Proteomics finding heat shock protein 27 as a biomarker for resistance of pancreatic cancer cells to gemcitabine

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Abstract. Pancreatic cancer remains a devastating disease and >96% of patients with pancreatic cancer do not survive for more than 5 years. Gemcitabine (2'-deoxy-2'-difluoro-deoxycytidine: Gemzar) appears to be the only clinically effective drug for pancreatic cancer, but it has little impact on outcome. Proteomic analysis of gemcitabine-sensitive cells (KLM1) and resistant pancreatic cells (KLM1-R) was performed to identify target proteins of the gemcitabine. We found seven proteins, HSP27, peroxiredoxin 2, endoplasmic reticulum protein ERp29 precursor, 6-phosphogluconolactonase, triosphospate isomerase, α enolase, and nucleophosmine that could play a role in determining the sensitivity of pancreatic cancer to gemcitabine. We knocked down HSP27 in KLM1-R and the sensitivity to gemcitabine was restored. In addition, increased HSP27 expression in tumor specimens was related to higher resistibility to gemcitabine in patients of pancreatic cancer. HSP27 may play an important role in the resistibility to gemcitabine, and it could also be a possible biomarker for predicting the response of pancreatic cancer patients to treatment with gemcitabine.

Introduction

Pancreatic cancer is characterized by difficulties in diagnosis, its aggressiveness, and the lack of effective systemic therapy, Only 4% of patients with adenocarcinoma of the pancreas survive for more than 5 years after diagnosis (1,2). Surgical resection is the sole curative treatment that is currently available, but only 10-15% of patients are free from metastasis at the time of diagnosis. Gemcitabine (2'-deoxy-2'-difluoro-deoxycytidine: Gemzar) is a deoxycytidine analogue with structural and metabolic similarities to cytarabine. Currently, this nucleoside analogue appears to be the only clinically effective drug for pancreatic cancer (3). However, the median survival time of patients treated with gemcitabine is only 6.3 months (range: 1.6-19.2 months) (4). Intrinsic or acquired resistance of pancreatic cancer to apoptosis is an important factor in the failure of this treatment (5). Better understanding of the cellular and molecular mechanisms of gemcitabine resistance is required to allow this drug to be used more effectively.

There have been reports that selenoprotein P contributes to gemcitabine resistance (6), that apoptosis-regulating genes control tumor sensitivity to gemcitabine (5,7), and that deoxycytidine kinase (dCK) deficiency is responsible for gemcitabine resistance (8). However, there has been no comprehensive study on protein expression in tumors that have developed gemcitabine resistance. The combination of two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) is powerful for high-throughput analysis of proteomic profiling of cancer.

In this study, we investigated the differential expression of proteins in a gemcitabine-sensitive and gemcitabine-resistant pancreatic cancer cell line, and identified a protein participating in gemcitabine sensitivity. The protein was knocked down and we examined gemcitabine sensitivity. In addition we studied whether the protein can become a biomarker of gemcitabine sensitivity clinically with tumor specimens obtained by endoscopic ultrasound-guided fine needle aspiration (EUS-FNA).

Materials and methods

Tumor cell lines and culture conditions. Two human pancreatic cancer cell lines, gemcitabine-sensitive KLM1 cells and gemcitabine-resistant KLM1-R cells, were kindly 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 gemcitabine, as described previously (7). The tumor cells were cultured in RPMI-1640

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medium with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 10% FCS. All cells were kept in a water-saturated atmosphere containing 5% CO_2 at 37˚C and without endotoxin.

Cell proliferation assay. Cells were seeded onto 96-well plates at a density of 1,000 cells/well, incubated for 24 h, and exposed to different concentrations of gemcitabine for 72 h. After incubation with gemcitabine, 10 μl of a 5 mg/ml solution of MTT (3-[4,5-dimethythazol-2-yl]-2,5-diphenyl tetrazolium bromide) was added to each well and the plates were incubated for another 4 h. Then the formazan product was dissolved by adding 100 μl of DMSO and keeping it in the dark for 1 h 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). Absorbance showed a linear relationship with the number of cells and each experiment was repeated three times.

