Differentially expressed proteins in human breast cancer cells sensitive and resistant to paclitaxel

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Abstract. The resistance of cancer cells to chemotherapeutic drugs represents a major problem in cancer treatment. Despite all efforts, mechanisms of resistance have not yet been elucidated. To reveal proteins that could be involved in resistance to taxanes, we compared protein expression in whole cell lysates of SK-BR-3 breast cancer cells sensitive to paclitaxel and in lysates of the same line with acquired resistance to paclitaxel. The resistant SK-BR-3 cell line was established in our lab. Protein separation was achieved using high-resolution 2D-electrophoresis, computer analysis and mass spectrometry. With these techniques we identified four proteins with different expression in resistant SK-BR-3 cells, i.e., serpin B3, serpin B4, heat shock protein 27 (all three upregulated) and cytokeratin 18 (downregulated). Observed changes were confirmed using western blot analysis. This study suggests new directions worthy of further study in the effort to reveal the mechanism of resistance to paclitaxel in breast cancer cells.

Introduction

Taxanes, diterpenes originally isolated from the bark of *Taxus brevifolia*, represent a relatively new group of anticancer drugs. Paclitaxel, the first taxane used in chemotherapy, came into clinical use in the early nineties and has become one of the main anticancer drugs, especially for the treatment of ovarian, breast and lung cancer, and Kaposi's sarcoma. In rapidly dividing cancer cells, paclitaxel and other taxanes bind to β-tubulin subunit and stabilize it. In doing so, they destroy the dynamic instability of microtubules, which is necessary for their proper function. As a result, cells are arrested in M phase of the cell cycle and subsequently undergo apoptosis (1). Nevertheless, cancer cell resistance (either intrinsic or acquired) to these compounds often reverses the benefits of taxane treatment.

The last decade has seen enormous efforts to elucidated the mechanisms of resistance to anticancer drugs (2-7). Nonetheless, elucidation is far from complete; although some mechanisms of resistance have been described that could provide useful markers of taxane resistance.

Drug efflux pumps often top the list of basic mechanisms of resistance that are common for many anticancer compounds. Upregulation of the ABC transporter P-glycoprotein (Pgp) (encoded by the ABCB1 gene) results in enhanced efflux of anticancer drugs from cells. The mechanism has been well established in resistance to taxanes (8-11). Other significant ABC transporters connected with taxane resistance include multidrug resistance-associated proteins 1 (ABCC1/MRP1) (12), 2 (ABCC2/MRP2) (13) and 3 (ABCC3/MRP3) (14). Oddly, upregulation of these proteins has not always been detected in taxane resistant cells (15-17), which conflicts with expectations.

Enhanced drug metabolism within the cell is considered to be another possible explanation for reduced taxane effectiveness in cancer cells. Two isoforms of cytochrome p450 are mainly responsible for paclitaxel utilization, i.e. cyp3A4 and cyp2C8 (18). In some cases, overexpression of these isoforms have been found in taxane resistant cancer cells (19,20). Nevertheless, individual patient genetic variability could account for the differences (21). Also phase II metabolic enzyme glutathione S-transferase P1 (GSTP1) has been reported to be connected with paclitaxel resistance. This enzyme conjugates glutathione with various drugs causing their deactivation. Localized inside the nucleus, GSTP1 can also protect DNA against damage caused by anticancer drugs (22). Some studies have suggested that expression of GSTP1 could be associated with higher survival rates of cancer cells after taxane treatment (23-25). Interestingly, GSTP1 has been shown to co-localize with another possible marker of taxane resistance, Bcl-2, within the nucleus (26-28).

Another mechanism of taxane resistance involve various β-tubulin mutations which can weaken the binding of taxane to β-tubulin and change the dynamics of the microtubule system (29-31). Mutations of α-tubulin are also believed to be connected with taxane resistance, but to a lesser extent; their importance lies more in influencing the binding of microtubule-associated proteins (MAPs) (32,33).

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Molecules directly connected with cell survival and apoptosis can also be involved. Expression of proteins, such as survivin, an inhibitor of apoptosis known to interact with tubulin (34,35), cyclin-dependent kinase inhibitor p21 (35,36) or p53, a factor inducing its expression (37,38), have been reported to influence cell sensitivity to taxanes.

Even though the mechanisms and compounds mentioned here is a heterogeneous group, there have been attempts to find connections between various mechanisms and markers of taxane resistance. De Hoon *et al* (22) have suggested that some mechanisms of resistance in breast cancer cells might be connected to expression and signaling of human epidermal growth factor receptor 2 (HER2) and subsequent activation of STAT3 as well as other downstream effectors.

Despite the current knowledge concerning resistance to taxanes, more studies are required to elucidate this phenomenon and provide reliable biomarkers to reveal tumors where taxane treatment (with all its side-effects) would have no impact. Indeed, investigation of resistance mechanisms (insights into treatment failure) remains a key task in cancer treatment (7,39).

