Biomarker Discovery by Tissue Microdissection and ProteinChip® Array Analysis

Identifizierung von Biomarkern über Gewebemikrodissektion und ProteinChip® Array Analyse

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Summary: In recent years, the search for new cancer biomarkers received a strong impulse from genomic and proteomic high throughput techniques. Biomarkers or biomarker patterns should enable scientists or medical staff to make a more reliable early diagnosis of certain human diseases, especially malignant tumors, and to make prediction of their progression, which could further contribute to a more differentiated, individually orientated tumor therapy. However, until now relevant markers have been established for only a few tumor diseases. One of the most promising proteomic tools for the detection of new cancer biomarkers is the ProteinChip® System technology. This mass spectrometry based system (SELDI; Surface Enhanced Laser Desorption and Ionization) appears to have a high potential for biomarker discovery and may serve in future as a clinical diagnostic assay platform.
Herein, we will review the current scientific literature dealing with cancer biomarker discovery by users of the ProteinChip System. Special emphasis is given to the investigation of microdissected tumor material. Further, we will discuss bioinformatic tools, suitable for the localisation and assessment of potential biomarkers.

Keywords: ProteinChip Arrays; SELDI; tumor; biomarker; microdissection; bioinformatics.

Zusammenfassung: Die Suche nach neuen Biomarkern in der Krebsforschung wurde in den letzten Jahren stark durch genomische und proteomische Hochdurchsatztechniken beeinflußt. Biomarker oder Biomarker-Muster werden zukünftig Wissenschaftler und medizinisches Personal in die Lage versetzen, frühzeitig verläßliche Diagnosen zu stellen. Dies gilt insbesondere für maligne Tumoren, da hier das Wissen um die Progression der Erkrankungen zu einer individuellen und tumororientierten Therapie führen kann. Bis heute konnten entsprechende Marker nur für wenige Tumorerkrankungen etabliert werden. Ein vielversprechendes proteomisches Werkzeug zur Entdeckung neuer Tumormarker ist derzeit die ProteinChip-Technologie. Dieses auf Massenspektrometrie beruhende Verfahren (SELDI; Surface Enhanced Laser Desorption and Ionization), scheint ein hohes Potential für die Biomarker-Indentifizierung zu beinhalten und könnte in Zukunft als Untersuchungs-Plattform für die klinische Diagnostik fungieren.
In dieser Übersichtsarbeit berichten wir über die aktuelle Literatur, die sich mit der Erkennung von Tumor-Biomarkern mit Hilfe des ProteinChip-System beschäftigt. Die Untersuchung an mikrosezierten Tumormaterial wird dabei besonders hervorgehoben. Weiterhin wird der Einsatz bioinformatischer Hilfsmittel diskutiert, die für die Lokalisation und Bewertung von möglichen Biomarkern unumgänglich sind.

Schlüsselwörter: ProteinChip Arrays; SELDI; Tumor; Biomarker; Mikrodissektion; Bioinformatik.

After the genomic gold rush, proteomics research came more into scientific focus over the last years. Some of the proteomic techniques were newly developed, although most of them are based on already existing procedures developed more than 20 years ago but ignored by the majority of the scientific community. The archetype of the proteomic techniques is the two-dimensional polyacrylamide gel electrophoresis, the 2-D PAGE [1]. This technique has matured to a state that allows high-resolution separation of proteins, good reproducibility, and sufficient sensitivity, but it is neither a large-scale nor a high throughput technology. Nevertheless, in combination with mass spectrometry (MS), 2-D PAGE can be a powerful method for separation and subsequent identification of proteins of interest. In particular, matrix assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF) [2] is commonly used for the accurate measurement of the molecular masses of peptides derived from in-gel digested proteins and subsequent identification by peptide fingerprinting [3, 4]. In contrast, the direct analysis of non pre-fractionated biological samples is beyond the scope of traditional MALDI-TOF, limited by the complexity of the analyte, the presence of buffer components like salts or deter-

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gents, and presence of non-protein components such as lipids and carbohydrates.

