Comparative Proteomic Analysis of Two Stress-management Strategies in Pancreatic Cancer

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Abstract. *Background: It is known that cancers adopt different strategies to cope with stress and overcome adverse micro-environmental conditions. Such strategies are also applicable to chemo-therapeutic treatment, which could subsequently result in chemo-resistance. Materials and Methods: In order to investigate known stress-evasion strategies observed in pancreatic cancer, the stress-resistant KLM1-derived cell lines KLM1-R (Gemcitabine (GEM)-induced stress) and KLM1-S (growth factor restriction-induced stress) were employed. Comparative proteomics were employed between for the two cell lines that were also compared against the parent cell line KLM1. Results: Proteomic analysis revealed changes in the expression levels of 6 proteins, namely: transitional endoplasmic reticulum ATPase, lamin A/C, PDZ and LIM protein 1, calmodulin, heat shock protein 60 and alpha enolase. Resistance to GEM of KLM1-R and KLM1-S was found to be comparable, with KLM1-S cells exhibiting close to 1.5-fold higher half-maximal inhibitory concentration (IC_{50}) compared to KLM1-R cells. Conclusion: These results suggest that KLM1-R can be used as a model of directly-acquired chemoresistance (responding directly to evade GEM treatment), while KLM1-S is a good model of indirectly-acquired chemoresistance (formed in response to having to survive with less availability of growth factors), additionally gaining a selective advantage upon GEM treatment.*

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Gemcitabine (GEM) is currently the standard chemotherapeutic agent used for patients with pancreatic cancer (1, 2). However, the clinical success of GEM is low due to a high degree of inherent and acquired chemoresistance (3, 4). GEM is known to be affected by various metabolic pathways, with multiple mechanisms contributing to its cytotoxicity and/or chemoresistance (5, 6). Molecular markers related to chemoresistance to GEM are generally related to apoptosis or DNA repair. In the case of apoptosis-related markers, these include genes activating the phosphatidylinositol 3-kinase (PI3K)/Akt or nuclear factor-kappa B (NF-κB) pathways controlling cell cycle regulation, proliferation and apoptosis, while in the case of DNA repair-related markers, these include genes involved in nucleoside transport and metabolism, such as DNA repair enzymes and nucleoside transporters (7-10).

In this study, proteomics analysis was employed in an attemp to understand how different chemoresistant populations of pancreatic cancer diverge in their strategies to survive chemotherapy treatment. The proteomic profiles of two cell lines derived from the pancreatic cancer cell line KLM1, produced through different stress mechanisms, were compared. The two cell lines used were KLM1-R, developed by extended exposure to GEM (considered to have directly-aquired chemoresistance) and KLM1-S, developed *via* culture with restricted growth factor availability (considered to have indirectly-aquired chemoresistance).

Materials and Methods

Cell culture. Human KLM1 (provided by the Department of Surgery and Science at Kyushyu University Graduate School of Medical Science), KLM1-R (provided by the Department of Surgery and Science at Kyushyu University Graduate School of Medical Science) and KLM1-S (generated in our lab) pancreatic cancer cell lines were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) with L-glutamate, supplemented with 10% (KLM1 and KLM1-R) or 1% (KLM1-S) heat-inactivated fetal

bovine serum (FBS), at 37˚C and 5% CO_2. KLM1-R cells were treated with 1 μg/ml for 3 passages before the start of the experiment (11).

Proteomic analysis. KLM1 and KLM1-S cell samples were homogenized in ice-cold 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 (PMSF), 10 mM ethylene-diaminetetraacetic acid (EDTA), 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1% NP-40). Suspensions were incubated with shaking for 1 h at 4˚C, centrifuged at 21,500 × *g* for 30 min at 4˚C to pellet any cell debris and the supernatant was stored at –80˚C until required (12).

Two-dimensional gel electrophoresis (2-DE) was performed following the same method described previously (13). In short, isoelectric focusing (IEF) was performed for the first dimension using an IPGphor 3 IEF unit (GE Healthcare, Buckinghamshire, UK) on 11 cm, immobilised, pH 3-10 linear gradient strips (Bio-Rad, Hercules, CA, USA) at 50 μA/strip. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed for the second dimension using precast 5-20% linear concentration gradient polyacrylamide gel (Bio-Rad), run at 200 V for 45 min. The gels were washed with Milli-Q (Millipore, Bedford, MA, USA) water three times before being stained overnight with See Pico™ (Benebiosis Co., Ltd, Seoul, Korea). After staining, the excess stain was removed by washing the gels with Milli-Q water containing 0.1% Tween-20, three times.