To study further the mechanisms of resistance to taxanes, we employed a proteomic approach using the SK-BR-3 cell line (human breast adenocarcinoma) with acquired resistance to paclitaxel. We compared protein expression patterns from paclitaxel resistant cells to patterns from non-resistant cells and searched for proteins with different expression.

Materials and methods

Materials. Immobiline DryStrips pH 3-11NL 18 cm, Protein Extraction Buffer-III, 2-D Clean-Up Kit, 2-D Quant Kit, DeStreak Reagent and IPG Buffer 3-11NL were obtained from GE Healthcare (Uppsala, Sweden). Rabbit polyclonal antibody against serpin B4 (anti-SERPINB4 antibody defined by manufacturer also as 'SCCA2/SCCA1 fusion protein antibody': ab104338), mouse monoclonal antibody against cytokeratin 18 (anti-cytokeratin 18 antibody: ab55395), mouse monoclonal antibody against heat shock protein 27 [anti-Hsp27 antibody (8A7): ab78436] and rabbit polyclonal antibody against GAPDH (anti-GAPDH antibody: ab9485) were obtained from Abcam (Cambridge, UK). Mouse monoclonal antibody anti-actin (clone AC-40: A3853) against human actin was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cells and cell culture conditions. Human breast carcinoma cell line SK-BR-3 was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Resistant SK-BR-3 cells were derived in our lab from original sensitive SK-BR-3 cells by gradual adaptation to paclitaxel. The concentration of paclitaxel in the culture medium was increased approximately every 20th passage. Resistant SK-BR-3 cells display long-term proliferation in culture medium containing 100 nM paclitaxel. At this taxane concentration original sensitive SK-BR-3 cells die within 72 h. This concentration of paclitaxel approximates concentrations used in clinical practice (26,40).

The culture medium consisted of basic medium supplemented with 10% heat-inactivated fetal bovine serum. The basic medium was RPMI-1640 based medium containing extra L-glutamine (300 μg/ml), sodium pyruvate (110 μg/ml), HEPES (15 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml), as previously described (41-43). Cells were maintained at 37˚C in a humidified atmosphere of 5% CO_2 in air. For maintaining taxane-resistant cells, the medium was supplemented with paclitaxel (in DMSO) to a final concentration of 100 nM (final concentration of DMSO in medium was below 0.1%) just prior to use. For experiments, cells were harvested and seeded (approximately $5x10^6$ in 15 ml of medium per sample). After 24-h preincubation the culture medium was replaced with a fresh medium and after another 24 h the cells were harvested.

Sample preparation for 2D-electrophoresis. Cells were trypsinized, washed with ice-cold PBS and resuspended in Protein Extraction Buffer-III (GE Healthcare) (urea, thiourea, ASB-16, CHAPS) containing 1% Protease-Inhibitor Mix G (SERVA Electrophoresis GmbH, Heidelberg, Germany). A 2-D Clean-Up Kit (GE Healthcare) was used for sample purification, per manufacturer's instructions. Briefly, the sample was precipitated using Precipitant and Co-precipitant, incubated for 15 min at 4˚C and centrifuged. The pellet was then washed with Wash Buffer with Wash Additive, incubated at -20˚C for 30 min and centrifuged. After brief air drying, the pellet was dissolved again in Protein Extraction Buffer-III as described above. Protein concentrations were determined using a 2-D Quant Kit (GE Healthcare), which is compatible with both the detergents and thiourea present in Protein Extraction Buffer-III.

2D-electrophoresis: isoelectric focusing. Isoelectric focusing was carried out in an IPGphor focusing unit (GE Healthcare, former Amersham Biosciences) using 3-11NL Immobiline DryStrips 18 cm (GE Healthcare). Each strip was rehydrated for at least 24 h with 340 μl of the diluted protein sample containing 400 μg of protein, 6.8 μl of bromophenol blue solution (0.1%), 4 μl of DeStreak Reagent (GE Healthcare) and 6.8 μl of IPG buffer (pH 3.0-11.0; GE Healthcare) in Protein Extraction Buffer-III as described above. After rehydration, strips were focused at 20˚C with current limited to 50 μA/strip using the following conditions: 150 V for 1 h, gradient 150-300 V for 10 min, 300 V for 2 h, gradient 300-1,000 V for 10 min, 1,000 V for 2 h, gradient 1,000-8,000 V for 1 h, 8,000 V for 15 h.

2D-electrophoresis: equilibration. After focusing, strips were equilibrated for 20 min in equilibration buffer containing 6 M urea, 30% glycerol, 2% SDS, 50 mM Tris (stock solution of 1.5 M Tris-HCl, pH 8.8), 2% dithiothreitol and then equilibrated for another 20 min in the same buffer with 2.5% iodoacetamide instead of dithiothreitol. Then the strips were placed on the top of gels and sealed using 0.5% agarose solution containing bromophenol blue.

