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Plasma Protein Profiling of Breast Cancer Patients of North Indian Population: A Potential Approach to Early Detection

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Abstract

Study background: Over the last two decades worldwide the mortality due to breast cancer (BC) raises catastrophically. Unfortunately, clinicians are still seeking for reliable resources for early speculations. Recently, the approach of investigative plasma based molecular markers builds a new hope for the early stage diseases.

Methods: Blood samples of healthy and BC individuals from North Indian women (n=25) were collected. In order to increase the detecting sensitivity of protein of interest, the pooled plasma samples were subjected to Multiple affinity removal system (MARS) Hu-14 and PROTEOPREP 20 and further processed by 2D-PAGE/LC-MS/MS analysis. Finally, the levels of selected proteins were independently estimated by western blotting and immunohistochemistry.

Results: The comparative 1D-SDS page and 2DE gel analysis showed the high proficiency of Hu-14 MARS for diminution of HAPs. The proteome profile of pooled plasma sample yielded total 24 differentially expressed spots in BC over control. Among them, the identified 3 up-regulated proteins, Serpin peptidase inhibitor clade A (privilege 92%; 95%CI 79.36-104.63), Apolipoprotein A IV (privilege 72%; 95%CI 52.4-91.6), Apolipoprotein AI (privilege 92%; 95%CI 79.36-104.63), and 1 down-regulated α-2-HS glycoprotein (privilege 76%; 95%CI 57.26-94.74) spots were of high significance ($p<0.05$). These proteins are involved in pathogenesis of cancer and play an important role in the regulation of metastasis. However their discerned molecular mechanisms evaluation related to BC is ongoing to scrutinize its clinical utility. Importantly, the results of immunochemical quantifications were found consistent with the changes in 2-DE check for all candidates apart from α-2-HS glycoprotein, where the immunohistochemistry analysis was not correlated well with 2DE and immunoblotting.

Conclusion: This preliminary work presents a panel of differential plasma proteins in BC patients, validated by combining routine clinical immunochemical reactions for the first time in North India. Further, in order to establish a cause and effect relationship of these highly modulated proteins with BC needs large scale sample size investigation.

Keywords: Breast cancer; North India; Proteomics

Abbreviations: MALDI-TOF: Matrix-Assisted Laser Desorption Time-of-Flight; 2D Nano LC-MS: Two-dimensional Online Liquid Chromatography-Mass Spectrometry; m/z: Mass/Charge; SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; 2DE: Two Dimensional Gel Electrophoresis; APO AI: Apolipoprotein A I; APO A IV: Apolipoprotein AIV; SERPINA: Serpin peptidase inhibitor clade A; AHSG: Alpha-2-HS-Glycoprotein

Introduction

Carcinoma of the breast remains one of the leading causes of female deaths world over [1-4]. Despite the substantiation of molecular deformities in biological specimens, therapeutics in BC are hampered by the scarcity of clinical markers. Majority of BC patients is diagnosed at an advanced stage of disease, which reduces their chances of survival. This situation is further exasperated only because BC is non-symptomatic at early stages and the treatment response is unpredictable. Thus, there is vital need for diagnostic modalities that would be both specific and sensitive to disease onset, as well as progression.

In the past several-large scale studies using the platforms of genomics, proteomics and other advance biological systems have massively contributed to the detection and establishment of coercive diagnostic tools for cancer management [5]. After the successful completion of Human Genome Project, the outlining of proteins within a single experiment called proteome profiling has gained much attention. However, mapping the accurate inflections (quantitatively) during the progression of the disease is crucial part, because such profiling requires the comprehensive strategies and resources that are able to efficiently provide reproducible differential expression values for proteins in two or more distinct biological samples [6].

The assessment of circulatory diagnostic protein markers is one of the most promising and dynamic areas of cancer research today. The applicability of MS-coupled with bioinformatics technology in these researches has come into prominence, as they are one of the means to translate the experimental data into clinical applications [7,8]. Since the blood examination is least invasive to patients and gives important information about any abnormality, it could provide a better clinical coordination for a holistic understanding of the

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disease process than any other resource. Previously, by monitoring the protein expression pattern in biological specimens, some studies have revealed that members of MUC-1 family i.e. mucin glycoproteins (e.g., CA 15-3, CA 27.29), carcinoembryonic antigen, oncoproteins (e.g., HER-2/c-erbB-2), and cytokeratins (e.g., tissue polypeptide antigen, tissue polypeptide–specific antigen) and BC1, BC2, and BC3 [9-13] as possible markers for BC but because of low reproducibility and non-organ specificity, only a few have made their way into clinical practice. As BC is a complex disease which can gain a more invasive and resistant character by numerous molecular changes [14], poses a challenge for the clinical administration. Moreover, the marked genetic heterogeneity of BC which involves mutation of BRCA1, BRCA2, RAD51, CHEK2, AR, ARHI and ERBB2 genes during tumorogenesis [15-17] makes its précised assessment difficult at early onset. It might require a panel of multiple biomarkers in order to achieve sufficient clinical efficacy.

In the present study, we employed 2D gel electrophoresis followed by LC-MALDI TOF MS to investigate protein expression alterations between BC and normal plasma of North Indian women. The information at the body fluid proteome level is highly conserved across species and holds a significant association among epidemiological conditions. However, such in-depth proteomic analysis is challenging due to the dynamic range of proteins in plasma. Therefore we deployed depletion for reduction of dominant HAPs that barricade the potential target proteins. Comparative proteomic profiling of immunodepleted plasma of healthy and of BC individuals has revealed that four modulated proteins apolipoprotein A I, apolipoprotein A IV, serpin peptidase inhibitor clade A and α-2-HS glycoprotein are highly reproducible candidate proteins which are known to be linked to the progression of breast carcinogenesis. Finally the selected aspirant proteins were independently validated through routine immunological measurements.

Materials and Methods

Sample collection and patient inclusion criteria

All consecutive control and patients (stage III) were selected from the North Indian population. Patients were admitted to the surgery of breast cancer and given written informed consent for research which approved by the ethics committee of the King George's Medical University (Lucknow, India.) The information about patients and controls are listed in Table 1. Based on the most common group of patients seen in clinic setting, we chose to focus on females aged 25–60 years. The patients were grouped according to age and menopause status. Additionally, women were excluded from this pilot project if they were pregnant, cigarette smoker or had certain conditions such as cardiovascular disease, tuberculosis. Twenty five Blood samples of each healthy as well as diseased subjects were collected in EDTA coated vials, centrifuged (4 °C, 2500 × g, 5 min) within 2 hours of collection and after assessing protein concentration through Lowry's method [18], the supernatants were stored at -80 °C, till further used.

Formalin-fixed paraffin-embedded Breast cancer metastatic axillary lymph node and reactive nonspecific lymphadenitis tissue slides (in 10 set pairs) were acquired from the Department of Pathology of King George's Medical University, Lucknow. The histology of specimens was further reviewed by an expert pathologist to re-examine the original diagnosis. Haematoxylin and eosin stained slides of cases were used for conformational analysis of histopathological changing pattern.

