

# Identification of 5-fluorouracil response proteins in colorectal carcinoma cell line SW480 by two-dimensional electrophoresis and MALDI-TOF mass spectrometry

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**Abstract.** Colorectal cancer (CRC) is the second most prevalent cause of cancer-related deaths in the Western world. 5-Fluorouracil (5-FU) is a standard chemotherapeutic drug to treat CRC. However, the response rate is less than 20% and patients who have responded to 5-FU may become resistant. Therefore there is an urgent need to examine the 5-FU response proteins so that patients with no response to 5-FU can change to other treatment strategies promptly. In this study, the proteomic expression profile in a CRC cell line SW480 before and after 5-FU treatment was examined using 2-dimensional electrophoresis technology. Fourteen proteins with differential expression were identified using mass spectrometry and 7 of them were validated using immunocytochemical (ICC) staining. Protein identification indicated that cyclophilin A, cytokeratin 19 (CK19), cytokeratin 8 (CK8), ras-related nuclear protein, heat shock protein 27 (hsp27) and peroxiredoxin 6 (Prx 6) were upregulated whereas heat shock protein 60 (hsp60), cytokeratin 18 (CK18), cytokeratin 9 (CK9), carbamoylphosphate synthetase I,  $\alpha$ -enolase, heat shock protein 70 (hsp70), nm23 and  $\beta$ -actin were down-regulated. Seven of the 14 proteins detected were validated by ICC staining, which showed that the expression of hsp27, Prx 6 and hsp70 correlated with that from proteomics profiling. Our results suggest that hsp27, Prx 6 and hsp70 are potential 5-FU response proteins and they may represent potential targets for further evaluation in other 5-FU-sensitive and -resistant CRC cell lines.

## Introduction

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in the Western world (1). Despite curative surgery, approximately 40% of patients still experience disease relapse leading to morbidity and eventual mortality (2). 5-Fluorouracil (5-FU) has been used for more than 40 years in the treatment of various cancers and remains the standard first-line chemotherapeutic drug for CRC, although the response rate as a single agent is usually less than 20% (3). In addition, patients who respond to 5-FU ultimately become resistant, which has been a major obstacle in advanced CRC chemotherapy (4). Therefore there is an urgent need to develop markers that can identify patients who would benefit from 5-FU therapy.

Recent improvements in 2-dimensional (2D) electrophoresis, image analysis, mass spectrometry, and the development of advanced bio-informatic databases and analysis software, have enabled the proteomics technology to identify disease-associated protein markers that are helpful in diagnosis or prognosis (5). Previously, this technology has been applied to analyze many cancers such as prostate (6), ovary (7), breast (8) and lung (9). Currently, there is a growing body of evidence revealing the great potential of proteomics technology in the analysis of the response and resistance of tumor cells to cytotoxic drugs (10-12). Therefore this study plans to investigate the effect of 5-FU in the human CRC cell line SW480 using proteomics profiling. The information obtained should be important for us to have a better understanding of CRC cell response to 5-FU, and possibly help to discover novel predictive markers to 5-FU treatment in patients with CRC.

## Materials and methods

**Cell line.** The SW480 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI medium 1640 (Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin.

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**Dose response curve of SW480 cells to 5-FU.** Cells were seeded into 96-well plates and allowed to attach for 24 h. Various concentrations (0, 0.22, 2.2, 22, 220 and 2200  $\mu$ M) of 5-FU (Ebewe, Unterach, Austria) were prepared by dissolving in double distilled water and added to fresh RPMI medium 1640. They were then added to the cells and incubated for up to 72 h. Cell viability was assessed after 0, 24, 48 and 72 h using 3-[4,5-dimethyl(thiazol-2-yl)-3,5-diphenyl] tetradium bromide (MTT) assay (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. The number of viable cells was expressed as a percentage relative to control cells incubated without 5-FU. Triplicate tests were performed for each concentration of 5-FU and time point.

**Treatment of SW480 cells with 5-FU.** 5-FU was dissolved in double distilled water and added to fresh culture medium at a final concentration of 16.3  $\mu$ M ( $IC_{50}$  was 16.3 $\pm$ 2.1  $\mu$ M at 72 h) whereas the same volume of double distilled water was added to the culture medium for control. The culture media with SW480 cells in the test and control culture plates were replaced with 16.3  $\mu$ M 5-FU and control culture media, respectively after incubating for 24 h. All culture plates were then incubated for 72 h at 37°C with 5% carbon dioxide in a humidified incubator.

