

A proteomic profiling of gemcitabine resistance in pancreatic cancer cell lines

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Abstract. Pancreatic cancer is one of the most highly fatal cancers and is generally resistant to chemotherapy. Currently, gemcitabine appears to be the only effective agent for its treatment and is the preferred first-line therapy. However, the clinical impact of gemcitabine remains modest due to a high level of inherent and acquired tumor resistance. We investigated protein expression in gemcitabine-resistant and -sensitive human pancreatic adenocarcinoma cell lines by proteomics. Tumor cell proteins were separated by two-dimensional gel electrophoresis, then the protein spots that showed increased or decreased expression in gemcitabine-sensitive cell lines were identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and Western blotting. Nine out of ten proteins showing differential expression in gemcitabine-resistant and -sensitive cell lines were identified, confirming an increase in heat shock protein 27 (HSP27) and a decrease in nucleophosmin (NPM) in the resistant lines. These results suggest that HSP27 and NPM may play a role in the poor response of pancreatic cancer to gemcitabine.

Introduction

Pancreatic cancer is characterized by its difficulty of diagnosis, aggressiveness and lack of effective nonsurgical therapy, so that only 1-4% of patients with adenocarcinoma of the pancreas survive for more than 5 years after diagnosis (1,2). Surgical resection is the only curative treatment currently available, but only 10-15% of patients have no metastases at the time of tumor detection. Gemcitabine (2'-deoxy-2'-difluorodeoxycytidine, Gemzar) is a deoxycytidine analogue with structural and metabolic similarities to cytarabine. Currently, this nucle-

oside analogue appears to be the only drug to exhibit activity against pancreatic cancer (3). However, the median survival time of patients treated with gemcitabine is only 6.3 months (range 1.6-19.2) (4). Intrinsic or acquired resistance to apoptosis is an important factor in the failure of this agent to control pancreatic cancer (5,6). Better understanding of the cellular and molecular mechanisms involved in gemcitabine resistance is necessary for this drug to be used more effectively; indeed, some reports concerning the mechanism of its resistance have already been published.

Overexpression of *mdr-1*, the gene which encodes P-glycoprotein (P-gP), has been shown to induce resistance to various anticancer drugs (7-9). However, it has been reported that gemcitabine resistance is not mediated by P-gP (10), and that P-gP-overexpressing cells are actually more sensitive to gemcitabine than parental cells (11). Other studies have indicated that the loss of BNIP3 expression is a late event that contributes to the chemoresistance of pancreatic cancer (12), that equilibrative-sensitive nucleoside transporter plays an important role in gemcitabine sensitivity (13) while the overexpression of carcinoembryonic antigen-related cell adhesion molecule 6 can protect pancreatic adenocarcinoma cells against gemcitabine-induced cytotoxicity (14), that Selenoprotein P reduces the intracellular level of reactive oxygen species, resulting in the loss of sensitivity to gemcitabine (15), and that an increase in Src tyrosine kinase activity represents a potential mechanism of chemoresistance (16). However, the results of these studies are controversial.

Proteomic analysis is a powerful tool for the identification of overall differences in protein expression between drug-resistant and -sensitive cells. The aim of this study was to identify proteins showing differential expression in gemcitabine-resistant and -sensitive pancreatic cancer cell lines by using proteomics. Such proteins may potentially play a role in the chemoresistance of pancreatic cancer.

Materials and methods

Cell lines and culture. Three human pancreatic adenocarcinoma cell lines (MiaPaCa-2, BxPC-3 and AsPC-1) were purchased from the American Type Culture Collection. Panc-1, PK45p and PK59 were kindly provided by the Institute of Development, Aging and Cancer at Tohoku University. Panc-1

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Table I. The cytotoxicity of gemcitabine in the pancreatic cancer cell lines.

