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RESEARCH PAPER

Alteration in protein expression in estrogen receptor alpha-negative human breast cancer tissues indicates a malignant and metastatic phenotype

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Abstract Ductal carcinoma in situ (DCIS) represents the earliest identifiable breast cancer lesion. Disruption of the myoepithelial cell layer and basement membrane is a prerequisite for DCIS to initiate invasion into the stroma. The majority of epithelial cells overlying a focally-disrupted myoepithelial cell layer are estrogen receptor-alpha negative (ER(−)); however, adjacent cells within the same duct confined by an intact myoepithelial cell layer express high levels of ER. These ER (+) and ER (−) cells were microdissected from the same ducts of breast cancer patients. Differential proteins expressed by ER(+) and ER(−) cells were identified using two-dimensional gel electrophoresis followed by mass spectrometry and Western blot analysis. ER(−) cells express lower levels of superoxide dismutase, RalA binding protein, galectin-1, uridine phosphorylase 2, cellular retinoic acid-binding protein 1, S100 calcium binding protein A11, and nucleoside diphosphate kinase A or non-metastasis protein 23-H1 (nm23-H1). The upregulated protein, Rho GDP-dissociation inhibitor 1 alpha, may induce chemotherapy resistance. The significant findings are that the microdissected ER(−) cells express 12.6 times less cellular retinoic acid-binding protein 1, a protein involved in cellular differentiation, and 4.1 times less nucleoside diphosphate kinase A or nm23-H1, a metastasis suppressor, and express fewer proteins than adjacent ER(+) cells. The collective role of the alterations of protein expression in ER(−) cells may be to promote a more malignant phenotype than adjacent ER(+) cells, including a decreased ability to undergo apoptosis and differentiation, and an increased potential to damage DNA, metastasize, and resist to chemotherapy.

Keywords Apoptosis · Cancer heterogeneity · Estrogen receptor · Human breast cancer · Invasion · Metastasis suppressor · Mass spectrometry · Protein biomarker · Proteomics · Tumor progression

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Abbreviations

CRABP1	Cellular retinoic acid-binding protein 1
DCIS	Ductal carcinoma in situ
2-DE	Two-dimensional gel electrophoresis
DTT	Dithiothreitol
ERα	Estrogen receptor alpha
IPG	Immobilized pH gradient
MALDI-TOF-MS	Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry
ME cells	Myoepithelial cells
M_r	Molecular mass

NDPK-A (or nm23-H1)	Nucleoside diphosphate kinase A (non-metastasis protein 23-H1)
OG	Octylglucopyranoside
pI	Isoelectric point
RhoGDIα	Rho GDP-dissociation inhibitor 1 alpha
S100A11	S100 calcium binding protein A11
UPase	Uridine phosphorylase
SMA	Smooth muscle actin

Introduction

Human ductal carcinoma in situ (DCIS) is the earliest identifiable breast cancer lesion, in which tumor cells are confined to the duct and surrounded by a layer of myoepithelial (ME) cells and the basement membrane, with no evidence of invasion into the surrounding stroma [1]. While approximately half of DCIS cases may eventually progress to an invasive cancer [2], many are likely to remain non-threatening lesions, which complicates determination of the best treatment regimen for breast cancer patients. In human breast ducts, the ME cell layer and basement membrane form a physical barrier between the stroma and the luminal epithelium that serve to isolate in situ breast tumors. The ME cells themselves are joined by intercellular junctions and adhesion molecules, forming a continuous layer that encircles the entire duct system, and a discontinuous layer or a basket-like structure that covers a vast majority of lobules and terminal duct-lobular units [3–5]. The basement membrane is composed of laminins, type IV collagen, entactin, heparan sulfate proteoglycans, and glycosaminoglycans, forming a continuous lining surrounding and attaching to ME cells via hemidesmosomes and focal adhesion complexes [3–5]. These structural features confer two essential functions upon the ME cells and the basement membrane. First, as the epithelium is normally devoid of lymph and blood vessels, and is therefore dependent upon the stroma for its metabolic and survival needs, the ME cells and basement membrane function as gate-keepers, directly regulating the communication between these two compartments. Second, due to the physical interposition of the ME cell layer and basement membrane between the stroma and epithelium, ductal tumor cells must first break their way through the ME cell layer, then the basement membrane, to reach the stroma and initiate invasion and subsequent metastases [6].

Estrogens play a major role in both the development of sexual glands and in the reproductive cycle [7], and the biological effects of estrogen are mediated through the estrogen receptor (ER). ERα, cloned in 1986 [8, 9], was believed to be the sole form of this receptor until 1996, when a second ER, called ERβ, was also cloned [10, 11]. The exact roles of ERα and ERβ in breast cancer are not fully understood, though it has been reported that estrogens are involved in the promotion of human breast cancer, possibly by way of their mitogenic activity [12].