Materials

The immunodepletion kit Human 14 Multiple Affinity Removal System (MARS Hu-14) and 5K MWCO concentrator were purchased from AGILANT TECHNOLOGIES INC. IPG strips and 0.5% pH 3-10 IPG buffer were purchased from Bio-Rad Laboratories (Hercules, CA, USA). ProteoPrep® 20 immunodepletion kit, 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), Dithiothreitol DTT, iodoacetamide (IAA), urea, acrylamide, Bis, TEMED, protease inhibitor, methanol, chloroform, ammonium persulfate and antigoat IgG-TRITC conjugate were from SIGMA-ALDRICH (MISSOURI, USA). The Novex® Sharp Unstained Protein Standard marker was from Life technologies (Invitrogen) (Bangalore, India). The mouse monoclonal antibody apolipoprotein AI and goat antirabbit IgG–FITC antibody from SANTA CRUZ BIOTECHNOLOGY INC, EUROPE, mouse monoclonal antibody alpha-1-antitrypsin, mouse monoclonal β-actin, rabbit polyclonal antibody alpha 2 HS glycoprotein and goat polyclonal antibody aplipoprotein A IV from ABCAM (CAMBRIDGE, MA, USA) and the rabbit antigoat IgG, rabbit antimouse IgG, rabbit antimouse IgG-FITC, goat anti-rabbit IgG-FITC from GENEi, (Bangalore, India) were procured. The rest of the chemicals used in the study were of analytical grade of purity and procured locally.

Samples pooling into 3 groups according to age and menopausal status

Twenty five samples of patients were chosen to the exclusion of samples, which underwent hemolysis. The integrity of each plasma sample was further verified by running 1D SDS-PAGE and stained with R-250 to check for massive protein degradation. Samples that did not satisfy the specified time frame or integrity check were stored away and not taken for analysis. Patient and control (n=25) samples were divided into three groups according to age and matched with menopausal status (Table 1).

Enrichment of low abundant proteins (LAPs)

For diminution of high abundance proteins the delipidated plasma samples were subjected to Human 14 Multiple Affinity Removal System (MARS Hu-14) and Proteoprep-20 plasma immunodepletion columns. All the steps of depletion were followed according to manufacturer's instructions. The depletion process was repeated for five to six times to get sufficient amounts of protein for further purposes. Sample was concentrated with an Ultrafree-MC microcentrifuge (5K MWCO) filter and spun at 7500 rpm until the sample volume was reduced to 150 µL. This step was repeated six times, after pooling the protein concentration of the sample was estimated by Lowry's method [18]. The efficiency of the depletion of the proteins from plasma of two columns was resolved by SDS-PAGE and 2DE. Where possible, equal loading of (60µg) protein per well for gel analysis was employed in SDS-PAGE. After electrophoresis, the gel was Coomasie R-250 stained and imaged. Similarly in 2DE 300-350µg of protein is loaded to check the effectiveness of depletion among the columns.

Age (years)	Patient	Control	Menopausal status
20-35	10 (2batches)	10	pre
35-50	10 (2batches)	10	pre
>50	5 (1 batch)	5	post
Total	25	25	

Table 1: Twenty five plasma samples were grouped according to age and menopausal status of the subjects.

2-DE profiling

2-DE analysis was performed using a standard protocol with some modifications [19]. Briefly, an equivalent amount of 250 µg of diseased and control plasma proteins were taken and precipitated with the ratio of protein:MeOH:chloroform:water 1:4:1:3 for quantitative recovery of protein in diluted solution [20]. Sucked off supernatant completely (carefully) and a protein pellet was air dried. Re-suspended protein pellet in rehydration buffer (7M Urea, 2M Thiourea, 4% CHAPS, 65mM DTT, 0.5%pH 3-10 IPG buffer) was diluted to 300 µL and applied to 17cm IPG strips of pH4-7 (Bio-Rad, Hercules, CA, USA) by dissolving it for passive adsorption. After overnight rehydration, IEF was carried out for 60kVh at 20°C following the manufacturer's instructions within the Protein IEF cell apparatus (Biorad). The limit of current was 50mA/gel. Once IEF was completed, the IPG strips were equilibrated immediately in SDS equilibration buffer (6M urea, 20%v/v glycerol, 2%w/v SDS, and 0.01%w/v bromophenol blue in 1.5 M Tris-HCl; pH 8.8) plus 1% w/v DTT for the first 15 min reduction step, followed by the second 15 min alkylation step with the aforementioned SDS equilibration buffer plus 2.5% w/v iodoacetamide (each strip in 6 ml solution). The strips were then transferred on to 12% SDS-PAGE gels using Protean II xi electrophoresis equipment (Biorad, Hercules, CA, USA) for the second dimensional separation. The IPG strips were sealed with 0.5% low melting agarose overlaying containing 0.1% SDS and 37.5 mM Tris (pH 8.8) for avoiding the interruption of the running electrophoresis buffer (Tris-glycine-SDS, pH 8.3) in sample. Molecular weight marker obtained from Bangalore Genei was placed onto the gel by pipetting 8-10µl onto a piece of blotting paper which was then loaded onto the gel surface. The gels were run at 15 mA/gel until the bromophenol blue dye marker had reached at the bottom of the gel. For reproducible results, 5 replicates per pooled group 2DE gels were run.

Protein staining, image acquisition and analysis

After electrophoresis, the gels were stained with colloidal coomassie blue stain (G250). The stain solution contain 10%(w/v) ammonium sulphate, 10%(v/v) phosphoric acid, 0.12%(w/v) Coomasie G250 (Fluka analytical) and 20%(v/v) methanol [21]. Destaining was done until the proteins appeared blue on a clear background. The gels were scanned and analyzed for background subtraction, spot detection, volume normalization and differences in protein expression levels among samples by using the PD Quest software version 7.4 (Bio-Rad Hercules, CA, USA). Protein spots in gels were identified and the densities of these spots were determined after normalization based on the total spot volumes on the gel. The variable spot, either increased or decreased (>2 fold change) and were specific for the BC and the control group were manually labelled and considered for further identification through MS analysis.

