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PROTEOMIC ANALYSIS OF INFILTRATING DUCTAL CARCINOMA TISSUES BY COUPLED 2-D DIGE/MS/MS ANALYSIS

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There is a growing interest in protein expression profiling aiming to identify novel diagnostic markers in breast cancer. Proteomic approaches such as two-dimensional differential gel electrophoresis coupled with tandem mass spectrometry analysis (2-D DIGE/MS/MS) have been used successfully for the identification of candidate biomarkers for screening, diagnosis, prognosis and monitoring of treatment response in various types of cancer. Identifying previously unknown proteins of potential clinical relevance will ultimately help in reaching effective ways to manage the disease. We analyzed breast cancer tissues from five tumor and five normal tissue samples from ten breast cancer subjects with infiltrating ductal carcinoma (IDC) by 2-D DIGE using two types of immobilized pH gradient (IPG) strips: pH 3–10 and pH 4–7. From all the spots detected, differentially expressed ($p < 0.05$ and ratio > 2) were 50 spots. Of these, 39 proteins were successfully identified by MS, representing 29 different proteins. Ten proteins were overexpressed in the tumor samples. The 2-D DIGE/MS/MS analysis revealed an increase in the expression levels in tumor samples of several proteins not previously associated with breast cancer, such as: macrophage-capping protein (CAPG), phosphomannomutase 2 (PMM2), ATPase ASN1, methylthioribose-1-phosphate isomerase (MRI1), peptidyl-prolyl cis-trans isomerase FKBP4, cellular retinoic acid-binding protein 2 (CRABP2), lamin B1 and keratin, type II cytoskeletal 8 (KRT8). Ingenuity Pathway Analysis (IPA) revealed highly significant ($p = 10^{-26}$) interactions between the identified proteins and their association with cancer. These proteins are involved in many diverse pathways and have established roles in cellular metabolism. It remains the goal of future work to test the suitability of the identified proteins in samples of larger and independent patient groups.

Keywords: **2-D DIGE, biomarker, breast cancer, infiltrating ductal carcinoma, MS/MS.**

Breast cancer is among the most commonly diagnosed invasive malignancies and the second leading cause of cancer death in women globally [1]. Breast cancer is a complex disease. Accumulation of numerous molecular alterations causes gaining of an increasingly invasive and resistant phenotype [2]. The variability of the host background and heterogeneity of malignant cells create distinct subgroups of tumors with different phenotypes and clinical outcomes.

Improvement of breast-cancer detection at its early stages through identification of diagnostic biomarkers is of critical importance. Also, classification of the disease heterogeneity by defining more reliable diagnostic/prognostic factors and developing molecular therapies for selective targeting of the tumor cells is of great importance. One way to identify such biomarkers is through analysis of alterations that occur at the gene or protein level in tumor tissue as compared to normal tissue. Proteomics has several advantages over genomics since proteins are more reflective of the current state of the cell's microenvironment, can undergo a vast range of posttranslational modifications affecting protein stability, localization, interactions, and functions, and represent more accessible and relevant therapeutic targets [3].

Probably the most widely used proteomic technology for identification of alterations in protein expression between different types of samples is two-dimensional gel electrophoresis (2-D PAGE) [4] or a variant of the technique with improved sensitivity, reproducibility and rapidity – two-dimensional differential gel electrophoresis (2-D DIGE) [5] coupled with mass spectrometry (MS) analysis. In order to identify and evaluate cancer-related proteins tumor tissues, blood plasma, or secreted body fluids (such as urine, sweat and saliva) are used as a source of biological material. Out of these, the tumor tissue itself, backed up by full

Abbreviations: 2-D DIGE/MS/MS – two-dimensional differential gel electrophoresis coupled with tandem mass spectrometry analysis; IDC – infiltrating ductal carcinoma; IPG – immobilized pH gradient.

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Table 1. Characterization of breast tissues used in this study

No	Age	Histology	Stage	ER/PR	Her 2	pTNM[a]			G[b]	Tissue type
						T	N	M		
1	66	Carcinoma ductale invasivum	IIA	3+/+	+	pT2	pN1a	pMx	G2	Nontumor
2	65	Carcinoma ductale invasivum	IIA	2+/1+	+	pT2	pN1a	pMx	G2	Nontumor
3	63	Carcinoma ductale invasivum	IIA	3+/+	+	pT2	pN1a	pMx	G2	Nontumor
4	68	Carcinoma ductale invasivum	IIA	3+/2+	+	pT2	pN1a	pMx	G2	Nontumor
5	69	Carcinoma ductale invasivum	IIA	2+/1+	+	pT2	pN1a	pMx	G2	Nontumor
6	65	Carcinoma ductale invasivum	IIA	3+/2+	+	pT2	pN1a	pMx	G2	Tumor
7	64	Carcinoma ductale invasivum	IIA	3+/+	3+	pT2	pN0	pMx	G2	Tumor
8	63	Carcinoma ductale invasivum	IIA	3+/2+	+	pT2	pN1a	pMx	G2	Tumor
9	66	Carcinoma ductale invasivum	IIA	3+/+	+	pT2	pN1a	pMx	G2	Tumor
10	65	Carcinoma ductale invasivum	IIA	2+/1+	3+	pT2	pN1a	pMx	G2	Tumor

[a]The TNM Classification of Malignant Tumors (TNM) is a cancer staging system that describes the extent of cancer in a patient's body. T – describes the size of the tumor and whether it has invaded nearby tissue; N – describes regional lymph nodes that are involved; M – describes distant metastasis (spread of cancer from one body part to another).