	Cell lines					
	MiaPaCa-2	Panc-1	BxPC-3	AsPC-1	PK45p	PK59
IC ₅₀ (μ g/ml)	6.81	8.07	6.67	1.05	417.45	294.72

The cytotoxicity of gemcitabine in the pancreatic cancer cell lines was evaluated by MTT assay. IC₅₀ values were calculated based on the assay results. MiaPaCa-2, Panc-1, AsPC-1 and BxPC-3 showed high sensitivity to gemcitabine (gemcitabine-sensitive), whereas PK45p and PK59 were much less sensitive (gemcitabine-resistant). Comparable results were obtained from three repetitions of the experiment.

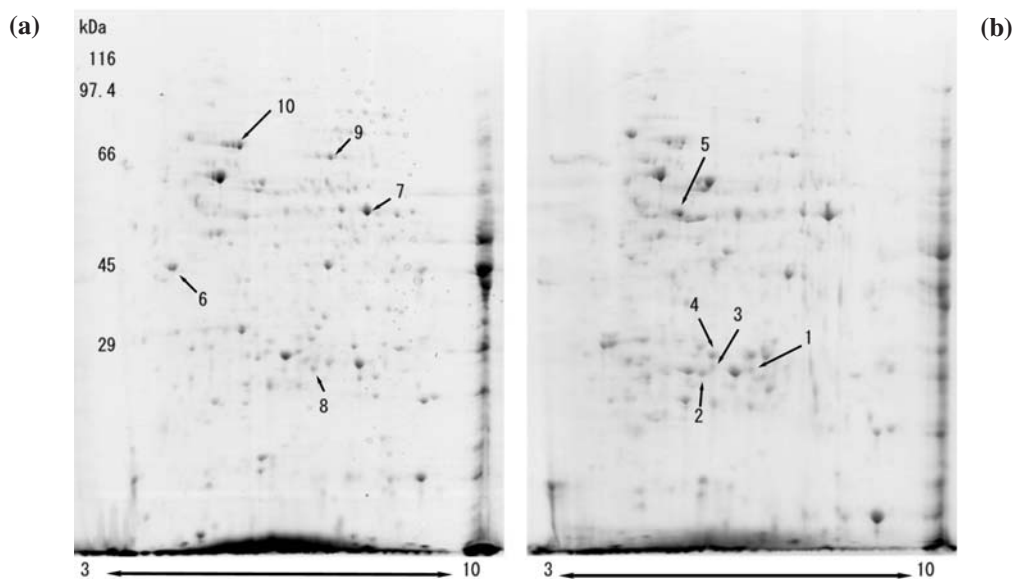


Figure 1. 2-DE gel pattern of (a) MiaPaCa-2 (gemcitabine-sensitive cell line) and (b) PK59 (gemcitabine-resistant cell line). Proteins (300 μ g) were separated on 12.5% non-gradient polyacrylamide gels and stained with CBB. Five spots (nos. 6-10) were more weakly expressed in gemcitabine-resistant than -sensitive cells. Five spots (nos. 1-5) had higher expression levels in gemcitabine-resistant than -sensitive cells.

and MiaPaCa-2 cells were grown in Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose and 10% FCS. BxPC-3, AsPC-1, PK45p and PK59 cells were grown in RPMI-1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, 1.0 mM sodium pyruvate and 10% FCS. Cells were incubated in a humidified atmosphere containing 5% CO₂ at 37°C. Culturing was conducted without endotoxin.

Cell proliferation assay. Cells from each cell line (MiaPaCa-2, Panc-1, BxPC-3, AsPC-1, PK45p and PK59) were seeded into 96-well plates at a density of 1,000-5,000/well, incubated for 24 h and exposed to various concentrations of gemcitabine for 72 h. Then, 10 μ l of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay) was added to each well, and further incubation was carried out for 4 h. Next, the formazan crystal product was dissolved by adding 100 μ l of DMSO and incubating the plates for 1 h with protection from light to completely dissolve the crystals. Finally, the absorbance was measured at a wavelength of 570 nm with an ELISA plate reader (Model 550 Microplate Reader; Bio-Rad, Hercules, CA). It was confirmed that the absorbance showed a linear relationship with the number of cells. The experiments were repeated three times.

