Proteomic profiling of MCF-7 breast cancer cells with chemoresistance to different types of anti-cancer drugs

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Received January 24, 2007; Accepted March 5, 2007

Abstract. Chemoresistance is a poor prognostic factor in breast cancer and, thus, presents a significant clinical challenge. The mechanisms of chemoresistance involve multiple complex biological processes. This study aims to identify common contributory factors to chemoresistance in breast cancer by comparing protein expression profiles of chemosensitive MCF-7 breast cancer cells and cells resistant to two different commonly used anti-cancer drugs (adriamycin and paclitaxel). Expression of the ATP binding cassette transporter, P-glycoprotein (P-gp), in breast tumours has previously been found to correlate with poor prognosis *in vivo* and, accordingly, we confirmed overexpression of P-gp in both adriamycin- and paclitaxel-resistant MCF-7 cells. Using two-dimensional gel electrophoresis and MALDI-TOF peptide mass fingerprinting, we identified 20 proteins differentially expressed between chemosensitive, adriamycin-resistant and paclitaxel-resistant MCF-7 cells. Cytokeratin-8, keratin-19, Hsp-27, 14-3-3 epsilon, annexin-A2 and phosphoglycerate kinase-1 showed altered expression in both adriamycin- and paclitaxel-resistant cells. Validation of a number of these changes was confirmed by Western blotting. Our findings provide further insights into the complex mechanisms of chemoresistance, as well as representing an attractive starting point for the identification of potential protein biomarkers to predict response to chemotherapy in breast cancer *in vivo*.

Introduction

Adriamycin and paclitaxel are two anti-cancer drugs commonly used as neoadjuvant chemotherapy (NAC) in women with breast cancer and result in up to a 30% complete pathological response (cPR) rate (1,2). However, a significant number of patients (up to 70%) do not achieve a cPR in the breast and, more importantly, those that do may have an incomplete pathological response in the regional tumour-draining lymph nodes (3,4). The ability to predict a response before commencing chemotherapy would be beneficial to patients who are unlikely to respond to the drugs and could save them from unnecessary drug toxicity. The mechanisms responsible for the variable responses to chemotherapy are, as yet, poorly defined. A range of factors contributing to this chemoresistance have been proposed, including drug efflux, drug detoxification and resistance to drug-induced apoptosis (5). Understanding these mechanisms will ultimately lead to improved treatments with enhanced tumour responses in the clinic.

Proteomic techniques can be used to determine relative expression levels of complex mixtures of proteins on a global scale. This technology has been applied to a wide range of cancer studies including analyses of drug resistance. A number of investigations involving protein profiling of various types of drug-resistant cancer cells have been documented previously including adriamycin-resistant breast cancer cells (6-13). However, there have been no proteomic studies of paclitaxel-resistant breast cancer cells or studies of breast cancer cell lines with different drug resistance in parallel. The objective of the study was, therefore, to apply proteomic technology [two-dimensional gel electrophoresis (2DE) and mass spectrometry (MS)] to document changes in the expression of abundant proteins in adriamycin- and paclitaxel-resistant breast cancer cells, relative to drug-sensitive cells. We reasoned that by analyzing these protein expression changes in cell lines resistant to different classes of anti-cancer drugs, common contributory factors to chemoresistance may be established. In addition, candidate biomarkers to predict clinical response to chemotherapy may be determined.

Materials and methods

Cell lines and cell culture. The parental, drug-sensitive MCF-7 (MCF-7/DS), human breast adenocarcinoma cell line was purchased from the European Collection of Cell Cultures (ATCC Number HTB-22). The MCF-7 adriamycin-resistant

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Key words: breast cancer, proteomics, adriamycin-resistant MCF-7 breast cancer cells, paclitaxel-resistant MCF-7 breast cancer cells, P-glycoprotein