Sample preparation. Suspensions of cultured cells were centrifuged at 1,500 rpm for 5 min. The pellet was washed three times with 10 mM PBS(-), pH 7.4, and then 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 μg/ml leupeptin, and 10 μg/ml aprotinin) at 4˚C for 1 h. The lysate was centrifuged at 15,000 x g for 30 min to obtain the supernatant, which was stored at -80˚C. Samples of the cell lines were prepared and stored three times each.

Two-dimensional gel electrophoresis (2-DE). Three hundred micrograms of protein was used for each 2-DE assay. First-dimension IEF was performed on 7-cm immobilized pH gradient strips with a linear pH gradient from 3 to 10 (GE Healthcare Bio-Science Corp., Piscataway, NJ) at 20˚C and 50 mA. Then the strips were rehydrated with 125 μl of sample solution (8 M urea, 2% CHAPS, and 0.5% IPG buffer) for 14 h. IEF was performed in three steps, which were 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 24 h. Subsequently, the gels were destained with 10% acetic acid in water containing 30% methanol for 30 min and then destained with 7% acetic acid and used for in-gel digestion.

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 showed at different intensities were excised from the gels and stored in 100 μl of ultrapure water at -80˚C as samples for MS analysis.

In-gel digestion. After cutting out 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% ACN, 50 mM ammonium bicarbonate, and 5 mM DTT for 10 min. The gel pieces were dehydrated in 100% acetonitrile twice for 30 min, and then rehydrated with an in-gel digestion reagent containing 10 μg/ml of sequencing grade modified trypsin (Promega, Madison, WI) in 30% acetonitrile, 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.

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

Immunoblot analysis. Samples (30 μg) were separated by SDS-PAGE at 15 mA, and then transferred electrophoretically from the gels to PVDF membranes (Immobilon-P; Millipore, Bedford, MA) and blocked overnight at 4˚C with TBS containing 5% skim milk. The primary antibody was an anti-heat shock protein (HSP) 27 monoclonal antibody (1:600, Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were incubated with this antibody for 1 h at room temperature, washed three times with TBS containing 0.05% Tween-20 and once with TBS, then incubated for 1 h at room temperature with the horseradish peroxidase-conjugated secondary antibody (1:2000, ICN Pharmaceuticals, Aurora, OH). The reaction products were visualized with a chemiluminescence reagent (ECL Western blotting detection reagents; GE Healthcare).

Transfection with siRNA. KLM1-R cells were seeded in 6-well plates at a density of approximately 50% in 2.0 ml of complete medium. At 24 h after seeding, either specific HSP27 siRNA (Santa Cruz Biotechnology) or control siRNA (Santa Cruz Biotechnology) was added at a final concentration of 520 nM and incubation was performed for 30 h. Then the medium was exchanged for 2.0 ml of fresh growth medium containing 10% FCS and cells were incubated for 24 h. For the MTT assay, cells were trypsinized and transferred to 96-well plates. For protein extraction, cells were kept in the 6-well plates and used for Western blot analysis at 48 h after transfection. All experiments were repeated three times.

EUS-FNA. All procedures were carried out by one endosonographer using a linear endoscopic ultrasound (EUS) scanner (GF UCT240; Olympus Optical Co., Tokyo, Japan) with an EU-C2000 ultrasound platform (Olympus Optical Co.). EUS was performed to localize the pancreatic tumor and to obtain samples for histological diagnosis. Fine needle aspiration (FNA) was performed with a 22-gauge needle (EchoTip; Wilson-Cook Medical Inc., Winston-Salem, NC) under direct EUS guidance.

