Proteomics Approaches: New Technologies And Clinical Applications In Breast Carcinomas

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Abstract

Breast cancer is the most diagnosed cancer in women, accounting for approximately 40,000 deaths annually in the USA. In Tunisia, the incidence of breast cancer is approximately 19 new cases per 100,000 women per year. Significant advances have been made in the areas of detection and treatment, but a significant number of breast cancers are detected late. The advent of proteomics provides the hope of discovering novel biological markers that can be used for early detection, prognosis, diagnosis, and therapy. Several proteomics technologies have been used to uncover molecular mechanisms associated with breast carcinoma at the global level to discover protein patterns that distinguish disease and disease-free states with high sensitivity and specificity. Two dimensional gel electrophoresis coupled with mass spectrometry constitute a new proteomics-based paradigm for detecting disease in pathology specimens and monitoring disease response to therapy. This review describes these proteomics technologies and their application in the analysis of breast carcinoma.

Keywords

Breast cancer, Proteomics, Markers, 2-DE, MALDI-TOF

Introduction

Breast cancer is a major health problem and one of the leading causes of death among women worldwide. Its incidence is steadily rising in developing countries. In Tunisia, the incidence of breast cancer is approximately 19 new cases per 100,000 women per year¹. Invasive carcinomas represent 70-80% of all breast cancer and among these, infiltrating ductal carcinomas (IDCA) are the most aggressive forms and have a poor prognosis². Histopathologically identical breast cancers show a different biological behavior in terms of aggressiveness, progression, and response to therapy. Thus, there is a great need for new breast cancer biomarkers that might help detect this cancer at an earlier stage, to uncover prognostically distinct subclasses, and to provide best individual treatment². Currently, the search for specific cancer-related alterations are largely focused on clinically relevant biological fluids such as serum and tissue².

Proteomics with the recent advances in mass spectrometry is considered as a powerful analytical method for deciphering protein expression alterations as a function of disease progression². Recently, proteomics-based analyses of breast serum and tissue lysates have resulted in the finding of a number of potential tumor biomarkers providing a basis for a better understanding of breast-cancer development and progression, and eventually serving as diagnostic and prognostic markers³. Probably the most widely used proteomic technology is the identification of alterations in protein expression between two different samples through comparative two-dimensional gel electrophoresis (2-DE) which provides high-resolution separation of proteins and offers a powerful method for their identification and characterization²,³. In such investigation a biomarker is defined as a protein having more or less intensity on one gel compared with the
other and should be particularly associated with the disease.

The aim of this review is to illustrate the proteomic technologies that have emerged for comprehensive and high-throughput protein analysis (two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS)) and to provide more details of their application in breast cancer research.

**The Proteomic Tools**

Proteomic analysis involves the study of complex mixtures of proteins and their interactions. The term proteome dates back to 1994 when Marc Wilkins defined it as “the total protein content of a genome”. Proteomic analysis can be viewed as an experimental approach to explain the information contained in genomic sequences in terms of the structure, function, and control of biological processes and pathways. Therefore, the proteome reflects the cellular state or the external conditions encountered by a cell. In addition, proteomic analysis can be viewed as a genome-wide assay to differentiate distinct cellular states and to determine the molecular mechanisms that control them. It is now generally recognized that expression analysis directly at the protein level as well as chemical modifications of expressed proteins are necessary to unravel the critical changes that occur as part of disease pathogenesis. Quantitative proteomic analyses can be used to identify the protein content in complex samples such as serum, plasma, cell and tissue extracts or subcellular fractions, and to determine the quantitative difference in abundance for each polypeptide contained in different samples. It is expected that the proteomic profiling patterns resulting from such analyses define comprehensive molecular signatures in health and disease. Analyses of the proteomic profiles would impact a wide range of biological and clinical research questions, such as the systematic study of biological processes and the discovery of clinical biomarkers for detection, diagnosis, and assessment of treatment outcome. The exploitation of a proteomic approach for the study of different diseases has led to the hypothesis that multiple biomarkers or a panel of biomarkers shown by proteomic profiling may correlate more reliably with a specific disease than a single biomarker or protein.