**Cell lysis and preparation for 2D electrophoresis.** Triplicate experiments from cell lysis to 2D electrophoresis were performed. After 72-h incubation, the culture medium was discarded and the cells were rinsed with ice-cold isotonic buffer (250 mM sucrose, 10 mM Tris-HCl, pH 7.2) for 4 times on ice. Lysis buffer composed of 9 M urea, 4% CHAPS, 10 mM Tris-HCl, 50 mM dithiothreitol (DTT), 0.6% pH 3-10 NL immobilized pH gradient (IPG) buffer, and 1X Complete protease inhibitor (Roche Diagnostics), pH 8.8, was then added to each culture dish. The cells were scraped thoroughly with a scraper and allowed to lyse on ice for 15 min. The cell lysates were collected and centrifuged at 10000  $\times$  g for 20 min at 4°C in order to obtain the clear supernatant without insoluble cell debris. The protein concentration of each cell line was measured using an RC DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Cell lysates were then reduced with 50 mM DTT for 2 h at 23°C followed by alkylation with 150 mM iodoacetamide at 23°C for a further 2 h so that the cell lysates of each cell line were ready for 2D electrophoresis.

**2D electrophoresis.** Fifty micrograms of reduced and alkylated proteins from each cell line were made up to a volume of 180  $\mu$ l with rehydration buffer (9 M Urea, 4% CHAPS, 10 mM Tris-HCl, 50 mM DTT, 0.6% pH 3-10 NL IPG buffer, pH 8.8). Passive rehydration was performed, after which isoelectric focusing (IEF) on 11-cm ReadyStrip™ IPG strips (Bio-Rad) with a non-linear pH range of 3-10 was performed for 20000 volt-hours. After IEF, the IPG strips were incubated with equilibration buffer (6 M Urea, 375 mM Tris-HCl, 20% glycerol, 2% sodium dodecyl sulfate (SDS), 2% DTT, pH 8.8) for 30 min with shaking at room temperature. SDS-polyacrylamide gel electrophoresis (PAGE) was then performed for 220 volt-hours in 4%-12% Bis-Tris Criterion™ XT precast gels (Bio-Rad). The proteins were fixed in the gel

with fixative solution (40% methanol, 10% acetic acid) for 30 min and silver staining was performed using the PlusOne™ Silver Staining kit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to the manufacturer's instructions.

**Image analysis.** The stained gels were scanned using a Model GS-700 imaging densitometer (Bio-Rad) and analyzed using PDQuest™ 2D analysis software (Bio-Rad). Total intensity in valid spots was used as the normalization method in the analysis. Spots differing by  $\geq$ 2-fold with an intra-spot covariance <20% were considered as proteins with differential expression.

**In-gel protein digestion.** Fourteen differentially expressed protein spots with the highest fold change were selected for protein identification. The selected spots were excised from the gel and transferred to siliconized tubes pre-rinsed 3 times with a solution containing 50% acetonitrile (ACN), 40% methanol and 0.1% trifluoroacetic acid (TFA). After SDS removal and destaining of silver, the gel pieces were incubated with a trypsin solution containing 25  $\mu$ g/ml sequencing-grade trypsin (Promega Corporation, Madison, WI, USA), 40 mM ammonium bicarbonate and 10% ACN. Trypsin digestion was allowed to proceed at 37°C for 15 h and peptides were harvested twice with 20  $\mu$ l 1% TFA solution followed by 20  $\mu$ l ACN solution.

**Peptide mass fingerprinting (PMF) using mass spectrometry.** Peptides were purified using ZipTip™ C18 microcolumns (Millipore Corporation, Billerica, MA, USA) according to the manufacturer's instructions and eluted in 2  $\mu$ l solution containing 80% ACN and 0.1% TFA. The eluted peptides were then spotted onto a 400-spot Teflon-coated sample plate (Applied Biosystems, Foster City, CA, USA) with  $\alpha$ -cyano-4-hydroxycinnamic acid as a matrix using the 'Sandwich' method. Samples were irradiated with a laser using a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (Voyager DE™ -Pro, Applied Biosystems) in reflector mode with external calibration using a Sequazyme™ peptide mass standards kit (Applied Biosystems) and mass spectra were acquired. Monoisotopic mass values from the spectra were submitted to the Aldente (<http://www.expasy.org/tools/aldente/>) and Mascot (<http://www.matrixscience.com/>) search engines for protein identification.

**Agar block preparation for the SW480 cell line.** The cells harvested after trypsinization were fixed in 10% buffered formalin for 18 h followed by centrifuging at 1560  $\times$  g for 10 min. The supernatant was discarded and 500  $\mu$ l of 2% agar was added to the cell pellet. The mixture was then placed on ice to solidify the agar with the cells followed by routine tissue processing to produce paraffin-embedded blocks for immunocytochemical (ICC) staining.

**Antibodies.** Rabbit polyclonal anti-cyclophilin A antibody (PC270, Calbiochem, San Diego, CA, USA); mouse monoclonal anti-cytokeratin 8 antibody (M0631, Clone 35BH11, DakoCytomation, Glostrup, Denmark); mouse monoclonal anti-heat shock protein 27 antibody (SPA-800, Clone G3.1, Stressgen Bioreagents Corporation, Victoria, BC,

