Isolation of a Growth Factor Stress-induced Pancreatic Cancer Sub-population: Insight into Changes Due to Micro-environment

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Abstract. *Background: Micro-environment plays a crucial role in determining the phenotypes within a tumor. Materials and Methods: In order to understand how the micro-environment affects pancreatic cancer, KLM1 cells were cultured under growth factor stress by culturing in foetal bovine serum (FBS)-free and reduced (1%) medium over several passages to mimic the core of a solid tumor with low vascularisation. Results: Proteomic analysis on these conditioned pancreatic cancer cells, called KLM1-S, compared to the parent cell line KLM1 revealed that a number of proteins including α-enolase, GAPDH, GRP78, HSP60 and STIP-1 were dysregulated. Additionally, KLM1-S cells exhibited a 250-fold increase in half-maximal inhibitory concentration (IC_{50}) over the parent cell line KLM1. Conclusion: By decreasing their replication rate and levels of intracellular reactive oxygen species (ROS), KLM1-S cells are able to resist gemcitabine (GEM). The results obtained suggest that in KLM1 different phenotypes are a result of cellular plasticity rather than a committed transformation.*

One of the major hurdles in cancer treatment is the presence of various sub-populations that respond differently to chemotherapy and the micro-environment within a tumor that plays a significant role in the phenotypes produced. The micro-environment of any solid tumor is composed of cancer cells and stromal cells, surrounded by an extracellular matrix and supported by a vascular network in a disorganised manner leading to micro-environment heterogeneity. The tumor stroma plays a key role by synthesising growth factors, chemokines and adhesion molecules (1), performing matrix remodelling (2), aiding malignant transformation, invasion and metastasis (3, 4). However, deep inside a solid tumor, cancer cells experience hypoxia and receive a decreased supply of critical compounds, such as glucose, essential amino acids and growth factors.

Under *in vitro* conditions, foetal bovine serum (FBS) provides cancer cells with all the factors provided *in vivo* by stromal cells. In this study, the effect of serum deprivation of pancreatic cancer phenotype was analyzed by comparative proteomics in an attempt to understand how a sub-set of pancreatic cancer cells can survive such conditions through modulation of biochemical pathways, also affecting their sensitivity to chemotherapy.

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Materials and Methods

Cell culture. Human KLM1 (provided by the Department of Surgery and Science at Kyushyu University Graduate School of Medical Science), PK-59 (provided by the Institute of Development, Aging and Cancer at Tohoku University) and MIAPaCa2 (provided by the Institute of Development, Aging and Cancer at Tohoku University) pancreatic cancer cell lines were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) with L-glutamate, supplemented with 10% heat-inactivated FBS, at 37˚C and 5% CO_2 for 3 passages. The next 3 passages were maintained in the same conditions except for the lack of FBS. Neither PK-59 nor MIAPaca2 managed to survive without FBS for the entirety of this period and, thus, no further conditioning was carried out. The surviving KLM1 cells were cultured for 3 passages without FBS and the small proportion of cells that survived this conditioning were subsequently cultured for all consecutive passages with 1% FBS, exhibiting a relatively stable condition with respect to morphology; from this point on, these cells were considered to be KLM1-S (stress-resistant).

Wound healing assay. KLM1 and KLM1-S cells were cultured in 12-well plates using DMEM supplemented with 10% and 1% FBS, respectively, until confluent. A 1 mm-wide wound was then created using a sterile yellow pipette tip and the progress of cell growth was followed for 72 h.

Proteomic analysis. KLM1 and KLM1-S cell samples were homogenised 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 (PMSF), 10 mM ethylenediaminetetraacetic acid (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 (5).

Two-dimensional gel electrophoresis (2-DE) was then carried out according to a method described previously (6). In summary, 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 on a precast polyacrylamide gel with a linear concentration gradient of 5-20% (Bio-Rad), run at 200 V. 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 gels were washed with Milli-Q water with 0.1% Tween-20, three times to remove excess stain.