The positions of the proteins on the gel were marked using the ProEXPRESS 2D Proteomic Imaging System (PerkinElmer Inc., Waltham, MA, USA) and the intensity of each spot was subsequently analyzed using the Progenesis SameSpot software (Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK) to quantify the protein expression levels (13). The analysis of variance (ANOVA) test was used to determine the statistical difference in expression between KLM1-R and KLM1-S (in triplicates). Spots that appeared to be significantly different in expression between KLM1-R and KLM1-S cells were cut out from the gel for mass spectrometry (MS) analysis.

In-gel digestion was carried out as previously described (13). In summary, the cut gel pieces were reduced twice in 50% acetonitrile (ACN), 50 mM ammonium bicarbonate, and 5 mM dithiothreitol (DTT) for 10 min. Then, they were dehydrated in 100% ACN twice for 30 min and finally rehydrated in a solution of 30% ACN, 50 mM ammonium bicarbonate and 5 mM DTT, containing 10 μg/ml sequencing grade-modified trypsin (Promega, Madison, WI, USA).

High-performance liquid chromatography (HPLC) and tandem mass spectrometry (MS/MS) were carried out on an Agilent 1100 LC/MSD Trap XCT (Agilent Technologies, Palo Alto, CA, USA), separating 25 μl of each sample on a Zorbax 300SB-C18 column (75 μm, 150 mm, Agilent Technologies). Protein identification was performed using 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) (14, 15).

Western blotting. Proteins were separated by SDS-PAGE electrophoresis and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore, Bedford, MA, USA). Membranes were blocked for 2 h at room temperature with TBS containing 5% skimmed milk. The primary antibodies used were goat polyclonal anti-lamin A/C (sc-6215; 1:200 dilution) (Santa Cruz Biotechnology, Dallas, TX, USA), rabbit polyclonal anti-HSP60 (ab46798; 1:5000 dilution) (Abcam, Cambridge, UK), goat polyclonal anti-alpha-enolase (sc-7455; 1:200 dilution) (Santa Cruz Biotechnology, Dallas, TX, USA) and goat polyclonal anti-actin (sc-1616; 1:1000 dilution) (Santa Cruz Biotechnology, Dallas, TX, USA).

Following incubation with the primary antibodies overnight at 4˚C, membranes were washed three times with TBS and then incubated with a complimentary horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution; Jackson ImmunoResearch Lab., West Grove, PA, USA) for 1 h at room temperature. Protein bands were visualized by exposure to a chemiluminescent reagent (ImmunoStar Long Detection; Wako, Osaka, Japan) and detected using an Image Reader LAS-1000 Pro (Fujifilm Corporation, Tokyo, Japan).

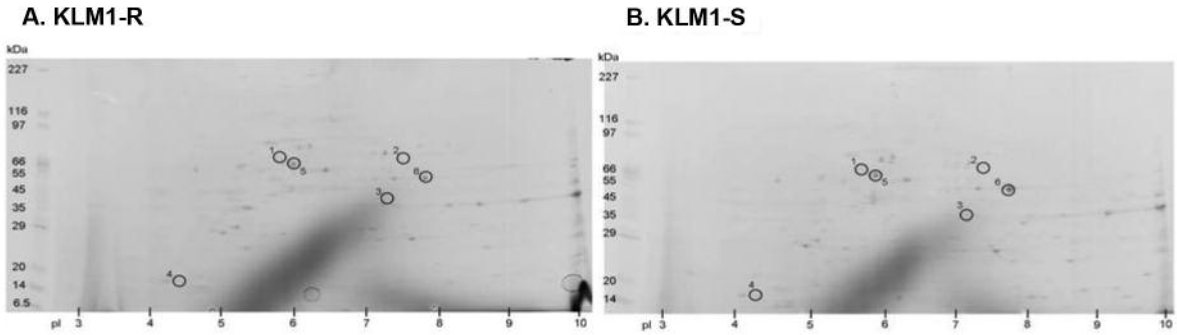
Cell Viability Assay. To determine the ability of the two cell lines to survive chemotherapy treatment by GEM, KLM1-R and KLM1-S cells were cultured to >80% confluency in flat-bottomed 96-well plates and treated with GEM at varying concentrations (from 0.01 to 20 μg/ml). Cell proliferation assays using 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS; CellTiter 96® Aqueous One Solution Cell Proliferation Assay; Promega) were carried out after 72h. Absorbance readings at 490nm were taken after 1h incubation with MTS reagent.

