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Advances in clinical cancer proteomics: SELDI-ToF-mass spectrometry and biomarker discovery

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Abstract

For most cancers, survival rates depend on the early detection of the disease. So far, no biomarkers exist to cope with this difficult task. New proteomic technologies have brought the hope of discovering novel early cancer-specific biomarkers in complex biological samples and/or of the setting up of new clinically relevant test systems. Novel mass spectrometry-(MS) based technologies in particular, such as surface-enhanced laser desorption/ionisation time of flight (SELDI-ToF-MS), have shown promising results in the recent literature. Here, proteomic profiles of control and disease states are compared to find biomarkers for diagnosis. This paper aims to address the authors' own work and that of other groups in clinical cancer proteomics based on SELDI-ToF-MS. Shortcomings and hopes for the future are discussed.

INTRODUCTION

For most cancers, survival rates depend on the early detection of the disease. Typically, the earlier a cancer is detected and diagnosed, the more successful the treatment, thus enhancing the survival rate. Early detection increases chances that the tumour is still localised. For colorectal cancer, the five-year survival rate is 90 per cent for localised cancers, compared with 61 per cent for cancers that have progressed or formed metastases.¹ Similar observations can be made for breast cancer (96 per cent and 86 per cent, respectively²) and lung cancer (48 per cent and 15 per cent, respectively³). Despite the availability of routine screening methods such as mammography for breast cancer, or colonoscopy, sigmoidoscopy or faecal occult blood test for colorectal cancer, these cancers are still a leading cause of death.

It is now necessary, therefore, to identify new tumour-associated serum proteins with a clear advantage over the markers that have been previously been assessed in clinical settings. These new biomarkers can be used clinically as

markers to detect early cancer and to monitor therapy or recurrence of disease. A suitable cancer biomarker is a substance found in an altered amount in the body, implying that a certain type of cancer is present. Ideally, a cancer biomarker should be detectable in the blood or other body fluids that can be accessed in a non-invasive manner. Unfortunately, the development of tumour-associated serum protein biomarkers over the past few decades has not been effective for diagnosing primary cancer. Clinical blood tests based on serum markers (proteins), such as CA19-9 for colorectal and pancreatic cancer,^{4,5} CA15-3 for breast cancer^{6,7} and CA125 for ovarian cancer,^{8,9} exhibit rather low positive predictive values. As a result, none of these biomarkers met the original goal of discovering cancer at an early stage. One reason for the low sensitivity and specificity is the presence of these markers in the serum of individuals without cancer or with non-malignant disease. Not only did these biomarkers fail to detect many early cancers, however, they also lacked specificity, even for the detection of

advanced diseases. Finding cancer-related biomarkers is proving problematic, and over the past decade the Food and Drug Administration (FDA) has approved only a few new diagnostic biomarkers. Only one marker (prostate-specific antigen¹⁰) has been discovered to be useful in testing for early cancer. As a consequence, the US cancer-related death rate between 1950 and 2001 did not change significantly¹¹ (Figure 1).

Because of the failure to identify new single biomarkers for the detection of early cancer, it has become more and more obvious that the simple cause and effect scenario no longer holds promise and that most physiological systems and diseases are multi-factorial. Moreover, because of the genetic heterogeneity among populations, one biomarker might indicate disease in one group but be statistically non-significant in another. Hence, focusing on proteomic patterns might have a higher level of prognostic or diagnostic accuracy than existing diagnostic assays (eg enzyme-linked immunosorbent assays [ELISAs]), which examine protein biomarkers one at a time. Although this 'multiplex assay' technology is a relatively new addition to clinical diagnostics, the utilisation of laboratory tests based on biomarker

patterns will give a more accurate diagnosis in routine clinical practice.

