Proteomic approach for the detection of breast cancer biomarkers using two dimensional gel electrophoresis and mass spectrometry

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Abstract

Aims: The major tool of the proteomic approch in breast cancer is to identify the differentially secreted proteins, which may work as a potentiel biological markers. We examined the protein expression patterns of infiltrating ductal carcinoma of the breast (IDCA) tissues and serum from Tunisian women using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and matrix-assisted laser desorption/ionization-time of fight (MALDI-TOF) mass spectrometer.
Methods: Serum protein and tumor protein tissues were solubilized and analysed with 2D-PAGE and visualised by a sensitive Colloidal Coomassie G250 stain. Protein expression was identified using MALDI-TOF MS/MS and evaluated using PDQuest 2-D softwarer. The proteins spectrums were identified by searching NCBI and Swiss Prot databases.
Results: Comparaisons of the protein spots identified on the 2D-PAGE maps from human serum and breast tumor tissues showed that Apolipoprotein AI were up-expressed in both tumor tissue and pre-treatmant serum compared with their counterparts.
Conclusion: 2-DE and MALDI-TOF/MS offers total protein expression profiles of breast cancer tissues and serum and will give a chance to identify tumor-specific diagnostic markers for breast cancer. The differentially up expressed of Apolipoprotein A I may play a key role during tumorigenesis of breast cancer.
Abbreviations: Apolipoprotein AI,(Apo AI),Isoelectrofocalisation ,(IEF), Matrix Assested Laser Desorption Ionisation Time of Flight, (MALDI-TOF),Mass spectrometry,(MS), Two Dimensional Gel Electrophoresis, (2D-PAGE).

Introduction

Breast cancer is a leading cause of death among women and a major problem of public health, considering the number of women who are diagnosed and who die annually of this pathology. This high mortality rate is usually ascribed to late diagnosis of this tumor, which lacks early symptoms 1. As with other cancers, metastasis in breast cancer is the leading cause for mortality. Therefore, Early detection can greatly reduce breast cancer mortality and there is an urgent need for reliable biomarkers pathologies. Biomarkers have the potential to aid in the diagnosis, prognosis, detection and treatment of breast cancer. Some current proteomic technologies are particularly suitable for protein profiling in the search for new biomarkers 1. Recent improvement in mass spectrometry technologies has increased the accuracy and sensitivity of this tool. It can use for the analysis of complexes mixtures such as tumor tissues or serum 2. Mass Spectrometry has been applied to breast cancer tissue, serum, serum and nipple aspirate fluid (NAF). Two dimensional electrophoresis (2D-PAGE) is a valuable tool for the separation and characterization of proteins from complex biological samples prior to MS analysis 1, 2. Its widely used for separing proteins since 20 years ago. The combination of high-resolution protein separation by two-dimensional gel electrophoresis and mass spectrometry has proven to be an essential tool for proteomics to identify proteins 3. In such investigation, a biomarker is defined as a protein having more or less intensity on one gel compared with the other as well as between different diseases stages, is, thererfore of crucial importance. Searching for human protein serum and tumor tissue alterations using 2D-PAGE with regard to neoplastic disease has been extensively investigated in many authors report. For more than three decade, 2D-PAGE was carried to look analysis the protein profils in cancerous versus normal tissues with the goal of identifying the protein markers that are differentially expressed between benign and malignant tissues or plama. Increased levels of molecular markers such as prostate specific antigen (PSA) and CA 125 are now routinely used for the detection of cancer in the prostate and ovary respectively. In a more focused study, Cho WC et al (2004), reported an increased level of serum amyloid A and it could be useful a biomarker for the nasopharyngeal cancer 4. Other markers like carcinoembryonic antigen (CEA) are used for detecting colorectal cancer, Her2/neu, CA 15-3 and RS/DJ-1 for advanced breast cancer 5. In the same way, Ostergaard et al (1997) analysed the bladder tumor, using 2D-PAGE, and observed a decreased level of galactine and stratifin 6. In other studies, using 2D-PAGE and MALDI-TOF/MS, Franzen et al (1993) identified a decreased level of tropomyosin3 in malignant breast tumor as compared to benign lesions 7. Furthermore, 2D-PAGE analyses of breast tumor (invasive carcinomas) exhibited the presence of proliferating cell nuclear antigen. Kallikreins, a family of secreted serine proteases were highly associated with ovarian carcinoma as well as with breast and prostate cancers. By the same proteomic approach, Vercoutter- Edouart et al (2001) found that the 14-3-3γ chaperone protein, down-regulated in primary breast carcinoma and

