Proteomic patterns for cancer diagnosis—promise and challenges

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Proteomic patterns have been discovered for a variety of cancers and cancer related diseases. The platforms used have been both mass spectrometry and microarrays and the incorporation of computer informatics has resulted in innovative possibilities for novel diagnostics.

Introduction

The early diagnosis of cancer has been a research goal for many years in the hope early treatment will provide a positive outcome for patients. For many cancers such as ovarian cancer it is well known that early stage diagnosis is the key to survival.[1] However, the search for diagnostic markers for cancer has been and continues to be a long and sometimes frustrating struggle. The early promise of serum markers such as carcinoembryonic antigen (CEA)[2,3] has turned to disappointment as this marker and several others proved to be useful only for monitoring diagnosed patients for recurrence. Indeed, the only test that has been approved for screening is prostate specific antigen (PSA) for prostate cancer and even this marker has proven to be problematic.[4]

The concept of using more than one test in a multiplexed fashion is not new. It has been used for many years for thyroid testing, testing for Down's syndrome risk and cholesterol testing but has been limited in use for the diagnosis of cancer as is evident in the information provided by many clinical labs (www.labcorp.com, www.questdiagnostics.com/hcp/topics/genetictesting/mss_t1.html). Recent studies have suggested that even the performance of PSA may be improved when a panel of tests to determine total, free and complexed PSA is used as a screening test.[4,5] Up until now, the tests used to detect and monitor cancer have been either imaging tests or immunoassay tests. The immunoassay tests depend heavily on the specificity of the antibodies used for the performance of the test as well as the selection of the protein antigen to which the antibodies are raised and subtle differences in the markers are sometimes difficult to detect. Thus, panels of tests that involve variations in a molecule have not been easy to formulate.

The term proteomics has been around since 1994 but it was not until the late 1990's that the term became commonly used in the scientific literature.[6] Proteomics was defined as referring to "the study of the proteome using technologies of large-scale protein separation and identification". At about that time, several developments occurred in the areas of mass spectrometry, bioinformatics and computer design and function that came together and resulted in the first report of a proteomic pattern that had the potential for a diagnostic test technology.[7] Since that time, there have been several reports of the use of the technology in classification of samples through pattern analysis.[8–10] The development of various forms of microarrays for protein detection and quantitation[11] and further refinements and advancements in equipment, software, sample processing and understanding of the basis of some of these potential markers have led to an explosion of information in the area of proteomics. This article will focus on the incredible possibilities that these proteomic technologies present along with the equally incredible challenges faced in making them available to the clinical laboratory.

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Microarrays

The miniaturization and development of gene arrays provided a technological basis for a similar trend for proteins and peptides. These arrays still have many of the basic operating principles of traditional assays but the platforms allow for high throughput screening utilizing low volumes of reagents and test samples. This has become important especially in the investigation of the proteome in samples where very low volumes are available such as tumor cell lysates.

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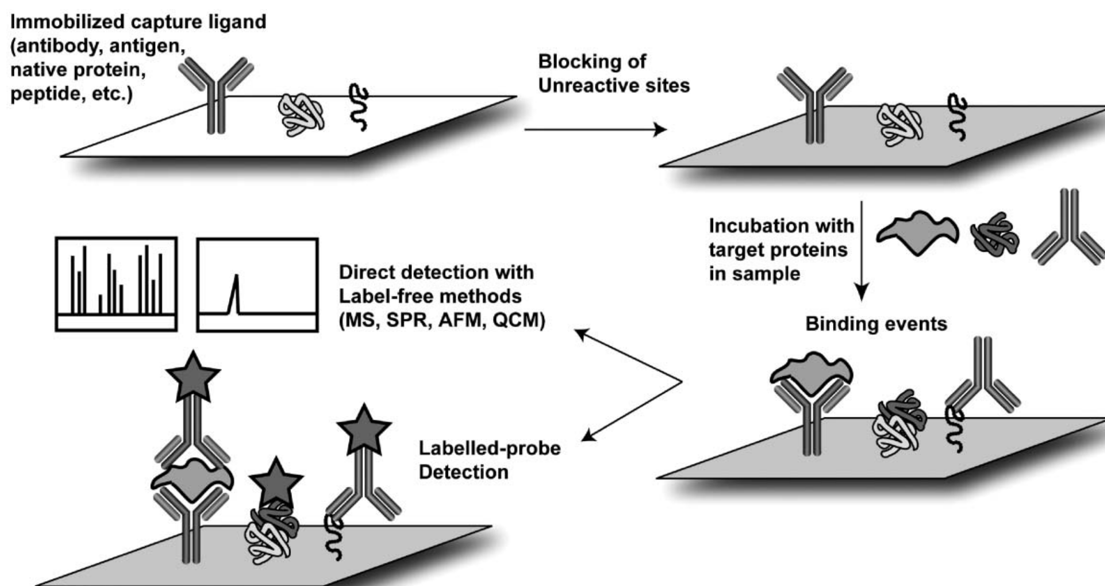

