GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Proteomic analysis of non-tumoral breast tissue

G.G. Costa[1], R. Kaviski[1], L.E.R. Souza[2], C.A. Urban[3], R.S. Lima[3], I.J. Cavalli[1] and E.M.S.F. Ribeiro[1]

[1]Departamento de Genética, Universidade Federal do Paraná, Curitiba, PR, Brasil
[2]Departamento de Patologia Básica, Universidade Federal do Paraná, Curitiba, PR, Brasil
[3]Unidade da Mama, Hospital Nossa Senhora das Graças, Curitiba, PR, Brasil

Corresponding author: E.M.S.F. Ribeiro
E-mail: eribeiro@ufpr.br

Genet. Mol. Res. 10 (4): 2430-2442 (2011)
Received March 24, 2011
Accepted September 6, 2011
Published October 3, 2011
DOI http://dx.doi.org/10.4238/2011.October.3.3

ABSTRACT. Breast cancer is a complex and heterogeneous disease. In spite of the advances made in recent decades, a better understanding of the intrinsic mechanisms of this disease is crucial. The development of new biomarkers is absolutely necessary to improve diagnosis and prognosis. Research using the proteomic approach has generated interesting results; however, the complexity of the mammary gland and of breast tumors remains a major limitation to the development of new markers. An initial step is to characterize non-tumoral human breast tissue. We present data from classical proteomic analysis based on 2-D electrophoresis and peptide mass fingerprinting identification, which were performed on six non-tumoral samples from patients with invasive ductal breast carcinomas. Forty-four different proteins from 70 spots were identified and classified according to their biological function. Cytoskeleton and associated proteins represent the largest class (30%) followed by the proteins with binding function (27%). Several of the proteins have been described in breast tumors, such as vimentin, endoplasmin, small heat shock beta-6, disulfide isomerase

and some cell growth, and proliferation regulators, suggesting the importance of including data on the characterization of non-tumoral breast and to studies on differential expression in cancer tissue.

Key words: Non-tumoral breast tissue; Proteomics; Biomarkers

INTRODUCTION

Breast tissue is controlled by a complex system of hormones (estrogens, progesterone) acting through their respective receptors, as well as a number of local factors from its microenvironment (growth factors). During life, mammary epithelium undergoes repeated cycles of growth, differentiation, and regression, with correlated changes in morphology and metabolism. The anatomic structures are modified by age, menstrual state, pregnancy, and hormonal replacement therapy. The repeated development and involutional changes of menstruation and pregnancy that occur between 15 and 50 years of age create abundant opportunities for minor aberrations to occur (Courtillot et al., 2005). The normal human breast gland is comprised of a branching ductal-lobular system lined by an inner layer of luminal epithelial cells and an outer layer of myoepithelial cells that are separated from the interstitial stroma by an intact basement membrane. The luminal epithelial cells are polarized glandular cells with specialized apical and basolateral membrane domains expressing sialomucin and cell-cell adhesion molecules, respectively. The myoepithelial cells contribute significantly to the formation of basement membrane, and their myogenic differentiation is responsible for the contractile function (Toillon et al., 2007). Because alterations in breast structure and function throughout life can predispose this tissue to the development of breast cancer (Navarrete et al., 2005), understanding the factors and mechanisms that regulate hormone-related changes in the normal human breast is essential. Proteomics is a promising approach for addressing both the development of complementary methods to detect and classify breast cancer in early stages and molecular markers to determine the outcome (Hondermarck et al., 2008, Moreira et al., 2010). A first step towards achieving this goal will be the definition of the phenotypes that exist in the normal breast (Lopez-Garcia et al., 2010). In order to contribute to the knowledge of the non-tumoral profile, we gathered data on six samples; 44 proteins were identified from 70 spots and grouped into categories based on their major biological functions (Pucci-Minafra et al., 2006): cytoskeleton and associated proteins, proteins with binding function, metabolic enzymes, molecular chaperones/heat shock proteins, detoxification and redox proteins, membrane-associated proteins with multiple activities, cell growth and proliferation regulators, protein degradation, and other functions.

MATERIAL AND METHODS

Sample characterization and protein extraction

Aliquots of non-tumoral breast tissue samples were obtained during the surgeries of six female patients (mean age 60.16 ± 10.94 years). All patients were affected with invasive ductal breast cancer, five were classified as grade II and one as grade I. The study was approved by the local Ethics Committee from the Nossa Senhora das Graças Hospital (Curitiba, Brazil) and the patients signed an informed consent.