The ProEXPRESS 2D Proteomic Imaging System (PerkinElmer Inc., Waltham, MA, USA) was used to record the positions of the proteins on the gel. The intensity of each spot was analyzed using the Progenesis SameSpot software (Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK) to quantify the protein expression levels (6). The differences in expression between the KLM1 and KLM1-S cells were analysed statistically using the analysis of variance (ANOVA) test. The 2-DE analysis was performed in triplicate. After statistical analysis, those spots that appeared to be significantly different in expression between KLM1 and KLM1-S cells were cut out from the gel for mass spectrometry (MS) analysis.

In-gel digestion was carried out according to a method described previously (6). Briefly, each set of cut-out gel pieces was reduced twice in 50% acetonitrile (ACN), 50 mM ammonium bicarbonate, and 5 mM dithiothreitol (DTT) for 10 min. The gel pieces were 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.

An Agilent 1100 LC/MSD Trap XCT (Agilent Technologies, Palo Alto, CA, USA) was used for high-performance liquid chromatography (HPLC) and tandem mass spectrometry (MS/MS). For each sample, 25 μl were applied and separated on a column (Zorbax 300SB-C18, 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) (7, 8).

Western blotting. After SDS-PAGE electrophoresis, gels were electroblotted onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore, Bedford, MA, USA) and blocked for 2 h at room temperature with TBS containing 5% skimmed milk. The primary antibodies used were rabbit polyclonal anti-STIP-1 (CST 4464; 1:1000 dilution) (Cell Signalling Technology, Boston, MA, USA), rabbit polyclonal anti-HSP60 (ab46798; 1:5000 dilution) (Abcam, Cambridge, UK), goat polyclonal anti-GRP78 (sc-1050; 1:200 dilution) (Santa Cruz Biotechnology, Dallas, TX, USA), mouse monoclonal anti-14-3-3σ (sc-100638; 1:1000 dilution) (Santa Cruz Biotechnology, Dallas, TX, USA) and goat polyclonal anti-actin (sc-1616; 1:1000 dilution) (Santa Cruz Biotechnology, Dallas, TX, USA).

Membranes were incubated with the primary antibodies overnight at 4˚C, washed three times with TBS containing 0.05% Tween-20 and once with TBS and then incubated with an adequate horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution; Jackson ImmunoResearch Lab., West Grove, PA, USA) for 1 h at room temperature. Membranes were then treated with a chemiluminescent reagent (ImmunoStar Long Detection; Wako, Osaka, Japan) and proteins were detected by using Image Reader LAS-1000 Pro (Fujifilm Corporation, Tokyo, Japan).

Cell viability assay. KLM1 and KLM1-S cells were cultured in flat-bottomed 96-well plates. After reaching >80% confluency, cells were treated with gemcitabine (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 72 h to determine the ability of KLM1-S cells to survive chemotherapy treatment compared to the parent cell line KLM1. Absorbance readings at 490nm were taken after 1h incubation with MTS reagent.

Results

Morphology. KLM1 cells are epithelial-like in morphology, presenting a regular polygonal shape and even size. However, upon exposure to serum-deprivation stress, the resultant KLM1-S cells tend to lose the regular polygonal shape and exhibit varying sizes. Particularly, after the third passage of conditioning, about 10% of the surviving KLM1 cells showed the hallmarks of senescence, *i.e.* polynucleation and enlargement due to flattening of the cytoplasm. However, these cells died off, over subsequent passages and the proliferating culture gave rise to regular diploid cells but still maintaining irregular shape and size (Figure 1).

Wound healing assay. To understand whether the morphological changes observed in the KLM1-S culture also affected the physical properties of these cells, a wound healing assay was carried out to compare the migration rates of KLM1-S cells with the parent KLM1 culture. The difference in migration was found to only be slightly less significance in KLM1-S compared to KLM1 and in both cell lines the wound was completely healed within 72 h. The kind of cells that filled the wound, however, varied markedly. As previously observed, while the KLM1 proliferating cells were all regular, epithelial-type cells, KLM1-S cells filling the wound included spindle-shaped and polynucleated cells (Figure 2; arrows).

