Proteomic analysis identifies candidate proteins associated with distant recurrences in breast cancer after adjuvant chemotherapy

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Abstract

Breast cancer is a heterogenous disease and it is of importance to select patients with regard to different prognosis and treatment sensitivity to individualize treatment regimes. In this study we successfully adapted a protein extraction protocol from mRNA extracted tumor samples enabling two-dimensional gel electrophoresis (2-DE) analysis of samples previously analyzed by cDNA microarray. The aim was to find candidate proteins that distinguish breast cancer patients with or without recurrences after adjuvant CMF (cyclophosphamide, methotrexate and 5-FU) treatment within four years to follow-up. We identified several proteins distinguishing the recurrence group from the non-recurrence group, especially in the ER and PgR positive subgroup ($n = 7$). The induced proteins were involved in translation/folding, iron ion binding, and protease inhibition, whereas proteins involved in signaling, ubiquitination, and splicing were decreased in expression. These results show that it is possible to use 2-DE to separate high abundant proteins in breast cancer tissue and to find discriminating proteins to identify patients with different prognosis after adjuvant CMF treatment.

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Keywords: Breast cancer; Two-dimensional gel electrophoresis; Drug resistance; Prognostic markers

1. Introduction

Breast cancer is the most common malignancy among women in the Western world, affecting approximately every tenth woman. After the primary local treatment the patients are typically divided into risk groups based on prognostic factors, such as stage (tumor size, lymph node status, and metastases), histological grade, age, and estrogen (ER) and progesterone receptor (PgR) status. Markers of proliferation, i.e. S-phase fraction (SPF), and invasive factors within the urokinase plasminogen activator system are sometimes also used.

Based on these prognostic factors, patients with a high risk of relapse receive adjuvant systemic therapy, either cytotoxic, endocrine, monoclonal antibodies and/or combination. Examples of adjuvant cytotoxic treatments are CMF (cyclophosphamide, methotrexate and 5-fluorouracil), antracyclin- and taxane-based drug combinations. The overall positive effect of adjuvant cytotoxic therapy is limited with only an increased survival of approximately 10% [1]. The remaining patients are either already cured by the primary local treatment or recur in spite of the treatment given and thus do not benefit from the adjuvant cytotoxic therapy. Possible mechanisms for recurrence despite treatment are low initial drug sensitivity or an acquired drug resistance, which are common clinical problems in cancer treatment.

Useful markers for chemotherapy resistance and/or sensitivity have not so far successfully been found, even though some markers show promising results in a limited number of studies, such as thymidylate synthase, thymidine kinase [2–4], c-erbB-2 [5], multidrug resistance-associated protein [6], and

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p53 [7–10]. The development of gene expression analyses and techniques within proteomics enables extensive characterization of malignant tumors, which may help us to understand treatment resistance and/or treatment sensitivity. Gene expression studies on mRNA level have shown to be able to detect differences between sporadic and hereditary breast cancer [11], and between ER positive and ER negative breast cancer [12].

Promising results for predicting clinical outcome have also been obtained [13–16]. However, several aspects in tumor biology cannot be captured by gene expression analysis only, such as protein expression levels, protein degradation and posttranslational modifications, emphasizing the need for complementary analysis at the protein level. Proteomic studies using two-dimensional electrophoresis (2-DE) analysis of breast cancer have found differences between ductal carcinoma and non-neoplastic tissue [17], the identification of proteins associated with c-erbB-2-expression [18], and evaluation of certain known prognostic factors [19]. In other malignancies (ovarian, prostate, vaginal, and cervical cancer), 2-DE has been used to discriminate between normal/benign and cancer tissue [20–22]. Chemotherapy resistance has also been studied in 2-DE using cell lines from melanoma [23]. To achieve more effective chemotherapeutic treatment of breast cancer patients it is essential to define reliable indicators of response to treatment in individual patients and to establish which mechanisms are responsible for drug resistance. In this study, our aim was to identify proteins that can be used to distinguish tumors from patients later developing distant recurrences after adjuvant CMF from patients without distant recurrence during the follow-up period.