to be involved in the transition of breast epithelial cells to neoplasia 8. In the present study we tried to identify new proteins in breast tumor (serum or tissue) by 2D-PAGE with mass spectrometry (MS) and to make a standard 2D-PAGE of human breast cancer tissue and serum. Here, by comparing 2 D -PAGE profiles of human serum and tumor proteins and using MALDI-TOF mass spectrometry of their trypsinized fragments, we have found an increased level of Apolipoprotein AI among Tunisian breast carcinoma women.

Material and methods

Serum and tissues samples

Serum and tumor tissues were obtained between September 2008 and October 2009 from the department of Gynecological oncology (Sousse Hospital, Tunisia) at the time of diagnosis. Sera were obtained from 40 women with untreated breast cancer (IDCA) and we collected 42 womens control subjects (having no evidence of any personal or family history of cancer or other serious illness). Peripheral venous blood samples were obtained and after centrifugation the serum was alicoted in 100 µl frozen and stored at -80 °C until analysed.Breast tumor tissue were obtained during surgical resection from the same serum samples patients (10 biopsies) during the same moment of serum levying.After excision, sample tissue were conserved in RNA later, and frozen immediately at -80°C and stored until use.

Protein sample preparation

Samples Handling (serum, tumor tissues)

To the serum, four volumes of cold acetone (-20°C) were added and the solution was incubated for 1 Hour at -20°C. The pellet was washed with cold acetone (80%), dried under partial vacuum and solubilised in 150 µl of 2D-PAGE buffer containing 7.0 M urea, 2.0 M Thiourea, 4% (w/v) CHAPS, 0,5% w/v DTT and 2% ampholytes (1 part pH 3/10, 1 part pH 5/7, 2 parts pH 6/8). Tissue samples were snap-frozen in liquid nitrogen and stored at -80˚C until used. Prior to analysis by 2D-PAGE, sections (100 mg) of tumor and adjacent normal breast tissue, erified by histological analysis, were suspended and mechanically homogenized in 200 µl of the same serum 2D-PAGE lysis buffer. The extracts were incubated at room temperature for 10 min with vortexing. The homogenate was centrifuged at 12500 g for 15 min and the yellow lipids were discarded from the supernatant and the clear supernatant transferred to a sterile microcentrifuge tube.

Protein assay

Protein contents were determined according to the procedure described by Bradford and modified by Ramagli and Rodriguez 9,10. Bovine serum albumin (Fraction V, Sigma) was used as a standard. Analytical 2D-PAGE was carried out in a Bio-Rad system (Miniprotean II). Equal amounts of proteins issued from control breast cancer samples proteins were subjected simultaneously to isoelectrofocalisation (IEF) and SDS-PAGE analysis. Extraction of proteins, solubilisation, IEF, SDS-PAGE and staining were carried under very similar conditions for the different samples. Each experiment was repeated for at least three times. Focused strips were equilibrated in SDS equilibration buffer and were then loaded onto SDS gel slabs for separation in the second dimension 11.

2D-PAGE and protein quantification

Two dimensional gel electrophoresis: gel staining, maldi-tof/ms and protein identification