LC-MALDI-TOF MS for protein identification

Differential protein spots of interest were excised manually and washed 3 times with milli-Q water. Each spot was placed into a 1.5 mL microtube filled with milli-Q water. In-gel digestion for LC-MS/MS analysis was performed at The Centre for Genomic Applications, New Delhi (India). In brief, trypsinized peptide samples (6 µL) lyophilized and suspended in a matrix solution containing α-cyano-4-hydroxy cinnamic acid (CHCA) in 50% ACN and 0.1% TFA were injected to NanoLC-1100 system (Agilent, Palo Alto, CA, USA), which comes combined with a microwell-plate sampler and thermostat column compartment for preconcentration (LC Packings, Agilent). The samples were loaded on the column (Zorbax 300SB-C18, 150 mm X 75 µm, 3.5 µm) using a pre-concentration step in a micro pre-column cartridge (Zorbax 300SB-C18, 5 mm X 300 µm, 5 µm). After 5 min, the pre-column was connected with the separating column, and multistep gradient (3% till 5 min, 15% for 5-8 min, 45% for 8-50 min, 90% for 50-55 min, 90% for 55-70 min, then again 3% for 70 min) was run.. An LC/MSD Trap XCT with a nano-electrospary interface (Agilent, Palo Alto, CA, USA) operated in the positive ion mode was used for MS. Ionization (1.5 kV ionization potential) was performed with a liquid junction and a non-coated capillary probe (New Objective, Cambridge, USA). Peptide ions were analyzed by the data-dependent method, the full MS scan. The scan sequence consists of 1 full MS scan followed by 4MS/MS scans of the most abundant ions. The peptides were spotted on ground steel plate and subjected to Bruker Ultraflex MALDI-TOF/TOF. Proteins were identified against the SWISS-PROT (GeneBio, Geneva, Switzerland) database (http://www.matrixscience.com) using Mascot Daemon (Matrix Science, London, UK), a client attached to the Mascot search protocol. The search parameters were as follows: Mass Tolerance: 2Da; monoisotopic values of 50 ppm/100 ppm; Fixed modifications: carbamidomethylation (cysteine); Variable modifications: Oxidation (methionine); Enzyme: Trypsin; Max Missed Cleavages: 1; Taxonomy: Homo sapiens. Probability based MOWSE score was calculated in terms of ion score −10*Log (P), where P is the probability and observed match was considered as a random event. Data was analyzed using Agilent Ion Trap Analysis Software Version 5.2.

Western blotting

Western blotting of the samples was performed as described earlier [22]. Briefly, samples were separated into 12% SDS gel, and then electro transferred onto nitrocellulose membrane at 250mA for 2 h. After blocking with 5% non fat dry milk in PBST contains 0.1% Tween 20 at the 4 °C overnight with gentle rocking, membranes were probed with antibodies. Primary antibodies used in our study include mouse monoclonal antibody apolipoprotein A I, mouse monoclonal antibody alpha 1 antitrypsin, rabbit polyclonal antibody alpha 2 HS glycoprotein and goat polyclonal antibody aplipoprotein A IV. Mouse monoclonal β-actin was chosen as a control. Membranes were incubated with corresponding primary antibody at dilutions recommended by the suppliers. Horse radish peroxidase-conjugated secondary antibodies and chemiluminescence kit (Millipore) were used for detection. Proteins expression was visualized by Versa Doc 4000 MP Imaging System (Bio-Rad Hercules, CA and USA).The densitometry of bands was measured by UNSCAN-IT software.

Immunofluorescence staining

Immunofluorescence staining of Apolipoprotein A I, alpha 1 antitrypsin, alpha 2 HS glycoprotein and aplipoprotein A IV was performed on formalin-fixed and paraffin-embedded lymphoid tissue sections. Slides were deparaffinised in xylene, rehydrated in a graded ethanol series, and treated with an antigen retrieval solution (10 mmol/L sodium citrate buffer, pH 6.0). Endogenous peroxidase activity was quenched with methanol: hydrogen peroxide solution and non-specific binding was minimized with normal sheep serum. The sections were incubated overnight in a moist chamber with respective primary antibodies at dilutions recommended by the suppliers. After incubation, the sections were incubated with TRITC and FITC-conjugated respective secondary antibodies for 40 min in dark. The slides were washed with phosphate buffer saline-Triton X-100 in between the incubations. Slides were lightly counter-stained with

Hoechst before imaging. The immunostained slides were photographed using fluorescence microscope Olympus IX51 (Olympus America, Center Valley, PA, USA).

Statistical analysis

The protein expression was analyzed by one-way analysis of variance (ANOVA) test in healthy control and diseased subjects, $p<0.05$ value was considered as significant. Protein expression data for healthy and diseased patient groups were expressed as the mean ± SD of 25 gels for fold-changes of normalized spot volumes. For the statistical analysis of data, Student-t-test was used and $p<0.05$ was considered as significant. The prevalence was calculated at crude rate and 95% confidence interval (CIs) of proteins was calculated by the asymptotic method with continuity correction [23].

Results

Patient information

This study was undertaken to identify the breast cancer associated plasma proteins in North Indian women. Twenty five healthy and patient women volunteer aged 20-60 were classified into three groups, 20-35, 35-50 and age older than 50, and matched with menopausal status (Table 1). In group A and B, we pooled the plasma of 10 samples of control and patient (5 in each batch) in two batches respectively. In group C we pooled 5 plasma samples in one batch. In all three age groups, standard samples were run in 5 replicates on 5 gels (5 gels per batch) to generate 25 images. The overall protein expression patterns between patient and control were evaluated by combining the data from all three age groups.

The clinicopathological characteristics of reactive nonspecific lymphadenitis and malignant breast cancer lymph node tissue (haematoxylin and eosin stained) slides were analyzed. The observation of microscopic analysis has clearly specified the differential characteristics of both the cases and reconfirmed the previous diagnosis. Briefly, in case of reactive lymphadenitis: the congested lymphatic and vessels were found in the cortex region with the prominent lymph follicle containing few germinal centers. Medullary sinuses are dilated and are filled with histiocytes/ macrophages and inflammatory cells. Mononuclear cell infiltration with patchy areas of fibrosis was also observed throughout the oedematous tissue. While in case of BC metastasis: the region of cortex and medulla showed the subcapsular and medullary dilated lymphatic sinuses and were effaced with metastasizing tumor. The tumor is comprised of proliferated atypical round to polygonal duct epithelial cell having enlarged round hyperchromatic nuclei with conspicuous nucleoli. Variable amount of scanty non-discernible cytoplasm showing high grade dysplasia was also observed. Areas of necrosis, fibrosis and hemorrhages with the increase number of abnormal mitotic figures and bizarre tumor giant cells were in prominence. The representative HE stained images of the cases, reactive lymphadenitis and BC metastasis lymph node tissue are given in (Figure 1 A-L).

Depletion of high abundance proteins

In order to detect low-abundance proteins, initial studies were performed to deplete plasma of the most abundant proteins using Hu-14 multi affinity reverse spin (MARS) column and PROTEOPREP-20 columns. The depleted samples were further concentrated and desalted using ultracentrifuge filters excluding proteins with a molecular mass lower than 5 kDa and then run on a Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis (SDS-page). Comparative SDS page analysis showed that among them depletion through the HU-14 MARS column is more proficient in terms of recovery and gives approximately 79% depletion of total protein content in the flow through fraction (Figure 2). Moreover the comparison of 2DE images of plasma processed with these two columns evidently confirm that the HU-14

