Identification of Up- and Down-regulated Proteins in Gemcitabine-resistant Pancreatic Cancer Cells Using Two-dimensional Gel Electrophoresis and Mass Spectrometry

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Abstract. *The prognosis of patients with pancreatic cancer is very poor because of late diagnosis and the lack of response to various therapies. Pancreatic cancer is generally resistant to chemotherapy and is highly fatal. Gemcitabine (GEM) appears to be the only effective agent for treatment of pancreatic cancer. However, a high level of inherent and acquired tumor resistance makes the clinical impact of GEM modest. Proteomic differential display analysis for GEM-sensitive human pancreatic adenocarcinoma cell line KLM1 and GEM-resistant KLM1-R cells by using two-dimensional gel electrophoresis and liquid chromatography tandem mass spectrometry produced 33 protein spots. Of these, 23 were up-regulated and 10 were down-regulated in KLM1-R compared to KLM1 cells. The up-regulated proteins include acidic leucine-rich nuclear phosphoprotein 32 family member A, reticulocalbin-1, gamma-synuclein, microtubule-associated protein RP/EB family, sialic acid synthase, peptidyl-prolyl cis-trans isomerase A, far upstream element-binding protein 2 and catalase. The down-regulated proteins include far upstream element-binding protein 1, gamma-synuclein, galectin-1 and stathmin. Two spots of heat-shock protein 27 were up-regulated in KLM1-R cells. These results suggest an important complementary role for proteomics in the identification of proteins which may play a role in the poor response of pancreatic cancer to GEM.*

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The prognosis of patients with pancreatic cancer is still very poor because of its aggressiveness and lack of early diagnosis and effective therapies (1). Surgical resection is the only curative therapy, but the disease has usually already progressed by the time of diagnosis, hence only 1-4% of patients with adenocarcinoma of the pancreas survive for more than 5 years after diagnosis (2, 3). Gemcitabine (2'-deoxy-2'-difluoro-deoxycytidine, Gemzar) (GEM) is a deoxycytidine analogue with structural and metabolic similarities to cytarabine. Although currently GEM has been demonstrated as the only drug having cytotoxic activity against pancreatic cancer (4), the median survival time of GEM-treated patients is only 6 months (5). Intrinsic or acquired resistance to apoptosis induced by GEM is an important factor in the failure to control pancreatic cancer (6, 7).

It is very important to understand the cellular and molecular mechanisms involved in GEM resistance to make GEM more effective. P-Glycoprotein has been shown to induce resistance to various anticancer drugs (8, 9). However, it has been reported that GEM resistance is not mediated by P-glycoprotein (10, 11). Rauchwerger *et al*. reported that equilibrative-sensitive nucleoside transporter plays an important role in GEM sensitivity (12). Duxbury *et al*. showed the overexpression of carcinoembryonic antigen-related cell adhesion molecule 6 protected pancreatic adenocarcinoma cells against GEM-induced cytotoxicity (13). Maehara *et al*. used acquired GEM-resistant pancreatic cancer cells which established by exposing parent sensitive cells to GEM and showed selenoprotein P reduced the intracellular level of reactive oxygen species, resulting in the loss of sensitivity to GEM (14).

Proteomics is a powerful tool for identifying proteins whose expressions are different between drug-resistant and drug-sensitive cells. Our previous reports used proteomics and identified heat-shock protein 27 (HSP27) as a key molecule playing an important role in GEM resistance (15).

However, a knock-down experiment showed that HSP27 was not the only protein concerned with GEM resistance.

The aim of this study was to identify proteins besides HSP27 showing differential expression in GEM-resistant and -sensitive pancreatic cancer cell lines by using proteomics with a large scale two-dimensional gel electrophoresis (2-DE) system.

Materials and Methods

Tumor cell lines and culture conditions. Two human pancreatic cancer cell lines, GEM-sensitive KLM1 cells and GEM-resistant KLM1-R cells, were provided by the Department of Surgery and Science at Kyushyu University Graduate School of Medical Science. KLM1-R was established by exposing KLM1 cells to GEM, as described previously (14). The 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% fetal bovine serum (FBS). Cells were incubated in a humidified atmosphere containing 5% CO_2 at 37˚C.

Sample preparation. Cells were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.5, 165 mM sodium chloride, 10 mM sodium fluoride, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% NP-40) on ice. Suspensions were incubated for 1 h at 4˚C, centrifuged at 21,500 ×g for 30 min at 4˚C, and the supernatants were stored at –80˚C until use (15).

