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RESEARCH ARTICLE

Proteomic profiling of 13 paired ductal infiltrating breast carcinomas and non-tumoral adjacent counterparts

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According to recent statistics, breast cancer remains one of the leading causes of death among women in Western countries. Breast cancer is a complex and heterogeneous disease, presently classified into several subtypes according to their cellular origin. Among breast cancer histotypes, infiltrating ductal carcinoma represents the most common and potentially aggressive form. Despite the current progress achieved in early cancer detection and treatment, including the new generation of molecular therapies, there is still need for identification of multiparametric biomarkers capable of discriminating between cancer subtypes and predicting cancer progression for personalized therapies. One established step in this direction is the proteomic strategy, expected to provide enough information on breast cancer profiling. To this aim, in the present study we analyzed 13 breast cancer tissues and their matched non-tumoral tissues by 2-DE. Collectively, we identified 51 protein spots, corresponding to 34 differentially expressed proteins, which may represent promising candidate biomarkers for molecular-based diagnosis of breast cancer and for pattern discovery. The relevance of these proteins as factors contributing to breast carcinogenesis is discussed.

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1 Introduction

The application of proteomic strategies to cancer detection and other clinical purposes is now holding a focal position. The main reason is the complexity of cancer biology, which results in the consistently observed heterogeneous responses to therapies across patient populations. Within female populations of Western countries, breast cancer is the most frequent type of cancer. Breast cancer is not a single disease, but includes several different forms that can be grouped into invasive and non-invasive histotypes. Among the invasive ones, ductal infiltrating carcinoma is the most common and aggressive form [1]. The evolution to a malignant phenotype involves mutation and/or misexpression of a variety of genes controlling cell proliferation, differentiation and death. However, since the transcriptional activity of a gene does not necessarily reflect cellular protein expression, the identification and quantification of proteins are essential steps for the understanding of molecular events leading to malignant transformation. In fact, different protein factors can be over-/underexpressed simultaneously and can activate/deactivate distinct cell functions. Consequently, biomarker searching and tumor profiling of breast cancer are the ultimate goals in the scientific community, necessary for defining phenotypic characteristics of individual cancers and for a better predic-

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Abbreviations: BCT, breast cancer tissues; **NAT**, non-tumoral adjacent tissues

tion of the clinical outcome. The standard clinical and pathological approaches to breast cancer staging are the AJCC (American Joint Committee on Cancer) criteria of tumor size, axillary lymph node status, and presence or absence of distant metastases. Other validated predictive factors include the assessment of the estrogen receptor, progesterone receptor, and epidermal growth factor receptor 2 (ErbB2). Unfortunately, no single marker has sufficient predictive value for breast cancer evolution. Indeed, patients with the same type and stage of disease often display significantly different clinical typologies and responses to therapy. This emphasizes the need to identify multiparametric biological markers for more accurate cancer detection and management. The proteomic approach, based on 2-DE combined with protein sequencing, is one of the most promising techniques for the identification of protein species related to malignancy, and has provided powerful analytical tools for identifying the differentially expressed and/or post-translationally modified proteins as potential biomarkers in tumors (reviewed in [2]).

With this aim, in the present study we analyzed 13 breast cancer tissues and their matched non-tumoral tissues by 2-DE. Collectively, we identified 51 protein spots, corresponding to 34 differentially expressed proteins. These were grouped into eight categories based on the closest affinity for their major biological functions, namely: (i) cytoskeleton and associated proteins; (ii) metabolic enzymes; (iii) molecular chaperones; (iv) proliferation and differentiation regulators; (v) detoxification and redox proteins; (vi) protein degradation; (vii) other proteins; and (viii) serum proteins. The seventh group contains proteins which have no clear classification at present.

Relative expression levels of differentially expressed proteins between tumoral and non-tumoral tissues were considered statistically significant according to the Student's *t*-test.

We suggest that this new approach of generating differential proteomic profiles, based on functional categories, is of interest for two reasons: firstly, for its obvious contribution to the biomarker detection, and secondly because it offers new insights into the molecular biology of breast cancer.

2 Materials and methods

2.1 Clinical specimens

Sample management was performed according to the bioethical recommendations. Aliquots of breast cancer and its adjacent non-tumoral tissues were obtained during surgical intervention and immediately frozen in liquid nitrogen and stored at –80°C until use. The patients did not receive any cytotoxic/endocrine treatment prior to surgery. In each case, non-tumoral tissue was located at least 5 cm away from the primary tumor. Diagnosis of ductal breast cancer (G2/G3) was confirmed histopathologically.

2.2 Sample preparations

The frozen breast tissue samples were washed several times with PBS and homogenized in RIPA buffer (50 mM Tris pH 7.5, 0.1% Nonidet P-40, 0.1% deoxycholate, 150 mM NaCl, 4 mM EDTA) and a mixture of protease inhibitors (0.01% aprotinin, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM PMSF). The extraction was carried out overnight at 4°C with the same buffer. The total cellular lysate was centrifuged at 15 000 rpm for 20 min to clear debris and the supernatant was dialyzed against ultrapure distilled water, lyophilized and stored at –80°C until analysis. Protein concentration in the cellular extracts was determined using the Bradford method [3].

2.3 2-DE

The proteins extracted from breast cancer tissue and normal adjacent tissue were solubilized in a buffer containing 4% CHAPS, 40 mM Tris, 65 mM DTE (1, 4-Dichioerythritol) in 8 M urea. Aliquots of 45 μg (analytical gels) or 1.5 mg (preparative gels) of total proteins were separately mixed with 350 μL of rehydration solution containing 8 M urea, 2% CHAPS, 10 mM DTE and 0.5% carrier ampholytes (Resolyte 3.5–10), and were applied for IEF using commercial sigmoidal IPG strips, 18 cm long with a pH range 3.5–10. The second dimension was carried out on 9–16% linear gradient polyacrylamide gels (SDS-PAGE), and the separated proteins were visualized by ammoniacal silver staining.

2.4 Image acquisition and data analysis

Silver-stained gels were digitized using a computing densitometer and analyzed with ImageMaster 2D Platinum software (Amersham Biosciences, Sweden). Gel calibration was carried out using an internal standard and the support of the ExPASy molecular biology server, as described elsewhere [4]. Quantitative variations in protein expression levels were calculated as the volume of the spots (*i.e.*, integration of OD over the spot area). In order to correct for differences in gel staining, spot volumes relative to the sum of the volume of all spots on each gel (%Vol) were calculated by the software. The differences in expression between breast cancer and normal adjacent tissues were analyzed by the Student's *t*-test; p values <0.05 were considered significant (*), $p<0.01$ highly significant (**) and $p<0.001$ very highly significant (***).

