Biomarker Discovery and Identification in Laser Microdissected Head and Neck Squamous Cell Carcinoma with ProteinChip® Technology, Two-dimensional Gel Electrophoresis, Tandem Mass Spectrometry, and Immunohistochemistry*

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Head and neck cancer is a frequent malignancy with a complex, and up to now not clear etiology. Therefore, despite of improvements in diagnosis and therapy, the survival rate with head and neck squamous-cell carcinomas is poor. For a better understanding of the molecular mechanisms behind the process of tumorigenesis and tumor progression, we have analyzed changes of protein expression between microdissected normal pharyngeal epithelium and tumor tissue by ProteinChip® technology. For this, cryostat sections from head and neck tumors (n = 57) and adjacent mucosa (n = 44) were laser-microdissected and analyzed on ProteinChip arrays. The derived mass spectrometry profiles exhibited numerous statistical differences. One peak significantly higher expressed in the tumor (p = 0.000029) was isolated by two-dimensional gel electrophoresis and identified as annexin V by in-gel proteolytic digestion, peptide mapping, tandem mass spectrometry analysis, and immuno-deplete assay. The relevance of this single marker protein was further evaluated by immunohistochemistry. Annexin-positive tissue areas were re-analyzed on ProteinChip arrays to confirm the identity of this protein. In this study, we could show that biomarker in head and neck cancer can be found, identified, and assessed by combination of ProteinChip technology, two-dimensional gel electrophoresis, and immunohistochemistry. In our experience, however, such studies only make sense if a relatively pure microdissected tumor tissue is used. Only then minute changes in protein expression between normal pharyngeal epithelium and tumor tissue can be detected, and it will become possible to educe a tumor-associated protein pattern that might be used as a marker for tumorigenesis and progression. Molecular & Cellular Proteomics 2: 443–452, 2003.

Head and neck cancer, mainly squamous cell carcinoma of the oral cavity, pharynx, and larynx, is a common human malignancy that affects ~500,000 patients per year worldwide (1). Despite the improvements in surgical techniques and chemo- and radiotherapies (2), the overall 5-year survival rate for patients with head and neck cancer is among the lowest of the major tumor types (3). The lack of progress in head and neck oncology emphasizes the search for molecular markers associated with the initiation and biological behavior of an individual tumor with the aim to benefit the patients with diagnostic and prognostic information regarding clinical course and outcome.

Head and neck squamous-cell carcinoma (HNSCCs) are thought to progress through a series of genetic alterations (4). These single DNA alterations (e.g. loss of heterozygosity and microsatellite instability (5), p16 (6), p53 (7, 8), telomerase reactivation (9–12), and Stat3 (13)) were investigated in detail in the last 10 years for a better understanding of molecular processes, but with low benefit to a better diagnosis or therapy (14). Therefore, highly parallel genomic and proteomic techniques now have to be applied to give a more detailed understanding of the molecular processes involved in head and neck cancer.

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insight into the complex genesis and progression of HNSCC as well as to identify new cancer-specific signatures.

Such an arising technique is the surface-enhanced laser desorption/ionization (SELDI) mass spectrometry (MS)-based ProteinChip technology (15–17). First described by Hutchens and Yip (18), it utilizes affinity surfaces to retain proteins based on their physico-chemical characteristics, followed by direct analysis by time of flight (TOF)-MS. Proteins being retained on a chromatographic surface can be easily purified from contaminants such as buffer salts or detergents prior to MS analysis, thus eliminating the need for preseparation techniques. Furthermore, the low sample requirements of this technique are ideal for small biopsies or microdissected tissue used to produce homogenous tissue samples in cancer research (19–21). Today, it is indisputable that microdissected tissue material, free of contaminating, unwanted tissue components, is extremely important to find reliable biomarkers for cancer diagnosis (22). In the case of epithelial tumors, the epithelial cells have to be separated from all surrounding tissue constituents. Because in normal tissue the lining epithelium consists only of one or few cell rows, and in tumor tissues the boundaries to normal pharyngeal tissue are irregular, this can only be done with an extremely precise technique such as laser-based microdissection. Its compatibility with ProteinChip technology has been shown in different small studies (16, 20, 21, 23). Up to now no studies with a statistically relevant number of cases have been performed, nor were differentially expressed proteins identified after microdissection.

