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Identification of Potential Tumor Markers in Sudanese Breast Cancer Patients Using a Proteomic Approach

Amany Elamin[1,2], Thomas Franz[3] and Muntaser E Ibrahim[1]*

[1]*Instiitute of Endemic Diseases, University of Khartoum, Sudan*
[2]*Commission for Biotechnology and Genetic Engineering, National Center for Research, Sudan*
[3]*Proteomics Core Facility, European Molecular Biology Laboratory (EMBL), Heidelberg, Germany*

Abstract

Breast cancer (BC) is considered as a major health problem in Sudan, being the most frequent hospital treated malignancy and the commonest cancer affecting Sudanese females (34%). Proteomics provides tools that investigate the precise molecular defect(s) in breast cancer tissue. Moreover, it plays a growingly important role in tumor markers discovery. In this study, a proteomic assay (2D-PAGE) was used to investigate the protein profiles of a panel of 12 Sudanese breast malignant tissues and 12 matched controls. Protein spots of interest were excised manually, and in-gel digested using trypsin. Proteins were identified using mass spectrometry (MALDI-TOF). Mascot program was used to search protein databases for matching peptides from known proteins. The overall profile was relatively similar, the preliminary results identified three proteins to be differentially expressed, suggesting that they may perform a role in breast neoplasia. Although the number of samples investigated in this study is comparatively small, to allow authoritative conclusions, the study provided three proteins that might be potential tumor markers in Sudanese breast cancer patients requiring further investigations and validation.

Keywords: Breast cancer; Tumor markers; Proteomics; 2D-PAGE; Mass spectrometry

Abbreviations: 2DE: Two-Dimensional Gel Electrophoresis; α1-AT: Alpha-1 Anti-Trypsin; DTT: Dithiothreitol; Pin1: Peptidylprolyl Cis-Trans Isomerase; PMF: Peptide Mass Fingerprinting; PrdxV: Peroxiredoxin V; ROS Reactive Oxygen Species; SDS PAGE: Sodium Dodecyl SulfatePolyacrylamide Gel Electrophoresis; MALDI TOF: Matrix Assisted Laser Desorption Ionization - Time of Flight; Mass Spectrometry; IPG: Immobilized pH Gradient; IEF: Iso-Electric Focusing

Introduction

Worldwide, breast cancer (BC) ranks as the top cancer among women, accounting for 23% of the total cancer cases and the principal cause of cancer death (14%) among females [1]. In Sudan, breast cancer is considered as the most frequent hospital treated malignancy, accounting for about one-fifth of all cancers reported in both sexes [2]. Among Sudanese women, breast and cervical Cancers account for about 50% of all cancers [3].

Proteomic techniques are powerful tools to help in the identification of tumor markers. These markers are thought to be helpful in the clinical management of cancer patients, assisting in diagnostic, staging, evaluation of therapeutic response, detection of recurrence and metastasis, and the development of new treatment modalities [4]. There are few breast tumor markers being used routinely in clinics, such as follow up molecular markers (CA 15-3, CA 27.29 and CEA), markers of prognostic and/or therapeutic value (hormone receptors, HER-2, Ki-67, p53 proteins), and the genes for hereditary breast cancer [5]. Nevertheless, the number of biomarkers reaching routine clinical use remains unacceptably low. Therefore, the search is still ongoing for biomarkers to improve early and specific detection and disease monitoring [6]. The present study aims to investigate the protein profiles of a panel of Sudanese breast cancer tissues and controls, with the target of identifying proteins that could potentially be used as tumor markers.

Materials and Methods

This is a case-control hospital-based study. A panel of 12 Sudanese breast cancer patients (12 tumor-control pairs) were recruited in this study. Tissue biopsies were collected by Breast Cancer Research Group members at the Institute of Endemic Disease, University of Khartoum, from hospitals around Khartoum State. Both fresh normal and biopsy tissues had been obtained from breast cancer patients 20-30 min after surgery from patients who did not receive preoperative treatment following the appropriate ethical procedure. Tissue samples were snap frozen in liquid nitrogen prior to transport to the Molecular Biology lab at the Institute of Endemic Disease. The samples were stored in a -80°C freezer. A fraction of each sample had been stored as archival material to be used for future needs.