Immunohistochemistry. Formalin-fixed and paraffin-embedded samples were cut into 4-μm thick sections, which were treated with anti-HSP27 monoclonal antibody (1:200, Santa Cruz Biotechnology). Then avidin-biotin (Vector, Burlingame, CA) and 3,3-diaminobenzidine (Dojindo, Kumamoto, Japan) were used for detection.

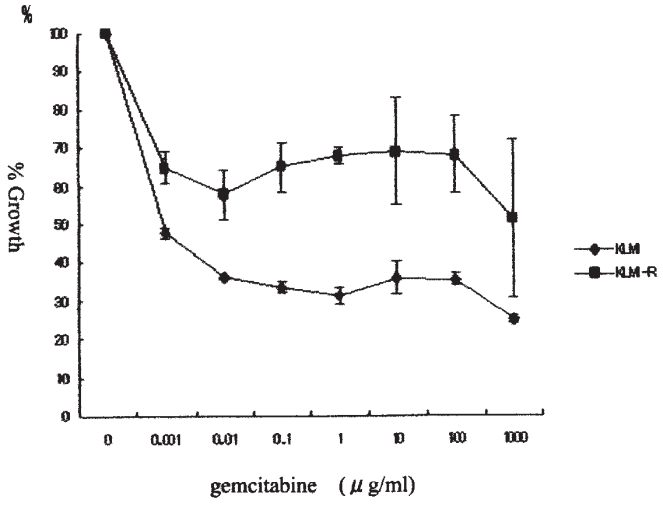

Figure 1. Cytotoxicity of gemcitabine against KLM1 and KLM1-R cells. KLM1 and KLM1-R cells were continuously exposed to various concentrations of gemcitabine for 72 h. KLM1-R cells were much less sensitive to gemcitabine.

Evaluation of HSP27 staining. HSP27 protein was stained brown in the cytoplasm of cancer cells. We counted the percentage of section with immunostaining signals of tumor in three different fields at a magnification of x400. We calculated the ratio of immunopositive area to cancerous area in three arbitrary fields of vision.

Results

Cytotoxicity of gemcitabine for KLM1 and KLM1-R cells. To evaluate the cytotoxicity of gemcitabine for KLM1 and KLM1-R cells, continuous exposure to various concentrations of the gemcitabine was performed for 72 h (Fig. 1). KLM1-R cells exhibited 2.2-fold (1 μg/ml gemcitabine) and 1.9-fold (10 μg/ml gemcitabine) greater viability compared with KLM1 cells ($p<0.05$), so the KLM1-R cell line was much less sensitive to gemcitabine.

Detection and identification of proteins. Protein expression was assessed using three samples each of KLM1 and KLM1-R cells cultured under the same conditions. More than 1,000 protein spots were visualized on the 2-DE gels. Differences in the intensity of these spots between KLM1 and KLM1-R were compared visually and analyzed with Progenesis PG240. As a result, 5 spots showed increased intensity on gels from KLM1-R cells (spots No. 1-5) and three spots showed decreased intensity (spots No. 6-8) (Fig. 2). The above-mentioned eight spots were excised from each gel, and identified by LC-MS/MS analysis. Information about the eight proteins thus identified is summarized in Table I. HSP27 was identified at three spots whose ratio was significantly different between the two cell lines. Expression comparisons of three spots of HSP27 by CBB dye are shown in Fig. 3.

Verification of protein expression by Western blot analysis. The expression of HSP27 was confirmed by immunoblot analysis, and it was shown to be up-regulated in KLM1-R cells (Fig. 3).

Effect of HSP27 on the response of KLM1-R cells to gemcitabine. To determine whether a decrease of HSP27 expression affected the sensitivity of pancreatic cancer cells to gemcitabine, we knocked down HSP27 using siRNA. When gemcitabine-resistant KLM1-R cells were treated with specific siRNA targeting HSP27, a significant reduction of HSP27 protein expression was detected compared with negative control siRNA-treated KLM1-R cells (Fig. 3). When normal KLM1 cells, normal KLM1-R cells, control KLM1-R cells (treated with control siRNA), and HSP27-silenced KLM1-R cells were exposed to various concentrations of gemcitabine for 72 h, the HSP27-silenced KLM1-R cells showed increased gemcitabine sensitivity (Fig. 4).