When specific alterations between the protein profile of microdissected tumor and normal pharyngeal epithelium tissue are found by ProteinChip technology, single peaks can be isolated and identified. For the isolation, different techniques are in use. As well as the two-dimensional gel electrophoresis (2-DE) (24) applied in this study, the ProteinChip system can also be used for the on-spot development of purification protocols and the monitoring of the subsequent purification process (25). In either case, the isolated protein is digested by enzymatic cleavage. Subsequently, the mass values of the generated fragments are used for peptide mapping to identify the protein of interest by data base searches. Furthermore, for unambiguous identification selected peptides can be sequenced by collision-induced dissociation (CID) using a ProteinChip interface coupled to a tandem mass spectrometer (26, 27).

In the study presented here, pure microdissected populations of normal pharyngeal epithelium and tumor squamous epithelial cells were analyzed on ProteinChip arrays. One differentially expressed peak showed a calculated mass (m/z value) of 35.90 kDa. Protein extracts of the same specimens were prepared and analyzed by 2-DE. Differentially expressed spots showing an appropriate mass were cut out of the second dimension gels and identified by in-gel digestion, peptide mapping, and tandem mass spectrometry (MS/MS) as human annexin V. The assumption that annexin V is identical to the differentially expressed peak found by ProteinChip analysis was confirmed with an immuno-deplete assay. The localization of annexin V in tissue was subsequently verified on cryostat sections of the HNSCCs by immunohistochemistry using a monoclonal anti-annexin V antibody. Positive tissue areas were microdissected in corresponding unstained sections and re-analyzed on ProteinChip arrays to show that annexin is matching to the differentially expressed peak found in the prior analysis.

Therefore, as shown for the first time on a high number of cases, combinatorial use of laser microdissection of tumorous tissue, ProteinChip technology, and immunohistochemistry show promise to find, isolate, and identify cancer-associated markers.

**EXPERIMENTAL PROCEDURES**

*Patients and Specimens—* 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; they had been collected fresh, snap frozen in liquid nitrogen, and were stored at −80°C. Tumor specimens were categorized according their International Union Against Cancer tumor nodes metastasis classification. All were classified as squamous cell carcinoma M0, G2.

*Laser Microdissection of Tissue Sections—* For the microdissection procedure, native air-dried cryostat tissue sections (8 μm thickness) on microscope slides coated with a 1.35-μm membrane (polyethylene naphthalate; Palm, Bernried, Germany) and a laser microdissection and pressure catapulting microscope (LMPC; Palm) were used. Tumor cell areas and normal pharyngeal epithelium were located in comparison with a hematoxylin and eosin (HE)-stained serial section, marked and cut out by a pathologist (Fig. 1). Stained sections revealed no or poor protein profile (data not shown).

We microdissected approximately 10 tissue areas containing several hundred cells each per sample. When tissue areas of this size were too large to transfer by laser pressure catapulting in a proper time, we manually transferred these samples by lancet needle into a

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**Fig. 1.** HE-stained section of a head and neck tumor sample. The white line demonstrates the course of the laser cuts during microdissection of normal pharyngeal epithelium (A) and tumor cell complexes (B).
tube. In this way samples that contained about 3000 to 5000 cells each were collected in at most 20 min. In the tube, proteins were extracted by a lysis buffer (100 mM sodium-phosphate (pH 7.5), 5 mM EDTA, 2 mM MgCl$_2$, 3 mM 2-β-mercaptoethanol, 0.1% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid (CHAPS), 500 μM leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)) for 50 min on ice. After centrifugation for 15 min at 15,000 rpm, supernatant was immediately analyzed or frozen in liquid nitrogen for a maximum of 1 day.