Biomarkers can be defined as cellular, biochemical, and molecular alterations by which normal, abnormal, or simply biologic processes can be recognized or monitored. These alterations should be able to objectively measure and evaluate normal biological process, pathogenic processes (like cancer), or pharmacologic responses to a therapeutic intervention. Therefore, proteomic profiling is valuable in the discovery of biomarkers as the proteome reflects both the intrinsic genetic program of the cell and the impact of its immediate environment. Protein expression and function are subject to modulation through transcription as well as through translational and post-translational events. In addition, biomarkers can be subtle changes in molecular structures, for instance alterations of post-translation modifications, which often can only be examined at the protein level.

Currently investigators are pursuing three different approaches to develop a technology to study biomarkers with increased sensitivity and specificity. The first is to improve on currently used or known biomarkers. The second approach is to discover and validate novel biomarkers with greater sensitivity and specificity. The third approach is to use a panel of biomarkers, either by combining several individually identified biomarkers or by using mass spectrometry to identify a pattern of protein peaks in sera that can be used to predict the presence of cancer. Expression pattern of a known biomarker or correlation of expression of several known biomarkers can be a valuable research and clinical tool for monitoring or treatment of cancer progression. High-throughput proteomic methodologies have the potential to revolutionize protein biomarker discovery and would allow for multiple proteins markers to be assayed simultaneously. With the significant advances in 2-DE and mass spectrometry, protein biomarker discovery has become one of the central applications of proteomics.
Two-Dimensional Polyacrylamide Gel Electrophoresis and Mass Spectrometry

During the early years of proteomics and until recently, profiling of protein expression in cancer relied primarily on the use of two-dimensional polyacrylamide gel electrophoresis (2-DE), which was later combined with mass spectrometry (MS). Most studies followed an approach in which a cocktail was used to solubilize the protein contents of an entire cell population, tissue or biological fluid (serum, plasma), followed by separation of the protein contents of the lysate using 2-DE gels and visualization of the separated proteins using silver staining. This approach is used to find new biomarkers and treatment targets for various disease conditions, including breast carcinomas\(^{2,4,6}\).

2-DE

2-D poly-acrylamide gel electrophoresis (2-DE) is a powerful technique for protein separation, first according to pH (isoelectric point) and then according to size (molecular weight). Electrophoresis gels are stained with colloidal Coomassie Brilliant Blue G (G-250), silver stain or a fluorescent stain. Coomassie brilliant blue stain will detect protein amounts greater than 100 ng; silver stain can detect proteins in the 1 ng range. Commonly between 3000 and 10000 spots can be detected on large gels (Figure 1). The digestion of spots, obtained by gel excision, directly in the gel makes it possible to further analyse proteins with Mass Spectrometry. Peptide spectra obtained in this way can be used to search protein sequence databases (for example, NCBI and SWISSPROT) for identification. Since the technique has been used for over 20 years, there is potentially a vast amount of information on proteins in different databases, but exploitation is hampered by difficulties with large-scale data analysis. Comparison of the results of only two experiments for a differential expression profile can be difficult.

Mass Spectrometry: Matrix-Assisted Laser Desorption/Ionization (MALDI-TOF/MS)

For further protein analysis, protein spots have to be excised from the 2-DE gels for identification by peptide-mass fingerprinting in a mass spectrometer. For this procedure, Matrix-Assisted Laser Desorption Time-Of-Flight (MALDI-TOF) MS is commonly applied. This technique is highly specific and sensitive to the atomic level.

MALDI-TOF is a user-friendly technique to identify proteins by peptide mass fingerprinting. In this technique the masses of tryptic peptides, generated after enzymatic degradation of gel-separated proteins, are measured at high accuracy (100 ppm). The mixture is then sublimated with a laser, electrical charges are conferred to the molecules and the sample ions are then introduced in an analyzer that resolves the fragments on the basis of their mass-to-charge \((m/z)\) ratio. Mass spectra allow identification of proteins by matching the experimental calculated peptide masses with spectra deposited in a protein database\(^7\).