Fig. 1 Configurations of microarrays (adapted with permission from Cretich *et al*[12] copyright 2006, American Chemical Society). Immobilization of capture ligands on a surface in an array on an appropriate surface is followed by blocking, incubating with samples for a binding event to occur. The binding is then detected either with a variety of label free methods or with the addition of a secondary label such as a fluorescent label or an enzyme.

Arrays have been adapted for many purposes[12–14] and the general formats are outlined in Fig. 1. They have been made for the evaluation of protein function such as substrate activity, protein–drug interactions and epitope mapping studies. Protein detection arrays utilize affinity reagents such as antigens, aptamers or antibodies as binding reagents to study specificity of proteins. Reverse phase arrays generally use a single binding reagent to detect proteins or peptides in complex mixtures and can be quantitative thus providing both specificity and relative quantitative data for cell lysates, tissues and serum samples. While many of these techniques come directly from traditional clinical assays, there are some that have evolved into sophisticated and powerful methods. One such method involved the use of differential biopanning.[15] Using the antibodies in sera from clinically defined patients (cancer and normal), phage libraries were screened and clones selected based on differential binding to antibodies in the cancer patient's sera as compared to antibodies in normal sera. The cloned proteins are then arrayed and evaluated as potential biomarkers to which antibodies could be found in patients as markers of disease. Only with the microarray technique can this type of process be scaled up to the level where biomarker discovery becomes possible and this method has been used to find potential biomarkers for ovarian cancer.[16]

Advances in detection technologies have also contributed to the use of microarrays in proteomics.[17] Some of these techniques are label free such as surface plasmon resonance but others are the result of more sensitive substrates and detection methods as well as amplification techniques such as secondary binding events or enzyme cascades. The use of fluorescent Quantum Dots® for reverse microarrays showed great promise in detection in a second antibody/avidin–biotin amplified format (Fig. 2). This simple format allowed for lower abundance proteins to be detected in even smaller volumes and, in conjunction with quantitation, has given insight into protein pathways.[18]

The complex nature of proteins, their modifications during activation and the wide concentration range have presented substantial challenges to protein arrays that were not encountered with nucleic acid arrays. However, advances in the

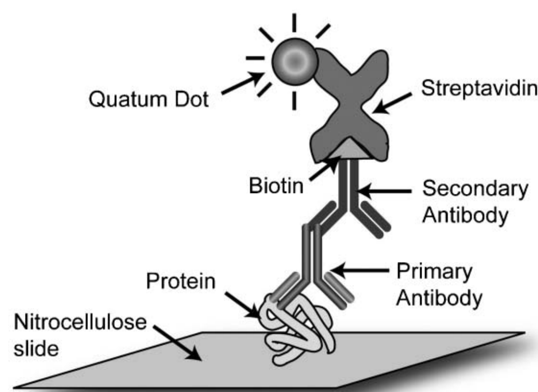

Fig. 2 Reverse microarray configuration using quantum dots for detection (adapted from Geho *et al*[18] copyright 2005 with permission from Elsevier). Sample is arrayed on a solid surface such as nitrocellulose. After probing with a specific primary antibody, a biotinylated second antibody is added. Strepavidin decorated with quantum dots is added as a sensitive label. This can then be detected with a high sensitivity CCD camera measuring the fluorescence of the sample and using image analysis a quantity can be estimated.

technology, manufacture of arrays and bioinformatics software to assist in the interpretation of data have led to their widespread use in the research setting. These small arrays will provide great contributions to our knowledge of disease and its diagnosis in the coming years through their use in the study of the human proteome.