2D-electrophoresis: SDS-PAGE. The second dimension was performed using an Ettan DaltSix Electrophoresis System (GE Healthcare, former Amersham Biosciences). A total of 10% polyacrylamide gel (pH 8.8) with a 4% stacking gel (pH 8.8) was used for protein separation. Gels were run at a constant current starting with 35 mA/gel for 1 h and then 65 mA/gel till the blue line reached the bottom of the gels (approximately 4 h). After running the second dimension, each gel was washed 3x10 min in distilled water and stained overnight in 500 ml of colloidal Coomassie Blue solution per gel according to (44).

Gel image and analysis. Stained gels were scanned using a calibrated UMAX PowerLook 1120 scanner employing LabScan software (both from GE Healthcare). Gel couples were analyzed using Image Master™ 2D Platinum 6.0 software (GE Healthcare, former Amersham Biosciences). We analyzed differences in intensity between corresponding spots on each set of gels (each set contained gel with proteins from sensitive SK-BR-3 cells and gel with proteins from resistant SK-BR-3). Spots with at least 2-fold average difference in expression between sensitive and resistant cell lysates were selected. Statistical significance of difference in intensity of these spots was determined using the Student's t-test.

Enzymatic digestions. CBB-stained protein spots were excised from the gel, cut into small pieces and destained using 50 mM 4-ethylmorpholine acetate (pH 8.1) in 50% acetonitrile (MeCN). After complete destaining, gels were washed with water, reduced in size by dehydration in MeCN and reconstituted again in water. The supernatant was removed and the gel was partly dried in a SpeedVac concentrator. Gel pieces were then incubated overnight at 37˚C in a cleavage buffer containing 25 mM 4-ethylmorpholine acetate, 5% MeCN and trypsin (100 ng; Promega). The resulting peptides were extracted with 40% MeCN/0.1% trifluoroacetic acid (TFA).

MALDI mass spectrometry and protein identification. An aqueous 50% MeCN/0.1% TFA solution of α-cyano-4-hydroxycinnamic acid (5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) was used as a MALDI matrix. Peptide mixture (1 μl) was deposited on a MALDI plate, allowed to air-dry at room temperature and overlaid with 0.4 μl of matrix. Mass spectra were measured using an Ultraflex III MALDI-TOF (Bruker Daltonics, Bremen, Germany); mass range of 700-4,000 Da, calibrated internally using the monoisotopic $[M+H]^+$ ions of trypsin auto-proteolytic fragments (842.5 and 2,211.1 Da). The peak lists created using the flexAnalysis 3.3 program were searched using an in-house MASCOT search engine against SwissProt 2013_12 database subset of human proteins with the following search settings: peptide tolerance of 30 ppm, missed cleavage site value set to one, variable carbamidomethylation of cysteine, oxidation of methionine and protein N-term acetylation. Proteins with MOWSE scores over the threshold, 56 (calculated for the used settings) were considered as identified. The identity of protein candidate was confirmed using MS/MS analysis.

Western blot analysis. Western blot was carried as described previously (41,43,45) with minor modification. Briefly, whole cell lysates were separated by SDS-PAGE using 9% acrylamide gels and blotted into 0.2 μm nitrocellulose membrane for 2 h at 0.25 A using a Mini-Protean II blotting apparatus (Bio-Rad). The membrane was blocked with 5% low fat milk in TBS for 30 min. Tween-20 (0.1%) in TBS was used for washing. The washed membrane was then incubated with the relevant primary antibody. After incubation (overnight, room temperature), the washed membrane was incubated for 1 h with the corresponding horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The horseradish peroxidase-conjugated secondary antibody was detected by enhanced chemiluminescence using the SuperSignal reagent from Pierce (Rockford, IL, USA) and a Gel Logic 4000 PRO device. Optical density of bands was analyzed using Carestream Molecular Imaging Software v. 5.2.0 (Carestream Health, Berlin, Germany).

Statistical analysis. Statistics was performed using Sigma Plot, version 11.00. Significant differences in intensity of spots (2-D gels) and bends (western blot) were analyzed by Student's t-test.

Results

Detection of proteins with changed expression. In order to compare protein expression in the human breast adenocarcinoma cell line (SK-BR-3) sensitive to paclitaxel and in SK-BR-3 cells with acquired resistance to paclitaxel, we prepared whole cell lysates of these cells. Lysates were prepared using Protein Extraction Buffer. Samples were purified using TCA-based precipitation and 400 μg of proteins of each sample were used for two-dimensional electrophoresis (2-DE) (see Materials and methods).

