic Identification and Characterization of Cancer Biomarkers

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ABSTRACT

Biomarkers are needed to elucidate the biological background and to improve the detection of cancer. Therefore, we have analyzed laser-microdissected cryostat sections from head and neck tumors and adjacent mucosa on ProteinChip arrays. Two differentially expressed proteins \(P = 3.34 \times 10^{-8}\) and \(4.6 \times 10^{-7}\) were isolated by two-dimensional gel electrophoresis and identified as S100A8 (calgranulin A) and S100A9 (calgranulin B) by in-gel proteolytic digestion, peptide mapping, tandem mass spectrometry analysis, and immunodepletion assay. The relevance of these single marker proteins was evaluated by immunohistochemistry. Positive tissue areas were reanalyzed on ProteinChip arrays to confirm the identity of these proteins. As a control, a peak with low \(P\) was identified as calcigizarin (S100A11) and characterized in the same way. This technical triade of tissue microdissection, ProteinChip technology, and immunohistochemistry opens up the possibility to find, identify, and characterize tumor relevant biomarkers, which will allow the movement toward the clonal heterogeneity of malignant tumors. Taking this approach, proteins were identified that might be responsible for invasion and metastasis.

INTRODUCTION

Despite enormous efforts, in only a few tumor diseases have relevant markers been established that can be used for early diagnosis or improved therapy in cancer (1, 2). This remains the case, although many new parallel genomic and proteomic techniques have been introduced in the last 5 years. The strategy of how to search for biomarkers therefore has to be reconsidered. One point might be that the in situ situation in tumors is neglected because results from starting material such as serum and nonmicrodissected tissue cannot be traced back to the biological properties or the heterogeneity of the tumor itself. Hence, microdissection, proteomic techniques, and immunohistochemistry (IHC) for the characterization have to be combined in a technical triade.

In this study, the proteomic technique surface-enhanced laser desorption/ionization-mass spectrometry (MS)-based ProteinChip technology has been used (3–5). First described by Hutchens and Yip (6), the technology makes use of affinity surfaces to retain proteins based on their physicochemical characteristics, followed by direct analysis by time of flight-MS. Proteins being retained on chromatographic surfaces can be easily purified from contaminants such as buffer salts or detergents, thus eliminating the need for preseparation techniques, as required with other MS techniques. Furthermore, the low sample requirements of this technique are ideal for small biopsies or microdissected tissue, which are required to produce the homogeneous tissue samples typically used in cancer research (7–10). Microdissected tissue material, free of contaminating and unwanted tissue components, is extremely important for finding reliable biomarkers in cancer diagnosis (11) and in elucidating clonal heterogeneity of tumors. In the case of epithelial tumors, the epithelial cells are separated from all surrounding tissue constituents. In normal tissue, the lining epithelium consists of only one or a few cell rows, whereas in tumor tissues, the boundaries to normal pharyngeal tissue are irregular and therefore can only be isolated with an extremely precise technique such as laser-based microdissection. The compatibility of laser-based microdissection with ProteinChip technology has been shown in a number of small studies (4, 8, 9, 12), but until now, only very few studies with a statistically relevant number of cases have been performed (10, 13).

When specific changes between the protein profile of microdissected tumor and normal pharyngeal epithelium tissue are found by ProteinChip technology, single peaks can be isolated and identified. Isolation and identification can be performed by either two-dimensional electrophoresis (14) or ProteinChip technology (15), where the isolated protein is digested by proteolytic enzyme cleavage, and the mass values of the fragments generated are used for peptide mapping to identify the protein of interest by a database search. Furthermore, for confirmatory identification, selected peptides can be sequenced by collision-induced dissociation using a ProteinChip Interface coupled to a tandem mass spectrometer (16, 17). Although successful identifications of surface-enhanced laser desorption/ionization-detected protein markers are frequently reported for other biological samples (18–21), those from microdissected materials are very rare (10). After identification, IHC with a specific antibody opens up the possibility to determine tissue distribution and localization of the identified proteins. By locating expression to specific tissue areas, insight into clonal heterogeneity and functional differentiation of the tumor can be obtained.