Results

Proteomic analysis. In order to link the observed chemoresistance to biochemical changes within these two KLM1-derived cell lines, comparative 2D-analysis was performed. The Progenesis SameSpot software was used to analyze differential protein expression in 2D-resolved KLM1-R and KLM1-S samples. This resulted in the identification of 10 protein spots differentially expressed in KLM1-S compared to KLM1-R cells ($p<0.05$) of which 6 (Figure 1A and B; indicated by circles) were successfully identified using the Agilent Spectrum MILL MS proteomics workbench against the Swiss-Prot protein database search engine and the MASCOT MS/MS ions search engine, following peptide detection using the Agilent 1100 LC-MS/MS Trap XCT system in positive ion mode (Figure 1C).

Western blotting. The proteins identified by proteomic analysis, lamin A/C, heat shock protein 60 (HSP60) and alpha enolase, were confirmed by western blotting using actin as a loading control (Figure 2). These three proteins showed significant up-regulation, as expected based on the comparative proteomic analysis.

Cell viability assay. The effect of the different stress-survival strategies of KLM1-R and KLM1-S on their chemoresistance was tested by exposing KLM1-R and KLM1-S cells to GEM ranging from 0.01 to 20 μg/ml for 72 h, followed by an MTS assay. The results (Figure 3) show that the half-maximal inhibitory concentration (IC_{50}), *i.e.* the concentration of GEM causing 50% growth



C

Spot No.	Protein Name	Accession No.	Fold Change (KLM1-S/KLM1-R)
1	Transitional ER ATPase	P55072	1.8 (increase)
2	Prelamin A/C	P02545	1.8 (increase)
3	PDZ and LIM domain protein 1	O00151	1.7 (decrease)
4	Calmodulin	P62158	2.7 (increase)
5	Heat Shock Protein 60	P10809	1.2 (increase)
6	Alpha enolase	P06733	3.0 (increase)

Figure 1. *2D-PAGE gels for A: KLM1-R and B: KLM1-S showing 6 differentially expressed protein spots, determined by Progenesis SameSpot software, which were subsequently identified by mass spectrometry. The protein names and accession numbers of these 6 successfully identified proteins are listed in C.*

inhibition, for the two cell lines is comparable with KLM1-S (IC_{50}≈30 μg/ml GEM), being only 1.5-fold more resistant to GEM than KLM1-R (IC_{50}=20 μg/ml GEM).

Discussion

Through proteomic analysis it was possible to isolate and identify six proteins, namely transitional endoplasmic reticulum ATPase, PDZ and LIM protein 1, calmodulin, lamin A/C, HSP60 and alpha enolase, out of which the latter three were further validated by western blotting. In all three cases, the protein level was up-regulated in KLM1-S compared to KLM1-R indicating a higher reliance on survival mechanisms that appear to centre on the PI3K/Akt pathway.

Alpha-enolase is commonly over-expressed in pancreatic cancer (16) supporting anaerobic proliferation and, when present on the cell surface, can act as a surface plasminogen receptor (17). Once bound, plasminogen is more efficiently activated to plasmin and, subsequently, contributes to tumor processes, such as invasion, metastasis and inflammatory responses (18). In pancreatic cancer, the post-translational modifications of alpha-enolase, namely acetylation, methylation and phosphorylation, also play a role (19). Furthermore, IgG autoantibodies to phosphorylated alpha-enolase were found in the sera from pancreatic cancer patients (20, 21). The overall role of alpha-enolase towards pancreatic cancer progression appears to be through increased phosphorylation of PI3K and Akt, thus modulating cell cycle and epithelial to mesenchymal transition (EMT) process (22).

In the case of lamin-A/C, its association to carcinogenesis is still not clearly understood. Lamin proteins fulfill a key structural role in nuclear architecture and play a role in chromatin remodeling, DNA replication, DNA repair, gene transcription and silencing (23, 24). Related to malignancies, increased lamin A/C protein expression has been associated with increased tumor invasiveness in colorectal cancer (25) and high-risk prostate cancer (24). Furthermore, the epigenetic silencing of the lamin A/C gene has been associated with poor outcomes in diffuse large B cell lymphoma (26). The mechanistic explanation for the role of lamin A/C in invasion is through modulation of the signaling pathway involving PI3K, Akt and PTEN (24).