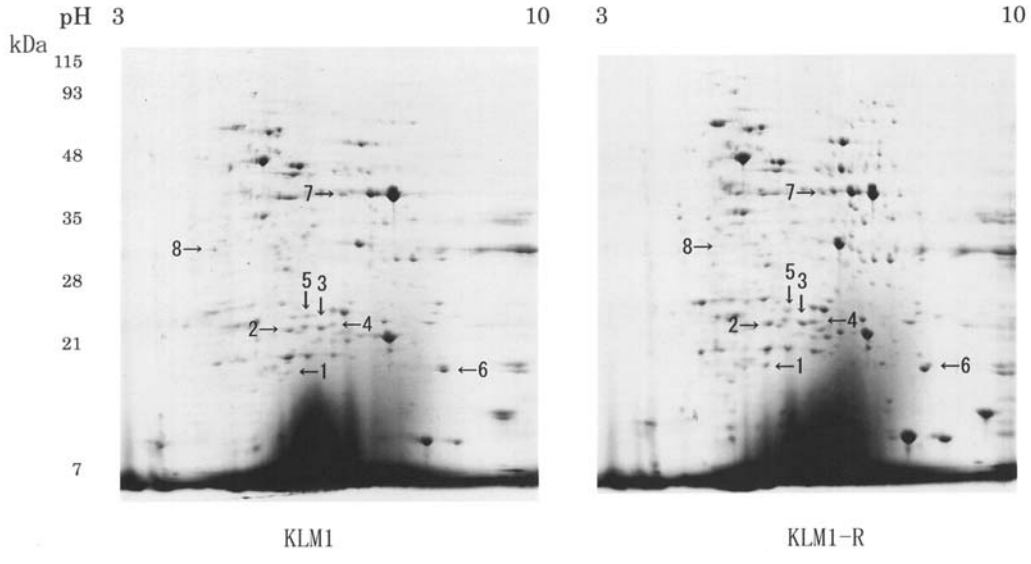

Figure 2. Two-dimensional gel images of KLM1 and KLM1-R cells. More than 1,000 protein spots were visualized on the 2-DE gels. Five spots showed increased intensity on gels from KLM1-R cells (spots No. 1-5) and three spots showed decreased intensity (spots No. 6-8).

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

Protein number[a]	Molecular mass (Da)	pI	Protein identification	Spot intensity[b] (% average ± SD)	p-value[c]
1	21892	5.66	Peroxiredoxin 2	2.69±0.69	0.013
2	22782	5.98	Heat shock protein 27	2.50±1.51	0.16
3	22482	5.98	Heat shock protein 27	2.13±1.00	0.12
4	28993	6.77	Endoplasmic reticulum protein ERp29 precursor	1.84±0.19	0.0014
	22782	5.98	Heat shock protein 27		
5	27547	5.70	6-phosphogluconolactonase	1.83±0.32	0.011
6	26538	6.51	Triosphosphate isomerase	0.40±0.068	0.0002
7	47038	6.99	α enolase	0.60±0.28	0.068
8	32575	4.64	Nucleophosmine	0.74±0.049	0.0008

[a]Spot numbers correspond to those in Fig. 2. [b]Percentage of spot intensitiy of KLM1-R to KLM1. [c]The differences in expression between KLM1and KLM1-R were analyzed by Student's t-test.