We prepared three pairs of 2-DE gels from three independent sets of samples containing SK-BR-3 cells resistant to paclitaxel and SK-BR-3 cells sensitive to paclitaxel. Gels were stained using colloidal coomassie blue. The stained gels were scanned and differences in intensity of corresponding spots in each pair of gels were analyzed using ImageMaster 2D Platinum 6.0 software (see Materials and methods).

Approximately 700 stable spots were detected on each 2-DE gel. The expression profiles of proteins with isoelectric point within pH range 3.0-11.0 and molecular mass of 20-150 kD were highly reproducible for both sensitive and resistant cells as well as for individual sets of samples. Differences in intensity of corresponding spots were analyzed for each pair of gels and spots with 2-fold higher or lower intensity, when comparing sensitive and resistant cells, and considered as proteins with changed expression. Eight spots with significantly changed expression were detected (Figs. 1 and 2). These spots were cut from the gels and subjected to MS analysis (see Materials and methods).

Identification of proteins with changed expression. The identified proteins (Table I) with changed expression in resistant SK-BR-3 cells were: serpin B3 (spots 1, 2, 3 with intensity increased to 235, 264 and 387%, respectively), serpin B4 (spots 4 and 5 with intensity increased to 190 and 195%, respectively), heat shock protein 27 (HSP27) (spots 6 and 7 with intensity increased to 218 and 340%, respectively) and cytokeratin 18 (spot 8 with intensity decreased to 41%) (Fig. 2).

Western blot analysis of the new sets of samples confirmed the detected changes in expression of all these proteins. Serpin B3 and serpin B4 were detected as one protein. These two proteins have a high homology (92%) and thus cross reactivity of primary antibodies against them is highly probable if not straightly admitted by manufacturer. The expression of serpin B3+B4 in SK-BR-3 cells resistant to paclitaxel detected using western blotting was elevated up to 158% when compared to cells sensitive to paclitaxel ($p<0.01$) (Fig. 3). The expression of HSP27 in resistant

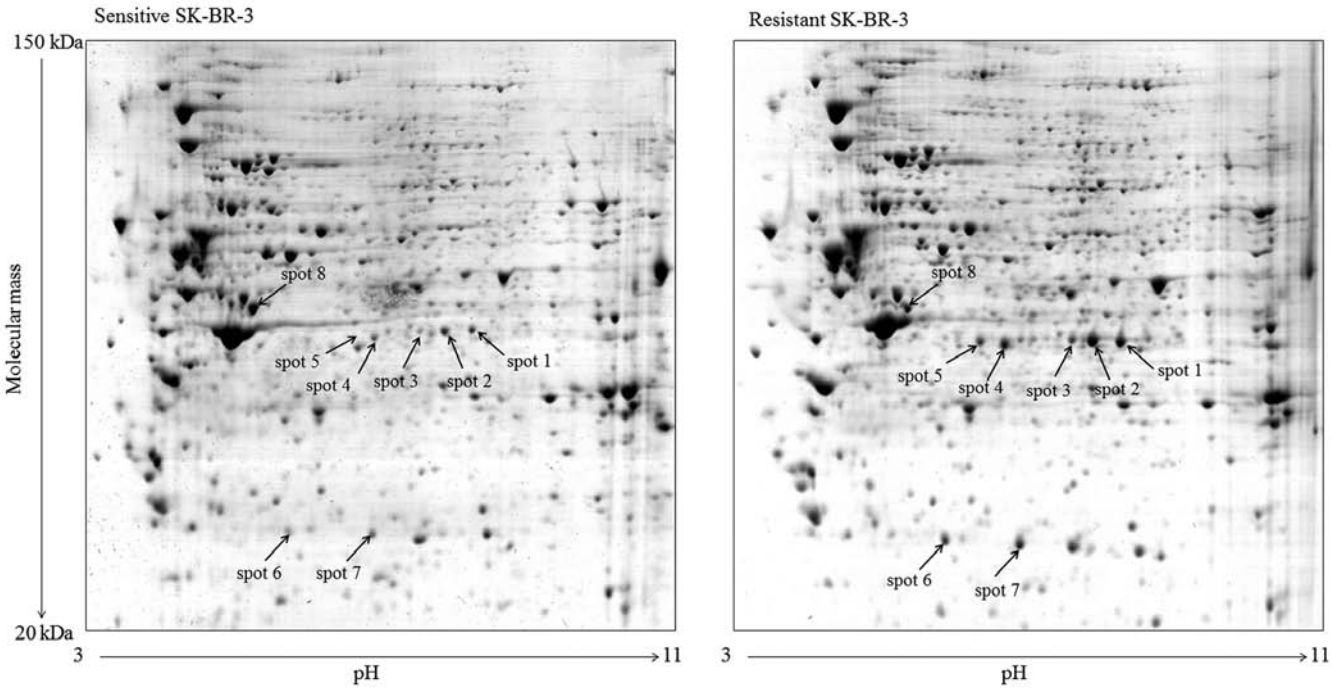

Figure 1. Representative 2-DE gels. Expression of proteins (whole cell lysates) in SK-BR-3 cells resistant to paclitaxel (100 nM) compared to the expression of proteins in SK-BR-3 cells sensitive to paclitaxel (100 nM). Representative 2-DE gels with eight spots representing detected proteins with different expression are shown (at least 2-fold higher or lower expression in resistant cells). These 8 spots were identified as serpin B3 (spots 1-3), serpin B4 (spots 4 and 5), heat shock protein 27 (spots 6 and 7) and cytokeratin 18 (spot 8).