Proteomic analysis becomes a valuable tool in determining the presence of biomarkers or in mapping biomarker profiles within different sample groups, for example in healthy and diseased individuals.¹²⁻¹⁴ The final result of such an experiment is typically a list of peptides/proteins that are up- or down-regulated between the two groups. Determination of changes in relative or absolute concentration is fundamental to the discovery of valid biomarkers. Traditional proteomics tools for protein profiling, such as two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) have so far not helped in discovering the urgently needed diagnostic biomarkers for the early detection of cancer. New proteomics technologies have brought the hope of discovering novel early cancer specific biomarkers in complex biological samples and/or of the setting up of new clinically relevant test systems. Protein arrays can be used to measure the abundance of particular biomarkers in the blood;¹⁵⁻¹⁷ however, mass spectroscopy (MS)-based approaches seem to dominate the search for biomarkers to overcome the existing dilemma.¹⁸⁻²⁰ Development of new MS techniques, such as surface-enhanced laser desorption/ionisation-time-of-flight (SELDI-ToF)-MS will hopefully help in the search for new biomarkers. The SELDI-ToF-MS technology is not only able to find single protein biomarkers but is also able to identify biomarker expression patterns. Proteomic pattern analysis is a novel approach for the diagnosis of diseases.²¹⁻²³

In summary, there is considerable hope that this new serum proteomic technology will contribute significantly to screening-test development and its translation into routine clinical practice.

OBSTACLES TO FINDING SERUM BIOMARKERS

Why is it so difficult to identify cancer serum biomarkers? First, protein

Biomarker patterns rather than single biomarker, will give a more accurate diagnosis of a disease

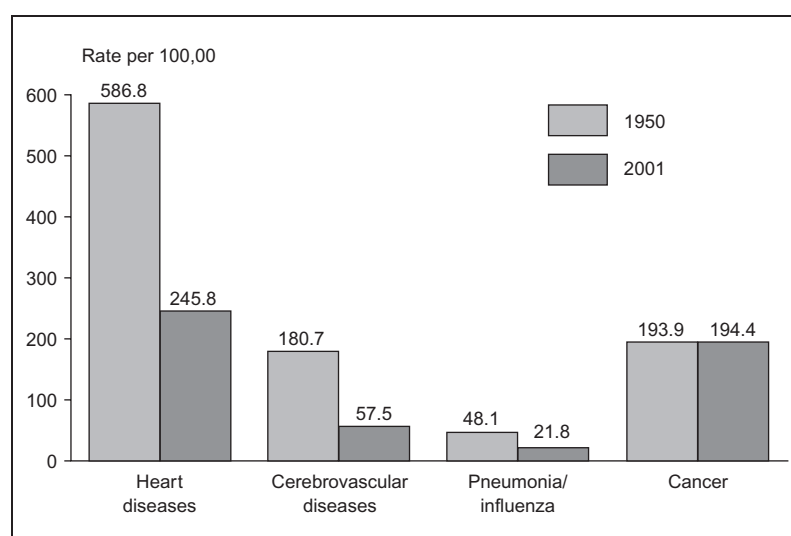


Figure 1: Change in the US death rates by cause, 1950 and 2001. Rates are age-adjusted to 2000 US standard population