LCM

The development of laser capture microdissection (LCM) in 1996[19] was a further advancement giving the ability to dissect cells of known pathological state from tissue. A commercial LCM system has been developed by Arcturus Bioscience, Inc. (www.arctur.com) and has made this technology widely available. The technique utilizes stained pathology slides and a coverslip with a laser activated adhesive. Cells of interest are identified by a pathologist and a laser is fired to activate the adhesive adjacent to the cell. When the coverslip is removed, the cells of interest are also lifted from the slide leaving behind the remaining cells. Using extracts from cancer cells and comparing them to neighboring normal cells, a pattern was seen that showed differences in the protein content and concentration of proteins between these two cell groups.[20] The technique of LCM has since spread to other researchers and, in conjunction with microarrays or mass spectrometry, has expanded our knowledge of the proteome within the cell.

SELDI-TOF

One of the key developments in the area of mass spectrometry was the commercialization of surface enhanced laser desorption ionization (SELDI) technology by Ciphergen (www.ciphergen.com). This technology incorporated the fractionation of samples on a surface that was then used as the target for matrix assisted laser desorption ionization (MALDI). In the commercialization process, Ciphergen developed a simple system that included a holder for arrays that was in the familiar 96 position microtiter plate format. This made sample processing simple and utilized equipment already in the biology laboratory. The Ciphergen instrument was also very simple to operate with software that was user friendly and included data analysis in formats such as a "gel view" that was also familiar to biologists. Although the data was of a lower resolution than larger mass spectrometers, the sensitivity was similar and the ease of use and familiarity of the entire system opened mass spectrometry to a whole new group of scientists who quickly began to explore the proteome using this tool.

Biologists soon learned that the combination of LCM and SELDI provided a powerful tool in exploring the proteome of cells. In 2000, a study of LCM from prostate cancer showed clear differences between cancer cells and neighboring cells by 2D gel electrophoresis. The difference was demonstrated by the dissection of cancer cells and normal cells from a single pathology slide and then solubilizing the cells. The resulting solution was then applied to the 2 dimensional gels.[20] Using LCM and SELDI, differences were also evident but the experiment was faster and easier to perform and the data was easier to analyze. Similar experiments to solubilize cells but then apply the solution to SELDI surfaces and generate mass spectra showed patterns that were different for several cancers including colon, breast and prostate.[21] The extension of this finding to serum was done using sera from groups of cancer patients and groups of patients who were known not to have disease at the time of blood draw. In a 2002 publication,[7] a series of sera from ovarian cancer patients and cancer free patients were examined by SELDI and the resulting spectra were used to train a computer algorithm to recognize the differences between these two groups. The pattern was then used to classify 116 additional patient samples—50 with cancer and 66 from cancer free women including women with benign disease such as ovarian cysts. The results of this study were remarkable with a 100% sensitivity and 95% specificity. It became very clear that the potential for diagnosis based on multiple parameters was possible but there was much to be done before it could be validated for use in patients. This was soon followed by similar findings for a wide range of diseases such as prostate cancer, breast cancer, renal cancer, and pancreatic cancer.[22–24] However, the controversy over this technology began almost immediately with questions being raised over the technology itself, its reproducibility, the bioinformatics used and the possibility of over-fitting, the potential bias in the samples as well as how this could possibly fit into a routine diagnostic lab.[25–28] It was clear that the technology was complex involving biology, mass spectrometry and computer bioinformatics and analysis and the interaction of these diverse areas had not been studied enough to say if it could be used for diagnostic purposes and studies regarding this continue today.

As biologists gained confidence in their ability to utilize mass spectrometry for proteomic investigations, so did they explore more complex instruments and systems for their research. The migration from the Ciphergen system to a hybrid higher mass resolution and accuracy system (the ABI Q-Star with a Ciphergen source) gave even richer data to mine. Indeed, this system demonstrated that it could give absolute classification in an ovarian cancer *vs.* cancer free serum sample set.[29] Such a finding raised hopes even more that a mass spectrometry pattern could be used as a diagnostic test. However, the issues of reproducibility, robustness and consequences of a misclassification raised concerns especially when considering a new technology.[28]