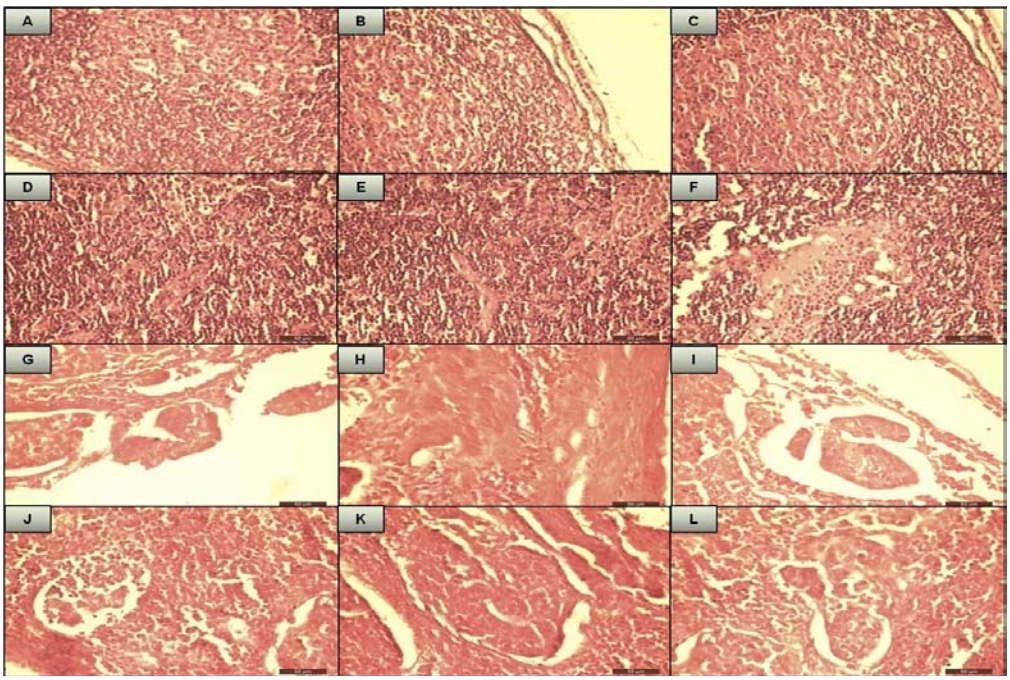

Figure 1: Representative images of histopathological features of reactive lymphadenitis and BC metastasis lymph node tissue (stained with haematoxylin and eosin) (A-F) reactive lymphadenitis, (G-L) BC metastasis lymph node. (*n=3*) All magnifications are at 40 X.

MARS column deplete HAPS more efficiently enhancing opportunities to visualize LAPS for mounting biomarkers (Supplementary Figure 1).

2DE profiling and protein identification

For screening the differential expressed proteins in the proteome of cancer patients, we used 2DE based profiling. Approximately 94–428 protein spots were visualized in the depleted plasma of BC and control samples (Figure 3). The quantity of each spot in a gel was normalized as a percentage of the total quantity of all spots in the gel. As shown in the scatter plot (Figure 4), the volume of paired protein spots showed a linear distribution. The correlation coefficient between cancer and control was 0.70. Further the comparative analysis of 2-DE gels revealed total 24 differentially expressed spots with statistically significance (*t* test, $p<0.05$). Fourteen spots were up- and ten were downregulated in patient plasma. Spot members of these classification models were identified by LC MS/MS followed MALDI TOF/TOF and further confirmed by MS/MS. The nature of 24 identified spots, the measured 2-DE coordinates and relative sequence coverage are listed in Table 2. Most of these identified proteins functionally involved in human immune system (79%) and transport (21%) and almost 84% of the differentially expressed proteins identified were secreted proteins (Figure 5 and Figure 6).

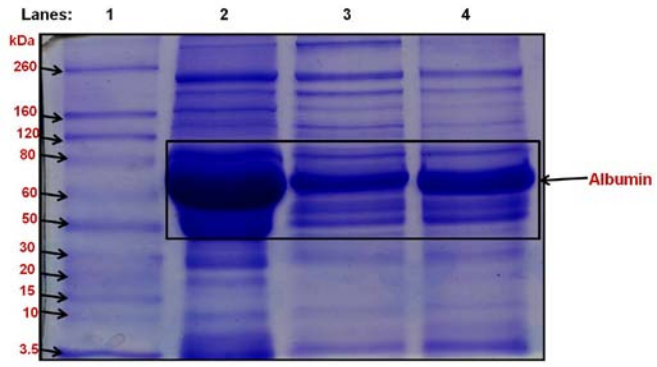

Figure 2: SDS PAGE of human plasma protein fractions after depletion through Hu-14 MARS and PROTEOPREP columns. Lane1: Unstained Marker (3.5-260 kDa); 2: Crude plasma; 3: Depleted plasma (MARS column); 4: Depleted plasma (Proteoprep-20 column). Total protein load was 60 µg/lane. The gels were stained with Coomassie R-250 stain.

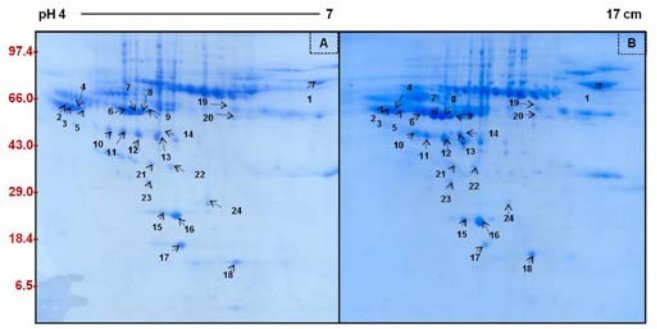

Figure 3: Representative 2-DE maps of control and BC patients (A) Control human plasma sample (B) BC. The gels were visualized with Coomassie G-250 staining. The pH gradient is indicated on the top of the gels horizontally, and migration positions of molecular weight markers are indicated on vertical axis. 24 significantly and constantly modulated proteins selected by quantitative analysis are indicated by arrows and numbered as 1-24 at their spot positions in the gels.

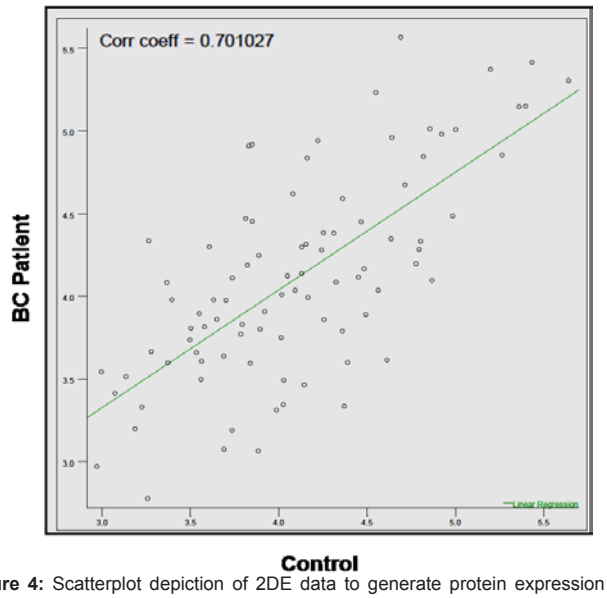

Figure 4: Scatterplot depiction of 2DE data to generate protein expression profile. The x axis indicates the volumes of protein spots in normal control whereas the y axis represents those in cancer patients. Corresponding correlation coefficient is included in the diagram.