[b]G (1–4) is the grade of the cancer cells (i.e. they are "low grade" if they appear similar to normal cells, and "high grade" if they appear poorly differentiated).

clinical and histological evaluation, is the most reliable source for identification of new cancer-specific protein changes. A large number of 2-D PAGE and recently a growing number of 2-D DIGE-based procedures have been applied to preclinical models of breast cancer, however, data generated from clinical samples is still limited [6–17].

From all classified breast cancers, invasive carcinomas represent 70–80% and include more than 10 different types. Among these, infiltrating ductal carcinomas (IDC) are the most aggressive forms associated with poor prognosis [18]. The aim of this study was to identify specific proteins in IDC, with distorted expression. For this, the 2-D DIGE/MS/MS technology was used. The results of this study may aid in identification of novel candidate biomarkers for IDC and in expanding the database of candidate proteins that could be used as diagnostic and therapeutic targets in breast cancer research.

EXPERIMENTAL

Tissue samples. Normal and tumor breast tissues were obtained from the Department of Thoracic and Vascular Surgery, Clinical Center, Skopje. The tissues were histologicaly characterized at the Institute of Pathology, Medical Faculty, University "St. Cyril and Methodius", Skopje. An informed consent for the use of these tissues for research purposes was obtained from the patients.

Patients' clinical records including age at diagnosis, histology, tumor size and lymph node status were reviewed to preselect the tissue samples. All of the tumor samples contained more than 50% tumor cells and were stored at –80°C before use. Five tumor samples and five normal tissue samples were chosen from the tissue archive based on the type of tumor, TNM classification, estrogen receptor/progesterone receptor (ER/PR) and human epidermal growth factor receptor 2 (Her2) status (table 1), and pooled separately for the 2-D DIGE analysis. In order to minimize the influence of individual variations, all 10 samples were from different individuals. The decision to work with proteins pooled from a small number of well matched tumors was made to minimize the misinterpretation of protein profiles arising from random differences of gene expression of different tumors.

Preparation of total protein extract from tissue. The frozen tissues were pulverized with liquid nitrogen in a mortar. The resultant tissue powder was weighed and 15 volumes of UTCD solution (8 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 50 mM dithiothreitol (DTT)) were added. The samples were thoroughly mixed and allowed to dissolve on ice for 30 min. If the samples were too viscous, they were put through a 21G needle for few times. The samples were centrifuged at 14000 rpm for 10 min at 4°C, and the supernatant was transferred into a clean tube. The protein concentration in all of the samples was measured using the Bradford method [19] in duplicate against a standard curve of bovine serum albumin. Equal amounts of proteins from each sample (150 μg) were pooled and purified with the 2-D Clean-UP Kit ("GE Helthcare") according to the manufacturer's instructions. The pellets were dissolved in 40 μL of UTC buff-

er (8 M urea, 2 M thiourea, 4% (w/v) CHAPS), quantified by Bradford method and stored at –80°C.

Fluorescent labeling with CyDye DIGE fluor minimal dyes. The pH of protein samples was adjusted to 8.5 with 1.5 M Tris-HCl. Proteins were labeled with the CyDye DIGE Fluor minimal dyes ("GE Healthcare") according to the manufacturer's instructions. Forty five micrograms of protein from each sample were minimally labeled with 400 pmol of Cy3 or Cy5, respectively. Cy2 was used to label an equivalent amount of the internal standard containing equal amounts of all samples. The samples were randomized between gels to ensure an even distribution between those labeled with Cy3 and Cy5 minimal dyes and to avoid repetitive linking of the same sample type with the same dye on multiple gels.

2-D DIGE. The 2D-DIGE analysis was performed in duplicate, on two types of Immobiline Drystrip gels: broad range (pH 3–10) (DIGE analysis 1) and medium range (pH 4–7) (DIGE analysis 2). The separate CyDyes labeling reactions were combined and rehydration buffer was added to a final volume of 450 μL. For the Immobiline Drystrip gels pH 3–10, we used Rehydration buffer 1 (8 M urea, 2 M thiourea, 2% (w/v) CHAPS, 1.2% (v/v) DeStreak Reagent, 0.5% (v/v) immobilized pH gradient (IPG) buffer pH 3–10, trace of bromophenol blue), while for the Immobiline DryStrip gels pH 4–7, rehydration buffer 2 (8 M urea, 2 M thiourea, 2% (w/v) CHAPS, 10 mM DTT, 1.2% (v/v) IPG-buffer pH4–7, trace of bromophenol blue) was used. Isoelectric focusing (IEF) of rehydrated 24-cm Immobiline Drystrip gels was performed on an Ettan IPGphor 3 System ("GE Helthcare"). The Immobiline Drystrip gels pH 3–10 and pH 4–7 were focused until a total of 52.6 kV and 64.5 kV was reached, respectively. The focused proteins in the IPG-strips were immediately equilibrated in two incubation steps, each lasting 15 min, at room temperature. In the first step, the equilibration buffer was supplemented with 62.5 mM DTT for reduction, followed by alkylation in the same buffer containing 2.5% (w/v) iodoacetamide instead of DTT. For protein resolution in the second dimension, the strips were applied onto homogeneous 12%-polyacrylamide gels, and SDS-PAGE was carried out on three gels simultaneously (Ettan DALTsix system; "GE Healthcare"), at 2.5 W per gel for 30 min, followed by 16 W/gel for 4 h.

DIGE imaging and analysis. The five 2-D DIGE gel images were scanned on an Ettan DIGE imager ("GE Healthcare"). Gel images with approximately the same pixel value without any saturation were obtained by adjusting the exposure time. All of the gels were scanned at 100-dpi resolution. Images were cropped using Image QuantTL software ("GE Healthcare") to remove the areas extraneous to the gel image. DIGE images were analyzed using Image Master Platinum Software 7.1 ("GE Healthcare"). Protein abundance was expressed as "volume ratio" according to the normalization method provided by Image Master Platinum Software. The ratio values for a given spot between normal and tumor samples were calculated from the volume ratio mean (VolRatio Mean) values for normal and tumor samples. Statistically significant, differentially expressed proteins were selected basing on two criteria: ANOVA (which in this case corresponds to Student's *t*-test) <0.05 and ratio >2. Protein spots with a significant difference were excised from a preparative gel stained with Coomassie G-250 (CBB) and analyzed by MALDI-TOF-TOF MS.