MALDI

As biologists became more familiar with mass spectrometry platforms and began to investigate the source of patterns, many began venturing beyond the world of SELDI into platforms that were more complex and with potential for both rich data mining and peptide isolation and identification. With the MALDI systems, the sample fractionation process takes place before presentation to the surface that is interrogated by the mass spectrometer whereas the SELDI system uses the same surface for fractionation and mass spectrometer interrogation. Thus the fractionation possibilities for MALDI are more complex but varied and have potential for concentration of low abundance materials. One of the significant findings discovered with some of these techniques was that diagnostic information could be found associated with carrier proteins such as albumin.[30]

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This discovery also helped explain the principle of what had been seen earlier in SELDI experiments where patterns were most clearly observed when undiluted sera were used as the sample.[7,29] It appeared that albumin, the major protein in serum, was "stuck" to surfaces rather than being specifically bound. Not only did this explain the source of the diagnostic ions but also it explained why many of these low molecular weight markers were not excreted in the urine and were not too low in abundance for detection. Many investigators had depleted albumin from samples before looking for diagnostic biomarkers. However, this appeared to be eliminating a rich source of potential markers.[31] A system for capturing carrier proteins and examining their cargo for potential markers has recently been introduced into the research market. The BioExpression kits produced by PerkinElmer (www.perkinelmer.com) utilize Cibachrome blue immobilized on a microwell plate to capture albumin from serum. Following washing, the albumin and its cargo is eluted in a basic solution. The peptides and peptide fragments are then captured on a C18 reverse phase surface in a Zip plate and eluted with matrix to a disposable MALDI source (Fig. 3). This is then read on the mass spectrometer to yield high resolution and high content spectra that can be examined for patterns. Using this system, a proteomic pattern was discovered for Alzheimer's disease[32] thus opening up the possibility of a diagnostic test that could detect early stage disease when treatment intervention is most useful. Patterns for cancers such as ovarian cancer and cutaneous t-cell lymphoma are currently being investigated using this system (PerkinElmer, personal communication). Therefore, the study of this part of the proteome is raising the hope for diagnostic markers and tests for those diseases where none currently exists beyond traditional biopsy.

Identification of biomarkers

Proteomic patterns have been shown to exist for several diseases. However, the use of mass spectrometry as a routine clinical tool for diagnosis of diseases such as cancer, cardiovascular disease, Alzheimer's disease and other complex diseases is still a long way off. In the view of some scientists, it is necessary to know the identity of the diagnostic ions in order to associate them with disease.[25] With all of the issues surrounding the technology,[28] it appeared that the path of least resistance was to identify the peptides or peptide fragments and utilize this knowledge to develop panels of traditional tests where many of the potentially problematic factors are understood.

Some investigators have capitalized on the fact that mass spectrometry patterns indicating a panel of peptides reflecting differences in the proteome can be used to classify disease and have adapted this approach using traditional immunoassays. This has been done for ovarian cancer where the sensitivity and specificity of CA125 (the traditional assay for monitoring disease) in detecting disease could be improved with the addition of three more assays.[33] Another approach was to survey currently available research assays thought to be associated with a particular disease and use them in a panel approach. The results showed promise with a combination of leptin, prolactin, osteopontin and insulin-like growth factor-II (IGF-II) giving a sensitivity and specificity of 95% in this pilot study of 86 women.[1]

The identification of the peptides or proteins within the proteome that are the basis of a potential diagnostic pattern is not a trivial matter. While the ion values composing the pattern are known and can guide discovery, the complexity of serum is too great for direct identification. Therefore, fractionation is the first necessary step. This can be done with a variety of techniques or combination of techniques such as 2D gels and liquid chromatography or newer techniques such as free flow electrophoresis that allow for concentration of low abundance proteins and peptides.[34,35] The identification can be done using either a targeted approach (looking for a protein that fits a particular ion value) or identification of all peptides in the mixture and then selecting the one(s) of interest and can be done by liquid chromatography and tandem mass spectrometry (LC-MS/MS).[36] The development of small but powerful computers along with large databases has allowed for analysis of more complex data to give identifications not possible a few years ago. The identification strategies have evolved along with highly sophisticated and ultra-high resolution instruments such as TOF-TOF and Fourier transform ion cyclotron resonance machines, fractionation equipment and software and databases.[37] All of these provide scientists with tools for exploring and defining the proteome.