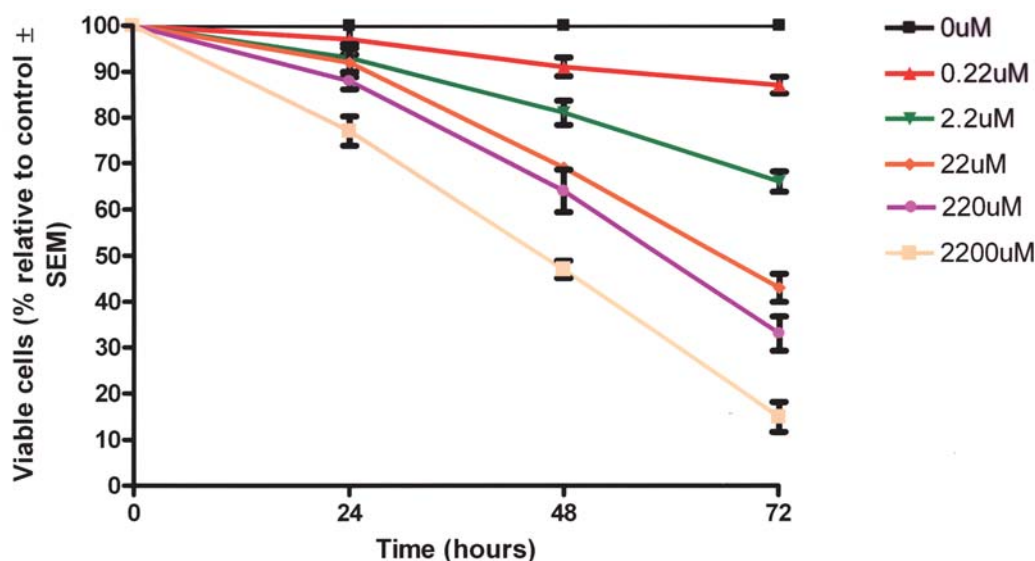


Figure 1. Dose response curve of SW480 cells to 5-FU.

Canada); mouse anti-peroxiredoxin 6 antibody (MAB3478, Clone 8H11, Chemicon International Inc, Temecula, CA, USA); mouse monoclonal anti-cytokeratin 18 antibody (M7010, Clone DC 10, DakoCytomation); mouse monoclonal anti- $\alpha$ -enolase antibody (H00002023-M01, Clone 8G8, Abnova Corporation, Taipei, Taiwan); and mouse monoclonal anti-heat shock protein 70 antibody (SPA-810, Clone C92F3A-5, Stressgen Bioreagents Corporation) were used.

**Immunocytochemical staining and evaluation.** Serial tissue sections (4- $\mu$ m thick) were cut and antigen retrieval was performed using Bond Epitope Retrieval Solution 2 on the Bond-max automated immunostainer (Vision BioSystems, Mount Waverley, Australia) at 100°C for 25 min. Primary antibody dilutions were prepared as follows: cyclophilin A, 1:150; cytokeratin 8 (CK8), 1:100; heat shock protein 27 (hsp27), 1:400; peroxiredoxin 6 (Prx 6), 1:250; cytokeratin 18 (CK18), 1:100;  $\alpha$ -enolase (ENO-1), 1:15000 and heat shock protein 70 (hsp70), 1:300. Staining was performed according to a standard protocol in the immunostainer. The polymer detection system was selected to avoid the problem of non-specific endogenous biotin staining. Appropriate positive controls were used: non-small cell lung cancer cells for cyclophilin A; benign colon epithelial cells for CK8 and CK18; breast cancer cells for hsp27, ENO-1 and hsp70; and benign prostate glandular epithelial cells for Prx 6. A positive control tissue was mounted on every test slide and negative controls were performed by replacing the antibody with Tris buffered saline. The stained slides were evaluated in 5 fields under a light microscope at x400 magnification by 2 independent observers. All slides were scored semi-quantitatively and expressed as an ICC score by multiplying the percentage of positive cells and the staining intensity, as described previously (12). Staining intensity was scored as follows: 0, negative; 1, weak; 2, moderate; 3, strong; and 4, very strong. The ICC score ranged from 0 to 400.

**Statistical analysis.** The  $IC_{50}$  value of 5-FU in SW480 cells was calculated using the log (inhibitor) against response equation.

The differences in ICC scores of anti-cyclophilin A, anti-CK8, anti-hsp27, anti-Prx 6, anti-CK18, anti-ENO-1 and anti-hsp70 stainings between 5-FU-treated and untreated SW480 cells were studied using the Wilcoxon matched pairs test which is a nonparametric test that compares 2 paired groups. GraphPad Prism software version 5.0 (GraphPad, Software Inc, San Diego, CA, USA) was used for all statistical analyses and  $P < 0.05$  was considered significant.

## Results

**Dose response curve of SW480 cells to 5-FU.** 5-FU induced both time- and dose-dependent growth inhibition in SW480 cells (Fig. 1). The calculated  $IC_{50}$  value after 72-h incubation with 5-FU was  $16.3 \pm 2.1 \mu$ M. Therefore the SW480 cells were incubated with  $16.3 \mu$ M of 5-FU for 72 h in all experiments.