The tissue was removed from the contralateral side of the tumor respecting the safety margin, which is at least 5 cm from the tumor. The absence of tumor cells, as well as the histological diagnosis and tumor grade, were confirmed by a pathologist.

The samples were immediately transferred to the laboratory, where 400 mg tissue was submerged in a lysis solution (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris, and 0.2% PMSF). Lysates were kept on ice for 30 min, followed by homogenization with an electric tissue disruptor, and 10 min in a refrigerated centrifuge at 12,000 rpm. The supernatants were collected and stored at -80°C until use. The protein concentration was determined by the Bradford method (Bradford, 1976).

2-D gel electrophoresis

Isoelectric focusing (IEF) was performed with precast 13 cm IPG strips (pH 4-7, 130 x 3 x 0.5 mm; GE Healthcare) using an Ettan IPGphor II (GE Healthcare). The cell lysate (1000 µg proteins) was mixed with rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, adding 50 mM DTT and 0.5% IPG buffer) totaling 250 µL. Rehydration occurred at room temperature for 16 h in the Immobiline DryStrip Reswelling Tray (GE Healthcare). The strips were placed on a ceramic plate (Manifold/GE Healthcare) and IEF was performed under the following conditions: 1) 500 V for 1 h; 2) 100 V for 1 h; 3) 8000 V for 2:30 h; 4) 8000 V for 30 min. After IEF, the strips were equilibrated for 15 min in a buffer containing 50 mM Tris-HCl, pH 8.8; 6 M urea; 30% (w/v) glycerol and 2% (w/v) SDS and 50 mM DTT with traces of bromophenol blue. Free thiol groups were alkylated for 15 min in the same solution containing 4.5% iodoacetamide. Equilibrated strips were placed on the top of an SDS polyacrylamide gel (10%, 18 x 16 cm) and sealed with 1% agarose containing traces of bromophenol blue.

SDS-PAGE was performed at Hoefer SE 600 Ruby in a running buffer (25 mM Tris, 192 mM glycine and 0.1% SDS) (GE Healthcare) at 11°C for 30 min at 15 mA and for about 4.5 h at 30 mA until the bromophenol blue line was around 1 cm from the bottom.

Gel staining, scanning and image analysis

After 2-D gel electrophoresis, gels were kept about 1 h in a fixation solution (1.3% orthophosphoric acid (85%) and 20% methanol). Gels were stained with a solution containing 1.5% orthophosphoric acid (85%), 7.7% ammonium sulfate and 0.01% Coomassie G-250. The stained gels were scanned with ImageScanner™ II (GE Healthcare) and analyzed with ImageMaster™ 2D Platinum v6.0 (GE Healthcare).

MS analysis and protein identification

The selected stained spots were excised from the gel followed by destaining with 50 mM NH_4HCO_3 and 50% acetonitrile. The supernatant was removed and gel pieces were completely dried at room temperature. The excised spots were then rehydrated in 15 µL digestion buffer containing 40 mM NH_4HCO_3, 10% acetonitrile and 15 ng/µL trypsin (Sequencing Grade Modified Trypsin; Promega) in an ice-cold bath for 30 min. The digestion was performed at 37°C overnight. The resulting peptides from the digests were mixed (1:1) with 50% acetonitrile and 0.1% trifluoroacetic acid saturated with HCCA matrix and spotted onto the MALDI targets.

MALDI-TOF mass spectra were recorded on a MALDI-TOF/TOF/MS/MS AutoflexII (Bruker Daltonics, Leipzig, Germany) mass spectrometer, in the 800-3200 Da mass range using a minimum of 250 shots of laser per spectrum. Delayed extraction source (150 ns) and reflector equipment allowed sufficient resolution to consider MH^+ of monoisotopic peptide masses. The TOF acceleration voltage was 20 kV. MALDI mass data were externally calibrated with a peptide calibration standard kit. Internal calibration was done using trypsin autolysis fragments at m/z of 842.52, 1045.56 and 2211.10 Da. Peptide mass fingerprinting was compared to the theoretical masses from the Swiss-Prot database using MASCOT (http://www.expasy.org/tools/). The search was done by restricting the taxonomic category to *Homo sapiens*. Typical search parameters were as follows: maximum 200 ppm of mass tolerance; carbamidemethylation of cysteine residues; one missed enzymatic cleavage for trypsin; a minimum of four-peptide mass hits were required for a match; methionine residues were considered to be in an oxidized form. Protein identifications were considered to be reliable when the protein score of the hit exceeded the threshold significance score of 56 ($P < 0.05$) and no less than four peptides were recognized.