Setting up of preparative 2-D gels for spot picking. For preparative colloidal CBB-stained gels, 45 μg of each of the 10 protein samples were combined, to give a total of 450 μg of protein. The volume was adjusted to 450 μL with the appropriate rehydration buffer depending on the pH range of the strip. The IEF and second dimension SDS-PAGE were run according to standard procedures. Gels were fixed in 30% (v/v) ethanol, 2% (v/v) phosphoric acid for 30 min, balanced in pre-staining buffer: 12% (w/v) $(NH_4)_2SO_4$, 2% (v/v) phosphoric acid, 18% (v/v) ethanol – for another 30 min and stained in staining solution: 1% (w/v) CBB, 12% (w/v) $(NH_4)_2SO_4$, 2% (v/v) phosphoric acid, 18% (v/v) ethanol – for 72 h. The gels were stored in the staining solution until the spots of interests were manually picked.

Mass spectrometry: in-gel tryptic cleavage. In-gel digestion was carried out with trypsin as described by Schevchenko et al. [20] with minor modifications and using for all steps a Freedom EVO 100 digester/spotter robot ("Tecan", Switzerland). Spots were first destained two times with a mixture of 100 mM ammonium bicarbonate (ABC) and 50% (v/v) acetonitrile (ACN) for 45 min at 22°C and then dried using 100% ACN for 15 min. Protein spots were then reduced with 25 mM ABC containing 10 mM DTT for 1 h at 60°C and then alkylated with 55 mM iodoacetamide in 25 mM ABC for 30 min in the dark at 22°C. Gels pieces were washed twice with 25 mM ABC and finally shrunk two times with 100% ACN for 15 min and dried using 100% ACN for 10 min. Bands were finally completely dehydrated after 1 h at 60°C. Gel pieces were incubated with 13 μL of sequencing grade modified trypsin ("Promega", USA; 12.5 μg/mL in 40 mM ABC with 10% ACN, pH 8.0) overnight at 40°C. After digestion, peptides were washed with 30 μL of 25 mM ABC, shrunk with 100% ACN and extracted twice with a mixture of 50% ACN-5% formic acid. Extracts were dried using a vacuum centrifuge "Concentrator plus" ("Eppendorf").

Mass spectrometry: identification. For MS and MS/MS analysis, peptides were redissolved in 4 μL of alpha-CHCA (2.5 mg/mL in 70% ACN-0.1% trifluoroacetic acid). One microliter and a half of each sample were spotted directly onto a MALDI plate ("Applied Biosystems", USA). Droplets were allowed to dry at room temperature. Samples analysis was per-

formed using a MALDI-TOF-TOF 4800 mass spectrometer ("Applied Biosystems"). Spectra acquisition and processing was performed using the 4000 series explorer software (ABI) version 3.5.28193 in positive reflectron mode at fixed laser fluency with a low mass gate and delayed extraction. External plate calibration was performed using 4 calibration points spotted onto the 4 corners of the plate using a mixture of five external standards (PepMix 1; "LaserBio Labs", France). Peptide masses were acquired by steps of 50 spectra in the range of 900 to 4000 Da. MS spectra were summed from 500 laser shots from an Nd-YAG laser operating at 355 nm and 200 Hz. After filtering tryptic-, keratin- and matrix-contaminant peaks up to 15 parent ions were selected for subsequent MS/MS fragmentation according to mass range, signal intensity, signal to noise ratio, and absence of neighboring masses in the MS spectrum. MS/MS spectra were acquired in 1-kV positive mode and 1 000 shots were summed by steps of 50. Database searches were carried out using Mascot version 2.2 ("MatrixScience", UK) via GPS explorer software (ABI) version 3.6 combining MS and MS/MS inquiries on human proteins from Swiss-Prot Databank containing 20325 sequences (January 2011) (www.expasy.org). The search parameters were as follows: carbamidomethylation as a variable modification for cysteins and oxidation as a variable modification for methionines. Up to 1 missed tryptic cleavage was permitted and mass accuracy tolerance of 30 ppm for precursors and 0.3 Da for fragments were used for all tryptic mass searches. Positive identification was based on a Mascot score above the significance level (i.e. <5%).The reported proteins were always those with the highest number of peptide matches. Under our identification criteria, no result was found to match to multiple members of a protein family.

Network analysis. Pathway analysis was carried out for proteins found to be differently expressed in tumor and control samples. International protein index accession numbers were imported into Ingenuity Pathway Analysis (IPA) version 7.5-2202 ("Ingenuity Systems", Mountain View, CA; http://www.ingenuity.com). The identified proteins were mapped to the most significant networks generated from previous publications and public protein interaction databases. A p value calculated with the right-tailed Fisher's exact test was used to yield a network's score and to rank networks according to their degree of association with our data set.

RESULTS

Analysis of differentially expressed proteins by 2-D DIGE

To identify the differentially expressed proteins which are strongly associated with IDC, we compared the protein profiles between pooled IDC and normal breast tissues. DIGE 1 analysis was used in order to obtain a full protein profile for the breast cancer tissue, while DIGE 2 analysis was used to achieve higher resolution in the pI range between 4 and 7, where most of the breast cancer-related protein changes would be expected according to previous studies [11, 21]. Representative 2-D gel images of DIGE 1 and DIGE 2 protein profiles are shown in figure 1*a*.