Immunohistochemical staining for ERβ in normal human breast tissues, DCIS, invasive cancers, and lymph node metastases has revealed a gradual reduction in ERβ expression during the transition from normal tissue to preinvasive lesions to invasive cancers, and ERβ was lost in 21% of the invasive cancers studied [13]. Another study utilizing similar techniques revealed that the percentage of cells positive for ERβ was high in normal mammary glands and in non-proliferative benign breast disease, but the percentage of positive cells was decreased significantly in proliferative benign breast disease and in carcinoma in situ [14]. These results are in agreement with results obtained when analyzing mRNA levels of ERβ in normal mammary, benign breast disease, breast cancer, and metastatic breast cancer in lymph nodes [15], where it was shown that ERβ mRNA expression was significantly decreased in breast cancer and lymph node metastatic tissues when compared with normal mammary and benign breast disease tissues. All of these results suggest that ERβ exerts a protective effect against the mitogenic activity of estrogens mediated by ERα, and may function as a tumor suppressor, as the loss of ERβ expression seems to correlate with the progression of breast carcinomas.

To identify potential precursors of invasive lesions, we have carried out studies focusing on the correlation between the structural integrity in ME cell layers and the immunohistochemical and genetic profiles in adjacent epithelial cells. In 5,698 duct cross-sections from 220 patients with ERα (+), non-invasive breast tumors, we detected a total of 405 focal ME cell layer disruptions, defined as the absence of ME cells resulting in a gap equal to or greater than the combined size of 3 ME or epithelial cells [16]. Of these focal disruptions, 350 (86.4%) were overlaid by epithelial cell clusters with no or substantially reduced ERα expression, in contrast to adjacent cells within the same duct, which expressed high levels of ERα and overlaid an intact ME cell layer [16]. Additional studies revealed that cell clusters overlying focally disrupted ME layers generally showed a substantially higher proliferation rate when compared to adjacent cells within the same duct, as assessed by immunohistochemical staining for Ki-67 [17, 18]. These features potentially signal the formation of a biologically more aggressive cell clone, with a protein expression profile that has yet to be fully examined, while focal breakdowns in the ME cell layer are likely to serve as portals for tumor progression and early invasion.

To identify new breast cancer biomarkers, the protein profiles of nipple aspirate fluids [19], or the serum of breast cancer patients [20], have been compared to that of healthy donors. Identifying biomarkers by these less invasive methods is of great importance, but questions remain as to what early differences in protein profiles of these samples might manifest to detectable levels, as the low abundance of proteins secreted in the serum and other biological fluids as a result of the disease is often masked by the high abundance of albumin and other highly abundant proteins [21]. The protein profiles of breast cancer cell lines have also been compared to the profiles of normal breast cell lines [22]. While tumor-derived cell lines can be useful for initial studies [23], each line might display a unique evolution that may not truly mimic real in vivo conditions. A proteomics study performed on prostate cells shows the discrepancies between microdissected prostate tissues and in vitro prostate lines [24]. Comparisons have also been made between microdissected breast tissues composed of either normal ductal epithelium or ductal epithelium containing DCIS lesions [25]. However, the samples collected for this study, composed of 50,000–100,000 cells, were unlikely to be have been homogeneous, and were likely composed of multiple cell types with divergent expression profiles.

Many diseases manifest themselves through severe changes in human pathophysiology, which forms the basis for clinical chemistry and provides its value in diagnoses and subsequent therapeutic interventions [26]. Proteomics includes the global analysis of proteins expressed by the genome of an organism, an organ, a tissue, or a group of cells with the typical aim being the evaluation of quantitative changes that occur as a function of disease, treatment, or environment [27]. Proteomics strategies are used to identify cancer-specific protein markers that could provide the basis for the development of new diagnostic methodologies, early disease detection, and monitoring the efficacy of treatments [27, 28]. Marker detection and correlation with tumor growth is less obstinate for advanced tumors, but the true value of these developing applications lies in early tumor detection and the prompt identification of invasive phenotypes [26].

To identify candidate markers specific to the initiation of invasion by DCIS cells, we here identify proteins that are differentially expressed by the ERα (−) cell clusters overlying focally disrupted ME cell layer when compared to adjacent ERα (+) cells overlying non-disrupted ME cell layer within the same duct (Fig. 1). Proteins extracted from ERα (+) and ERα (−) tissue samples were subjected to two-dimensional gel electrophoresis (2-DE) followed by MALDI-TOF-MS analysis of trypsin-digested spots. Differentially-expressed proteins that were identified by this method were investigated in ERα (+) and ERα (−) tissues microdissected from the ducts of the same patients (Fig. 1) using Western blot. We report the differential expression of proteins of ERα (−) cells overlying a focally disrupted ME layer compared to adjacent ERα (+) cells bound by an intact ME layer.