Proteomic analysis. In order to link the observed morphological changes to the stress-induced biochemical

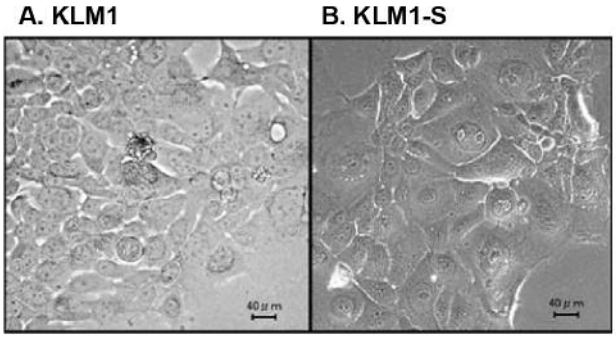


Figure 1. *Light microscope (magnification ×200) images showing the different morphologies of A, KLM1; and B, KLM1-S cells. KLM1 cells show a typical epithelial-like morphology, having a regular polygonal shape and even size. KLM1-S cells exhibit an irregular shape and varying sizes, including a small number of polynucleated cells with enlarged, flattened cytoplasm.*

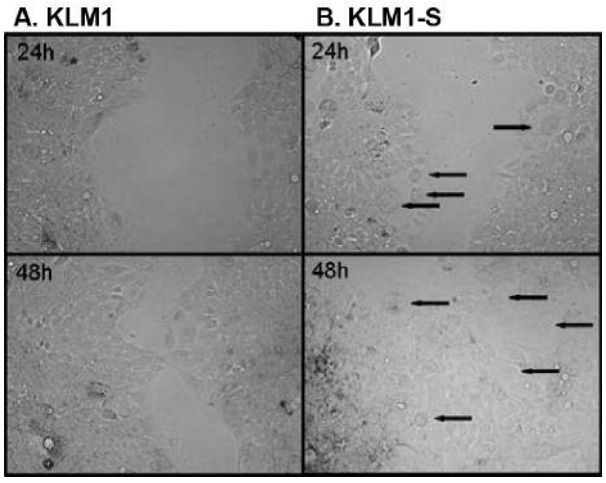


Figure 2. *Wound healing assay showing the difference in the rate of outgrowth over the first 48 h after the incision of a 5 mm wound in a confluent plate of A, KLM1; and B, KLM1-S cells. Polynucleated and spindle-shaped KLM1-S cells filling the wound are marked by arrows.*

changes within these newly-established cells, a comparative 2D-analysis was carried out. Differential protein expression in 2D-resolved KLM1 and KLM1-S was analyzed by the Progenesis SameSpot software. Differences in spot intensities between the two cell lines were found for 20 protein spots (Figure 3; circles), with 14 protein spots being up-regulated and 6 protein spots being down-regulated in KLM1-S compared to KLM1 cells ($p<0.05$). Out of these 20 protein spots, 9 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 3).

Western blotting. The stress-related proteins identified by proteomic analysis were confirmed by western blotting, using actin as a loading control (Figure 4). The stress-related proteins STIP-1, HSP60, 14-3-3σ and GRP78 showed a significant up-regulation as was expected based on the comparative proteomic analysis.