2. Method and patients

2.1. Patients

According to treatment guidelines in the Regional Care program for breast cancer in Southern Sweden issued in 1991, premenopausal lymph node positive (N+) breast cancer patients were recommended radiotherapy and postoperative adjuvant chemotherapy. Patients in the present study were selected in a stepwise manner to fulfill the following criteria: premenopausal women with primary breast carcinoma, stage T1-3N1-2M0, diagnosed 1992–97, for whom frozen primary tumor samples were still available, referred to the Department of Oncology in Lund or Malmö for adjuvant radiotherapy, treatment with nine cycles of CMF, and either distant recurrence within 40 months after completion of CMF or remaining free from distant recurrence for 40 months or longer. This cohort consisted of 85 patients (29 recurrences and 56 recurrence-free patients). Out of these, 20 patients were selected based on recurrence status and ER/PgR status, thus making up four groups with five patients in each: (1) distant recurrence and ER−/PgR−; (2) distant recurrence and ER+/PgR+; (3) no distant recurrence and ER−/PgR−; and (4) no distant recurrence and ER+/PgR+. The study was approved by the ethics committee at Lund University. The data from time to recurrence and the conventional clinical markers (e.g. ER, PgR, SPF, DNA ploidy status, histological grade, histological type, tumor size, number of tumor-involved lymph nodes, age at diagnosis, and location of distant recurrence) are summarized in Table 1.

2.2. Treatment

Patients were treated with an intravenous CMF schedule; cyclophosphamide 600 mg/m^2, methotrexate 40 mg/m^2 and 5-fluorouracil 600 mg/m^2, on day 1, every 3 weeks, for 9 cycles.

Radiotherapy was delivered to ipsilateral axillary and supraclavicular lymph nodes, and the remaining breast parenchyma after breast conservation surgery, or thoracic wall after mastectomy. The absorbed target dose was 50 Gy in 25 fractions in one series for five weeks. During the five-week radiotherapy session, cyclophosphamide was given at a dose of 850 mg/m^2 every three weeks, while methotrexate and 5-fluorouracil were omitted.

2.3. Methods

2.3.1. Conventional clinical markers

ER and PgR were analyzed at the time of the primary operation with enzyme immunoassay according to kit instructions (Abbott Laboratories, Diagnostic Division, Chicago, IL, USA), and expressed as fmol per mg cytosol protein. Receptor values above or equal to 25 fmol/mg protein were considered positive.

Flow cytometric DNA analysis was also performed routinely at the time of the primary operation in an Ortho Cytoron Absolute flow cytometer (Ortho Diagnostic Systems, Raritan, NJ, USA). Ploidy status was defined as follows: one DNA cell population is equal to diploid and two or more cell populations are equal to non-diploid. Samples with an SPF ≥ 12% were classified as high SPF, and those samples with values below these levels as low SPF [24]. Histological grade was re-evaluated for all the samples by the same observer according to Elston and Ellis [25]. The grading procedure consisted of judgment of tubule formation, nuclear plemorphism, and mitotic count. Each of these morphological features was given a score of 1 to 3 points. The overall histological grade was obtained by adding these points, and was categorized as follows: grade 1, 3–5 points, grade 2, 6–7 points, and grade 3, 8–9 points. Histological type was re-evaluated according to WHO [26].

2.3.2. Protein isolation for 2-DE

The tumor tissue was obtained from the tumor bank at the Department of Oncology, consisting of residual tumor samples after routine analyses of ER, PgR, DNA ploidy status, and SPF. From this tissue, the mRNA pool was isolated from the top layer of a Trizol extraction. The layers beneath the mRNA pool (interphase and organic phase) contained the extracted proteins. The DNA was precipitated from the interphase and organic phase with 40% ethanol without precipitating the proteins, and the proteins were then precipitated from the supernate with isopropyl alcohol. The supernatant was removed and the protein pellet was washed in 0.3 M guanidine hydrochloride in 95% ethanol followed by a final wash in 75% ethanol. Extensive washing proved to be necessary to remove interfering substances from the protein pool, such as lipids and large insoluble particles. The protocol for protein extraction was optimized using only

Table 1
Conventional clinical parameters for 20 breast cancer patients, subdivided as follows