Sample preparation. The suspensions of cultured cells were centrifuged at 1,500 rpm for 5 min. Each pellet was then washed three times with 10 mM PBS (-), pH 7.4, and was lysed in lysis buffer (1% NP-40, 1 mM sodium vanadate, 1 mM PMSF, 50 mM Tris, 10 mM NaF, 10 mM EDTA, 165 mM NaCl, 10 μ g/ml leupeptin and 10 μ g/ml aprotinin) using a Potter homogenizer with a teflon coating at 4°C for 1 h. The lysate was separated by centrifugation at 15,000 \times g for 30 min, and the supernatant was stored at -80°C. Protein samples for each cell line were obtained three times.

Two-dimensional gel electrophoresis. Protein (300 μ g) was used for each two-dimensional gel electrophoresis (2-DE) assay. First-dimension IEF was performed on 7-cm immobilized strips with a linear pH gradient of 3-10 (GE Healthcare Bio-Science Corp., Piscataway, NJ) at 20°C and 50 mA. The strips were then rehydrated with 125 μ l of sample solution (8 M urea, 2% CHAPS and 0.5% IPG buffer) for 14 h. IEF was performed in three steps; 500 V for 1 h, 1,000 V for 1 h and 8,000 V for 2 h. Voltage increases were carried out according to a gradient. The second-dimension was run on precast polyacrylamide gels (2-D homogeneous 12.5; GE Healthcare) in two steps; 600 V, 20 mA for 30 min and 600 V, 50 mA for 70 min. After electrophoresis, the gels were stained with CBB R-250 (Nacalai Tesque, Kyoto, Japan) for

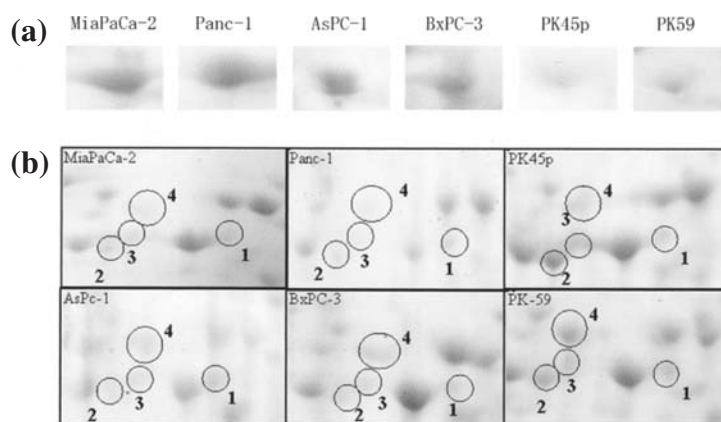


Figure 2. Comparison of spots in gemcitabine-sensitive cells (MiaPaCa-2, Panc-1, BxPC-3, AsPC-1) and gemcitabine-resistant cells (PK45p, PK59). The spot numbers correspond to those in Fig. 1. (a) Downregulated expression spots in gemcitabine-resistant cells. This protein was nucleophosmin. (b) Upregulated expression spots in gemcitabine-sensitive cells. Spots 1-3 were heat shock protein. Spot 4 was 6-phosphogluconolactonase.

24 h. Subsequently, they were de-stained with 10% acetic acid in water containing 30% methanol for 30 min and then stained with 7% acetic acid and used for in-gel digestion. Similar experiments for all cell lines were repeated in triplicate.

Image analysis. The positions of the protein spots on the gels obtained using samples of KLM1 and KLM1-R cells were recorded with an Agfa Arcus 1200 Image Scanner (Agfa-Gevaert N.V., Mortsel, Belgium) and were analyzed with Progenesis software (Progenesis PG240; Perkin-Elmer Inc., Wellesley, MA). Spots that were visible at different intensities were excised from the gels and stored in 100 μ l of ultrapure water at 80°C as samples for MS analysis.

In-gel digestion. After cutting the target protein spots from the gels, CBB dye was removed by rinsing three times in 60% methanol, 50 mM ammonium bicarbonate and 5 mM DTT for 15 min, and twice in 50% CAN, 50 mM ammonium bicarbonate and 5 mM DTT for 10 min. The gel pieces were dehydrated in 100% acetonitrile (ACN) twice for 30 min each, and then rehydrated with an in-gel digestion reagent containing 10 μ g/ml of sequencing grade modified trypsin (Promega, Madison, WI) in 30% ACN, 50 mM ammonium bicarbonate and 5 mM DTT. In-gel digestion was performed overnight at 30°C. The samples were rinsed in 30% ACN, 50 mM ammonium bicarbonate and 5 mM DTT for 2 h and lyophilized overnight at -30°C.