In the study presented here, pure microdissected populations of normal pharyngeal epithelium and tumor squamous epithelial cells were analyzed using ProteinChip arrays. The two differentially expressed peaks with the best \(P\) values, along with a control peak showing a low \(P\), were identified using two-dimensional electrophoresis and in-gel digestion, peptide mapping, and tandem MS. The assumption that these proteins are identical to the differentially expressed peaks found by ProteinChip analysis was confirmed with an immunodepletion assay. The localization of these proteins in tissue was subsequently verified on cryostat sections of the squamous cell carcinomas of the head and neck (HNSCC) by IHC, using the corresponding monoclonal antibodies or antisera, respectively. Positive tissue areas were microdissected in corresponding serial unstained tissue sections and reanalyzed using ProteinChip arrays to show that these proteins are matching to the differentially expressed peaks found in the prior analysis. Thereby, the relevance of statistically significant proteins could be traced back to the in situ situation in the tumor.

MATERIALS AND METHODS

Laser Microdissection of Tissue Sections. All 57 head and neck tumor samples and matched normal mucosa \(n = 44\) were obtained after surgical
resection at the ENT Department of the Friedrich-Schiller-University (Jena, Germany); these were collected fresh, snap-frozen in liquid nitrogen, and stored at −80°C. Tumor specimens were categorized according their Union International Contre Cancer-Tumor-Node-Metastasis classification. All were classified as squamous cell carcinoma G2, M0.

Microdissection was performed with a laser microdissection and pressure-catapulting microscope (Palm, Bernried, Germany) as described elsewhere (10). In brief, we microdissected on native air-dried cryostat tissue sections ~3000–5000 cells each in a maximum of 20–30 min. Proteins were extracted by a lysis buffer [100 mM sodium phosphate (pH 7.5), 5 mM EDTA, 2 mM MgCl2, 3 mM 2-β-mercaptoethanol, 0.1% 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, 500 μM leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride] for 30 min on ice. After centrifugation (15 min; 15,000 rpm), the supernatant was immediately analyzed or frozen in liquid nitrogen for a maximum of 1 day.

### Profiling of Microdissected Normal Pharyngeal Epithelium and Tumor Tissue

The protein lysates from both microdissected tissues were analyzed on a strong anion exchange array (SAX2; Ciphergen Biosystems, Inc., Fremont, CA) as described elsewhere (10). In brief, array spots were preincubated by a washing/loading buffer containing 100 mM Tris-buffer (pH 8.5), with 0.05% Triton X-100 followed by an application of 2 μl of sample extract on ProteinChip arrays, which were incubated at room temperature for 90 min in a humidity chamber. After washing twice and application of 2×0.5 μl sinapinic acid (saturated solution in 0.5% trifluoroacetic acid/50% acetonitrile), mass analysis was performed in a ProteinChip Reader (model PBS II; Ciphergen Biosystems, Inc.) according to an automated data collection protocol. Cluster analysis of the detected signals and the determination of the respective P values for normal and tumor tissue were carried out with the Biomarker Wizard Program (Version 3.0; Ciphergen Biosystems, Inc.). For P calculation, spectra with at least 10 signals in the range between 2 and 20 kDa displaying a signal-to-noise ratio of at least 5 were selected and analyzed with the Mann-Whitney U test for nonparametric data sets.

### Identification of Differential-Expressed Protein Peaks

Samples for two-dimensional electrophoresis were prepared directly from surgical material of ProteinChip System-analyzed HNSCC and corresponding normal tissue. Proteins were isolated and two-dimensional electrophoresis was performed as described elsewhere (10). In brief, isoelectric focusing was carried out on a PROTEAN IEF Cell (Bio-Rad) using 17-cm immobilized pH gradient strips. Vertical SDS-PAGE was performed in a cooled PROTEAN II Multi Cell (Bio-Rad) using linear gradient gels with total acrylamide concentrations ranging from 7 to 20%. Analytical gels were silver stained using the Vorum protocol (22), and semipreparative gels were stained with Coomassie brilliant blue G-250.