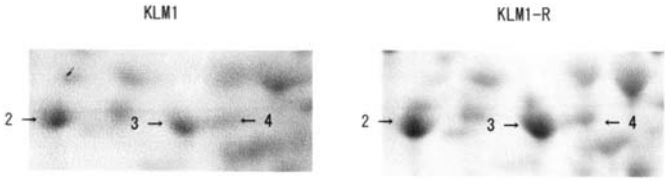

Figure 3. Comparison of spots of HSP27 between KLM1 and KLM1-R. The 2-DE pattern of KLM1 is on the left and that of KLM1-R is on the right. The spot numbers correspond to those in Fig. 2. HSP27 was shown to be up-regulated in KLM1-R cells compared with KLM1 cells.

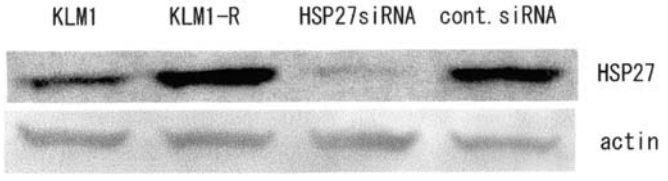

Figure 4. Immunoblotting of HSP27. HSP27 was shown to be up-regulated in KLM1-R cells compared with KLM1 cells. When gemcitabine-resistant KLM1-R cells were treated with specific siRNA targeting HSP27, a significant reduction of HSP27 protein expression was detected compared with negative control siRNA-treated KLM1-R cells and normal KLM1-R cells.

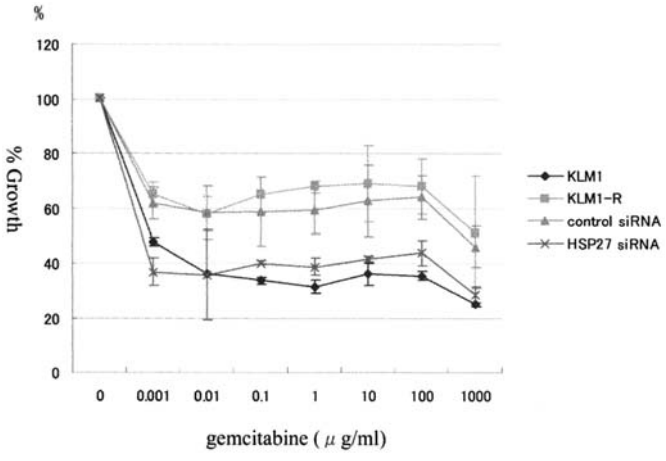

Figure 5. Sensitivity of HSP27-silenced KLM1-R cells to gemcitabine. The HSP27-silenced KLM1-R cells showed increased drug sensitivity as well as KLM1.

Immunohistochemistry of HSP27 in pancreatic cancer tissues and correlation with gemcitabine effects and survival. To evaluate the expression of HSP27 in clinical specimens, we performed immunohistochemical analysis of pancreatic cancer tissues that were obtained by EUS-FNA. Connective tissue was prominent in the specimens obtained by EUS-FNA. We observed the tumors under a microscope (x400) in three arbitrary fields of vision. The rate of HSP27-stained cancer area to total cancer area was calculated. The mean value in three views was calculated. The therapeutic gain of gemcitabine was judged according to guidelines of Response Evaluation Criteria in Splid Tumor (RECIST) (9). We examined the eleven patients who were diagnosed with EUS-FNA as having pancreatic cancer and treated with gemcitabine. Seven patients had progressive disease (PD) (Fig. 6a), and 4 patients had stable disease (SD) (Fig. 6b) in 11 patients. The PD group's positive ratio for HSP27 was higher than that of the SD group (p=0.0066) (Fig.6c). When the overall survival analysis of these patients was performed according to the HSP27 immunoreactivity, a shorter survival of pancreatic cancer patients correlated with high HSP27 expression (positive rate >30%) rather than with low HSP27 expression (positive rate <30%) (p=0.0025) (Fig. 6d).