**Identification of differentially expressed proteins between 5-FU treated and control SW480 cells by 2D electrophoresis and mass spectrometry.** One hundred and fourteen spots were upregulated whereas 90 spots were downregulated with at least 2-fold differential differences between the 5-FU-treated and control SW480 cell line after 2D electrophoresis and image analysis. Among these, 6 upregulated spots and 8 downregulated spots were digested by trypsin followed by protein identification using mass spectrometry. Identified upregulated proteins were cyclophilin A (2.8-fold), CK19 (2.8-fold), CK8 (4.5-fold), ras-related nuclear protein (RAN) (5.3-fold), hsp27 (8.6-fold) and Prx 6 (10.9-fold). Downregulated proteins included hsp60 (3.2-fold), CK18 (3.6-fold), CK9 (4.0-fold), carbamoylphosphate synthetase I (CPS1) (4.0-fold), ENO-1 (4.2-fold), hsp70 (8.9-fold), nm23 (5.9-fold) and  $\beta$ -actin protein (10.0-fold) (Fig. 2). The identity of proteins was the same from the Aldente and Mascot search engines and their biological properties and functions are shown in Table I.

**Confirmation of proteomic findings using immunocytochemical stainings in paraffin-embedded sections of 5-FU-treated and control SW480 cells.** Seven antibodies, including



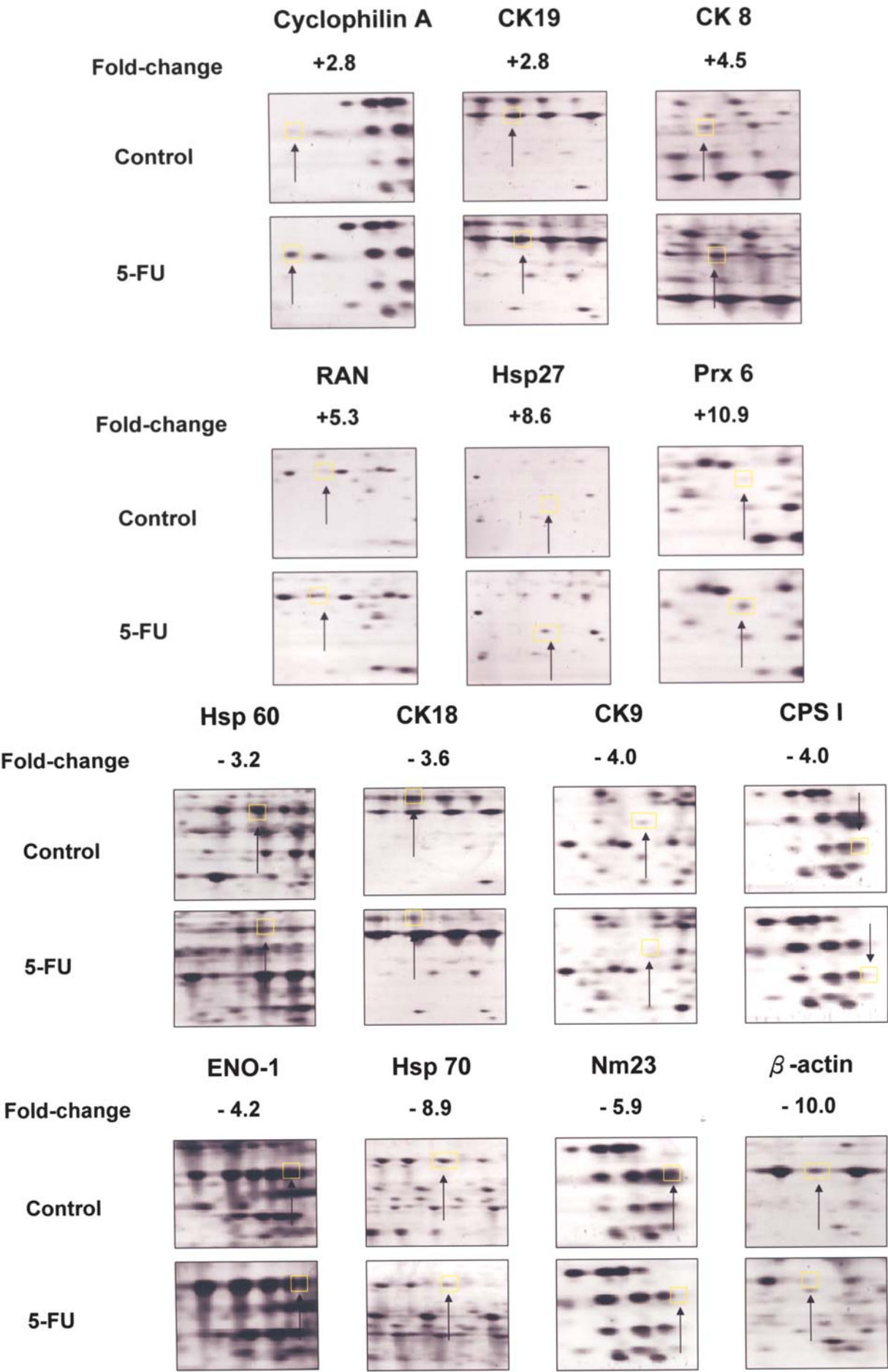


Figure 2. Differentially expressed proteins between 5-FU-treated and control SW480 cells after 2D electrophoresis in triplicate gels.