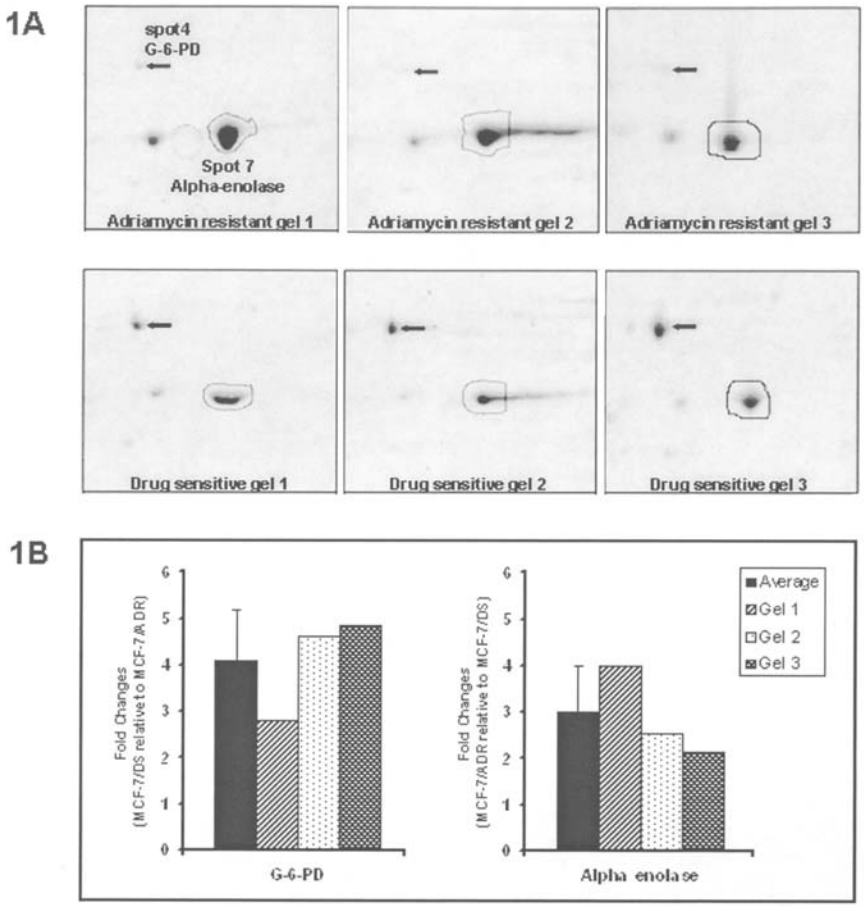

Figure 1. Example of 2DE gel spot analysis by the image analysis software. (A) Magnification area of two sets of three independent gels indicates two spots that showed different expression of normalized spot volume: spot 4 (with arrow, shows underexpression in MCF-7/ADR) and spot 7 (in circle, shows overexpression in MCF-7/ADR). (B) Statistical analysis of fold changes in normalized volume among triplicate gels is shown. The mean fold change with standard deviation and fold change among the individual gels of two spots in A are demonstrated in histogram form.

subline (MCF-7/ADR) and the MCF-7 paclitaxel-resistant subline (MCF-7/PAC) were kind gifts from Dr Timothy Gant (MRC Toxicology Unit, University of Leicester, UK) (14) and Dr Susan Bates (National Cancer Institute, USA) (15), respectively. These cell lines were grown in RPMI-1640 culture media (Invitrogen, Paisley, UK), supplemented with 10% (v/v) fetal bovine serum and 100 U/ml of penicillin/0.1 mg/ml of streptomycin (Invitrogen). Additionally, in order to maintain drug-resistant activity, MCF-7/ADR and MCF-7/PAC were exposed to 0.5 μM adriamycin and 200 nM paclitaxel (both Sigma-Aldrich, Poole, UK), respectively, for 5-7 days before the experiments started. All cells were grown in identical culture conditions in a 37˚C, 5% CO_2 incubator; culture media were changed twice weekly.

