Proteomics-based identification of serum albumin precursor autoantibody as serum marker in infiltrating ductal breast carcinomas

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Abstract

The identification of tumor antigens that elicit an antibody response may have utility in breast cancer screening, diagnosis and in establishing prognosis. Until now, autoimmunity in cancer has been mainly revealed in solid tumors. This study was to apply the proteomic approach to the identification of proteins that commonly elicit a humoral response in women Infiltrating Ductal Carcinomas. Sera from 40 newly diagnosed patients with breast cancer and 42 healthy individuals as controls were analyzed by two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization-time of fight mass spectrometer (MALDI-TOF/MS). Protein expression was evaluated using PDQuest 2-D software. As a result, autoantibody against serum albumin precursor was detected in breast cancer patient's sera. The immunoproteomic approach implemented here offers a powerful tool for determining novel tumor antigens that elicit a humoral immune response in patients with invasive breast cancer. This antigen and/or their related circulating antibody may display clinical usefulness as potential diagnostic markers and provide a means for a better understanding of the molecular mechanisms underlying breast cancer development.

Introduction

Breast cancer is a leading cause of death among women and a major problem of public health. This high mortality rate is usually ascribed to late diagnosis of this tumor, which lacks early symptoms. The incidence rate of this malignancy is steadily rising in developing countries. It is expected to account for 31% of all new cancer cases among women in USA 1. In Tunisia, the incidence of breast cancer is approximately 19 new cases per 100 000 women per year 2. Breast tumors have proven challenging to treat and manage due to their poor sensitivity to conventional therapies and our inability to detect early tumor formation. 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 cancer. Some current proteomic technologies are particularly suitable for protein profiling in the search for new biomarkers. Recent improvement in mass spectrometry technologies and two dimensional gel electrophoresis are valuable tools for the separation and identification of proteins from complex biological samples 3. For these purposes, a challenge for proteomics is to display and analyze all the proteins in a particular proteome. A new proteomic approaches, named immunomics (SERPA: SERological Proteomics Analysis), was considered the best for the detection of antigens that elicit a humoral immune responses and generate autoantibodies. SERPA analysis is a serological approach that detects tumor antigens that react with untreated patient's sera. Interestingly, tumor antigens released in the bloodstream by cancer cells may induce a humoral immune response and generate autoantibodies that could be useful for cancer screening, diagnosis, as well as, in immunotherapy 4. Although the mechanisms by which these antigens elicit autoantibody formation in cancer remain unknown. Numerous autoantibodies found in the sera of patients with breast cancer have been proposed as diagnostic or prognostic markers of the disease.

Until now, autoimmunity has been reported against a number of intracellular and surface antigens in patients with different kinds of tumors 5. Using proteomics approaches, autoantibodies against triosephosphate isomerase, superoxide dismutase, annexins I and II and PGP9.5 have been reported as useful markers for screening and diagnosis of human lung cancer 6. Using the same way, Sugita et al (2004) have shown the presence of autoantibodies against NY-ESO-1 in breast cancer 7. Autoantibodies against HSP70 and peroxiredoxin showed significantly higher frequency immunoreaction in patients with hepatocellular carcinomas 8. A number of autoantigens in breast cancer patient sera have also been reported using serological screening of tumor-derived cDNA expression libraries and phage display libraries 9. Using proteomics-based methods, autoantibodies against an oncogenic protein named RS/DJ-1 have been reported as a useful diagnostic marker in breast cancer 10.

The goal of this study was to apply a serum proteomics analysis (SERPA) approach to the identification of novel tumor antigens that may induce a humoral immune response in sera from patients with infiltrating ductal carcinomas of the breast. Cellular proteins from MCF-7 tumor breast cell line were separated by 2-DE, transferred onto PVDF membranes and then sera from breast cancer patients and controls were screened individually by western blot analysis for antibodies that react against separated proteins. Tumor antigens eliciting a humoral immune response by sera from cancer patients were isolated and subsequently identified by MALDI-TOF mass spectrometry.

Materials and Methods

Serum samples

Patients and controls were selected from the same population living in the middle coast of Tunisia. Sera were obtained at the time of diagnosis prior to any therapy from 40 patients with histologically diagnosed breast cancer at the department of gynecology at Sousse Hospital after informed consent was given. Tumors classified as infiltrating ductal carcinomas were pathologically staged according to the tumor-node-metastasis classification system of UICC. Histological grade was assessed according to the system of Elston and Ellis. All patients were divided into four groups according to their disease stage (stages I-IV). Stage grouping was based on TNM formula of the patient, depending on tumor size, presence of regional metastatic lymph nodes and presence of distant metastases. This patient population consisted of 40 females with an age range of 29-85 years (median age, 49 years). Of the 40 cases, 10 were of clinical stage I, 15 were of clinical stage II and 15 were of clinical stage III.The majority of patients express the estrogen receptor. Sera from 42 age-matched healthy volunteer women who visited the general health check-up division at Sousse Hospital (age range: 27-74; median age, 45 years) and having no history of cancer or autoimmune disease were collected and used as controls. Aliquots of sera were immediately frozen at -80°C until used and were never refrozen.