RESULTS AND DISCUSSION

The main objective of cancer proteome science is to define patterns of protein expression from tissues originating under different conditions (healthy, benign diseases, different types and stages of cancer, metastasis) that are useful for basic and applied research applications (Pucci-Minafra et al., 2006). We examined six samples obtained from patients with invasive ductal breast cancer, which were collected respecting the safety margin (at least 5 cm from the tumor) and free of cancer cells. Three bidimensional gels were made for each sample and a reference gel was chosen based on the representation and resolution of the spots. The six reference gels were then analyzed to choose the master reference gel (Figure 1), in which the protein spots are identified with the abbreviated name of the Swiss-Prot database. Using the ImageMaster™ 2D Platinum v6.0 software, we detected 118 spots, which were present in the master and in at least two of the other five reference gels. Of the 118 spots, 70 were identified, corresponding to 44 different proteins (Table 1). Comparing the theoretical and the observed value of the molecular weight (MW) and pI of each protein, we noticed that the majority (76%) was identified at the expected position on the gels ($\Delta MW < 20\%$ and $\Delta pI < 0.6$, according to Dupont et al., 2005). Approximately, 30% of the 44 identified proteins were represented by more than one spot, suggesting the occurrence of post-translational modifications, such as acetylation, phosphorylation and glycosylation (Ramos and Bora, 2004; Mayr et al., 2005; Zhu et al., 2005; Löster and Kannicht, 2008).

Through the information obtained from literature and NCBI (http://www.ncbi.nlm.nih.gov/) and UniProtKB/Swiss-Prot (http://www.expasy.ch/) databases, the 44 identified proteins were separated according to their main biological functions into nine classes (Figure 2): 1) cytoskeleton and associated proteins; 2) proteins with binding function; 3) metabolic enzymes; 4) molecular chaperones/heat shock proteins; 5) detoxification and redox proteins; 6) membrane-associated proteins with multiple activities; 7) cell growth and proliferation regulators; 8) protein degradation, and 9) other functions. These classes were based on a study by Pucci-Minafra et al. (2006). In spite of this functional classification, these classes should be treated with caution. We agree with the authors that the organization of proteins into functional categories is fundamental to better understanding the mechanisms and the biochemical functions of cells and their involvement in cancer.

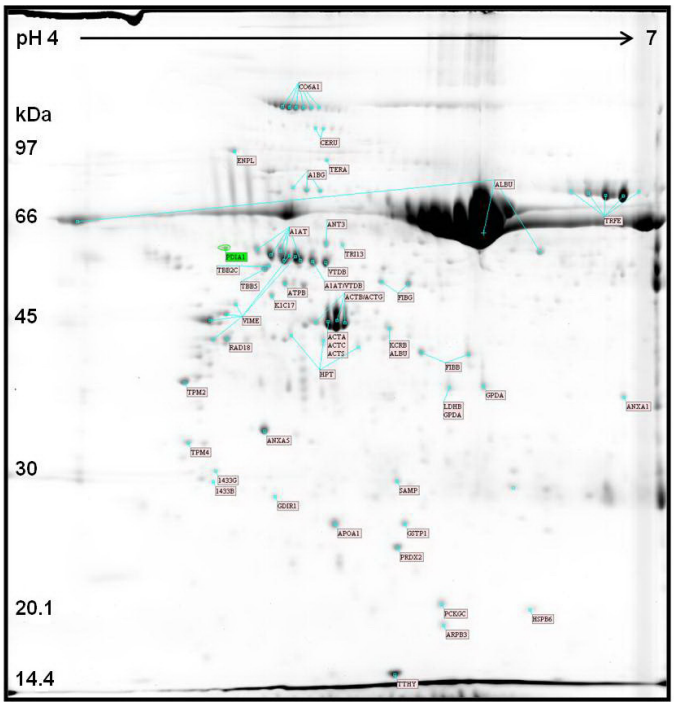

Figure 1. Reference gel obtained after 2-D electrophoresis. The protein spots are identified with the abbreviated name of the Swiss-Prot database.