2.5 Protein identification

N-terminal microsequencing was performed by automated Edman degradation in a protein sequencer (Procise, 419 Applied Biosystems), as described elsewhere [5].

3 Results and discussion

Although many studies in the last few years have been performed to detect phenotypic changes occurring in the multistage carcinogenesis of the mammary gland, more information is still needed concerning extensive proteomic profiling of cancer tissues as distinguished from non-tumoral counterparts to be used for patient stratification and monitoring. In the present study we report the comparative proteomic profiles of 13 pairs of surgical samples obtained from patients with ductal infiltrating breast cancer, histologically diagnosed as G2/G3 grade. Figure 1 shows the miniatures of

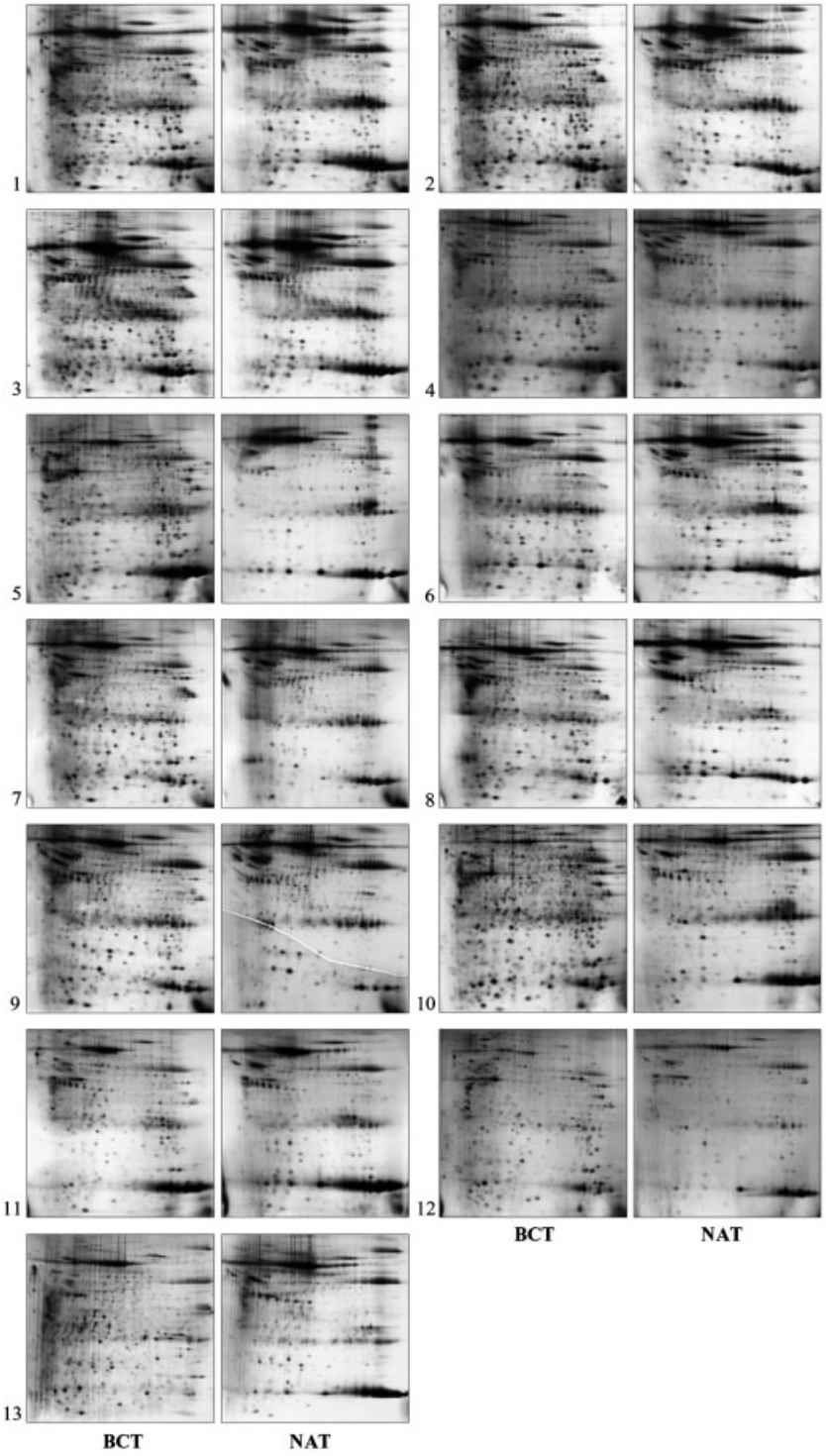

Figure 1. Panel showing the miniatures of the 2-D matching maps from 13 surgical specimens of BCT and its NAT. 2-D separation was performed on IPG gel strips (18 cm, 3.5–10 NL) followed by the SDS-PAGE on a vertical linear-gradient slab gel (9–16 %T).

the proteomic maps of the 13 paired samples. Here it is possible to observe that the cancer-derived proteomics display a far higher level of complexity than non-tumoral tissues: around 1200 protein spots were detected by the ImageMaster software in the non-tumoral tissues, while an average of 1500 were detectable in the cancer tissues.

Figure 2 shows one representative proteomic pair, among the 13 matched pairs of breast cancer and non-tumoral adjacent tissue. The protein identities are marked with labels corresponding to the abbreviated name of the Swiss-Prot database. Fifty-one protein spots, corresponding to 34 distinct proteins, were identified in the maps. The protein identity was assessed by N-terminal sequencing and by gel matching with reference maps previously obtained in our laboratory, where identification was performed by N-terminal microsequencing and by MALDI-TOF [5]. Matching validation was made by Western blotting with anti-actin and anti-enolase (not shown), which occupy a strategic position on proteomic maps and are among the proteins used as internal standard for map calibration, and by N-terminal sequencing of six randomly selected protein spots, among the ones previously identified, (namely, TPIS acidic form, GSTP1, SODM, THIO, and two isoforms of UBIQ).

The identified proteins, listed in Table 1, are grouped into eight functional categories, according to our previously described criteria [6]: (i) cytoskeleton and associated proteins (ii) metabolic enzymes; (iii) molecular chaperones; (iv) proliferation and differentiation regulators; (v) detoxification and redox proteins; (vi) protein degradation; (vii) other proteins; and (viii) serum proteins.

To compare the pattern and intensity of protein expression between the paired samples of breast cancer and non-tumoral tissues we applied the densitometry algorithm of the ImageMaster software, using the %Vol parameter in order to avoid interfering staining differences between the maps [7].

Figures 3–10 show the collection of differentially expressed protein spots between breast cancer tissues (BCT) and non-tumoral adjacent tissues (NAT). The panel displays the cropped images from 2-D gels alongside the densitometric graphs, analyzed using the Student's *t*-test; $*p<0.05$ was considered significant; $**p<0.01$ was considered highly significant, and $***p<0.001$ very highly significant. The data in the graphs are expressed as mean number ± SD.