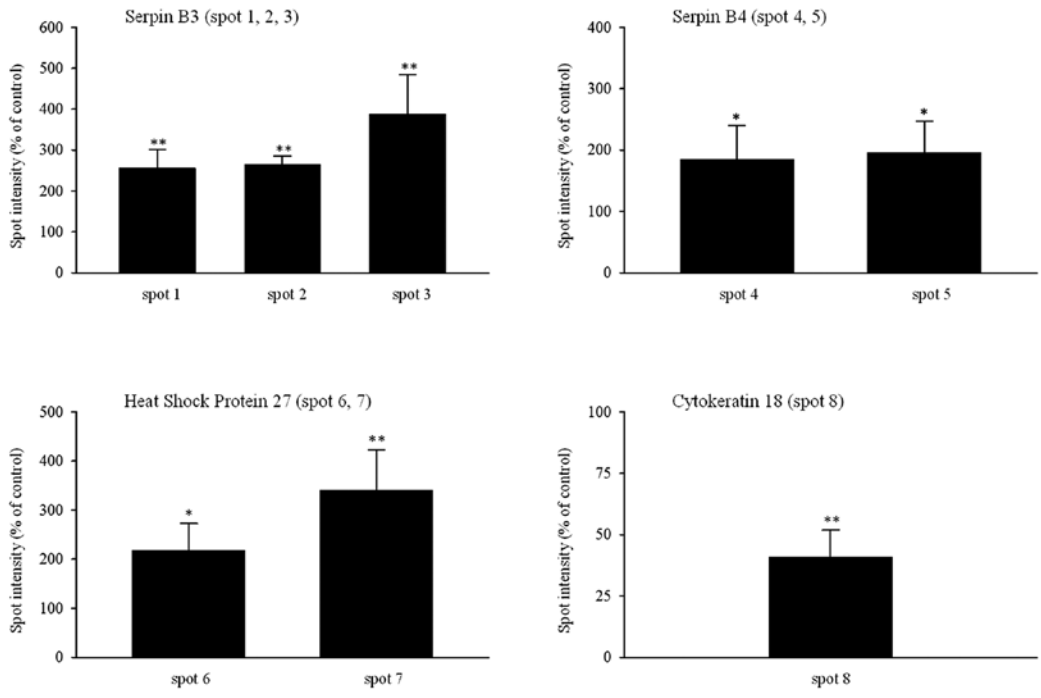

Figure 2. Differences in protein expression. Expression of detected proteins (spots 1-8 on 2-DE gel) in SK-BR-3 cells resistant to paclitaxel compared to expression in SK-BR-3 cells sensitive to paclitaxel (control). Columns represent mean values ± SD of intensity of corresponding spots from three independent sets of gels. $^{*}p<0.05$ means statistically significant difference when compared to the control, $^{**}p<0.01$ means statistically significant difference when compared to the control (Student's t-test).

cells was increased to 144% in resistant cells compared to sensitive cells ($p<0.01$) (Fig. 3). Western blot analysis of cytokeratin 18 showed three bands very close together. We searched for western blots of whole SK-BR-3 lysates using cytokeratin 18 antibody and found that more bands or one very wide band for cytokeratin 18 has been previously

Table I. Results of MS analysis.

No.	Protein name	SwissProt no.	M	UM	SC (%)	MS (score)	MW (kD)	pI-value
1	Serpin B3	SPB3_HUMAN	19	7	47	FMFDLFQQFR (55)	45	6.4
2	Serpin B3	SPB3_HUMAN	16	6	47	FHCNHPFLFFIR (41)	45	6.4
3	Serpin B3	SPB3_HUMAN	9	5	25	SVDFANAPEESR (63)	45	6.4
4	Serpin B4	SPB4_HUMAN	11	4	31	STDFANAPEESR (66) TYQFLQEYLDAIKK (59)	45	5.9
5	Serpin B4	SPB4_HUMAN	8	3	16	STDFANAPEESR (55) TYQFLQEYLDAIKK (57)	45	5.9
6	Heat shock protein beta-1	HSPB1_HUMAN	8	4	41	LFDQAFGLPR (56)	23	6.0
7	Heat shock protein beta-1	HSPB1_HUMAN	8	3	41	LFDQAFGLPR (48) SNEITIPVTFESR (49)	23	6.0
8	Keratin, type I cytoskeletal 18	K1C18_HUMAN	19	4	44	TVQSLEIDLDSMR (51) SLGSVQAPSYGAR (43)	48	5.3

Differentially expressed proteins identified from 2-DE experiment. Table includes spot number, protein name, SwissProt database number, number of peptides matched to the identified protein, number of unassigned peaks, sequence coverage, peptide sequences confirmed by MS/MS (MASCOT score of individual peptides is given in parenthesis), MW and pI-values. No, spot number; M, matched peaks; U, unmatched peaks; SC, sequence coverage (%), MS, MS/MS confirmation - AA sequence (MASCOT score); MW, molecular weight of protein (kD).