Protein identified	Spot No.	Fold change	Patient Responders (n)	Prevalence 95% CI
Alpha-1-antitrypsin	9	4.7	23	92%(79.36-104.63)
Apo A I	16	6.0	23	92%(79.36-104.63)
Apo A IV	14	5.9	18	72%(52.40-91.60)
Alpha-2-HS Glycoprotein	4	-2.3	19	76%(57.26-94.74)
Human Serum Amyloid P Component	24	-2.2	15	60%(38.79-81.20)
Human Plasma Retinol-Binding Protein	17	-2.0	11	44%(22.54-65.46)
Haptoglobin protein	12	4.5	12	48%(26.41-69.58)
Apo-Human Serum Transferrin	1	4.8	11	44%(22.54-65.46)

Table 2: Prevalence (95% CI) of BC related proteins identified by 2D-LC-MALDI–TOF MS.

Protein spots that varied >2 fold change and appeared in more than 18 gels of BC group over control subjects were taken as granted (Table 2). Based on this criterion we have selected five frequently expressed identified protein spots (4, 9, 14, 15 and 16) for further proceedings. These spots were identified as alpha-2HS glycoprotein (spot no. 4), serpin peptidase inhibitor clade A (SERPINA1) (spot no. 9), apolipoprotein A-IV (spot no. 14) and lipid free apolipoprotein A-I (spot no. 15-16). The close up views and fold change expression of each are shown in (Figure 6 A and B). To eliminate the redundancy of proteins with multiple isoforms under different accession numbers, only a particular isoform belonging to the species Homo sapiens with the highest MASCOT score were selected. The alpha 2-HS glycoprotein had Mascot score of 75, sequence coverage of 28% and 9 matched peptides where as SERPINA had Mascot score of 151, sequence coverage of 33% and 14 matched peptides. The apolipoprotein A-IV precursor had Mascot score of 220, sequence coverage of 51%, 33

matched peptides and apolipoprotein A-I had Mascot score of 97-110, sequence coverage of 36-46%, 9-11 matched peptides (Figure 7 A-D).

Validation of proteins using western blotting

In order to ensure the reliability of the proteome results of 2DE, the expression pattern were validated independently by Western blotting confirmations. The result of densitometry analysis showed that the expressions of Apolipoprotein A I (83%), Apolipoprotein A IV (10%), SERPINA (85%) were increased significantly and that of Alpha HS Glycoprotein decreased (49%) in BC patients as compared with control subjects. The reassessments of expression patterns of the selected proteins and their percentage increment in BC patients are shown in Figure 8 A and B.

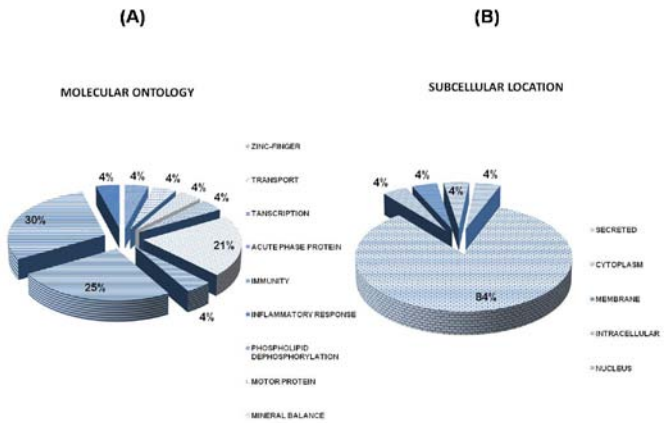

Figure 5: Percentage of plasma proteins identified by 2D-LC-MALDI–TOF MS for BC according to (A) Biological functions and (B) Sub-cellular location.

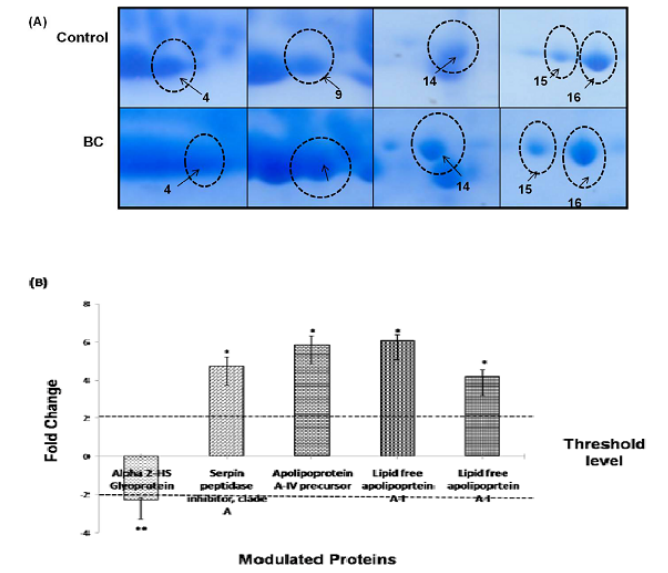

Figure 6: (A) Close-up view of the selected differential proteins indicated with their respective spot no. showing significantly ($p<0.05$) up-regulation and down-regulation. (B) Quantitative fold changes of selected proteins, calculated with respect to control on the basis of volume measured by PD QUEST Software. Values are expressed as fold changes of the control values, indicated by the horizontal control level line. Values represent mean ± SD of 3 sets of experiments. *: Significantly up-regulated protein ($p<0.05$). **: Significantly down-regulated protein ($p<0.05$).

Validation of proteome data with immunohistochemistry

To further confirm the altered level of selected proteins and their correlation with BC progression, we followed immunohistochemistry analysis in reactive lymphanitis and BC malignant lymph node tissues. The Immunofluorescence expression of APO AI, APO AIV and Alpha 1 antitrypsin were consistent and showed significantly higher prevalence of high level expression of these three proteins in malignancy state (Figures 9 A-C). However, the result of Alpha 2 HS glycoprotein immunofluorescence stain was not interrelated with the results of 2DE and western blotting (Supplementary Figure 2). Each protein was tested in 5 pairs of sections and the staining was scored using a scale of negative, weak, moderate and positive, according to the staining intensity. Cases with negative and weak staining intensity were considered as negative while cases with moderate and strong staining intensity were considered as positive.

Discussion

The intricacy of cancer proteome far exceeds the range of any single analytical approach or a sample resource. In this study, we have executed the strategy of immunoaffinity depletion prior to 2DE-LC-MS/MS to study the unbound fractions of elute consisting of important low abundance proteins. Based on affinity-purified polyclonal antibody binders, these columns contain low molecular weight antibody ligands for the depletion of HAPs. The ligand selection ensures optimal specificity and removes 97-98% of the total protein mass in human plasma or serum allows proficient analysis of less abundant target proteins. Our results showed that after depletion of HAPs the elution of MARS column was conceptually less complex than PROTEOPREP and thus it provides a conspicuous sample for 2DE-LC-MALDI TOF MS analysis. We applied this scheme to a sample set collected from female patients with breast carcinoma, and compared their plasma proteome profile with healthy (control) group. We also employed a sample pooling method to first index as many as possible proteins in the disease groups in a high throughput approach. The comparative 2DE proteome profiling coupled with LC-MS/MS identification of group samples showed total 24 differentially modulated (>2fold) spots in BC patients (Table 3, Figure4). Of these, Apolipoprotein A I, Apolipoprotein A IV, Alpha-2- HS glycoprotein, Serpin peptidase inhibitor clade A (SERPINA1), Retinol binding protein (CHAIN A), Human Serum Amyloid P Component, Apo Human Serum Transferrin appeared to be of a particular importance, since the database search and previous findings for these altered proteins indicated their possible role in regulation of pathogenesis of breast cancer metastasis. As the probability of Apolipoprotein A I, Apolipoprotein A IV, Alpha-2-HS glycoprotein, SERPINA1 were comparatively high and showed reproducible results in all patient groups (Table 3), we have selected them for our further investigations of clinical validation. Further, the result of western blot analysis in other individuals and immunofluorescence staining of BC positive axillary lymph node tissue also confirms the similar modulated expressions and depict the strong linkage of these selected candidate proteins with breast cancer metastasis progression (excluding Alpha 2HS glycoprotein).