In DIGE 1 analysis the master gel contained 1679 spots, 1 245 of which were detected and matched on all three gels. In DIGE 2 analysis the master gel contained 1803 spots, 1399 of which were detected and matched on all three gels. Of all detected spots 26 spots were found to be differentially expressed (ANOVA < 0.05 and ratio > 2) in DIGE 1, and 24 spots were differentially expressed in DIGE 2 (table 2). In DIGE 1 only 4 proteins were overexpressed in tumor samples (tab. 2, spots *7*, *8*, *17* and *21*) and 9 proteins were overexpressed in tumor samples in DIGE 2 (tab. 2, spots *3*, *7*, *11*, *14*, *15*, *16*, *17*, *18* and *24*).

MS identification of differentially expressed proteins

Of the 50 combined differentially expressed proteins in the two assays, 39 were successfully identified by MS (results summarized in table 3). Unidentified spots were those that had low concentrations and/or low molecular mass. Among the identified spots, 9 were found in both DIGE 1 and DIGE 2 (fig. 1*b*). One protein was identified in two different spots in DIGE 2 (spot *9* and *10*). Overall, a total of 29 different proteins were identified by MS. With IPG strip pH 3–10, in the pI range between 3 and 7, we have found 12 differentially expressed proteins compared to 19 proteins found when IPG strip pH 4–7 was used. However, IPG strip pH 3–10 allowed us to detect 7 differentially expressed proteins in the pI range between 7 and 10, one of which was overexpressed in tumor tissues. All differentially expressed proteins identified both in DIGE 1 and DIGE 2 displayed the same expression trends in both assays.

Protein networks

The highest ranked network generated by the Ingenuity software (fig. 2) had an F-score of 26 ($p = 10^{-26}$) and was associated with the following functions: cellular growth and proliferation, cancer, cell cycle. Most of the differentially expressed proteins in IDC are closely connected within the network through two major nodes: beta-estradiol and tumor necrosis factor (TNF). Beta-estradiol is involved in a number of cellular processes that favor increased proliferation [22], stimulate G/S-phase transition [23] and suppress apoptosis [24] in breast cancer and numerous other types of cancer as well. TNF is involved in the regulation of a wide spectrum of biological processes including cell proliferation [25, 26] and apoptosis [27]. This cytokine has been implicated in a variety of diseases, includ-

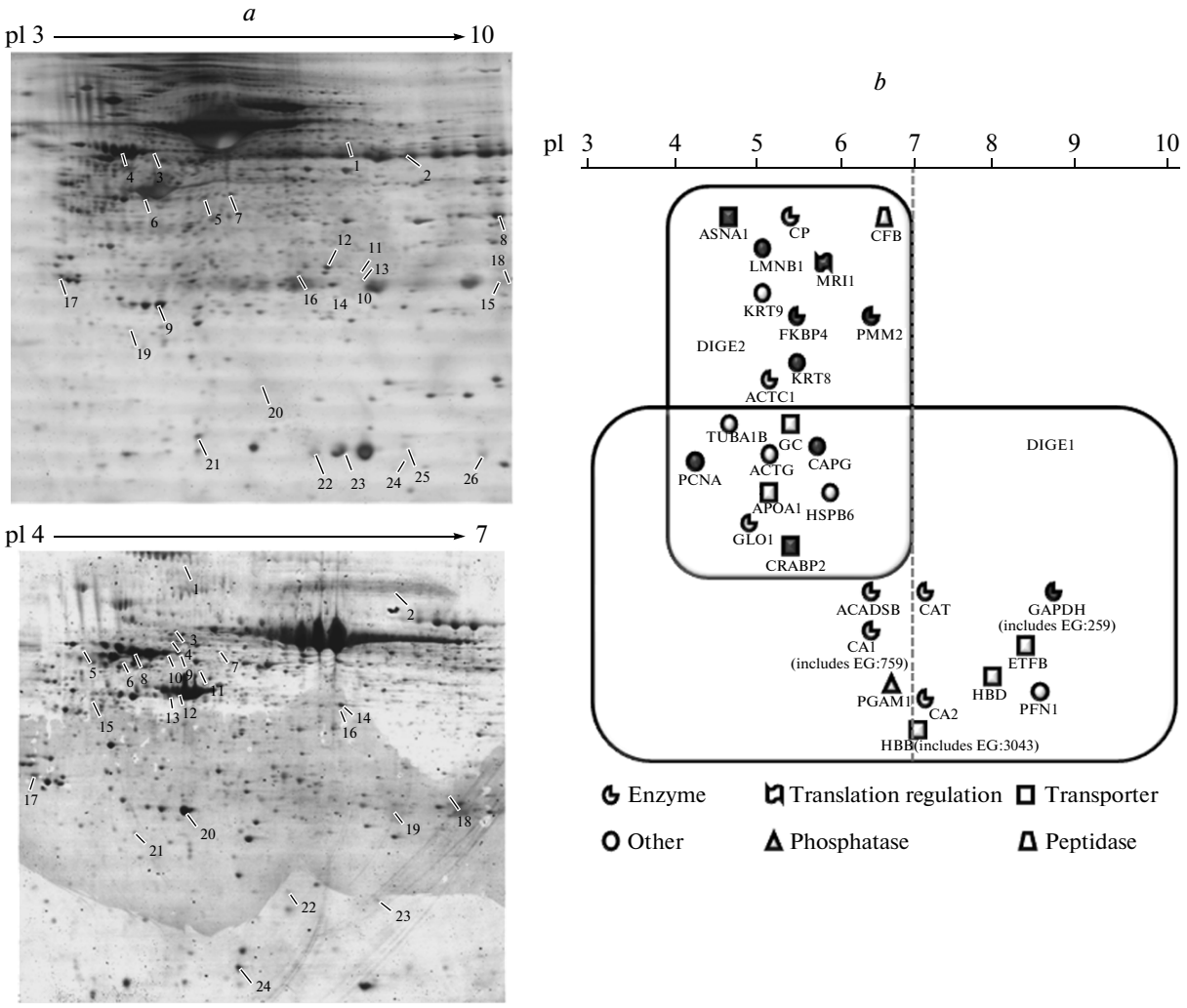

Fig. 1. 2-D map of the proteome obtained from breast tissues. *a* – Typical preparative colloidal Coomassie G-250 stained gels with differentially expressed proteins marked with numbered arrows. *b* – A map of differentially expressed proteins in IDC, based on type of IPG strip used. Different shapes represent the functional classes of proteins, while their color indicates the degree of overexpression (grey) or underexpression (white) of the corresponding protein in tumor samples as compared to normal ones.