Table I. Identity of proteins with their biological properties and functions.

Swiss-Prot accession number	Protein identified	M.W. (Da)	Pi	Fold-change	Function
Upregulated proteins					
P62937	Cyclophilin A	18,217	7.68	2.8	Peptidyl-prolyl isomerase
P08727	CK19	44,079	5.04	2.8	Cytoskeleton protein
P05787	CK8	53,529	5.52	4.5	Cytoskeleton protein
P62826	RAN	24,609	7.01	5.3	GTPase
P04792	Hsp27	22,427	7.83	8.6	Molecular chaperone
P30041	Prx6	25,133	6.00	10.9	Anti-oxidant enzyme
Downregulated proteins					
P10809	Hsp60	61,157	5.70	3.2	Molecular chaperone
P05783	CK18	47,305	5.27	3.6	Cytoskeleton protein
P35527	CK9	62,320	5.19	4.0	Cytoskeleton protein
P31327	CPSI	139,341	5.16	4.0	Urea cycle enzyme
P06733	ENO-I	47,481	7.00	4.2	Glycolytic enzyme
P11142	Hsp70	53,580	5.62	8.9	Molecular chaperone
P22392	Nm23	17,270	8.55	5.9	Nucleoside diphosphate kinase
P60709	B-actin	40,536	5.55	10.0	Cytoskeleton protein

M.W., molecular weight; Da, Dalton; pi, isoelectric point.

anti-cyclophilin A, anti-CK8, anti-hsp27, anti-Prx 6, anti-CK18, anti-ENO-1 and anti-hsp70, were successfully optimized with strong signals and a clean background. Cellular localization of the 7 proteins was as follows: nucleus and cytoplasmic expression for cyclophilin A, ENO-1 and hsp70; cytoplasmic expression for hsp27 and Prx 6; and cytoskeletal network for CK8 and CK18. In general, the percentage of positive cells and staining intensity of anti-hsp27 and anti-Prx 6 were higher in 5-FU-treated than control SW480 cells (Fig. 3A-D). On the other hand, the percentage of positive cells and staining intensity of anti-hsp70 were lower in 5-FU-treated than control SW480 cells (Fig. 3E and F). For anti-cyclophilin A, anti-CK8, anti-CK18 and anti-ENO-1 staining, a similar staining pattern was observed (Fig. 3G-N). The ICC scores for these 7 antibody stainings in 5-FU-treated and control SW480 cells are shown in Fig. 4. The differences in ICC scores between 5-FU-treated and control SW480 cells for hsp27, Prx 6 and hsp70 stainings were significant ( $P < 0.05$ , Wilcoxon matched pairs test). For CK19, RAN, hsp60, CK9, CPS1, nm23 and  $\beta$ -actin proteins, stainings were not performed because either antibodies were not available or they could not be optimized well in the immunostainers.

## Discussion

Early detection of 5-FU response proteins would greatly enhance the effectiveness of treatment strategies and CRC patients with no response to 5-FU could be administered a different chemotherapeutic drug promptly. In the past, research at the protein level was hampered by the complexity of the proteome. Rapid development of the proteomic technologies integrated with advanced bioinformatics tools in recent years

has changed the situation and allows the comparison of thousands of proteins simultaneously. In this study, the change in protein expression in the CRC cell line SW480 after 5-FU treatment was studied using 2D electrophoresis and the identity of proteins was examined using mass spectrometry. Six up-regulated and 8 downregulated proteins were identified which belong to various families with different functions including cytoskeleton (CK19, CK8, CK18, CK9 and  $\beta$ -actin), molecular chaperones (hsp27, hsp60 and hsp70), peptidyl-prolyl isomerase (cyclophilin A), GTPase (RAN), anti-oxidant enzyme (Prx 6), urea cycle enzyme (CPS I), glycolytic enzyme ( $\alpha$ -enolase) and nucleoside diphosphate kinase (nm23). Using ICC staining, three (hsp27, Prx 6 and hsp70) of the 7 proteins were found to have the same expression pattern as that from 2D electrophoresis whereas the remaining 4 proteins (cyclophilin A, CK8, CK18 and ENO-1) did not have any significant difference after 5-FU treatment. The fold changes, indicated by 2D electrophoresis, of the 3 proteins with significant differences in percentage of positive cells and staining intensity ranged from 8.6 to 10.9 (hsp27, Prx 6, hsp70) whereas the fold changes from the other 4 proteins without any significant difference in ICC staining ranged from 2.8 to 4.5 (cyclophilin A, CK8, CK18 and ENO-1). Therefore we speculate that ICC staining may not be sensitive enough to detect a smaller fold change indicated by 2D electrophoresis and a more sensitive ICC detection system should be developed in the future so that proteins with smaller fold change can be confirmed. ICC detection is very important for confirmation of differentially expressed proteins detected from proteomics because not only the percentage of positive cells and the staining intensity can be assessed, but also the cellular origin with the localization of the differentially expressed protein

