Journal of Proteomics & Bioinformatics - Open Access

JPB/Vol.3 Issue 3

Research Article | OPEN ACCESS Freely available online | doi:10.4172/jpb.1000126

Proteomic Analysis of Human Breast Cancer: New Technologies and Clinical Applications for Biomarker Profiling

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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 enormous progress in proteomics, enabled by recent advances in MS (mass spectrometry), has brought protein analysis back into the limelight of breast cancer research, reviving old areas as well as opening new fields of study like 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. Breast cancer proteomics has already identified markers of potential clinical interest (such as the molecular chaperone alpha B-crystallin) and technological innovations such as large scale and high throughput analysis are now driving the field. In this review, we discuss the basic features of proteomic technologies, including MS, and we consider the main current applications and challenges of proteomics in breast cancer research, including (i) protein expression profiling of breast tumours, tumour cells, tumour fluids and the auto-immune response of the breast cancer cells. All of these applications continue to benefit from further technological advances, such as the development of proteomics methods, high-resolution, high-sensitivity MS, SERPA approach, and advanced bioinformatics for data handling and interpretation.

Keywords: Breast markers; Proteomics; 2-DE; Mass spectrometry; SERPA

Abbreviations: 2DE: Two-Dimensional Electrophoresis; SDS-PAGE: Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis; IEF: Isoelectric Focusing; RBP: Retinol Binding Protein; TTR: Transthyretin; 2D-blotting; western blotting following 2-DE; BC: Breast Cancer; IDCA: Infiltrating Ductal Carcinoma; MALDITOF- MS: Matrix Assisted Laser Desorption/Ionization Time Of Flight Mass Spectrometry; SERPA: Serological Proteome Analysis.

Introduction

Breast cancer is a leading cause of death among women and a major health problem of public, considering the number of women who are diagnosed and who die annually of this pathology. Its incidence is steadily rising in developing countries. For example, in the USA, breast cancer is estimated to be the most commonly diagnosed neoplasm in women in 2008, as it will account for 26% of all new female cancer cases (Jemal et al., 2008). In addition, it is expected to be the second leading cause of USA cancer deaths in 2008 (Jemal et al., 2008). Age is the most important risk factor, and the incidence of breast cancer is increasing 0.5% annually as the population in the West ages. Other risk factors have been reported including parity, age at the first pregnancy, breastfeeding, age at menarche and age at menopause, oestrogen treatment after menopause, environment, stress, and nutrition. Familial history of breast cancer is another major risk factor, emphasising the role of genetics in this pathology (Hondermarck et al., 2001). The large majority of malignant breast tumors are carcinomas which are divided into two classes: *in situ* and invasive carcinomas. 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 prognosis (Hondermarck et al., 2001). 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 to detect this cancer at an earlier stage, to uncover prognostically distinct subclasses, and to provide best individual treatment (Hondermarck et al., 2001). Currently, the search for specific cancer-related alterations largely focus upon clinically relevant biologically fluids such as serum, plasma, cell and tissue (Hondermarck et al., 2001).

Proteomics with the recent advances in mass spectrometry is considered as a powerful analytical method for deciphering proteins expressions alterations as a function of disease progression (Hondermarck et al., 2001). Recently, proteomics-based analyses of breast serum and tissue lysates have resulted in the finding of a number of potential tumor biomarkers providing, therefore, a basis for a better understanding of the breast-cancer development and progression, and eventually serving as diagnostic and prognostic markers (Hondermarck, 2003). 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

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Received February 11, 2010; **Accepted** March 15, 2010; **Published** March 15, 2010

Citation: Hamrita B, Nasr HB, Chahed K, Kabbage M, Chouchane L (2010) Proteomic Analysis of Human Breast Cancer: New Technologies and Clinical Applications for Biomarker Profiling. J Proteomics Bioinform 3: 091-098. doi:10.4172/jpb.1000126

separation of proteins and offers a powerful method for their identification and characterization (Anderson et al., 1996). Proteins of interest can be then characterized by mass spectrometry (Hondermarck, 2003). The goals of these efforts are to improve diagnostic methods by either discovering new serological tests or biomarkers, or to improve pathological analysis using tissue proteomics. Such data would provide the knowledge base for the identification of therapeutic targets and the development of new strategies against breast cancer (Hondermarck et al., 2001). The aim of this review is to illustrate the proteomic technologies that have emerged for comprehensive and high-throughput protein analysis and to provide more detailed of their application in breast cancer research and treatments.