Cell culture and 2-D immunoblot analysis

The source of antigens was the human breast adenocarcinoma MCF-7 cell line and was purchased from the American Type Culture Collection (ATCC). It was cultivated in DMEM supplemented with 1.0 mM sodium pyruvate, 0.1 mM nonessential aminoacids (L-aspartic acid, L-asparagine, glycine, L-proline, L-glutamic acid, Lserine,L-hydroxyproline), 1.5 g/l sodium bicarbonate, 0.01 mg/ml insulin and 10% fetal bovine serum at 37°C in an ambient containing 5% CO2 and 95% humidified air. Cells were collected at 80-90% confluence using ice-cold PBS.

After washing twice with cold PBS, cells were collected by centrifugation at 500g and stored at -80°C. For proteomic analyses, 2.106 cells were washed in PBS and then harvested in 250µl of 2-DE solubilization buffer containing 7M urea, 2M thiourea, 4% CHAPS, 0.5% DTT and 2% ampholytes (1 part pH 3/10, 1 part pH 5/7, 2 parts pH 6/8). The clear supernatant issued after centrifugation of the homogenate at 12500 g for 15 minutes was transferred to a sterile microcentrifuge tube and immediately subjected to 2-DE analysis. Protein contents were determined according to the procedure described by Bradford. Bovine serum albumin (Fraction V, Sigma) was used as a standard. 2-DE was carried out in a Bio-Rad system (Miniprotean II) as described previously 11. Briefly, MCF-7 protein extract (100µg) was applied to the first dimension and isoelectrofocalisation (IEF) was performed on 7cm IEF rod gels (pH 4.0-8.0) at 200 volts for 15 min, 300 volts for 15 min and 400 volts for 18h. 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) and were then loaded onto 12% SDS gel slabs for separation in the second dimension. For each experiment, extraction of proteins, solubilization, IEF and SDS-PAGE were carried under similar conditions. Protein patterns were visualized by Coomassie blue G-250 staining or by silver staining 12. For 2D-blotting, fractionated proteins (100 µg) were transferred using the Semi-Dry apparatus (Bio-Rad) onto PVDF membrane (Immobilon-P). Transfer efficiency was checked by staining of the membranes with Ponceau S and the protein pattern on the membranes was the same as that of 2-DE gel visualized by Coomassie G250. For hybridization with sera, the membranes (2D-blot) were blocked for 1h at 4°C with 5% nonfat dry milk in TBS containing 0.1% Tween 20 (TBST) for 2h at room temperature. The membranes were then washed three times with TBST for 10 min each time. The membranes were subsequently incubated overnight at 4°C with either patient sera or control sera as a source of primary antibody at a 1:300 dilution. Following four washes with TBST, the membranes were incubated for 1h at room temperature with horseradish peroxidase-conjugated anti-human IgG antibody (1:2000 dilution, Sigma A8775) and then washed as mentioned previously. Signals were detected using 3,3'-Diaminobenzidine (DAB) according to the manufacturer's protocols. As negative controls, additional blots were incubated with TBST instead of serum and then with the secondary antibody and no spots were detected. After matching spots on the PVDF membrane with the 2-DE map of the same sample, relevant proteins reacting with sera from breast cancer patients were excised from the Coomassie blue-stained gels, destained and in-gel digested.

Spot picking and in-gel digestion

After separation, the proteins were visualized by a sensitive colloidal Coomassie G250 stain. Briefly, the dye solution containing 17% (w/v) ammonium sulfate, 3% (v/v) phosphoric acid, 0,1% (w/v) Coomassie G250 and 34% (v/v) methanol was changed once after 12 hours staining and the gel slabs subjected to a 24-hour cycle for increasing dye deposition on low abundance proteins. The detection was then increased by placing the gel into 1% v/v acetic acid for producing a better contrast between spots and gel.