Time[a]	ER[a]	PgR[a]	Lymph nodes[a]	SPF[a]	Ploidy[a]	Size[a]	Hist grade[a]	Hist type	Age[a]	Rec.-location
(A) Group 1, distant recurrences, ER/PgR negative										
14	0.9	1.5	1	13	Non-dip	25	3	Ductal-UNS	47	Retina/lungs
−3	1.9	3.3	2	8.6	Non-dip	35	3	Ductal-UNS	48	Lungs/liver
26	20	12	1	30	Non-dip	22	3	Ductal-UNS	43	Lungs/bone
35	0	0	21	7.3	Non-dip	21	3	Tubuloductal	52	Pleura
10	1.4	0	21	23	Non-dip	15	3	Ductal-medullar	37	CNS
14[b]	*1.4*	*1.5*	*2*	*13*		*22*	*3*		*47*	
(B) Group 2, distant recurrences, ER/PgR positive										
19	89	250	6	5.8	Diploid	37	3	Ductal-UNS	45	Bone
30	200	280	12	24	Non-dip	50	2	Lobular	49	Bone
38	42	100	1	6.2	Diploid	11	1	Tubuloductal	37	Liver/bone
16	47	150	2	14	Non-dip	21	3	Ductal-UNS	45	Liver
30	160	26	2	18	Diploid	25	3	Ductal-UNS	46	Bone/pleura/liver
30	*89*	*150*	*2*	*14*		*25*	*3*		*45*	
(C) Group 3, no distant recurrences, ER/PgR negative										
55	6.6	6.5	5	16	Non-dip	35	3	Ductal-UNS	50	
94	0.7	1.3	5	21	Non-dip	18	3	Ductal-medullar	41	
58	0	0	2	21	Non-dip	36	3	Ductal-UNS	48	
69	1.1	2.8	2	28	Non-dip	22	3	Ductal-UNS	48	
58	0	0	2	14	Diploid	25	3	Ductal-medullar	46	
58	*0.7*	*1.3*	*2*	*21*		*25*	*3*		*48*	
(D) Group 4, no distant recurrences, ER/PgR positive										
54	210	340	2	4.6	Non-dip	15	2	Ductal-UNS	49	
85	190	1300	8	8.4	Non-dip	21	3	Ductal-UNS	50	
55	100	330	1	9	Non-dip	12	1	Tubuloductal	50	
59	350	420	2	12	Non-dip	21	3	Ductal-UNS	50	
82	210	370	5	2.8	Diploid	20	2	Ductal-UNS	48	
59	*210*	*370*	*2*	*8.4*		*20*	*2*		*50*	

Time is the number of months to recurrence, evaluated from the day when the CMF treatment was accomplished, or follow-up time for the patients with no recurrences. The median for each parameter is also calculated and shown in italics.

[a] =Time to recurrence (0 = after 6 months' treatment) or follow-up time for patients in the non-recurrence groups (months), ER and PgR (fmol/mg protein), lymph nodes (number of tumor-involved lymph nodes, SPF (%), ploidy (diploid population, non-diploid population), size (tumor size, mm), hist grade (histological grade 1–3), hist type (histological type), age (age at diagnosis, years).

[b] Median value.

15% of the mRNA extracted leftover, resulting in the possibility of running multiple gels from the same sample.

2.3.3. Sample preparations and gel electrophoresis

Immobiline Dry strips (180 mm, pH 4–7, non-linear) were rehydrated in 350 μl of the solubilization solution containing 8 M urea and 2% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate), 10 mM DTT (dithiothreitol), and 0.5% immobilized pH gradient (IPG) 4–7 buffer. The isoelectrophofocusing (IEF) step was performed at 20 °C in an IPGphor^{TM} (Amersham Pharmacia Biotech, Uppsala, Sweden) and run according to the following gradient schedule: (1) 0–300 V for 1 min; (2) 300–3500 V for 1.5 h; (3) 3500 V until approximately 45,000 V h were reached. The strips were equilibrated for 10 min in a solution containing 65 mM DTT, 6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS (sodium dodecyl sulfate), and 50 mM Tris–HCl, pH 8.8. A second equilibration step was also carried out for 10 min in the same solution except for DTT, which was replaced by 259 mM iodoacetamide. The strips were soaked in electrophoresis buffer (24 mM Tris base, 0.2 M glycine and 0.1% SDS) just before the molecular weight separation, and applied on 14% homogeneous Duracryl slabgel and overlaid with a solution of 1% agarose in electrophoresis buffer (kept at 60 °C). Electrophoresis was carried out in a Hoefer^{TM} DALT gel apparatus (Amersham Pharmacia Biotech, San Francisco, CA, USA) at 20 °C and constant 100 V for 18 h.