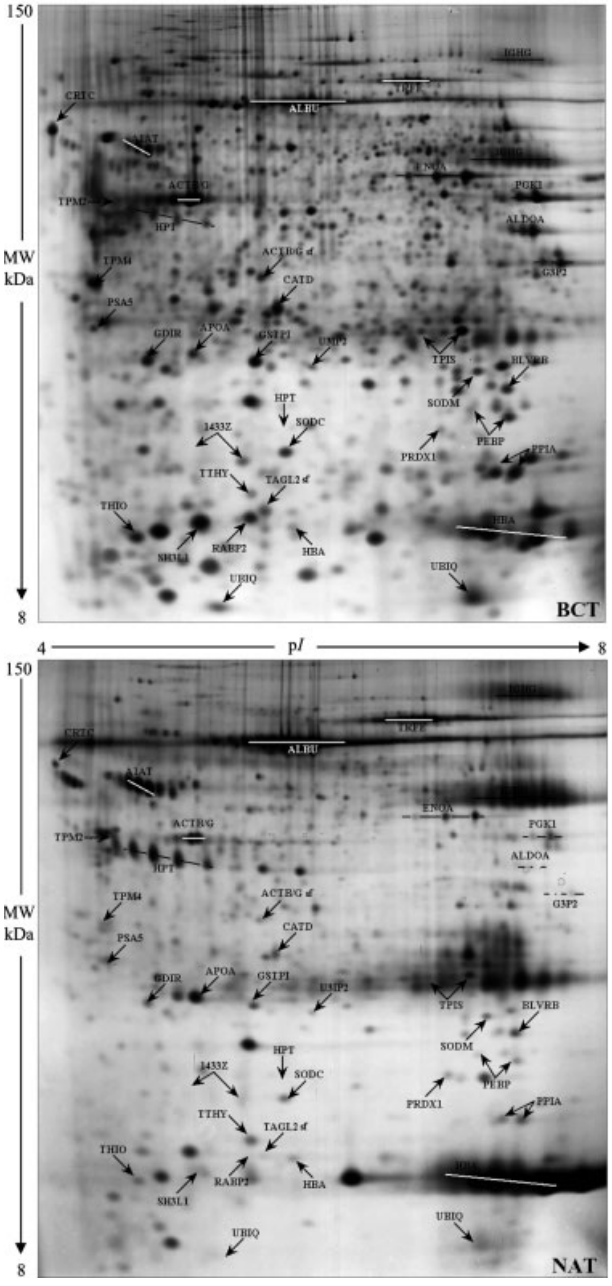

Figure 2. Representative proteomic maps of the matched BCT and NAT derived from a random select patient. Protein spots of known identity are labeled with the abbreviated name of the Swiss-Prot database. When present, different isoforms of the same protein are jointly labeled. sf = short form.

3.1 Cytoskeleton and associated proteins (Fig. 3)

The proteins identified within this category are ACTB/G, TPM2, TPM4 and a short form of ACTB/G and of TAGL2. As already reported [6], beta and gamma actin are not distinguishable in the map, since they are highly homologous, differing by only four amino acids at the amino-terminal region. In both proteins, the mature forms are N-terminal blocked and this precludes their N-terminal sequencing by Edman degradation, which would have offered unequivocal identity of each isoelectric form and the possibility of discriminating between the two gene products. Therefore, we applied both designations, B and G, to the actin forms detected in the present maps. Generally, we detected three isoelectric variants of ACTB/G in the silver-stained gels. Interestingly, two of them appear significantly overexpressed in all the tumor tissues, while one of three shows no significance level, indicating a high variability among the patient's proteomic profiles.

Table 1. Catalogue of the protein spots identified in the proteomic maps of the 13 pairs of breast cancer and healthy tissues, whose prototypes are reported in Fig. 2. The protein names, accession numbers (AC) and abbreviated names correspond to the nomenclature used in the Swiss-Prot database.

Protein name	AC number	Abbreviated name	Exp. p*I*/MW (theoretical)	ID methods[a)]	N-terminal residues
1. Cytoskeleton and associated proteins					
Actin, cytoplasmic 1,2	P60709/P63261	ACTB/G	5.16/42 000 (5.29/41 736)	1, 3	
Actin, cytoplasmic 1,2	P60709/P63261	ACTB/G	5.20/42 000	1, 3	
Actin, cytoplasmic 1,2	P60709/P63261	ACTB/G	5.23/42 000	1, 3	
Actin, cytoplasmic 1,2 sf	P60709/P63261	ACTB/G sf	5.56/36 548	2	res. 62–71
Transgelin-2 sf	P37802	TAGL2 sf	5.61/13 186 (6.63/21 117)	2	res. 12–26
Tropomyosin alpha 4 chain	P67936	TPM4	4.78/33 145 (4.67/28 391)	3	
Tropomyosin beta chain	P07951	TPM2	4.81/41 527 (4.66/32 851)	3	
2. Metabolic enzymes					
Alpha enolase	P06733	ENOA	6.46/46703 (6.99/47 037)	1, 3	
Alpha enolase	P06733	ENOA	6.66/45 935	1, 3	
Alpha enolase	P06733	ENOA	6.82/45 631	1, 3	
Fructose-bisphosphate aldolase A	P04075	ALDOA	7.29/37 688 (8.39/39 289)	3	
Fructose-bisphosphate aldolase A	P04075	ALDOA	7.34/37 627	3	
Glyceraldehyde-3-phosphate dehydrogenase	P04406	G3P2	7.39/35 500 (8.58/35 922)	2	res. 2–7
Glyceraldehyde-3-phosphate dehydrogenase	P04406	G3P2	7.52/35 500	2	res. 2–11
Glyceraldehyde-3-phosphate dehydrogenase	P04406	G3P2	7.59/31 720	2	res. 2–21
Phosphoglycerate kinase 1	P00558	PGK1	7.32/41 323 (8.30/44 483)	3	
Phosphoglycerate kinase 1	P00558	PGK1	7.31/41 326	3	
Triosephosphate isomerase	P60174	TPIS	6.53/25 000 (6.51/26 538)	2	res. 1–5
Triosephosphate isomerase	P60174	TPIS	6.81/25 000	2	res. 1–10
3. Molecular chaperones					
Calreticulin	P27797	CRTC	4.52/59 300 (4.29/48 142)	3	
Peptidyl-prolyl *cis-trans* isomerase A	P62937	PPIA a	7.04/14 500 (7.82/17 881)	3	
Peptidyl-prolyl *cis-trans* isomerase A	P62937	PPIA b	7.19/14 500	3	
4. Proliferation and differentiation regulators					
14-3-3 protein zeta/delta	P63104	1433Z a	5.23/16 955 (4.73/27 745)	3	
14-3-3 protein zeta/delta	P63104	1433Z b	5.50/16 124	3	
SH3 domain-binding glutamic acid-rich-like protein	O75368	SH3L1	5.28/11 931 (5.22/12 643)	2	res. 2–11
Cellular retinoic acid binding protein 2	P29373	RABP2	5.54/12 859 (4.73/27 745)	2	res. 1–10
Rho GDP-dissociation inhibitor 1	P52565	GDIR	5.03/23 907 (5.03/23 207)	3	
5. Detoxification and redox proteins					
Glutathione *S*-transferase P	P09211	GSTP1	5.56/23 256 (5.44/23 225)	2	res. 1–10
Peroxiredoxin 1	Q06830	PRDX1	6.64/17 189 (8.27/22 110)	3	
Superoxide dismutase [Cu-Zn]	P00441	SODC	5.74/16 024 (5.70/15 804)	3	
Superoxide dismutase [Mn]	P04179	SODM	6.89/21 380 (6.89/22 204)	2	res. 25–34
Thioredoxin	P10599	THIO	5.07/17 122 (4.82/11 606)	2	res. 1–10
6. Degradation proteins					
Cathepsin D	P07339	CATD	5.69/30 247 (5.56/26 628)	2	Res. 169–178
Proteasome subunit alpha type 5	P28066	PSA5	4.77/28 148 (4.74/26 411)	2	res. 4–13
Ubiquitin	P62988	UBIQ a	5.44/8 260 (6.55/8565)	2	res. 1–10
Ubiquitin	P62988	UBIQ b	6.86/8 313	2	res. 1–15
7. Other proteins					
Flavin reductase	P30043	BLVRB	7.14/19 993 (7.31/21 988)	2	res.1–9
Phosphatidylethanolamine-binding protein	P30086	PEBP a	6.82/18 386 (7.43/20 965)	2	res. 1–10
Phosphatidylethanolamine-binding protein	P30086	PEBP b	7.15/17 930	2	res. 1–10
U3 small nucleolar RNA-interacting protein 2	O43818	U3IP2	5.90/22 608 (6.74/50 443)	2	res. 15–24
8. Serum proteins					
Serum albumin	P02768	ALBU	multiple forms (5.92/69 367)	3	
Serotransferrin	P02787	TFRE	multiple forms (6.81/77 050)	4	