Serum is a challenging task for proteomic analysis using current technologies

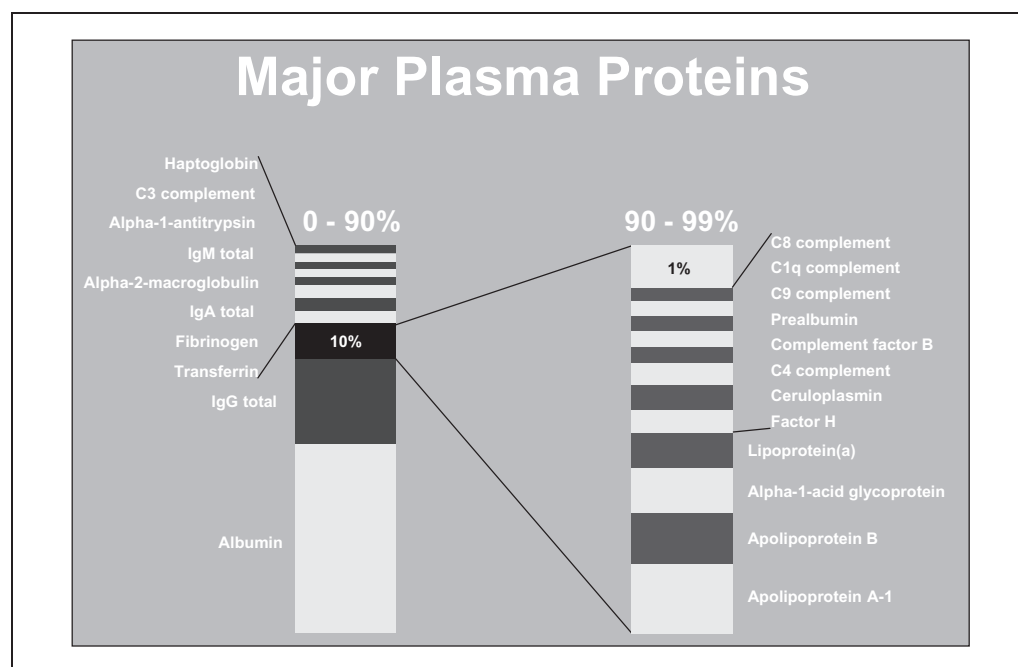
concentrations vary enormously, perhaps up to ten orders of magnitude in serum, making the characterisation of the proteins within the lower 1 per cent of protein abundance an analytically challenging task using current technologies.²⁴ Unfortunately, 99 per cent of this protein concentration is made up of only 22 proteins, with albumin making up approximately 50 per cent of the protein content of the serum (Figure 2). Therefore, most observed proteins are typically of high abundance. Many potentially valuable biomarkers are expressed at very low levels and are difficult to detect. Finding new and better methods for detecting and identifying these low-abundant proteins represents a new challenge for routine diagnostics. Secondly, protein concentrations are dynamic,²⁴ sometimes changing markedly with stress, disease or treatment. Thirdly, proteins can be modified by cleavage or by addition of new functional groups (eg phosphorylation or glycosylation), changes that may affect detection. It should also be kept in mind that the proteome is much more complex than the genome. The human genome contains only about 33,000 genes, but

these genes code for more than 200,000 proteins, not to mention post-translational modifications, which expand that number even further.

TRADITIONAL PROTEOMICS TOOLS FOR BIOMARKER PROFILING

A well-established technology for the detection of serum biomarkers through changes in serum protein concentration is 2D-PAGE. Many laboratories throughout the world are performing such proteomic profiling. Protein spots that appear to be differentially abundant by staining techniques can be excised from the gel and digested. Usually, 2D-PAGE is coupled to MS to identify the proteins.²⁵⁻²⁷ Unfortunately, the method is labour intensive and requires large amounts of samples; it is therefore not suitable for large-scale screening programmes or clinical testing. As the identification of reliable and clinically useful biomarkers may require the comparison of thousands of samples, this is a major problem. In spite of all possible future improvements to such a technique, 2D-PAGE will probably remain rather a low-throughput

Figure 2: Chart representing the relative contribution of proteins within plasma. Twenty-two proteins constitute approximately 99 per cent of the protein content of plasma. (Source of data: Homepage Plasma Proteome Institute – The Plasma Proteome: Enabling a Revolution in Diagnostics. Leigh Anderson; <http://www.plasmaproteome.org>)



approach to proteomic analysis. Limitations also exist in the isolation of, for example, the low molecular weight region of the proteome, low-abundant proteins or proteins with extreme pI values.