Protein patterns of the two-dimensional gels from normal pharyngeal epithelium and tumor tissue were compared, and consistent differentially expressed proteins with a size of ~5–20 kDa were excised. In-gel digestion of proteins was performed as described elsewhere (10). In brief, excised gel pieces were destained and dried. After rehydration and digestion with 10 μl of a trypsin solution (0.04 μg/μl; Roche) at 37°C for 7 h, supernatants were applied directly on a ProteinChip array with a hydrophobic surface (H4; Ciphergen Biosystems, Inc.). After digestion, peptides were analyzed using the PBS II instrument. The spectra for the peptide mapping experiments were internally calibrated using three common trypsin autolysis products. Proteins were identified using the fragment masses generated through trypsin digestion by searching in a publicly available database.

The criteria for positive identification of proteins were as follows: (a) probability index should be >1.0e-0000; (b) Z score for the protein should be >2; and (c) molecular weight and isoelectric point of identified proteins should match estimated values obtained from two-dimensional gel electrophoresis.

### RESULTS

#### Profiling of Microdissected Normal Pharyngeal Epithelium and Tumor Tissue

For this study, areas corresponding to ~3000–5000 cells/tissue probe were excised, and 101 tissue sections (57 tumor and 44 normal pharyngeal epithelium tissues) were successfully dissected by a pathologist. All protein lysates from the microdissected tissues were applied to SAX2 arrays and analyzed on a PBS II instrument. In the low range (2–20 kDa), up to 96 peaks were detected with normalized intensities.

After evaluation with Biomarker Wizard Program, the peak masses with the two best P values and down-regulated in epithelial tumor tissue, 10.84 kDa (P = 3.34 × 10−5) and 13.23 kDa (P = 4.6 × 10−5), were selected for additional characterization and identification. As a control, one peak (11.78 kDa) with no significant P (P = 0.379) was investigated in parallel. First, we used the TagIdent tool from ExPaSy by entering the mass of these unknown proteins. This tool searches for proteins similar in size and in isoelectric point in the SWISS-Prot and TrEMBL Translation of EMBL (European Molecular Biology Laboratory) databases, which can give some indication about possible candidates. The latter identified calgranulin A (accession number P05109), calgranulin B (accession number P06702), and calgizzarin (accession number P31949) were among the proteins listed.

#### Identification of Differential-Expressed Protein Peaks

Historically assessed tumor pieces and biopsies from normal tissue were subjected to two-dimensional electrophoresis to identify the detected peaks at 10.84, 13.23, and 11.78 kDa. Numerous protein spots showed...
ing differential expression in both specimens were observed. Because of the binding of the unknown protein species to a strong anion exchange surface at pH 8.5 in our ProteinChip analysis, we expected the isoelectric point of this protein candidate to be ≤8.5. We therefore decided to concentrate on 19 spots in range of 5–20 kDa exhibiting an isoelectric point in the range of 4.5–7 in our two-dimensional electrophoresis. Selected spots were cut out from the second dimension gels and subsequently subjected to in-gel digestion with trypsin and protein identification. An empty gel piece underwent the same treatment as a control. The digest solution was spotted on a hydrophobic H4 array and the masses of the fragments determined by the PBS II instrument. Database searches revealed calgranulin A, calgranulin B, and calgizzarin with high Z-score (2.34, 2.37, and 2.31, respectively) and good sequence coverage (Profound)6 as the best candidates (Table 1).

These results were further confirmed by tandem MS analysis. The H4 array with the tryptic digests was transferred to a tandem MS equipped with a surface-enhanced laser desorption/ionization Protein-Chip Interface. The peptides generated were selected and fragmented into smaller ions by collision-induced dissociation. Sequences of the peptides are given in Table 2. These results confirmed the identification of the proteins as calgranulin A, calgranulin B, and calgizzarin.

To confirm that calgranulin A, calgranulin B, and calgizzarin are matching to the differentially expressed peaks at 10.84, 13.23, and 11.78 kDa found by ProteinChip analysis, an immunodepletion assay was performed with microdissected tumor and normal pharyngeal epithelium tissue. Analysis of the supernatant of the immunodepletion assay by ProteinChip arrays showed that the peaks corresponding to calgranulin A, calgranulin B, and calgizzarin were significantly reduced. In the negative control (immunodepletion process with no antibody), the corresponding peaks were clearly detectable (Fig. 1).
Calgranulin A and calgranulin B showed an identical reactivity in tissue, with a strong immune reactivity in the normal epithelium, except in the basal and parabasal cells. In tumor tissue, no expression could be detected for either protein (Fig. 2, A and C). Normal and tumor tissue components such as collagenic fibers, fibrocytes, fibroblasts, and macrophages were positive, whereas glandular ductal cells and endothelial cells were negative for calgranulin A/B. Calgizzarin showed a positive immune reaction with all layers of normal and tumor epithelium (Fig. 2, B and D). In contrast to calgranulin A/B, endothelial cells and glandular ductal cells were positive. Table 3 summarizes all immunohistological results for calgranulin A/B and calgizzarin.