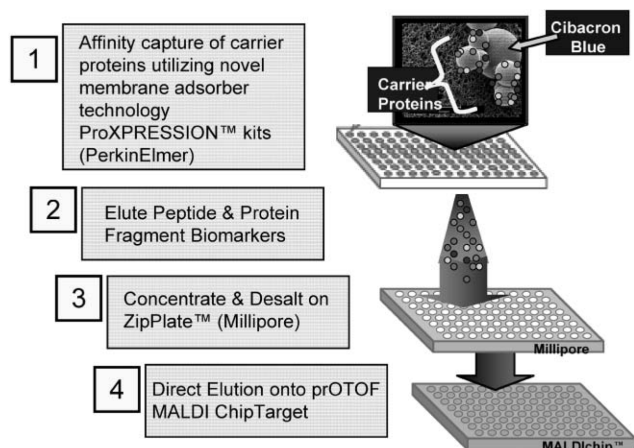

Fig. 3 Procedure for ProXpression® Peptide Fractionation (Courtesy of Dr Mary Lopez, PerkinElmer Life Sciences).

Bioinformatics

The evolution of proteomic software has come out of many of the pattern recognition techniques that were originally developed for other purposes. The initial patterns published were based on genetic algorithms and self-organizing maps.[7] Since this time, several other techniques have confirmed the presence of patterns and these techniques were different in their approach. Well-exercised machine learning software such as decision trees (www.spss.com), the

support vector machine (Chih-Chung Chang and Chih-Jen Lin, LIBSVM: a library for support vector machines, 2001. Software available at http://www.csie.ntu.edu.tw/~cjlin/libsvm) and partial least square regression (www.sas.com) have also been used to confirm that patterns do exist. Recently, these three methods have been used in a "voting" scheme for classification giving further confidence in the results.[38] In cases where the complexity of the spectra are too great for the computer, visualization tools can be used to guide the computer and parse data for processing.[39] Such an approach was used for a particularly complex data set and yielded promising results in the detection of a pattern in the serum proteome following radiation exposure.[40]

For biologists interested in proteomic patterns, some instrumentation include bioinformatics software as part of a system package. The Ciphergen Biomarker Wizard (www.Ciphergen.com) package and the PG600 software from PerkinElmer (www.perkinelmer.com) are examples of pre-packaged bioinformatics software that are relatively simple to operate and provide the tools for biomarker discovery.

The future

The use of proteomic patterns as potential diagnostic technology is evolving with the strides that are being made in the areas of understanding patient sample acquisition and sampling, sample processing, sample fractionation and preparation, robotic processing, mass spectrometry, bioinformatics and data analysis and interpretation. However, there is much to be done before these techniques can come to the clinical lab. The basics of the source of the diagnostic ions must be confirmed and validated. Understanding the principle of the test procedure is a necessary part of any clinical test. In addition, there are many factors that must be addressed before mass spectrometry technology can be considered for clinical use (Table 1). Meanwhile, the pattern approach is being adapted to and used in traditional immunoassays some of which have been incorporated into microarrays.[1,33] This approach is one that could allow transition to the clinical laboratory because the technology is better understood. However, the manufacturing issues of a multiplexed assay system are challenging and these will need to be resolved. The combination of the microarray approach using binding partners such as antibodies along with mass spectrometry is an exciting possibility. As shown in Fig. 1, antibodies would be used to capture proteins or peptides of interest when they could be detected by such methods as surface plasmon resonance.[41] MALDI interrogation of the bound protein or peptide could then be used to "fine tune" the specificity of the antibody binding event. This could be used to detect subtle differences in disease such as phosphorylation events that are difficult to detect using antibodies but are detectable by mass spectrometry.

The combination of proteomic techniques—mass spectrometry, microarrays and bioinformatics—will not only provide powerful diagnostic information but could provide insight into disease mechanisms. All of this needs enormous development but the results for patient care will be substantial.

