



Comparative assessment of proteins between cell lines and tissues representing carcinomas of the breast using 2-dimensional gel electrophoresis

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ABSTRACT

Incidence of breast cancer in the age group of 30 to 50 is increasing at an alarming rate globally. Moreover, existing treatments are either marginally effective or resistance developed, hence development of safe and potent pharmacological agents is immediately required. However, establishment of a disease progression marker for early detection, development of safe and effective treatment agents requires identification of key proteins that are exclusively expressed in advanced malignant breast tumors. 2-Dimensional gel electrophoresis (2-DGE) is one of the early methods used for the identification of deregulated proteins in cancers. Therefore, the proteins of benign and malignant tumors as well as breast cancer cell lines MCF-7, SKBR-3 and MDA-MB-468, were subjected to 2-DGE and relative expression calculated. Analysis of the data showed a significant increase in the intensity of 7 protein spots compared to malignant tissues and benign ones. Further, comparison of cell line proteins with tissue lysates revealed that SKBR-3 is much closer to malignant tumors, hence, may be considered for screening drug targets as well as for evaluating the efficacy of pharmacological agents. In conclusion, our 2-DGE identified key differences and similarities in the expression of proteins between breast cancer cell lines, benign and malignant tissues.

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INTRODUCTION

Breast cancer is one of the leading causes of cancer related deaths in women (DeSantis *et al.*, 2019). The incidence of breast cancer has increased significantly in the past 50 years, with 1.7 million new cases being added to the existing pool every year (Dey *et al.*, 2016). According to the 2018 report by the World Health Organization (WHO), breast cancer accounts for about 11.2 % of the total female cancers (Bray *et al.*, 2018; Martín-Sánchez *et al.*, 2018). Although breast cancer is more prevalent

in women (1 in 8 women), it is also seen in men (1 in 1000) (Arem *et al.*, 2015). In India, 155,000 women were diagnosed with breast cancer (Rangarajan *et al.*, 2016; Madhav *et al.*, 2018). The more alarming fact is that, in the year 2012, India had the highest number of deaths (70,218 deaths) due to breast cancer compared with any other countries (Donepudi *et al.*, 2014). Analysis of recent statistics has revealed that the incidence and mortality rates of breast cancers are >20% and 14% respectively, with more incidence rates in the developed countries, while mortality rates are significantly high in the less developed countries (Youliden *et al.*, 2014; Ghoncheh *et al.*, 2016).

Lack of facilities for early detection, the advent of quadruple negative breast cancer types, and poor access to various treatment methods are the major contributors to increased mortality rates in developing countries (Angajala *et al.*, 2019). Although existing therapies are efficient, a number of drawbacks and side effects were reported. For example, chemotherapy and radiation treatment are not target specific. Hence, they damage the normal cells and cause systemic toxicity (Bhatt *et al.*, 2010; Nurgali *et al.*, 2018). For some breast cancers such as TNBCs, no selective treatment agents exist as they lack any hormone receptors. Moreover, recent studies have reported the development of resistance to chemotherapeutic agents (Moiseenko *et al.*, 2017; Marquette and Nabell, 2012). Therefore, a search to identify selective therapeutic targets still continues. Proteomics is one tool to identify deregulated proteins in cancers (Shruthi *et al.*, 2016; Hanash *et al.*, 2012; Shukla, 2017)