Fig. 1: Example of 2-DE separation. Total protein extracts from the MCF-7 breast cell line were separated by 2-DE and visualized by silver staining\(^{21}\).
When MALDI-TOF fails to unequivocally identify a protein, ESI-MS/MS can be used, a powerful separation tool which, even in the presence of many other co-detected peptides, can select, isolate, fragment and sequence a single peptide ion. ESI methodology generates intact molecular ions of molecules directly from solution by coupling a high-performance liquid chromatography (HPLC) column directly to the mass spectrometer. Further improvements in instrumentation, such as the triple quadrupole analyzers with robust ion sources and fast scanning mass analyzers allow the identification and quantification of protein with great sensitivity in a highly reproducible manner.

Examples of proteome analysis in breast carcinomas

Plasma samples

The accessibility of blood samples and the routine drawing of blood for other analyses make the use of plasma and blood ideal candidates for the identification of biomarkers for clinical studies. One of the studies to suggest that 2-DE could be used to distinguish protein spot patterns between disease states and control was by Chahed et al. Plasma was compared between blood donor controls and from Tunisian patients with breast carcinoma. Despite the complexity of the protein profiles, differences were detected between disease and control samples.

Several proteins were up-regulated in all of the breast cancer samples compared to that of healthy controls. The majority of the protein identifications appeared to represent differences in overall abundance. 2-DE investigations showed elevated levels of acute phase proteins such as haptoglobin (β-chain), serum amyloid P, α1-antitrypsin, α1-antichymotrypsin and α1-acidic glycoprotein in plasma from patients diagnosed with breast cancer (Figure 2). Two other proteins, highly elevated in cancer plasma, were identified as Retinol Binding Protein (RBP) and transthyretine (TTR).

Tissue samples

Separation and analysis of proteins from tissue samples and breast tumour biopsies have proved very successful in identifying novel markers. In our laboratory, Kabbage et al. successfully identified the α-B crystallin, Hsp27 and MnSOD, which were elevated in breast tumor samples. The molecular chaperone HSP27 and α-B-crystallin, which is a small heat shock protein (HSP) are two dysregulated proteins in tumor tissues. The concomitant upregulation of these HSPs together with α-B-crystallin and HSP27 is not surprising since chaperones are thought to work cooperatively to fulfill their functions. Due to the capacity of HSPs to prevent stress-accumulated, unfolded, and nascent protein aggregation, their expression has proven to have important pathological implications such as cell proliferation and disease prognosis. The HSP27 is a molecular chaperone whose rate of synthesis increases many folds in response to environmental stress and during malignant transformation. Although no evidence of post-translational alterations was pointed out, these isoforms as reported in renal cell carcinomas might reflect phosphorylation or other post-translational modifications. The role of α-B-crystallin in cancer pathology has been widely discussed with regard to its potential oncogenic role. Previous studies unveiled that this small HSP may constitute a good target for modulating cell death pathways. Its expression has been shown to inhibit both the mitochondrial and the receptor death activation pathways of caspase 3 and correlates with TRAIL resistance in a panel of cancer cell lines. This protein may also be an interesting molecular target for exploring the evolution and the origin of breast tumors since higher α-B-crystallin levels were reported in ductal carcinoma in situ, which is an earliest form of detectable breast cancer. The data reported herein appear to confirm this for invasive carcinomas as well. Although further studies are needed to answer how this oncoprotein contributes to breast cancer, the data reported herein highlight the importance of this molecular chaperone in invasive carcinomas as a biomarker that may play a distinctive role in the process of carcinogenesis. The MnSOD is a mitochondrial enzyme that has been reported to protect cells against oxidative stress by increasing the dismutation rate of superoxide anion (O−2).
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to hydrogen peroxide (H2O2) which is then converted into water by catalase and glutathione peroxidase. The role of this antioxidant enzyme in carcinogenesis is still however controversial and unclear. In fact although, it has been reported to suppress apoptosis and protect cells against several insults, under some circumstances, the Mn-SOD may prevent cell proliferation\(^{(18)}\). Although further studies are needed, the present elevation of Mn-SOD may indicate that the