NEWLY DEVELOPED MS-BASED TOOLS

Novel MS-based technologies are a potential alternative to the 2D-PAGE approach to multi-dimensional separation of proteins. In regard to the main problem, the complexity of the sample, the past two decades have seen a growth of significantly improved spectrometric devices, allowing precise analysis of biomolecules.^{19,20,28,29} In general, the instruments are made up of three primary components: the source, which produces ions for analysis; the mass analyser, which identifies the ions based on their mass-to-charge ratios; and the detector, which quantifies the ions resolved by the analyser. The technique is sensitive to the picomole to femtomole range required for the detection of low-abundant cancer biomarker. MS-detectable biomarkers can consist of, for example, oligonucleotides, small polar molecules, peptides, proteins and post-translationally modified proteins, such as glycoproteins and phosphoproteins.^{26,27} Currently, several different MS-based proteomics approaches exist that can be used for the discovery of novel biomarkers. Ciphergen's (Fremont, CA, USA) ProteinChip[®] system (SELDI-ToF-MS), which has enabled clinically relevant proteomics studies to be performed, is described below. Other techniques for differential analysis — such as isotope-coded affinity tags (ICAT),³⁰ in which proteins from different sources are labelled with isotopic variants of a cysteine-specific tag, or multi-dimensional liquid chromatography-MS platforms³¹ — are used successfully in the discovery of new biomarkers but are not reviewed here.

SELDI-ToF-MS detection of serum proteins: Strategy for identifying cancer-associated serum proteins

The SELDI-ToF-MS technology implemented in the ProteinChip[®] system from Ciphergen Biosystems Inc. is designed to perform MS analysis of protein mixtures retained on chromatographic array surfaces.^{21–23} Easily obtainable clinical biofluid samples, such as blood, urine or nipple aspirate, can be directly (raw) applied to the ProteinChip[®] array surface. An advantage of SELDI-ToF-MS is its relatively high tolerance for salts and other impurities. The sample requirement is low (1–10 µg total protein per spot) and sample volume can be freely chosen from 0.5 µl up to around 400 µl.²² The mass limits for detection of biomarkers lies between 1,500 and 20,000 Da, according to the authors' experience. Chromatographic surfaces with certain physicochemical characteristics (hydrophobic, cationic, anionic, metal ion presenting or hydrophilic) or preactivated for the coupling of capture molecules (protein, DNA or RNA) prior to sample loading can be used. After a series of binding and washing steps, proteins and any contaminants that do not bind to the spot surface are removed. This reduces the complexity of the samples. This, in turn, results in an increased probability of detecting markers that are present at lower concentrations. Applying serum pre-fractionation can further reduce serum complexity. The removal or separation of high-abundance proteins by efficient pre-fractionation enables improved detection of lower-abundance proteins. By depleting high-abundant proteins such as albumin, however, it should be borne in mind that other proteins of interest might also be excluded from analysis by co-depletion. Moreover, pre-fractionations contribute to lower reproducibility. Subsequently, an energy-absorbing matrix is applied to each sample and a laser is used to desorb and ionise bound species, enabling their mass-to-

SELDI-ToF-MS is a promising approach for the discovery of novel biomarkers

Good experimental design is important for biomarker identification

charge ratios to be measured by ToF-MS. The spectra obtained in a single experiment consist of many different biomarkers. Differentially expressed biomarkers may be determined from these biomarker profiles by comparing peak intensities between two different groups. Thus, the entire process can be achieved in a quick, simple procedure, consisting of spectral pre-processing (baseline subtraction, normalisation and calibration), cluster and a statistical analysis. Comparisons of the protein peak patterns or single proteins obtained from samples representing different populations are expected to provide detailed diagnostic patterns classifying pathological states. Utilising this technology, hundreds of clinical samples can be analysed per day. This method has already yielded biologically and clinically promising results. Several studies dealing with the early detection of cancer biomarkers have already been published.^{32–35}

The SELDI-ToF-MS analysis is divided into a pattern discovery and a pattern-matching phase. Bioinformatics tools are required to detect these differences and play a crucial role in the analysis of these data. The successful use of the sensitive ProteinChip[®] system depends on a good experimental design and high-quality biological samples³⁶ (also discussed at the European CIPHERGEN User Meeting in Dublin, 2004 and confirmed by the authors' own findings). The following considerations should be kept in mind before starting a SELDI experiment:

- The training samples and the validation samples (test set) should each include at least 110 randomly selected subjects without cancer and 70 subjects with cancer. The test set is needed to evaluate a positive biomarker pattern independently. Training and test set populations should be treated identically.
- Analytical reproducibility, including sample treatment (eg storage) and preparation, method of analysis and

processing of results, has to be controlled. Since, for example, sample treatment is often specific to individual hospitals, the use of samples from multiple institutions is the preferred approach for biomarker discovery. Ideally, algorithms employed for serum proteomic profiling should filter out temporal fluctuations in the serum proteome which are unrelated to the disease being considered, such as stress, medical treatment or other interventions that influence serum biomarker levels.

- Because malignancies are often connected to an inflammatory response from the host, the inclusion of benign diseases connected to an inflammatory response is crucial for obtaining a cancer profile rather than a generalised systemic reaction to disease.
- By using ProteinChips[®] from the same batch, variability between chips can be minimised. Random spotting of samples onto ProteinChips[®] will also decrease variability.
- Stringent quality controls to evaluate performance over an experiment/time are required. The present authors use a pooled human serum, randomly spotted on each ProteinChip[®]. Any variability arising from pre-analytical factors (eg instrument performance) can be evaluated in this way.

Once the best fitting key mass-to-charge ratios values are selected, identification of these biomarkers can proceed or the biomarker pattern can be used for screening.

APPLICATION EXAMPLES

In the next two subsections, two examples of clinical cancer proteomics (for ovarian and gastric cancer) based on SELDI-ToF-MS will be given.

Serum markers for ovarian cancer

Recently, several groups have demonstrated the viability of using serum

Promising studies for the detection of serum biomarkers in ovarian and stomach cancer were performed

proteomic pattern analysis for the diagnosis of ovarian cancer.^{32,35,37} In 1995–2000, 44 per cent of women diagnosed with the disease were five-year survivors. Women diagnosed with stage IV ovarian cancer had a much shorter five-year survival (20 per cent) than women diagnosed with stage I disease (85 per cent).³⁸ The multicentre study from Zhang *et al.*³⁵ used SELDI-ToF-MS to analyse blood proteins from 361 patients and 142 healthy blood donors. The initial assessment included 195 women with known ovarian cancer, 166 with benign disease and 142 women without disease, and enabled the creation of distinct proteomic patterns which distinguished cancer patients from healthy individuals. These biomarker/patterns were then used to analyse an independent set of blinded samples, 41 from women with ovarian cancer, 41 from healthy women and 20 each from patients with breast, colon and prostate cancers. The expression levels of the three most promising biomarkers were statistically significantly different between the healthy controls and the early-stage ovarian cancer patients ($p < 0.000001$ for all three of the markers). For discrimination between healthy controls and stages I/II invasive ovarian cancer, the three biomarkers and CA125 showed a sensitivity of 83 per cent (95 per cent confidence interval [CI] 61–95 per cent) and a specificity of 94 per cent. This is statistically significantly better than CA125 alone, which had, at the same sensitivity, a specificity of only 52 per cent (95 per cent CI 39–65 per cent). The detected proteomic patterns have the potential to contribute substantially to a decrease in the mortality rate of ovarian cancer.