Ciphergen's ProteinChip® technology was developed to overcome the limitations of MALDI and to enable the direct mass spectrometric analysis of crude biological samples by combining another well established method, namely the solid phase chromatography, with mass spectrometry [5–7]. The so-called Surface Enhanced Laser Desorption and Ionization Time-of-Flight (SELDI-TOF) process was firstly described by Hutchens and Yip [8]. It utilizes array-based affinity surfaces to retain proteins based on their physical or chemical characteristics, followed by direct analysis by TOF-MS. Because proteins are retained on the surface, contaminants such as buffer salts or detergents can easily be washed off prior to MS analysis, thus eliminating the need for other pre-separation techniques. Furthermore, the small sample requirements, typically 1–2 µl per analysis, are ideal for small biopsies or micro-dissected tissues widely used to produce high-quality homogenous tissue samples in cancer research [9–11], where only minute amounts of sample can be easily obtained. Today, it is undisputable that microdissected tissue material, free of contaminating, unwanted cells, is extremely important to find reliable biomarkers for cancer diagnosis [12]. To date, the SELDI-based technique has been successfully applied to a lot of basic research and medical topics [13–16].

Nevertheless, besides these proteomic tools, DNA arrays are still of importance to detect biomarkers on the RNA expression level. As an example, van't Veer and coworkers [17] analysed thousands of genes by multivariate data analysis and successfully predicted the clinical outcome of breast cancer, that may also lead to a more efficient therapy. However, these methods provide no information whether the gene products, i. e. the proteins, have been really expressed or not [18]. The gap of information about changes in protein expression levels, post-translational modifications, or degradations prevents a real understanding of pathway networks responsible for the regulation of physiological and pathological processes. As most of the diagnostic biomarkers and drug targets are proteins, protein-based large-scale technologies like the SELDI-based ProteinChip technology appear to be an ideal tool for identifying cancer-associated biomarkers.

It must be realized, however, that proteome research is magnitudes more complex and needs more efforts than those applied to decipher the genomic information. Among the 1 billion possibly existing protein molecules, about 20 000 different entities are present in one cell. Furthermore, for the yeast proteins, for example, it is known that 84 % of the proteins are present in multi protein complexes [19]. Thus, the detection of proteomic biomarkers, the identification and the enlightenment of the biological role, will be the most exciting challenge for the future.

In this review, we will discuss ProteinChip technology-based approaches in cancer research, especially in conjunction with laser microdissection. Other possible fields of application for the ProteinChip System as well as a more concise explanation of the technique can be found in this issue, Wiesner [20], or in other reviews [7].

Proteomic techniques used in cancer research

Most proteomic techniques are based on or linked to MS-approaches [21]. As reviewed by Srinivas [22], this is true for the 'Isotope Affinity Tags', where different isotopes are used to label two different cell states [23]; MALDI-derived techniques, allowing the ionization of larger proteins [24]; 'reverse liquid chromatography tandem mass spectrometry' (LC-MS/MS), for the separation and sequencing of peptides in the low femto-molar range [25]; and 'Imaging MS', where direct mapping of proteins on the surface of tissue sections or individual cells can be performed [26].

Analogous to DNA arrays, microarray-based technologies for proteins are forthcoming. Antibody arrays are frequently used, which allow the selective profiling of whole proteomes [27, 28]. Another interesting array-based approach is represented by the so-called 'tissue arrays'. Here, cylindrically sliced biopsies from multiple (n = 200) individual tumors are arrayed on a slide, to provide information on all specimen by a single antibody-based experiment [29].

The ProteinChip System makes an important difference here, as it has the capability to detect whole ranges of differentially expressed proteins without limitations by pre-selections as is the case with antibodies or other capture molecules based microarray approaches. That means, the SELDI process allows the detection of whole protein pattern as a tissue-specific signature. This aim was originally attempted with the 2D-PAGE method but was never reached because of its limitations in reproducibility and speed. In this sense, SELDI can be understood as complementary to the 2D-PAGE approach with lower detection capability in the higher mass range but much faster throughput, higher reproducibility, and sample requirements lower by orders of magnitude.

Biomarker discovery in body fluids

Until now, biomarker discovery with the ProteinChip System was mostly done by analyzing body fluids like serum or urine. In contrast to the later reviewed work with microdissected tissues, body fluid analyses are fast and easy to perform by direct application on the arrays. Nevertheless, we know from our own experiments that intra-individual changes in serum are high. For instance sex, hormone levels, nutrition states, or inflammations 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. As this is not necessarily the case, in our opinion, a promising way could be to search for biomarkers in serum samples, but not before their presence has been ascertained in the diseased tissue itself. The subsequent marker detection in body fluids could then be more focused by applying antibody-based assays (e. g. array-bound antibodies) which allow the detection of minute amounts of proteins in the haystack of the proteome.