2.3.4. Gel staining and spot analysis

Gels were silver stained [27] and scanned using a Fluor-S^{TM} MultiImager (Bio-Rad Laboratories, Sundbyberg, Sweden) and Quantity One (version 4.0.3, Bio-Rad Laboratories, Sundbyberg, Sweden). Spot analysis was performed using the PDQUEST (version 6.1.0) two-dimensional gel analysis system (Bio-Rad discovery series, Bio-Rad Laboratories, Sundbyberg, Sweden). After spot detection and matching, every spot on the gel was given an integrated optical density (IOD) value by the software program. This value was compared to the total IOD of all of valid spots and thus every spot is shown as ppm (parts per million) of the total IOD of the valid spots. The average spot intensity of every spot on the gels from the early distant recurrences group was compared to the average spot intensity to corresponding spots on the gels from the no recurrences group. The data sets were analyzed using Ludesi Interpreter^{TM}, http://www.ludesi.com. The significant differentially expressed

spots were further filtered based on spot quality. Several comparisons were made both including all samples in recurrence and no recurrence group or subdivided into ER/PgR positive or negative subgroups. In addition the ER/PgR positive/negative samples were compared when including all samples as well as after further subdivision on to the recurrence/no recurrence group.

2.3.5. Identification of the protein spots

Thirty-nine spots with a p-value less than 0.05 and eleven landmark proteins were sliced out and transferred to small Eppendof tubes and washed three times with a wash-solution (40% acetonitrile, 60 mM ammonium hydrogen carbonate, pH 7.8). The protein spots were dried down in a vacuum concentrator for 15 min and digested with trypsin (Promega Porcine) in 25 mM ammonium bicarbonate and incubated overnight at 37 °C. The digest was stopped by adding 0.2% TFA (trifluoro acetic acid) and Ziptips were used to concentrate and desalt the protein digests according to the manufacture's instructions (Millipore, Bedford, MA, USA). The peptides were thereafter spotted on polished stainless steel target plates together with 7.5 mg/mL α-cyano-4-hydroxycinnamic acid dissolved in 60:40 acetonitrile–water. The MALDI (matrix assisted laser desorption ionization) plates were analyzed in automated mode on the AB4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA) with 1000 laser shots in MS mode and with internal two-point calibration on trypsin peptides with a resulting mass accuracy of <10 ppm. Peaks with a signal-to-noise ratio above 50 passing the exclusion filter of trypsin autolysis peaks and matrix peaks were subjected to MS/MS analysis using up to 3000 laser shots/precursor unless the pre-defined signal-to-noise level in the MS/MS acquisitions was achieved sooner. The MS/MS data were submitted for data base to Mascot (http://www.matrixscience.com/) with a parent mass error tolerance of 50 parts per million and mass fragments with an error tolerance of 0.2 Da.

2.3.6. Statistics of conventional markers

The statistical analysis of the conventional clinical markers was performed in Stata 7.0 (StataCorp. 2001. Stata Statistical Software: Release 7.0. College Station, TX: Stata Corporation). Mann–Whitney U-test and Kruskall–Wallis was used to test the null hypothesis of equal distribution in two subgroups. The level of significance was set to 5%.

3. Results

3.1. Description of patient cohort

Twenty patients were selected all with measured clinical makers summarized in Section 2 (see Table 1). Patients were selected to be as similar as possible with regard to the conventional clinical markers to rule out any influence associated with these markers. However, small but statistically significant differences were found in the ER/PgR positive subgroup after comparison recurring to non-recurring tumors. The expression of ER and PgR was higher in the non-rercurring subgroup, (89 vs. 210,

$p = 0.047$, 150 vs. 370 $p = 0.009$) also the age at diagnosis was statistically significant lower in the recurring group (45 vs. 50, $p = 0.02$). In the ER/PgR negative subgroup, the corresponding comparisons showed no statistical significant difference (all p-values >0.24).