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**Fig. 2**: Two dimensional gel electrophoresis analyses of plasma proteins derived from (A) a healthy donor and (B) a breast cancer patient. Partial 2-DE images from a control gel (A) and from a breast cancer sample (B) are shown. Abbreviation: STF: serotransferrin; ALB: albumin; ACT: anti-chymotrypsin; ATR: anti-trypsin; AGP: acidic glycoprotein; Hp: haptoglobin; Fb: fibrinogen beta chain; ApoAI: ApoAI lipoprotein; SAP: serum amyloid P; RBP: retinol binding protein\(^{(9)}\).
antioxidant defense system has been stimulated in invasive carcinomas of the breast, highlighting the ability of tumor cells to prevent damage due to reactive oxygen species.

Utility and recent advancements in the proteomics approaches

In recent years, the combination of 2-DE and MS has been utilized extensively for proteomics research in medicine. The power of the 2-DE-based technology was recognized by the research community early on, and scientists from various disciplines were attracted to the field of proteomics.

The information obtained by the 2-DE-based approach is high because a number of specific protein attributes can be determined. Thousands of proteins can be resolved and visualized simultaneously on a single 2-D gel; for each protein, the isoelectric point, MW, and the relative quantity can be measured (Figure 2). With MS, each protein can be characterized via a unique peptide-mass fingerprint and/or amino-acid sequence tag (Figure 3).

High-resolution capabilities of 2-DE allow the separation and detection of post-translationally modified proteins. In many instances, post-translationally modified proteins can be readily located in 2-D gels because they appear as distinctive horizontal or vertical clusters of spots. In addition, modified proteins can be revealed by MS analysis, when multiple spots of the same protein are identified.

In terms of equipment, the 2-DE-based technology is well suited for research conducted in an academic setting. Most scientists engaged in biological research are familiar with one-dimensional gel electrophoresis; 2-DE, while more complex and labor-intensive, is a natural extension of their expertise. In addition, 2-DE equipment is relatively inexpensive and can therefore be supported by individual project grants. Access to other essential components,

A.

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RHFQWQDEPP  QSPWDRVKDL  ATVYVDVLKD  SGRDYVSQFE  GSALGKQLNL
KLDDNWSVT  STFSKLREQL  GFPTQEFWDN  LKEKETGRLQ  EMDKDLQKV
AKVQPYLDDF  QKKEWQEEMEL  VRIQKVPEMLRA  ELMQEGARQKI  HELQEQKLSPL
GEEMRDRARA  HVDALRTHLA  PYSDELRORL  AARLEALKEN  GGARLAEYHA
KATEHLSTLS  EKAKPALEDL  ROQLLPVLES  FKVFLSASE  EYTKKLNTQ
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B.

Fig. 3: Representative example of MALDI-TOF spectrum for Apolipoprotein AI (A) and the matched peptide sequences were underlined (B) showing differential expression in the serum and tissues of the breast (IDCA)(10).
such as mass spectrometers and bioinformatics resources, can be obtained through shared-instrumentation and/or fee-for-service facilities, which are in place at many academic institutions. Thus, many investigators from various scientific disciplines can incorporate proteomics into their research programs. A number of modifications to the 2-DE-based methodology have been introduced and explored.

- Pre-fractionation of proteins prior to 2-DE separation can be carried out to reduce the complexity of the protein mixtures and/or to isolate specific sub-sets of proteins, like the albumin and immunoglobulin in the serum or in the plasma\(^{(19)}\).