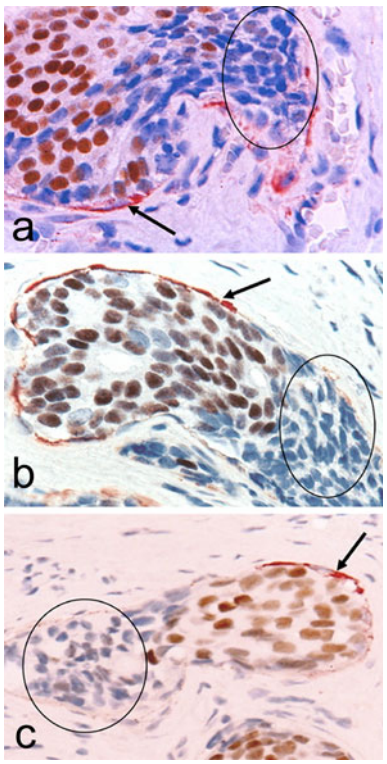

Fig. 1 ERα expression status in cell clusters overlying focally-disrupted myoepithelial cell layers. Human breast tissue sections from DCIS (**a**), hyperplastic (**b**), and normal-appearing (**c**) ducts were double-immunostained for ERα (*brown*) and SMA (*red*). *Arrows* identify myoepithelial cell layers. *Circles* identify ERα-negative cell clusters overlying focal myoepithelial cell layer disruptions (300×) (Color figure online)

Materials and methods

Research involving human subjects was performed in accordance with the principles outlined in the declaration of Helsinki (1964). All the research projects involved in using human tissues and cells were approved by the Institutional Human Subject Review Board or Committees. Freshly-frozen DCIS breast tissue microdissected samples and corresponding slide-mounted, serial-sectioned paraffin-embedded tissues were provided by co-author Yan-Gao Man, M.D., Ph.D., Director of the Gynecologic and Breast Research Laboratory at the Armed Forces Institute of Pathology and American Registry of Pathology in Washington, D.C.

Tissue microdissection and protein extraction

Serial sections of 5 to 7 μm thickness were cut and placed on positively-charged microscopic slides and rehydrated by ethanol gradient. Sections were immunohistochemically stained for ERα and different proliferation markers. Non-specific antibody binding was blocked with blocking buffer (0.2% Triton X-100, 5% normal goat serum, and 3% bovine serum albumin in Tris-buffered saline) for 1 h at room temperature prior to overnight incubation at 4°C with a monoclonal mouse anti-human ERα antibody (NCL-ER-6F11, Vector, Burlingame, CA) diluted into the same buffer (10 μg/mL). Samples were then incubated with an alkaline phosphatase-conjugated secondary antibody diluted 1:5000 in blocking buffer for 4 h at room temperature, and positive signals were detected using Fast-Red (Sigma). Clusters of cells overlying myoepithelial disruptions, composed primarily of ERα (–) cells, were excised by laser-capture microdissection from adjacent, unstained serial sections. Clusters of ERα (+) cells from the same duct, but bound by an intact ME layer, were similarly obtained. Microdissected tissues were then homogenized in a tissue lysis buffer composed of 10 mM Tris, 130 mM NaCl, 1% octylglucopyranoside (OG), 1% CHAPS, 10 mM sodium phosphate, and 10 mM sodium fluoride (pH 7.5). The ratio of the volume of tissue lysis buffer added to the weight of the tissue was 1/1 (v/v). This was followed by vortexing for 10 min and then by centrifugation at 17000 × g for 20 min. This resulted in the formation of 3 layers: The top layer was composed primarily of non-soluble lipids, the middle layer contained the soluble proteins, and the bottom layer was composed of cell debris. The middle layer was recovered and used for subsequent analyses.

Protein extraction from whole human breast tissue samples

ERα (+) and ERα (−) whole human breast tissue samples were minced using a scalpel, and proteins were extracted using the procedure outlined above.

Protein quantitation

Protein concentrations of the tissue lysates were determined using a bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL) using bovine serum albumin (BSA) as a standard according to the manufacturer's instructions. The concentrations of proteins in the tissue lysates from the microdissected cells and the tissue samples were determined using the standard curve generated by the absorbance of the standards at 562 nm in the Y axis versus the known concentration of the standards in the X axis.

Two-dimensional gel electrophoresis

2-DE was performed as described previously [29]. Briefly, proteins were reconstituted in a rehydration buffer composed of 5 M urea, 2 M thiourea, 1% OG, 0.25% v/v Biolyte 3-10 ampholyte, 12.5% water-saturated isobutanol, 10% isopropanol, 5% glycerol, and 50 mM dithiothreitol (DTT). The samples were rehydrated at 50 V for 16 h at 20°C using 11 cm Immobilized pH Gradient (IPG) strips, pH 4 to 7 (Bio-Rad). Proteins were focused at 250 V for 15 min, and then 8000 V was maintained for a total of 60,000 Vh per gel. The strips were then equilibrated for 10 min in 2.5 mL of a solution composed of 375 mM Tris–HCl pH 8.8, 6 M urea, 2% SDS, and 2% DTT. After this first equilibration, the strips were equilibrated for another 10 min in 2.5 mL of a second equilibration buffer composed of 375 mM Tris–HCl pH 8.8, 6 M urea, 2% SDS, and 2% iodoacetamide. The equilibrated IPG strips were washed with cathode buffer (0.1 M Tricine, 0.1 M Tris–HCl pH 8.2, and 0.1% SDS) and placed onto a 10% Tris–HCl polyacrylamide gel. The anode buffer consisted of 0.2 M Tris–HCl pH 8.9. Gels were electrophoresed at 50 V for 30 min, then at 100 mA/gel until the end of the separation. Gels were stained with SYPRO Ruby (Bio-Rad) according to the following procedure: Gels were fixed in 50% methanol (v/v) and 12% acetic acid (v/v) for 2 h, then incubated with SYPRO Ruby in the dark overnight. Gels were then washed with ddH_2O for 3 h. Gels were scanned using Typhoon 9410 Scanning Systems (GE Healthcare). The excitation wavelength was 457 nm and the signals were detected at 610 nm. Spot detection and densitometry were performed using PDQuest 8.0 (Bio-Rad, Hercules, CA) and ImageJ (http://rsb.info.nih.gov/ij).