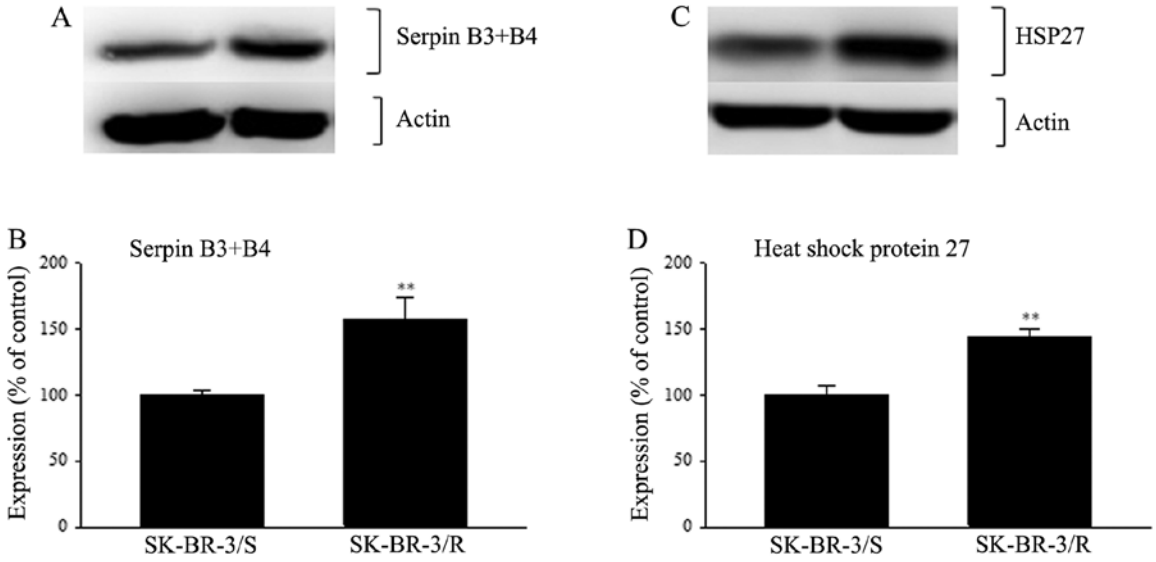

Figure 3. Confirmation of results by western blot. Expression of serpin B3+B4 and heat shock protein 27 (HSP27) in SK-BR-3 cells resistant to paclitaxel (SK-BR-3/R) compared to expression in SK-BR-3 cells sensitive to paclitaxel (SK-BR-3/S) (control). The levels of tested proteins were determined using western blot analysis. (A) Representative western blot of serpin B3+B4 is shown. Actin was used to confirm equal protein loading. (B) Densitometric analysis of serpin B3+B4 western blots of three independent experiments is also shown. (C) Representative western blot of HSP27 is shown. Actin was used to confirm equal protein loading. (D) Densitometric analysis of HSP27 western blots of three independent experiments is also shown. Columns represent mean values ± SD of protein levels from three independent sets of experiments. **Statistically significant difference ($p<0.01$) when compared to the control (Student's t-test).

published (http://www.scbt.com/datasheet-32722-cytokeratin-18-rck106-antibody.html; accessed June 7, 2013). Thus, for densitometry analysis we took these three bands as one. We detected decreased expression of cytokeratin 18 down to 68% in resistant cells compared to sensitive cells ($p<0.01$) (Fig. 4).

For serpin B3+B4 and HSP27, actin was used as a loading control. For cytokeratin 18 GAPDH was used. The reason for the different loading controls was that cytokeratin 18 has approximately the same size as actin and both these proteins are very strongly expressed. Therefore stripping off either cytokeratin 18 or actin failed to yield satisfactory results.

Discussion

Using western blot analysis, we found a significant increase (158%) in the expression of serpin B3/B4 in human SK-BR-3 breast cancer cells resistant to paclitaxel compared to sensitive SK-BR-3 cells (Fig. 3). The increase is lower compared to 2-DE gels analysis, but still significant (analyzed by Student's t-test, $p<0.01$) (Fig. 2).