Discussion

In the present study, proteomic analysis revealed that expression of HSP27 was increased in a gemcitabine-resistant pancreatic cancer cell line, while HSP27-silenced cells showed increased sensitivity to gemcitabine. These findings suggest that increase of expression of HSP27 by advanced pancreatic cancer might contribute to gemcitabine resistance, and silenced

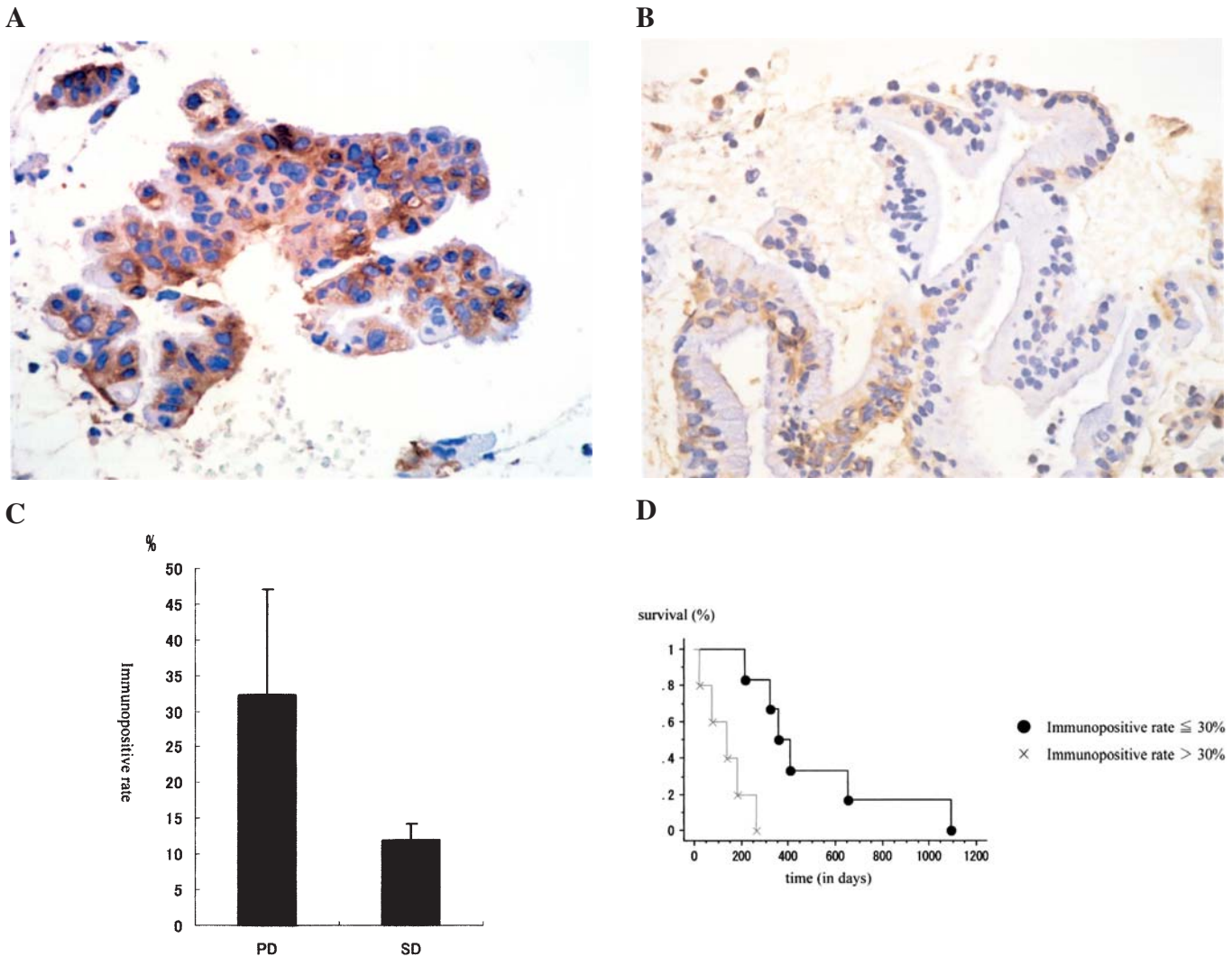

Figure 6. Immunohistochemistry of HSP27 in pancreatic cancer tissues and its correlation with survival rate of patients. (a) Tissue from a patient with progressive disease (PD). Fifty-one percent of the cancerous area was immunopositive (x400). (b) Tissue from a patient with stable disease (SD). Eleven percent of the cancerous area was immunopositive (x400). (c) The positive rate of HSP27 in PD and SD. The PD group's ratio of positive for HSP27 was higher than that of the SD group. (d) Immunohistochemistry positive rate of HSP27 and patient survival. Patients whose positive rate was >30% had a shorter survival than those with a rate <30%.

expression of HSP27 of gemcitabine-resistant pancreatic cancer might increase the gemcitabine sensitivity.

The mechanisms of gemcitabine resistance are still controversial, although many studies have been performed. The apoptosis-regulating proteins of the bcl-2 family and P-glycoprotein have been reported to have a role in resistance to chemotherapy (7,10-14), as well as various other proteins. However, none of these proteins showed any difference between gemcitabin-sensitive and -resistant tumor cells in our proteomic analysis. One possible reason for this may be that we used a cell line with acquired gemcitabine resistance rather than intrinsic resistance, while the other reason would be the limitations of 2DE. However, we found that gemcitabine-resistant cells showed increased expression of 4 other proteins in addition to HSP27 and decreased expression of 3 proteins, suggesting that various proteins may participate in gemcitabine resistance as well as HSP27. By knocking down HSP27 using siRNA, the gemcitabine sensitivity of pancreatic cancer cells was increased, confirming that HSP27 has a role in gemcitabine resistance.

