Human Pancreatic Cancer Cells with Acquired Gemcitabine Resistance Exhibit Significant Up-regulation of Peroxiredoxin-2 Compared to Sensitive Parental Cells

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Abstract. *Gemcitabine (2'-deoxy-2'-difluorodeoxycytidine) is the only clinically effective drug for pancreatic cancer. However, high levels of inherent and acquired tumor resistance to gemcitabine lead to difficulty of chemotherapy for pancreatic cancer. We have reported on a proteomic study of gemcitabine-sensitive KLM1 and -resistant KLM1-R pancreatic cancer cells, and identified some proteins which were shown to be up-regulated in KLM1-R compared to KLM1 cells. In those proteomic studies, peroxiredoxin-2 was listed as an up-regulated protein in KLM1-R cells. Peroxiredoxin-2 is a member of a family of peroxiredoxins providing a protective role for redox damage. In this study, the expression of peroxiredoxin-2 in KLM1 and KLM1-R cells was compared. It was found that peroxiredoxin-2 was significantly up-regulated in KLM1-R cells compared to KLM1 cells ($p<0.001$). However, peroxiredoxin-1 expression was significantly down-regulated in KLM1-R cells ($p<0.001$). These results suggest that peroxiredoxin-2 is a possible candidate biomarker for predicting the response of patients with pancreatic cancer to treatment with gemcitabine.*

Gemcitabine (2'-deoxy-2'-difluorodeoxycytidine), a deoxycytidine analog with structural and metabolic similarities to cytarabine, is one of the most effective chemotherapeutic drugs for pancreatic cancer (1). However, many patients with pancreatic cancer exhibit intrinsic or acquired resistance to gemcitabine (2). To identify the proteins with differential expression between pancreatic cancer cells sensitive and resistant to gemcitabine, we used KLM1 and KLM1-R cells, respectively. KLM1 is highly sensitive to gemcitabine (3); KLM1-R was established from KLM1 by exposure to 10 μg/ml of gemcitabine, with a half-maximal (50%) inhibitory concentration (IC_{50}) value for gemcitabine a 20-fold greater compared to KLM1.

Our recent proteomic studies revealed many proteins whose expressions were different between KLM1 and KLM1-R cells, and a validation study using western blotting and knock-down identified heat-shock protein-27 (HSP27) as a key molecule playing an important role in gemcitabine resistance (4-6). Peroxiredoxin-2 was included in these candidate proteins. Peroxiredoxin-2 is a member of a family of thiol-specific antioxidant proteins. The peroxiredoxin family has six isoforms (peroxiredoxin-1, to 6). Peroxiredoxin-1 protects cells from cell death by scavenging reactive oxygen species (ROS). Peroxiredoxin-3 is a mitochondrial antioxidant protein which scavenges H_2O_2 in cooperation with thiol and peroxynitrite, and detoxifies peroxynitrite. Tsutsui *et al.* demonstrated that the overexpression of peroxiredoxin-3 protected the heart against post-myocardial infarction remodeling and failure in mice (7). Peroxiredoxin-4 is a ubiquitously expressed endoplasmic reticulum (ER)-localized peroxiredoxin. This peroxiredoxin plays roles in both H_2O_2 removal and disulfide formation (8). Peroxiredoxin-5 plays roles in reducing alkyl hydroperoxides or peroxynitrite using cytosolic or mitochondrial thioredoxins (9). Peroxiredoxin-6 plays roles in both glutathione (GSH) peroxidase and phospholipase A2 activities, and uses GSH as an electron donor to reduce H_2O_2 (10, 11).