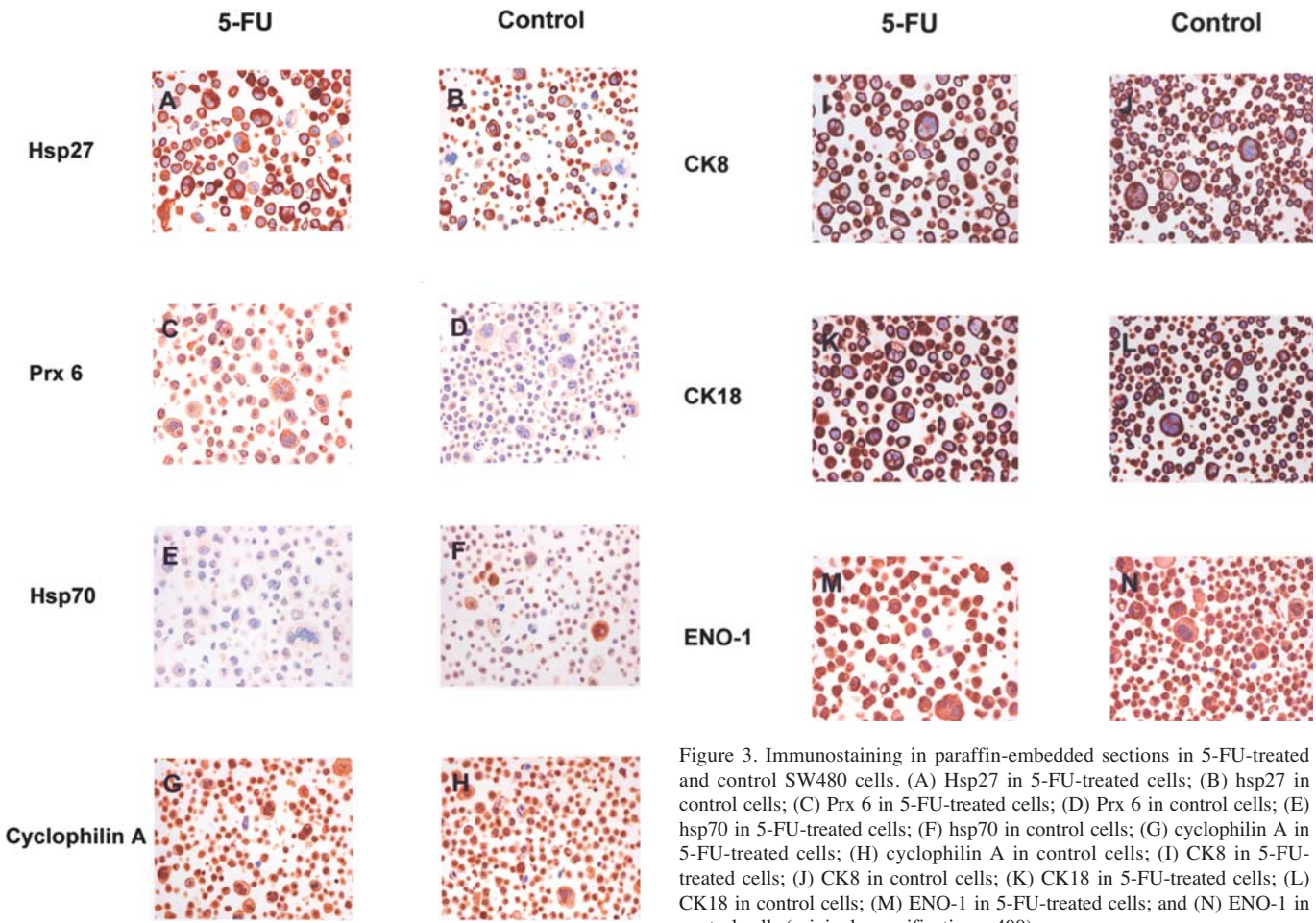


Figure 3. Immunostaining in paraffin-embedded sections in 5-FU-treated and control SW480 cells. (A) Hsp27 in 5-FU-treated cells; (B) hsp27 in control cells; (C) Prx 6 in 5-FU-treated cells; (D) Prx 6 in control cells; (E) hsp70 in 5-FU-treated cells; (F) hsp70 in control cells; (G) cyclophilin A in 5-FU-treated cells; (H) cyclophilin A in control cells; (I) CK8 in 5-FU-treated cells; (J) CK8 in control cells; (K) CK18 in 5-FU-treated cells; (L) CK18 in control cells; (M) ENO-1 in 5-FU-treated cells; and (N) ENO-1 in control cells (original magnification, x400).