HSP27 belongs to the family of small heat shock proteins, which are molecular chaperones that modulate the ability of cells to respond to several types of injury and are expressed in virtually all organisms from prokaryotes to mammals (15). Evidence has been obtained that HSP27 regulates apoptosis by interacting with key components of the apoptotic signaling pathway (16). HSP27 inhibits etoposide-induced apoptosis by preventing cytochrome c and dATP-triggered activation of caspase-9, which occurs downstream of cytochrome c release (17,18). Increased expression of antiapoptotic factor enhances the resistance of tumor cells to chemotherapy. Thus, the overexpression of HSP27 inhibits doxorubicin-induced apoptosis of human breast cancer cells (19), as well as apoptosis of prostate cancer cells induced by etoposide, diethyl-maleate, cycloheximide, or radiation (20), and etoposide-induced apoptosis of neuroblastoma cells (21). In the present study, HSP27 was shown to be overexpressed by KLM1-R cells, as is the case with the above-mentioned cancers. It is suggested that pancreatic cancer develops resistance through the antiapoptotic action of HSP27, and that

this is an important component of resistance to gemcitabine. In fact, HSP27-silenced KLM1-R cells showed an increase of sensitivity to gemcitabine, which reached the same level as that of parental KLM1 cells.

EUS-FNA has come into widespread use, mainly in Western countries, as an efficient and safe method for the cytologic or histologic diagnosis of pancreatic cancer (22-26). Although EUS-FNA is only employed to make a histological diagnosis of pancreatic cancer at present, it may also contribute to tailor-made medicine in future by evaluating gemcitabine sensitivity. We can possibly expect an improved response to gemcitabine by combining it with a method of reducing HSP27 expression in pancreatic cancer.

Proteomic analysis was useful for finding intracellular proteins with differential expression between pancreatic adenocarcinoma cell lines showing sensitivity and resistance to gemcitabine. HSP27 may be involved in the mechanism of resistance to gemcitabine, and it could also be a possible biomarker for predicting the response of pancreatic cancer to treatment.

Acknowledgements

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