The proteomic tools for identifying molecular markers of the breast

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 (Anderson et al., 1996). Quantitative proteomic analyses can be used to identify the protein content in complex samples such as serum, plasma, cell and tissue extracts and to determine the quantitative difference in abundance for each polypeptide contained in different samples (Anderson et al., 1996). 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 and diagnosis. Biomarkers can be defined as cellular, biochemical, and molecular alterations by which normal, abnormal, or simply biologic processes can be recognized or monitored (Vercoutter-Edouart et al., 2001; Hondermarck, 2003). These alterations should be able to objectively measure and evaluate normal biological process, pathogenic processes (like breast cancer), 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 posttranslational events. In addition, breast markers can be subtle changes in molecular structures, for instance alterations of post-translation modifications, which often can only be examined at the protein level (Shevchenko et al., 1996; François et al., 2001). 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 (François et al., 2001).

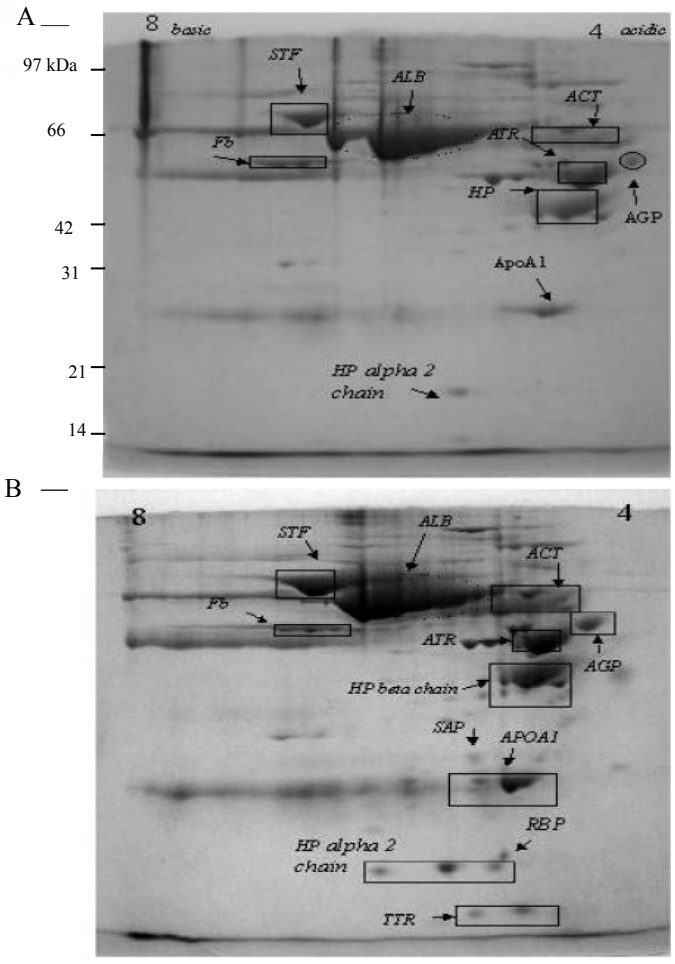

Figure 1: Two dimensional gel electrophoresis analyses of plasma proteins derived from (A) a healthy donor and (B) a breast cancer patient (Chahed et al., 2004). Partial 2-DE images from a control gel (A) and from a breast cancer sample (B) are shown. Abr: STF: serotransferrin; ALB: albumin; ACT: anti-chymotrypsin; ATR: anti-trypsin; AGP: acidic glycoprotein; Hp: haptoglobin; Fb: fibrinogen beta chain; ApoAI: ApoAI lipoprotein; SAP: serum amyloid P; RBP: retinol binding protein; TTR: Transthyretine.