ing autoimmune diseases, insulin resistance, and cancer, including breast cancer [28, 29].

DISCUSSION

We applied proteomic techniques to protein expression profiling of IDC tumor and normal tissues. A comparative analysis of the human breast tissue proteome by 2-D DIGE in combination with MS/MS by MALDI-TOF-TOF enabled us to identify 29 proteins that showed significant changes in their expression levels between tumor and normal tissues.

Fourteen proteins from those highlighted in the study were reported to be associated with at least one type of cancer, while six ones (apolipoprotein A-I [11], catalase [30], proliferating cell nuclear antigen [28], glyoxalase I [21], hemoglobin beta [31] and profilin 1 [32]) were associated specifically with breast cancer. The fact that in this study a number of proteins already known to be associated with breast cancer has been identified corroborates the approach used, indicating that it successfully exposes differentially expressed proteins in an *ex vivo* system.

The rest of the differentially expressed proteins with relation to some type of cancer were the following: actin, cytoplasmic 2 and profilin 1 are upregulated and downregulated in liver cancer, respectively [33]; carbonic anhydrase 2 is downregulated in liver cancer [33] and colon cancer [34]; vitamin D-binding protein is downregulated in liver cancer [35]; ceruloplasmin is downregulated in liver cancer [35] and upregulated in ovarian cancer [36]; proliferating cell nuclear antigen is upregulated in colon cancer [34, 37] and prostate cancer [38]; cellular retinoic acid-binding protein 2 is downregulated in colon cancer [39] and upregulated in leiomyosarcoma [40]; complement factor B and ac-

Table 2. The statistical significance ($p < 0.05$) and calculation of ratios of differentially expressed spots with more than two-fold change in the protein level

Experiment	Reference spot[a]	ANOVA <0.05	VolRatio Mean (normal)	SD (normal)	VolRatio Mean (cancer)	SD (cancer)	Ratio (N/C)	Fold change[b)]
DIGE1	1	0.042	1.86	0.67	0.62	0.22	3.00	3.0
	2	0.015	1.71	0.40	0.59	0.22	2.88	2.9
	3	0.032	1.82	0.42	0.65	0.19	2.82	2.8
	4	0.014	1.76	0.62	0.38	0.11	4.63	4.6
	5	0.042	1.59	0.47	0.69	0.22	2.29	2.3
	6	0.018	1.49	0.18	0.45	0.12	3.34	3.3
	7	0.010	0.15	0.08	1.74	0.52	0.09	–11.3
	8	0.021	0.71	0.08	1.58	0.40	0.45	–2.2
	9	0.017	2.07	0.55	0.68	0.25	3.05	3.1
	10	0.012	2.23	0.70	0.31	0.17	7.12	7.1
	11	0.007	2.52	0.72	0.28	0.05	9.14	9.1
	12	0.037	0.88	1.31	0.30	0.12	2.96	3.0
	13	0.020	2.10	0.75	0.36	0.03	5.78	5.8
	14	0.016	1.70	0.57	0.30	0.17	5.63	5.6
	15	0.036	1.91	0.75	0.47	0.23	4.04	4.0
	16	0.001	1.97	0.12	0.42	0.14	4.66	4.7
	17	0.006	0.28	0.04	1.49	0.39	0.19	–5.3
	18	0.025	1.83	0.47	0.75	0.06	2.43	2.4
	19	0.003	2.86	0.82	0.73	0.35	3.91	3.9
	20	0.023	2.37	0.63	0.30	0.16	7.94	7.9
	21	0.035	0.33	0.15	1.37	0.49	0.24	–4.1
	22	0.002	2.03	0.42	0.19	0.11	10.79	10.8
	23	0.008	1.89	0.59	0.18	0.10	10.39	10.4
	24	0.008	1.88	0.51	0.22	0.12	8.71	8.7
	25	0.016	2.11	0.71	0.36	0.21	5.86	5.9
	26	0.003	1.89	0.41	0.23	0.11	8.10	8.1
DIGE2	1	0.033	1.57	0.52	0.55	0.07	2.88	2.9
	2	0.035	1.68	0.46	0.78	0.18	2.16	2.2
	3	0.043	0.55	0.14	1.45	0.50	0.36	–2.6
	4	0.011	1.64	0.37	0.60	0.06	2.75	2.7
	5	0.048	1.75	0.71	0.50	0.06	3.49	3.5
	6	0.007	0.83	0.79	0.28	0.11	2.93	2.9
	7	0.028	0.49	0.20	1.63	0.50	0.30	–3.3
	8	0.025	1.87	0.67	0.46	0.16	4.06	4.1
	9	0.029	1.74	0.52	0.67	0.18	2.61	2.6
	10	0.042	1.68	0.55	0.70	0.15	2.39	2.4
	11	0.028	0.33	0.04	1.47	0.54	0.22	–4.5
	12	0.021	1.61	0.43	0.66	0.13	2.45	2.4
	13	0.026	1.55	0.38	0.65	0.19	2.40	2.4
	14	0.008	0.11	0.05	1.67	0.50	0.07	–14.9
	15	0.047	0.51	0.10	1.43	0.48	0.36	–2.8
	16	0.049	0.49	0.20	1.44	0.55	0.34	–2.9
	17	0.008	0.39	0.06	1.63	0.39	0.24	–4.2
	18	0.010	0.69	0.13	1.60	0.28	0.43	–2.3
	19	0.004	1.65	0.29	0.47	0.16	3.50	3.5
	20	0.032	1.70	0.53	0.61	0.20	2.78	2.8
	21	0.008	2.69	0.62	0.67	0.25	4.00	4.0
	22	0.047	1.71	0.61	0.52	0.29	3.31	3.3
	23	0.014	2.28	0.72	0.28	0.23	8.09	8.1
	24	0.023	0.24	0.08	1.43	0.49	0.17	–6.0