Serum markers for gastric cancer

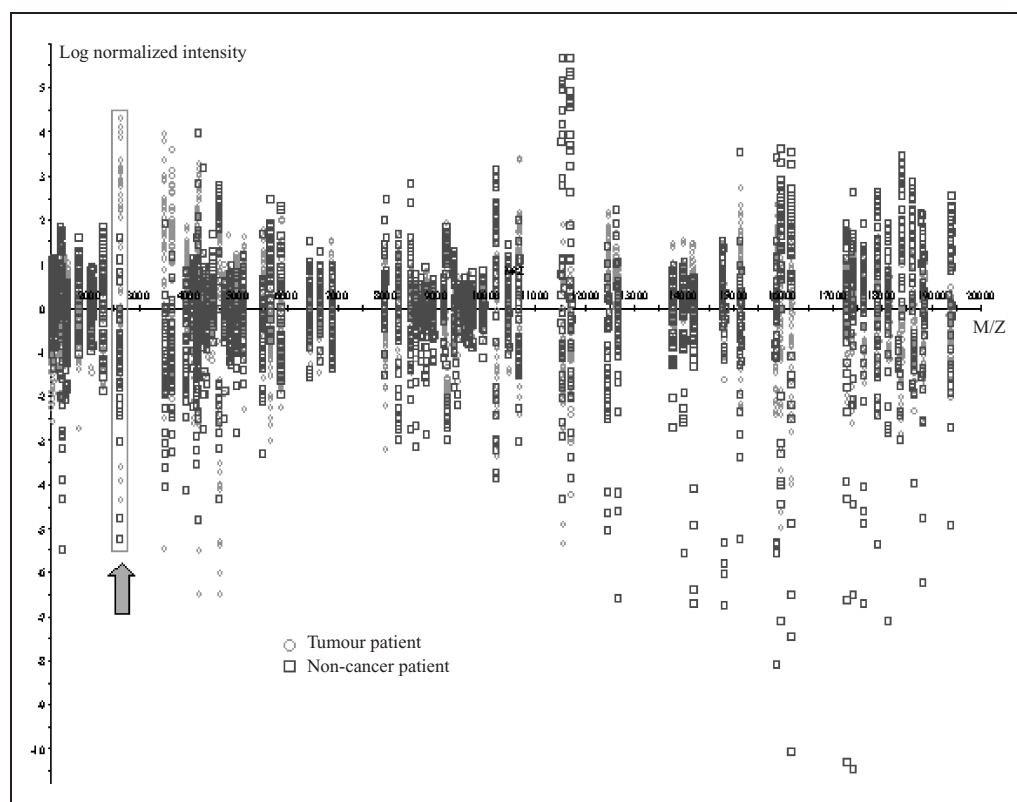
A study to identify potential biomarkers for gastric cancer was recently performed by the authors.³⁹ Serum samples from 50 patients with gastric cancer and from 60 control patients without gastric cancer were screened for protein patterns that differentiated gastric cancer patients from

healthy patients. A classifier ensemble, consisting of 50 decision trees, correctly classified all gastric cancers and all controls in a training set (100 per cent sensitivity and 100 per cent specificity). The classifier proved its high value in identifying even early gastric cancers; in an independent test set, eight out of nine stage I gastric cancers (88.9 per cent sensitivity) were correctly classified. Moreover, all 11 previously unseen and randomly selected control sera obtained from patients with dyspeptic symptoms, but without gastric cancer (100 per cent specificity), were classified correctly. Furthermore, 28 sera from gastric cancer patients (including five additional stage I cancers) taken in different hospitals were correctly classified (100 per cent sensitivity) and 29 of 30 healthy blood donors were classified as being free of cancer. The ProteinChip[®] system will enable the identification of cancer biomarkers which will facilitate the development of a diagnostic test for gastric cancer, even in its early stages. Using this approach, the pattern itself — independent of the identity of the proteins or peptides — is the discriminating factor. Nevertheless, there exist single molecular masses with high discriminatory power, which could be used as promising cancer biomarkers. Promising molecular masses with different peak intensities between the two sample groups are shown in Figure 3.

Critical consideration

It is clear that this technology will provide new insights. It is also clear that the use of this technology is at an early stage. Despite the exceptional analytical power of the ProteinChip[®] system, systematic limitations of the approach at the present state of the technology have become apparent. First, as with any other analytical technique, not all proteins can be visualised equally well. Whereas the range below 20 kDa is especially well resolved, sensitivity for higher molecular weight proteins is clearly lower, resulting in a smaller number of signals.

Figure 3: Biomarker Wizard Software (CIPHERGEN, Inc.) Scatter Plot. Shown are cancer biomarkers obtained by a study from Ebert *et al.*³⁹ The two groups of 'non-cancer' and 'tumour' patients (square and circle, respectively) can be best separated by looking at well separated clusters. One cluster example is enframed by a rectangle.