MALDI-TOF-MS

Proteins were identified utilizing Matrix-Assisted Laser Desorption-Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF–MS) [30–34] as described previously [35]. Briefly, after four iterations of 2-DE for each sample, protein spots were excised under UV light, combined, and incubated with 0.5 μg/mL TPCK-treated trypsin. Peptides generated from the trypsin digestion were then mixed with a matrix (α-cyano-hydroxycinnamic acid) [36, 37] at a 100–1000-fold molar excess over the analyte [38]. This mixture was then spotted onto a target plate and allowed to evaporate until crystals were formed. The MALDI-TOF used for this analysis was a Kratos PC Axima CFR+ (Shimadzu

Biotech) that operated in the reflectron positive ion mode with an operating voltage of 20 kV, an extraction voltage of 19.98 kV, and a pulse voltage of 2000 V. Spectra were acquired over the mass range of 500 to 3000 Da. MASCOT software was used to identify the proteins. The search was performed against the SwissProt database for the *Homo sapiens* taxonomy. The following parameters were used: one missed cleavage, mass tolerance of 1.3 Da, oxidation of methionine (variable modification), and carbamidomethylation (fixed modification).

Western blotting

Proteins from microdissected ERα (+) and ERα (−) cells were reconstituted with SDS–PAGE buffer and 10 μg of each sample were loaded onto a 10% polyacrylamide gel. The gel was electrophoresed at 30 V for 30 min, then at 50 mA until the end of the separation. Proteins contained within the gel were then electroblotted onto a nitrocellulose membrane (50 V for 50 min). Western blot analyses were accomplished utilizing a 1 μg/mL dilution of primary antibody followed by incubation with a horseradish peroxidase-conjugated secondary antibody against the appropriate species. Visualization of the bands was then accomplished by the addition of a 1:1 ratio of Super Signal West Pico-Stable Peroxidase Solution and Luminol/Enhancer Solution (Pierce, Rockford, IL) and by developing the chemiluminescent signal in the dark using Kodak Scientific Imaging Film (Kodak cat. 1651496), Fixer and Replenisher/Developer and Replenisher (Kodak cat. 1901859) according to the manufacturer's instructions.

Results and discussions

2-DE of microdissected ERα (+) and ERα (−) tissues

2-DE of proteins extracted from 150,000 microdissected ERα (+) and ERα (−) breast cancer cells shows that ERα (−) cells have reduced protein expression (Fig. 2a), although there are 3 proteins exclusively expressed by the ERα (−) cells (Fig. 2b *Blue spots*). PDQuest analysis of the images revealed the presence of 28 common proteins differentially-expressed between these two cell clusters (Fig. 2b *Green spots*). ERα (+) cells express 83 proteins that are not expressed by ERα (−) cells (Fig. 2**b** *Red spots*).

Differential protein expression between ERα (+) and ERα (−) human breast cancer tissue samples (whole tissue homogenates)

2-DE of ERα (+) invasive breast cancer tissue samples versus ERα (−) samples (Fig. 3a) followed by MALDI-TOF analysis