Table 1. Continued

Protein name	AC number	Abbreviated name	Exp. p*I*/MW (theoretical)	ID methods[a)]	N-terminal residues
Ig/gamma-1- chain C region	P01857	IGHG1	multiple forms (8.46/36 106)	4	
Ig/gamma-1- chain C region	P01857	IGHG1	multiple forms (8.46/36 106)	4	
Haptoglobin	P00738	HPT a	5.74/17 422 (6.13/45 205)	2	res. 19–28
Haptoglobin	P00738	HPT b	multiple forms (6.13/45 205)	4	
Alpha-1-antitrypsin	P01009	A1AT	multiple forms (5.37/46 736)	4	
Hemoglobin alpha subunit	P69905	HBA	5.83/12 007 (8.73/15 126)	2	res. 1–7
Hemoglobin alpha subunit	P69905	HBA	multiple forms (8.73/15 126)	4	
Transthyretin	P02766	TTHY	5.54/14 022 (5.35/13 761)	2	res. 21–27
Apolipoprotein A-I	P02647	APOA1	5.22/24 311 (5.27/28 079)	2	res. 25–32

a) Identification methods: 1, Western blotting; 2, N-terminal sequencing by automated Edman degradation; 3, gel matching with previously identified protein spots by MALDI-TOF and N-terminal sequencing (see [6]); 4, gel matching with human plasma of SWISS-2D PAGE.

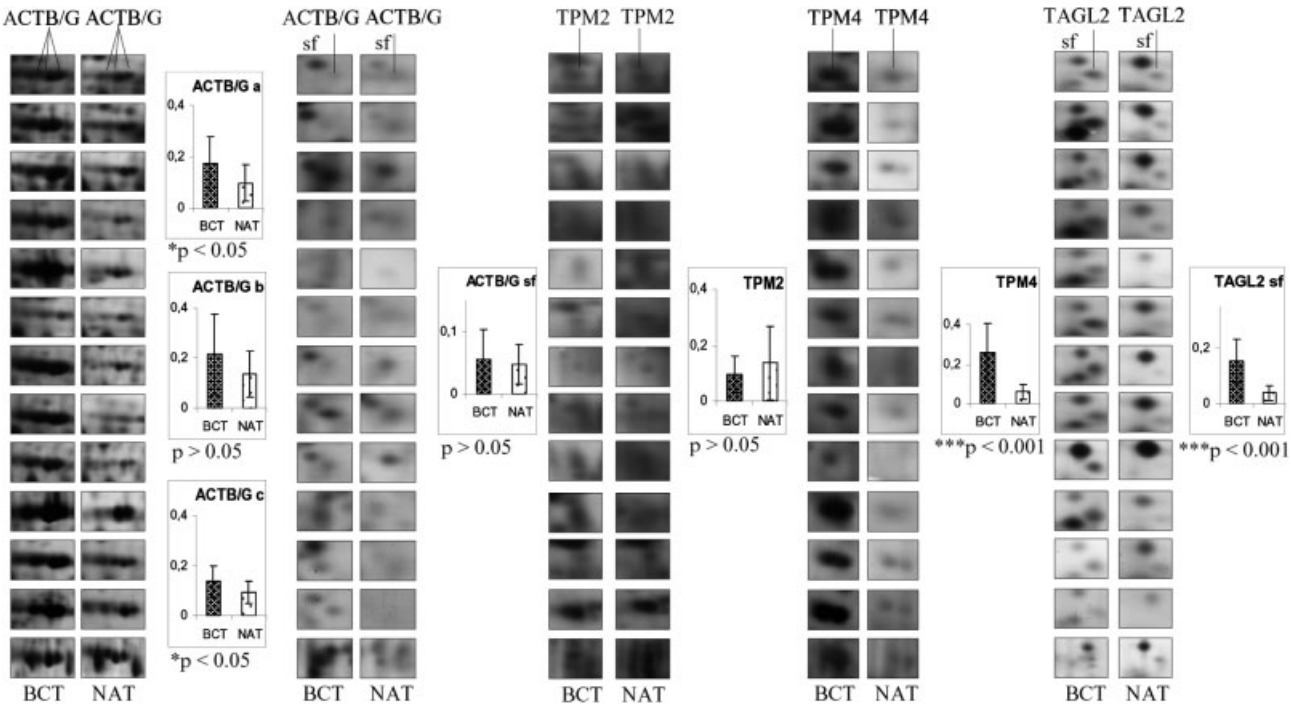

Figure 3. Cytoskeleton and associated proteins. The panel shows differences of spot features between BCT and NAT. Differential expression of spot density was calculated as Vol%. The differences in expression between breast cancer and normal adjacent tissues were analyzed by the Student's *t*-test: *$p<0.05$ was considered significant; **$p<0.01$ highly significant; ***$p<0.001$ very highly significant. The data in the graphs are expressed as mean number ± SD.