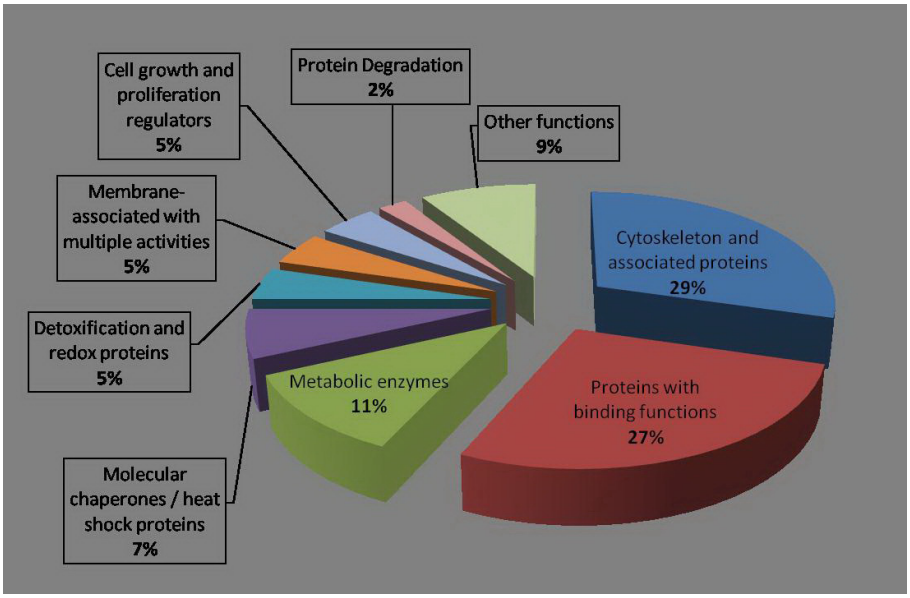

Figure 2. Functional classification of the identified proteins.

Table 1. Data from the proteins grouped by respective functional class.

Spot number	Accession number	Protein name	Abbreviated name	Experimental MW/pI (theoretical)	ΔMW/ΔpI	% Masses matched	Sequence coverage	MASCOT score/ 2nd score
Cytoskeleton and associated proteins								
361	P62736/	Actin, aortic smooth muscle/	ACTA/	45.0/5.34	5.8%/0.11/	7/29 (24%)	20%	75/59
	P68032/	Actin, alpha cardiac muscle/	ACTC/	(42.4/5.23/42.3/5.23)	5.6%/0.11			
	P68133	Actin, alpha skeletal muscle	ACTS					
359	P60709/P63261	Actin, cytoplasmic 1/2	ACTB/G	45.0/5.27 (42.0/5.29)	6.7%/0.02/6.4%/0.04	6/7 (86%)	23%	96/41
362	P60709/P63261	Actin, cytoplasmic 1/2	ACTB/G	45.0/5.42	6.7%/0.13/6.4%/0.11	10/37 (27%)	32%	79/-
367	P60709/P63261	Actin, cytoplasmic 1/2	ACTB/G	44.0/5.38	4.5%/0.09/4.3%/0.07	9/25 (36%)	28%	88/33
497	Q9P1U1	Actin-related protein 3B	ARP3B	20.0/5.91 (48.1/5.61)	58.4%/0.3	5/21 (24%)	14%	62/25
333	Q04695	Keratin, type I cytoskeletal 17	K1C17	50.0/5.06 (48.4/4.97)	3.2%/0.09	7/28 (25%)	20%	60/20
470	P52565	Rho GDP-dissociation inhibitor 1	GDIR1	28.0/5.07 (23.2/5.02)	17.1%/0.05	5/22 (23%)	29%	57/25
411	P07951	Tropomyosin beta chain	TPM2	38.0/4.62 (32.9/4.66)	13.4%/0.04	7/13 (54%)	19%	86/43
441	P67936	Tropomyosin alpha-4 chain	TPM4	33.0/4.63 (28.6/4.67)	13.3%/0.04	8/12 (67%)	20%	109/49
298	P07437	Tubulin beta chain	TBB5	56.0/5.01 (50.1/4.78)	10.5%/0.23	19/29 (66%)	52%	216/162
292	P68371	Tubulin beta-2C chain	TBB2C	57.0/5.04 (50.2/4.79)	11.9%/0.25	12/29 (41%)	31%	99/70
275	P08670	Vimentin	VIME	60.0/5.13 (53.7/5.06)	10.5%/0.07	14/31 (45%)	35%	115/28
276	P08670	Vimentin	VIME	60.0/5.17	10.5%/0.11	12/52 (23%)	25%	66/44