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-DE gel; for each protein, the isoelectric point, MW, and the relative quantity can be measured (Figure 1, Figure 2). 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-DE 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

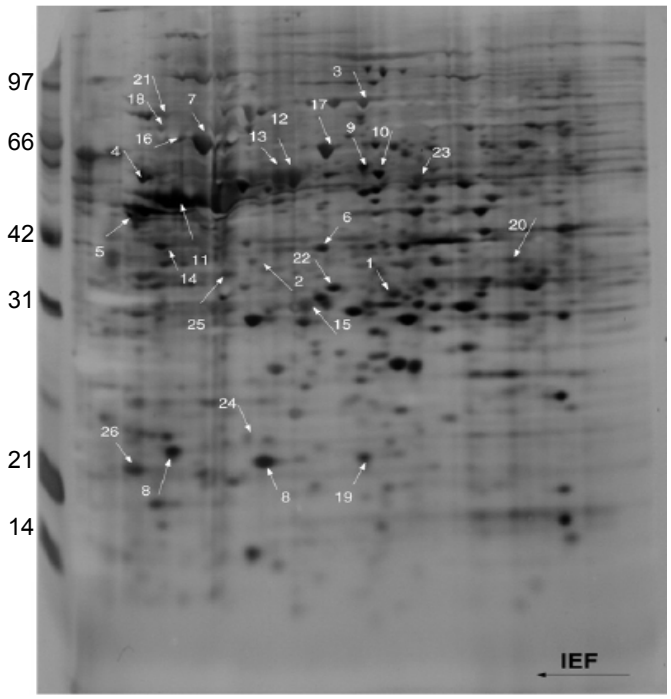

Figure 2: Total protein extracts from the MCF-7 cell line were separated by 2-DE and visualized by silver staining (Hamrita et al., 2008). The location of proteins reacting with patient sera is indicated with arrows on the 2-DE gel. 1: enoyl-CoA hydratase; 2: breast cancer patient sera is indicated with arrows on the 2-DE gel. 1: enoyl-CoA hydratase; 5: cytokeratin 8; 6: hnRNPA2B1; 7: HSP60; 8: peroxiredoxin-2; 9,10: hnRNPH; 11: cytokeratin 18; 12,13: cytokeratin 8; 14: β-tubulin; 15: prohibitin; 16: hnRNPK; 17: protein disulfide isomerase; 18: hnRNPK; 19: nucleoside diphosphate kinase A; 20: hnRNPH3; 21: transitional endoplasmic reticulum ATPase; 22: F-actin; 23: ornithine aminotransferase; 24: Mn-SOD; 25: haptoglobin-related protein; 26: B-crystallin.

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, 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, like:

Introduction of immobilized pH gradient gels (IPG strips) for IEF has played a major role for the widespread application of 2-DE gels (Hanash et al., 2002). However, this strategy, which increases sample requirements and offers lower throughput, may not be practical for many proteomics studies (Hondermarck et al., 2001).

Recently, laser capture microdissection (LCM) technology has been introduced to enable the isolation of pure cell populations (Cowherd et al., 2004). The compatibility of LCM with 2-DE-based proteomic analysis of human tumors has been demonstrated (Mathélin et al., 2006).

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 (Hamrita et al., 2009; Bjorhall et al., 2005).

MALDI-TOF-MS remains an important tool for protein identification because of its high throughput, sensitivity, and high mass accuracy (Domon et al., 2006). 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 (Chaurand et al., 2006). 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.