ProteinChip Array Preparation and Analysis—The protein lysates from microdissected HNSCC tissue were analyzed on a strong anionic exchanger array (SAX2; Ciphergen Biosystems, Fremont, CA). First, sample targets on the arrays were equilibrated three times by applying 5 μl binding/washing buffer (100 mM Tris buffer, pH 8.5, containing 0.05% Triton X-100) for 20 min, respectively. After equilibration, the buffer was removed and 5 μl fresh buffer was added to each spot. Into this, 2 μl of sample extract was spiked and the ProteinChip array was incubated in a humidity chamber for 90 min at 20 °C. Afterward, sample droplets were removed and each target was washed three times with 5 μl binding/washing buffer. The targets were then washed two times with 5 μl water to remove the buffer salts and subsequently dried on air. After application of 2 × 0.5 μl saturated sinapinic acid (Sigma) dissolved in 50% acetonitrile (Sigma) containing 0.5% trifluoroacetic acid (Sigma), mass analysis was performed in a ProteinChip Reader (PBS-II; Ciphergen Biosystems) according to an automated data collection protocol. This includes an average of 195 laser shots to each spot with a laser intensity of 200 and 270, respectively, dependent on measured region (low = 2–20 kDa and high = 20–200 kDa, respectively). The detector was run at a sensitivity of 9. Normalization of all spectra was performed using total ion count. Cluster analysis of the detected signals and the determination of respective p values were carried out with the Biomarker Wizard Program (Version 3.0; Ciphergen Biosystems). For p value calculation, spectra with at least 10 signals in the range between 2 kDa and 20 kDa exhibiting a signal-to-noise ratio of at least 5 were selected and analyzed with the Mann-Whitney U test for non-parametric data sets.

Two-dimensional Gel Electrophoresis—Samples for 2-DE were prepared directly from surgical material of ProteinChip system-analyzed HNSCC and corresponding normal tissue. A piece of tissue (about 3 mm$^3$) was minced with a lancet and was then homogenized in liquid nitrogen with a mortar. The obtained powder was dissolved in ice-cold lysis buffer (100 mM sodium-phosphate, pH 7.5, 5 mM EDTA, 2 mM MgCl$_2$, 3 mM 2-β-mercaptoethanol, 0.1% CHAPS, 500 μM leupeptin, and 0.1 mM PMSF) and was further mechanically homogenized by means in a douncer (Roth, Karlsruhe, Germany). Tissue homogenate was centrifuged (15 min at 15,000 rpm), and protein concentrations in the supernatant were measured using the Coomassie brilliant blue G-250.

In-gel Digestion—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 ~35 kDa were excised manually by a scalpel and transferred into a 1.5-ml reaction tube. Excised gel pieces were treated twice with 400 μl of 50% methanol containing 10% acetic acid and agitation for 45 min; followed by incubation in 400 μl of a buffer containing 100 mM ammonium bicarbonate (pH 8.0) with agitation for 30 min; followed by incubation in 400 μl of 50% acetonitrile containing 100 mM ammonium bicarbonate with agitation for 1 h and finally by incubation in 50 μl 100% acetonitrile with agitation for 15 min. Afterward, gel pieces were centrifuged in a SpeedVac (Savant) to complete dryness. For rehydration and digestion, individual gel pieces were covered with 10 μl of a trypsin solution (0.04 μg/μl; Roche) for 10 min followed by the addition of up to 20 μl 25 mM ammonium bicarbonate and incubated at 37 °C for 7 h. Digested samples were centrifuged for 1 min at 13,000 rpm, and 3 μl of the supernatants were applied directly on spots of a ProteinChip array with a hydrophobic surface (H4; Ciphergen Biosystems), which was preactivated with 30% acetonitrile containing 100 mM NaCl. After samples were dried, spots were covered twice with 0.5 μl of 20% α-cyano-4-hydroxy cinnamic acid (Ciphergen Biosystems) in 50% acetonitrile containing 0.5% trifluoroacetic acid. Peptide fragment masses were analyzed with an average of 110 laser shots to each spot with a laser intensity of 180 and a sensitivity of 95% percentile; 3) molecular mass and isoelectric point of identified proteins should match estimated values obtained from 2-DE.

Tandem Mass Spectrometry—MS/MS data were acquired on a Micromass QTOF™ II (Manchester, UK) tandem quadrupole-TOF mass spectrometer equipped with a Ciphergen PCI 1000 ProteinChip array interface. Ions were created using a pulsed nitrogen laser operated at 50 pulses per second. Nitrogen gas, at 18 mtorr, was used for collisional cooling of formed ions, and argon gas was used for all low-energy CID experiments. Applied collision energy usually followed the rule of 50eV/kDa. The system was externally calibrated in MS/MS mode using the parent ion and selected fragments of ACTH human fragment 18–39 (m/z = 2465.1933). MS/MS spectra were exported as Sequest files and used for data base searches with Mascot (www.matrixscience.com) using NCBI and SwissProt data bases.