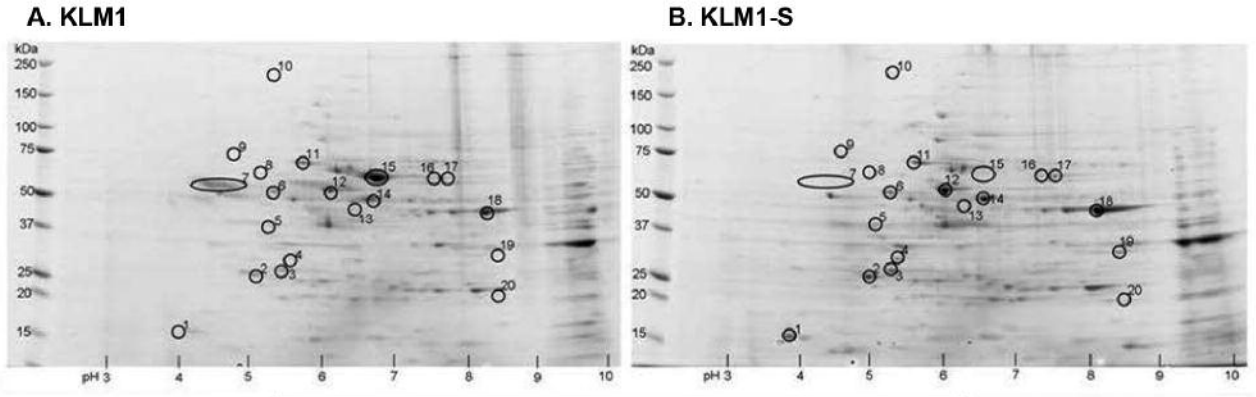
Cell viability assay. To check if these biochemical changes supporting the stress-survival ability of KLM1-S cells also influenced their chemoresistance, KLM1 and KLM1-S were exposed to GEM ranging from 0.01 to 20 μg/ml for 72 h and analyzed by the MTS assay. The results clearly show that while the half-maximal inhibitory concentration (IC_{50}), *i.e.* the concentration of GEM causing 50% growth inhibition, for KLM1 is slightly over 0.1 μg/ml GEM, KLM1-S does not reach its IC_{50} even at a concentration of 20 μg/ml GEM, which means it is over 250-fold more resistant to GEM (Figure 5).

Discussion

When tumors present an aggressive phenotype, there is often an associated insufficient blood supply. This is due to the fact that such tumor cells proliferate at a faster rate than the endothelial cells generating the vascular system and even, if produced, tumor vascularisation is poorly organised. This inevitably leads to reduced delivery of oxygen, nutrients and important factors together with reduced elimination of metabolic products and secretions (9-11). This is particularly true for pancreatic cancer and has an impact on the limited effectiveness of chemotherapy observed in various pancreatic cancers.

The limited supply of oxygen and restricted glucose availability within the tumor micro-environment and their link to resistance to therapy has been intensively studied over the past two decades (reviewed in 12 and 13). Hypoxia has been linked with worse prognosis, through the action of the transcription factor hypoxia-inducible factor-1α HIF-1α (14, 15), partly *via* the activation of the PI3K/Akt pathway, which is thought to induce genes to function as factors towards apoptotic suppression induced by hypoxia and glucose deprivation (16-18).

However, restricted serum availability (for factors other than glucose and amino acids) in pancreatic cancer has not been so well-characterised with only a single study focusing on the prolonged resistance to serum deprivation at the protein level (17). In the present study, this issue was addressed by starting with three cell lines, which show varying resistance to GEM. MIAPaCa2 is GEM-sensitive,



C

Spot No.	Protein Name	Accession No.	Fold Change (KLM1-S/KLM1)
2	14-3-3 sigma	P31947	3.3 (increase)
5	Reticulocalbin-1	Q15293	3.8 (increase)
6	Protein Disulphide Isomerase	Q15084	4.8 (increase)
11	78kDa Glucose-Regulated Protein	P11021	3.1 (increase)
12	Heat Shock Protein 60	P10809	5.4 (increase)
17	Stress-Induced Phosphoprotein 1	P31948	5.2 (increase)
18	Alpha-enolase	P06733	2.4 (decrease)
19	Glyceraldehyde-3-Phosphate Dehydrogenase	P04406	2.2 (decrease)
20	Glyceraldehyde-3-Phosphate Dehydrogenase	P04406	4.0 (decrease)

Figure 3. *2D-PAGE gels for A, KLM1; and B, KLM1-S showing 20 differentially expressed protein spots, determined by the Progenesis SameSpot software. Out of these 20 spots 14 were up-regulated and 6 were down-regulated in KLM1-S compared to KLM1 cells. The protein names and accession numbers of the 9 spots that were successfully identified are listed in C.*

PK-59 is GEM-resistant and KLM1 is GEM-sensitive but known to become resistant to GEM, if conditioned.