Protein signatures: 2-DE and Mass spectrometry

High-throughput proteomic methodologies have the potential to revolutionize protein biomarker discovery and to 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 (Srinivas et al., 2001). 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 (Shevchenko et al., 1996). This approach is used to find new biomarkers and treatment targets for various disease conditions, including breast carcinomas (Anderson et al., 1996; François et al., 2001; Hondermarck et al., 2001). New methods for rapid identification of both known and unknown proteins are under development. Matrix-assisted laser desorption and ionization with time-of-flight detection mass spectrometry (MS) (MALDI-TOF) and surface-enhanced laser desorption and ionization with time-of-flight spectrometry (SELDI-TOF) are two of the methods currently being employed. MALDI techniques immobilize protein samples in an energy absorbing matrix (chemical) on a chip or plate. The entire repertoire of proteins in the sample interacts with the matrix from which a selected subset of proteins is bound to, a function of the composition of the selected matrix. MALDI analysis is well suited for resolution of proteins <20 kDa, the low molecular weight proteome, a heretofore poorly dissected information reserve. Conversely, SELDI technology uses selective surfaces for binding a subset of proteins based on absorption, partition, electrostatic interaction or affinity chromatography on a solid-phase protein chip surface (Issaq et al., 2002). The detector plate records the intensity of the signal at a given m/z value, and a spectrum is generated (Wright et al., 1999). The different peaks in the spectrum correspond to different m/z protein species. This

datastream of information can be coupled with datastreams from a series of test subjects and complex bioinformatics to define discriminants for cancer detection. A variety of artificial intelligence bioinformatic tools have been demonstrated to successfully develop discriminating signatures for different cancers, and early work is ongoing to demonstrate the applicability of this concept to other diseases.

Markers Identified Using Proteomics

Sera samples

The accessibility of blood samples and the routine drawing of blood for other analyses make the use of plasma and serum ideal candidates for the identification of biomarkers for clinical studies (Hanash et al., 2008; Zhang et al., 2002). Searching for human plasma/serum alterations using 2-DE with regard to neoplastic disease has been extensively investigated. As early as 1974, 2-DE was carried to look for differences between protein patterns of individuals suffering cancer (Wright, 1974). Since, several markers were characterized and are currently used for diagnosis. As an example, kallikreins, a family of secreted serine proteases were highly associated with ovarian carcinoma as well as with breast and prostate cancers (Yousef and Diamandis, 2001). Other markers are effective for diagnosing primary or advanced neoplastic diseases. The carcinoembryonic antigen (CEA) is used for detecting colorectal cancer, Her2/neu, CA 15-3 and CA 27-29 for advanced breast cancer (Diamandis, 1996; Buzdor and Hortobagy, 1999). 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. (2004). Plasma was compared between blood donor controls and from patients with breast carcinoma. Several proteins were up-regulated in all of the breast cancer samples compared to that of healthy controls. 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 1). Two other proteins, highly elevated in cancer plasma, were identified as Retinol Binding Protein (RBP) and transthyretine (TTR) (Chahed et al., 2004). In an effort to identify other potential serum markers for breast cancer, proteomic analysis with 2-DE and MS has been used. Protein extracts expressed in the serum of breast cancer patients after depletion of high abundance proteins (with AffiGel-blue) were compared to sera from healthy women using proteomic approaches. By comparing 2-DE profiles between tumor and non-tumor samples and using MALDI-TOF mass spectrometry of their trypsinized fragments, we report herein the identification of two proteins of interest, namely haptoglobin precursor and alpha-1- antitrypsin precursor, whose expression was altered in sera from infiltrating ductal breast carcinoma patients (Hamrita et al., 2009). Several others studies have been reported to differentiate between serum or plasma of breast cancer patients, patients with benign breast disease and/ or healthy controls (Tomaiuolo et al., 2009; François et al., 2001). Becker et al, (2004), investigated whether the BRCA-1 mutation was reflected by the serum proteome. Multiple SELDI-TOF MS peaks were significantly different in expression between breast cancer patients with and without the BRCA-1 mutation (Becker et al., 2004). However, as none of these peaks were structurally identified, their association to the BRCA-1 gene remains unclear. In other study, Li et al, (2002) observed three serum peaks to distinguish patients from controls: one (4.3 kDa) decreased and two (8.1 and 8.9 kDa) increased in patients. These peaks were structurally identified as a fragment of inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4, 4.3 kDa), C3a des-arginine (C3adesArg, 8.9 kDa) and a C-terminal truncated form thereof (C3adesArgD8, 8.1 kDa) (Li et al., 2002; Li et al., 2005). The latter study also reported a decreased 8.9 kDa C3adesArg expression in breast cancer, whereas in all previous studies, this fragment was found increased (Li et al., 2002; Li et al., 2005). In addition, the 4.3 kDa ITIH4 fragment was one of the several ITIH4 fragments found increased in breast cancer by Regarding the inconsistent regulation observed across multiple studies, the definitive value of the different ITIH4 fragments, C3adesArg, and C3adesArgD8 in the diagnosis of breast cancer cannot be determined yet. The fibrinogen fragment, though increased in the breast cancer serum peptidome, was found decreased in breast cancer plasma and reverted to normal values after surgical extirpation of the tumour (Shi et al., 2006). The difference between study results most likely originates from the biological matrix investigated, as plasma differs from serum by inhibition of the coagulation cascade, by which fibrinogen is generated. Other recently identified breast cancer biomarkers using SELDI include Hsp27, 14-3-3 sigma, and mammaglobin/ lipophilin B complex (Belluco et al., 2007).