Serpin B3 and B4 share the same molecular weight (92% identical sequence of amino acids) but have different pI (serpin B3 represents a neutral form with pI>6.2, serpin B4 represents an acidic form with pI<6.2) (46). This corresponded

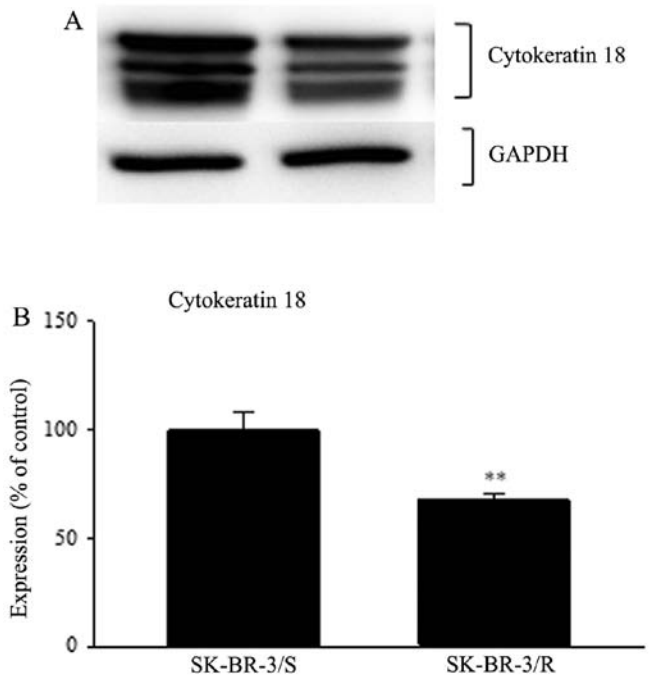

Figure 4. Confirmation of results by western blot analysis. Expression of cytokeratin 18 in SK-BR-3 cells resistant to paclitaxel (SK-BR-3/R) compared to expression in SK-BR-3 cells sensitive to paclitaxel (SK-BR-3/S) (control). The levels of tested proteins were determined using western blot analysis. (A) Representative western blot of cytokeratin 18 is shown. GAPDH was used to confirm equal protein loading. (B) Densitometric analysis of cytokeratin 18 western blots of three independent experiments is also shown. Columns represent mean values ± SD of protein levels from three independent sets of experiments. **Significant difference ($p<0.01$) when compared to the control (Student's t-test).

with position of their spots on our 2-DE gels (Fig. 1). Our gels revealed three spots for serpin B3 and two spots for serpin B4. A higher number of serpin B3 protein species on 2-DE gels was described previously by Ho *et al* (47). Spots of the same protein with different pI, which we found in our study, could be the result of various levels of phosphorylation of serpins. Because of their almost identical sequence of amino acids, serpin B3 and serpin B4 are often analyzed together as serpin B3+B4.

Serpins (serine protease inhibitors) represent a large group of proteins, most of them capable of inhibiting proteases (serine proteases and in some cases also cysteine proteases) (48). Serpin B3 and B4 are clade B serpins or ovoalbumin serpins (ov-serpins). Serpin B3 inhibits papain-like cysteine proteases (e.g., cathepsin-S, -K and -L), while serpin B4 inhibits both serine proteases (e.g., cathepsin G) and cysteine proteases (e.g., Der p 1 and Der f 1) (49). Both these serpins are often overexpressed in cancer cells of epithelial origin (46,50).

Upregulation of serpin B3 and B4 has previously been described as protective in cells exposed to radiation (51). Suppression of these proteins has been shown to suppress tumor growth (52). Recently, serpin B3 was suggested as a prognostic tool for docetaxel resistance in breast cancer (53) and platinum resistance in epithelial ovarian cancer (54) and lung cancer (55).

The mechanism of pro-survival effect of serpin B3 and B4 on cancer cells is likely to be connected with their ability to inhibit proteases. Cathepsin S, a known target for serpin B3, is essential for proper presentation of antigens to immune cells. Inhibition of its activity could lead to impaired recognition of cancer cells by the immune system. Another cathepsin, cathepsin L, promotes production of endostatin which has an anti-angiogenic effect (56). Therefore, inhibition of cathepsin L by serpin B3 could support angiogenesis. Cathepsin G has been described as an agent opposing tumor cell-cell adhesion (57). Thus, its inhibition would benefit cancer cells. It is worth noting that some of these cathepsins have been reported to have pro-cancer effects, as well (58,59).

More complex explanations of upregulation of serpin B3 and B4 in resistant cancer cells have also been suggested. Serpin B3 is believed to be able to inhibit the lysosomal cell death pathway (LCDP) induced by microtubule-stabilizing agents, including paclitaxel (60), through inhibition of LCDP mediators, e.g. cathepsins. Interestingly, serpin B4 has been described to inhibit also granzyme M-induced cell death, which is the main pathway used by cytotoxic lymphocytes to kill tumor cells (61).