Cell cytotoxicity assay. The tetrazolium salt MTT (3-[4,5,-dimethylthiazol-2]-2,5-diphenyltetrazolium bromide), as previously described, was used to test the capacity of the drugs in growth inhibition of MCF-7/DS, MCF-7/ADR and MCF-7/PAC cells (16). Briefly, cells were seeded into 96-well cell culture plates. After incubation at 37˚C, in a 5% CO_2 incubator for 24 h, drug containing culture medium at various concentrations was added and left for 48 h. Then, after incubation in MTT solution dissolved in phosphate-buffered saline (PBS, 1 mg/ml) for 4 h, dimethylsulfoxide (DMSO) was added to solubilize the reduced tetrazolium salts. Finally, measurement of the absorbance at 570 nm in a microtitre plate reader was performed. The absorbance at 570 nm was plotted against the concentration of drugs to calculate inhibition concentration at 50% (IC_{50}) using GraphPad Prism 2.0 (GraphPad Software, San Diego, USA). The experiments were repeated in triplicate.

Two-dimensional gel electrophoresis (2DE) and MALDI-TOF peptide mass fingerprinting. Proteins were extracted from intact cell pellets (~6x10^5 cells), using a buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 12 μl/ml Destreak™ (Amersham Biosciences, Little Chalfont, UK), 5 μl/ml Biolyte 3/10 Ampholytes™ (Bio-Rad, Herts, UK) and bromophenol blue and loaded onto 11-cm non-linear gradient/pH 3-10 ReadyStrip™ IPG strips (BioRad). First dimension isoelectric focusing (IEF) was performed at 20˚C, 8000 V for 35000 Vh using a PROTEAN IEF® cell (Bio-Rad). The second dimension gel electrophoresis was performed using 5-20% gradient SDS-polyacrylamide gels with buffer running solutions containing 0.25 M Tris, 0.19 M glycine and 0.1% (w/v) SDS. Gels were stained with 0.12% (w/v) Coomassie Brilliant Blue (Sigma-Aldrich) in 50% (v/v) methanol and 20% (v/v) acetic acid overnight; and destained with a solution containing 10% (v/v) methanol, 10% (v/v) acetic acid in deionised water. Image

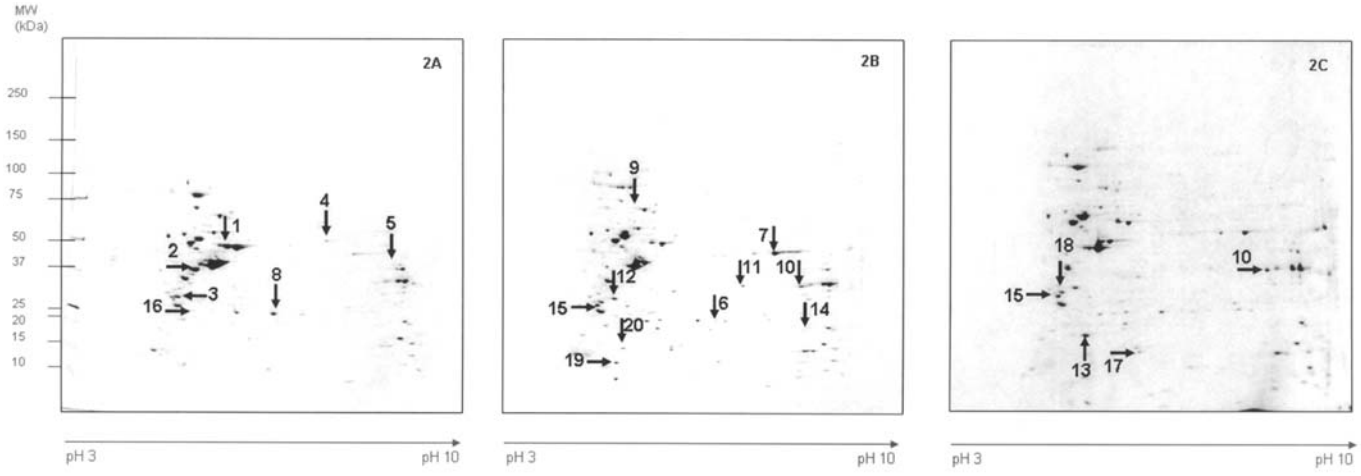

Figure 2. Representative example of differential protein expression profiles in MCF-7 drug-sensitive and -resistant cell lines. Proteins from MCF-7 drug-sensitive (A), adriamycin-resistant (B) and paclitaxel-resistant (C) breast cancer cells were extracted from $6x10^5$ cells, resolved by 2DE and visualised by staining with Coomassie Brilliant Blue. The denatured molecular weight of markers is indicated on the left hand side and the estimated isoelectric point on the bottom. Numbered protein spots relate to those in Table I.