Cells and Tissue samples

Separation and analysis of proteins from cells, tissue samples and breast tumour biopsies has proved very successful in identifying novel markers. In our laboratory, we have used an immunoproteomic approach named SERPA to identify tumor antigens that elicit a humoral immune response in patients with breast cancer. Using this methodology, we detected twenty six immunoreactive proteins (antigens) against which sera from newly diagnosed patients with infiltrating ductal carcinomas exhibited reactivity. These protein spots were targeted by mass spectrometry. Among these antigens, peroxiredoxin-2 (Prx-2) belongs to a family of thiol-specific antioxidant proteins that control intracellular H2O2 by reducing reactive oxygen species (ROS) issued from free radicals. Such proteins may have an important role and protect the breast tumor cells against oxidative injury and modulate cell proliferation and apoptosis of malignant cells (Chung et al., 2001). To our best knowledge, thus far, there have been no data on Prx-2 as a factor eliciting humoral immune responses in cancer patients. Mn-SOD and PDI are both antigens that significantly demonstrate a high frequency immunoreaction in breast cancer sera. PDI is often upregulated under stress conditions and is involved in anti-oxidative reactions and during the folding of secretory proteins, as well as, in the catalysis of the formation and isomerization of disulfide bonds. Although the mechanisms for the development of these antibodies in cancer patients remain unknown, their occurrence has been suggested as depending on the amount of their respective antigenic proteins in tumor tissues (Yang et al., 2007). In our hands, expression levels of Mn-SOD and PDI proteins were significantly higher in tumor tissues, suggesting that overexpression of these proteins may be a contributing factor to their immunogenicity (Chahed et al., 2005). This finding, although preliminary, may be further used as a starting point to better understand the mechanisms of generation of theses antibodies in breast carcinogenesis. HSP60