Sample preparation

Tissue protein extraction had been accomplished at the Department of Molecular Biology, Institute of Endemic Disease, University of Khartoum, Sudan. Tissue Samples were homogenized by freezing with liquid nitrogen and hand grinding with a cooled mortar and pestle to a fine powder. An adequate amount of sample was suspended in lysis buffer (9 M Urea, 4% CHAPS, 100 Mm DTT, 1% Pharmalyte pH 3-10, and Protease inhibitor cocktail). The suspension was vortexed for 30 seconds to facilitate protein solubilization, incubated for 1 hour at room temperature, and centrifuged (16000 g, 30 minutes). The supernatant (crude extract) was transferred to a clean tube, stored at 20°C.

***Corresponding author:** Muntaser E. Ibrahim, Institute of Endemic Diseases, University of Khartoum, Sudan, Tel: 24911793267; E-mail: mibrahim@iend.org

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2D-PAGE

Protein precipitation and quantification: All tissue protein samples were precipitated using acetone-methanol precipitation procedure. Estimation of protein concentration was done using Smart spec™ and Bio-Rad protein assay (Bradford standard assay) which measures protein concentrations in the range 200 µg to 1400 µg. This in order to enable accurate comparison of the measurements of proteins from different 2D runs.

IEF with IPG gel strips rehydration and equilibration: Equal amount of protein samples were diluted in rehydration buffer (8 M Urea, 10 Mm DTT, 1% CHAPS, 0.25% Biolyte pH 3-10), to make a total volume of 125 µl of sample buffer per IPG gel strip (pH 3-10 NL, 7 cm), dry strips were actively rehydrated in this solution for 16 hours for 50 V at 20°C and then focused using Protean IEF Cell System. Following IEF gels were equilibrated by gentle shaking for 15 minutes in 5 ml equilibration buffer (6 M Urea, 1% DTT, 30% Glycerin, 5% SDS, 0.05 M Tris and a trace of bromophenol blue) followed by 5 ml of the same solution containing 2 Iodoacetamide instead of DTT for 15 min.

SDS-PAGE

The second dimension was performed using the Laemmli method [7]. The IEF gel was positioned on top of a 12.5% w/v SDS-poly-acrylamide gel (70 mm × 70 mm × 1 mm) and sealed with boiling 1% agarose in running buffer (1.5 M Tris, pH 8.8). Electrophoresis was run in a BioRad mini protean 3 cooling temperature of 20°C. Some samples were separated in the second dimension using 12.5% w/v SDS-poly-acrylamide gel (170 mm × 170 mm × 1 mm) and run using Bio-Rad protean II xi equipment.

Staining and visualization: Gels were stained using Coomassie staining [8]. After destaining with deionized water; gels were scanned using GS-800 calibrated densitometer, processed using Quantity one program, spot detection and quantification were carried out using PDQUEST™ version 8.1 software from (Bio-Rad).

In-gel digestion, target spotting and MALDI-TOF analysis of proteins: Gels of interest were rinsed with water and spots of interest were cut from the gel manually with a clean scalpel and chopped into small pieces and placed into microcentrifuge tubes. The gel pieces were washed two times with 100-150 µl water (HPLC grade) for 5 minutes, acetonitrile twice in order to destain Coomassie stained gel pieces, they were rehydrated into 100-150 µl 100 mM NH_4HCO_3, equal volume of acetonitrile had been added for 10-15 min, all liquid was removed, and the gel pieces were shrunk in double amount acetonitrile. This step was repeated three times until the gel pieces are colorless.