[a]Spot numbers are indicated in fig. 1*a*.

[b] "+" and "–" indicate the factor by which the spot intensity of normal tissue increases or decreases, respectively, relative to the cancer tissue.

Proteins overexpressed in cancer tissues are highlighted in grey.

Table 3. List of differentially expressed proteins in IDC identified by MS

	Reference spot[a)]	Name of the protein in SwissProt or NCBI	Number of the protein in SwissProt	Total ion score	Best ion score	No of peptides identified	pI teor	Mw theor (kDa)	pI Exper	Mw Exper (kDa)	% of sequence coverage
DICE 1	1	Catalase	CATA_HUMAN	334	72	13	6.9	60	7.3	61	29
	2	*no protein identified*	–	–	–	–	–	–	*8.0*	53	–
	3	Vitamin D-binding protein	VTDB_HUMAN	732	75	18	5.4	53	5.3	52	55
	4	Tubulin alpha-1B chain	TBA1B_HUMAN	185	78	15	4.9	50	5.0	52	45
	5	Short/branched cliain specific acyl-CoA dehydrogenase, mitochondrial	ACDSB_HUMAN	99	52	3	6.5	47	5.7	39	6
	6	Actin, cytoplasmic 2	ACTG_HUMAN	329	115	10	5.3	42	5.2	40	38
	7	Macrophage-capping protein	CAPG_HUMAN	358	135	11	5.8	38	6.0	32	42
	8	Glyceraldehyde-3-phosphate dehydrogenase	G3P_HUMAN	318	126	8	8.6	36	8.9	32	38
	9	Apolipoprotein A-1	APOA1_HUMAN	430	86	20	5.6	31	5.3	25	66
	10	Carbonic anhydrase 2	CAH2_HUMAN	180	90	6	6.9	29	7.3	30	28
	11	*no protein identified*	–	–	–	–	–	–	7.4	20	
	12	Carbonic anbydrase 1	CAH1_HUMAN	529	126	8	6.6	29	7.1	30	45
	13	*no protein identified*	–	–	–	–	–	–	7.5	20	–
	14	*no protein identified*	–	–	–	–	–	–	*7.3*	*20*	–
	15	*no protein identified*	–	–	–	–	–	–	*9.0*	*20*	–
	16	Phosphoglycerate mutase 1	PGAM1_HUMAN	290	92	8	6.7	29	6.7	30	46
	17	Proliferating cell nuclear antigen	PCNA_HUMAN	423	96	12	4.6	29	4.6	30	48
	18	Electron transfer flavoprofein subunit beta	*ETFB_HUMAN*	*44*	*44*	*1*	*8.2*	*28*	*9.0*	*30*	*5*
	19	Lactoylglutathionc lyase	LGUL_HUMAN	425	97	8	5.1	21	5.0	18	46
	20	Heat shock protein beta-6	HSPB6_HUMAN	254	68	4	6.0	17	6.2	16	21
	21	Cellular retinoic acid-binding protein 2	RABP2_HUMAN	330	71	6	5.4	16	5.6	15	52
	22	*no protein identified*	–	–	–	–	–	–	7.0	15	–
	23	Hemoglobin subunit beta	HBB_HUMAN	597	136	11	6.8	16	7.2	15	93
	24	Hemoglobin subunit delta	HBD_HUMAN	50	22	4	7.8	16	7.9	14	29
	25	Profilin-1	*PROF1_HUMAN*	*24*	*24*	*4*	*8.4*	*15*	*7.9*	*15*	*37*
	26	*no protein identified*	–	–	–	–	–	–	*8.7*	*14*	–

Table 3. (Contd.)