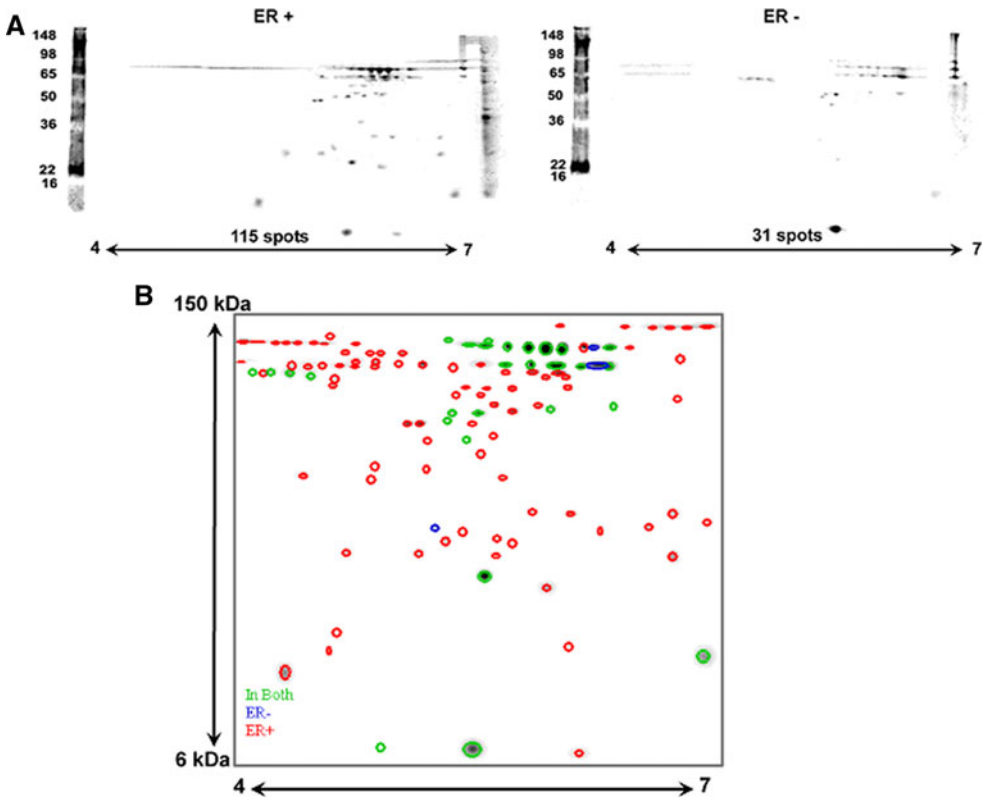

Fig. 2 **a** 2-DE of proteins extracted from 150,000 ERα (+) and ERα (−) cells, respectively, microdissected from human DCIS tissues originating from 3 patients. Proteins were rehydrated at 50 V for 16 h at 20°C using 11 cm Immobilized pH Gradient (IPG) strips, pH 4–7. A 10% polyacrylamide gel was used in the second dimension. Proteins were stained by SYPRO Ruby and visualized using an excitation wavelength of 457 nm. The signals were detected at 610 nm. **b** PDQuest imaging analysis of 2-DE representing proteins extracted from 150 thousand ERα (+) and ERα (−) cells microdissected from human DCIS tissues originating from 3 patients shows 28 differentially-expressed proteins