Protein contents were determined according to the procedure described by Bradford and the Bovine serum albumin (Fraction V, Sigma; www.sigmaaldrich.com) was used as a standard. Analytical 2D-PAGE was carried out in a Bio-Rad system (Miniprotean II, (www.biorad.com)) according to O'Farrell 12. Equal amounts of proteins (150 µg) issued from patients serum and controls, tumoral and adjacent normal breast tissues were subjected simultaneously to isoelectrofocalisation (IEF) and SDS-PAGE analysis. Proteins were fractionated on 7-cm IEF rod gels (pHi 4.0-8.0) with a low voltage at 200 V for 15 min, followed by 300 V for 15 min and 400 V for 20 h. After IEF was terminated , Focused strips were equilibrated in SDS equilibration buffer [125 mM Tris-Hcl pH 6.8, 2.5% (w/v) SDS, 10% (w/v) glycerol, 0.025% (w/v) bromophenol blue].The second dimension of SDS-PAGE (11%)was performed on vertical system. The upper and lower electrophoresis buffers contained (Tris 25 mmol/L ; 192 mmol/l glycine; 0,25% SDS adjusted to pH : 8,8). Electrophoresis run at 80V for the 15 min and then a maximum of voltage at 140V for 1 Hour.The temperature of the cooling plate was set at 22°C. Isoelectric points and molecular weights of individual proteins were evaluated with polypeptide SDS-PAGE standard. After SDS-PAGE the gels were stained with a protocol compatible with mass spectrometry. After separation in SDS-PAGE, gels were stained overnight by using a sensitive colloidal coomassie G-250 stain and destained in 1% acetic acid 13. The dye solution contained 14% (w/v) ammonium sulfate, 3% (v/v) phosphoric acid, 0,1% (w/v) coomassie G250 and 34% (v/v) methanol. The colloidal coomassie G-250 stain were scanned into adobe photoshop 6.0. Differential protein levels among the normal breast, breast tumor tissue and serum were confirmed and piked out by comparaison of 2D-PAGE images using melanie 3.0 software tools and analyzed by mass spectrometry. The protein spots of interest were excised manually and washed three times with milli-Q water. The excised spots were destained with the destaining solution (15 mM potassium ferricyanide, 50 mM sodium thiosulfate) and washed with 25 mM ammonium bicarbonate/ 50% acetonitrile till the gels were changed opaque and colorless. After having been dried with a vacuum concentrator (SpeedVac Plus), the gel was rehydrated with 3-10 Al of trypsin solution (10 ng/Al) at 4-C for 30 min and then incubated overnight at 37-C. The tryptic peptides were extracted with 60% acetonitrile, 0.1% trifluoroacetic acid, and dried with a vacuum concentraton. Tryptic peptide mixtures were dissolved in 0.5% trifluoroacetic acid (TFA). After digestion the peptides were treated by elution from Zip Tip-C18 reversed phase pipette tips (Millipore). Recovered peptides were prepared for MALDI-TOF mass spectrometry by mixing with a saturated solution of alpha-cyano-4-hydroxy cinnamic acid in 50% acetonitile as a matrix, and droplets were allowed to dry on the MALDI sample plate. Internal calibration of the MALDI mass data was applied using the masses of two trypsin autolysis products : [M + H] + 842.51 and [M + H] + 2211.10 Da. Data generated were screened in databases using a mass tolerance <50 ppm. Peptide mass maps were obtained using a Voyager DE (Applied Biosystems, Foster City, CA) MALDI-TOF mass spectrometer operated in positive ion reflectron mode. Proteins were identified from the peptide masse maps using MASCOT program (http ://www.matrixscience.com) to search the nonredundant protein data base Swiss-prot or NCBInr.

Results

The breast cancer marker:

Subjects

To optimize protein identification, the 2D-PAGE, was performed on serum and tissue from subjects without breast cancer. The total proteins were separated by 2D-PAGE using a pH range of 4-to 8 in the first and 12% SDS-PAGE in the second dimension. Gels were stained overnight by using colloidal Coomassie Brillant Blue (G 250) Fig 1. Each experiment was repeated for at least three times. After optimization, we tested with 2D-PAGE, 82 serum samples and 10 tumor tissues (with their control tissues) from the same women patient. The major goal of this

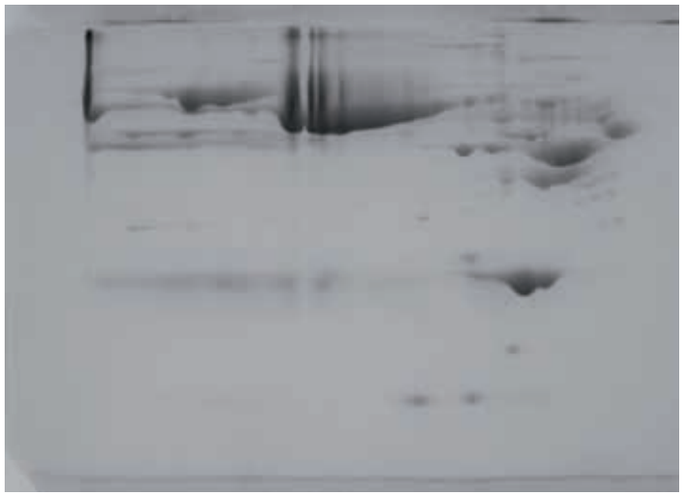
study is to identify a protein marker that is differentially expressed from serum or tissues versus controls.