Gel pieces were then rehydrated into 15-20 µl digestion buffer (100 mM NH_4HCO_3, H_2O, 100 mM $CaCl_2$) at 4°C for 15-20 minutes, this step had been repeated twice and then all remaining buffer was removed and 15-20 µl of the same buffer (without trypsin) had been added to cover the gel pieces and keep them wet during enzymatic digestion. Samples were left in a heating block overnight 37°C, gel pieces were spun down, 80 µl extraction buffer was added containing 10% (v/v) formic acid and 100% (v/v) acetonitrile 1:1. After incubation for 15 minutes at 37°C in sonication bath gel pieces were spun down and the supernatant was collected into a separate 0.5 ml tube and double the volume of acetonitrile was added and incubated for 15 minutes in a sonication bath. Then gel pieces were spun down and the supernatant was collected, and the extracts were pooled and dried down using "speed-vac" (at 30°C). After the drying step, the peptides were washed three times by zip tipping with 10 µl washing solution containing 50% acetonitrile, 0.1% TFA (trifluoroacetic acid). Peptides bound to the column were re-suspended in 2 µl of elution buffer and then mixed with 1-2 µl of the α-cyano-4-hydroxycinnamic acid matrix. This mixture was then added to MALDI/TOF target plate and left to dry in a 37°C heating plate in order to evaporate the solution and left the peptides mixed with matrix crystals on the target plate. Control digests were performed on gel piece that did not contain any protein and were naturally found at the edge of the gel. Extracted peptides were then analyzed by MALDI/TOF MS (Bio-Rad). The laser wavelength was 120 nm. The MALDI spectra were averaged over 100 laser shots. External calibration pepmix was employed.

Database searches for protein identification: A program available on the internet (http://www.matrixscience.com) was used to search the protein databases. The program Mascot uses peptide mass fingerprints (PMFs) to search databases for matching peptides from known proteins. The following parameters were used in the searches: protein mass ranged from 1000-100000 Dalton, trypsin digest (one missed cleavage allowed), Variable modifications: Oxidation (M), mass accuracy 120 ppm; species: *Homo sapiens*.

Results

Differences between normal and neoplastic breast cancer tissue were identified using 2D-PAGE. The overall profile was relatively similar. Proteins that were reproducibly differentially expressed between tumors and controls were circled and numbered some proteins were altered quantitatively whilst some were qualitatively different in gels.

Only six proteins were identified to be differentially expressed between tumors and controls in most (>60%) of samples analyzed. Three of which were identified with MALDI-TOF by PMF and matched with known proteins in MASCOT database. These proteins were (Figure 1) (Table 1): (1) Alpha-1-antitrypsin (*α1-AT*) precursor (Alpha-1 protease inhibitor) which was identified to have a molecular mass of 46,878 KDa, an isoelectric point (pI) of 5.37 and a score of 85 whereas protein scores greater than 54 are normally significant ($P<0.05$) (Figure 2), (2) *PeroxiredoxinV*, mitochondrial precursor (*PrdxV*), which identified to have a molecular mass of 22,298 KDa, an isoelectric point (pI) of 8.85 and a score of 61 (Figure 3), and (3) Peptidyl-prolyl cis-trans isomerase A (*Pin1)*, with a molecular mass of 18,098 KDa, an isoelectric point (pI) of 7.82 and a score of 61 (Figure 4).

Besides these three protein spots that were successfully identified, a total of three spots in the malignant group of gels were considered

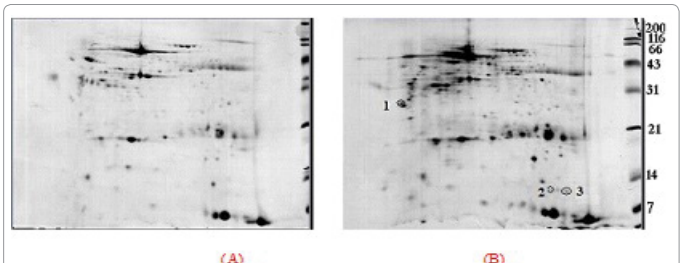

Figure 1: Representative Coomassie-stained 2D gels of normal (A) and malignant (B) samples, respectively. One hundred twenty-five micrograms of protein had been loaded onto non- linear IPG strips (pI 3^10) and isoelectric focusing were performed. The second-dimensional run was carried out on 12.5% SDS^PAGE gels. Both gels were processed in the same batch. Proteins that were reproducibly differentially expressed are circled and numbered A1AT (1), Peroxiredoxin-5, mitochondrial precursor (2), and Peptidyl-prolyl cis-trans isomerase A (3).