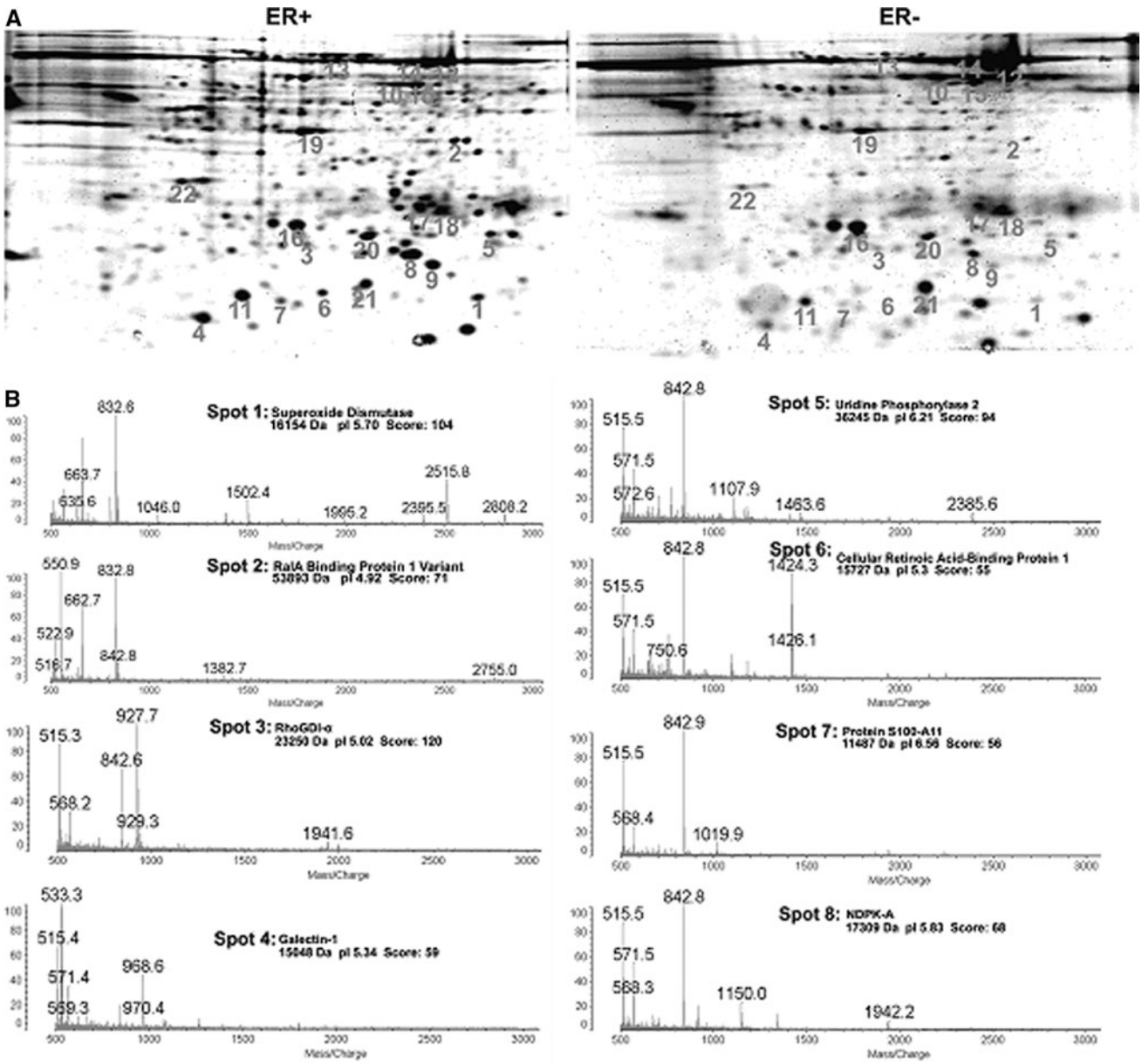

Fig. 3 **a** 2-DE of proteins extracted from entire ERα (+) and ERα (−) tissue samples. Proteins were rehydrated at 50 V for 16 h at 20°C using 11 cm Immobilized pH Gradient (IPG) strips, pH 4–7. A 10% polyacrylamide gel was used in the second dimension. Proteins were stained by SYPRO Ruby and visualized using an excitation wavelength of 457 nm. The signals were detected at 610 nm. Proteins were identified using MALDI-TOF-MS. **b** MALDI-TOF mass spectra of tryptic digests obtained on a Kratos PC Axima CFR+ (Shimadzu Biotech) operated in the reflectron positive ion mode with an operating voltage of 20 kV, an extraction voltage of 19.98 kV, and a pulse voltage of 2000 V. Spectra were acquired over the mass range of 500 to 3000 Da

of tryptic digests (Fig. 3b) revealed the differential expression of 247 proteins, 22 of which were identified (Table 1). Eight of these proteins were selected to be analyzed and validated in the microdissected ERα (+) and ERα (−) tissues by Western blotting: Superoxide Dismutase (Mr 16154; pI 5.7), RalA binding protein 1 variant (Mr 53893; pI 4.92), Rho GDP-Dissociation Inhibitor 1 (RhoGDIα) (Mr 23250; pI 5.02), Galectin-1 (Mr 15048; pI 5.34), Uridine Phosphorylase 2 (UPase2) (Mr 36245; pI 6.21), Cellular Retionic Acid-binding Protein 1 (CRABP1) (Mr 15727; pI 5.3), S100 calcium binding protein A11 (S100-A11) (Mr 11487; pI 6.56), and Nucleoside Diphosphate Kinase A (NDPK-A) (Mr 17309; pI 5.83). The selection was based primarily on the role these proteins play in cellular distribution, apoptosis, phosphorolysis, tumorigenesis, invasion, or metastasis, as well as the availability of commercial antibodies.

Table 1 Differentially expressed proteins between ERα (+) and ERα (−) tissue samples. Index numbers correspond to Fig. 3a

Index no.	Accession	M*r*	p*I*	Cov.(%)	Peptide match	MOWSE	Protein description	ER+/ER− ratio
1	SODC_HUMAN	16154	5.7	72	11	104	Superoxide dismutase	2.47
2	Q59E87_HUMAN	53893	4.92	45	36	71	Ral A binding protein	14.5
3	GDIR_HUMAN	23250	5.02	34	16	120	Rho GDP-dissociation inhibitor 1	0.7
4	LEG1_HUMAN	15048	5.34	42	6	59	Galectin-1	2.21
5	UPP2_HUMAN	36245	6.21	26	10	94	Uridine phosphorylase 2	5.92
6	RABP1_HUMAN	15727	5.3	39	5	55	Cellular retionic acid-binding protein 1	24
7	S10AB_HUMAN	11487	6.56	49	5	56	Protein S100-A11	3.01
8	NDKA_HUMAN	17309	5.83	31	6	68	Nucleoside diphosphate kinase A	3.26
9	METL5_HUMAN	24046	6.23	65	25	258	Methyl transferase-like protein 5	4.3
10	DIXC1_HUMAN	77886	5.85	34	33	134	Dixin	0.36
11	A1AG2_HUMAN	23873	5.03	54	18	99	Alpha-1-acid glycoprotein precursor	2.84
12	ALBU_HUMAN	71317	5.92	36	21	144	Serum albumin precursor	0.17
13	GRP75_HUMAN	73920	5.87	28	17	85	Stress-70 protein, mitochondrial precursor	2.21
14	ALBU_HUMAN	71317	5.92	20	13	66	Serum albumin precursor	0.23
15	EGLN1_HUMAN	46847	8.83	28	11	91	Egl nine homolog 1	0.32
16	APOA1_HUMAN	30759	5.56	42	12	96	Apolipoprotein A-1 precursor	0.87
17	FBXL8_HUMAN	41403	6.96	42	14	95	F-box/LRR-repeat protein 8	1.31
18	ITM2A_HUMAN	30121	5.65	23	6	72	Integral membrane protein 2A	0.73
19	ACTB_HUMAN	42052	5.29	26	9	83	Actin, cytoplasmic 1	1.64
20	TUSC4_HUMAN	44429	6.1	20	9	66	Tumor suppressor candidate 4	1.75
21	SSX2_HUMAN	21721	5.61	23	6	58	Protein SSX2	0.46
22	TPM4_HUMAN	28619	4.67	26	8	73	Tropomyosin alpha-4 chain	3.27