2D-PAGE analysis (serum and tumor proteins)

To identify a breast markers from the serum and tissues, a comparaison of proteome by two dimensional gel electrophoresis on control serum sample and tissue with the proteome patient. The proteins extracted from serum and tumor tissues were separated and localized in the pH range of 4-8. The molecular masse ranging from 10 to 140 kDa. Fig 1 is annotated to show the localization of the Coomassie blue stained 2D-PAGE image. In 98% of the detected protein spot there were no difference in abundance between the gels.Only one spot were up expressed in all of the breast cancer samples compared to that of healthy controls. The proteins spots were excited from the gels, digested with trypsin and analysed using MALDI-TOF/MS .Peptide mass fingerprints from the protein were obtained and the resulting spectra were used to identify the protein with the Mascot Search program. These two proteins identified was the same one, Apolipoprotein AI Fig 2. The mass spectrum of the identified proteins (Apolipoprotein AI) and the sequences of the assigned peptides were presented Fig 3.

A.

ph8 ◄——————————————— ph4



B.

ph4 ———————————————► ph8

Fig 1: Two dimensional gel electrophoresis analyses of serum and tumor proteins derived from (A), a serum and (B) tumor healthy donor.

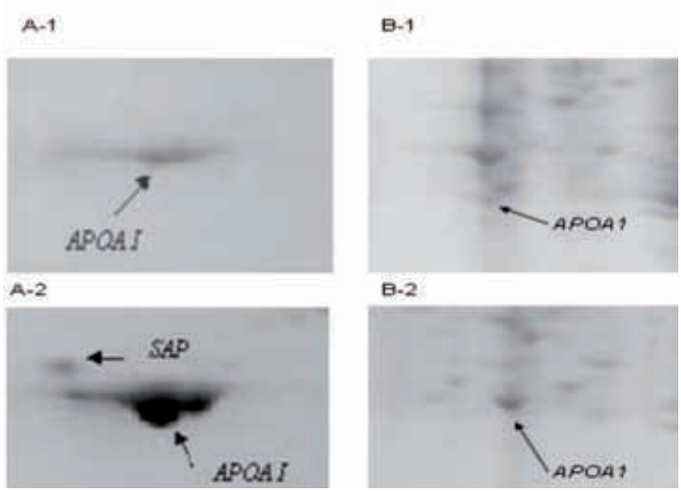
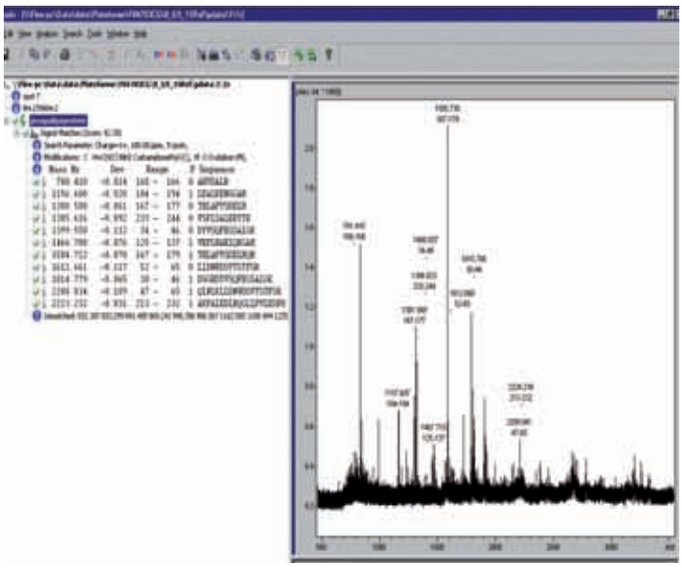


Fig 2: Two dimensional gel electrophoresis patterns of proteins focusing on areas containing ApolipoproteinA1.(A): serum proteins (A1: normal sample, A2: tumor sample), SAP: serum amyloid P. (B): proteins issued from normal and tumor tissues (respectively B1,B2).