	Reference spot[a)]	Name of the protein in SwissProt or NCBI	Number of the protein in SwissProt	Total ion score	Best ion score	No of peptides identified	pI teor	Mw theor (kDa)	pI Exper	Mw Exper (kDa)	% of sequence coverage
	1	Ceruloplasmin	CERU_HUMAN	239	50	18	5.4	122	5.4	210	18
	2	Complement factor B	CFAB_HUMAN	73	50	8	6.7	85	6.2	120	13
	3	Lamin-B1	LMNB1_HUMAN	443	72	12	5.1	66	5.3	72	22
	4	Keratin, type I cytoskeletal 9	KIC9_HUMAN	353	164	14	5.1	62	5.2	62	30
	5	*not identified*	–	–	–	–	–	–	4.8	60	–
	6	*not identified*	–	–	–	–	–	–	4.9	55	–
	7	Peptidyl-prolyl cis-trans isomerase FKBP4	FKBP4_HUMAN	575	129	12	5.4	52	5.5	52	36
	8	Tubulin alpha-1B chain	TBA1B_HUMAN	363	86	12	4.9	50	5.0	52	38
	9	Vitamin D)-binding protein	VTDB_HUMAN	765	91	23	5.4	53	5.3	52	61
	10	Vitamin D-bindlng protein	VTDB_HUMAN	491	123	17	5.4	53	5.3	52	51
	11	Keratin, type II cytoskeletol 8	K2C8_HUMAN	151	47	15	5.5	54	5.4	50	29
DIGE 2	12	Actin, alpha cardiac muscle 1	ACTC_HUMAN	632	162	11	5.2	42	5.3	40	40
	13	Actin, cytoplasmic 2	ACTC_HUMAN	458	135	9	5.3	42	5.2	40	30
	14	Macrophage-capping protein	CAPG_HUMAN	440	152	10	5.8	38	6.0	32	38
	I5	ATPase ASNA1	ASNA_HUMAN	211	90	4	4.8	39	4.8	25	13
	16	Methylthioribose-1-phosphate isomerase	*MTNA_HUMAN*	*34*	*34*	*4*	*3.9*	*39*	*6.0*	*40*	*19*
	17	Proliferating cell nuclear antigen	PCNA_HUMAN	327	76	8	4.6	29	4.6	30	42
	18	Phosphomannomutase 2	PMM2_HUMAN	220	60	11	6.4	28	6.5	25	51
	19	*not identified*	–	–	–	–	–	–	6.3	20	–
	20	Apolipoprotein A-I	APOA1_HUMAN	859	126	13	5.3	28	5.4	20	47
	21	Lactoylglutathione lyase	LGUL_HUMAN	349	81	7	5.l	21	5.0	18	43
	22	*not identified*	–	–	–	–	–	–	5.8	16	–
	23	Heat shock protein beta-6	HSPB6_HUMAN	119	49	3	6.0	17	6.2	16	23
	24	Cellular retinoic acid-binding profein 2	RABP2_HUMAN	302	77	6	5.4	16	5.7	12	52

[a]Spot numbers are indicated in fig. 1*a*.

Proteins overexpressed in cancer tissues are highlighted in grey.

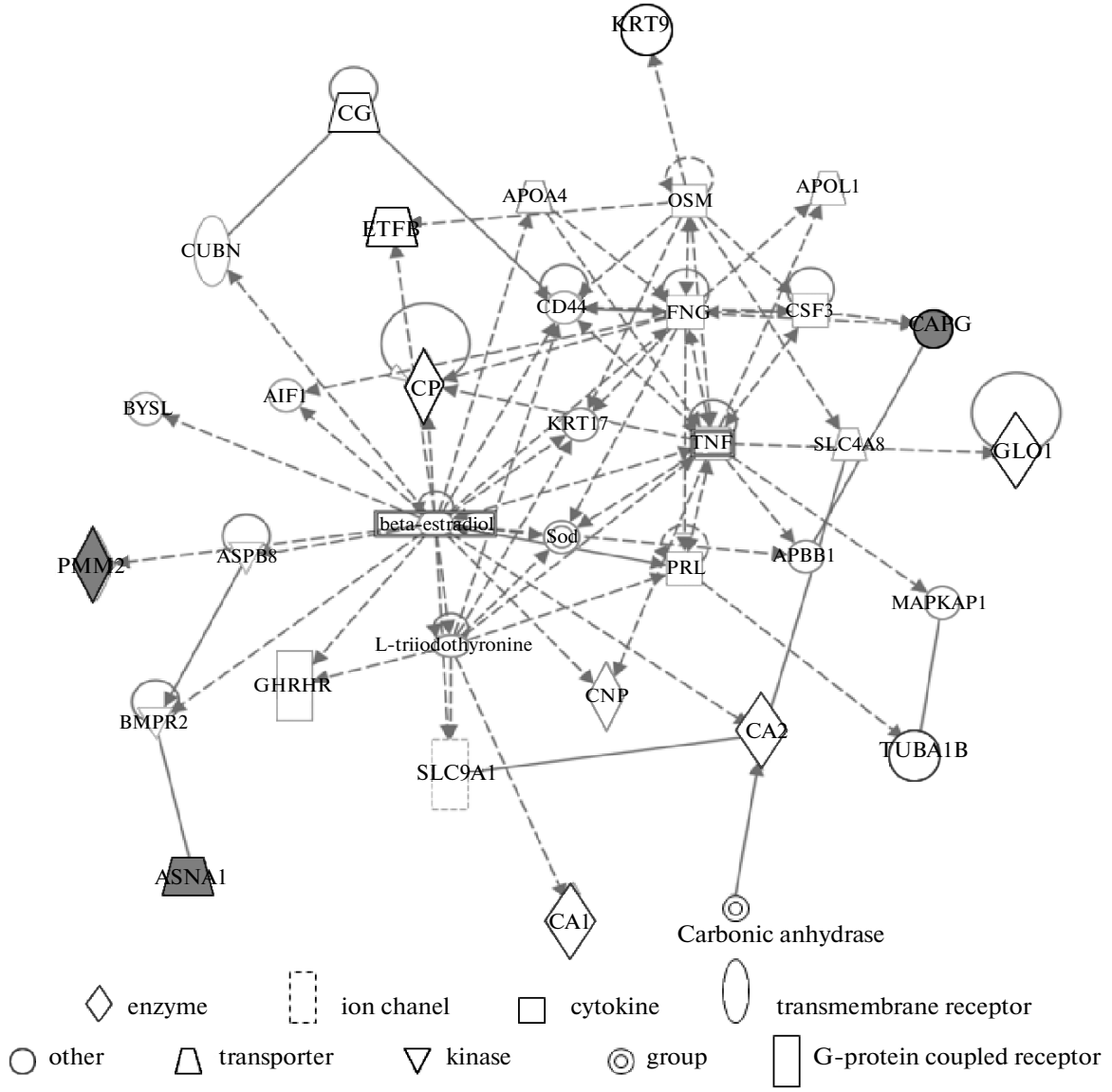

Fig. 2. Highest ranked protein network after IPA of functional associations between the breast cancer proteins identified by MS. The F-score for this network (cellular growth and proliferation, cancer and cell cycle) was 26 ($p = 10^{-26}$). Most of the differentially expressed proteins in IDC are closely connected in the network through two major nodes, beta-estradiol and TNF. The network is graphically displayed with proteins as nodes and the biological relationships between the nodes as lines. Different shapes represent the functional classes of proteins. The length of a line reflects published evidence supporting the node-to-node relationship concerned. The color of the shapes indicates the degree of overexpression (grey) or underexpression (white) of the corresponding protein in tumor samples as compared to normal ones.