While the identification of early detection markers and therapeutic targets using tissue samples helps in clinical diagnosis and treatment decisions, it is not known whether a similar pattern of protein expression is also observed in established cell lines representing benign and metastatic breast tumors. This information is key as the cell lines, but not tissues, are widely used in drug discovery and development research (Malorni *et al.*, 2006; Valle *et al.*, 2011). However, it is not currently known, which among these cell lines better represent breast cancer tumors (Xu *et al.*, 2010; Dai *et al.*, 2017). Addressing this, a recent study first measured and compared the expression of proteins among the breast cancer cell lines T47D and MCF7 (Aka and Lin, 2012). Analysis of the data showed upregulation of (a) cell growth-stimulating G1/S-specific cyclin-D3 and prohibitin; (b) anti-apoptotic and carcinogenic proteins in T47D compared to MCF7 (Aka and Lin, 2012). However, proteins involved in transcription repression and apoptosis regulation such

as NF-X1, nitrilase homolog 2, and interleukin-10 reported to be heavily expressed in MCF7 compared to T47D (Aka and Lin, 2012; Mooney *et al.*, 2002). Hence, cell lines widely vary in terms of their proteome (Geiger *et al.*, 2012). Knowing the expression of proteins of cell lines is essential as the majority of drug screening and target identification experiments are being carried out using cell lines. In addition, it is also important to identify the cell line that better represents the malignant and benign tissues. Therefore, in this present investigation, an attempt is made to isolate and compare proteins from cell lines widely used in breast cancer research with the proteins collected from breast carcinoma tumors using 2-dimensional gel electrophoresis.

MATERIALS AND METHODS

Cell lines

MCF7 (ER+, PR+, HER2-), SKBR3 (ER-, PR-, HER2+), MDA-MB-468 (ER-, PR-, and HER2 +/-), cells lines were procured from National Center for Cell Science, Pune, Maharashtra, India.

Benign and Malignant Tissues

Biopsies from benign and malignant tumors were collected from patients after receiving the approval from the Institutional Ethics Committee of JSS Medical College (Approval # JSSMC/IEC/14/1991/2017-18, Dated 05.06.2017), JSS Academy of Higher Education & Research, Mysuru, Karnataka, India

Reagents

Dulbecco's Modified Eagle's Media (DMEM), Fetal Bovine Serum (FBS), Glutamax, Dulbecco's Phosphate Buffered Saline (DPBS), Trypsin-EDTA (0.25%), Pen-Strep, reagents were from Thermo Fisher Scientific, Waltham, MA, USA. All cell culture plastics were from Techno Plastic Products (TPP) Pvt Ltd, Bengaluru, Karnataka, India. Tris HCl, sodium dodecyl sulfate (SDS), Bromophenol blue, Dithiothreitol (DTT), Glycine, Urea, Thiourea, CHAPS, Methanol, Acetic acid, Coomassie brilliant blue R-250 were from Sisco Research Laboratories Pvt. Ltd. Mumbai, Maharashtra, India.

IPG strips (Nonlinear pH: 3-10, Length: 11cms) and IPG buffer were from Bio-Rad Laboratories India Pvt. Ltd., EMAAR Digital Greens, Haryana, India.

5X running buffer (pH-8.3) was prepared by dissolving 0.18M Tris base and 1.44M Glycine and 0.75% SDS

Rehydration buffer was prepared by 8M urea, 1M Thiourea, 2% CHAPS, 15mM DTT, 2% IPG buffer, traces of Bromophenol Blue (0.005%).

Equilibrium buffer was prepared by first making

1. Equilibrium solution 1: 6M Urea, 50mM Tris pH6.8, 30% Glycerol, 4% SDS, 20mM DTT
2. Equilibrium solution 2: 6M Urea, 50mM Tris pH6.8, 30% Glycerol, 4% SDS, 4.5% Iodoacetamide

The staining solution was made by dissolving 20% Acetic acid, 40% Methanol, and 0.2% Coomassie brilliant blue R-250.

The destaining solution was prepared by 20% Acetic acid and 20% Methanol.