Immuno-deplete Assay—Two microliters of anti-human annexin V
Monoclonal antibody (Vac-α<sub>V</sub>; Bender MedSystems, Vienna, Austria) were incubated with 10 μl protein A-agarose (Sigma) for 15 min on ice. A pellet was generated by centrifugation, and the supernatant was discarded. The pellet was washed twice with a buffer containing 20 mM HEPES, pH 7.8, 25 mM KCl, 5 mM MgCl<sub>2</sub>, 0.1 mM EDTA, and 0.05% Nonidet P-40. Afterward, 5 μl of a lysate from laser-dissected tumor were incubated with this pellet for 45 min on ice. As a negative control, 5 μl of the lysate were incubated with protein A-agarose without antibody for 45 min on ice. After incubation, samples were cleared by centrifugation, and 3 μl of each supernatant were analyzed by ProteinChip arrays.

**Immunohistochemistry**—Eight micrometers cryostat sections of frozen head and neck cancer tissue containing both normal pharyngeal epithelium as well as and head and neck cancer cells placed on charged slides, dried for ~60 min at 20 °C followed by fixation in 2% paraformaldehyde containing 0.1 M cacodylate buffer, pH 7.2 (Fluka) for 25 min at 20 °C. After fixation slides were rinsed twice with aqua dest and then treated with 10% methanol in Tris-buffered saline (TBS) containing 1% H<sub>2</sub>O<sub>2</sub> for 30 min to inhibit endogenous peroxidatic activity. They were subsequently rinsed twice in TBS, and incubated with monoclonal antibody anti-annexin V (Vac-α<sub>V</sub>; Bender MedSystems), diluted 1:500 in 10% fish gelatin in TBS containing 10% Triton X-100 overnight. Slides were rinsed 3× 10 min in TBS and the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) and 3,3'-diaminobenzidine. Jenchrom pxbl-kit (MoBiTec, Göttingen, Germany) was used according manufacturer’s instructions to visualize antibody localization. Negative controls were incubated only with the labeled secondary antibody provided in the ABC kit. Sections cut in parallel to the immunohistochemistry (IHC)-treated sections were stained by HE for identification of different tissue areas. IHC staining was evaluated by a pathologist and an anatomist.

**Laser Scanning Microscopy**—The laser scanning microscopy was performed with a LSM 310 (Carl Zeiss, Oberkochen-Jena, Germany), preferably in the transmission mode using an argon ion laser at 488 nm wavelength. In most cases, Zeiss objective Plan-NEO FLUAR 40×/1.30 Oil was employed at a scanning time of ~60 s. The nonprocessed gray values of the typical transmission images were converted to inverted values and then further processed to obtain red-green-blue images out of 3× 28 color values. The chosen false color table was stored as a LUT (Look Up Table) and finally saved as a TIFF file (30).

RESULTS

**LMPC Procedure**—The LMPC microscope (Palm) used here allows a real dissection of defined tissue areas without heating and denaturing proteins. The membrane between the section and slide did not influence the protein profile (data not shown) and it was helpful for better handling of the laser-cut section. Due to the small size, and to avoid the impact caused by pressure catapulating, it was preferable to manually transfer the laser-excised areas of tissue slices into test tubes with a lancet needle. By analyzing a tissue lysate dilution series, we defined the minimal required quantity of tissue that still generates satisfying results by SELDI analysis as about 700 cells (data not shown). Nevertheless, for this study areas corresponding altogether to about 3000 to 5000 cells were excised. In this way, all 101 tissue sections (57 tumor and 44 normal pharyngeal epithelium tissues) could be successfully dissected by a pathologist.

**Profiling of Microdissected Normal Pharyngeal Epithelium and Tumor Tissue**—All protein lysates from the microdissected tissues were applied to strong anionic exchanger arrays and analyzed on a PBS-II system. The arrays were run with two different laser intensities to achieve better resolution for low (2–20 kDa) and high molecular mass (20–100 kDa) proteins. In the low range up to 96 peaks and in high range up to 57 protein peaks (signal-to-noise = 3) were detected with normalized intensities. In the high range 47 tumor and 41 normal tissue lysate resulted in profiles that could be evaluated. For p<sub>p</sub> value calculation, 21 tumor and 22 normal samples were selected according to the criteria given above. An in-tumor up-regulated signal at 35.9 kDa was one out of four peaks with extremely low p values of < 0.0001. This one was selected for further characterization and identification (Fig. 2). First, we used the TagIdent tool from ExPASsy (www.ex-pasy.ch/tools/tagident) by entering the mass of this unknown protein. This tool searches for proteins similar in size and in isoelectric point in the SWISS-Prot and TrEMBL data bases, which can give some indication about possible candidates. The highest score yielded annexin V (accession number P08758).