3.2. The development of assay conditions

We then developed an extraction protocol allowing isolation of both mRNA and proteins from the tumor samples. The proteins were separated by 2-DE using both p*I* (isoelectric point) strips 3–10 and 4–7 to determine which p*I* range was the most suitable for analyzing the tumor material. Samples from six patients (three from the recurring group and three from the non-recurring group) were analyzed with both p*I* ranges, and samples from two patients were re-analyzed twice to study the reproducibility of the 2-DE. From these gel sets it was then possible to determine a correlation coefficient, which is a rough estimate of the reproducibility, of both the sample reproducibility within the same patient group, including sample similarity, extraction reproducibility and experimental reproducibility, as well as the reproducibility of experimental protocol from the repetitive analysis of the same sample. A correlation coefficient of 1 equals 100% reproducibility of the expression levels between two sets of sample and we found the intra sample reproducibility to be 0.9 whereas inter sample correlation coefficient was around 0.8 for both p*I* ranges, which is consistent with previously published studies on tumor material [28]. The 2-D gels from samples from the six patients, in both p*I* ranges, matched separately, resulted in approximately 800 matched spots in the 3–10 range (see Supplement 1 for comparison) and 1000 in the 4-7 range (Fig. 1). Since the p*I* range 4–7 contains a higher number of matched spots with better resolution, this p*I* range was used throughout the remaining analysis of the extended study of 20 breast cancer patients.

After spot matching and statistical analysis, spots of interest were analyzed by tandem mass spectrometry and the identified proteins are shown in Fig. 1 and corresponding protein identifications and expression levels in Tables 2–4 (see Supplement 2 for zoom-segments of gel spots of interest). In addition landmarks were identified in order to have reference points concerning the p*I* and molecular weight.

3.3. Recurrences versus no recurrences

Thioredoxin domain containing protein 5 (similar to glucose regulated protein) was significantly increased (p < 0.05) in the recurrence group (n = 10) compared to the group without recurrences (n = 10; Table 2A).

Comparison after subdivision of the recurring (n = 5)/no recurring (n = 5) group with regard to ER/PgR status, resulted in the identification of seven differentially expressed proteins (p < 0.05) from the ER/PgR positive subgroup (Table 2B). Proteins with increased expression in the recurrence group were involved in translation/folding, iron ion binding, and protease inhibitor, whereas those with a lower expression were involved in signaling, ubiquitination, and splicing. Additional

Table 2
Proteins with different expression in breast cancer samples from patients with recurrences vs. no recurrences

Spot#	Acc#	Protein name	Mwt[a]	Score[b]	#pep	Functional group	Ratio
(A) All tumors							
1	Q9BVH9	Thioredoxin domain containing protein 5 (Similar to glucose-regulated protein)	36725	90	2	Unknown	2.4
(B) ER/PgR positive tumors							
2	P09525	ANNEXIN IV	35729	245	5	Signaling	0.6
3	Q14240	EUKARYOTIC INITIATION FACTOR 4A-II	46593	177	6	Translation/protein folding	2.4
4	P15374	UBIQUITIN CARBOXYL-TERMINAL HYDROLASE ISOZYME L3	26337	50	1	Ubiquitination	0.5
5	Q07955	PRE-MRNA SPLICING FACTOR SF2, P33 SUBUNIT	27711	83	2	Splicing	0.2
6	P47813	EUKARYOTIC TRANSLATION INITIATION FACTOR 1A	16433	58	1	Translation/protein folding	5.7
7	P02792	FERRITIN LIGHT CHAIN	19933	89	1	Iron ion binding	6.8
8	P01009	ALPHA-1-ANTITRYPSIN PRECURSOR	46878	72	1	Protease inhibitor	1.1
(C) ER/PgR negative tumors							
9	P08670	VIMENTIN	53579	68	1	Cytoskeletal	2.0
10	P20774	OSTEOINDUCTIVE FACTOR PRECURSOR	34243	44	2	ECM	1.1

The spot number is correlated to the numbers found marked on the gel in Fig. 1. Accession numbers from Swissprot (http://us.expasy.org/sprot/). A score >50 was considered a significant hit by the search engine. Number of peptides is matched peptides to the corresponding protein. The column "functional group" represents the function of the proteins. The ratio is calculated from the recurrence/no recurrence.