Resolving gel (12%) for 2D-SDS-PAGE was prepared by mixing 15.0mL of 1.5M Tris HCl (pH-8.8), 24.0mL 30% acrylamide, 3.0mL 1.5% ammonium persulfate, 0.6mL 10% sodium dodecyl sulfate, 0.03mL TEMED followed by adding 17.37mL water (Final volume of the mixture is 60.0mL)

Collection of breast cancer tissues

First, Institutional Ethics Committee approval for collecting tumor samples from patients suffering from breast cancer (who had visited JSS Hospital and referred for a pathological examination) was obtained (JSSMC/IEC/1991/2017-2018, dated 5th June 2017) to conduct this study. Next, the consent for using the tumor tissues for research purposes was obtained from each patient. The tumor tissue was washed with PBS and processed for whole-cell protein lysate isolation.

Preparation of whole-cell lysates

First, homogenization of the cells and tissues was carried out by freeze-thawing method or by grinding the samples using liquid nitrogen, respectively. Next, 0.5ml lysis buffer was added to the homogenized samples, and the sonication carried out by following a 3sec ON and 5sec OFF cycle (for 10 times). Samples were centrifuged at 14500rpm for 1hr at 4°C and to the supernatant 20µl, DNase (1mg/ml DNase I in a buffer, which contains Sodium chloride, Magnesium chloride and Glycerol) was added to initiate the degradation of DNA. The reaction was carried out for about 3.0h at 4°C.

Estimation of total protein by BCA

Total protein in the tissue and cell lysates was estimated using a BCA kit from Pierce (Thermo Fisher Scientific, Rockford, IL). Experimentally, a microplate procedure designed to estimate protein content in the 96-well plate, was followed as detailed by Smith. et al. (Smith et al., 1985). First, 10µL of each standard (ranging from 125µg/ml to 2,000µg/ml) or sample was added into a microplate well. Next, a 200µL reagent mixture (A and B solutions mixed in 1:50 ratio) was added and incubated

at 37°C for 30.0minutes. Absorbance was measured at 562nm using a multimode plate reader from Perkin Elmer. A standard graph was plotted and the concentration of samples estimated.

2-Dimensional Gel Electrophoresis

Iso-electric focusing: In isoelectric focusing, the separation of proteins occurs in the horizontal direction based on pI values of the protein (1-12). The net charge of the protein depends on the pH of the local environment. When the total protein was placed in the medium with a linear pH gradient and subjected to an electric field. The proteins migrated to the opposite electrode. During migration, the protein gains or lose a proton and reaches a point where it becomes uncharged (pI value) and stops. In this way, the proteins condense or focus into sharp bands in the pH gradient based on their PI values. This process was carried out using commercially available IPG strips

First dimension separation by Iso Electric Focusing-Cup loading

1. Using the rehydration buffer, the IPG strips were rehydrated. Necessary care was taken to avoid air bubbles, which otherwise may hinder the distribution of the protein sample in the IPG strip.
2. The plastic strip was peeled and the IEF strip placed on the focusing tray in such a way that the gel side is facing down. The "+" and pH range were marked on the IPG strip legibly
3. After 1h of absorption of rehydration buffer, mineral oil was overlaid on each of the IPG strips to prevent evaporation.
4. The tray was covered with the plastic lid and left on the leveled ground for about 11-16h for rehydration of strip to occur.
5. The rehydrated IPG strip was removed and excess mineral oil blotted.
6. The rehydrated strip was kept on the cup loading tray with the gel side up.
7. Connected the electrodes and protein samples loaded. Each sample was overlaid with mineral oil.
8. Next, the tray was carefully placed on the PROTEAN IEF platform, and the run was carried out using a protocol as detailed: 500V-Rapid→30min; 8000V Slow→1hr; 8000V-Rapid→20000Vh; 500V-→Hold

Next, inter dimension equilibration (equilibration buffer-1 & 2) was carried out (to coat the proteins with SDS) before separating the proteins by SDS-PAGE (O'Farrell PH, 1975). Next, proteins were separated by SDS-PAGE in a vertical direction based on the mass-to-charge ratio.