- An important issue relevant to sample preparation for proteomics is procurement of targeted cell populations from tissue specimens. The method commonly employed to prepare enriched tumor samples involve extraction of tumor cells by scraping frozen tissue sections. Recently, laser capture microdissection (LCM) technology has been introduced to enable the isolation of pure cell populations. The compatibility of LCM with 2DE-based proteomic analysis of human tumors has been demonstrated\(^{(20)}\).

- Introduction of immobilized pH gradient gels (IPG strips) for IEF has played a major role for the widespread application of 2-DE gels. Recent advancements include improvements in the separation of alkaline proteins, and design of narrow-range IPG strips covering a single pH unit\(^{(3)}\). However, this strategy, which increases sample requirements and offers lower throughput, may not be practical for many proteomics studies\(^{(2)}\).

- Horizontal SDS-PAGE units in combination with ready-made gels and buffer strips are commonly used for the second dimension of 2-DE. However, vertical SDS-PAGE systems may be more suitable for high-throughput comparative proteomics. With vertical systems, multiple SDS-PAGE separations can be run in parallel, and the quality of the resulting 2D-spot patterns is superior to that obtained with horizontal systems. Reproducible, high-quality 2D patterns are essential for the success of computer-assisted 2-DE-gel analyses.

- MALDI-TOF-MS remains an important tool for protein identification because of its high throughput, sensitivity, and high mass accuracy. Numerous advancements have been made in MALDI-TOF instrumentation and new-generation, automated MALDI-TOF mass spectrometers are commercially available. These high-throughput systems are run without operator intervention, and incorporate algorithms for iterative optimization of instrument parameters during data acquisition. Improved software tools for the detection of monoisotopic peaks in MALDI-TOF spectra have also been developed. Another type of newly developed MS instrumentation combines electrospray ionization (ESI) with a quadrupole time-of-flight (QTOF) analyzer. The QTOF analyzer can be coupled with MALDI, and MALDI-QTOF-MS was shown to be a promising new tool for proteomics. The latest generation of proteomics instrumentation also includes the MALDI tandem-time-of-flight (MALDI-TOF/TOF) mass spectrometer. The major advantages of the MALDI-TOF/TOF instrument are ultra-high throughput, high sensitivity, and high-energy collision-induced dissociation capabilities that provide enhanced peptide-sequence information.

### Proteomics: Future Perspectives

From the above literature review it is clear that proteomics plays a primary role in the investigation and the identification of potential diagnostic and prognostic markers. More and more studies are involving proteomic based technologies, thus the number of publications involving proteomics is expected to increase over the coming years. Despite its success, there are a number of challenges faced by proteomic technologies, which are partly due to the complexity of the human proteome. With
regards to 2-DE, currently this is labour intensive, relatively low throughput, variable and biased towards the separation of certain classes of proteins. Regarding SELDI-TOF-MS (Surface Enhanced Laser Desorption Ionisation- Time Of Flight), most studies to date have been small and need validation by larger scale multi-centre studies. In addition, the algorithms used to select discriminatory peaks need to be standardised if the technology is to become clinically viable. Efforts are also needed to obtain the identity of some of the molecules that comprise the discriminatory peaks. However, attempts are currently underway at combining the SELDI-TOF-MS with a high performance MS/MS analyser capable of peptide sequencing.

**Conclusion**

The identification of reliable biomarkers to track breast cancer, which could provide a better classification of tumors and allow for personalized therapy presents an exciting challenge for the scientific and medical community. Analysis of proteins expressed by tumors, using novel concepts and methods, could accelerate our quest to attain this goal and bring to light a better and more comprehensive view of the molecular heterogeneity of cancers. In this way the proteomics approaches provides powerful tools to study pathological processes or clinically important problems at the molecular level and will have a major impact in the future. Since the introduction of proteomics, 2-DE and MS have been successfully used in a large number of studies in many biological fields. Recent advancements have significantly enhanced the capabilities of the 2-DE-based technology, and it is likely that 2-DE will remain an important tool for proteomics research in the near future.

**References**