Upstream regulation of serpin B3 and B4 expression has been suggested. Serpins B3+B4 were described as being activated through STAT3 activation (62). De Hoon *et al* have suggested signaling pathways connected with resistance to paclitaxel (22). They proceed from human epidermal growth factor receptor 2 (HER2) through PI3K/Akt to i) activation of STAT3 and ii) upregulation of P-glycoprotein and survivin, resulting in an overall increase in cancer cell survival (22). Based on these two studies, we suspect that increased expression of serpins B3+B4 is connected to paclitaxel resistance via the mechanism suggested by De Hoon *et al* (22).

In this study we confirmed that increased expression of serpin B3 and B4 can potentially affect resistance to paclitaxel and we hypothesize that the mechanism of serpin B3 and B4 upregulation involves activation of STAT3.

Another protein found to have significantly higher expression (144%) in resistant SK-BR-3 cells compared to sensitive cells was heat shock protein 27 (HSP27). Again, the increase in HSP27 expression detected using western blot analysis was lower than the increase found using 2D gels analysis (Figs. 2 and 3), however, it was nonetheless statistically significant (Student's t-test, $p<0.01$).

Heat shock protein 27 is an ATP-independent chaperon and functions as a protective agent against protein aggregation (63). It is highly expressed even in normal cells under stress conditions like heat, oxidative stress or exposure to chemotherapeutic agents (64). In some studies, HSP27 was thought to protect cancer cells against cell death. Its overexpression was reported to inhibit activation of procaspase-3 and procaspase-9 (65). It antagonizes Bax-mediated mitochondrial changes (66) and it is generally thought to block programmed cell death by directly sequestering intermediates in the caspase-dependent apoptosis pathway (67).

Thus, we hypothesize that HSP27 can positively affect cell survival through inhibition of the inner (mitochondrial) apoptotic pathway. Development of the ability to prevent activation of this pathway could be crucial for survival of SK-BR-3 cells when exposed to paclitaxel and thus could be part of the resistance mechanism to taxanes in certain types of cancer cells.

Cytokeratin 18 was the only protein with reduced expression in resistant SK-BR-3 cells compared to sensitive cells. The cytokeratin 18 molecule (together with cytokeratin 8) is a component of the most common intermediate filaments in cells of epithelial origin. Besides the many functions connected with its role in intracellular scaffolding (e.g., structuring cytoplasm, providing resistance against external stresses) and cellular processes like mitosis or apoptosis, it also has a role in tumor cell behavior (68). Additionally, some studies have suggested the involvement of cytokeratin 18 in certain signaling pathways (68,69).

Cytokeratin 18 was reported to be involved in pro-survival PI3K/Akt pathway (69), which can counteract Fas-mediated apoptosis (70). Fortier *et al* reported overexpression of cytokeratin 18 (and cytokeratin 8) as a result of overexpression of Akt1 (71). This would suggest that higher expression of cytokeratin 18 could be a marker of reduced susceptibility to cell death (i.e., resistant cells); however, our findings do not support this conclusion.

The tumor necrosis factor receptor 2 (TNFR2) is another signaling pathway with which cytokeratin 18 can interact. Binding of cytokeratin 18 to the cytoplasmic domain of tumor necrosis factor receptor 2 (TNRF2) leads to changes in JNK signaling and NF-κB activation. Cytokeratin 18 is also able to negatively influence TNF-induced apoptosis (72). However, our results showed a different pattern, which could mean that TNF-induced cell death does not play as important role in paclitaxel-induced cell death in SK-BR-3 cells.

Relative to our results, Meng *et al* (73) found a lower expression of cytokeratin 18 promoting proliferation of breast cancer cells MCF-7; however, they connected this effect to the modulation of estrogen receptor-α pathway. Nevertheless, SK-BR-3 cells do not express estrogen receptor-α. A trend similar to that in our study was found in some primary breast carcinoma screenings where lower expression of cytokeratin 18 as associated with a worse prognosis (74,75).

The function and significance of cytokeratin 18 in cancer cells needs to be elucidated more thoroughly; more precisely we need to determine why upregulation of cytokeratin 18 indicates reduced susceptibility to treatment in some cases, but not in others.

Our study found four proteins (serpin B3, serpin B4, heat shock protein 27, cytokeratin 18) with changed expression in breast cancer cells (the type not expressing estrogen receptor but overexpressing HER2) resistant to paclitaxel. These proteins represent a heterogeneous group and do not seem to be directly connected with each other (with serpin B3 and B4 as an exception). This suggests that processes involved in cancer cells surviving normally lethal doses of paclitaxel are both numerous and complex. Nevertheless, even if revealed proteins interact with different pathways of cell signaling, they still represent a group of potential biomarkers that should be probably seen as a kind of whole and tested together rather than considered and treated as four individual biomarkers.

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