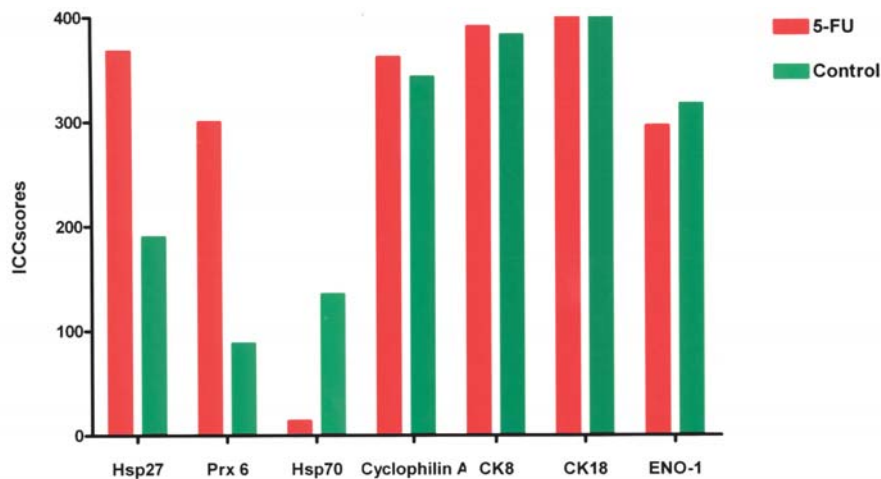


Figure 4. ICC scores of hsp27, Prx 6, hsp70, cyclophilin A, CK8, CK18 and ENO-1 in 5-FU-treated and control SW480 cells.

can be examined under a microscope. A description of each of the differentially expressed proteins indicated from mass spectrometry is shown below.

*Upregulated proteins.* Cyclophilin A is a protein that possesses peptidyl-prolyl isomerase activity (13). It is essential for T-cell activation and can form strong interactions with HIV-1 gag proteins (14). It is implicated in many cellular processes

including trafficking, signal transduction, cell cycle regulation, differentiation, transcriptional regulation and stabilization of multiprotein complexes (15). Cyclophilin A is reported to be overexpressed in CRC, small-cell lung cancer and pancreatic cancer (16,17).

CK19 is a member of the family of intermediate filaments which is expressed in all epithelial cells including pancreatic islet cells from 12 to 16 weeks of fetal development. Thereafter,



its expression decreases and eventually disappears from most islet cells but remains strong in pancreatic ductal cells (18). Recent study has indicated that CK19 is a powerful predictor of survival in pancreatic endocrine tumors (19) and is helpful in the differential diagnosis of true papillary carcinoma of thyroid and papillary carcinoma-like changes in Graves' disease (20). On the other hand, circulating CK19 protein or mRNA fragments have been used as a prognostic marker in non-small cell lung (21), breast (22), ovarian (23), bladder (24) and liver (25) cancer.

CK8 is not expressed in healthy epithelium except for a weak expression in the liver and heart (26). During mitosis, membrane CK8 can exhibit a re-distribution intracellularly by undergoing phosphorylation at serine 73 and it relocates to the plasma membrane of the cancer cells, which represents a valuable target for anti-cancer therapy (27). CK8 is homogeneously expressed in head and neck, lung, breast, cervix, and colon carcinoma cells (26) and circulating CK8 protein expression is significantly associated with tumor progression and decreased survival in patients with non-small cell lung cancer (28).

The RAN protein is the most abundant small GTPase in the cell and is best known for its function in nucleocytoplasmic transport of both RNA and proteins (29). Although overexpression of RAN may greatly alter cell cycle kinetics and cell viability (30), its involvement in cancer is still being explored. A recent report has indicated that RAN is up-regulated in over 80% of nasopharyngeal carcinoma tissues (31). Moreover, it has been suggested that RAN may be involved in prostate cancer formation (32).

Hsp27 is expressed in response to a wide variety of physiological and environmental stress conditions including anticancer therapy (33). It is a molecular chaperone that can prevent cell death caused by toxic agents to promote apoptosis (34). Another cytoprotective effect of hsp27 is to increase the tumorigenic potential of cancer cells (34). Previous reports have shown that hsp27 would increase the risk of malignant progression in benign proliferative breast lesions (35) and it is a prognostic marker in breast (36), prostate (36), liver (37), and esophageal cancer (38). Our results indicate that hsp27 is a potential 5-FU response protein and further investigation of hsp27 expression in CRC specimens before and after 5-FU treatment should be carried out. In fact, hsp27 is a good predictor of response for esophageal squamous cell carcinoma (ESCC) using 5-FU in chemo-radiotherapy (39).

Prxs are a recently characterized group of stress inducible, thiol-containing proteins with efficient antioxidant capacity by reducing hydrogen peroxide to water (39). The mammalian Prx family is divided into 6 groups (Prx I-VI) located in the cytoplasm and involved in the cell signaling system (40). Prx 6 is overexpressed in mesothelioma (41) and correlates with the development, recurrence and progression of bladder cancer (42). In fact, using the same proteomics-based approach, Prx 6 is identified as a novel serum marker in ESCC (43). To our knowledge, this is the first report of an upregulation of Prx 6 after 5-FU treatment in SW480 cells and this study confirms that Prx 6 is a potential 5-FU response protein. Further evaluation of Prx 6 expression in 5-FU-treated CRC patient samples is highly recommended in order to explore its potential in chemotherapeutic applications.