Second dimension separation by SDS-PAGE

SDS-PAGE was carried out using a resolving gel (12%) followed by placing the IEF strip carefully and sealing the strip with embedding gel. The marker was loaded and the run carried out at 100V till the tracker dye reaches the edge of the plate

Staining and de-staining of the gel

After the electrophoretic run, the gel was separated and stained overnight using staining solution as detailed by (Bovilla *et al.*, 2016). The stained gels were destained using the destaining solution, and images captured using GelDoc. The captured images were analyzed using PDQuest software (Bovilla *et al.*, 2016).

RESULTS AND DISCUSSION

Analysis of cell lines and tissue protein lysates using 2-Dimensional gel electrophoresis identified key protein expression signatures in breast cancers

To identify the breast cancer cell line(s) that best represents the tumor tissue, proteomes of 3 breast cancer cell lines (MCF7, SKBR and MDA-MB-468), a benign tumor and 3 malignant tumors were compared using 2-dimensional gel electrophoresis. The protein lysates were collected from exponentially growing cells (cultured in T75 flasks) as well as tissues using lysis buffer (1mM Tris, 1mM PMSE, pH 8.0) as detailed in materials and methods. The protein concentration was estimated using the BCA method and lysates subjected to 1D gel electrophoresis to check the quality of isolated proteins. Once the quality is confirmed (no degradation; data not shown), 250 μ g total protein was subjected to 2D gel electrophoresis.

2D Gel electrophoresis

After confirming the quality of protein preparation, the protein lysates (250 μ g) collected from cell lines, benign and malignant tissues were subjected for analysis using 2D gel electrophoresis using the method described earlier. The data shown in Figure 1 indicate the presence of about 200 different proteins in the pH range of 3 to 10 and the molecular mass range of 14-80kDa. Comparison of protein spots among cell lines and tissues identified (a) common proteins (169) as well as (b) unique proteins.

Comparative assessment of cell line Proteome

In order to determine the cell line that closely resembles the benign and or malignant tissue specimens, first, cell lines representing carcinomas of breast were analyzed by 2-dimensional electrophoresis and the spots (based on position) compared. ER^{+ve}, PR^{+ve} but HER2^{-ve} (MCF-7) and ER^{-ve}, PR^{-ve}, but HER2^{+ve} (SKBR3), ER^{-ve}, PR^{-ve} and HER2^{-ve} (MDA-MB-468) cell lines were chosen for this study.

Comparison of protein spots based on fold change differences identified more similarities between MCF7 and SKBR3 cell lines, despite differences in their ER, PR and HER2 status (Figure 1 and Table 1). For example, out of 169 proteins that are present in MCF7 as well as in SKBR3 cell lines, 147 proteins found <2 fold difference. However, only 125 proteins showed <2 fold change when the MDA-MB-468 cell line was compared with SKBR3 (Table 1)

Comparison of these three cell lines with benign and malignant tissues showed more similarities between SKBR3 and the malignant tissues, indicating that SKBR3 might be a good representative cell line to consider for in vitro cell-based assays (Figure 1 and Table 2). Between the two other cell lines, MDA-MB-468 showed more similarities with malignant tissue samples compared to MCF-7.

Analysis of the protein spots of cell lines and malignant tissues compared to benign tissue proteins showed that among the seven up-regulated proteins, the spot numbers 7 (29.43fold to 54.39 fold), 29 (8.52 fold to 50.18 fold) and 62 (22.73 fold to 329.22 fold) showed maximum fold increase in metastatic tumors (Figure 1 and Table 3). Hence, these proteins may be considered to distinguish malignant tissues from benign tissues. However, it is currently unknown about the nature of these proteins. Moreover, it is also currently unknown whether a similar expression pattern is observed with all the malignant tissues. Therefore, further studies are warranted.