[a] Mwt (molecular weight) (Da).
[b] Mascot (www.matrixscience.com).

two proteins were identified in the ER negative subgroup with higher expression in the recurrence group. These proteins are involved in cytoskeletal processes and extracellular matrix (Table 2C).
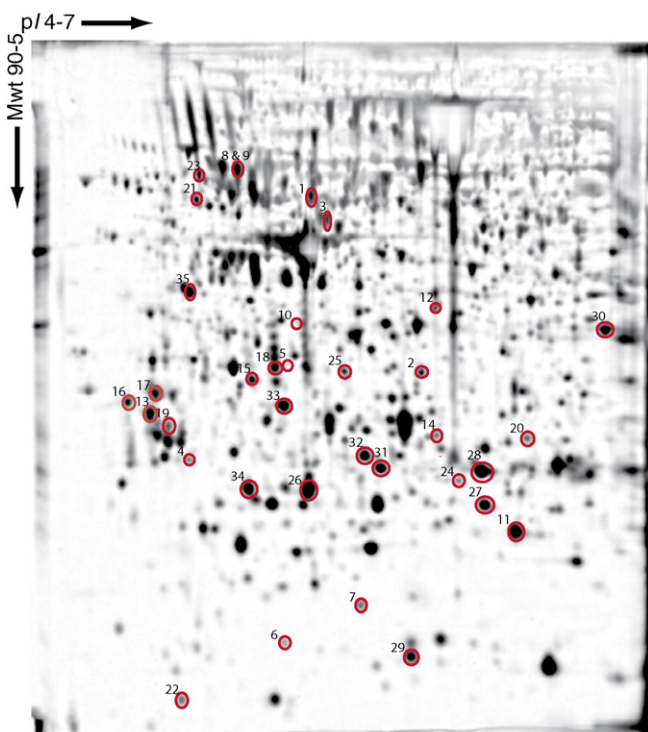

Fig. 1. Proteins were separated by 2-DE and differentially expressed proteins and landmarks are marked with a spot number, corresponding to the spot number in Tables 2–4. Spots number 8 and 9 (alpha-1-antitrypsin precursor and vimentin) were identified as a co-migration in the same spot. The actual gel used in this picture is from a patient with an early distant recurrence and an ER/PgR positive tumor.

3.4. ER/PgR negative versus ER/PgR positive tumors

An apoptosis-associated speck-like protein showed a lower expression in the ER negative group ($n = 10$) than in the ER positive group ($n = 10$; Table 3A).

After subdividing with regard to recurrence status, and in the same way comparing ER/PgR negative and ER/PgR positive tumors, we found six proteins (see Table 3B) with significantly different expression in the recurrence group and eight proteins (see Table 3C) in the non-recurrence group. Proteins with a higher expression in the ER/PgR negative subgroup were found to be involved in translation/protein folding, signaling, and N-acetylglucosamine metabolism. Proteins with a lower expression were found to be involved in cytoskeleton, DNA repair, ECM (extracellular matrix), signaling, translation/folding, protease inhibitor, and cytochrome C oxidase.

4. Discussion

Our aim was to identify candidate proteins to predict the clinical outcome after adjuvant CMF treatment. It has previously been shown that tumors with different receptor status have large differences in gene expression patterns [12,14]. In order to obtain more homogeneous groups we therefore divided the total series of 20 patients into 4 subgroups with different combinations of distant recurrence (yes or no) and ER/PgR status (negative or positive). We used an extraction method allowing purification of both mRNA and proteins for the analysis of cDNA microarray and 2-DE. Two different p*I* ranges were investigated in order to establish expression maps with the highest number of uniquely resolved spots. Even though the 3–10 p*I* range is broader, the 4–7 range resulted in a higher number of matched spots, most likely since only few proteins are present in the extreme edges of the 3–10 p*I*, and that an increased separation of the more

Table 3
Proteins with different expression when comparing ER/PgR positive breast cancer samples to ER/PgR negative