**Downregulated proteins.** Hsp60 is a mitochondrial protein involved in protein folding, transport, secretion and can activate apoptosis (44). Its overexpression has been reported in exocervix, prostate and colorectal carcinogenesis (44). Hsp60 can be induced by photofrin-mediated photodynamic therapy (PDT) in a PDT-induced resistant colon cancer cell line (45) and is a good prognostic marker to ESCC (44) and neoadjuvant chemoradiation in bladder cancer (46).

CK18 is a cytokeratin expressed in embryogenesis and in gall bladder epithelium, mucosa of the small intestine and colon, hepatocytes, eccrine sweat glands, fallopian tube, cervix uteri and endometrium in adults (47). In addition, CK18 is overexpressed in proliferating tissues such as endometriosis (48), liver cirrhosis (49), and malignant tumors in colon (50), breast (51), ovarian, endometrium and cervix (52).

CK9 is expressed in most epidermal keratinocytes of soles and palms but only scanty keratinocytes in other body sites (53). Report on CK9 is very rare and previous study indicates that the function of CK9 is to provide additional reinforcement in the stress-bearing palmo-plantar epidermis (54).

CPS1 is the first mitochondrial enzyme in the urea cycle and it functions to convert toxic ammonia into urea in the liver (55). A genetic mutation of CPS1 has been identified as the cause of neonatal hyperammonemia (55). Apart from liver, CPS1 is only expressed in the enterocytes of the small intestine where it plays a key role in the synthesis of citrulline (55). Previous studies indicate that CPS1 is overexpressed in gastric cancer patients (56) whereas it is underexpressed in hepatocellular carcinoma patients (57).

ENO-1 is 1 of the 3 isoforms in the enolase enzyme family that is involved in glycolytic metabolism (58). ENO-1 is a major form of enolase present in the early stages of embryonic development and is expressed ubiquitously in different types of tissue (58). Recent evidence has shown that ENO-1 is important in tumorigenesis because ENO-1 overexpression is associated with poor prognosis in patients with non-small cell lung cancer (58) and may be involved in cancer invasion and metastasis (59). In contrast, it also has tumor suppressor activity by inducing apoptosis after transfection of ENO-1 mRNA into neuroblastoma cells (60).

Hsp70 is a major stress inducible, cancer-associated, anti-apoptotic protein (61). Increased expression of hsp70 has been reported in renal cell cancer (62), gastric cancer (63), hepatocellular cancer (64), CRC (65) and endometrial cancer (66). Hsp70 expression is associated to imatinib resistance in chronic myeloid leukemia (67) and to curcumin resistance in SW480 cells (68). Similar to hsp27, hsp70 is a good response predictor for ESCC using 5-FU as a chemotherapeutic drug in chemo-radiotherapy and hsp70 is also a biomarker of cellular resistance in the multimodal approaches combining 5-FU and hyperthermia in the treatment of locally advanced prostate carcinoma (39,69). The present data in this study shows that hsp70 is a potential 5-FU response protein and the expression of hsp70 in CRC specimens before and after 5-FU treatment should be examined.

The Nm23 tumor metastasis suppressor gene is found to encode a protein identical to nucleoside diphosphate kinase (NDP kinase), of which 8 different genes (*nm23-H1* to *nm23-H8*) have been identified in humans (70). NDP kinase is

responsible for transferring the  $\gamma$ -phosphates between di- and tri-phosphonucleosides in order to provide cells with high-energy nucleosides other than ATP, and is involved in a variety of different cellular processes (70). The role of nm23 in cancer is still poorly understood. Nm23-H1 is overexpressed in early-stage CRC but not in advanced tumor stages (71). Nm23-H4, nm23-H6 and nm23-H7 are involved in tumor development as they are overexpressed in colon and gastric cancer when compared to the adjacent normal tissues (72).

$\beta$ -actin is the isoform of actin that is expressed in non-muscle cells (73). There is scanty literature reporting  $\beta$ -actin's expression in cancer because it is usually used as a house-keeping gene or protein (74). However, recent studies have indicated that  $\beta$ -actin is overexpressed in many tumor cells especially in actively moving, highly invasive cells and therefore  $\beta$ -actin expression may be related to the metastatic potential of the cancer cell (73,75,76).

In summary, this study is the first to show the effect of 5-FU in CRC SW480 cells. Two hundred and thirty proteins were modulated by 5-FU in SW480 cells. This finding is important because it demonstrates the application of a powerful global proteomic and ICC approach which successfully discovered the upregulation of hsp27 and Prx 6, and down-regulation of hsp70 as 5-FU response proteins. Further study to detect their expression in other 5-FU-sensitive CRC cell lines such as SNU-C4 and LoVo and in 5-FU-resistant cell lines such as HT-29 and ContinB would enable us to determine their potential in predicting 5-FU treatment response. Moreover, detailed investigation of the functional role of the molecular targets identified in this study would improve our understanding of the chemotherapeutic effects of 5-FU and, in the long run, may lead to a more effective chemotherapeutic treatment to this common cancer.

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