Elucidation and analysis of the proteomes of breast cancer cell lines and tissues are very key in developing effective markers as well as identifying key therapeutic targets. The proteomic studies carried out in breast cancer assisted in significantly improving tumor molecular profiling, grouping of patients, diagnosis, screening and in the development of personalized therapeutics (Qin and Ling, 2012; Breuer and Murph, 2011). For instance, a study conducted on the post mitochondrial and cytosolic polypeptides isolated from breast tumors and non-malignant breast tissue demonstrated a general increase in the polypeptide expression in malignant tumors compared to normal tissues (Wirth *et al.*,

2D protein profiling of breast cancer cell lines and breast cancer tissues

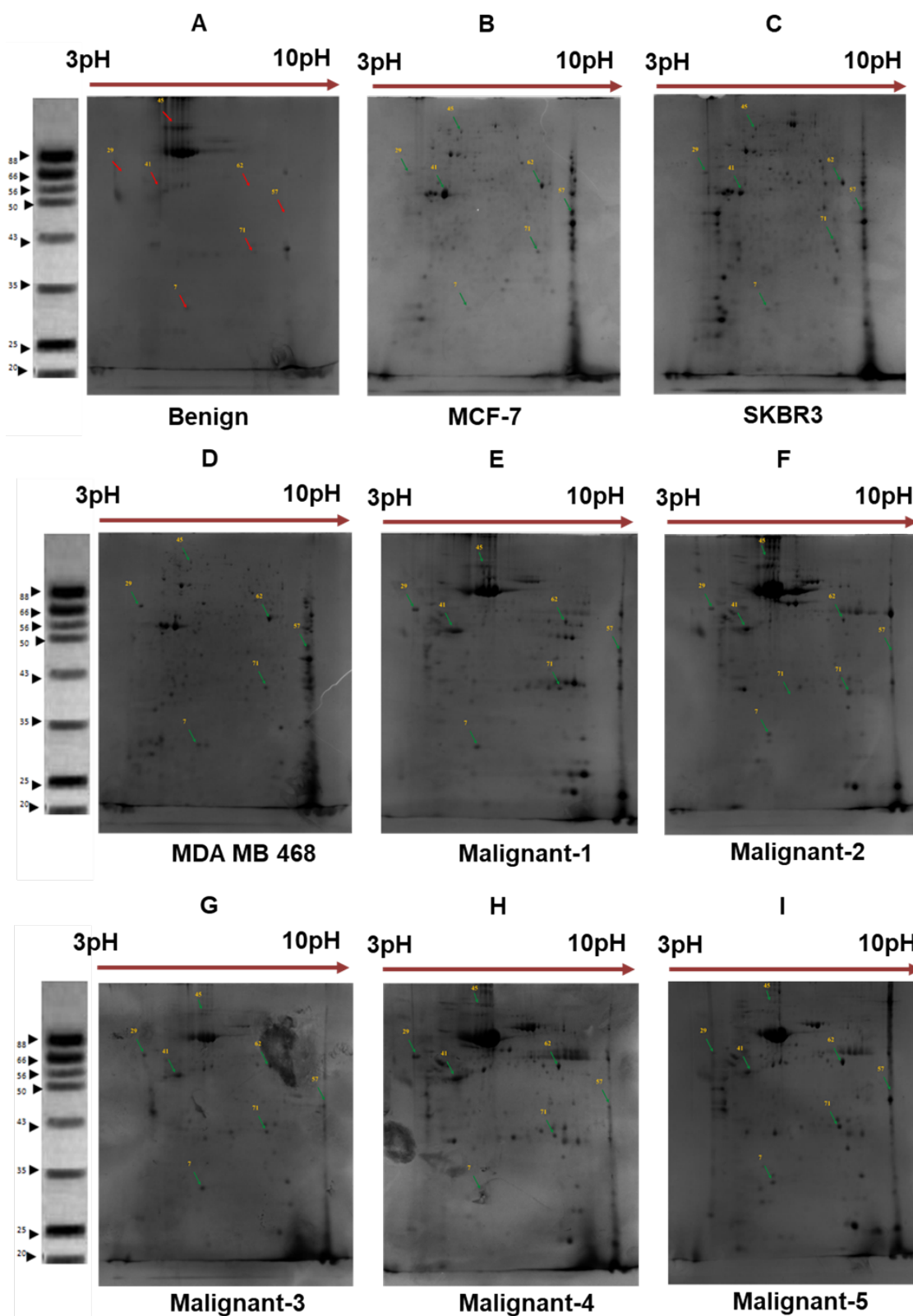


Figure 1: 2D gel images of cell lines (B, C, D), benign (A) and malignant tissues (E, F, G, H, I). Protein spot numbers 7, 29, 41, 45, 57, 62 and 71 (marked with arrows) had shown elevated expression in cell lines as well as malignant tissues when non-cancerous benign samples were considered as reference.

Table 1: Comparison of the number of proteins (spots) that have more than 2 fold variation in the expression levels compared to (ref) reference cell line

	MCF7	SKBR3	MDA-MB-468
MCF7	Ref	103	99
SKBR3	147	Ref	114
MDA-MB-468	144	125	Ref

Table 2: Comparison of the number of proteins (spots) with more than 2-fold variation in the expression levels compared to the reference cell line

	MCF-7	SKBR3	MDA-MB-468	Benign	M1	M2	M3	M4	M5
MCF-7	ref	103	99	75	107	106	110	100	116
SKBR3	147	ref	114	88	122	122	143	107	146