Table 1 Recommended practices for clinical applications of protein profiling by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. (Adapted from Hortin[28]. Reprinted with permission. Copyright 2005, AACC)

Preanalytical
Evaluate optimum patient preparation
Identify optimum procedures for specimen collection and processing
Analyze specimen stability
Develop criteria for specimen acceptability
Analytical
Prepare calibrators for mass, resolution, and detector sensitivity
Use internal standards
Automate specimen preparation
Optimize methods to yield highest possible signals for peaks of interest
Identify sequences of peaks of interest
Develop calibration materials for components of interest
QC: prepare/identify at least two concentrations of control material
Evaluate reproducibility (precision)
Evaluate limits of detection and linearity
Evaluate reference intervals
Evaluate interferences such as hemolysis, lipemia, renal failure, acute-phase responses
Develop materials or programs for external comparison/proficiency testing of analyzers
Postanalytical
Analyze each spectrum to identify peaks before applying diagnostic algorithms
Develop criteria for the acceptability of each spectrum based on peak characteristics
Use peaks rather than raw data as the basis for diagnostic analysis
Use caution in interpretation of peaks with $m/z < 1200$
Select peaks with high intensities and sample stability for diagnosis
Select approximately equal numbers of peaks that increase and decrease in intensity as diagnostic discriminators
In developing a training set for diagnosis, careful clinical classification of patients is essential
Clinical validity depends on having a typical rather than highly selected population of patients
The number of training specimens should be at least 10 times the number of measured values
Any clinical application should use a fixed training set and algorithm for analysis
Any analysis should provide a numerical value
Diagnostic performance should be evaluated with ROC curves to select cutoffs
A sensitivity analysis should be performed of the necessary precision for accurate diagnostic performance
There should be QC procedures for daily verification of software performance

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References

1 G. Mor, I. Visintin, Y. Lai, H. Ahao, P. Schwartz, T. Rutherford, L. Yue, P. Bray-Ward and D. C. Ward, Serum protein markers for early detection of ovarian cancer, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 7677–7682.
2 H. M. Meyer, Status of the CEA test, *Hum. Pathol.*, 1973, **4**, 298.
3 D. P. Stevens, I. R. Mackay and K. J. Cullen, Carcinoembryonic antigen in an unselected elderly population: a four year follow up, *Br. J. Cancer*, 1975, **32**, 1477–151.