2-DE. Eighty micrograms of protein were used for 2-DE. For the first dimension, isoelectric focusing (IEF) was performed in an IPGphor 3 IEF unit (GE Healthcare, Buckinghamshire, UK) on 11 cm, immobilized, pH 3-10 linear gradient strips (BIO RAD, Hercules, CA, USA) at 50 μA/strip. Samples were mixed with 200 μl of rehydration buffer (8 M urea, 2% CHAPS, 0.01% bromophenol blue, 1.2% Destreak reagent (GE Healthcare) and 0.5% IPG buffer (GE Healthcare) and loaded into the IPGphor strip holder (GE Healthcare). IEF was performed using the following voltage program: rehydration for 10 h (no voltage), a stepwise increase from 0 to 500 V for 4 h, 500 to 1,000 V for 1 h, 1,000 to 8,000 V for 4 h, a linear increase from 8,000 V for 20 min, and a final phase of 500 V from 20,000 to 30,000 Vh. In the second dimension, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a precast polyacrylamide gel with a linear concentration gradient of 5-20% (BIO RAD), run at 200 V (16).

Fluorescent gel staining. Agitation was carried out at all stages. After 2-DE, the gels were subjected to fixing with 40% ethanol and 10% acetic acid for 2 h. The gels were stained with Flamingo™ Fluorescent Gel Stain (BIO RAD) overnight. Stained gels were washed with Milli-Q water three times (17).

Image analysis and spot selection. The positions of the protein spots on the gels were recorded using the ProEXPRESS 2D Proteomic Imaging System (PerkinElmer Inc., Waltham, MA, USA). Expression levels of the proteins were quantified by analyzing the intensity of each spot with Progenesis PG240 software (PerkinElmer) (16). The differences in expression between KLM1 and KLM1-R was analyzed statistically by Student's *t*-test. 2-DE analysis was performed three times. After statistical analysis, the gels were re-stained with See Pico™ (Benebiosis Co., Ltd, Seoul, Korea), and the selected spots whose expression was significantly different between KLM1 and KLM1-R were cut and removed for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

In-gel digestion. The See Pico dye was removed from the gel piece by rinsing three times in 60% methanol, 50 mM ammonium bicarbonate, and 5 mM dithiothreitol (DTT) for 15 min. The sample in the gel piece was reduced twice in 50% acetonitrile (ACN), 50 mM ammonium bicarbonate, and 5 mM DTT for 10 min. The gel piece was dehydrated in 100% ACN twice for 30 min, and then rehydrated with an in-gel digestion reagent containing 10 μg/ml sequencing-grade-modified trypsin (Promega, Madison, WI, USA) in 30% ACN, 50 mM ammonium bicarbonate, and 5 mM DTT. This procedure for in-gel digestion was performed overnight at 30˚C. The samples were lyophilized overnight with the use of Labconco Lyph-lock 1L Model 77400 (Labconco, Kansas, MO, USA). Lyophilized samples were dissolved in 0.1% formic acid (18, 19).

LC-MS/MS. Protein samples dissolved in 0.1% formic acid were centrifuged at 21,500 xg for 5 min and the supernatant was stored at –80˚C until use. An Agilent 1100 LC/MSD Trap XCT (Agilent Technologies, Palo Alto, CA, USA) was used for HPLC and MS/MS. Twenty-five microliters of each sample were applied and separated on a column (Zorbax 300SB-C18, 75 μm, 150 mm; Agilent Technologies). The Agilent 1100 capillary pump was operated under the following conditions: solvent A: 0.1% formic acid, solvent B: ACN in 0.1% formic acid; column flow: 0.3 μl/min, primary flow: 300 μl/min; gradient: 0-5 min 2% solvent B, 60 min 60% solvent B; stop time: 60 min. Protein identification was performed in the Agilent Spectrum MILL MS proteomics workbench against the Swiss-Prot protein database search engine (http://kr.expasy.org/sprot/) and the MASCOT MS/MS Ions search engine (http://www.matrixscience.com/search_form_select.html). The criteria for positive identification of proteins were set as follows: filter by protein score >10.0, and filter peptide by score >8, % scored peak intensity. The Spectrum Mill workbench can search MS/MS spectra using an MS/MS ion search (20, 21).