analysis software (Progenesis 200 and Progenesis Same Spots software; Nonlinear Dynamics, Newcastle, UK) was used to construct average gels from three independent experiments and comparison between those average gels was performed. Protein spots which showed more than a 1.5-fold difference in normalized spot volume between drug-sensitive and drug-resistant cell lines (or which were 'undetectable' in one of the cell lines) were chosen for further analysis. Fig. 1 shows an example of two protein spots with differences in normalized spot volume between MCF-7/DS and MCF-7/ADR. The protein spots of interest were then excised and processed robotically using the MassPREP Workstation (Waters-Micromass, Manchester, UK). The excised spots were in-gel digested with trypsin in 96-well plates. Digested aliquots were subjected to a desalting and concentration step on ZipTipC_{18} (Millipore Corp., Bedford, USA) before being analyzed with a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer (Waters-Micromass). Peptide sequence matching was performed using the MASCOT interface to SWISS-PROT.

Western blot analysis. In order to validate data obtained from 2DE, the expression of six selected proteins was determined by Western blot analysis. In brief, 10 or 20 μg of total protein (as indicated) was resolved on 12% (w/v) SDS-polyacrylamide gels. Proteins were transferred onto nitrocellulose membranes, which were then blocked with 5% (w/v) non-fat milk in PBS for 1 h before incubation with primary antibodies [mouse anti-cytokeratin 19 monoclonal antibody (1:1500, Abcam, Cambridge, UK), mouse anti-Hsp27 monoclonal antibody (1:800, Abcam), rabbit anti-annexin-A2 polyclonal antibody (1:400, Santa Cruz Biotechnology, Santa Cruz, USA), rabbit anti-stathmin polyclonal antibody (1:2000, Santa Cruz Biotechnology), rabbit anti-14-3-3 epsilon polyclonal antibody (1:300, Santa Cruz Biotechnology), mouse anti-sorcin monoclonal antibody (1:400, Zymed laboratories, San Francisco, USA) and mouse anti-P-gp (C219, 1:2000, Merck Biosciences, Nottingham, UK)] diluted with 5% (w/v) non-fat milk in PBS. Membranes were then probed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies [rabbit anti-mouse (1:2000, Dako, Denmark) or goat anti-rabbit (1:8000, Sigma-Aldrich)] diluted with 5% (w/v) non-fat milk in PBS for 1 h. Western blots were developed using an enhanced chemiluminescence reagent (ECL Western Blotting Substrate, Pierce, Rockford, USA). Coomassie staining of gels run in parallel confirmed equal protein loading.

Results

In vitro cytotoxicity assays demonstrated that the MCF-7/ADR cells exhibited a 200-fold higher resistance to adriamycin, compared with the MCF-7/DS line (49.6±3.0 vs 0.2±0.1 μM), which is comparable to previous studies (17). In addition, the MCF-7/PAC cells showed a 43-fold higher resistance to paclitaxel, compared with MCF-7/DS (433.7±265.5 vs 10.1±6.8 nM). In agreement with previous analyses (18), the overexpression of the ATP binding cassette transporter, P-gp, was confirmed in our cells by Western blotting (Fig. 4A).