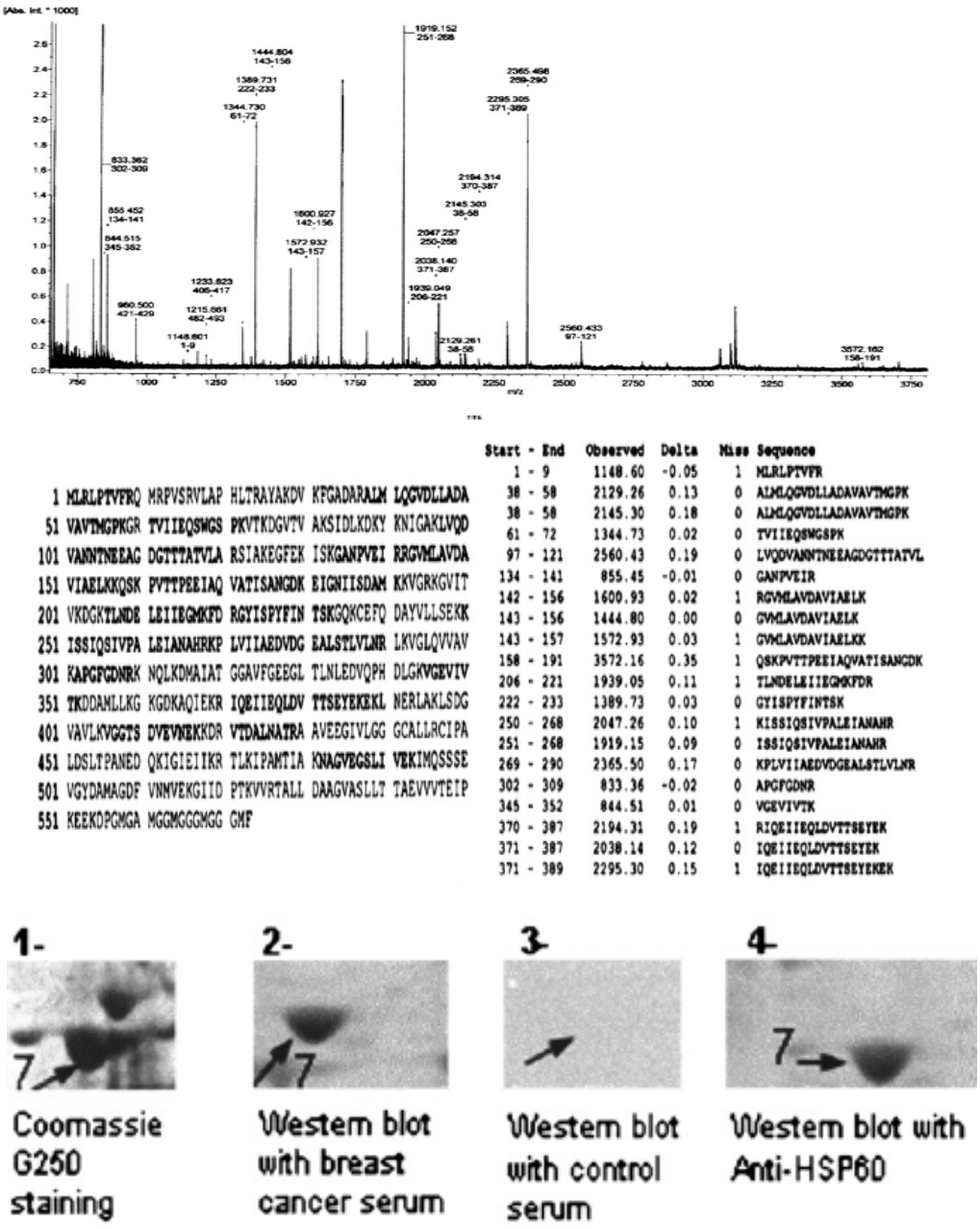

Figure 3: Identification of reactive protein spot as HSP60 by MALDI-TOF mass spectrometry and by western blot analysis (Hamrita et al., 2008). Upper panel: MALDI-MS spectrum obtained after trypsin digestion and peptide sequences from HSP60 matching with peaks obtained from MALDI-MS spectra (bottom).Lower panel: Close-up of a Coomassie blue-stained 2-DE gel in the location of HSP60 protein is shown (1). MCF-7 cell lysate proteins were separated by 2-DE, transferred to PVDF membranes and then immunoblotted with either a sera from a breast cancer patient (2) and from a healthy individual (3) or with an anti-HSP60 antibody (4).