Microdissected tissues versus whole tissue samples

The low amount of proteins extracted from the ERα (+) and ERα (−) microdissected tissues did not allow for the identification of differentially-expressed proteins between these two cell clusters, and we therefore proceeded by analyzing differential protein expression from tissue samples composed exclusively of ERα (+) or ERα (−) invasive breast cancer cells as described in the pathology reports of these tissues and verified by Western blots. More than 1 mg of proteins was extracted from each tissue sample to allow for the protein identification using MALDI-TOF analysis on trypsin-digested spots. Western blotting was then performed to validate differential protein expression in 3 ERα (+) and ERα (−) pairs of tissue that were microdissected from the ducts of 3 patients.

Differential protein expression between microdissected ERα (+) and ERα (−) cells

Western Blotting was performed on lysates of the ERα (+) and ERα (−) tissues microdissected from the same duct of DCIS microinvasive cancer patients (Fig. 4). The expression levels of Superoxide Dismutase, RalA binding protein, Galectin-1, UPase, CRABP1, S100-A11, and NDPK-A were lower in ERα (−) cell clusters than their ERα (+) counterparts. Rho GDP-Dissociation Inhibitor 1 (RhoGDIα) was the only protein upregulated in the ERα (−) cells (Table 2).

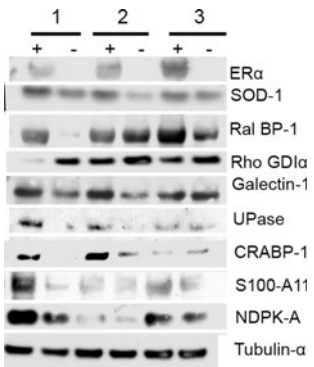

Fig. 4 Western Blots performed on microdissected ERα (+) and ERα (−) cells. Numbers 1–3 refer to the 3 pairs of samples microdissected from the ducts of 3 different patients that are either positive (+) or negative (−) for ERα expression. Individual rows include findings for Estrogen Receptor-α, superoxide dismutase, RalA binding protein 1, RhoGDIα, Galectin-1, Uridine Phosphorylase, cellular retinoic acid-binding protein 1, S100-A11, nucleoside diphosphate kinase A, and tubulin-α. Lanes represent 5 μg of total protein. Western blot analyses were accomplished utilizing a 1 μg/mL dilution of primary antibody followed by incubation with a horseradish peroxidase-conjugated secondary antibody against the appropriate species with positive signals detected by chemiluminescence

Table 2 Western blot measurement of differentially-expressed proteins between microdissected human breast DCIS ERα (+) and ERα (−) cells

Protein description	ER+/ER− ratio	Protein role
Superoxide Dismutase	2.52	Anti-oxidant [39, 40]
Ral A binding protein	4.5	Tumorigenesis–Metastasis [41–47]
Rho GDP-dissociation inhibitor 1	0.59	Resistance to drugs [48–51]
Galectin-1	4.27	Apoptosis [52–55]
Uridine phosphorylase 2	5.1	Contributes to drug efficacy [56, 57]
Cellular retionic acid-binding protein 1	12.6	Cell growth and differentiation [58, 59]
Protein S100-A11	4.48	Tumorigenesis [60–62]
Nucleoside diphosphate kinase A	4.1	Metastasis suppressor [63, 64]

Superoxide dismutase plays a key anti-oxidant role in the cell by converting superoxide radicals to hydrogen peroxide and oxygen [39]. Its cellular role is to provide a defense against the potentially damaging activities of superoxide radicals; diminished superoxide dismutase enzyme activity leads to many of the observed properties in cancer cells such as damage to the mitochondrial and plasma membranes, modulating key enzyme activities by oxidizing sulfhydryl groups to disulfides, and causing DNA double-strand breakage and possibly mutation and genomic instability. Downregulation of this enzyme has been found in many cancers [40]. The superoxide dismutase protein expression level was 2.52 fold higher in ERα (+) than ERα (−) epithelial cells belonging to the same patient.

RalA protein is a Ras-related small GTPase. The Ral GTPase family of proteins plays a major role in intracellular membrane trafficking, as well as tumorigenesis, invasion, and metastasis [41, 42]. Because RalA binding protein interacts with RalA and binds directly to RalB [43], which is associated with cancer metastasis [44], and RalA binding protein activates the GTPase activity of Cdc42 and partially Rac1 [45], both proteins are involved in cell migration [46]. Furthermore, given the role of RalA binding protein in cancer cell chemoresistance [47], RalA binding protein may also be critical in tumor invasion and metastasis. RalA binding protein was found to be 4.5 times higher in ERα (+) than ERα (−).

RhoGDIα is a cellular protein that controls the cellular distribution and activity of RhoGTPases [48, 49]. RhoGDIα is elevated in breast cancer cell lines and breast tumor tissues [50]. RhoGDIα has also been reported to promote the resistance of cancer cells to drug-induced toxicity, thus playing an anti-apoptotic role [51]. RhoGDIα was the only protein that was identified as elevated in ERα (−) cells when compared to its ERα (+) cells.