There exist limitations for biomarker discovery via the SELDI-ToF-MS approach

Secondly, detection of high intensities of protein peaks of a specific molecular mass may not necessarily mean that high levels of the corresponding protein product will be present in body fluids (due to the suppression of signals through higher-affinity binding, so that absolute quantitation is not possible). As long as similar biological fluids are compared, however, this is not an issue for biomarker discovery. This would only be a problem if one tried directly to compare the quantity of a protein in, for example, urine and serum.

Thirdly, a limiting factor of most profiling experiments has been the consensus that low magnitude changes in expression are not meaningful, or perhaps are immeasurable. This ensures that confirmed biomarkers must have a certain level of intensity difference before being recognised as reliable, and will therefore be more trustworthy predictors than those exhibiting lower differences only.

Another drawback of this new technology remains the problem of

creating reliable protein profiles from biological samples. In many cases, mass resolution and mass drift is found to be too low. As a result, data comparison, exchange and verification between different laboratories is difficult, hence the ProteinChip[®] system is not at a stage where it is sufficiently robust to be introduced into a diagnostic setting. The goal is reproducibility.

Another problem is obtaining clinical samples of sufficiently high quality (including collection and handling), which is essential for successful experiments. A strict quality control for the samples being analysed and the analytical procedure within different laboratories is vital for any screening, to omit systematic biases from pre-analytical variables (eg sub-phenotypes/systematic differences of study population, instrument condition or sample handling). Efficient data analysis systems (bioinformatics) to assimilate the vast amounts of protein expression data from healthy and diseased individuals into clinically relevant datasets is essential.⁴⁰

Although the ProteinChip[®] system is currently used for a variety of research applications, the technology has not yet been adopted as a routine method for clinical testing.

At this stage in the development of proteomic pattern analysis, it is debatable whether it is worth the effort to identify the features/biomarkers. The identification of these features/biomarkers is not absolutely necessary for their use as biomarkers and may provide little aid in developing this diagnostic platform. For the detection of disease, it seems sufficient that disease-related patterns can be reproduced reliably in a clinical setting. MS eliminates both the need to purify and identify biomarkers and the need to develop antibodies to proteins prior to clinical use. Conversely, the identification of these diagnostic biomarkers is of considerable interest to the medical community and it will help to elucidate mechanistic and therapeutic insights into a particular cancer. With the current state of technology, the purification of marker proteins can be time consuming and might need considerable biochemical background knowledge and experience. In a critical comment, Diamandis⁴¹ stated that he suspects that the molecules constituting the distinguishing pattern are 'epiphenomena' of cancer and that they are produced by other organs in response either to the presence of cancer or to a generalised condition of the cancer patient. He remains doubtful as to whether they are able to distinguish between the varieties of disease that could potentially generate them. To overcome this criticism, identification of the peptides and proteins corresponding to particular mass-to-charge ratios generated by SELDI-ToF-MS analysis will be necessary. This may be achieved by using various systems, such as tryptic digestion followed by MS identification, MS-MS identification or database searching (Figure 4). In addition, by identifying molecular masses and tagging these with protein names, other high-throughput assays (eg ELISAs) can be developed.

Another major criticism is that in studies using the same technology to develop a diagnostic test for the same cancer, different peaks are recognised by the algorithms as being crucial in distinguishing serum from healthy and diseased individuals. Therefore, independent studies need to be performed as an important requirement for clinical validation. For example, three different prostate cancer detection studies reported different distinguishing biomarkers, sensitivities and specificities.^{34,42,43} One explanation for this discrepancy is that the technology is very sensitive to experimental details, such as how the serum is collected, stored and processed (see experimental design above). Moreover, one has to keep in mind that the equipment, such as the laser or detector, has a limited life span. For this reason, obtaining identical spectra over time is difficult, even when using the same machine. Any slight deviations from a standard protocol could result in changes in the proteomic pattern provided by a particular serum sample. The algorithm used to identify these discriminatory biomarkers is also fundamental. A well suited statistical examination will even be able to reveal the existence of biases through non-disease related variables. Different instruments, analytical procedures and computer algorithms should not, however, be a limitation for a robust system that is able to be used in a clinical setting. Until the problems mentioned above are solved, one solution for the near future will be the identification of relevant biomarkers for particular disease states. This information can be used to generate various types of affinity reagent (eg an antibody), which can then be incorporated into an ELISA-based platform to screen serum samples for the presence of the biomarker originally identified by MS.