In order to identify other proteins showing changes in expression between drug-sensitive and -resistant cell lines, we employed 2DE analysis of MCF-7/DS, MCF-7/ADR and MCF-7/PAC cell extracts. This enabled the detection of 21 protein spots differentially expressed and meeting our criteria for further analysis (see Materials and methods) between MCF-7/DS and MCF-7/ADR cells. Comparison of MCF-7/DS and MCF-7/PAC cells identified 9 such spots (Fig. 2). These were excised and digested with trypsin prior to mass spectrometry protein analysis. Of the 30 spots in total, 26 were successfully identified using MALDI-TOF peptide mass fingerprinting. Six proteins were common to both MCF-7/ ADR and MCF-7/PAC, when compared with the drug-sensitive cells. Thus, we have identified 20 unique proteins differentially expressed between MCF-7/DS and resistant cell lines. These 20 proteins are listed in Table I with statistical analysis of their fold-changes in expression between drug-resistant, and MCF-7/DS cells. The identified proteins may be classified into 7 functional groups: cytoskeletal proteins; glucose metabolism-related proteins; molecular chaperones; calcium binding; detoxification; cell

Table I. Identification of proteins differentially expressed in MCF-7 cell lines.

	No[a]	Protein	MCF-7/ADR compared with MCF-7/DS (fold change ± SD)	MCF-7/PAC compared with MCF-7/DS (fold change ± SD)	Denatured MW (kDa)	Access number (MASCOT)	MASCOT score (or z-score)[b]	p-value[b]
Cytoskeletal	1	Cytokeratin-8	-6.1±5.3	-1.7±0.1	53	gi/181573	105	4.5e-06
	2	*Keratin-19*	Undetectable in ADR	Undetectable in PAC	44	gi/14043271	283	7.1e-24
	3	Tropomyosin-3	-2.8±0.8		29	gi/55665782	114	5.6e-07
Glucose	4	Glucose-6-phosphate dehydrogenase	-4.1±1.1		59	gi/12653141	138	2.2e-09
metabolism	5	Phosphoglycerate kinase-1	-3.1±2.2	-4.0±1.1	45	gi/48145549	146	3.5e-10
	6	Triosephosphate isomerase	2.4±0.9		27	gi/39932641	(7.59)	3.5e-27
	7	Alpha-enolase	3.0±0.9		45	gi/119339	(8.7)	4.7e-35
Molecular	8	*Heat-shock protein 27*	-2.0±02	-2.5±0.3	22	gi/662841	115	4.4e-07
chaperones	9	Heat-shock protein 70	1.9±0.6		71	gi/62897129	102	8.8e-06
Calcium-	10	*Annexin-A2*	5.4±1.3	1.8±0.1	38	gi/16306978	210	1.4e-16
binding	11	Annexin-I	Undetectable in DS		39	gi/113944	(8.71)	3.7e-35
	12	Annexin-V	Undetectable in DS		36	gi/999937	203	7.0e-16
	13	*Sorcin*		4.4±0.8	21	gi/38679884	149	1.8e-10
Detoxification	14	Peroxiredoxin-I	3.8±0.6		19	gi/55959887	113	7.0e-07
Cell cycle-	15	*14-3-3 epsilon*	2.7±0.8	2.3±0.2	27	gi/67464424	168	2.2e-12
and apoptotis-	16	Rho GDP dissociation inhibitor alpha	Undetectable in ADR		23	gi/76780069	124	5.5e-08
related	17	*Stathmin*		2.2±0.3	17	gi/2914387	(7.16)	2.2e-24
	18	Proliferation cell nuclear antigen		2.2±0.5	29	gi/2914387	103	7.1e-06
Others	19	Galectin-1	2.5±0.2		15	gi/126155	(5.38)	1.4e-14
	20	Eukaryotic translation initiation factor-5A	4.4±2.7		17	gi/54037409	(5.52)	2.9e-15

[a]Protein spot numbers are highlighted in Fig. 2. Fold-changes in protein expression are indicated as overexpression (+) or underexpression (-), relative to MCF-7/DS and shown with mean and standard deviation. These proteins are classified into 7 categories according to their functions. Those proteins in italics were selected to be validated by Western blotting (data shown in Fig. 4A). [b]MASCOT score, z-score and p-values are parameters that correlate with the quality of the search result on the MASCOT and SWISS-PROT. High scores and low p-value are indicative of successful, valid identifications.