**Two-dimensional Gel Electrophoresis**—Histologically checked tumor pieces and biopsies from normal tissue were subjected to 2-DE. Numerous protein spots showing differential expression in both specimens were observed. Due to the binding of the unknown protein species to a strong anion exchanger surface at pH 8.5 in our ProteinChip analysis, we expected the isoelectric point of this protein candidate to be below 8.5. We therefore decided to concentrate on 27 spots in range of 25–40 kDa exhibiting a isoelectric point of 4–6.5 in our 2-DE. Selected spots were cut out from the second dimension gels and were subsequently subjected to in-gel digestion and protein identification. In this analysis, 3 spots were successfully identified. One of the identified spots was annexin V (Fig. 3).
In-gel Digestion and Protein Identification—The cut out spots were in-gel digested with trypsin. An empty gel piece underwent the same treatment as a control. The digest solution was spotted on a hydrophobic H4 array, and the size of obtained fragments was determined by the PBS II instrument. Data base searches revealed annexin V with high Z-score (2.42) (Profound; 129.85.19.192/profound_bin/WebProFound.exe) as the best candidate, exhibiting a good sequence coverage of 58% corresponding to 14 matching peptides (Table I). The calculated mass of human annexin V is 35980 Da (Profound) and thus slightly higher (0.25%) than the average mass of about 35900 Da found in the profiling experiments. The result was further confirmed by MS/MS analysis. The H4 array with the tryptic digests was transferred to a MS/MS equipped with a ProteinChip interface. Fig. 4A shows an overview of the MS spectrum. Four of the peptides were selected and fragmented into smaller ions by CID. The sequence of the four peptides is given in Table II. These results confirmed the identification of the protein as annexin V (Fig. 4B).

Immuno-deplete Assay—The reassurance that annexin V is matching to the differentially expressed peak at 35.90 kDa found by ProteinChip analysis was done with an immuno-deplete assay using also microdissected tumor and normal pharyngeal epithelium tissue as starting material. Analysis of the supernatant of the immuno-deplete assay by ProteinChip arrays showed that the peak at 35.90 kDa was absent. In the negative control without antibody, the peak at 35.90 kDa was clearly detectable (Fig. 5).

TABLE I

| Measured mass (Da) | Computed mass (Da) | Residues | Peptide sequence |
|-------------------|-------------------|----------|-----------------|
| 954.4             | 954.137           | 194–201  | FITIFGTR        |
| 1106.7            | 1106.244          | 277–285  | SEIDLFNIR       |
| 1234.9            | 1234.418          | 277–286  | SEIDLFNIRK      |
| 1274.8            | 1274.503          | 291–301  | NFATSLYSMIK     |
| 1340.9            | 1340.412          | 7–18     | GTVTFPGFDER     |
| 1705.5            | 1704.895          | 30–45    | GLGTDEEESILTLTTSR |
| 1734.7            | 1734.046          | 246–260  | SIPAYAETLYYAMK  |
| 1800.8            | 1800.001          | 187–201  | WGTDEKFITIFGTR  |
| 1851.9            | 1851.083          | 64–79    | DLLDDKSELTKFEK  |
| 2097.8            | 2098.318          | 291–309  | NFATSLYSMIKGDTSGDYK |
| 2097.8            | 2097.226          | 7–25     | GTVTFPGFDERADAETLR |
| 2421.3            | 2420.769          | 208–227  | KVFDKYMTISGFQIEETIDR |
| 2889.7            | 2888.948          | 127–151  | QVYEEEYGSSLEDVVDGTSGYQQR |
| 3399.6            | 3398.879          | 213–242  | YMTISGFQIEETIDRETSGNLEQQLAVVK |

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Immunohistochemistry—To confirm the identification and to localize annexin V in tissue sections, we examined its expression by immunohistochemistry in five head and neck cancer tissue sections that were also analyzed with ProteinChip technology. Negative controls without the primary or with no antibody at all demonstrated negative results.

In the squamous cell carcinoma, clear immune reactivity could be seen in the periphery of the invasive growing regions (Fig. 6A). In the inner parts, only weak labeling could be detected (Fig. 6A). The normal cells of the squamous and ciliated epithelium of the pharynx showed no reactivity, with the exception of sporadic basal cells. Also, in the fibroblasts of the connective tissue as well as in collagen fibers infrequent expression of annexin V could be detected (Fig. 6, A and C). A strong labeling could be found in the epithelia of the mucous glands (data not shown). Inside the cells, annexin V is localized in the cytoplasm (Fig. 6, B–D).