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Many studies have reported on relations between peroxiredoxins and resistance to induced cell death in tumor cells. An etoposide-resistant neuroblastoma cell line showed overexpression of peroxiredoxin-1 (12). Peroxiredoxin-2 was up-regulated in radiation-resistant MCF-7 breast cancer cells (13). Chung *et al*. reported that overexpression of peroxiredoxin-2 in SNU638 cells lead to greater resistant to cell death induced by cisplatin and H_2O_2 than control cells (14). Kubota *et al*. reported the higher expression of peroxiredoxin-2 in poor responders to therapy of osteosarcoma (15). Dai *et al*. reported the down-regulation of peroxiredoxin-3 in platinum-resistant ovarian cancer cell lines (16). On the other hand, Wang *et al*. reported peroxiredoxin-3 expression was significantly higher in patients with the platinum-resistant epithelial ovarian cancer (17). Peroxiredoxin-4 protected head-and-neck squamous cell carcinoma cells from radiation-induced apoptosis (18). On the other hand, Smith *et al*. reported that peroxiredoxin-4 was down-regulated in cisplatin-resistant breast cancer cells (19). Peroxiredoxin-6 was found to be strongly up-regulated in cisplatin-resistant cervical squamous cell carcinoma cells after cisplatin exposure (20). Knockdown of peroxiredoxin-6 in peroxide-induced cytotoxicity-resistant hepatoma cell line Hepa 1-6 increased apoptosis (21). Peroxiredoxin-6 was up-regulated in 5-fluorouracil-treated colorectal SW480 cancer cells (22).

In the present study, in order to validate the comparison of the expression of peroxiredoxin-2 between gemcitabine-sensitive and resistant pancreatic cancer cells, we performed western blot analysis of peroxiredoxin-1 and -2 in KLM1 and KLM1-R cells.

Materials and Methods

Cancer cell lines and culture conditions. KLM1 and KLM1-R, human pancreatic cancer cell lines, were kindly provided by the Department of Surgery and Science at the Kyushu University Graduate School of Medical Science. KLM1 is gemcitabine-sensitive, and resistant KLM1-R was established by exposing KLM1 cells to gemcitabine, as described previously (3). These cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (inactivated at 56˚C for 30 min), 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 10 mM HEPES, and 1.0 mM sodium pyruvate, and maintained in a humidified 5% carbon dioxide-95% air mixture at 37˚C.

Sample preparation. Sub-confluently growing cells were homogenized in ice-cold lysis buffer [50 mM Tris-HCl, pH 7.5, 165 mM NaCl, 10 mM NaF, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% NP40], and centrifuged at 15,000 ×*g* for 30 min at 4˚C. The supernatants were collected and used for western blotting after protein concentrations were measured by Lowry method (23). The samples from KLM1 and KLM1-R cells were prepared five times independently.

Western blotting. For western blot analysis, 15 μg of protein samples were used. Sodium dodecyl sulfate-polyaclylamide gel electrophoresis (SDS-PAGE) was carried out in precast gels (12% acrylamide; Mini-PROTEAN TGX Gels, Bio-Rad, Hercules, CA, USA). Proteins separated by SDS-PAGE were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA, USA) electrophoretically. After blotting, the membranes were blocked with Tris-buffered saline (TBS) containing 5% skimmed milk for 1 h at room temperature. Membranes were then incubated with rabbit polyclonal antibody to peroxiredoxin-1 (0.5 μg/ml, ab41906; Abcam, Cambridge, MA, USA), rabbit polyclonal antibody against peroxiredoxin-2 (0.5 μg/ml, R8656; Sigma-Aldrich, St. Louis, MO, USA) or goat polyclonal antibody to actin (1:500, #sc-1616; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4˚C. The membranes were reacted with horseradish peroxidase-conjugated secondary antibodies (dilution range 1:10,000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) for 1 h at room temperature after washing with TBS containing 0.05% Tween-20 three times and once with TBS. Bands of peroxiredoxin-1, peroxiredoxin-2 and actin were visualized by enhanced chemiluminescence system (ImmunoStar Long Detection; Wako, Osaka, Japan), and recorded by using Image Reader LAS-1000 Pro (Fujifilm Corporation, Tokyo, Japan) (24-26). Progenesis PG240 software (Nonlinear Dynamics Ltd., Newcastle-upon-Tyne, UK) quantified the expression levels of the bands of peroxiredoxin-1 and peroxiredoxin-2 (27).