Picked spots were washed with 100µl of 25 mM NH4HCO3 and dehydrated with 100 µl of acetonitrile (ACN). This operation was repeated twice and the pieces of gel were dried under vacuum for 10 min. Reduction was achieved by1-hour treatment with 10 mM DTT in NH4HCO3 buffer (100µl) at 56°C. After discarding the DTT solution, alkylation reaction was performed by addition of 100µl of 25 mM iodoacetamide in 25 mM N 4HCO3 buffer for 1 hour at room temperature, protected from light. Finally, the excised gel pieces were again washed 3 times for 5 min with 25 mM NH4HCO3 and ACN alternately. Gel pieces were completely dried under vacuum before tryptic digestion. The dried gel volume was evaluated (about 1 to 2 µl) and three volumes of trypsin (Promega, V5111), 12.5 ng/µl, in 25 mM NH4HCO3 buffer (freshly diluted) were added. The digestion was performed at room temperature overnight. Afterwards 5 µl of 35% H2O/60% ACN/ 5% HCOOH were added and the mixture sonicated for 30 min and centrifuged in order to extract tryptic peptides.

Protein identification by MALDI-TOF-MS

Mass measurements were carried out on a BIFLEX III TM MALDI-TOF (Bruker, Daltonics, Bremen, Ge) equipped with the SCOUT TM high resolution Optics with X-Y multisample probe and gridless reflector. This instrument was used at a maximum accelerating potential of 19 kV (in positive mode) and was operated in reflector mode. A saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma, Saint Louis, MO) in acetone was used as a matrix. A first layer of fine matrix crystals was obtained by spreading and fast evaporation of 0.5 µl of matrix solution. On this fine layer of crystals, a droplet of 0.5 µl of aqueous HCOOH (5%) solution was deposited. Afterwards, 0.5 µl tryptic digest were added and mixed to a second 0.3 µl droplet of saturated matrix solution (in 50% H2O/50% ACN). The preparation was dried under vacuum. The sample was washed once by applying 0.7 µl of aqueous HCOOH (5%) solution on the target and then flushed after a few seconds. In positive mode, internal calibration was performed with tryptic peptides coming from autodigestion of trypsin, with monoisotopic masses at m/z = 842.510 and m/z = 2211.105. Monoisotopic peptide masses were assigned and used for databases searches. For the mass measurements, up to one missed tryptic cleavage and optional methionine oxidation were considered. In most cases the mass accuracy was less than 50 ppm, a value which is generally considered adequate for achieving statistically significant results for protein

identification. These files were then fed into the search engine MASCOT (Matrix Science, London, UK). The data were searched against the Swiss-Prot and NCBI non-redundant (NCBInr) protein sequence databases.

Statistical analysis

A comparison of the frequency of antibodies against each antigen was determined among breast cancer patients and healthy controls. The statistic analysis was performed using SPSS, version 13.0. The chi-square test was used to determine the differences between groups. For each statistical analysis, a P-value of <0.05 was considered significant.

Results

In the current study, we have used a serological proteome approach that combines two-dimensional gel electrophoresis and western blotting for the detection of autoantibodies in the sera of patients with breast cancer. Sera obtained at the time of diagnosis from 40 breast cancer patients and 42 matched healthy subjects were investigated for the presence of IgG-based immunoreactivity against MCF7 cytosolic proteins. For this purpose, proteins issued from a tumor cell line MCF-7 were separated by 2-DE, transferred onto polyvinylidene ifluoridemembranes and incubated with sera issued from patients with breast cancer or from healthy controls. To detect antibodies against proteins from MCF-7, each transferred PVDF membrane was incubated with one serum as the primary antibody and with horseradish peroxydase-conjugated rabbit anti-human as secondary antibody. Figure 1 displays a silver stain profile of the 2-DE of proteins issued from MCF-7 cells. Three immunoreactive proteins recognized by sera of breast cancer patients are highlighted with arrows. Positive sera were reactive against this group of proteins at the highest serum dilution tested which was 1:1000. Figure 2 shows a comparison of western blot profiles among the sera from an IDCA patient (T) and from a healthy control (N). The immunoreactive spots (antigens) were subsequently excised and subjected to in-gel tryptic digestion and MALDITOF mass spectrometry analyses. Protein identification was repeated at least twice using spots from different gels. The acquired peptide mass fingerprint (PMF) was used to search through the Swiss-Prot and National Center for Biotechnology Information non redundant (NCBInr) databases by the Mascot search engine. Figure 2 shows the MS spectra obtained from the protein in spot # 1. The identified protein involved in apoptosis cell signalling (human serum albumin precursor). Sera from 19 of 40 patients with breast cancer exhibited IgG-baed reactivity against a protein identified by mass spectrometry as HSA.

Discussion

In this study, 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 detect the human serum albumin precursor, an immunoreactive proteins against which sera from newly diagnosed patients with infiltrating ductal carcinomas. Human serum albumin (HAS) is produced by hepatocytes cells and is a multifunctional plasma protein, with ascribed ligand-binding, transport properties and enzymatic activities 13; 14 ;15. HSA is involved in the control of oncotic pressure, modulation of inflammatory pathways and is known to be involved in apoptosis 16;17;18. Serum albumin stimulates oxygen consumption and augments glucose oxidation 19. Moreover, HSA reduced activation of the transcriptional factor NF-ϰB, thus reducing oxidative stresses 26. The protective effect of HSA against inflammatory process and oxidative stress, probably due to its ability either to interact with circulating molecules or cells involved in reactive oxygen production 20.