Liquid chromatography-tandem mass spectrometry. Lyophilized samples were dissolved in 20 ml of 0.1% formic acid and centrifuged at 15,000 \times g for 5 min. Sequencing of the identified protein spots was performed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with a Spectrum Mill MS Proteomics Workbench (Agilent Technologies, Santa Clara, CA, USA).

Western blot analysis. Samples (30 μ g) were separated by SDS-PAGE at 15 mA, and then proteins on the gels were transferred electrophoretically to PVDF membranes (Immobilon-P; Millipore, Bedford, MA) that were blocked overnight at 4°C with TBS containing 5% skim milk. Primary antibodies were anti-heat shock protein 27 (HSP27) monoclonal antibody

(1:600) and anti-B23 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were incubated with the primary antibody for 1 h at room temperature, washed three times with TBS containing 0.05% Tween-20, washed once with TBS and then incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody (1:2000) (ICN Pharmaceuticals, Aurora, OH). Final development was performed with a chemiluminescence reagent (ECL Western Blotting Detection Reagents; Amersham Pharmacia Biotechnology).

Results

Selection of pancreatic cancer cell lines by gemcitabine exposure. To evaluate the cytotoxicity of gemcitabine in the pancreatic cancer cell lines, MiaPaCa-2, Panc-1, AsPC-1, BxPC-3, PK45p and PK59 cells were exposed to different concentrations of the drug for 72 h. Based on the measurements obtained, the IC₅₀ value was defined as the concentration of gemcitabine causing 50% inhibition of growth (Table I). Four cell lines, MiaPaCa-2, Panc-1, AsPC-1 and BxPC-3, showed a high sensitivity to gemcitabine with IC₅₀ values of, respectively, 6.81, 8.07, 1.05 and 6.67 μ g/ml (Table I). In contrast, PK45p and PK59 cells were much less sensitive. Their IC₅₀ values were 417.45 and 294.72 μ g/ml, respectively (Table I). The cell lines were classified into two groups: gemcitabine-sensitive cells (MiaPaCa-2, Panc-1, AsPC-1 and BxPC-3) and gemcitabine-resistant cells (PK45p and PK59).

Detection and identification of proteins in two-dimensional electrophoresis gels. Protein expression was assessed in three samples each from MiaPaCa-2, Panc-1, AsPC-1, BxPC-3, PK45p and PK59 cells cultured under the same conditions. More than 500 spots were visualized on the 2-DE gels, and differences in intensity between the gemcitabine-resistant and -sensitive cells were compared visually and analyzed with Image Master. Five of the spots showed a decrease in protein content in gemcitabine-resistant cell lines (nos. 6-10), while the other five showed an increase (nos. 1-5) (Fig. 1). The comparison between each spot is shown in Fig. 2. After the ten spots were cut out from each gel, analysis and identification were conducted by LC-MS/MS analysis. Information about the

Table II. Identification of differentially-expressed proteins in gemcitabine-resistant and -sensitive cells.

Protein no. ^a	Molecular mass (Da)	pI	Protein identification	Sensitive cells ^b (Rate average ± SD)	Resistant cells ^b (Rate average ± SD)	p-value ^c
1	22,782	5.98	Heat shock protein 27	0.97±0.75	1.41±0.87	0.310
	28,993	6.77	ERp29 precursor			
2	22,787	5.98	Heat shock protein 27	1.11±0.39	4.51±3.68	0.048
3	22,787	5.98	Heat shock protein 27	0.71±0.52	2.25±1.97	0.019
4	27,547	5.70	6-phosphogluconolactonase	0.51±0.48	1.48±0.79	0.001
5	69,267	5.95	Ezrin (p81)(cytovillin)(villin)	0.81±0.37	6.26±8.73	0.460
6	32,575	4.64	Nucleophosmin	0.93±0.41	0.37±0.28	0.008
7	47,038	6.99	α enolase	1.97±1.41	0.51±0.48	0.027
8	26,538	6.51	Triosephosphate isomerase	0.96±0.52	0.74±0.78	0.470
9	62,639	6.40	Stress-induced-phosphoprotein 1	1.22±0.62	0.73±0.42	1.000
	74,139	6.57	Lamin A/C (70 kDa lamin)			
10	-	-	Unidentified	-	-	-

