Comparative proteomic analysis of ductal breast carcinoma demonstrates an altered expression of chaperonins and cytoskeletal proteins

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Received January 7, 2013; Accepted March 7, 2013

DOI: 10.3892/mmr.2013.1375

Abstract. The aim of the present study was to analyze the protein composition of ductal breast carcinoma and the surrounding normal tissue in individual patients using comparative 2D proteomics and mass spectrometry to detect candidate disease biomarkers for diagnosis and prognosis. Samples of normal and cancerous tissue obtained form 28 patients were analyzed. Chaperonins and cytoskeletal proteins predominated among the 11 proteins for which major changes in abundance were detected. Of these 11 proteins with an altered expression, 2 had a decreased expression and 9 had an increased expression. In addition, the abundance of a few cytokeratins was also altered; however, they were not capable of serving as specific circulatory biomarkers. The proteins which we observed to exhibit an altered expression in infiltrating ductal breast carcinoma may be exploited as novel targets for therapeutic interventions or represent novel diagnostic/prognostic markers for the early detection of aggressive tumors, particularly those with multridrug-resistant phenotypes during the earlier stages of the disease.

Introduction

With an incidence of approximately 1 million new cases each year, breast cancer (BC) constitutes a major cause of mortality in females diagnosed at the stage of infiltrating disease (1). BC is classically classified as either lobular or ductal in form, with scirrhous, medullary and mucinous variants. Their biological and clinical heterogeneity and variable response to therapy lead to refined classifications based on receptor status (2) as luminal A and B type [estrogen receptor (ER)-positive]; human epidermal growth factor receptor (HER)2 overexpressing epidermal growth factor (EGF) receptors; and basal type [not expressing ER, progesterone (PR) and HER, also known as triple-negative breast cancer (TNBC)] and normal-like cancer. Attempts to devise additional classifications are based on markers for diagnosis and prognosis (cytokeratins and chaperonins), in relation to specific mutations detected by proteomic and cDNA microarray techniques (3). Such studies are currently in progress.

In the present study, we performed a proteomic analysis of normal and BC tissue from individual patients undergoing mastectomy at the Ferrara University Hospital (Ferrara, Italy), during the last 2 years. Changes in the expression of specific proteins in the majority of patients support the investigation of their role as tissue and serological markers for the identification of aggressive tumors and as targets for therapy refractory cases.

Materials and methods

Tissue specimens. Samples of normal and cancerous tissue were collected from 28 patients (represented as P1 to P28) with ductal BC for proteomic analysis. The study was approved by the Institutional Ethics Committee of the Ferrara University Hospital. Diagnosis was confirmed by histopathological analysis, which demonstrated that the tumor specimens contained >50% tumor cells. Samples from 10 patients providing large quantities of tissue were snap-frozen in liquid nitrogen and stored at -80˚C until proteomic analysis was performed.

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Key words: proteomics, breast cancer, cancer biomarkers