Serpin peptidase inhibitor clade A

The increased expression of a Serpin peptidase inhibitor clade A (SERPINA1) spot, as detected in the present study in BC patients by 2DE/MS and confirmed by immunoblotting and Immunofluorescence measurements, was in good agreement with previous findings in pancreatic and cutaneous squamous cell carcinoma [24,25]. It is

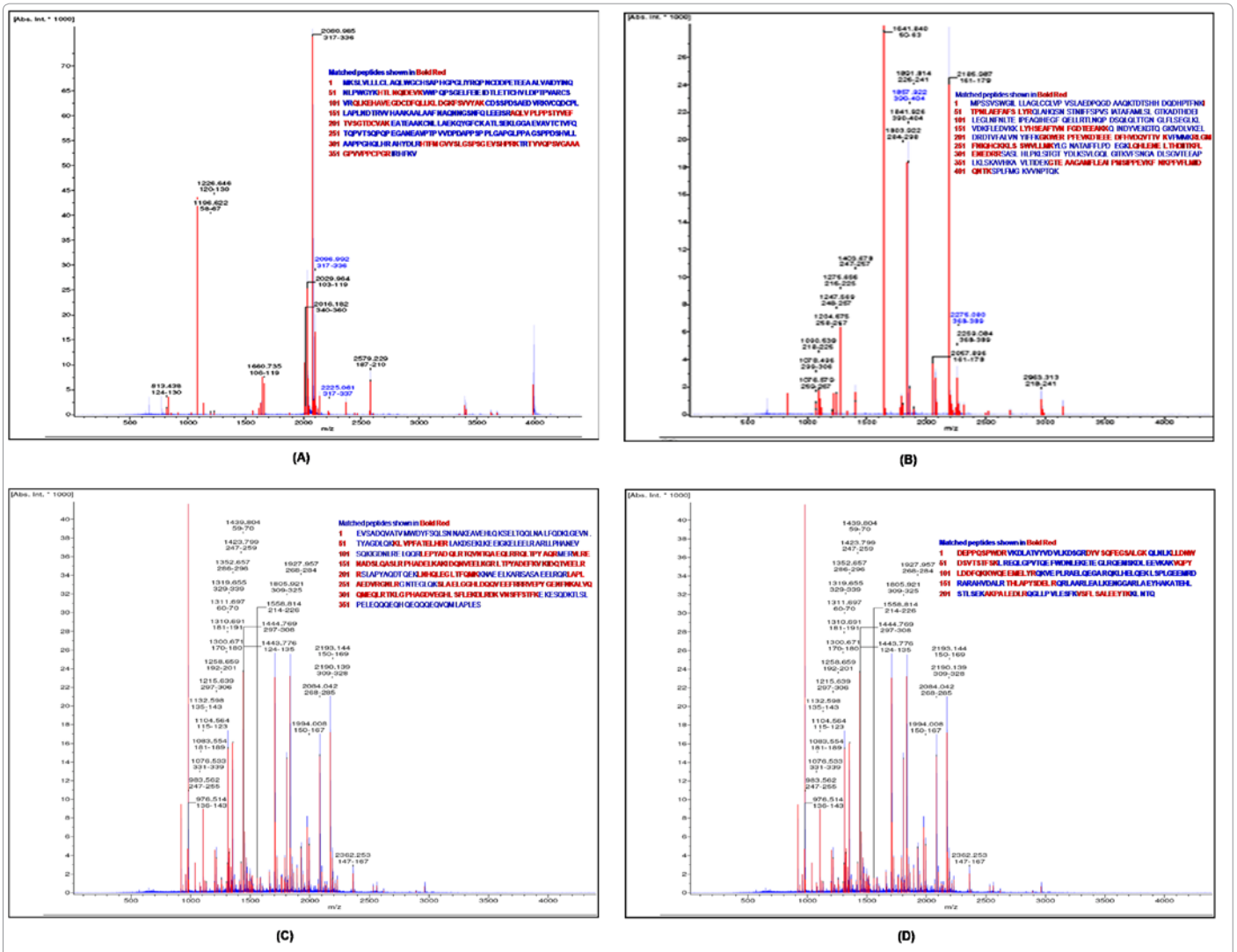

Figure 7: A representative peptide mass fingerprints of identified proteins (A) Alpha-2-HS-glycoprotein (B) Serpin peptidase inhibitor, clade A (C) Apolipoprotein A-IV precursor (D) Apolipoprotein A-I.

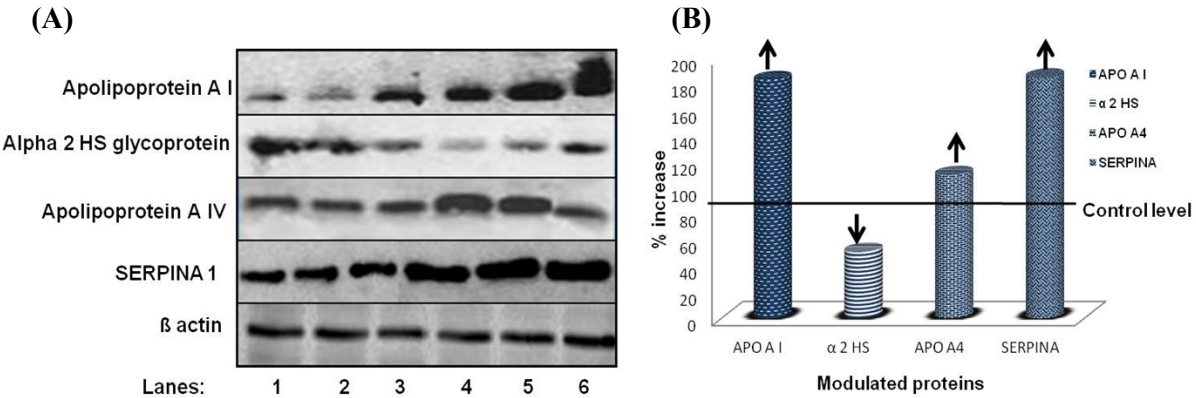

Figure 8: (A) Western blots showing expression of apolipoprotein A-I, alpha-2-HS-glycoprotein, apolipoprotein A-IV and Serpin peptidase inhibitor, clade A (SERPINA1) among control and BC groups. Equal loading of the samples was evaluated by reprobing the membranes with β-actin antibody. Lane1: Control 1; 2: Control 2; 3: Control 3; 4: BC1 5: BC2; 6: BC3. (B) Their percentage increment was calculated respect to control on the basis quantitative fold change of pixel density measured by UNSCAN- IT software.