346	P08670	Vimentin	VIME	48.0/4.87	10.6%/0.19	14/27 (52%)	37%	126/32
351	P08670	Vimentin	VIME	46.0/4.82	14.3%/0.24	14/24 (58%)	33%	132/33
355	P08670	Vimentin	VIME	45.0/4.75	16.2%/0.31	11/29 (38%)	29%	90/24
378	P08670	Vimentin	VIME	43.0/4.76	19.9%/0.3	21/24 (87%)	40%	261/30
Proteins with binding function								
267	P01009	Alpha-1-antitrypsin	A1AT	61.0/4.98 (46.9/5.37)	23.1%/0.39	10/25 (40%)	22%	106/31
272	P01009	Alpha-1-antitrypsin	A1AT	59.0/5.05	20.5%/0.32	13/35 (37%)	24%	123/19
283	P01009	Alpha-1-antitrypsin	A1AT	58.0/5.12	19.1%/0.25	15/33 (45%)	37%	162/31
617	P01009	Alpha-1-antitrypsin	A1AT	61.0/5.09	23.1%/0.28	8/31 (26%)	21%	62/24
618	P01009	Alpha-1-antitrypsin	A1AT	58.0/5.19	19.1%/0.18	9/36 (25%)	27%	67/-
285	P01009	Alpha-1-antitrypsin	A1AT	58.0/5.26	19.1%/0.11	9/29 (31%)	17%	162
260	P01008	Antithrombin-III	ANT3	61.0/5.32 (53.0/6.32)	13.1%/1	13/22 (59%)	29%	114/25
478	P02647	Apolipoprotein A-I	APOA1	26.0/5.37 (30.7/5.56)	15.3%/0.19	18/30 (60%)	62%	229/34
156	P00450	Ceruloplasmin	CERU	115.0/5.27 (123.0/5.44)	6.5%/0.17	9/27 (33%)	13%	58/22
157	P00450	Ceruloplasmin	CERU	115.0/5.31	6.5%/0.13	7/19 (37%)	10%	53/25
392	P02675	Fibrinogen beta chain	FIBB	41.0/5.8 (56.6/8.54)	27.6%/2.74	21/24 (87%)	38%	204/39
394	P02675	Fibrinogen beta chain	FIBB	41.0/6.04	27.6%/2.5	20/26 (77%)	39%	195/26
317	P02679	Fibrinogen gamma chain	FIBG	53.0/5.74 (52.1/5.37)	1.7%/0.37	8/22 (36%)	25%	82/25
320	P02679	Fibrinogen gamma chain	FIBG	53.0/5.60	1.7%/0.23	8/25 (32%)	28%	85/27
369	P00738	Haptoglobin	HPT	43.0/5.15 (45.9/6.13)	6.3%/1.02	10/16 (62%)	25%	130/57
379	P00738	Haptoglobin	HPT	43.0/5.25	6.3%/0.88	9/14 (64%)	24%	138/31
386	P00738	Haptoglobin	HPT	42.0/5.49	8.5%/0.64	8/13 (61%)	19%	87/27
230	P02768	Serum albumin	ALBU	70.0/4.08 (71.3/5.92)	1.85%/1.84	13/15 (87%)	25%	165/40