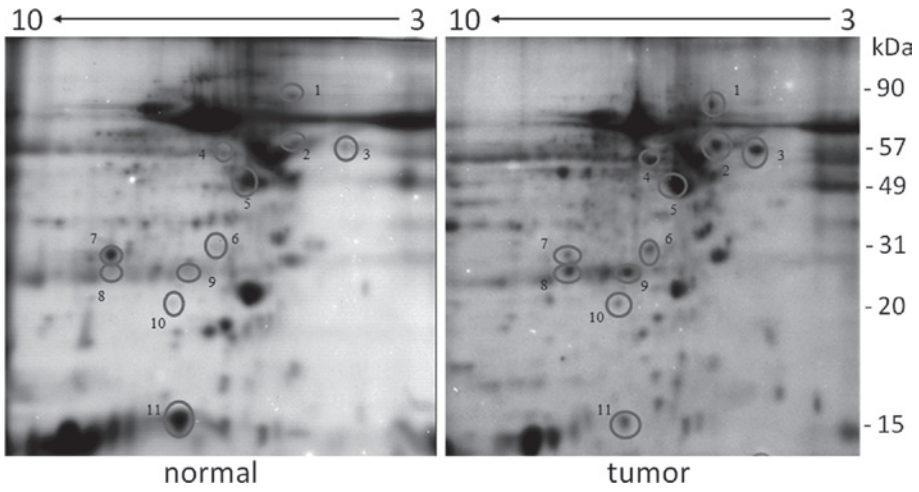

Figure 1. Two dimensional (2D) gel electrophoresis of mammary tissue proteins. Total protein from homogenates of tumor or normal breast tissues from the same patient were loaded onto each gel and separated by 2D gel electrophoresis. The spots circled in the image represent the relevant upregulated or downregulated proteins, which were excised and analyzed by liquid chromatography-mass spectrometry (LC-MS)/MS. The identified proteins are listed in Table I.

Proteomic analysis by 2 dimensional (2D) electrophoresis and mass spectrometry. Proteomic analysis of BC and normal tissue from individual patients was performed by homogenization in 2.5 volumes of 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris, 1 mM benzamidine, 1 mM iodacetamide and 1 mM EDTA, pH 8.8. Following centrifugation, portions of supernatant corresponding to 260 μg protein were separated by isoelectric focusing (IEF) on precast pH 3-10 linear IPG strips at 40,000 Vh, according to the manufacturer's instructions. Following thiol reduction by 1% dithiothreitol (DTT) and alkylation by 4% iodoacetamide, the strips were separated on 2D SDS-PAGE on 12.5% polyacrylamide gels for 1 h at 200 V. Gels were stained with colloidal Coomassie, scanned with the Molecular Imager PharosFX System and analyzed using the ProteomeWeaver 4 program (Bio-Rad, Hercules, CA, USA). Spots excised from the gels were processed by trypsin digestion for mass spectrometry-based peptide identification. The gel fragments were briefly rinsed in buffered acetonitrile and then dried. Following thiol group reduction and alkylation, the peptides were digested overnight with 12.5 ng/μl trypsin, resuspended in aqueous formic acid and analyzed with an Ultimate 3000 Nano/micro HPLC apparatus coupled with a LTQ-Orbitrap XL mass spectrometer (Thermo Scientific, Sunnyvale, CA, USA) (4,5). Using Excalibur 2.0.7 software, spectra were submitted for peptide identification to the MASCOT program against the NCBI database.

Results

The clinical characteristics of each individual patient associated with altered protein expression are summarized in Table I. The proteins with altered expression are also listed, as recognized by 2D gel electrophoresis. We achieved optimal resolution at a total load of 260 μg of protein on the IEF strip, running 2D electrophoresis at a constant voltage of 200 V for 1 h in order to avoid proteins <20 kDa running out of the gel. Typically, 2D electrophoresis of tumor and normal samples of individual patients (Fig. 1) resolved several hundreds of proteins. By visual inspection, 11 proteins had altered expression in BC tissue (circles, Fig. 1); 2 proteins had a decreased expression and 9 had an increased expression. These proteins were identified by mass spectrometry. The 9 proteins with an increased expression (Table I) were endoplasmin precursor (gp96), protein disulfide isomerase (PDI), TCP-1 subunit θ (TCP1-θ), F actin-capping protein subunit β (FACP-β), heat shock protein β 1 (namely, HSP27), triosephosphate isomerase (TPI), RS/DJ-1, β-tubulin (Tub-β) and β-actin (Act-β), while carbonic anhydrase-1 (CA-1) and adipocyte fatty acid binding-protein (A-FABP) were downregulated. These proteins included molecular chaperones, cytoskeletal proteins and metabolic enzymes. We also identified several cytokeratin peptides; however, these were ignored due to possible contamination during sample processing. Cytokeratins are relevant in BC immunocytochemical approaches, however, not when searching for circulating biomarker discovery (6).