To ensure that the localized calgranulin A, calgranulin B, and calgizzarin are identical to the peaks found by ProteinChip analysis, IHC-positive and -negative cell areas were obtained by tissue laser microdissection. In protein lysates from the positive fraction, a signal identical in mass to the peak obtained with the initial ProteinChip analysis was detected. In the protein lysate from the negative fraction, this peak was not visible (Fig. 1).

**DISCUSSION**

New biomarkers or biomarker patterns found by genomic or proteomic high-throughput techniques will enable scientists and medical staff to make a more reliable early diagnosis of certain human diseases, especially malignant tumors, and facilitate the prediction of their progression. In this way, biomarkers may contribute to a more differentiated, individually oriented tumor therapy. Despite enormous efforts, until now only in a few tumor disease relevant markers have been established (1).

One of the most promising proteomic tools for the detection of new proteomic cancer biomarkers is Ciphergen’s ProteinChip technology (for examples, see Refs. 25, 26). Until now, this technique has been predominantly used for body fluid analyses because they are fast and easy to analyze by direct application onto ProteinChip arrays. Nevertheless, it is known that inter- and intraindividual changes in serum depending on sex, hormone level, nutrition state, or inflammation are high and can change the protein profile drastically. Hence, biomarkers involved in the genesis and progression of cancer must be present at a high level to be observed above normal changes. Despite these concerns, a large number of studies using body fluids as starting material have been published [serum (25, 27); urine (28); nipple

**Table 3** Protein expression of calgranulin A, calgranulin B, and calgizzarin in different normal and tumor tissue components

<table>
<thead>
<tr>
<th>Component</th>
<th>Calgranulin A, B</th>
<th>Calgizzarin</th>
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<tbody>
<tr>
<td>Normal epithelium</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Basal layer</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Parabasal layers</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Superficial layer</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Epithelial tumor cells</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Other tissue or hematological components</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagenic fibers</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fibrocytes/fibroblasts</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glandular ductal cells</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Macrophages</td>
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<td>+</td>
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<td>Leukocytes</td>
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<td>Endothelial cells</td>
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aspirate fluid (29); and pancreatic juice (26)]. However, if after bioinformatic processing markers can be found, they would be ideal for screening high-risk individuals or even individuals without elevated risk, which is discussed by the latest study on ovarian cancer (30) or others (18, 19, 21).

In contrast to serum, the analysis of tissues is more time consuming because here microdissection is necessary to separate tumors from healthy cells, although the chance to find a reliable tumor marker might be higher than in serum. There is certainly a higher chance of obtaining more information about the biological mechanisms leading to the genesis and progression of cancer. Studies using tissue as a starting material have been underrepresented until now, and in most cases, a low number of samples were analyzed, which might be because even laser-based microdissection is tedious and has to be done by an experienced pathologist. To date, prostate cancer (4, 12, 31), melanomas (8, 32), lung tumors (13), renal cell carcinomas (9, 32), and HNSCC (10) have been assessed in this way.

After a significant protein has been detected by profiling experiments with ProteinChip arrays, two questions have to be addressed: first, how the protein can be enriched and identified, and second, whether this identified protein can be found and localized in the starting tissue. Localization may give insight to the heterogeneity of tissue and the tumor itself.