^aSpot numbers corresponding to those in Fig. 1. ^bThe percentage of spot intensity of each cell line to MiaPaCa-2. ^cDifferences in expression between gemcitabine-sensitive and -resistant cells analyzed by the Student's t-test.

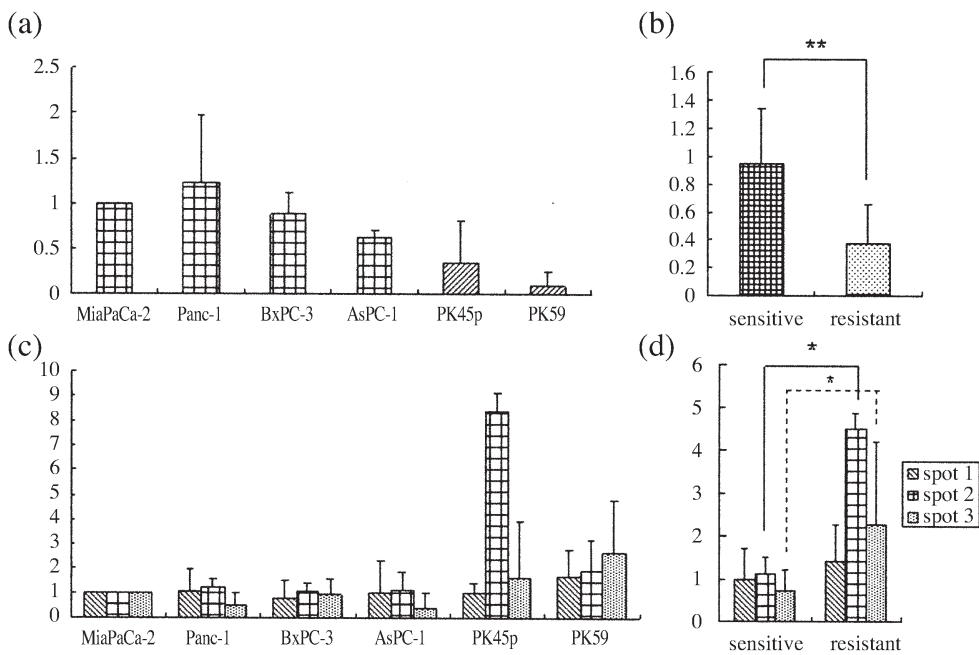


Figure 3. Expression analysis of nucleophosmin (a and b) and HSP27 (c and d). Expression levels of proteins were quantified by analyzing the percentage of volume, the intensity and the area of each spot (MiaPaCa-2 was 1.00). The y-axis shows the percentage of volume; the x-axis, each cell line. Differences in expression between sensitive and resistant cells were analyzed by the Student's t-test; *p<0.05, **p<0.01. Data are expressed as the mean of each protein ± SD.

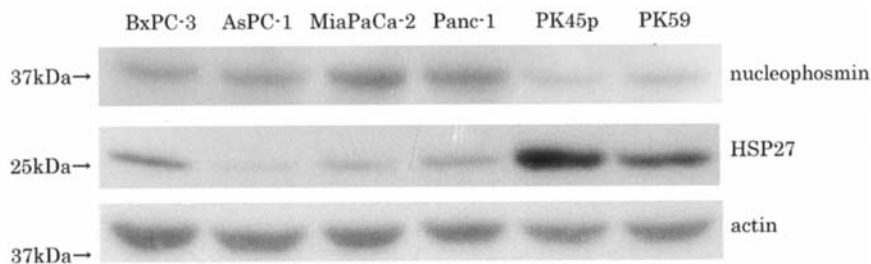



Figure 4. Immunoblot analysis of nucleophosmin and HSP27. Expression of nucleophosmin and HSP27 was confirmed. The intensity of the spot of nucleophosmin was weaker in gemcitabine-resistant cells, and the intensity of the spot of HSP27 was stronger.

 ins thus identified is summarized in Table II. HSP27 phosphoinositide 3-kinase (PI3K) demonstrated a significant change in their ratios, as shown in Fig. 3 (Student's t-test, $p < 0.05$).