ID	Protein identified	SWISS-PROT accession no.	Mass (kDa)/pI	Score*	Expect	Residues identified by MALDI-MS
1	Alpha-1-antitrypsin precursor (Alpha-1 protease inhibitor)	P01009	46,878/5.37	85	4.80E-05	35-64, 161-178, 248-250 127-159, 216-241,
2	Peroxiredo-xin5, mitochondrial precursor	P30044	22,298/8.85	61	0.012	58-75, 87-102, 103-116, 103-118, 160-176, 180-191, 181-191
3	Peptidyl-prolyl cistrans isomerase A	P62937	18,098/7.82	63	0.0074	1-18, 31-43, 37-48, 55-68, 76-90, 133-143
*Protein scores greater than 54 are significant at P<0.05. pI=Isoelectric point						

Table 1: Catalogue of the protein spots identified; given: Protein name accession numbers (the abbreviations used in the MASCOT database), isoelectric point and molecular weight and the matching score.

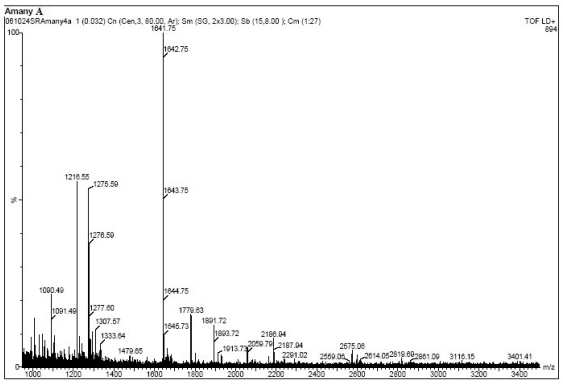

Figure 2: MALDI-TOF mass spectrum of the mixture of tryptic peptides derived from (1) protein spot. (Alpha-1-antitrypsin).

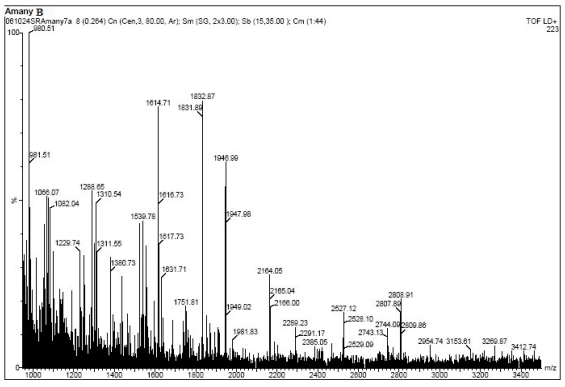

Figure 3: MALDI-TOF mass spectrum of the mixture of tryptic peptides derived from (2) protein spot (Peroxiredoxin-5).

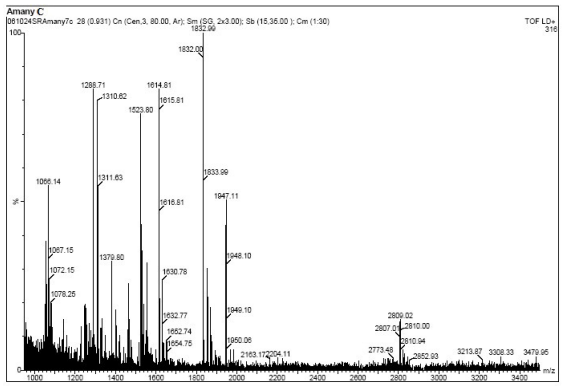

Figure 4: MALDI-TOF mass spectrum of the mixture of tryptic peptides derived from (3) protein spot (Peptidyl-prolyl cis-trans isomerase A).

differentially expressed but did not find a statistically acceptable match in the database.