Despite these concerns, a large number of studies using body fluids as starting material have been published. Petricoin and co-workers [30] described a study on ovarian cancer. They combined the biomarker discovery in serum samples with a sophisticated bioinformatic algorithm, which leads to a complete segregation between cancer and non-cancer. This paper was controversially discussed in a later issue of Lancet [31]. Main criticisms were the missing identification of biomarkers and the incorrectly interpreted positive prediction values. In addition to serum, urine was used for ProteinChip analysis to diagnose transitional cell carcinoma (TCC) of the bladder [32]. Five potential novel biomarkers and seven protein clusters were found, resulting in an improved sensitivity for detecting low grade TCC compared to bladder-washing cytology. Nipple aspirate fluid (NAF) has also been used as a potential non-invasive source for markers in the early diagnosis of breast cancer. Paweletz and coworkers [33] analysed 27 samples with the ProteinChip technology. They were able to differentiate NAF samples from breast cancer patients and healthy controls, including those with an abnormal mammogram who were later proven to be normal. Pancreatic ductal adenocarcinoma were investigated in another study [34]. Samples of pancreatic juice obtained from patients undergoing pancreatectomy were compared with juice samples from patients with other pancreatic diseases. Also, a protein identified as Hip/PAP-I was found at a significantly higher level in serum of carcinoma patients.

This research on body fluids is fascinating, because it offers the potential to detect biomarkers in material obtained non-invasively. This is especially true for body fluids like NAF or urine, which are in more direct contact to the tumor and therefore potential biomarkers concentrations are higher compared to serum. These markers would be ideal for screening in high risk individuals or even in individuals without elevated risk, provided that the costs were affordable. In our opinion, this goal will be reached only for a few tumor entities (e.g. TCC), whereas in many other cases, detectable markers will be present only in an advanced tumor stage, too late for an early and preventive diagnostic assay.

In any case, the level of confidence in a set of biomarkers for the early detection of cancer is highly dependent on the purity and homogeneity of the biological material, whether body fluids or microdissected or non-microdissected tumor material is used.

Biomarker discovery in microdissected tissue

In contrast to body fluids as starting material, another more research orientated approach is the direct analysis of diseased and normal tissue. Here, microdissection is indispensable to separate tumorous from healthy cells, e.g. in the case of epithelial tumors, the epithelial cells from the underlying or surrounding connective tissue. An extremely precise technique is needed because in normal tissue the lining epithelium consists only of one or few cell rows and in tumor tissues boundaries to normal tissue are irregular. Further, microdissection is essential prior to tumor analysis to separate epithelial or mesenchymal tumor cells from tumor stroma, preexistent tissue components, necrotic or apoptotic areals, or areals with inflammation (Fig. 1). Nevertheless, it should be considered that ProteinChip analysis of larger tumor areals containing both epithelial tumor cells and tumor stroma enables the synchronous analysis of proteomic changes in both components in the course of tumor development and progression.

As mentioned above, there are significant advantages in using homogenous cancer cell populations as they can be obtained by cell culture or by laser or needle microdissection. Tumor-derived cell lines can be used for initial studies [35] but have their own special evolution and cannot be seen as a real copy of the *in vivo* situation in every case [36].

Tissue microdissection can be done with a fine needle attached to a joystick-controlled micromanipulator [10]. This technique was successfully applied for chromosome microdissections, where even single bands could be cut [37]. Nevertheless, this technique is troublesome and needs highly skilled staff. It is especially difficult to cut out the tissue areas of interest because the tissue cohesion differs.

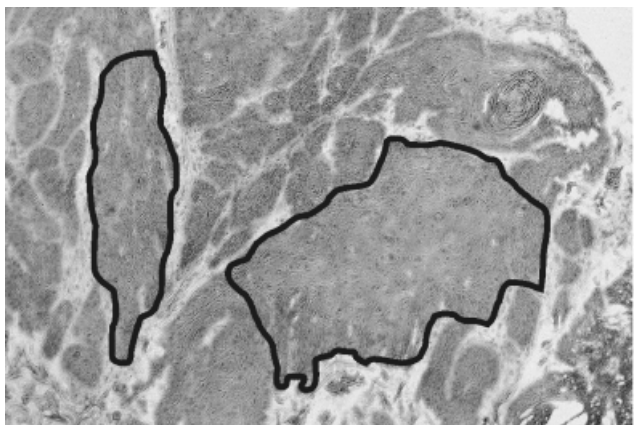


Figure 1 Histological section of a squamous cell carcinoma. The black line marks the laser excised areas in the corresponding unstained slice, to separate the epithelial areas from tumor stroma and preexistent tissue components.