The estimation of the abundance of proteins with altered expression was achieved through normalization of the intensity of spots from different gels against a virtual spot calculated as an average on the same gel of 5-6 spots with identical expression in all analyzed samples. Ratios of proteins present were obtained in spots of BC and corresponding normal tissue in 2D electrophoresis gels (Table I). Ratios >2 or <0.5 denote proteins significantly upregulated or downregulated in BC versus normal tissue, respectively. Thus, gp96 and TPI proteins were consistently modulated in at least 7 out of 10 patients, even if the score for TPI should be higher due to its expression not being constantly detectable in normal tissue, thus precluding the calculation of a precise ratio. PDI, FACP-β and Tub-β were highly expressed in 8 out of 10 tumors. HSP27, RS/DJ-1, CA-1 and A-FABP were consistently modulated in 9 out of 10 patients. Act-β and TCP1-θ were elevated in all tumors analyzed.

Discussion

In the present study, the abundance of numerous proteins in normal and cancerous breast tissue was analyzed, with 11 proteins exhibiting a consistently altered expression in

Table I. Population characteristics and identification of significantly altered protein expression levels in BC.

	Clinical setting									
	pT1c-pN0(i)(sn)	pT2-pN0(i-)(sn)	pT1c-pN0(i+)(sn)	pT1c-pN0(i-)(sn)	pT1mic-pN0(i-)(sn)	pT2-pN1a	pT1c-pN0(i+)(sn)	pT1c-pN0(i-)(sn)	pT1c-pN0(i-)(sn)	pT2-pN3a
Case	P10	P13	P18	P19	P20	P21	P22	P23	P27	P28
Cytology										
NCI	n.d.	4.5	3.3	3.32	n.d.	5.56	3.28	3.3	3.24	6.44
Grade	G2	G3	G2	G2	micro	G3	G2	G2	G2	G3
ER	90	0	96	97	0	21	99	72	99	52
PR	50	0	36	94	0	8	98	64	56	68
HER2	1+	1+	0	0	0	0	0	0	0	3+
Identified proteins										
gp96	2.43	2.00	4.86	8.13	5.00	4.67	**1.14**	5.00	**1.27**	**1.18**
PDI	8.25	3.38	6.00	4.33	n.d.	9.33	2	4.76	n.d.	4.55
TCP1-θ	2.22	2.00	2.11	4.33	2.89	7.19	4.80	12.68	3.86	5.38
FACP-β	3.50	**1.44**	2.75	2.00	2.00	2.92	**1.30**	3.33	2.89	2.00
HSP27	2.43	1.29	5.33	6.77	2.57	2.00	2.29	4.67	3.60	2.73
TPI	3.50	n.d.	5.75	2.41	**1.38**	3.50	n.d.	3.33	6.00	3.93
RS/DJ 1	2.25	0.91	4.00	2.53	3.58	2.33	2.80	4.80	6.00	2.42
TUB-β	2.53	**1.40**	6.50	2.00	2.02	3.50	**0.80**	4.98	4.32	2.18
ACT-β	6.25	3.60	8.00	2.28	2.75	3.89	2.00	13.33	3.52	3.64
CA-1	0.50	0.28	**1.11**	0.50	0.50	0.18	0.50	0.19	0.50	0.45
A-FABP	0.23	0.18	0.50	0.11	0.25	0.13	0.36	**0.89**	0.15	0.18

Ratios of the expression of individual proteins in normal and BC tissue are defined in the text. Numbers in bold indicate patients in whom expression deviates from the general trend observed in our cases. NCI, National Cancer Institute; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; PDI, protein disulfide isomerase; TCP1-θ, TCP-1 subunit θ; FACP-β, F actin-capping protein subunit β; HSP27, heat shock protein 27; TPI, triosephosphate isomerase; TUB-β, β-tubulin; ACT-β, β-actin; CA-1, carbonic anhydrase 1; A-FABP, adipocyte fatty acid binding protein; n.d., not defined; BC, breast cancer.