Discussion

The present work aimed to introduce a contribution to the understanding of proteomic profiling of breast cancer in Sudanese patients. A panel of normal and malignant breast tissue specimens were analyzed in order to identify proteins changes associated with breast carcinogenesis. Proteomic analysis identified a total of 3 differentially expressed proteins between malignant and control tissue samples. Moreover, a total of three spots were found considerably differentially expressed but did not find a statistically acceptable match in the database. This could be explained by the fact that proteins which are covalently modified (e.g., glycoproteins) may present a problem, since it is not easy to predict sites of modification, and thus some of the molecular weights may not match the expected sets of peptide molecular weights for proteins in the database [9].

Three proteins spots differentially expressed in cancer tissue gels were successfully identified with a statistically significant number of molecular weights match. These proteins were: Alpha-1-antitrypsin precursor (Alpha-1 protease inhibitor), *PeroxiredoxinV* (mitochondrial precursor), and Peptidyl-prolyl cis-trans isomerase A (*PPIA*).

Alpha-1-antitrypsin (*α1-AT*) is a broad-spectrum inhibitor of serine proteases, including trypsin-, chymotrypsin-, and elastase-like enzymes [10]. Its major physiological role is the inhibition of leukocyte elastases released at sites of inflammation. It is present at significant levels in blood and at lower levels in other extra-cellular fluids, including breast milk [11]. Several cultured human tumor cell lines have been shown to produce functionally active *α1-AT* including the breast cancer cell line MCF7 [12]. Elevated immuno-staining of *α1AT* has also been described in various tumors, including tumors of the breast [13,14].

The preliminary proteomics analysis included *PeroxiredoxinV* (*PrdxV*), also known as *PrxV*, *PMP20*, *AOEB166*, or *ACR1* [15-17]. It is a member of the *Prdxs* family of antioxidant proteins which is known to be overexpressed in several different carcinomas [18,19]. Many studies indicated that aberrant expression of *Prdxs* was found in various kinds of cancers. Moreover, some members of Prdxs were thought to be a biomarker of cancer cells [20]. The ubiquitous distribution of PrdxV and its electron donors suggests that they might play an important antioxidant role in these different sub-cellular compartments. It also prevents p53 dependent generation of Reactive Oxygen Species (ROS) [21].

The third molecule to be suggested is Peptidyl-prolyl cis-trans isomerase A (Pin1), which is known to play important roles in many cellular events, such as cell cycle progression and differentiation. Pin1 is thought to be overexpressed in breast cancer and cooperates with Ras signaling in increasing catalysis for oncogenesis [22,22]. Overexpression of Pin1 promotes tumor growth, while inhibition of Pin1 causes tumor

cell apoptosis. Therefore, it may serve as an effective diagnostic and therapeutic anticancer target acting by blocking cell cycle progression. Many inhibitors of Pin1 were discovered, including several classes of designed inhibitors (alkene isosteres, reduced amides, indanyl ketones) and natural products (juglone, pepticinnamin E analogues, PiB) [23]. Validation of identified markers is of utmost importance to ascertain reproducibility and prevent systematic bias and overfitting of data [24]. Validation studies need to be conducted in a separate cohort of breast cancer patients and controls using Immunohistochemistry. Also, the specificity of these markers should be tested in other types of cancers` tissues and controls. Immunohistochemistry [25] also, the specificity of these markers should be tested in other types of cancers` tissues and controls. Only a single conformational study was conducted out of this study up to date. Peroxiredoxin V expression was examined in a separate number of Sudanese breast cancer patients and matching controls using immunohistochemistry and *in situ* hybridization [26]. Further studies are needed to determine the potential of using identified markers of breast cancer in clinical management.

Conclusion

Although the number of samples investigated in this study is comparatively small, which constrain outlining authoritative conclusions; this preliminary proteomic study suggests a panel of tumor markers that could have diagnostic, prognostic and/or therapeutic values. Further investigations are needed to validate the data provided in this study and to draw a link between those markers and pathological parameters and therapy.

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Competing Interests

Authors have no competing interests.

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