MDA MB 468	144	125	Ref	107	122	117	130	110	136
Benign	137	141	138	Ref	137	136	142	123	141
M1	155	135	126	137	Ref	127	148	110	139
M2	134	128	118	147	135	ref	133	120	145
M3	128	115	111	147	123	122	Ref	107	127
M4	133	132	127	135	133	132	151	ref	142
M5	126	118	101	141	115	115	116	95	Ref

Table 3: Comparison of the number of proteins (spots) that have more than 2 fold variation in the expression levels compared to reference benign tissue

Spot no	Benign	MCF-7	SKBR3	MDA MB 468	M1	M2	M3	M4	M5
7	Ref	13.52	43.23	15.47	54.39	35.06	29.43	39.44	33.61
29	Ref	5.01	5.19	8.75	50.18	45.17	18.28	11.12	8.52
41	Ref	53.81	35.08	57.63	64.14	17.25	37.15	23.6	1.16
45	Ref	33.98	89.17	36.46	19.44	28.8	0.02	20.08	13.93
57	Ref	41.36	11.66	29.87	15.04	29.56	25.84	24.8	11.44
62	Ref	33.18	23.22	18.03	182.26	27.69	22.73	329.22	239.35
71	Ref	10.96	20.87	26.97	11.73	13.95	12.92	8.32	8.3

1987). More predominantly six polypeptides were expressed only in tumors. Further analysis showed the absence of a polypeptide in tumor tissues (Wirth *et al.*, 1987). Likewise, another study identified the overexpression of 22 polypeptides and down-regulation of a single peptide in tumors compared to normal breast tissues (Liang *et al.*, 2010). Similarly, a comparative study analyzing 123 primary breast tumors and matched normal tissues identified the up-regulation of a novel protein C7 or F24 (Gromov *et al.*, 2010). In our study, we found overexpression of 7 proteins in cell lines MCF-7, MDA-MB-468 and SKBR3 as well as in metastatic tumor tissues compared to benign tissues. Among 7 proteins, pro-

tein spots 7, 29 and 62 are more specifically overexpressed in malignant tissues.