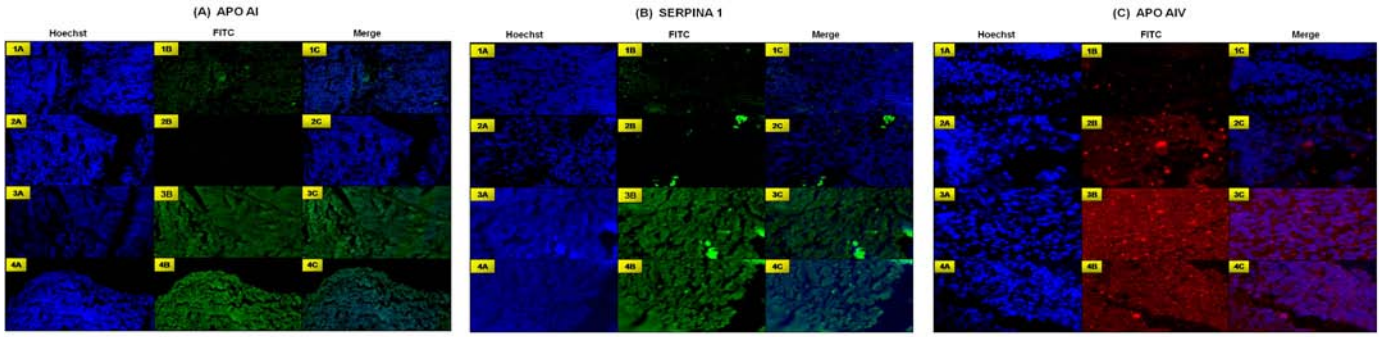

Figure 9: Representative images of immunofluorescence staining of (A) APO AI, (B) SERPINA 1, (C) APO AIV in reactive lymphadenitis (1,2) and BC metastasis lymph node (3,4). (*n=2*) The presence of these proteins in respective cases is indicated by the amount of fluorescence emitted. All magnifications are at 20 X.

commonly known as Alpha1-antitrypsin (A1AT) or alpha-1 proteinase inhibitor (A1PI) because it inhibits a wide variety of proteases [26]. Protease is a group of enzymes (including trypsin, chymotrypsin and elastase) found in body tissues and blood whose function is to breakdown the peptide bonds, controlling protein composition, turnover and ultimate destruction. Its role in fighting with the growth of cancerous tumors has been reported as it dissolves fibrin coating on the cancer cells and removes the dead and abnormal tissues [27]. However, its regulation is particularly important as sometimes it could attack and damage normal tissues rather than the intended target resulting in local tissue damage. SERPINA1, a commonest protease inhibitor targets protease enzyme to prevent its potentially unrestricted harmful activity. SERPINA1 play a significant role in controlling inflammation, coagulation and repair mechanisms in the body [28,29]. Its prime involvement in the regulation of growth processes and in the inhibition of apoptosis and lymphocyte cytotoxicity has also been demonstrated [30]. Earlier studies have suggested that α1-antitrypsin deficiency is associated with increased risk of different types of cancer, as it induces serine protease activities thus enhancing detachment of malignant cells from their original sites [31]. But later on in some reports the increased expression of α1-antitrypsin in cancer progression was also identified [32]. According to Kataoka et al. [33] the C-terminal fragment of α1-AT increases tumor growth, due to its modulatory effects on natural killer cytotoxicity against tumor cells. Moreover the critical role of SERPINA1 in regulation of ubiquitin-proteasome, cell survivality and growth of tumors by downregulating the TNF- α expression has also mentioned in various studies [34,35]. Recently, the high sensitivity of A1AT and autoantibodies against A1AT in breast cancer patients has been recognized by Eneida López-Árias et al. [36]. They found it may be useful as a serum biomarker for early-stage screening and diagnosis. Collectively, these findings are enough to sustain that our outcome with increased expression of α1-antitrypsin in BC patients will possibly be a reliable, reproducible and easy-to-obtain diagnostic marker among North Indian patients.

Apolipoprotein A I and apolipoprotein A IV

Among the identified differential proteins, the two upregulated proteins are of the family of Apolipoprotein (apolipoprotein AI, AIV), which are known to be involved in many biological functions as they are the main proteins of transport. Studies revealed that changes in plasma lipid and plasma lipoproteins play significant role in cardiovascular risks and proliferation of many cancer including breast [37-39].

Apo A–I is a major constituent of the high density lipoprotein in plasma [40]. It plays imperative roles in regulation of tumor growth, angiogenesis, invasion and metastasis [41] which is the key related factors of cancer progression. Formerly a significant association has been accounted between ApoA1polymorphisms and both the susceptibility to as well as the prognosis in breast cancer [42]. The modulated level of Apo A I could be specific to cancer diagnosis as it promotes the innate tumor cell recognition and lysis by emphasizing the association of F1F0 ATP synthase and gamma delta subtype antigen receptor [43]. However, APO A I generally has been characterized as acute phase reactant. Mechanistically it has the ability to bind proinflammatory phospholipids [44] which are known activators of proliferation in cancer cells [45] thus they could be possible potential targets for BC therapy. A negative association of APO A I to inflammation and antioxidant capability, oestrogen metabolism which is a crucial component of tumorigenicity has also been in the literature [46,47]. In numerous reports such as bladder cancer, pancreatic cancer and colon cancer the APO A–I levels are found up-regulated [48-50] however, reduced plasma Apo A–I level has been reported in cholangio-carcinoma, ovarian and breast cancer [51-53]. This current 2D-LC MS/ MS and immunochemical analysis revealed apolipoprotein A–I level is upregulated in BC patients of North India. Thus, these above mentioned studies and the current result verified that the regulation of apolipoprotein A–I level during BC malignancy is complicated and needs further clarification to conquering any conclusion.