Continued on next page

Table 1. Continued.

Spot number	Accession number	Protein name	Abbreviated name	Experimental MW/pI (theoretical)	ΔMW/ΔpI	% Masses matched	Sequence coverage	MASCOT score/ 2nd score
Proteins with binding function								
271	P02768	Serum albumin	ALBU	61.0/6.39	14.4%/0.47	15/20 (75%)	27%	180/27
364	P02768-2	Serum albumin (isoform 2)	ALBU	44.0/5.64	38.2%/0.28	11/23 (48%)	22%	210
459	P02743	Serum amyloid P-component	SAMP	30.0/5.69 (25.5/6.1)	15%/0.41	5/23 (22%)	25%	68/32
192	P02787	Serotransferrin	TRFE	78.0/6.80 (79.3/6.81)	1.7%/0.01	8/20 (40%)	13%	70/23
195	P02787	Serotransferrin	TRFE	80.0/6.55	0.9%/0.26	12/19 (63%)	18%	133/29
196	P02787	Serotransferrin	TRFE	81.0/6.64	0.9%/0.08	12/31 (39%)	21%	94/22
198	P02787	Serotransferrin	TRFE	80.0/6.73	2.1%/0.17	21/35 (60%)	29%	225/28
201	P02787	Serotransferrin	TRFE	80.0/6.89	0.9%/0.08	15/31 (48%)	22%	142/28
514	P02766	Transthyretin	TTHY	15.0/5.66 (16.0/5.52)	6.25%/0.14	4/19 (21%)	48%	69/26
285	P02774	Vitamin D-binding protein	VTDB	58.0/5.26 (54.5/5.40)	6%/0.14	10/29 (34%)	30%	162
286	P02774	Vitamin D-binding protein	VTDB	57.0/5.32	4.4%/0.08	8/23 (35%)	25%	84/18
Metabolic enzymes								
319	P06576	ATP synthase subunit beta, mitochondrial	ATPB	53.0/5.13 (56.5/5.26)	6.2%/0.13	16/21 (76%)	37%	194/40
364	P12277	Creatine kinase B-type	KCRB	44.0/5.64 (42.9/5.34)	2.5%/0.3	9/23 (39%)	32%	210
415	P21695	Glycerol-3-phosphate dehydrogenase [NAD+]	GPDA	38.0/6.1 (38.2/5.81)	0.5%/0.29	12/24 (50%)	43%	142/31
417	P21695	Glycerol-3-phosphate dehydrogenase [NAD+]	GPDA	38.0/5.94	0.5%/0.13	6/14 (43%)	18%	184
417	P07195	L-lactate dehydrogenase B chain	LDHB	38.0/5.94 (36.9/5.71)	2.9%/0.23	8/14 (57%)	25%	184
491	P35558	Phosphoenolpyruvate carboxykinase, cytosolic [GTP]	PCKGC	21.0/5.91 (69.9/5.8)	69.9%/0.11	6/30 (20%)	13%	56/28
Molecular chaperones/heat shock proteins								
164	P14625	Endoplasmin	ENPL	102.0/4.86 (92.7/4.76)	9.1%/0.1	10/22 (45%)	15%	72/24
492	O14558	Heat shock protein beta-6	HSPB6	21.0/6.34 (17.2/5.95)	18.1%/0.39	4/10 (40%)	29%	62/31
268	P07237	Protein disulfide-isomerase	PDIA1	60.0/4.82 (57.5/4.76)	4.2%/0.06	8/19 (42%)	18%	103/38
Detoxification and redox proteins								
479	P09211	Glutathione S-transferase P	GSTP1	26.0/5.71 (23.6/5.43)	9.2%/0.28	6/33 (18%)	41%	68/27
481	P32119	Peroxiredoxin-2	PRDX2	25.0/5.68 (22.0/5.66)	12%/0.02	5/20 (25%)	22%	76/27
Membrane-associated proteins with multiple activities								
422	P04083	Annexin A1	ANXA1	37.0/6.81 (38.9/6.57)	4.9%/0.24	9/22 (41%)	32%	104/27
433	P08758	Annexin A5	ANXA5	34.0/5.01 (36.0/4.94)	5.5%/0.07	16/30 (53%)	52%	193/52
Cell growth and proliferation regulators								
460	P31946	14-3-3 protein beta/alpha	1433B	30.0/4.75 (28.2/4.76)	6%/0.01	7/16 (44%)	34%	82/29
453	P61981	14-3-3 protein gamma	1433G	31.0/4.77 (28.4/4.8)	8.4%/0.03	6/22 (27%)	25%	60/26

Continued on next page

Table 1. Continued.

Spot number	Accession number	Protein name	Abbreviated name	Experimental MW/pI (theoretical)	ΔMW/ΔpI	% Masses matched	Sequence coverage	MASCOT score/ 2nd score
Protein degradation								
262	O60858	Tripartite motif-containing protein 13	TRI13	61.0/5.41 (47.8/5.75)	21.6%/0.34	6/23 (26%)	15%	58/32
Other functions								
185	P04217	Alpha-1B-glycoprotein	A1BG	82.0/5.17 (54.8/5.58)	33.1%/0.41	5/16 (31%)	17%	61/26
189	P04217	Alpha-1B-glycoprotein	A1BG	81.0/5.23	32.3%/0.35	11/17 (65%)	27%	143/33
197	P04217	Alpha-1B-glycoprotein	A1BG	81.0/5.30	32.3%/0.28	8/13 (61%)	24%	106/27
139	P12109	Collagen alpha-1(VI) chain	CO6A1	131.0/5.10 (109.6/5.26)	16.3%/0.16	10/22 (45%)	12%	71/25
140	P12109	Collagen alpha-1(VI) chain	CO6A1	130.0/5.14	15.7%/0.12	14/27 (52%)	17%	106/27
142	P12109	Collagen alpha-1(VI) chain	CO6A1	129.0/5.29	15%/0.03	10/20 (50%)	16%	99/30
144	P12109	Collagen alpha-1(VI) chain	CO6A1	129.0/5.17	15%/0.09	10/22 (45%)	11%	67/26
145	P12109	Collagen alpha-1(VI) chain	CO6A1	128.0/5.21	14.4%/0.05	14/18 (78%)	20%	150/42
146	P12109	Collagen alpha-1(VI) chain	CO6A1	129.0/5.25	15%/0.01	7/13 (54%)	12%	82/34
375	Q9NS91	E3 ubiquitin-protein ligase RAD18	RAD18	43.0/4.82 (57.0/7.17)	24.5%/2.35	6/23 (26%)	19%	92/33
170	P55072	Transitional endoplasmic reticulum ATPase	TERA	96.0/5.33 (89.9/5.14)	6.3%/0.19	10/13 (77%)	13%	57/25

MW = molecular weight; pI = isoelectric point.