Statistical analysis. Statistical significance of differences between KLM1 and KLM1-R cells was calculated by Student's *t*-test. A *p*-value of <0.05 was accepted as being significant.

Results

Western blot analysis of peroxiredoxin-1 and 2 in KLM1-R and KLM1 cells. Intracellular proteins from gemcitabine-resistant KLM1-R and -sensitive KLM1 cells were analyzed by western blotting with primary antibody against peroxiredoxin-1, peroxiredoxin-2 and actin. The protein expression of peroxiredoxin-2 was up-regulated significantly in KLM1-R cells compared to KLM1 cells (Figure 1). The expression of peroxiredoxin-2 was significantly up-regulated in KLM1-R compared to KLM1 cells ($p<0.001$ by Student's *t*-test) (Figure 2). On the other hand, peroxiredoxin-1 was significantly down-regulated in KLM1-R cells ($p<0.001$) (Figures 3 and 4).

Discussion

The present study validated the significant up-regulation of peroxiredoxin-2, which was identified as being up-regulated by a recent proteomic study (4), in gemcitabine-resistant KLM1-R cells by means of western blot analysis with a specific antibody to peroxiredoxin-2 ($p<0.001$ by Student's *t*-test). On the other hand, peroxiredoxin-1 was found to be down-regulated in KLM1-R cells.

Peroxiredoxins are thiol-specific antioxidant enzymes which regulate H_2O_2 levels and protect cells from oxidative stress. Peroxiredoxin-2 thus promotes survival of cells under oxidative stress. Overexpression of peroxiredoxin-2 was