Interestingly, the HAS, is a protein that has been reported as a tumor antigen, a mediator of malignant processes and an indicator of progression of cancer disease and possessed specific suppressive effect on mammary tumorigenesis 15; 21. In a previous study Renqing et al. (2007) have shown the expression of human serum albumin precursor in the human breast adenocarcinoma MCF-7 cell line 15. The localization of serum albumin precursor is predominantly in invasive ductal carcinomas tissues of the breast rather in situ but the molecular synthesis of serum albumin precursor in breast carcinoma cells has not been investigated 15.

Up to now, only few reports have mentioned the implication of human serum albumin precursor in malignant diseases and no previous studies have reported autoantibodies against this protein in any cancer 15. Although the mechanisms for the development of immunogenicity against human serum albumin precursor in cancer are not very clear. This finding may be used as a starting point to better understand the mechanisms of generation of this autoantibody in breast carcinogenesis. There is a growing tide supporting that such apoptosis-related proteins can undergo changes and elicit an autoimmune response in cancer and autoimmune diseases 22. Also the human serum albumin precursor may be 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 of serum albumin. It has been reported a molecular interaction between human serum albumin precursor and tubulin-β chain 15. Tubulin-β chain is well known to construct microtubules which are principal components of the mitotic spindle in eukaryotic cells. Antibodies against β-tubulin were also observed with breast cancer sera (Figure 1) 23.

Although to our knowledge, this is the first report of common occurrence of antibodies against β-tubulin in invasive breast carcinomas, there have been previous reports of immunoreactivity against this protein notably in sera of patients with neuroectodermal tumors, in acute leukemia patients and in young Tunisian nasopharyngeal carcinomas subjects 24, 25. It has been reported in this field that generation of this autoantibody may be related to the dramatic reorganization of cytokeratins that occurs during apoptosis. The mechanisms by which this antigen elicit autoantibody formation in breast cancer remain largely unknown, the high level of expression of a protein , post-translational modifications, and an unusual localization of the protein in the tumor have been suggested to trigger such humoral autoimmune responses in cancer 22.

This antigen and/or their related circulating antibodies may display clinical usefulness as potential diagnostic markers and provide a means for a better understanding of the molecular mechanisms underlying cancer development.

Conclusions

In summary, our data have shown the value of using an immunomics based proteomic approach in identifying potential antigens and/or their related circulating antibodies that may display clinical usefulness as potential diagnostic markers. Using this methodology, we detected an immunoreactive protein against serum albumin precursor that shown significantly high frequency immunoreaction in breast cancer sera raising the question of a possible role of this humoral immune response in the neoplasic process.

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Figures

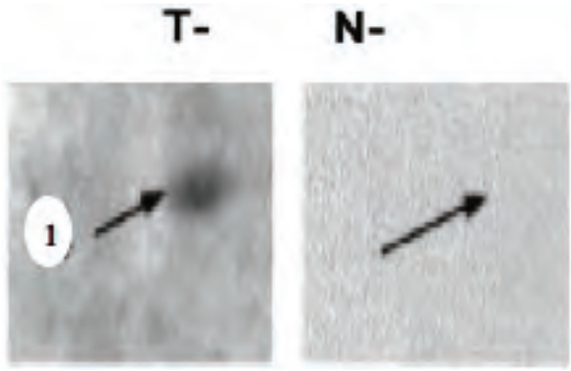
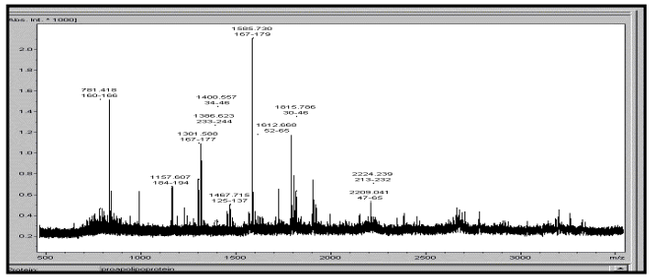


Fig 1. Close-up section of western blots showing IgG-based reactivity against serum albumin precursor (1), issued from MCF-7 cells with serum samples from breast cancer patients (T) or healthy controls (N).

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 2. Identification of reactive protein spot # 1 as serum albumin precursor (SAP) by MALDI-TOF mass spectrometry and by western blot analysis. Upper panel: MALDI-MS spectrum obtained from spot # 1 after trypsin digestion (top) and peptide sequences from SAP matching with peaks obtained from MALDI-MS spectra (bottom).