Spot#	Acc#	Protein name	Mwt[a]	Score[b]	#pep	Functional group	Ratio
(A) All tumors							
11	Q9ULZ3	APOPTOSIS-ASSOCIATED SPECK-LIKE PROTEIN	21670	76	3	Apoptosis	0.8
(B) Tumors from patients with distant recurrences							
12	Q9BVP0	N-ACETYLGLUCOSAMINE KINASE	37694	152	4	*N*-Acetylglucosamine metabolism	2.0
13	P42655	14-3-3 PROTEIN EPSILON	29155	113	5	Signaling	0.7
14	P30040	ENDOPLASMIC RETICULUM PROTEIN ERP29 PRECURSOR	29032	122	3	Translation/protein folding	2.2
15	Q99426	CYTOSKELETON-ASSOCIATED PROTEIN CKAPI	27594	84	1	Cytoskeletal	0.7
16	P24534	ELONGATION FACTOR 1-BETA	24788	84	2	Translation/protein folding	0.6
17	P07226	TROPOMYOSIN, FIBROBLAST NON-MUSCLE TYPE	28619	376	14	Cytoskeletal	0.5
(C) Tumors from patients with no recurrences							
18	P20774	OSTEOINDUCTIVE FACTOR PRECURSOR	34243	154	4	ECM	0.2
19	P29312	14-3-3 PROTEIN ZETA/DELTA	27899	215	4	Signaling	3.3
20	P30040	ENDOPLASMIC RETICULUM PROTEIN ERP29 PRECURSOR	29054	56	1	Translation/protein folding	1.8
21	P01009	ALPHA-1-ANTITRYPSIN PRECURSOR	46878	56	2	Protease inhibitor	0.3
22	P00167	CYTOCHROME B5	15189	234	3	Cytochrome *c* oxidase activity	0.4
23	P54727	UV EXCISION REPAIR PROTEIN PROTEIN RAD23 HOMOLOG B	43202	75	3	DNA repair	0.6
24	P29354	GROWTH FACTOR RECEPTOR-BOUND PROTEIN 2	25304	61	2	Signaling	1.9
25	Q9H3J8	My027 protein	33554	112	2	Unknown	0.5

The spot number is correlated to the numbers found marked on the gel in Fig. 1. Accession numbers from Swissprot (http://us.expasy.org/sprot/). A score >50 was considered a significant hit by the search engine. Number of peptides is matched peptides to the corresponding protein. The column "functional group" represents the function of the proteins. The ratio is calculated from the ER/PgR negative/ER/PgR positive.

[a] Mwt (molecular weight) (Da).
[b] Mascot (www.matrixscience.com).

crowded area 4–7 interval resulted in a increase of the number of matched spots.

In general, the number of proteins able to distinguish the different sample groups was rather small. However, this is not surprising since only the most highly expressed proteins are detected in the 2D gels and previous analysis of tumor samples have found similar numbers of discriminating proteins [20–22]. One protein, thioredoxin domain containing protein 5 (similar to glucose-regulated protein), was found to be increased in the tumors with distant recurrences, when comparing tumors from patients with recurrences versus. no recurrences. This protein is present in the endoplasmic reticulum lumen, although its function is not known in detail. When the tumors were furthermore subdivided in the ER/PgR positive subgroup, seven proteins showed significant differences in expression levels between

the recurrence and non-recurrence group. These proteins were involved in translation/protein folding, splicing, ubiquitination, and iron ion binding (ferritin), which are of importance in tumorgenesis. For example, recent findings have indicated that the ubiquitin conjugation leads to selective degradation of nuclear oncoproteins and suppressor gene products [29,30], and that ferritin is important for proliferation in many different neoplasms [31].

In the ER/PgR negative subgroup fewer differentially expressed proteins were found. This lower number of distinguishing in the ER/PgR negative is consistent with the findings in the comparison of conventional factors between the recurrence and the non-recurrence group. In the ER/PgR positive subgroup, ER, PgR, and age at diagnosis differed, whereas none of the factors differed in the ER/PgR negative subgroup. In another study

Table 4
Landmarks were identified as reference points for the p*I* and molecular weight