To make sure that the here-localized annexin V is identical to the peak found by ProteinChip analysis, IHC-positive and -negative cell areas were gained by tissue laser microdissection. In the protein lysates from the positive fraction, a signal identical in mass to the peak obtained by the prior performed ProteinChip analysis was detected. In the protein lysate from the negative fraction, this peak was not visible (Fig. 7).

Table II

Results obtained from the CID-MS/MS analysis of four selected peptides on the ProteinChip MS/MS interface

| Peptide (m/z) | Sequence | Residues in annexin V |
|--------------|----------|----------------------|
| 1106.56      | SEIDLFNIR | 277–285              |
| 1340.58      | GTVTDFPGFDER | 7–18              |
| 1704.86      | GLGTDEESILTLSR | 30–45          |
| 1850.87      | DLLDDLKSELTGKEK | 64–79         |

Fig. 4. A, MS/MS analysis on ProteinChip interface in single MS mode from the tryptic in-gel digest. The four tryptic fragments indicated by arrows were further selected for CID MS/MS analysis. In B, the CID-MS/MS of the peptide with m/z 1704.88 (*) is shown, confirming the identification of the protein as annexin V.
DISCUSSION

In recent years, the search for new cancer biomarkers received a strong impetus from genomic and proteomic high-throughput techniques. Biomarkers or biomarker patterns should enable scientists and medical staff to make a more reliable early diagnosis of certain human diseases, especially malignant tumors, and to facilitate the prediction of their progression. This could contribute to a more differentiated, individually orientated tumor therapy. But until now only in a few tumor diseases have relevant markers been established (31).

One of the most promising proteomic tools for the detection of new cancer biomarkers is the ProteinChip technology (e.g. Refs. 32 and 33). Until now, this technique has been preferentially used for body fluid analyses, because they are fast and easy to perform by direct application on the ProteinChip arrays. Nevertheless, it is known that intra-individual changes in serum are high. For instance, sex, hormone level, nutrition state, and inflammation can strongly change the protein profile. Hence, biomarkers responsible for the genesis and progression of cancer must be present at a high level to be observed above normal changes. Despite these concerns, a high number of studies using body fluids as starting material were published (serum (32, 34); urine (35); nipple aspirate fluid (36); pancreatic juice (33)). If markers, however, can be found, they would be ideal for screening in high-risk individuals or even in individuals without elevated risk.

The analysis of tissues is more time consuming because here microdissection is indispensable to separate tumors from healthy cells, but the chance to find a reliable tumor marker might be higher than in serum. Microdissection, however, has to be done with an extremely precise technique like

![Immunohistochemistry of annexin V visualized by laser scanning microscopy. A, annexin V in squamous cell carcinoma (overview; ×180). Positive staining (yellow-green) is visible especially in the periphery of the invasive growing tumor areas (arrow); slight deposition of annexin V could also been seen on collagenic fibers (arrowhead). B, Detail of an invasive growing tumor area in higher magnification (×1500). C, Detail of A; intracytoplasmatic localization and deposition on collagenic fibers (×1000). D, Detection of annexin V in the cytoplasm of single tumor cells (×1700).](image)

![Areas with positive and negative reaction in IHC were microdissected and analyzed on ProteinChip arrays. A signal with a molecular mass of 36.90 kDa (*) representing annexin V was detectable in protein lysates from positive areas and was absent in the negative areas.](image)
LMCP, because in the case of normal tissue the lining epithelium consists only of one or a few cell rows and boundaries between tumor tissue and normal tissue are irregular.

To our knowledge, only six small studies combining microdissection and ProteinChip technology are published up to now. This might be due to the fact that laser-based microdissection is tedious and has to be done by an experienced pathologist. Another reason for this low amount of studies may be that up to now the microdissection for ProteinChip system analyses should be carried out on unstained tissue sections for optimal results, where single constituents are hard to recognize and to differentiate. The first study by Wright and coworkers in 1999 (16) used the combination of microdissection and ProteinChip technology to procure defined pure cells from prostate cancer in order to find biomarkers and to develop a clinical assay. In a review article, Paweletz (37) stated that as few as 25 cells are sufficient for the establishment of reproducible protein profiles. In our former investigations published in 2000 and 2001 (20, 21), we worked with a fine glass needle attached to a joystick-controlled electric micromanipulator. Normal epithelia to malignant prostate cells. For nine prostatectomy specimens, they could clearly show that proteins were differentially regulated in the different disease states.