Although calmodulin was not confirmed by Western blotting, this protein has been shown to be closely correlated with the occurrence, growth and proliferation of tumors and directly linked to the PI3K/Akt pathway (27, 28). Calmodulin associates with the Src Homology 2 (SH2) domains of the 85-kDa regulatory subunit of PI3K leading to enhanced PI3K activity, which, in turn, modulates downstream signaling and induces Akt phosphorylation (29-31), thus playing a role in promoting tumor progression. It has been shown that in breast cancer, epidermal growth factor (EGF)-induced activation of Akt is mediated by

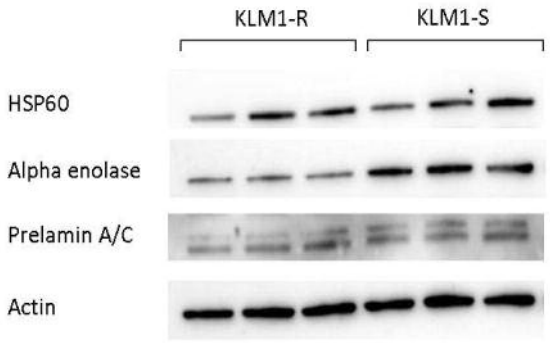


Figure 2. *Western blot analysis for the differentially expressed proteins identified by mass spectrometry.*

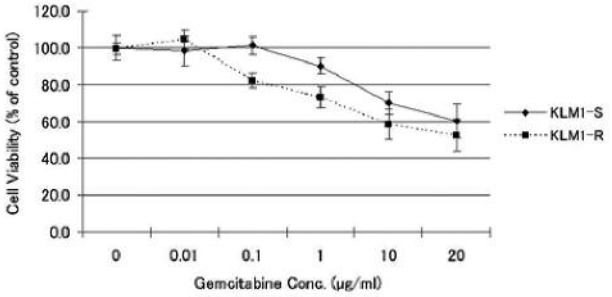


Figure 3. *Cell viability assay for KLM1-R and KLM1-S cells after 72 h exposure to concentrations of GEM ranging from 0.01 to 20 μg/ml. The IC_{50} for KLM1-R is close to 20 μg/ml GEM, while the IC_{50} of KLM1-S is beyond a concentration of 20 μg/ml GEM (extrapolated to 30 μg/ml GEM), which reflects a 1.5-fold greater GEM resistance in KLM1-S compared to KLM1-R.*

calmodulin possibly through the modulation of sub-cellular translocation to the membrane (32), while, in hepatocellular carcinoma, calmodulin activation of PI3K is correlated with disease progression (28). Calmodulin might also be the link between the PI3K/AKT pathway and the last two proteins identified in our proteomic analysis: transitional endoplasmic reticulum ATPase, PDZ and LIM protein 1.

While HSP60 is known to be over-expressed in a variety of tumors, this protein is not commonly over-expressed in pancreatic cancer (33). Thus far, the only reported observations related to pancreatic cancer are that HSP60 may be linked to metastasis *via* interaction with beta-catenin (34, 35) and that there is no apparent correlation between HSP60 levels and tumor differentiation grade (36).

Conclusion

- Different stress-mitigating strategies in pancreatic cancer cell lines were investigated in this study.
- The stress-resistant KLM1-derived cell lines KLM1-R (GEM-induced stress) and KLM1-S (growth factor restriction-induced stress) were employed.
- Proteomic analysis revealed changes in the expression levels of 6 proteins, namely, transitional endoplasmic reticulum ATPase, lamin A/C, PDZ and LIM protein 1, calmodulin, heat shock protein 60 and alpha enolase.
- Resistance to GEM of KLM1-R and KLM1-S was found to be comparable, with KLM1-S cells exhibiting close to 1.5-fold higher IC_{50} compared to KLM1-R cells.
- These results suggest that KLM1-R can be used as a model of directly-acquired chemoresistance (responding directly to evade GEM treatment), while KLM1-S is a good model of indirectly-acquired chemoresistance (formed in response to having to survive with less availability of growth factors) additionally gaining a selective advantage upon GEM treatment.

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Western blot detection by Image Reader LAS-1000 Pro was carried out at the Gene Research Centre of Yamaguchi University.

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