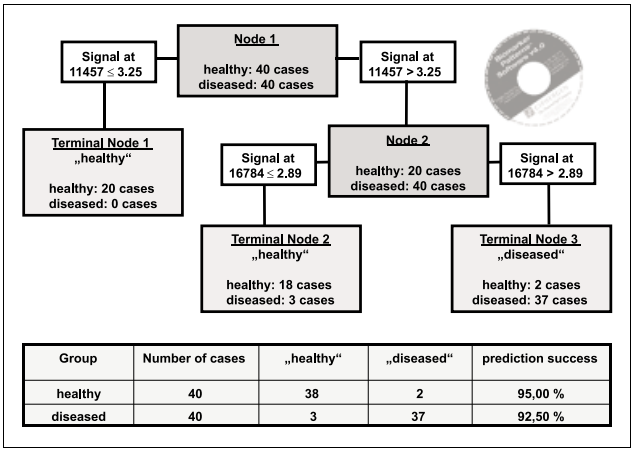
Currently, two distinct laser-assisted microdissection techniques are available. In the first method, special tissue areas or single cells from histological sections bound on polymer are cut out by a laser (laser microbeam microdissection; LMM) and subsequently transferred by laser pressure catapulting (LPC) in a vessel lid [38]. This system was commercialized by P.A.L.M. Microlaser Technology (Bernried, Germany). The second method was developed at the National Institutes of Health, USA and commercialised by Arcturus Enineering (Mountain View, CA, USA). Here, a thermo-labile coated lid is in direct contact with the tissue section, so that the laser can directly transfer selected cell areas to the repository [39]. But even with one of these techniques, it can be a time-consuming process to collect enough cells for conventional proteomic analysis such as 2-D PAGE, which requires about 50 000–100 000 cells per analysis. The combination of laser-assisted microdissection with the ProteinChip technology eliminates much of the sample size restrictions, and a typical analysis can be performed with 5000 cells or less [11]. This is due, in part, to the reduced sample requirements for ProteinChip technology (typically 1–5 µl) and to the development of optimized extraction protocols for many tissue types. The lack of proteins from other contaminating tissues assures a high degree of reproducibility, because inhomogeneity is eliminated during the collection step [12, 40]. Extraction of DNA/RNA from laser-microdissected samples provides the same benefits of enrichment for genomic analysis as protein extraction from laser-microdissected samples does for proteomic analysis. In order to gain the benefits of combined genomic and proteomic analysis, it would be ideal if standard protocols for DNA/RNA extraction, such as Trizol isolation, were to work. Optimized fixation and staining protocols are required, which preserve RNA [41, 42] and protein integrity and provide acceptable morphology in stained frozen sections even without coverslips.

To our knowledge, only five small studies combining microdissection and ProteinChip technology are published up to now. This might be due to the fact that even laser-based microdissection is tedious and has to be done by an experienced pathologist. Another reason for this low number 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, whereby single constituents are only hard to recognize and to differentiate. At least in our hands, all standard histological stainings impair the protein analysis of lower cell amounts (own unpublished data).