Galectin-1 can induce apoptosis in breast cancer cells by blocking the cell cycle at the S/G2 transition [52]. It has been shown to be involved in cancer invasion and metastasis by regulating cell adhesion and migration [53]. Galectin-1 is thought to be synthesized by the carcinoma cells and secreted into the stroma. A recent study demonstrated increased expression of Galectin-1 in human breast cancer cells and cancer-associated stroma and hypothesized that Galectin-1 plays a major role in tumor-stroma interaction in breast cancer [54]. The Galectin-1 protein level was 4.27 times higher in ERα (+) when compared to the ERα (−) expression. Uridine phosphorylase (UPase) catalyzes the phosphorolysis of uridine to uracil. It is also involved in fluoropyrimidine metabolism, playing a role in the intracellular activation of 5-fluorouracil [55].

UPase is elevated in various tumor tissues including breast cancer tissues. This elevation contributes to the therapeutic efficacy of fluoropyrimidines in cancer patients [56], and it has been demonstrated that elevated levels of UPase are correlated with favorable prognoses in cancer [57]. UPase was 5.1 times higher in ERα (+) than in ERα (−).

Cellular retinoic acid binding proteins are thought to play a role in shuttling retinoic acid (RA) from the cytoplasm to the nucleus, therefore playing a role in cell growth and differentiation regulation in fetal and adult tissues [58]. CRABP1 regulates RA activity by increasing its degradation rate by enhancing the production of RA-metabolizing enzymes [59]. CRABP1 levels were 12.6 times higher in ERα (+) than in neighboring ERα (−) cells.

S100-A11 is involved in tumorigenesis [60], although it has been shown to be upregulated in some cancers [61] and downregulated in others [62]. S100-A11 levels in ERα (+) samples were 4.48 more than that in ERα (−) samples.

Nucleoside diphosphate kinase-A (NDPK-A or nm23-H1) is a metastasis suppressor gene identified in 1988 [63]. Restoration of NDPK-A expression reduces metastasis of breast cancer [64]. The NDPK-A protein level was 4.1 times higher in ERα (+) cells than in nearby ERα (−) counterparts.

The loss of ERα expression, signaling the early signs of breast cancer invasion, was accompanied by the down-regulation of superoxide dismutase that plays an anti-oxidant role, galectin-1 known to induce apoptosis, uridine phosphorylase that contributes to drug efficacy, cellular retinoic acid-binding protein 1 that plays a role in cell growth and differentiation, S100-A11 involvement in

tumorigenesis, and a metastasis suppressor nucleoside diphosphate kinase A. RhoGDIα, known to induce the resistance of cancer cells to drug-induced toxicity, was the only protein that was elevated in ERα (−) cells when compared to adjacent ERα (+) cells. The level of RalA binding protein, involved in tumorigenesis and metastasis, was 4.5 times higher in the microdissected ERα (+) cells than in adjacent ERα (−) ones. The physiological and pathological roles of these differentially expressed proteins are complex, and may sometimes conflict with one another. Although the specific effects of the alteration of each of these identified proteins remains to be further investigated, their collective role may be to promote a malignant phenotype for ERα (−) cells, including a decreased ability to undergo programmed cell death and differentiate, an increased potential to damage DNA and generate genomic instability, a resistance to chemotherapy drugs, and ultimately to metastasize. Disruption of both the ME cell layer and the basement membrane is a prerequisite for tumorigenic DCIS cells to initiate invasion into the stroma. The majority of epithelial cells overlying focally-disrupted ME cell layers are ERα (−), while adjacent cells within the same duct confined by an intact ME cell layer express high levels of ERα. In this study, ERα (+) cells bound by an intact ME cell layer and ERα (−) cells overlying myoepithelial disruptions were microdissected from the same ducts of breast cancer patients, and proteomic analyses revealed great discrepancies in protein expression within the same breast cancer duct accompanied by non-expression of this hormonal receptor.

Conclusions

Our study shows that substantial alteration in protein expression was observed between clusters of adjacent ERα (+) and ERα (−) epithelial cells, although no estrogen-regulated genes were identified among the proteins exhibiting increased expression in ERα (+) cells. We propose two hypotheses to be further tested in the future. The first is that an ERα (−) cell cluster in DCIS with microinvasion may represent a clone from a genetically-altered cancer stem cell, explaining why many of the differentiation makers are not expressed or are expressed at a much lower level than those of adjacent ERα (+) cells. Second, an ERα (−) cell cluster may arise from dedifferentiated proliferating ERα (+) cells. The most significant facts are that the microdissected ERα (−) cells are pathologically more aggressive, overlay a focally-disrupted myoepithelial layer, express 12.6 times less cellular retinoic acid-binding protein 1, a protein involved in cellular differentiation, and 4.1 times less metastasis suppressor nucleoside diphosphate kinase A or nm23-H1, and express fewer proteins than adjacent ERα (+) cells in DCIS with microinvasion. Further research is required to test these two hypotheses and find out which one is the predominate case in the evolution of human breast DCIS to invasive carcinoma.

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