Recently, several authors [8] have suggested the existence of a relationship between actin organization and changes in actin isoform expression with the ability of cancer cells to form metastases.

Two other cytoskeletal proteins, TPM4 and TPM2, were identified in all tumor tissues, while small amounts or even traces were observed in the non-tumoral counterpart. TPM4 was differentially expressed at a higher significance level, while the TPM2 intensity level was more variable among patients. TPM is a major structural protein associated with the actin microfilaments. Multiple TPM isoforms have been reported in several cell lines, including breast cancer cells [6], and some of them have been thought to be associated with the metastatic potential of several primary tumors [9].

Another interesting protein significantly overexpressed in the tumor samples is transgelin 2. Recently, the overexpression of transgelin 2 mRNA was reported in a large percentage (69%) of hepatocellular carcinomas [10], which suggests its potential role as a diagnostic marker for cancer detection.

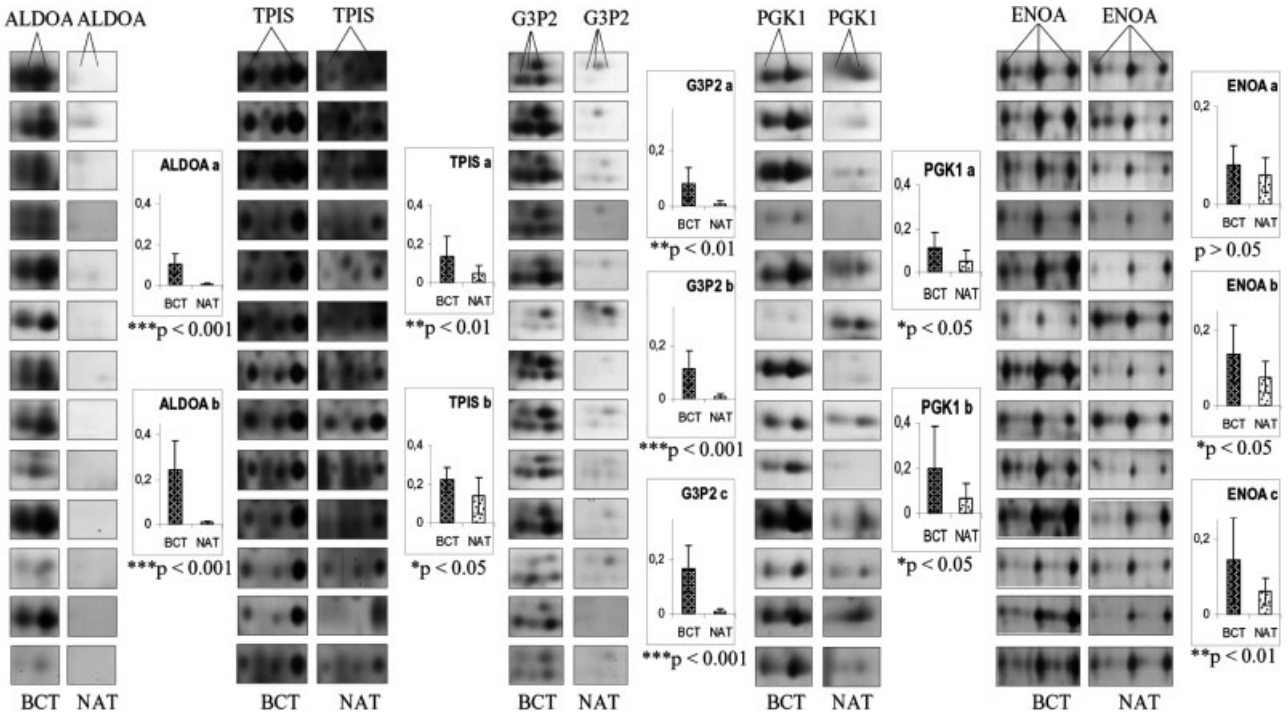

Figure 4. Metabolic enzymes. Spot comparison was performed as described in Fig. 3.

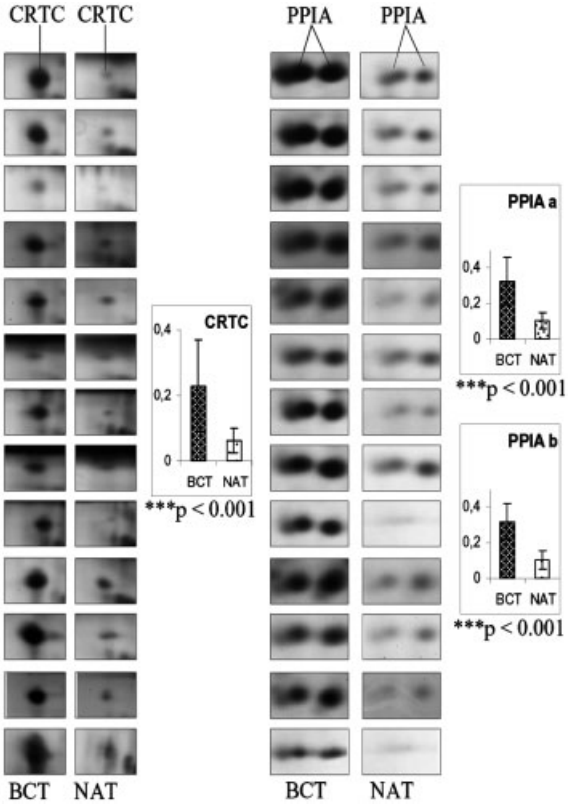

Figure 5. Molecular chaperones. Spot comparison was performed as described in Fig. 3.

3.2 Metabolic enzymes (Fig. 4)

In this category of proteins, we identified five enzymes of the glycolytic pathway: ALDOA, TPIS, G3P2, PGK1, ENOA, which are collectively overexpressed in the tumor tissue *vs.* the non-tumoral counterparts. This observation appears to be of particular interest when considering the anaerobic shift of the metabolism of cancer cells, already described in the pioneering work of Warburg [11] and presently used for clinical cancer detection by FDG-PET ((18)F-fluorodeoxyglucose-Positron Emission Tomography) imaging analyses [12].