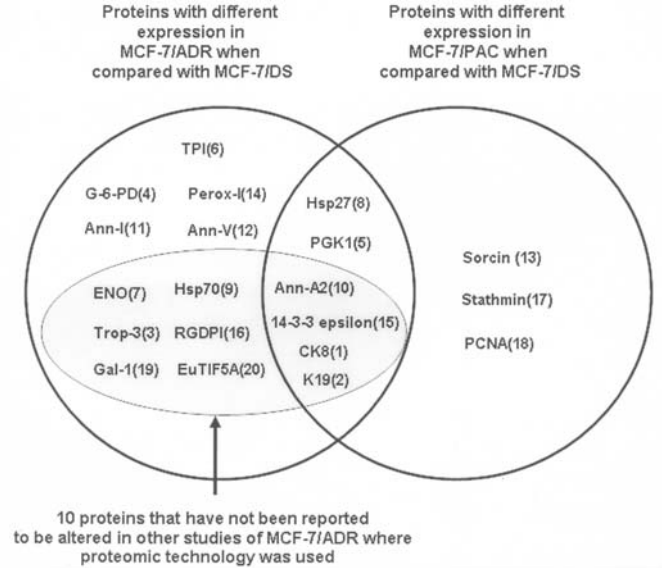

Figure 3. A schematic diagram summarizing the 20 proteins that were found to have altered expression, when comparing MCF-7/ADR and MCF-7/PAC cells with MCF-7/DS. Six proteins showed altered expression in both adriamycin and paclitaxel-resistant cells, and include Hsp27, PGK1, Ann-A2, 14-3-3 epsilon, CK8 and K19. Ten new proteins which have been documented to express differently in MCF-7/ADR, when compared with MCF-7/DS cells, are highlighted in the separated ellipse. Numbers in brackets refer to numbers in Fig. 2 and Table I.

cycle- and apoptotis-related; and others (Table I). The six common proteins that showed altered expression in both adriamycin- and paclitaxel-resistant cells were cytokeratin-8 (CK8), keratin-19 (K19), Hsp27, 14-3-3 epsilon, annexin-A2 (Ann-A2) and phosphoglycerate kinase 1 (PGK1) (Fig. 3).

Of the 20 proteins, six were selected for Western blotting analyses in order to validate the data on 2DE and MALDI-TOF peptide mass fingerprinting. These included proteins showing up- or down-regulation, proteins common to both chemo-resistant cell lines, as well as proteins which had not previously been identified in related studies (see Discussion and Fig. 4). Western blot results were entirely consistent with the 2DE and MALDI-TOF mass spectrometry data. In brief, K19 and Hsp27, which were found by analysis of 2DE gels to be over-expressed in drug-resistant wild-type cells, showed the same result on Western blotting. In addition, Ann-A2, 14-3-3 epsilon, sorcin and stathmin were determined to be overexpressed in drug-resistant breast cancer cells by Western blotting, in accordance with the 2DE results (Fig. 4A).

Discussion

This study reports the identification of 17 and 9 proteins differentially expressed in adriamycin-resistant and paclitaxel-resistant breast cancer cells, respectively, compared with