tumors. The proteins which exhibited an altered expression were as follows:

i) Chaperonins, which are notably involved in increased tumorigenicity, metastatic potential and resistance to chemotherapy. Chaperonins include HSP27 induced under unfavorable conditions to protect cells from death, preventing aggregation of denatured proteins, regulating caspase activity, intracellular redox state, polymerization of actin and cytoskeletal dynamics (7). HSP27 is a proposed immunocytochemical discriminator to refine C3 and C4 categories in suspect BC aspirates (8) and gp96 of the HSP90 family (9), which represents a tool for active immunization against tumors by associating with cell surface peptides for presentation to cytolytic T lymphocytes and cell destruction. Notably Vitespen, a peptide vaccine based on gp96, prolongs survival in patients with early-stage melanoma or renal cancer (10). The presence of gp96 in infiltrating ductal BC is attractive for vaccine treatment in TNBC patients resistant to classical therapy while maintaining the expression of gp96 (patient P20).

ii) The cytoskeletal proteins, Act-β, FACP-β, Tub-β and TCP1-θ, of which the latter protein assists in the ATP-dependent folding of actin and tubulin (11). Notably, some cytoskeletal proteins upregulated in BC are involved in ER activation: Act-β binds to the ER-α complex, contributing to ER nuclear functions (12); chaperonin TCP1-θ (spot 3) is involved in the folding of Act-β (13) and estrogen-regulated HSP27 controls the palmitoylation of ER, which is required for its interaction with membranes (14). This indicates that cytoskeletal rearrangement is a key step in the motility mechanisms leading to metastatic spreading; however, it may also be involved in hormone receptivity (15).

iii) Signaling proteins, including RS/DJ-1, TPI and A-FABP. RS/DJ-1 is an oncogene protein regulating RNA protein interaction, present in sera from BC patients but not from healthy patients, along with circulating antibodies against this protein (16). Similarly, autoantibodies against TPI are present in BC patients (17) and also in patients with oral cavity and lung squamous cell carcinoma (18,19). PDI catalyzes the formation and rearrangement of protein disulfide bonds, acting as a reductase at the cell surface cleaving disulfide bonds with structural modifications of cell-associated proteins and as chaperonin (inhibiting aggregation of misfolded proteins) or antichaperonin (facilitating aggregation) inside cells, depending on the concentration. In addition, PDI binds estrogen and thyroid hormones (20). Notably, the knockdown of PDI in MCF7 BC cells induces caspase-dependent apoptosis (21). A-FABP is another protein whose expression is negatively affected. It plays a role in intracellular lipid transport and metabolism as well as signaling. The prognostic value for A-FABP has been reported in bladder cancer since its decreased expression correlates with poor prognosis (22). It is induced by PPAR ligands [the promoter region of the A-FABP gene contains functional peroxisome proliferator-responsive elements (23)] and its overexpression is likely beneficial in the treatment of bladder cancer. Contrasting results have been reported in BC since serum levels of A-FABP have been associated with tumor risk and aggressive behavior (24); however, comparable values of expression have been reported in ductal infiltrating carcinoma and normal tissue (25). Our data instead report the decreased levels of A-FABP in ductal BC compared with normal tissues in 9 out of 10 patients, including the TNBC patient in our population. The association between BC and A-FABP, and if the data are confirmed in larger populations, the possible selective induction by PPAR ligands may impact on the development of future strategies for the treatment of TNBC.

In conclusion, despite the limited number of patients investigated in the present study, the robustness of our results suggests that similar results may be observed in future studies with a larger sample size, including other tumor types and their multidrug-resistant (MDR) counterparts. This is an aspect of particular interest, since one of the drawbacks of conventional anticancer therapy is the development of drug resistance. The proteins which we observed to exhibit an altered expression in infiltrating ductal BC may be exploited as novel targets for therapeutic interventions or represent novel diagnostic/prognostic markers for the early detection of aggressive tumors, particularly those with MDR phenotypes during the earlier stages of the disease.

Acknowledgements

This study was supported by grants from Fondazione Cassa Risparmio di Ferrara to Professor Paolo Carcoforo.

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