Spot#	Acc#	Protein name	Mwt[a]	Score[b]	#pep
26	P02647	APOLIPOPROTEIN A-I PRECURSOR	30759	288	7
27	P30048	MITOCHONDRIALTHIOREDOXIN-DEPENDENT PEROXIDE REDUCTASE PRECURSOR	28017	185	3
28	P04792	HEAT SHOCK 27 KD PROTEIN	22427	290	5
29	P00441	SUPEROXIDE DISMUTASE	16023	174	3
30	P04083	ANNEXIN I	38787	416	8
31	P04792	HEAT SHOCK 27 KD PROTEIN	22427	201	3
32	P02743	SERUM AMYLOID P-COMPONENT PRECURSOR	25485	99	2
33	O00299	CHLORIDE INTRACELLULAR CHANNEL PROTEIN 1	27249	125	1
34	P52565	RHO GDP-DISSOCIATION INHIBITOR 1	23250	109	2
35	P08865	40S RIBOSOMAL PROTEIN SA	32947	232	6

The spot number is correlated to the numbers found marked on the gel in Fig. 1. Accession numbers from Swissprot (http://us.expasy.org/sprot/) A score >50 was considered a significant hit by the search engine. Number of peptides is matched peptides to the corresponding protein.

[a] Mwt (molecular weight) (Da).
[b] Mascot (www.matrixscience.com).

from our group, we also found it easier to predict clinical outcome for the ER positive than for the ER negative cohort, based on gene expression data or conventional factors [32]. A possible explanation for this could be that the ER positive tumors are a more homogenous group than ER negative tumors. We have previously analyzed the same samples with cDNA microarray (Nimeus et al. in press in European Journal of Cancer, 2006). A list of genes distinguishing patients with distant recurrences from patients with no recurrences was created and the 4484 genes included were ranked according to their prognostic importance. When comparing the most important genes to the proteins with different expression, similarities to this study were found. As mentioned above, thioredoxin domain containing protein 5 (similar to glucose-regulated protein) was increased in the group of patients with distant recurrences and the corresponding gene was ranked the 59th most important gene and was also induced in the group with distant recurrences. Two proteins involved in the initiation of translation, eukaryotic translation initiation factor 4A-II and 1A, were found to be increased in tumors with distant recurrences. Genes with similar functions were also upregulated in the tumors with distant recurrences in the gene expression data set, exemplified by three different eukaryotic translation initiation factors (factor 5, 2 and 4A-I) ranked 125th, 288th and 367th, respectively and eukaryotic translation elongation factor 1 ranked 76th. Eukaryotic translation initiation factor 1A was downregulated in the tumors with distant recurrences and ranked 1596th most important gene. As has previously been shown [33], there is no absolute correlation between mRNA and protein expression, which may explain why not all proteins were detected on the gene list. In addition the 2-DE approach is hampered by the fact that only a limited number of proteins can readily be detected and identified. However, even though the cDNA microarray generates a more complete list of distinguishing genes, the 2-D gel approach allows quantification at the protein level as well as detection of posttranslational modifications, corroborating that these two techniques may be complementary.

Comparing the ER/PgR positive tumors and ER/PgR negative, one protein, apoptosis-associated speck-like protein, was found in lower amounts in the ER/PgR positive subgroup. This protein promotes caspase-mediated apoptosis and has previously been shown to be a target of methylation-induced gene silencing in human breast cancers [34]. In the subgroup with distant recurrences several proteins involved in translation/protein folding and proteins associated with cytoskeletal functions were differentially expressed, which indicates reorganization of highly abundant proteins in these tumors. Among the differentially expressed proteins in the subgroup with no recurrences, a UV-excision repair protein was found in lower amounts in the ER/PgR negative tumors than in the ER/PgR positive tumors. This protein is involved in DNA repair. Previously it has been shown that impaired DNA repair has been associated with poor clinical prognosis [35]. It is noteworthy that neither ER nor PgR were detected, which most likely depends on the low expression levels of these proteins, far below the sensitivity of the staining procedure.

In summary, by the use of 2-DE we were able to find candidate proteins involved in several different biological functions linked to tumorgenesis that were differentially expressed in primary tumors from patients later developing distant recurrences compared to those not developing recurrences. Even though the number of patients included in this study was relatively few we still may have found processes of importance for drug resistance/sensitivity in breast cancer. Independent verifications of these markers need to be accomplished in larger patient samples.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2006.09.019.

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