In our study, we addressed the first question by detecting differentially expressed proteins in microdissected tissue using ProteinChip technology and subsequent enrichment and identification of the proteins of interest by two-dimensional electrophoresis, in-gel digestion, peptide mapping, tandem MS, and immunodepletion assay. After profiling, the obtained masses of proteins were used for database searches, which gave some indication about possible candidates. Two-dimensional electrophoresis offers the opportunity to enrich and isolate putative candidates and to digest them with trypsin. The generated peptides could then be analyzed on the PBS II ProteinChip Reader and database searches pointed with a high probability to calgranulin A, calgranulin B and calgizzarin. To confirm that the isolated and digested proteins are identical to the differentially expressed peaks found with ProteinChip arrays, we performed an immunodepletion assay with the same starting material and corresponding antibodies. The respective peaks were absent in the analyzed supernatant and must therefore be depleted by the antibody. The identification was further confirmed by tandem MS analysis of selected peptides from the digest. The second question about the localization of calgranulin A, calgranulin B, and calgizzarin in tissue was then addressed by IHC. These proteins could be found in different normal and tumor tissue components. The reanalysis of calgranulin A-, calgranulin B-, and calgizzarin-positive and -negative tissue areas by microdissection and profiling confirms moreover their identities to the differentially expressed peaks. This process enabled the tissue heterogeneity of samples to be partly solved by laser microdissection, dividing epithelial tissue from connective tissue. The clonal heterogeneity of the tumor itself concerning the transcriptome and the proteome is morphologically hard to recognize and therefore cannot be completely solved by microdissection, unless by repeated cycles of microdissection, protein profiling, and immunohistochemical analyses with different antibodies.

In contrast to publications that show the protein profiles of specific tissues exclusively without an identification and characterization (12, 25, 30), we were able to identify significant signals in protein profiles from microdissected tissues. Calgranulin A, calgranulin B, and calgizzarin belong to the group of S100 proteins involved in the Ca\(^{2+}\) signaling network and regulate intracellular activities such as cell growth and motility, cell cycle progression, transcription, and cell differentiation (33, 34). This group of proteins has received increased attention because of their involvement in several human diseases such as rheumatoid arthritis, acute inflammatory lesions, cardiomyopathy, Alzheimer’s disease, and cancer (35–38). It is unique that the individual members of S100 proteins are located in specific cellular compartments from which they are able to relocate upon Ca\(^{2+}\) activation, transducing the Ca\(^{2+}\) signal in a temporal and spatial manner by interacting with different targets specific for each S100 protein (39). Another important aspect exclusive to the S100 protein family is that most genes of the members are located in a gene cluster on human chromosomal region 1q21 (40).

This region is characterized by several rearrangements that occurred during tumor development (41). This circumstance might be linked to the deregulation of some S100 gene expression in various tumor types and might be associated with tumor development and metastasis (33).

The proteins identified here have been described earlier in gene expression studies, e.g., in breast carcinoma (42), in murine epitelial skin tumors (43), and in a mouse model of a gastric B-cell mucosa-associated lymphoid tissue lymphoma (44) with increased gene expression of S100A8 (MRP8; calgranulin A), S100A9 (MRP14; calgranulin B), and S100A11 (S100C; calgizzarin), respectively. Also, protein expression studies have detected an increased level of these S100 proteins in different tumor tissues compared with their corresponding abundance in normal tissues (45–47). These observations are only consistent with our nonsignificant results concerning S100A11, calgizzarin. In our study, we detected signals of S100A11 by IHC that were distributed in both tissue types with a slightly stronger signal in HNSCC than in normal tissues.

On the other hand, our findings concerning the expression of the S100A8 and S100A9 proteins are contrary to the previously published studies. We estimate that a reason for the discrepancy to the other studies might be the fact that (a) our analysis is based on different tumor entities, (b) the level of mRNA does not necessarily correlate with protein expression level because of translational control mechanisms, and (c) we used microdissection and reanalysis of the found proteins by IHC to confirm our data. Only one recently published gene expression study on head and neck tumor used IHC for the confirmation of the results. Interestingly, these authors also found a decrease of the expression of the S100A9 gene (48).

In conclusion, it can be stated that a better estimation of the biological importance of certain cell populations and tumor clonal
REFERENCES


17. Zhang L, Yu W, He T, et al. Contribution of human alpha defensin-1, -2 and -3 to the understanding of tumor progression. The paradigmatic triade of miRNA, protein and metabolite: forming potential of a given cell population by biomarkers will be necessary prerequisites for providing a more detailed insight and understanding of tumor progression. The paradigmatic triade of microdissection, surface-enhanced laser desorption/ionization-based ProteinChip technology, and IHC (Fig. 3) opens up this possibility while reducing the complexity of the proteome by using a defined cell population.

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REFERENCES


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