Results

Detection of protein spots with different expression between KLM1 and KLM1-R on 2-DE gels. Protein expression was assessed in three samples each from GEM-sensitive KLM1 and GEM-resistant KLM1-R cells which separately cultured under the same conditions. More than 1000 spots were visualized on the 2-DE gels, and differences in intensity between the GEM-sensitive and GEM-resistant cells were compared visually and analyzed with Progenesis PG240 software for each gel. The expression of 33 protein spots differed between KLM1 (Figure 1A) and KLM1-R (Figure 1B). Of these, 23 protein spots appeared to have higher levels, and 10 protein spots appeared to have lower levels in KLM1-R than in KLM1. The spots on KLM1-R whose expression level significantly increased or decreased by more than 1.5-fold ($p<0.05$) compared with that of KLM1 are

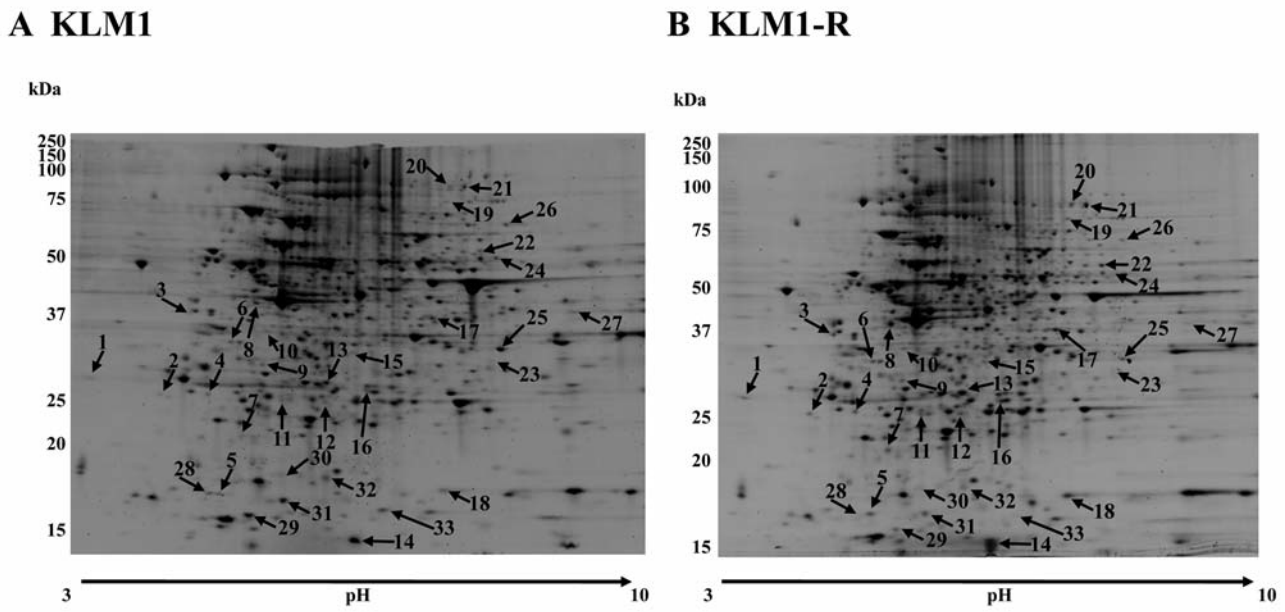

Figure 1. *2-DE gel pattern of A: KLM1 (gemcitabine-sensitive cell line), and B: KLM1-R (gemcitabine-resistant cell line) cells. Proteins (80 μg) were separated on precast polyacrylamide gel with a linear concentration gradient of 5-20%.*

indicated by arrows in Figure 1B. The identification of these 33 protein spots with different expression levels was accomplished by measuring tryptic peptide masses using the Agilent 1100 LC-MS/MS Trap XCT system in the positive ion mode and carrying out a database search in the Agilent Spectrum MILL MS proteomics workbench against the Swiss-Prot protein database search engine and the MASCOT MS/MS Ions Search engine, as summarized in Table I. Each sample provided good spectra of amino acid sequences.

Discussion

In present study, proteomic analysis by using large-scale 2-DE with 11 cm IPG strips showed that twenty-three protein spots including acidic leucine-rich nuclear phosphoprotein 32 family member A (ANP32A), reticulocalbin-1, basic gamma-synuclein, microtubule-associated protein RP/EB family, sialic acid synthase, peptidyl-prolyl *cis-trans* isomerase A, far upstream element-binding protein 2 (FAB-2), catalase, HSP-27 were up-regulated, and ten protein spots including far upstream element-binding protein 1 (FAB-1), acidic gamma-synuclein, galectin-1, stathmin were down-regulated in KLM1-R than in KLM1 cells.