Moreover, these data confirm our previous report showing increased levels of glycolytic enzymes in breast cancer cells *vs.* non-tumoral mammary-derived cells [4]. It is also likely that the incremented expression level of some glycolytic enzymes may be related to additional functions performed by the cells. As an example, G3P2, besides its pivotal role in the glycolytic pathway and energy production, fulfils a multiplicity of functions such as membrane fusion, microtubule bundling, phosphotransferase activity, and nucleic acid binding, all aspects deserving attention from the point of view of transformation [13, 14].

3.3 Molecular chaperones (Fig. 5)

Presently, we have identified only two proteins in this category: CRTC and PPIA. Both of them are significantly overexpressed in the tumor tissue *vs.* the non-tumoral counter-

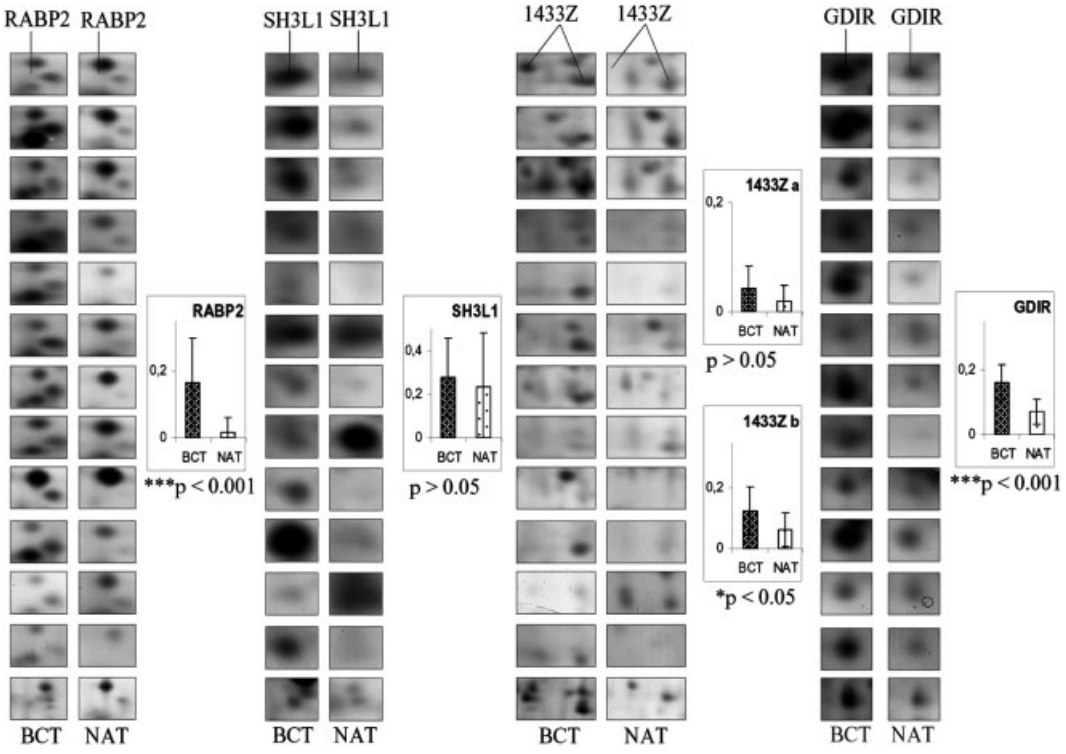

Figure 6. Cell cycle regulation and differentiation. Spot comparison was performed as described in Fig. 3.

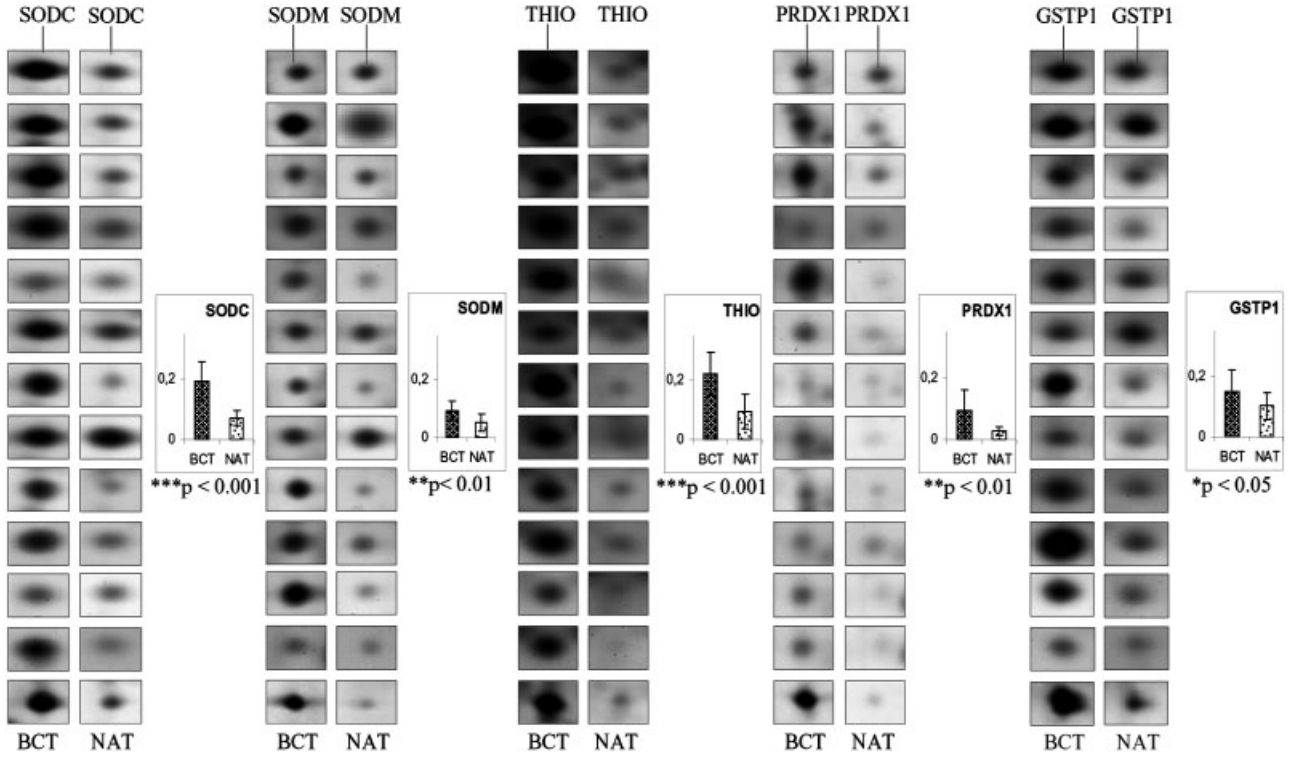

Figure 7. Detoxification and redox proteins. Spot comparison was performed as described in Fig. 3.