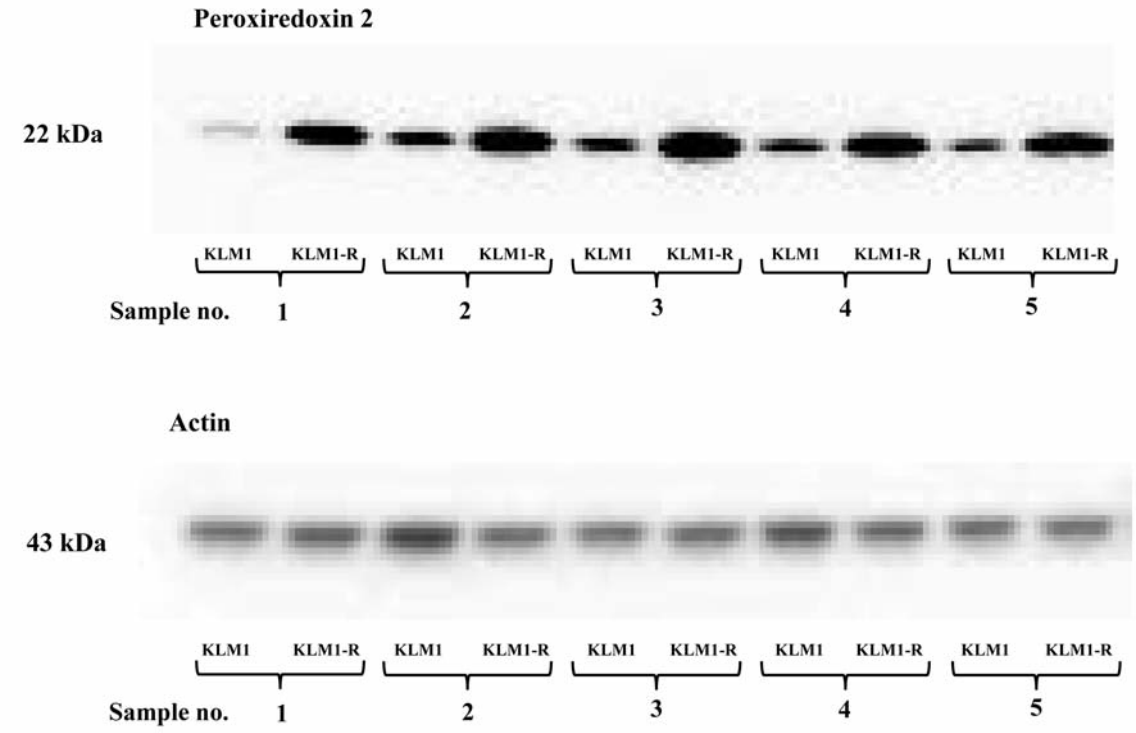

Figure 1. *Western blot analysis of peroxiredoxin-2 in gemcitabine-resistant human pancreatic cancer KLM1-R cells and parental gemcitabine-sensitive KLM1 cells. Fifteen micrograms of protein from KLM1-R cells and KLM1 cells were used. Bands of 22 kDa are peroxiredoxin-2, and 43 kDa bands are actin, as a loading control. The samples from KLM1-R and KLM1 were prepared five times independently.*

found to protect cells from oxidative stress-induced apoptosis (28), while down-regulation of peroxiredoxin-2 increased harmful effects of oxidative stress on cardiomyocytes (29). Peroxiredoxin-1 regulates H_2O_2 levels to maintain appropriate cellular redox levels as does peroxiredoxin-2. The expression of peroxiredoxin-1 is high in many types of cancer cells and tissues (30, 31). Suppression of peroxiredoxin-1 resulted in a modest increase in peroxide-induced cytotoxicity towards MCF-7 mammary adenocarcinoma cells (32). Knock-down of peroxiredoxin-1 significantly enhanced HeLa cell sensitivity to beta-lapachone, a potential anticancer agent (33). Kim *et al*. reported that peroxiredoxin-1 expression status predicted for recurrence and shorter survival in stage I non-small cell lung cancer after surgery (34, 35).

Many groups have reported on the relation between drug resistance and peroxiredoxin-1 or peroxiredoxin-2. Kalinina *et al*. reported that cisplatin-resistance was accompanied by a significant increase in the expression of peroxiredoxin-1 and 2 genes in cancer cell lines (36). Urbani *et al*. reported the overexpression of peroxiredoxin-1 in an etoposide-resistant neuroblastoma cell line (12). Peroxiredoxin-2-overexpressing SNU638 gastric cancer cells became more resistant to cisplatin (14). Not only the basic analysis of the relationship of

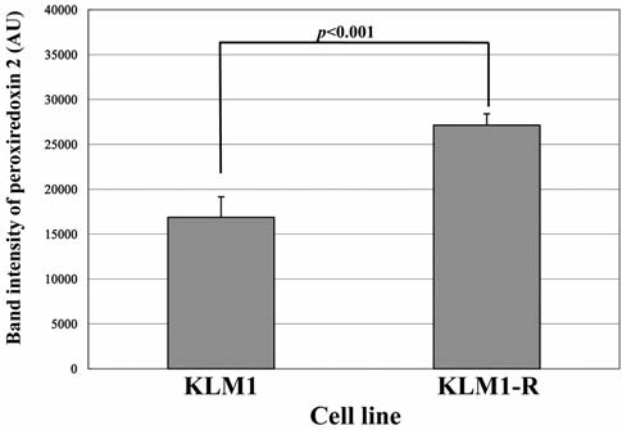

Figure 2. *Comparison of the intensities of bands of peroxiredoxin-2 between gemcitabine-resistant human pancreatic cancer KLM1-R cells and parental gemcitabine-sensitive KLM1. The intensities of peroxiredoxin-2 were significantly up-regulated in KLM1-R compared to KLM1 ($p<0.001$ by Student's t-test) (n=5).*

peroxiredoxin-1 and 2 and chemoresistance, but also the clinical analysis of the relationship between peroxiredoxin-2 and prognosis of patients was carried out by Kubota *et al*. They reported peroxiredoxin-2 to be a predictive biomarker of