ANP32A is one of a family of leucine-rich acidic nuclear proteins. It plays important roles in many cellular processes, including regulation of chromatin remodeling, transcription, RNA transport, transformation and apoptosis. Schafer *et al*. reported that ANP32A in breast cancer cells enhanced sensitivity to cytochrome *c*-induced apoptosis (22). Hoffarth *et al*. reported that they identified hypofunction of ANP32A as mechanism of resistance to apoptosis induced by cancer therapy and additional stresses *in vitro* and *in vivo* by combined biochemical and genetic studies. They further showed that the absence of immunohistochemically detectable ANP32A expression correlated with poor survival following chemotherapy for patients with non-small cell lung cancer (23). Their results showed that up-regulation of ANP32A related to the sensitivity of breast cancer and non-small cell lung cancer to chemotherapy. However, our results were different from these reports. In our study, ANP32A was up-regulated in resistant KLM1-R cells. However, the mechanism of its involvement in GEM-resistance remains insufficiently characterized to date. Altered ANP32A expression, in any case, is important for GEM resistance.

Reticulocalbin-1 localizes in the lumen of the endoplasmic reticulum of a number of different types of cell, and plays a role in synthesis, modification and intracellular transport of protein (24, 25). Tong *et al*. reported that reticulocalbin-1 and HSP27 were down-regulated in liver cancer cells treated with suberonylanilide hydroxamic acid, an orally administered histone deacetylase inhibitor that has shown significant antitumor activity in a variety of tumor cells. Down-regulation of reticulocalbin-1 and HSP27 may play a role in apoptosis induced by suberonylanilide hydroxamic acid. Although these results suggest the possible link between up-regulation of reticulocalbin-1 and HSP27, and

Table I. *Identification of nuclear proteins which are differentially expressed between KLM1 and KLM1-R.*

Spot no.	Protein name	Accession no.	Theoretical pI	Theoretical Mr	Distinct peptides	Sequence coverage (%)	MS/MS search score	Change in KLM1-R (fold)
1	Acidic leucine-rich nuclear phosphoprotein 32	P39687	4.00	28,585.5	3	16	48.35	Increased (6.44)
2	Heme-binding protein 2	Q9Y5Z4	4.58	22,875.5	1	10	16.62	Increased (2.85)
3	Reticulocalbin-1	Q15293	4.86	38,890.2	2	11	24.83	Increased (>100)
4	Protein FAM151B	Q6UXP7	5.60	31,354.7	1	10	11.36	Increased (2.75)
5	Gamma-synuclein (basic)	O76070	4.89	13,330.9	7	64	96.72	Increased (2.88)
6	Thyrotrophembryonic factor	Q10587	5.81	33,247.8	1	6	14.51	Increased (2.15)
7	Ubiquitincarboxy-terminal hydrolaseisozymeL3	P15374	4.84	26,182.7	1	13	12.24	Increased (2.79)
8	Putative HSP 90-alpha A5	Q58FG0	6.15	38,738.2	1	5	14.29	Increased (2.72)
9	Microtubule-associated protein RP/EB family	Q15691	5.02	29,999.2	5	29	66.06	Increased (2.04)
10	Thyrotrophembryonic factor	Q10587	5.81	33,247.8	1	6	13.36	Increased (2.10)
11	HSP27	P04792	5.98	22,782.6	3	20	32.41	Increased (3.02)
12	HSP27	P04792	5.98	22,782.6	6	47	80.84	Increased (3.29)
13	Proteasomeactivator complex subunit 1	Q06323	5.78	28,723.3	2	18	32.89	Increased (2.38)
14	Protein S100-A11	P31949	6.56	11,740.5	2	20	19.43	Increased (2.17)
15	Thyrotrophembryonic factor	Q10587	5.81	33,247.8	1	6	15.03	Increased (3.08)
16	Pyridoxine-5'-phosphate oxidase	Q9NVS9	6.62	29,988.2	2	15	30.68	Increased (4.60)
17	Sialicacid synthase	Q9NR45	6.29	40,307.8	6	27	83.02	Increased (1.51)
18	Peptidyl-prolyl *cis-trans* isomeraseA	P62937	7.68	18,012.6	6	29	76.75	Increased (3.62)
19	Far upstream element-binding protein 2	Q92945	6.84	73,146.8	5	13	77.19	Increased (2.07)
20	Elongation factor 2	P13639	6.41	95,338.7	2	3	34.03	Increased (3.15)
21	Elongation factor 2	P13639	6.41	95,338.7	2	2	22.98	Increased (1.63)
22	Catalase	P04040	6.90	59,756.5	4	10	67.60	Increased (4.31)
23	WD repeat-containing protein 5	P61964	8.54	36,588.7	1	9	10.72	Increased (4.00)
24	PRAME family member 4	O60810	8.69	55,467.1	1	5	12.16	Decreased (0.27)
25	Glyceraldehyde-3-phosphate dehydrogenase	P04406	8.57	36,053.4	1	5	14.81	Decreased (0.49)
26	Far upstream element-binding protein 1	Q96AE4	7.18	67,560.7	14	29	195.29	Decreased (0.40)
27	Centromereprotein U	Q71F23	9.19	47,521.9	1	2	11.58	Decreased (0.32)
28	Gamma-synuclein(acidic)	O76070	4.89	13,330.9	3	35	36.27	Decreased (<0.01)
29	Galectin-1	P09382	5.34	14,715.8	4	33	46.53	Decreased (0.31)
30	Thioredoxindomain-containing protein 12	O95881	5.25	19,205.9	1	12	14.64	Decreased (0.42)
31	Coactosin-like protein	Q14019	5.54	15,945.1	2	21	22.29	Decreased (0.39)
32	Stathmin	P16949	5.76	17,302.6	2	15	24.88	Decreased (0.42)
33	Fatty acid-binding protein, epidermal	Q01469	6.60	15,164.5	1	14	12.23	Decreased (0.05)