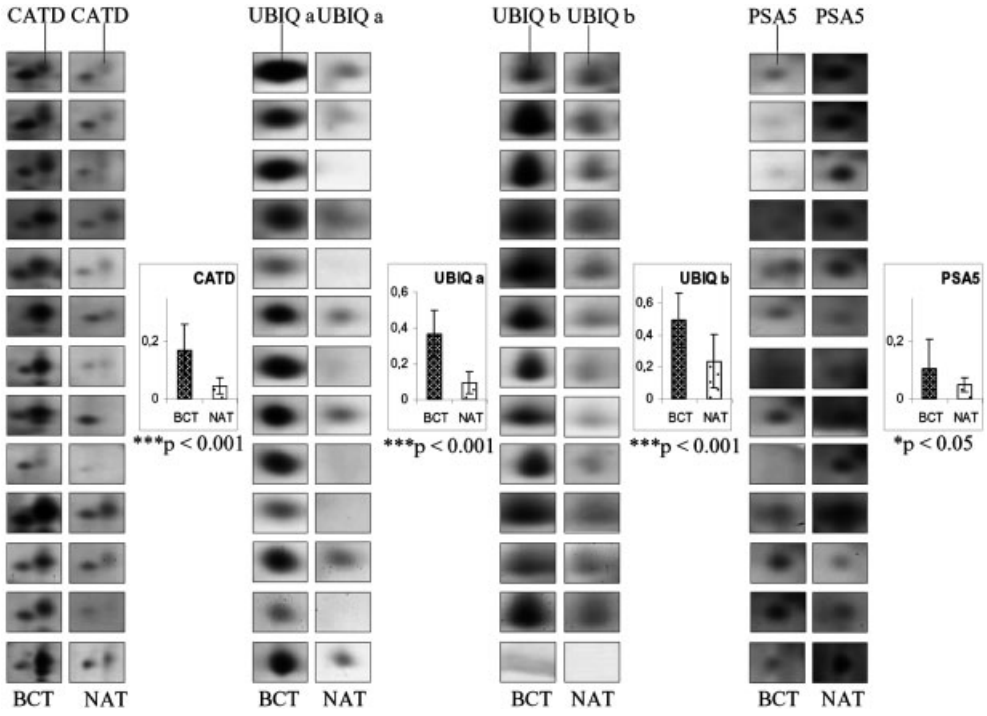

Figure 8. Protein degradation. Spot comparison was performed as described in Fig. 3.

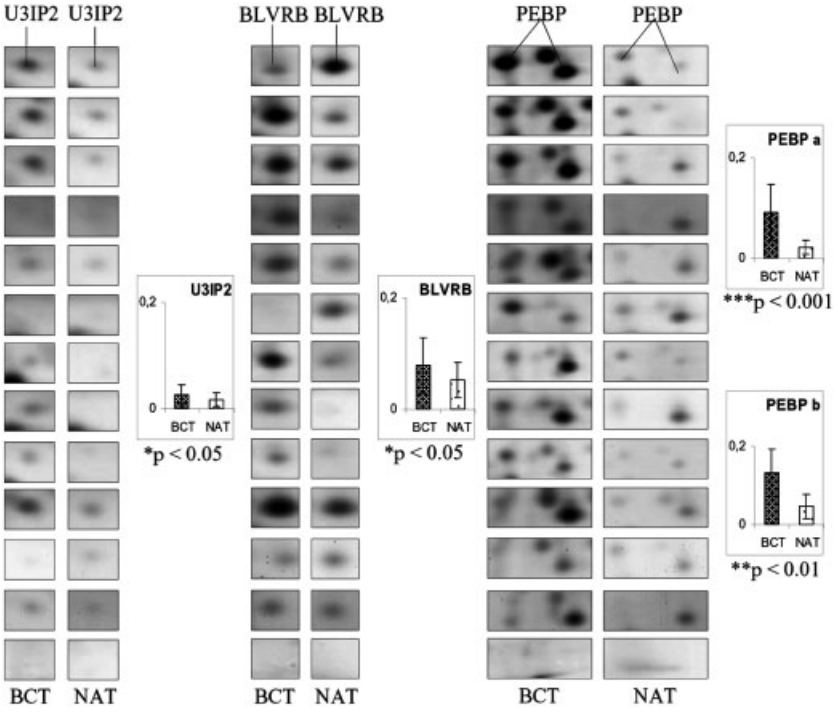

Figure 9. Other proteins. Spot comparison was performed as described in Fig. 3.

part, stressing the hypothesis that they have a function in cancer. CRTC has been shown to be overexpressed in human breast by other authors [15], and also in bladder carcinomas [16]. Recently, it has also been demonstrated that calreticulin has a role in regulating p53 function by affecting its rate of degradation and nuclear localization [17]. Likewise, PPIA has been found to be associated with the growth of colon cancer cells [18].

3.4 Proliferation and differentiation regulators (Fig. 6)

We identified four proteins in this category: RABP2, SH3L1, 1433Z and GDIR. RABP2 is a retinoid-binding protein, thought to regulate the access of retinoic acid to the nuclear retinoic acid receptors, and therefore to participate in a regulatory feedback mechanism to control the action of retinoic acid on cell differentiation. This protein was found among the most variable within the tumor samples: a result suggesting that its overexpression, when present, may be used to discriminate among subtypes of ductal infiltrating carcinomas.

The 1433Z family exhibits diverse biological activities, and may be involved in regulating cell division, differentiation, survival, apoptosis [19–21] and cancer [22, 23]. It is noteworthy that one of the two isoelectric forms is significantly overexpressed in the tumor samples, while the other is more variable. Similarly, the expression level of SH3L1, a gene product belonging to the SH3BGR (SH3 domain-binding glutamic acid-rich-like protein) family and structurally related to thioredoxin (Trx) super family [24], was found to be rather variable.

Conversely, GDIR (Rho GDP-dissociation inhibitor 1) was found significantly overexpressed in the tumor samples *vs*. the non-tumoral counterparts.

3.5 Detoxification and redox proteins (Fig. 7)

Detoxification and redox proteins are related enzymes performing important roles in cell catabolism and protection against metabolic stresses. Indeed, experimental evidence has suggested that oxidative stress mediates various cellular responses, and that, in turn, the control of reduction/oxidation (redox) is fundamental in maintaining the homeostasis of the whole organism. The group includes the following enzymes: SODC, SODM, THIO, PRDX1, GSTP1, which were collectively overexpressed in all of our cancer-derived proteomics. Currently, there is much interest in the thioredoxin and glutathione systems, due to their major role as redox systems in animal cells and putative targets for cancer therapy (see [25] for review). In recent years, the peroxiredoxin system has also received much attention for its high antioxidant efficiency. The mammalian Prdx gene family has six distinct members located both in the cytoplasm and in various subcellular locations, including peroxisomes and mitochondria. Some of the Prdx members also have effects on cell differentiation and apoptosis and have been found to be overexpressed in breast cancer [26].