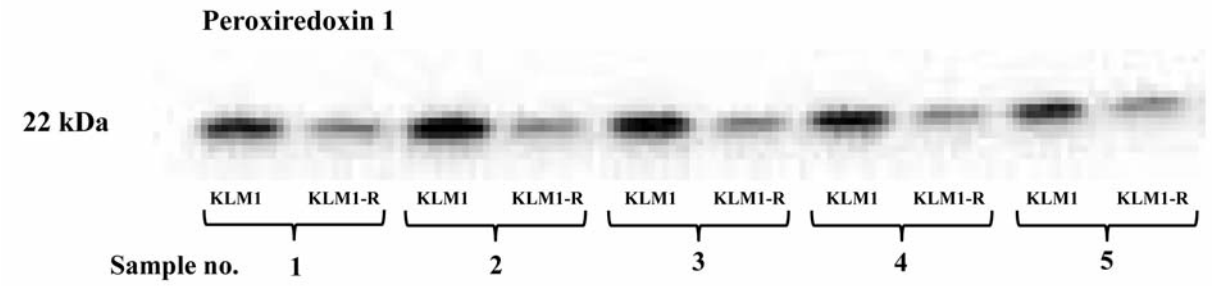

Figure 3. *Western blot analysis of peroxiredoxin-1 in gemcitabine-resistant human pancreatic cancer KLM1-R cells and parental gemcitabine-sensitive KLM1 cells. Fifteen micrograms of protein from KLM1-R cells and KLM1 cells were used. Bands of 22 kDa are peroxiredoxin-1. The samples from KLM1-R and KLM1 were prepared five times independently.*

response to induction chemotherapy in osteosarcoma (15). Not only peroxiredoxin-2, but also peroxiredoxin-1 was reported as a protein involved in drug resistance. Why in the case of gemcitabine-sensitive KLM1 and -resistant KLM1-R cells are peroxiredoxin-1 and 2 expressed differently? Human peroxiredoxin-1 and 2 are more than 90% homologous in their amino acid sequences (37); the 10% difference in sequence may have a key role in this differential effect on expression.

Westwood *et al*. reported on a peroxiredoxin inhibitor conoidin A. Conoidin A bound covalently to the peroxidatic cysteine of *Toxoplasma gondii* peroxiredoxin-2, inhibiting its enzymatic activity *in vitro*, and also inhibited hyperoxidation of human peroxiredoxin-2 (38, 39). Therefore, the clinical application of conoidin A for patients with pancreatic cancer who need treatment with gemcitabine might be expected.

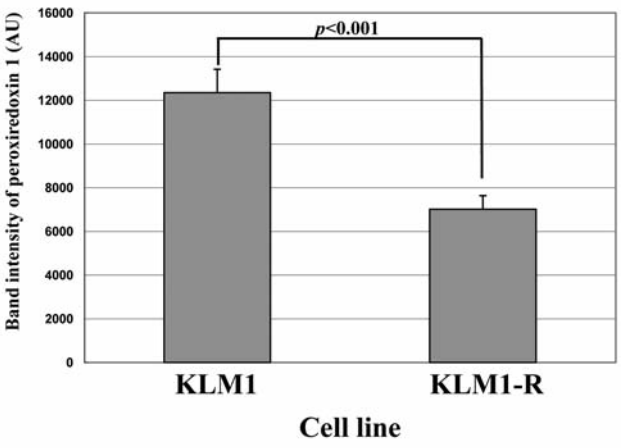

Figure 4. *Comparison of the intensities of bands of peroxiredoxin-1 between gemcitabine-resistant human pancreatic cancer KLM1-R cells and parental gemcitabine-sensitive KLM1 cells. The intensities of peroxiredoxin-1 were significantly down-regulated in KLM1-R compared to KLM1 ($p<0.001$ by Student's t-test) (n=5).*

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