Spot numbers refer to those in Figure 1. These spots were cut out and 33 spots were subsequently identified by MS.

GEM resistance, further studies are still necessary to clarify the possible crosstalk among them (26).

Gamma-synuclein is one of a family of highly conserved small proteins predominantly expressed in neurons. It plays a role in neurofilament network integrity. From some groups it has been reported that gamma-synuclein was associated with chemoresistance (27, 28). In the present study, two spots of gamma-synuclein were identified: the basic protein was up-regulated and the acidic one was down-regulated in GEM-resistant KLM1-R cells. Although we could not elucidate the post-translational modification of this acidic spot of gamma-synuclein, this may be phosphorylated in GEM-sensitive KLM1 cells. Also two up-regulated spots of HSP27 in KLM1-R cells were identified. The acidic spot may be phosphorylated.

Peptidyl-prolyl *cis-trans* isomerase A was originally identified as an intracellular receptor for cyclosporine A (29). The immunosuppressive activity of cyclosporine A is thought to be mediated by the engagement of calcineurin by the cyclosporin A–peptidyl-prolyl *cis-trans* isomerase A complex (30), an observation supported by the finding that peptidyl-prolyl *cis-trans* isomerase A knockout mice are resistant to immunosuppression by cyclosporin A (31). Wong *et al*. reported that peptidyl-prolyl *cis-trans* isomerase A was up-regulated in 5-fluorouracil-treated colorectal cancer cells (32). Choi *et al*. showed that overexpression of peptidyl-prolyl *cis-trans* isomerase A induced chemoresistance to cisplatin, and its knockdown increased the sensitivity to cisplatin (33).

Catalase occurs in almost all aerobically respiring organisms and serves to protect cells from the toxic effects of

hydrogen peroxide. Many reports showed the protective effect of catalase on cancer cells suffering from reactive oxygen species and chemotherapy (34, 35).

FBP-1 and -2 are FBP family members, and bind to the dendritic targeting element and may play a role in mRNA trafficking. Malz *et al*. reported that reduction of FBP-2 levels was associated with elevated FBP-1 expression (36). Interestingly, our present study also showed up-regulation of FBP-2 and down-regulation of FBP-1 in GEM-resistant KLM1-R cells.

Proteomic analysis with a large-scale 2-DE system was shown here to be useful for detecting intracellular proteins with differential expression in pancreatic adenocarcinoma cell lines that were sensitive or resistant to GEM. Such proteins may be involved in the mechanism of resistance to chemotherapy, and could also be indicators of the response to pancreatic cancer therapy.

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