For the analysis of proteins from tumor tissue, two problems have to be solved: first, the tissue heterogeneity of samples, and second, the heterogeneity of the tumor itself. Only the first could be solved by LMPC by dividing e.g. epithelial tissue from connective tissue. The tumor heterogeneity concerning the transscriptome and the proteome is morphologically hard to recognize and cannot therefore be completely solved by microdissection.

After a significant protein has been detected by profiling experiments with ProteinChip arrays, two additional questions have to be addressed. First, how the protein can be enriched and identified, and second, whether this identified protein can be found in the starting tissue and where the protein is exactly localized.

In our study, we addressed the first question by detecting differentially expressed proteins in microdissected tissue by ProteinChip technology and subsequently enriched and identified the protein of interest by 2-DE, in-gel digestion, peptide mapping, MS/MS, and immuno-depletion assay. As a first approach, after profiling the obtained masses of the intact protein can be used for data base search. This will give some indication about possible candidates, and in our case the later-identified annexin V was on the candidate list. 2-DE allowed us to enrich and isolate putative candidates and to digest them with trypsin. The generated peptides could then be analyzed again on the PBS II ProteinChip Reader, and a data base search pointed with a high probability to annexin V. To validate that digested protein isolated from two-dimensional gels is identical to the differentially expressed peak found with ProteinChip array, an immuno-depletion assay was performed with the same starting material and an annexin V antibody. In the analyzed supernatant, the peak at 35.90 kDa was missing and must therefore be depleted by the antibody. The identification was further confirmed by sequencing of selected peptides with MS/MS, a powerful but yet not widespread technique. The second question about the localization of annexin V in tissue was then addressed by IHC.

Annexin V could be found in different normal and tumor tissue components. Here as well in the re-analysis of annexin V-positive and -negative tissue areas on chip confirmed its identity to the differentially expressed peak at 35.90 kDa. In normal pharyngeal epithelium, annexin V shows the highest abundance in secretory cells of mucous glands that were excluded by microdissection. In carcinoma, it is exclusively expressed in the periphery of the invasively growing tumor areas. Therefore, annexin V might be connected to proliferation and/or cell mobility and could possess metastatic potential. For annexin I and annexin II, it was shown that their expression is associated to metastatic head and neck cell lines by using 2-DE (9).

An already described function of annexin V is the masking of phosphatidyserine on the outer cell membrane, protecting apoptotic cells from phagocytosis. The binding to phosphatidyserine is widely used for the detection of apoptotic cell (39). The localization of the immune reactivity in the periphery of invasive tumor areas does not support this function here. Annexin V belongs to a multigene family with at least 10 unique genes in mammals, but also homologues exist in Drosophila, Candida elegans, Hydra, Dictostelium, and several plant species (40, 41). Annexin V was first described in chick cartilage cells as anchoring CII (42). Annexin V shows the typical annexin structure consisting of the so called annexin core and the unique N-terminal domain. The annexin core is made up by four highly similar structural elements each containing five α-helices connected by short loops. This five-helix motif contains the typical calcium and phospholipid binding site of the annexins. The annexin core has also been proposed to form a pore that upon binding of the annexin molecule to the plasma membrane could serve as a calcium channel (43). Besides this and its function as a collagen receptor, several other characteristics have been described (44) that cannot help to explain the immunohistochemical data of this study.

Annexin V cannot be seen as a relevant marker for the whole tumor, but for clonal cell populations of tumor it seems to be characteristic for the invasively growing tumor cells.
Furthermore, the results stand for a paradigm of how to discover and identify potential tumor markers. The localization of annexin V shown with IHC is also an interesting hint to a previously unknown biological background of annexin V. Therefore, further tumor entities have to be analyzed for the relevance of this protein.

In conclusion, it can be stated that a better estimation of the biological importance of certain cell populations in regard to the progression from preneoplastic tissue alterations to malignant tumors and the prediction of the metastasis-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 SELDI-based ProteinChip technology in combination with microdissection and IHC opens up this possibility while reducing the complexity of the proteome by using a defined cell population and therefore allowing a better and earlier cancer diagnosis in future.

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