tin, alpha cardiac muscle 1 are downregulated in prostatic cancer [41], and hemoglobin beta is upregulated in squamous cell cancer [42].

Ten proteins investigated were overexpressed in tumor tissue and 9 of them were not associated with breast cancer so far. We suppose that at least some of these proteins could be used as candidate biomarkers in breast cancer.

Macrophage-capping protein (CAPG) (14.86-fold increase) is a member of the gelsolin/villin family of actin-regulatory proteins. The overexpression of CAPG was detected in colorectal cancer cell lines [43] and cervical cell lines [44]. Cellular retinoic acid binding protein 2 (CRABP2) (6-fold increase) is a member of the specific carrier proteins for the vitamin A family. Its precise function remains unknown, but the inducibility of the CRABP2 gene suggests that it is important in retinoic acid-mediated regulation of human skin growth and differentiation. CRABP2 associations with cancer has been observed in two studies. However, both upregulation [40] and downregulation [39] of CRABP2 in cancer tissues was reported. Peptidyl-prolyl cis-trans isomerase FKBP4 (4.51-fold increase)

plays a role in immunoregulation and basic cellular processes involving protein folding and trafficking. The overexpression of the FKBP4 coupled with relative differences in its expression in tumors may have important functional implications for estrogen receptor alpha and other steroid receptors in breast cancer [45]. Expression of human FKBP4 mRNA was also observed in human breast cancer cell lines expressing human Her2 [ERBB2] protein [46]. Methylthioribose-1-phosphate isomerase (MRI1) (3-fold increase) belongs to the family of isomerases, specifically those intramolecular oxidoreductases interconverting aldoses and ketoses. This enzyme participates in methionine biosynthetic processes. To the best of our knowledge, there are no published data linking MRI1 and cancer. However, Western-blotting must confirm the presence of MRI1 because it was identified by only one peptide (in MS/MS only 1 peptide was fragmented, but 4 peptides were seen in the simple MS, three of which were not fragmented). ATPase ASNA1 (2.78-fold increase) is an arsenite transmembrane transporter. So far, there is no report in the literature demonstrating the relation of ASNA1 and cancer. Keratins (KRTs) 8 and 9 are members of the cytokeratin family. In breast IDC keratins were found to be expressed both in normal and in tumor tissues but the level of their expression was not determined [47]. In our study, KRTs 8 and 9 are overexpressed and underexpressed in tumor tissues, respectively, by almost 3-times. Further studies are needed to elucidate the possible role of KRTs as markers for breast cancer. Lamin B1 (2.6-fold increase) belongs to the family of proteins, which make up the two-dimensional matrix of proteins located next to the inner nuclear membrane and are highly conserved in evolution. Lamin proteins are thought to be involved in nuclear stability, chromatin structure and gene expression, and are often aberrantly expressed or localized in tumors [48]. Lamin expression is variable between and even within cancer subtypes that limits their use as diagnostic biomarkers. Phosphomannomutase 2 (PMM2) (2.34-fold increase) catalyzes the isomerization of mannose-6-phosphate to mannose-1-phosphate. In MCF7 cells expressing human estrogen receptor alpha, 17-beta-estradiol were reported to be involved in expression of human PMM2 mRNA [49].

In conclusion, a use of a 2-D DIGE combined with MS/MS by MALDI-TOF-TOF allowed us to reveal the increased expression of several proteins, which previously were not associated to breast cancer, such as: CAPG, PMM2, ATPase ASN1, MRI1, FKBP4, CRABP2, lamin B1, and keratin of type II. Since we did not have the opportunity to use Laser Capture Microdissection (LCM) in order to obtain pure cell populations for analysis, the samples used here, in addition to tumor cells, would also contain other cell types and components of the stroma. This includes immune and inflammatory cells, blood vessel cells, fibroblasts as well as the extracellular matrix. Therefore, these other cellular components could be the source of some of the differences in the observed expression levels. However, it has been shown that these cell types may critically influence the multistep process of tumorogenesis [50, 51] and play a significant role in the overall cancer development [52], so all of the differentially expressed proteins found in this study could be considered as a product of the overall IDC condition regardless of their origin.

The proteins that have been identified in this study appear to be involved in multiple and diverse pathways and have established roles in cellular metabolism. The possible role of these diverse pathways and their connection with the signal transduction cascade of breast cancer remains to be solved in the future. Validation steps (Western blotting, immunochemistry, etc.) generally form an integral part of proteomic studies. Unfortunately, we have been unable to validate the data yet, but this work is on the way. We believe that the absence of a validation step for the obtained data could be at least partly compensated by the IPA results. The insertion of the data set in IPA yielded a p-value of 10^{-26} (score 26). Therefore, the fact that the proteins differentially expressed in IDC were clustered with a high rank in the IPA network (associating the functions of cellular growth and proliferation, cancer, cell cycle), indicates that the selected protein data set is nonrandom and connected to the cancer pathways. However, in the future, it is crucial to evaluate the suitability of these candidate biomarkers on samples of larger and independent patient cohorts by using independent techniques.

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