and alpha B-crystallin are two other immunoréactive proteins most commonly observed in the breast cancer cells (MCF-7) (Figure 3, Figure 4). The molecular chaperone HSP60 is involved in protein folding, as well as, in activation of α2β1 integrin which is a major contributing factor in breast cancer progression and metastasis (Barazi et al., 2002). Recently, it has been reported that increased expression of HSP60 in breast tumors may have a prognostic value since it correlates with the presence of lymph node metastasis (Li et al., 2007). The data reported herein appears to confirm this for breast cancer patients as well, and suggest that molecular alterations leading to an immune reaction directed toward antigens related to the system of protein folding may be an important marker in breast carcinogenesis. Prohibitin is another antigen that was recognized in breast cancer cell. Prohibitin is involved in cell cycle control, differentiation and in suppression of tumor progression. In addition, studies have shown that prohibitin interacts with cell cycle regulatory proteins and modulates Rb/E2F, as well as, p53 regulatory pathways (Fusaro et al., 2003). Besides prohibitin, other antigens identified in this study were also known to be involved in apoptosis such as heterogeneous nuclear ribonucleoproteins (hnRNP) K and A2B1. Interestingly, the hnRNPK is involved in the activation of the human c-Myc promoter and enhances cell proliferation and growth of breast cancer cells in an anchorage independent manner (Mandal et al., 2001). Although the mechanisms for the development of immunogenicity against these proteins in

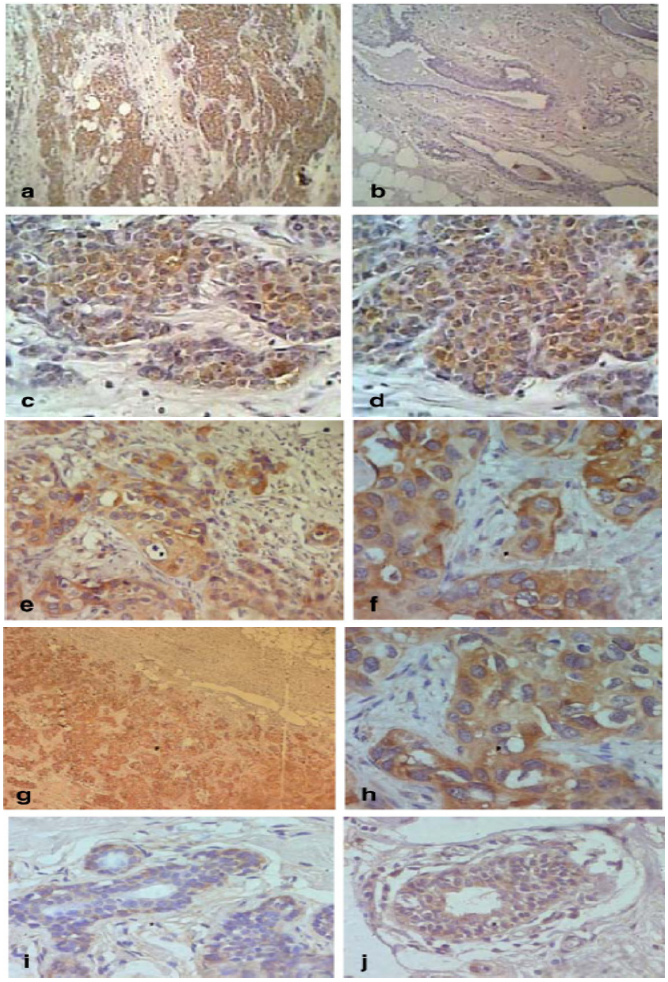

Figure 4: Overexpression of HSP60 and α-B crystallin in infiltrating ductal carcinoma tissues of the breast (Hamrita et al., 2008). Formalin-fixed, paraffin-embedded sections were immunostained with rabbit polyclonal antibody against α-B crystallin or antibody against HSP60. A strong staining (brown) in tumor cells has been observed, whereas α-B crystallin and HSP60 were expressed at lower levels in normal epithelial cells. a, c, d: HSP60 expression in IDCA tissues (a: 100× magnification; c,d:400× magnification). b: HSP60 expression in non-tumor tissues (100× magnification). E-h: α-B crystallin expression in IDCA tissues. i, j: α-B crystallin expression in non-tumor tissues (400× magnification).