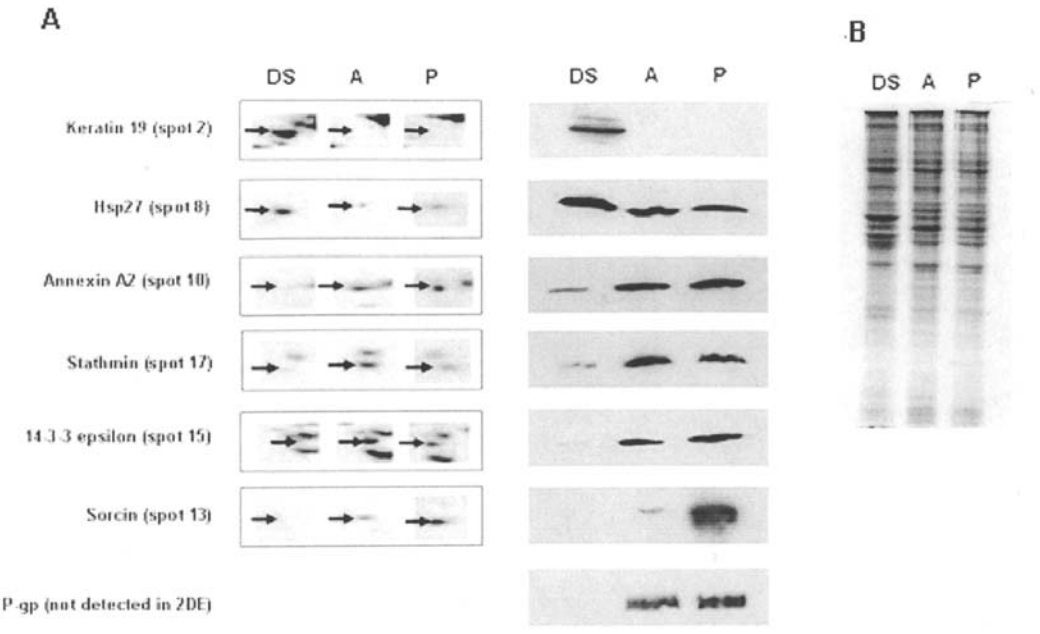

Figure 4. Western blot validation of 2DE and MALDI-TOF peptide mass fingerprinting data. (A) Proteins were resolved by SDS-PAGE and selected proteins detected by Western blotting as indicated (right panel). For comparison, magnified regions of the 2DE gels from which protein spots were identified are shown in the left panel with arrows indicating the relevant proteins. Spot numbers within brackets refer to those in Fig. 2. For the multidrug transporter P-gp, a representative Western blotting of crude cell membranes from drug-sensitive and -resistant MCF-7 cells (obtained from centrifugation of cell lysates at 100,000 x g for 1 h) is presented. Cell membranes (20 μg) were applied to each lane and resolved on 7% SDS-PAGE gels. DS, drug-sensitive MCF-7; A, adriamycin-resistant MCF-7; and P, paclitaxel-resistant MCF-7. (B) SDS-PAGE was employed to confirm equal protein loading in parallel with Western blotting. Twenty μg of total protein was loaded in each lane.

drug-sensitive MCF-7 cells. Although there have been previous reports, using proteomic technology to analyze protein expression in MCF-7/ADR (6,9,10), there are two significant advances described herein. Firstly, to the best of our knowledge, this is the only proteomic analysis of an MCF-7 paclitaxel-resistant cell line, and more importantly, we have performed the first study of protein expression profiles in cell lines with different drug resistance to determine possible common mechanisms (or potentially common cellular responses to different drugs). Additionally, we have performed analysis on total protein extracts, rather than analyzing cytosolic proteins as in previous studies (6,9,10). Our study was intentionally designed to demonstrate how proteomic technology can be used to identify expression changes in the most abundant proteins in drug-sensitive and resistant cell lines, which by extension may be representative of the protein expression changes in the rest of the proteome. The use of Coomassie Brilliant Blue staining ensures fuller compatibility with down-stream protein identification methods, resulting in our being able to positively identify all but four protein spots which met our criteria for further analysis. By identifying and validating a group of major abundant proteins, we present a panel of potential biomarkers for further clinical studies.

Consequently, we documented ten new proteins which showed different expression in MCF-7/ADR when compared with MCF-7/DS (Fig. 3). Although several of these proteins have been previously implicated in various types of drug-resistant cancer cells (7,11-13,19), they have never been linked with MCF-7/ADR, thus providing an interesting insight into the mechanisms of adriamycin resistance in breast cancer cells.

We have confirmed that our proteomic approach is a sensitive method to document protein expression changes in related cell lines. However, this approach has a bias against the detection of integral membrane proteins which are known to be poorly resolved by 2DE (20). For example, P-glycoprotein overexpression was not detected in the 2DE study, although Western blotting of cell membranes confirmed its overexpression in the two drug-resistant cell lines.

We have also performed validation of the proteomic data by Western blotting of a subset of six proteins and confirmed the robust nature of the results. The rationale of selection of individual proteins for validation is; firstly, four of these are detected in two cell lines that are resistant to different anti-cancer drug combinations (K19, Hsp27, Ann-A2, 14-3-3 epsilon) and, thus, can provide some insight into potentially common mechanisms of chemoresistance; and secondly, over-expression of stathmin or sorcin is associated with paclitaxel resistance in ovarian and breast cancer cell lines, emphasizing the considerable clinical relevance of our findings (21,22).