Western blot analysis of differentially-expressed proteins. The expression of two proteins (HSP27 and NPM) for which antibodies were available was confirmed by immunoblotting. HSP27 was upregulated and NPM downregulated in PK45p and PK59 (Fig. 4). The expression of ezrin, triosephosphate isomerase and α -enolase was not confirmed by immunoblotting (data not shown).

Discussion

In the present study, proteomic analysis showed that HSP27 expression was increased and NPM expression decreased in gemcitabine-resistant cell lines.

HSP27 belongs to the family of small heat shock proteins, molecular chaperones that modulate the response of cells to injury and are found in virtually all organisms, from prokaryotes to mammals (17). A previous study demonstrated that HSP27 regulates apoptosis by interacting with key components of the apoptotic signaling pathway (18), and inhibits etoposide-induced apoptosis by preventing cytochrome c and dATP-triggered activation of caspase-9, which occurs downstream of cytochrome c release (19,20). The increase of an anti-apoptotic factor such as HSP27 would enhance the ability of cells to resist chemotherapy. In fact, HSP27 overexpression inhibits the doxorubicin-induced apoptosis of human breast cancer cells (21), as well as inhibiting the etoposide-, diethylmaleate-, cycloheximide- or radiation-induced apoptosis of prostate cancer cells (22), and the etoposide-induced apoptosis of neuroblastoma cells (23). We did not examine expression with the regulation of HSP27, but it has been reported that when HSP27 is knocked down by siRNA 5-fluorouracil sensitivity in colon cancer is increased (24). As with these cancers, the present study revealed that HSP27 was also overexpressed by gemcitabine-resistant tumor cell lines, suggesting that pancreatic carcinoma may acquire gemcitabine resistance via the anti-apoptotic activity of HSP27. Accordingly, HSP27 might play an important role in tumor resistance to gemcitabine.

NPM is a multifunctional protein involved in several aspects of nucleolar function. This protein plays a role in the processing of pre-rRNA (25), and also acts as a histone chaperone (26). The expression of NPM is downregulated in cells undergoing differentiation or apoptosis (27). Berberine- and retinoic acid-induced apoptosis of human leukemia cells is associated with the downregulation of NPM expression and telomerase activity (28,29). In contrast, our study showed that NPM was decreased in gemcitabine-resistant cells. Gao *et al* reported that NPM regulates the nuclear translocation of GADD45a and contributes to GADD45a-induced cell cycle arrest in the G2-M phase (30), but that gemcitabine-induced apoptosis is not related to G2-M arrest. Thus, NPM may regulate the apoptosis of pancreatic adenocarcinoma cells by other mechanisms.

Apoptosis-regulating proteins from the bcl-2 family and P-gP have been reported to participate in resistance to chemotherapy (5,31-33), but differences in the expression of these proteins in gemcitabine-sensitive and -resistant cells were not

demonstrated in our study. Four proteins besides NPM were decreased and three proteins besides HSP27 were increased in the gemcitabine-resistant cell lines, suggesting that various proteins may participate in modulating gemcitabine sensitivity, in addition to NPM and HSP27. Therefore, further study of these proteins needs to be performed using surgical specimens, and blood levels of HSP27 should be examined in cancer patients as a possible marker for pancreatic cancer.

In conclusion, proteomic analysis was useful for the detection of intracellular proteins with differential expression in pancreatic adenocarcinoma cell lines sensitive or resistant to gemcitabine. Such proteins may be involved in the mechanism of resistance to chemotherapy, and could also be indicators of response to pancreatic cancer therapy.

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