The first study by Wright and coworkers in 1999 [6] used the combination of microdissection and ProteinChip Arrays to procure defined pure cells from prostate cancer in order to find biomarkers and to develop a clinical assay. In a review article, Paweletz [43]. Stated that as few as 25 cells are sufficient for reproducible protein profiles. In our investigations published in 2000 and 2001 [10, 11] we worked with a fine glass needle attached to a joystick-controlled electric micromanipulator. Normal epithelia to melanoma and cervical intraepithelial neoplasias (CIN) to cervix uteri carcinoma were compared. In both studies, at least 500–1000 cells were necessary for a reproducible profile. This cell number was also affirmed by Wellman and co-workers [44] who laser-microdissected stroma, epithelium, and tumor cells of the prostate to decipher the epithelial-mesenchymal cross talk. Extensive studies with microdissected material are surely in progress and will result in a wide number of potential markers, if a better understanding of biological heterogenity (e. g. the use of an exactly defined sample) and the presence of all clinical data will be given, as stated in the review "Cancer biomarkers: easier said than done" [45]. However, the classification of these markers represents the bottleneck of the ProteinChip technology approach because purification of these proteins – which is the prerequisite for the identification – is tedious and not successful in every case, especially with the low amount of samples available from microdissections. Although the direct identification of small proteins (< 3000 Da) is possible by reading the ProteinChip Arrays in an interface-equipped Tandem-MS instrument, and the same can be done for peptides deriving from enzymatically digested larger proteins, implementation of these technologies is currently not widespread. In most of the studies published, proteins were identified by their native mass in combination with ProteinChip Array based immunoassays [32].

Bioinformatics

Bioinformatic analysis of ProteinChip data comprises several pre-processing and post-processing steps [46]. Pre-processing ranges from the conversion of the time of flight (TOF) data to molecular weight (MW) values and normalization of intensities in the corresponding signals. It also includes baseline substraction, peak detection, signal to noise calculation, and mass calibration. Post-processing includes various bioinformatic tools which reveal single markers or patterns relevant for a certain biological state. The detection of single proteins responsible for differention between normal vs. diseased is the first step which can be done with the normal ProteinChip Software supplied with the system. The implemented Biomarker Wizard tool allows the calculation of p-values and the display of clusters (common peaks in different spectra) in a logarithmic scale or as a Box and Whisker Plot. If discrimination between normal and diseased spectra remains difficult based on single markers, multivariate tools can be applied. From the biological understanding, this will be true in most cases because genesis and progression of cancer is always dependent on protein networks rather than single molecules. Until now, several software packages have been used to analyse the results accessed with Protein-



Group	Number of cases	„healthy“	„diseased“	prediction success
healthy	40	38	2	95,00 %
diseased	40	3	37	92,50 %

Figure 2 Biomarker Patterns™ Analysis. Simplified presentation to demonstrate the general principle. The program calculates which signals and intensitities are best suited to split the samples into as pure as possible sub-groups, whereby the final number of nodes can be restricted by re-testing the tree during model building and by pre-definition of the costs for wrong assignments of certain groups. The prediction success is one of the most important results with regard to the practicability of the model which has to be tested for reliability by new data sets after this initial learning period.

Chip technology. The algorithms used can be classified in unsupervised learning in form of cluster analysis or supervised learning in form of classification methods. Supervised classification and regression tree methods are implemented in the Biomarker Patterns™ Software (Ciphergen). Based on an always newly created set of rules, peak intensities from protein signals of certain mass values are used for group separation and the generation of classification trees (Fig. 2). The procedure is comparable to a medical anamnesis in which, according to the patient's statements, the diagnosis is concluded. Here, it is decided whether protein profiles are from a diseased or normal specimen. Because of its clearness, this tree-based model is easy to understand and interpret and can also be used by the non-bioinformatically trained scientists (see Wiesner [20], this issue, for details and references).

In the near future, other statistical methods that will be ideal for all kind of studies, other analytic tools like self-organizing maps [47] unified maximum separability analysis (UMSA; [48]) or support vector machines (SVM; [49]) should also be evaluated as to their appropriateness in the detection of biomarkers with the ProteinChip System.

Conclusion

The discovery of biomarkers for a more differentiated individual tumor diagnosis and therapy will be the aim for the next decade. The better estimation of the biological importance of certain cell populations in regard to the progression from pre-neoplastic tissue alterations to malignant tumors and the prediction of the metastasis forming potential of a given cell population will be necessary prerequisites for providing a more detailed insight and understanding of tumor progression.

Among other approaches, the SELDI-based ProteinChip technology is a powerful tool in clinical proteomics. As with other technologies, it is dependent on accessory tools. In our case, microdissection offers the possibility of reducing the complexity of the proteome by using a defined cell population. As for all other high throughput technologies, multivariate exploitation by complex algorithms is essential. Without any doubt, the combination of ProteinChip technology, tissue microdissection and bioinformatic tools will lead to newly detected markers for earlier cancer diagnosis and will provide more information on progression and adequate therapy strategies.

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