cancer are not very clear, there is a growing tide supporting that such apoptosis-related proteins can undergo changes and elicit an autoimmune response in cancer and autoimmune diseases (Levine et al., 1999). Interestingly, the haptoglobin-related protein (Hpr) is another protein that has been reported as a tumor antigen, a mediator of malignant processes and an indicator of progression of disease and response to therapy. In a previous study, Hpr immunoreactivity within breast cancer cells has been shown to localize predominantly in invasive rather than in situ carcinomas and correlates with phenotypically aggressive neoplasia and shorter disease-free survival (Kuhajda et al., 1989). It has been suggested that synthesis and secretion of Hpr by cancer cells might be useful in screening and diagnostic procedures and that this may account for at least one of the mechanisms of developing autoantibodies against this protein (Epelbaum et al., 2008). In the other study, Kabbage et al, (2008) successfully identified the α-B crystallin, Hsp27 and Mn-SOD, which were elevated in breast tumor samples (Kabbage et al., 2008). 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 fulfil their functions (Kiang et al., 1998). 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 (He et al., 2004). The HSP27 is a molecular chaperone whose rate of synthesis increases many folds in response to environmental stress and during malignant transformation (Korneeva et al., 2000). Although no evidence of posttranslational alterations was pointed out, these isoforms as reported in renal cell carcinomas might reflect phosphorylation or other posttranslational modifications (Korneeva et al., 2000). 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 (Sarto et al., 2004). 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 (Parcellier et al., 2005). 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 (Kamradt et al., 2001; Kamradt et al., 2005). 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 ($O^{-}2$) 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 (Wheeler et al., 2003). Although further studies are needed, the present elevation of Mn-SOD may indicate that the 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.

Validation of the biomarkers

The end result of proteomic analysis is to have appropriate validation before the marker can reach clinical applications. Once a putative biomarker has been identified, validation using additional measurements and compound identification is necessary (Anderson and Anderson, 2002). For example, one can repeat the analysis at additional time points and determine the temporal correlation of putative biomarkers with the progression of the disease. Determining the timing of the appearance of a biomarker has been shown to be important in assessing a biomarker's prognostic utility. This fact illustrate that critical issues are needed to be addressed for the validation studies include the specificity and reproducibility of the marker.

In the case of cancer tissues and biological fluids, this is further complicated by intra- and inter-cell heterogeneity. The use of tumor tissues or needle biopsies may be problematic because multiple and representative tissue sampling is not always feasible, e.g. tumors in non-accessible sites. To address these problems, a proteomic study realised in our laboratory on breast cancer that combined proteomics and immunohistochemistry with clinical data and correlated the protein database to breast cancer cell heterogeneity within normal tissue with recurrence (Figure 4). More exciting methods are being developed that can compare proteins present in easily accessible biological fluids from patients, which are predictive of disease progression and/or therapeutic response. This approach has been reported for body fluids including serum. However, most current published studies are very preliminary and were conducted in a very small number of samples with no specific marker being carefully validated.

Conclusions

The identification of reliable biomarkers to track breast cancer, which should provide a better classification of tumors, allow for personalized therapy and exciting challenge for the scientific and medical community. Analysis of proteins expressed by serum, plasma and tumors, using novel concepts and methods, should accelerate our quest to attain this goal and bring to light a better and more comprehensive view of the molecular heterogeneity of breast 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, SERPA approach and MS have been successfully used in a large number of studies in many biological fields.

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