A.



B.

RHFWQQDEPP QSPWDRVKDL ATVYVDVLKD SGRDYVSQFE GSALGKQLNL KLLDNWDSVT STFSKLREQL GPVTQEFWDN LEKETEGLRQ EMSKDLEEVK AKVQPYLDDF QKKWQEEMEL YRQKVEPLRA ELQEGARQKL HELQEKLSPL GEEMRDRARA HVDALRTHLA PYSDELRORL AARLEALKEN GGARLAEYHA KATEHLSTLS EKAKPALEDL RQGLLPVLES FKVSFLSALE EYTKKLNTQ

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).

Discussion

There is a very need action to identify and discover tumor markers that may detect the breast cancer at an early stage. The objective of this study was to identify, using quantitative assessment with 2D-PAGE and mass spectrometry, proteins with altered serum and tissue expression in infiltrating ductal carcinoma of the breast. Serum and tumor proteomic analysis of malignant breast cancer and samples from human healthy donors were compared by high resolution two dimensional gel electrophoresis. One protein is up-regulated in all of the breast cancer samples compared to that of healthy controls. This protein identification appeared to represent differences in overall abundance. Serum 2D-PAGE investigations showed elevated level of Apolipoprotein AI in serum from patients diagnosed with breast cancer. An increased expression of the apolipoprotein A1, is identified as being significantly overexpressed in the tumor (Figure 2).To our knowledge, this study, is the first one that report an elevated of the same protein in the serum and tumor tissue.

Several other 2D-PAGE studies have identified an expression of apolipoprotein AI in metastasis colonic adenocarcinoma. These findings are consistent with the notion that expression of ApoAI is associated with colonic adenocarcinoma progression, and thus ApoAI is a potential marker of aggression. Kozak KR et al (2005) identified ApoAI, as a serum biomarker that could be useful in the diagnosis of ovarian cancer 14. We conclude that ApoAI combined with CA125 should significantly improve the detection of early stage ovarien cancer 14. However other study found decreased levels of apolipoprotein AI, Zhang et al (2004) reprted a dowen-regulated level of ApoAI with transthyretin in the serum of ovarian cancer patients 15.Additionally, Steel LF et al (2003), reported the same result in the serum study. A decreased levels of apolipoprotein AI in the hepatocellular carcinoma, detected with 2D-PAGE 16. Apolipoprotein AI, present a single polypeptide chain with 243 aminoacid residues.It's an excellent cofactor for leucethin cholesterol acyltransferas (LCAT), protein official for the formation of the cholestery esters in serum. Apolipoprotein AI is the major protein constituent of high density lipoproteins (HDL) and plays a crucial role in lipid transport and metabolism. Many atherosclerosis study, reported a relation ship between the incidence of coronary atherosclerosis disease (CAD) and lower expression of ApoA I. Overexpression, in the serum and tumor sample, of apolipoprotein A1 presursor (ApoA1), could be linked to its anti-inflammatory functions .The high density lipolipoprotein (HDL) associated apolipoprotein A I allow the specific inhibition of essential inflammatory cytokine production. The tumor necrosis factor alpha (TNFα) and interleukin-1β (IL-1 β) 17.This cytokines are the regulator of the immune response, induction of inflammatory acute phase and transition to chronic inflammation. A cellular contact between stimulated T cells and activated monocytes is bloked by Apolipoprotein A I 17. Bloking the production of these cytokines by Apolipoprotein A I, may constitue a new therapeutic approch. As well as to its potential inhibition of TNFα and interleukin-1β by bloking contact mediated activation of monocytes by T lymphocytes 17. This finding suggest that change of apolipoprotein A-I levels inversely correlate with disease activity. In conclusion, we have used a proteomic approach to analyze serum and tumor tissues of the breast. We have identified interesting tumor-associated proteins that may help in under-standing the carcinogenesis of this disease and eventually serve as potential diagnostic markers.

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