Another apolipoprotein identified as apolipoprotein A IV, which was found upregulated in cancer patients, was consistent with the previous findings that showed the increased expression of this protein in lung squamous cell carcinoma [54]. Apo A IV is a member of the apoAI/CIII/AIV/AV gene cluster (of 17 kb) located on the arm of human chromosome 11 [55]. Human apoA-IV is composed of 376-amino acid residues and modified by O-linked glycosylation. Although its exact function in carcinogenesis is not yet much explored but its considerable role in different cancers has been reported. Recent evidences from experimental models suggest to apolipoprotein A-IV as a protective agent against lipoprotein oxidation and atherosclerosis, which is highly associated with neoplastic diseases as they are related to the main event of thrombosis [56]. Previously, in a 2DE/Proteomics based study apolipoprotein A-IV plasma levels were found significantly higher in cancer-free BRCA1 mutation carriers compared with BRCA1 mutation carriers who developed breast cancer [57]. As human Apo-A IV secretion is stimulated by lipid absorption and is at least partly associated with HDL [58], therefore a prospect extensive research to examine the relationships between these factors and APO IV is required for enlightening the role of this protein in breast cancer. Since the role of lipid transport, HDL and oxidative stress are in the development

Spot No.	Protein Name	Score	Gene	Theoretical / Observed		Accession No.	Sequence coverage	No. of matched peptides	No. Of unmatched peptides	Fold change
				Mr (KDa)	pI					
1	Chain A, Apo-Human Serum Transferrin (Glycosylated)	197	PRO1400	97 / 76.9	6.8/6.85	P02787	35%	29	11	4.8
2	PRO2743 [Homo sapiens]	78	PRO2743	67 / 23.0	4.2/6.44	NP_001129513	38%	8	33	-2.6
3	Histone deacetylase 11 isoform 1	69	HDAC11	66/ 39.2	4.6/ 6.81	10438544	28%	8	33	-6.6
4	Alpha-2-HS-glycoprotein, isoform CRA_a	75	AHSG	62/ 39.9	4.8/ 5.43	P02765	28%	9	36	-2.3
5	Kinesin family member 19	76	KIF19	60/ 61.9	5.0/ 7.17	Q99PT9	26%	10	59	-2.5
6	Chain A, A 2.1 Angstrom Structure of An Uncleaved Alpha-1- Antitrypsin	166	PRO0684	60/ 44.3	5.4/ 5.35	P01009	38%	12	16	3.2
7	Chain A, A 2.1 Angstrom Structure of An Uncleaved Alpha-1- Antitrypsin	177	PRO0684	60/ 44.3	5.4/ 5.35	P01009	28%	10	28	3.8
8	Chain A, A 2.1 Angstrom Structure Of An Uncleaved Alpha-1- Antitrypsin	96	PRO0684	60/ 44.3	5.5/ 5.35	P01009	28%	12	57	4.2
9	Serpin peptidase inhibitor, clade A	151	PRO2209	60/ 46.8	5.6/ 5.36	P01009	33%	14	26	4.7
10	HP protein/hemoglobin binding	92	HP	57/ 31.6	5.3/ 8.48	P00738	36%	12	39	2.0
11	HP protein/hemoglobin binding	92	HP	57/ 31.6	5.4/ 8.48	P00738	39%	11	28	2.0
12	HP protein	95	HP	57/ 31.6	5.6/ 8.48	P00738	39%	8	44	4.5
13	HP protein	76	HP	57/ 31.6	5.8/ 8.48	P00738	32%	13	74	4.2
14	Apolipoprotein A-IV precursor	220	APOA4	58/ 43.3	5.7/ 5.2	P06727	51%	33	61	5.9
15	Apolipoprotein A-I	97	APOA1	43/ 28.0	5.7/ 5.7	P02647	36%	9	21	6.0
16	Apolipprotein A I	110	APOA1	43/ 28.0	5.8/ 5.27	P02647	46%	11	49	4.2
17	Chain A, Crystal Structure Of The Trigonal Form Of Human Plasma Retinol-Binding Protein	160	PRO2222	19/ 21.2	6.2/ 5.27	P02753	67%	10	52	-2.4
18	Phosphatidic acid phosphatase type 2 domain containing 1A	69	PPAPDC1A	14/ 29.0	7.0/ 8.5	Q5VZY2	48	6	26	2.1
19	Complement component 4B (Childo blood group)	91	C4B-1	75/ 74.5	6.8/ 5.81	A2BHY4	29	14	64	-2.1
20	Prepro-C3b/C4B inactivator	289	CFI	70/ 68.5	6.8/ 7.72	AAA52455.1	10	4	22	-2.7
21	Apolipoprotein J precursor	52	APOJ	50/ 49.3	5.8/ 6.27	178855	2	1	1	-2.2
22	Inter-alpha-trypsin inhibitor family heavy chain-related protein (IHRP)	221	PRO1851	51/ 103.5	6.1/ 6.51	Q14624	4	2	29	2.3
23	Tripartite motif-containing 31	16	TRIM31	48/ 31.1	5.7/ 7.11	C4B4E3	4	1	15	-2.2
24	Human Serum Amyloid P Component	71	PTX2	45/ 23.3	6.5/ 6.12	P02743	23	5	21	-2.2

Table 3: Mass spectrometry identification of 24 modulated proteins.

and progression of cancer, we hypothesized that the increased levels of ApoA-IV in BC patients may have been causal in disease mount. And with combination with apoAI this protein could be taken as decisive marker for early diagnosis of BC in North Indian women.

Alpha-2-HS glycoprotein

Furthermore, the spot identified as alpha 2-HS glycoprotein (AHSG) was found to be downregulated in the plasma of BC subjects. AHSG is also designated as a fetuin-A or α-2-ζ-globulin. Basically AHSG is a secreted plasma protein that is expressed in hepatocytes, monocyte/macrophages and in bone. During injury and inflammation its downregulated expression has been reported [59]. As an antagonist to transforming growth factor β, AHSG inhibits tumor progression i.e. shows negative role in cancer progression [60]. However the association of TGF-β with tumor progression and resistance to chemotherapy in recognizing cancers has been well published in the literature. Thus it may possible that down-regulation of AHSG enhances TGF-β-mediated immune suppression and could help in cancer progression. Further, the reports on down regulation of alpha-2 HS glycoprotein in the serum of lung cancer [54] and in both at the protein and the mRNA level of HCC patients [60-62] shows our data were concurrence with these findings. The utility of AHSG in the screening and staging of BC with anti-AHSG autoantibody as a potential serum biomarker has also been confirmed [63]. However some of the diverging results illustrated the increased level of alpha-2-HS-glycoprotein in the stage IV samples of BC [64] creates the need of additional aspect examination. As in our findings we also obtained some deviating results in immunohistochemistry analysis of the BC lymph node where the progressive expression of this protein was shown at a late metastasis stage which is in contrast to 2DE/Immunoblotting conclusions.

In conclusion, the present study is the first one to be carried out to develop the plasma protein based diagnostic markers in ethnic north Indian population which are recognized to the high risk carrier of BRCA1 mutation. As India has a rich multiplicity of cultural population

with different life styles, such a pilot study for characterizing the differential breast cancer responsive proteins might offer an impulsion to conduct larger prospective evaluations in different ethnic Indian populations. This early diagnostics based data could be helpful in engendering the information about the factors of disease grounds that possibly will useful to provide potential targets for therapy. Here, we have shown that Apo AI, Apo A IV, AHSG, SERPINA1 appeared differentially expressed in 2DLC-MS/MS analysis of BC plasma. As these findings are congruent with the results of western blotting and Immunofluorescence staining of BC positive axillary lymph node tissue (in different individuals) except for the alpha 2 HS Glycoprotein, supports the validity and reproducibility of our results. Since the clinical routine (blood based) diagnostics has ability to assess the estimation of protein concentration in an easy and reproducible way, thus with conjunction with 2DE-MS proteome profiling these findings may provide the successful strategy to determine the status of breast cancer progression at earliest. Hence, a large–scale study is needed to confirm and validate our current results.

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Conflict of interest

The authors declare that no conflicting financial interests exist.

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