Up-regulation of DDX39 in Human Pancreatic Cancer Cells with Acquired Gemcitabine Resistance Compared to Gemcitabine-sensitive Parental Cells

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Abstract. *Intrinsic or acquired resistance of pancreatic cancer to gemcitabine (2'-deoxy-2'-difluorodeoxycytidine) is an important factor in the failure of gemcitabine treatment. Proteomic analysis of gemcitabine-sensitive KLM1 pancreatic cancer cells and -resistant KLM1-R cells identified heat-shock protein-27(HSP27) as a biomarker protein which is involved in gemcitabine resistance. However, a knock-down experiment showed that HSP27 was not the only protein implicated with gemcitabine-resistance. Finding further candidate proteins is necessary for achieving effective gemcitabine therapy for patients with pancreatic cancer. DDX39 is an Asp-Glu-Ala-Asp (DEAD)-box RNA helicase reported to be overexpressed in tumor cells, such as lung squamous cell cancer, gastrointestinal stromal tumor, urinary bladder cancer and malignant pleural mesothelioma. In urinary bladder cancer cells, overexpression of this protein is intimately bound with tumorigenesis and poor prognosis. In the present study, the expression of DDX39 in gemcitabine-sensitive KLM1 and -resistant KLM1-R cells was compared. It was found that DDX39 was significantly up-regulated in gemcitabine-resistant KLM1-R cells compared to sensitive KLM1 cells. The ratio of expression of DDX39 to that of actin was significantly up-regulated in KLM1-R cells compared to KLM1 cells ($p=0.0072$ by Student's t-test). These results suggest that DDX39 is a possible candidate biomarker for predicting the response of patients with pancreatic cancer to treatment with gemcitabine.*

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Gemcitabine (2'-deoxy-2'-difluorodeoxycytidine) is a deoxycytidine analog with structural and metabolic similarities to cytarabine. Gemcitabine has therapeutic effects on pancreatic cancer (1). However, the efficacy of gemcitabine on patients with pancreatic cancer is inadequate. Intrinsic or acquired resistance of pancreatic cancer to gemcitabine is an important issue (2). KLM1 is a pancreatic cancer cell line with high sensitivity to gemcitabine. Gemcitabine-resistant KLM1-R cells were established by exposing KLM1 cells to gemcitabine, and exhibit a 20-fold half-maximal inhibitory concentration (IC_{50}) for gemcitabine compared to KLM1 cells. Since KLM1-R was established from KLM1, almost all features in this cell line other than sensitivity to gemcitabine are the same as the parental KLM1 cell line. Therefore, it is meaningful to analyze these two cell lines for important proteins playing a role in gemcitabine resistance.

Our previous studies, by using proteomics, identified heat-shock protein-27 (HSP27) as a key molecule playing an important role in gemcitabine resistance (3). Furthermore, 23 up-regulated proteins, such as catalase, and 10 down-regulated proteins, such as galectin-1, in KLM1-R cells compared to KLM1 cells were identified (4). However, the identified proteins do not explain gemcitabine resistance. It is necessary to find further candidate proteins for successful gemcitabine therapy for patients with pancreatic cancer.

DDX39 is an Asp-Glu-Ala-Asp (DEAD)-box RNA helicase which plays roles not only in unwinding double-stranded RNA molecules, but also in transcription, splicing, RNA transport, ribosome biogenesis, RNA editing, RNA decay and translation, and telomere protection and

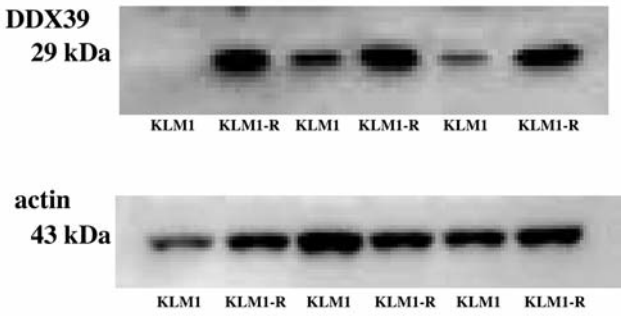

Figure 1. *Western blot analysis of DEAD (Asp-Glu-Ala-Asp) box polypeptide 39 (DDX39) in gemcitabine-resistant KLM1-R and parental gemcitabine-sensitive KLM1 human pancreatic cancer cells. Fifteen micrograms of protein were used from KLM1-R and KLM1 cells. Bands of 29 kDa are DDX39, and 43 kDa bands are actin, as a loading control.*

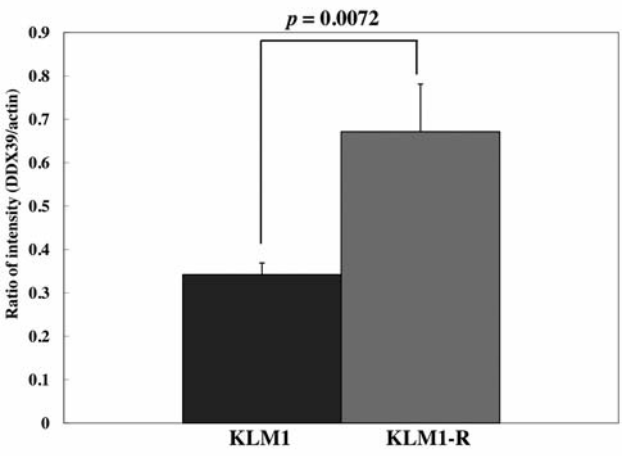

Figure 2. *Comparison of the intensities of bands between gemcitabine-resistant KLM1-R and parental gemcitabine-sensitive KLM1 human pancreatic cancer cells. The ratio of band intensity of DDX39 to actin was significantly up-regulated in KLM1-R cells compared to KLM1 cells (p=0.0072 by Student's t-test) (n=3).*