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4 R. Lee, A. R. Localio, K. Armstrong, S. B. Malkowicz and J. S. Schwartz, A meta-analysis of the performance characteristics of the free prostate-specific antigen test, *Adult Urol.*, 2006, **67**, 762–768.
5 R. J. Babaian, Y. Naya, C. Cheli and H. A. Fritsche, The detection and potential economic value of complexed prostate specific antigen as a first line test, *J. Urol.*, 2006, **175**, 897–901.
6 G. Abbott, Proteomics, transcriptomics; what's in a name?, *Nature*, 1999, **202**, 715–716.
7 E. F. Petricoin, A. M. Ardekani and B. A. Hitt, Use of proteomic patterns in serum to identify ovarian cancer, *Lancet*, 2002, **359**, 572–7.
8 K. R. Kozak, M. W. Amneus, S. M. Pusey, F. Su, M. N. Loung, S. A. Loung, S. T. Reddy and R. Farias-Eisner, Identification of biomarkers for ovarian cancer using strong anion-exchange ProteinChips: Potential use in diagnosis and prognosis, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 12343–12348.
9 D. K. Ornstein, W. Rayford and V. A. Fusaro, Serum proteomic profiling can discriminate prostate cancer from benign prostates in men with total prostate specific antigen levels between 2.5 and 15.0 ng ml^{-1}, *J. Urol.*, 2004, **172**, 1302–5.
10 J. Koopmann, Z. Zhang and N. White, Serum diagnosis of pancreatic adenocarcinoma using surface-enhanced laser desorption and ionization MS, *Clin. Cancer Res.*, 2004, **10**, 860–8.
11 L. L. Liotta, V. Espina, A. I. Mehta, V. Calvert, K. Rosenblatt, D. Geho, P. J. Munson, L. Young, J. Wulfkuhle and E. F. Petricoin, Protein microarrays: Meeting analytical challenges for clinical applications, *Cancer Cell*, 2003, **3**, 317–325.
12 M. Cretich, F. Damin, G. Pirri and M. Chiari, Protein and peptide arrays: Recent trends and new directions, *Biomol. Eng.*, 2006, **23**, 77–88.
13 Y. Hu, M. Uttamchandani and S. Q. Yao, Microarray: A versatile platform for high-throughput functional proteomics, *Comb. Chem. High Throughput Screening*, 2006, **9**, 203–212.
14 S. F. Kingsmore, Multiplexed protein measurement: technologies and applications of protein and antibody arrays, *Nat. Rev. Drug Discovery*, 2005, **5**, 310–321.
15 J. E. Nowak, M. Chatterjee, S. Mohapatra, S. C. Dryden and M. A. Tainsky, Direct production and purification of T7 phage display cloned proteins selected and analyzed on microarrays, *BioTechniques*, 2006, **40**, 220–227.
16 M. Chatterjee, S. Mohapatra, A. Ionan, G. Bawa, R. Ali-Fehmi, X. Wang, J. Nowak, B. Ye, F. A. Nahhas, K. Lu, S. S. Witkin, D. Fishman, A. Mankarah, R. Morris, N. K. Levin, N. N. Shirley, G. Tromp, J. Abrams, S. Braghici and M. A. Tainsky, Diagnostic markers of ovarian cancer by high-throughput antigen cloning and detection on arrays, *Cancer Res.*, 2006, **66**, 1181–1190.
17 V. Espina, E. C. Woodhouse, J. Wulfkuhle, H. D. Asmussen, E. F. Petricoin and L. A. Liotta, Protein microarray detection strategies: focus on direct detection technologies, *J. Immunol. Methods*, 2004, **290**, 121–133.
18 D. Geho, N. Lahar, P. Gurnani, M. Huebschman, P. Herrmann, V. Espina, A. Shi, J. Wulfkuhle, H. Garner, E. Petricoin, L. A. Liotta and K. P. Rosenblatt, Pegylated, streptavidin-conjugated quantum dots are effective detection elements for reverse-phase protein microarrays, *Bioconjugate Chem.*, 2005, **16**, 559–566.
19 M. R. Emmert-Buck, R. F. Bonner, P. D. Smith, R. F. Chauqui, Z. Zhuang, S. R. Goldstein, R. A. Weiss and L. A. Liotta, Laser capture microdissection, *Science*, 1996, **274**, 998–1001.
20 D. K. Ornstein, J. W. Gillespie, C. P. Paweletz, P. H. Buray, J. Herring, C. D. Vocke, S. L. Topalian, D. G. Bostwick, W. M. Linehan, E. F. Petricoin and M. R. Emmert-Buck, Proteomic analysis of laser capture microdissected human prostate cancer and in vitro prostate cell lines, *Electrophoresis*, 2000, **21**, 2235–2242.
21 C. P. Paweletz, J. W. Gillespie, D. K. Ornstein, N. L. Simmone, M. R. Brown, K. A. Cole, Q-H. Wang, J. Huang, N. Hu, T. T. Yipe, W. E. Rich, E. C. Kohn, W. M. Linehan, T. Weber, P. Taylor, M. R. Emmert-Buck, L. A. Liotta and E. F. Petricoin, Rapid protein display profiling of cancer progression directly from human tissue using a protein biochip, *Drug Dev. Res.*, 2000, **49**, 34–42.