Recent proteomic studies of biopsy tissues using 2D gels and Mass Spectrometry have also reported the identification of novel and specific proteins (Guo *et al.*, 2013). For instance, in HER2 positive breast cancers, proteins such as PGRMC1, G3BP, and hnRNP, CK19, FASN, HSP27, PGK1, and GLO1 were overexpressed while GRP78 and RKIP proteins were down-regulated (Gromov *et al.*, 2014). Likewise, a recent study used a microtissue array (TMA) proteomic approach to analyze 98 breast cancer tumors and 20 healthy specimens. Analysis of this study data revealed the overexpression of GLO1 in 79%

of advanced tumors (Fonseca-Sanchez *et al.*, 2012). A separate study using MALDI-TOF showed that HER2 positive cell line SKBR3 expressed various proteins associated with glycolysis, lipid synthesis, stress-related chaperonage, and antioxidant and detoxification pathways (Zhang *et al.*, 2005). For example, proteins such as AKR (36 kDa/7.7), P4HB (57.5 kDa/4.76), Haptoglobin (45.9 kDa/6.13), FASN (276 kDa/6.0), PGK1 (45.0 kDa/8.3), ENO1 (47.5 kDa/7.01), GLO (21.0 kDa/5.12), TPI (26.6 kDa/6.4), Hsp27 (22.8kDa/5.98) were upregulated in SKBr3 cell line (Zhang *et al.*, 2005). But, whether these proteins are also expressed in tumors harvested from patients is not known. Addressing these lacunae, data from our study demonstrated the overexpression of 7 proteins predominantly in malignant tissues. However, currently, these proteins were not identified, hence, require additional studies

2D-PAGE is one of the gel-based proteomic studies where the separation of proteins takes place orthogonally based on their isoelectric point and molecular weight (Rabilloud and Lelong, 2011). 2-DE was first introduced by O'Farrell and Klose in 1975 (Rabilloud and Lelong, 2011). The identification of spots on 2D Gels was developed by Edman sequencing (Komatsu, 2007). Although this method of identification of proteins is more labor-intensive and less sensitive than the Mass Spectroscopy method, the 2D-PAGE remains the method of choice to analyze complex tissue samples as well as biological fluid. One of the significant advantages of 2D-PAGE is the ability to visualize protein isoforms (Magdeldin *et al.*, 2014; Naryzhny, 2016). In addition, post-translational modifications that alter protein characteristics, including the protein charge and molecular masses (the parameters that affect the position of any given protein on a gel), can also be observed in the 2D gel system. Technical innovations in 2D-PAGE, such as the immobilized pH gradient gels, the 2D difference electrophoresis (2D-DIGE) system, multiple differential staining for phosphoproteins, glycoproteins and radioactive multiplex imaging, have made 2D gel electrophoresis a very powerful tool to analyze complex proteins. Therefore, in this study, we have used 2D gel electrophoresis to analyze the complex proteins of cell lines, benign and malignant tissues.

PD Quest 2-D analysis software offers comprehensive and flexible 2-D gel electrophoretic analysis. Powerful auto-matching algorithms quickly and accurately match gels with little or no manual intervention. PD Quest software's flexible annotation features make it a useful tool to establish a centralized information repository, which allows virtu-

ally any type of characterizing data to be linked to each spot on a master gel image. It is easy to view and share information associated with the identified protein. Key features of this software are that it automatically detects the spots and matches them, has sophisticated quantification and statistical analysis tools and efficient spot cutting configurations for high accuracy, high throughput, and flexibility in protein identification experiments. Hence, in this study, we have used PDQuest software to analyze 2D gels.

CONCLUSION

In conclusion, our study identified protein spots that are similar among breast cancer cell lines as well as benign and malignant tissues. In addition, our study has detected exclusive protein spots expressed in malignant tumors and cell lines compared to benign tissue. In summary, the data presented in this study provided key information pertaining to the relative expression patterns of proteins in benign and malignant tissues in comparison with widely used breast cancer cell lines.

Study limitations

1. Lack of normal breast cell line, and cell lines representing triple-positive breast cancers
2. The over expressed proteins have not been identified in this study. Hence, future studies should focus on identifying and characterizing these proteins
3. Currently, only 5 metastatic breast tissues were analyzed, but, analyzing more number of tissues (at least 100) will provide more confident data.

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