Cytoskeleton and associated proteins

Thirteen identified proteins from 17 spots composed this class. Some spots contained more than one protein, representing co-migrant proteins with similar primary structures or molecular weight. This is the largest class (30% of the proteins).

The cytoskeleton is a complex and dynamic system. In general, cytoskeletal proteins polymerize to form long chains, giving the appearance of strand-like or fibrous structures. It is directly or indirectly involved in several important cellular processes, including cell polarity, adhesion, intracellular transport, signal transduction, and cell movement (Pucci-Minafra et al., 2006), which in turn play important roles in embryogenesis, wound healing, inflammation, and cancer metastasis (Eriksson et al., 2009). There are three general classes of cytoskeletal fibers: 1) microtubules, 2) intermediate filaments, and 3) actin filaments. The actin proteins are extremely conserved evolutionarily and are essential for eukaryotic cells. Because they are very similar, distinctions by peptide mass fingerprinting are difficult, and they are commonly identified in a single spot (Dupont et al., 2005; Pucci-Minafra et al., 2006). We identified three spots as actin, cytoplasmatic 1/2 (ACTB/ACTG). They differ by just four amino acids and were detected together. The same occurred with actins in aortic smooth muscle, alpha cardiac muscle and alpha skeletal muscle (ACTA/ACTC/ACTS). All the different types of actins were considered in determining the total count of identified proteins.

Seven spots were identified as vimentin, an important structural component of the cytoskeleton and generally accepted as the cytoskeletal component responsible for maintaining cell integrity. Studies using knockout mice have revealed the involvement of this protein in a number of critical cellular processes related to the organization and regulation of proteins involved in adhesion, migration and cell signaling. Furthermore, it plays a significant role in supporting and anchoring the position of the organelles in the cytosol, providing flexibility to the cell (Eriksson et al., 2009).

Proteins with binding function

With 28 bands corresponding to 12 proteins and their isoforms, the class of proteins with binding functions (27%) was the second most abundant. The proteins identified in this group were: antithrombin, alpha-1-antitrypsin (A1AT, six spots), serotransferrin (five spots), haptoglobin (three spots), serum albumin (three spots, including the isoform 2), fibrinogen beta chain (two spots), fibrinogen gamma chain (two spots), vitamin D-binding protein (VTDB, two spots), ceruloplasmin (two spots), apolipoprotein A-I, serum amyloid P-component, and transthyretin. Many of these proteins have their binding function associated with the transport of molecules. A1AT and VTDB were identified in the same spot as a mixture.

The main function of human ceruloplasmin is to reduce molecular oxygen, transforming oxidized ferrous ions into a less toxic form, without releasing reactive oxygen species (Sokolov et al., 2006; Altamura et al., 2009). Serum albumin, which has as one of its principal functions regulation of osmotic blood pressure, is also the main zinc carrier in plasma (Deng et al., 2006; Lu et al., 2008). Serum albumin was present in a mixture with creatine kinase B-type (KCRB/albumin) above the acceptable range of 20% for the mass (38.2%). We suggest that this protein corresponds to isoform 2, generated by alternative splicing and with molecular

mass equal to 47.36 kDa (UniProtKB/SwissProt database). The MW variation for this isoform was 7%, remaining within the acceptable range.

Metabolic enzymes

The group of metabolic enzymes (11%) was composed of five spots corresponding to five enzymes: L-lactate dehydrogenase B chain (LDHB), KCRB, glycerol-3-phosphate dehydrogenase [NAD+] (GPDA), ATP synthase subunit beta, mitochondrial (ATPB) and phosphoenolpyruvate carboxykinase, cytosolic [GTP] (PCKGC). This last protein was identified with a score limit of 56 and sequence coverage of 13%. The variation between the theoretical and experimental MW was 70%. There are no reports of alternative splicing in the UniProtKB/SwissProt database. LDHB and GPDA were identified together as a mixture. Among these proteins, some are involved in the anaerobic glycolytic pathway and others are mitochondrial enzymes belonging to energy metabolism.