22 Z. Zhang, R. C. Bast and Y. Yu, Three biomarkers identified from serum proteomic analysis for the detection of early stage ovarian cancer, *Cancer Res.*, 2004, **4**, 5882–90.
23 D. K. Ornstein, W. Rayford and V. A. Fusaro, Serum proteomic profiling can discriminate prostate cancer from benign prostates in men with total prostate specific antigen levels between 2.5 and 15.0 ng ml^{-1}, *J. Urol.*, 2004, **172**, 1302–5.
24 J. Koopmann, Z. Zhang and N. White, Serum diagnosis of pancreatic adenocarcinoma using surface-enhanced laser desorption and ionization MS, *Clin. Cancer Res.*, 2004, **10**, 860–8.
25 E. F. Diamandis, Analysis of serum proteomic patterns for early cancer diagnosis: Drawing attention to potential problems, *J. Nat. Cancer Inst.*, 2004, **96**, 353–356.
26 K. A. Baggerly, J. S. Morris and K. R. Coombes, Reproducibility of SELDI-TOF protein patterns in serum: Comparing datasets from different experiments, *Bioinformatics*, 2004, **20**, 777–785.
27 L. A. Liotta, M. Lowenthal, A. Mehta, T. P. Conrads, T. D. Veenstra, D. A. Fishman and E. F. Petricoin, Importance of communication between producers and consumers of publicly available experimental data, *J. Nat. Cancer Inst.*, 2005, **97**, 310–314.
28 G. L. Horton, Can mass spectrometric protein profiling meet desired standards of clinical laboratory practice?, *Clin. Chem.*, 2005, **51**, 3–5.
29 T. P. Conrads, V. A. Fusaro, S. Ross, D. Johann, V. Rajapakse, B. A. Hitt, S. M. Steinberg, E. C. Kohn, D. A. Fishman, G. Whiteley, J. C. Barrett, L. A. Liotta, E. F. Petricoin and T. D. Veenstra, High-resolution serum proteomic features for ovarian cancer detection, *Endocr. Relat. Cancer*, 2004, **2**, 163–178.
30 A. Mehta, S. Ross and M. S. Lowenthal, Biomarker Amplification by Serum Carrier Protein Binding, *Dis. Markers*, 2004, **19**, 1–10.
31 L. A. Liotta, M. Ferrari and E. Petricoin, Clinical proteomics: written in blood, *Nature*, 2003, **425**, 6961, 905.
32 M. F. Lopez, A. Mikulskis, S. Kuzdzal, D. A. Bennett and J. Kelly, High-Resolution Serum proteomic Profilino of Alzheimer Disease Samples Reveals Disease-Specific, Carrier-Protein-Bound Mass Signatures, *Clin. Chem.*, **51**, 1946–1954.
33 A. J. Rai, Z. Zhang, J. Rosenzweig, L-M. Shih, T. Pham, E. T. Fung, L. J. Sokoll and D. W. Chan, Proteomic approaches to tumor marker discovery: Identification of Biomarkers for Ovarian Cancer, *Arch. Pathol. Lab. Med.*, 2002, **126**, 1518–1526.
34 R. L. Moritz and R. J. Simpson, Liquid-based free-flow electrophoresis reversed-phase HPLC: a proteomic tool, *Nat. Methods*, 2005, **2**, 863–873.
35 Y. Wang, W. S. Hancock, G. Weber, C. Eckerskorn and D. Palmer-Toy, Free flow electrophoresis coupled with liquid chromatography-mass spectrometry for a proteomic study of the human cell line (K562/CR3), *J. Chromatogr., A.*, 2004, **1053**, 269–278.
36 L. J. Zimmerman, G. R. Wernke, R. M. Caprioli and D. C. Liebler, Identification of protein fragments as pattern features in MALDI-MS analyses of serum, *J. Proteome Res.*, 2005, **4**, 1672–1680.
37 G. Siuzdak, *The Expanding Role of Mass Spectrometry in Biotechnology*, MCC Press, San Diego 2003; ISBN 0-9742451-0-0.
38 C. Belluco, E. Petricoin, C. Peschle, C. Liu, M. Lowenthal, D. Johann, L. Lasebikan, S. Ross-Rucker, M. Lise, C. DiMaggio, E. Mammano, F. Facchiano, D. Nitti, E. Garaci, G. B. Mills and G. Whiteley, LMW protein fragments may detect early stage breast cancer, *Cancer Res.* (submitted for publication).
39 D. J. Johann, M. D. McGuigan, S. Tomov, V. A. Fusaro, S. Ross, T. P. Conrads, T. D. Veenstra, D. A. Fishman, G. R. Whiteley, E. F. Petricoin and L. A. Liotta, Novel approaches to visualization and data mining reveals diagnostic information in the low amplitude region of serum mass spectra from ovarian cancer patients, *Dis. Markers*, 2003–2004, **19**, 197–207.
40 C. Menard, D. Johann, M. Lowenthal, T. Muanza, M. Sproull, S. Ross, J. Gulley, E. Petricoin, C. N. Coleman, G. Whiteley, L. Liotta and K. Camphausen, Discovering clinical biomarkers of ionizing radiation exposure with serum proteomic analysis, *Cancer Res.*, 2006, **66**, 1844–1850.
41 D. Nedelkov and R. W. Nelson, Surface plasmon resonance mass spectrometry: recent progress and outlooks, *Trends Biotechnol.*, 2003, **21**, 301–305.

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