In our study, three cytoskeletal proteins were shown to be underexpressed, two of which, CK8 and K19 were common to both adriamycin- and paclitaxel-resistant breast cancer cells. There is also evidence to support that the loss of cytokeratin expression, including CK8 and K19, is associated with aggressive breast tumours *in vivo* (23).

Our study documented four glucose metabolic-related proteins showing altered expression in MCF-7/ADR and MCF-7/PAC cells including glucose-6-phosphate dehydrogenase (G-6-PD), triosephosphate isomerase (TPI), enolase alpha (ENO) and PGK-1. All have been previously linked to multidrug resistance in cancer cells (9,10,24).

Heat-shock proteins (Hsps) are a family of ubiquitous proteins found in response to stressful stimuli, including chemotherapeutic agents. The function of Hsps in the context

of chemoresponse has not been completely clarified, but may relate to a balance between cell survival and death by preventing protein aggregation during cell-associated stress (25). Previous 2DE studies also reported the underexpression of Hsp27 and we further substantiated this by Western blotting (10).

We found overexpression of four calcium-binding proteins in drug-resistant MCF-7 cells. Of these, three are members of the annexin family (Ann-A2, -I and -V) and all are overexpressed in MCF-7/ADR cells [the latter two having been reported previously (9,10)]. The mechanism by which annexins confer drug resistance is not known, but may include a role in drug efflux through exocytosis of drug-filled vesicles (26). The other calcium-binding protein identified and validated in our study is sorcin, which is overexpressed in MCF-7/PAC cells. This protein has been previously linked to drug-resistant leukemia, ovarian and breast cancer cells (22,27,28).

Proteins involved in drug detoxification including glutathione reductase, glutathione S-transferase, peroxiredoxin-1 (Perox-1) and peroxiredoxin-6, have been reported to be associated with chemoresistance in human melanoma and cervical cancer cell lines (7,12). In this study, we found overexpression of Perox-1 in MCF-7/ADR cells, which has been reported previously (9,10).

A group of cell cycle- and apoptosis-related proteins that showed expression changes in chemoresistant cancer cells in our study includes Rho GDP dissociation inhibitor alpha (RGDPI), proliferation cell nuclear antigen (PCNA), 14-3-3 epsilon and stathmin. RGDPI and 14-3-3 epsilon have been previously reported in drug-resistant cervical and breast cancer cells (7,29). PCNA (found to be up-regulated in MCF-7/PAC) has been previously studied as a prognostic factor in breast cancer with variable results (30). Stathmin, a p53-regulated protein, was confirmed to be overexpressed in MCF-7/PAC. In fact, overexpression of stathmin has been shown to affect the polymerization of microtubules and sensitivity to anti-microtubule drugs (including paclitaxel) in breast cancer cell lines (21).

In conclusion, our *in vitro* study identified a group of proteins that are differentially expressed between chemoresistant and sensitive breast cancer MCF-7 cells, specifically to adriamycin and paclitaxel, which are commonly used chemotherapeutic drugs in women with breast cancer. Two further questions arise which require in depth study. Firstly, are any of the elements of this proteomic profile predictive of clinical responses to NAC in women receiving combination therapy of adriamycin and the taxanes? On-going research to correlate *in vivo* protein expression changes and clinical response will address this issue. Secondly, are the protein expression changes identified central to the mechanism of cellular drug resistance? Functional assays that relate differential expression of these novel proteins to the generation or maintenance of the drug-resistant phenotype will provide further information to improve our understanding of the roles of these proteins in cancer drug resistance.

Acknowledgements

We would like to thank Drs Timothy Gant and Susan Bates who kindly provided the MCF-7/ADR and MCF-7/PAC cell lines, Kevin Bailey and Susan Mason who provided expert help with the MALDI-TOF and gel analysis. Suebwong Chuthapisith is supported by Siriraj Medical School, Thailand.

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