maintenance (5-8). Recent studies reported the up-regulation of DDX39 in cancer tissues and cells, such as human lung squamous cell carcinoma (5), gastrointestinal stromal tumor tissues from patients with poor prognosis (9), urinary bladder cancer tissues (10) and human malignant pleural mesothelioma cells (11). Since DDX39 was found to predict poor prognosis of patients with gastrointestinal stromal tumor (9), we expected DDX39 to play a role in gemcitabine resistance. However, Kato *et al*. showed that DDX39 was a suppressor of invasion and loss of its function predicted disease progression in bladder cancer (10). Therefore, the precise role of DDX39 in cancer progression and drug resistance is still unclear.

In the present study, in order to compare the expression of DDX39 in gemcitabine-sensitive and resistant pancreatic cancer cells, we performed western blot analysis of DDX39 in KLM1 and KLM1-R cells.

Materials and Methods

Cancer cell lines and culture conditions. The human pancreatic cancer cell lines KLM1 and KLM1-R were kindly provided by the Department of Surgery and Science at Kyushu University Graduate School of Medical Science. KLM1-R was established by exposing KLM1 cells to gemcitabine, as described previously (12). These cells were cultured in RPMI-1640 medium with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 10 mM HEPES, 1.0 mM sodium pyruvate, and 10% fetal bovine serum. All cells were maintained in a water-saturated atmosphere containing 5% CO2 at 37˚C.

Sample preparation. When the cells grew sub-confluently they 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 as samples (3, 13, 14).

Western blotting. Fifteen micrograms of each protein sample were used for western blot analysis. Sodium dodecyl sulfate-polyaclylamide gel electrophoresis (SDS-PAGE) was carried out in 15% polyacrylamide slab gels (width: 85 mm, height: 60 mm, thickness: 1 mm). After SDS-PAGE, the proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA, USA) by electroblotting, and blocked for 1 h at room temperature with Tris-buffered saline (TBS) containing 5% skimmed milk. Membranes were then incubated with rabbit polyclonal antibody to DDX39 (1.25 μg/ml, #AV36384; Sigma-Aldrich, Saint Louis, MO, USA) or goat polyclonal antibody to actin (1:500, #sc-1616; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4˚C. After washing with TBS containing 0.05% Tween-20 three times and with TBS once, the membranes were incubated 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. Protein bands were visualized by enhanced chemiluminescence reagent (ImmunoStar Long Detection; Wako, Osaka, Japan), and detected by using Image Reader LAS-1000 Pro (Fujifilm Corporation, Tokyo, Japan) (15-17). Expression levels of the protein bands were quantified by analyzing the intensity of each band with Progenesis PG240 software (Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK), and the expression level of DDX39 in each cell line is presented as a ratio of DDX39 to actin.

Statistical analysis. Statistical significance of differences between cell lines was calculated by Student's *t*-test.

Results

Western blot analysis of DDX39 in gemcitabine-resistant KLM1-R and -sensitive KLM1 cells. The intracellular proteins from gemcitabine-resistant KLM1-R and -sensitive

KLM1 cells were analyzed by western blotting with a primary antibody against DDX39 and actin. The expression levels of DDX39 were elevated significantly in KLM1-R cells compared to KLM1 cells (Figure 1). The ratio of DDX39 to actin was significantly up-regulated in KLM1-R compared to KLM1 cells (p=0.0072 by Student's t-test) (Figure 2).

Discussion

Up-regulation of DDX39 in gemcitabine-resistant pancreatic cancer KLM1-R cells was observed on western blot analysis using a specific antibody against DDX39. The band intensity of DDX39 was significantly up-regulated in KLM1-R compared to KLM1 cells (p=0.0072 by Student's t-test).

KLM1-R was established from KLM1. KLM1 cells are gemcitabine-sensitive. The cells were treated with 10 μg/ml gemcitabine for one week, and then cultured in a gemcitabine-free medium for two weeks. After repeating the treatment four times cells exhibited stable drug resistance (12). This procedure for the induction of gemcitabine resistance is quite simple, and it is easy to believe almost all features in this cell other than sensitivity to gemcitabine are the same as these of KLM1 cells. Therefore, the proteins which exhibit different expression between KLM1 and KLM1-R cells may reveal possible roles in gemcitabine resistance.

Kikuta *et al*. identified DDX39 as a prognostic marker for patients with gastrointestinal stromal tumor. Kubota *et al*. reported the significant association of the expression of DDX39 with metastasis and poor clinical outcomes in patients with gastrointestinal stromal tumor (9, 18). Although if the patients were treated with gemcitabine or other chemotherapeutic agents, it can be correct that DDX39 plays an important role in gemcitabine resistance or drug resistance, they described that patients were not treated with adjuvant chemotherapy until distant metastasis was detected. What is the role of DDX39 in gemcitabine resistance? At this time there is no report showing the relation of up-regulated DDX39 and drug resistance. However, Yoo *et al*. showed the induction of progressive telomere elongation by overexpression of DDX39 in cancer cells (8). Telomere protection and maintenance induced by up-regulated DDX39 may protect cancer cells from apoptosis induced by gemcitabine. The relation and mechanism of gemcitabine resistance induced by overexpression of DDX39 in pancreatic cancer cells remains to be investigated.

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