3.6 Protein degradation (Fig. 8)

The protein-degradation machinery plays an important role in protein homeostasis and cellular health. Within this category, we found three enzymes significantly overexpressed in the cancer tissues, namely, CATD, UBIQ, and PSA5.

Cathepsin D is a lysosomal protease involved in protein catabolism and is supposed to play important roles in antigen processing, degenerative diseases, and cancer progression. In breast cancer it has been associated with an increased risk of relapse and metastasis [27].

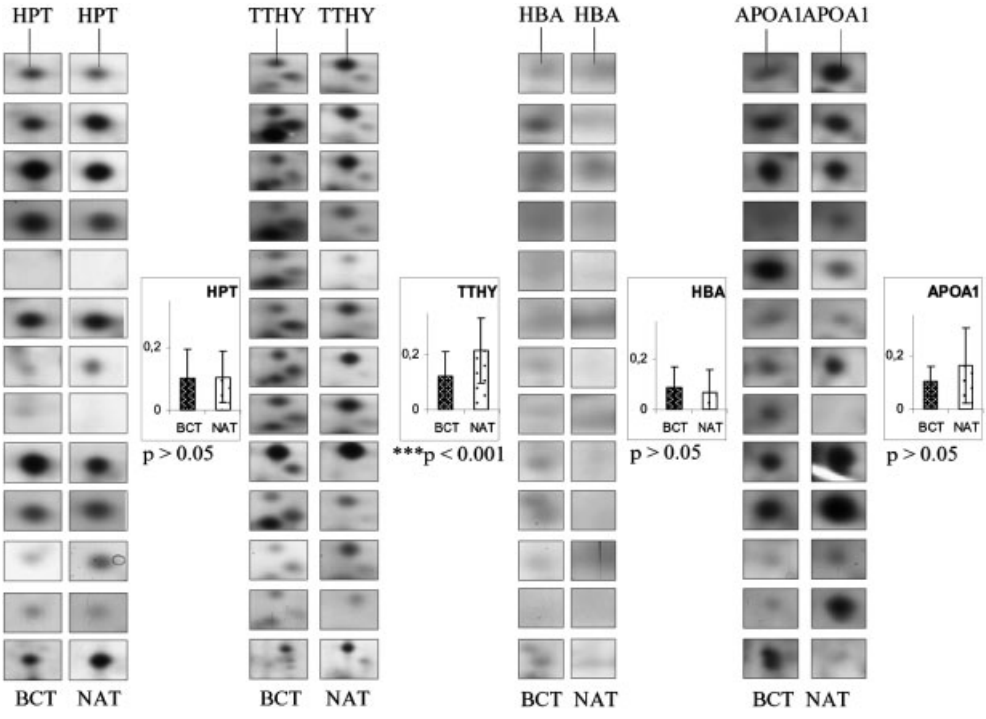

Figure 10. Serum proteins. Spot comparison was performed as described in Fig. 3.

Our present results, showing its consistent overexpression in the tumor samples *vs.* the non-tumoral counterparts, add meaning to the recent statement by Rochefort *et al.* [27] confirming "the clinical value of cathepsin D as a prognostic marker in breast cancer, when using well-standardized assays."

Similarly, our data confirm UBIQ and PSA5 as reliable candidate markers for breast carcinomas [28]. Indeed, the ubiquitin-proteasome pathway system is involved in the degradation of many key regulatory cellular proteins such as tumor suppressors and transcriptional regulators, and of misfolded/denatured proteins, thus regulating different cellular processes including apoptosis, proliferation, differentiation and stress response [29]. Thus, is not surprising that the pathogenesis of many malignancies and other disorders is correlated, to different extents, with aberrations in the system.

3.7 Other proteins (Fig. 9)

We have included in this category three proteins with miscellaneous functions, namely, U3IP2, BLVRB, and PEBP.

The protein U3IP2 is a component of a small nucleolar ribonucleoprotein particle (snoRNPs) thought to participate in the processing and modification of pre-ribosomal RNA and to regulate complex-associated protein shuttle between the nucleus and cytoplasm [30]. The occurrence and overexpression in cancer tissues renders intriguing its role in carcinogenesis, but at present more information is needed to clarify its function.

BLVRB, biliverdin reductase B (flavin reductase-NADPH), is a member of the insulin receptor substrate family with serine/threonine/tyrosine kinase activity, involved in the conversion of biliverdin to bilirubin [31]. Interestingly, BLVRB was also found to translocate to the nucleus in cells treated with cGMP [32] and to function as a transcription factor for activator protein 1-regulated genes and for activation of c-jun and CREB/ATF-2 [33, 34].

Finally, PEBP is a member of the phosphatidylethanolamine-binding protein family, also named RKIP (Raf kinase inhibitor protein). It has been identified in a wide variety of tissues and is thought to regulate several intracellular signaling pathways, while its deregulation may contribute to tissue pathology. Among the reported effects, it has been observed that loss of RKIP expression in prostate cancer cells confers a metastatic phenotype to them. This effect that may be reverted by restoring RKIP expression, suggesting a role of the metastasis suppressor gene for PEBP/RKIP [35].

3.8 Serum proteins (Fig. 10)

The serum proteins are clearly more abundant in the non-tumoral tissues than in the cancer fragments. This is not surprising, due to the lower amount of cells and the absence of neoplastic foci within the host matrix. The majority of them, *i.e.*, ALBU, A1AT, HPT, IGHG1, HBA, and TFRE, migrate in the 2-D gels as multiple isoelectric isoforms (see Table 1). Therefore, adequate evaluation and statistical comparison between samples were allowed only for other identified serum proteins migrating as individual spots (Fig. 10), one of which, the TTHY, showed a highly significant difference in expression in normal *vs.* cancer tissues.

4 Concluding remarks

This study demonstrates quantitative and qualitative differences in the proteomic profiles between breast cancer tissues, namely, ductal infiltrating carcinomas, and the non-tumoral adjacent tissues. The work is intended to offer a new contribution to breast cancer clinical proteomics from a functional point of view.

Appreciable differences of protein expression were detected in all the cancer tissues from the following categories: cytoskeleton and associated proteins, metabolic enzymes, molecular chaperones, detoxification and degradation, and cell cycle regulation proteins. Conversely, almost all serum proteins displayed lower levels in breast cancer tissue than in normal adjacent tissue.

The functional role of each category has been described in the result section.

In conclusion, we suggest that the present collection of differentially expressed proteins, while signifying a novel contribution to the molecular biology of breast cancer, may represent promising candidate biomarkers for molecular-based diagnosis of breast cancer and for pattern discovery.

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