Molecular chaperones/heat shock proteins

Protein folding in the cell is aided by enzymes that speed up the process and chaperones that prevent aggregation. Misfolded proteins can obviously result in loss of function, besides forming large insoluble aggregates that also interfere in cellular function (Wilkinson and Gilbert, 2004). Molecular chaperones are an important group of heat shock proteins, and their expression is induced by cellular stress (Pucci-Minafra et al., 2006). The molecular chaperones/heat shock proteins class comprised 7% of the identified proteins. Three spots were analyzed, each one corresponding to a different protein: endoplasmin, small heat shock protein beta-6, and protein disulfide-isomerase. This group is underrepresented if we compare our study with some cancer proteomics reports (Pucci-Minafra et al., 2007; Kim et al., 2011) in which several proteins of this group are commonly overexpressed and constitute a target for therapy.

Detoxification and redox proteins

Proteins related to detoxification and redox proteins (4%) are enzymes important for catabolism and cellular protection against metabolic stress (Pucci-Minafra et al., 2006). We found two proteins that represent this class: glutathione S-transferase P (GSTP1) and peroxiredoxin-2 (PRDX2). The GST family codes for enzymes essential to the detoxification process, reducing gluthatione and facilitating the excretion of many xenobiotics. *GSTP1*, located in chromosome 11q18, is polymorphic and may be of particular relevance in cancer susceptibility (Leichsenring et al., 2006). PRDX2 is a potent peroxide reductant; it is the third most abundant protein in the erythrocyte and plays a major role in the cell's oxidative defense, mainly in detoxifying endogenous-generated H_2O_2 (Johnson et al., 2010).

Membrane-associated proteins with multiple activities

Annexins I and V are the class of membrane-associated proteins with multiple

activities (5%). The annexins are involved in a wide range of functions both inside and outside cells. They belong to a class of proteins that interact with Ca^{2+} ions and with components of the membrane. These are properties that link annexins to many membrane-related events, such as the regulated organization of membrane domains and/or membrane/cytoskeleton linkages, certain exocytic and endocytic transport steps, and the regulation of ion fluxes across membranes (Gerke et al., 2005).

Cell growth and proliferation regulators

The category of proteins involved in cell growth and proliferation regulation (5%) was composed of two proteins, 14-3-3 beta/alpha (1433B) and gamma 14-3-3 (1433G), each corresponding to one spot. The 14-3-3 proteins exhibit a remarkable conservation of sequences between species and form a group of multifunctional proteins that bind to and modulate the function of several cellular proteins (Fu et al., 2000).

Protein degradation

The tripartite motif-containing protein 13 (TRI13) was the only protein identified in this class (2%). It is encoded by the *TRIM13* gene, which belongs to the *TRIM* family. Proteins of this family are involved in ubiquitination of damaged proteins or in excess. This signal causes the proteins to be routed to proteosomes (Meroni and Diez-Roux, 2005), a very important mechanism to ensure cell homeostasis.

Other functions

The class of proteins with other functions (9%) was composed of 11 spots corresponding to four proteins and their isoforms: collagen alpha-1(VI) chain (CO6A1) (six spots), alpha-1B-glycoprotein (A1BG) (three spots), transitional endoplasmic reticulum ATPase (TERA), and E3 ubiquitin-protein ligase RAD18 (RAD18).

The CO6A1 and other proteins of the collagens superfamily are responsible for maintaining the integrity of various tissues. The protein TERA belongs to the AAA family (ATPases associated with different cellular activities), which is associated with several cellular processes, such as membrane fusion, protein degradation, organelle biogenesis, and cell cycle regulation (Meyer et al., 2000). According to the NCBI database, the A1BG is a plasmatic glycoprotein whose function remains unknown. Furthermore, according to the NCBI database, RAD18 is involved in post-replication repair of DNA damaged by UV.

CONCLUSION

Breast tissue is very complex due to the influence of various factors throughout life, and therefore it is difficult to establish a proteomic profile. We identified several proteins, including possible isoforms that constitute an important basis for future studies. The identification of these proteins and their isoforms help to explain the post-translational modifications responsible for the observed variation in the proteomic analyses. Many of the proteins identi-

fied in this study have been reported as differentially expressed in relation to breast tumors such as vimentin, endoplasmin, small heat shock beta-6, disulfide isomerase and some cell growth, and proliferation regulators, suggesting the importance of including data on the characterization of non-tumoral breast contributing with novel information for subsequent studies on differential expression with tumor tissue.

ACKNOWLEDGMENTS

The authors are grateful to the Departamento de Bioquímica, UFPR for the assistance with the Maldi-Tof and to Dr Roseli Wassem for valuable technical suggestions. Research supported by CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil).

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