CONCLUSIONS

This paper has attempted to discuss critically the various technologies in the field of cancer diagnostics. It has become

For a routine use of SELDI-ToF-MS in clinics, first independent studies have to be performed

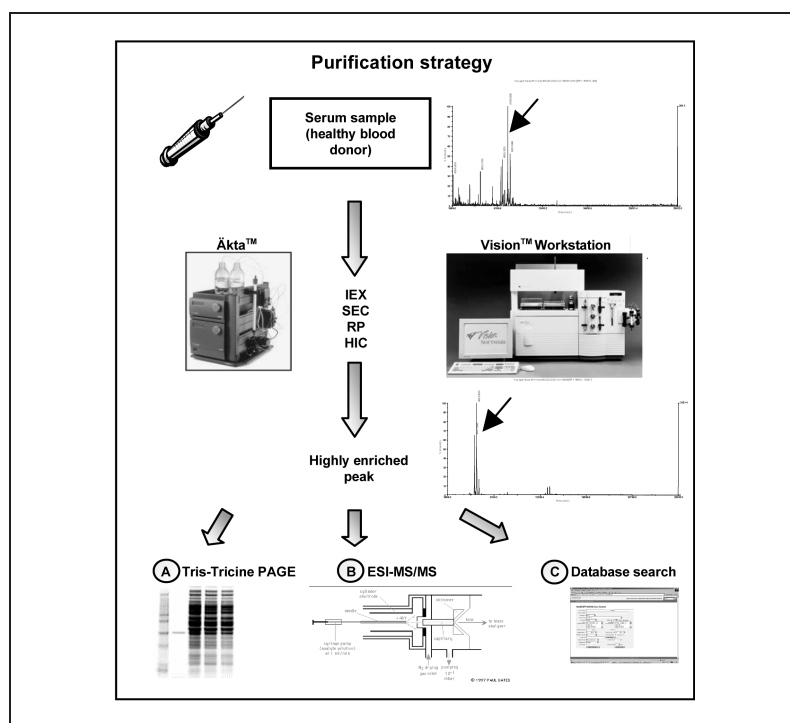


Figure 4: Various strategies for biomarker identification. (A) Protein of interest will be excised from the gel, digested and subsequently identified by mass spectrometry (MS) (peptide mass fingerprint). (B) Direct identification via electron spray ionisation (ESI)-MS/MS. (C) By knowing the exact molecular mass, a database search can be performed. Post-translational modification might be problematic
PAGE = polyacrylamide gel electrophoresis; IEX, ion exchange chromatography; SEC, size exclusion chromatography; RP, reversed phase chromatography; HIC, hydrophobic interaction chromatography.

MS-based technologies will revolutionise clinical diagnostics

clear that traditional technologies, such as 2D-PAGE, will not be suitable for leading diagnostics into the 21st century.

Although new MS-based technologies still have technical difficulties and other critical considerations to overcome, MS-based diagnostics have the potential to revolutionise molecular medicine. This technology is able to aid the systematic identification and characterisation of proteins as diagnostic and prognostic markers in tissue, serum and other body fluids. Using modern MS technologies, clinical tests can be developed that are practical, robust, accurate and inexpensive. As always, when dealing with the patient's life, safety and accuracy are of the highest importance. The road ahead for fulfilling these mandatory requirements will be fraught with

difficulties, but there is reason to be optimistic that these technologies will be of great advantage for every patient.

Acknowledgments

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