In the process of serum-depletion culture conditioning, only a small sub-population of KLM1 cells survived and upon extended culturing under serum restriction the cell line KLM1-S was obtained, which presented a different set of characteristics from its parent cell line, including more irregular cell morphology (with some senescent-like cells), increased resistance to stress, differential expression of at least 20 proteins (as defined by comparative proteomics) and chemoresistance.

What is particularly interesting about the small percentage of recurrent senescent cells is that they could be enhancing the proliferative rate, migration and invasion of KLM1-S. It has been shown that pre-malignant cells present enhanced proliferation, migration and invasion when co-cultured with, or grown in medium conditioned by, senescent fibroblasts (19-22).

Upon comparative proteomic analysis, the differences in protein profiles was clearly evident. The top 20 differentially expressed protein spots, determined by the Progenesis SameSpot software, included 14 up-regulated and 6 down-regulated protein spots in KLM1-S compared to KLM1 cells. Out of these, 9 spots were successfully identified and all have been previously linked to increased cancer survival under stress and chemoresistance.

Stress-induced phosphoprotein-1 (STIP-1) has been reported to be over-expressed in both invasive-type pancreatic cancer cell lines and malignant tissue samples from pancreatic cancer patients (23). This co-chaperone protein is known to bind both heat shock proteins HSP70 and HSP90 but its role in pancreatic cancer is not yet fully understood. However, its localisation has been determined to be an important factor in pancreatic tumors. Moreover, increased STIP-1 expression has been linked with the invasiveness of pancreatic cancer cells possibly through the modulation of HSP90 activity (24).

Over-expression of GRP78 in pancreatic adenocarcinoma has only been reported in one study (25). However, its over-expression has also been observed in various types of cancer cell lines and solid tumors, including lung, colon and liver cancers (26-28). It is known that GRP78 inhibits apoptotic signaling and protects the cell from apoptosis induced by ER or non-ER stress, such as those brought about by various chemotherapy agents (29-31).

HSP60 is not commonly over-expressed in pancreatic cancer and, so far, the only reported observation is the

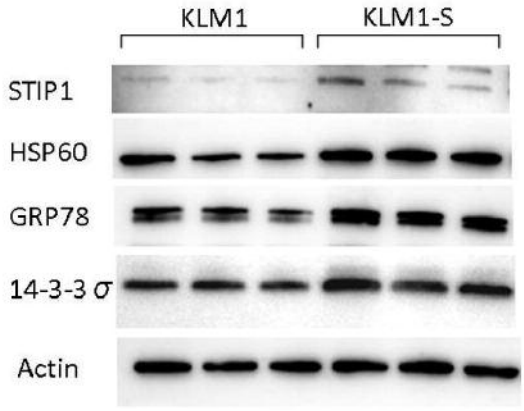


Figure 4. *Western blot analysis for the differentially expressed proteins identified by mass spectrometry.*

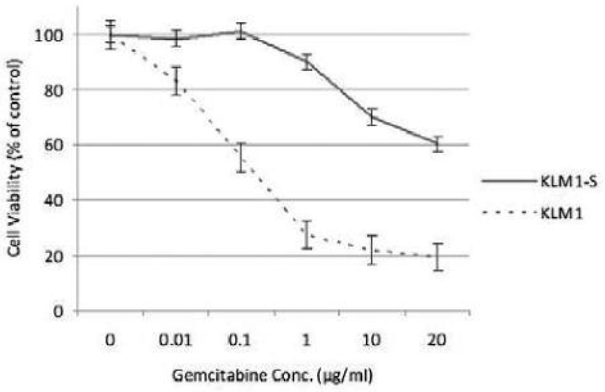


Figure 5. *Cell viability assay for KLM1 and KLM1-S cells after 72 h exposure to concentrations of GEM ranging from 0.01 to 20 μg/ml. The IC_{50} for KLM1 is slightly over 0.1 μg/ml GEM, while the IC_{50} of KLM1-S is beyond a concentration of 20 μg/ml GEM, which reflects a 250-fold increase in GEM resistance from KLM1 to KLM1-S.*

absence of correlation between HSP60 levels and tumor differentiation grade (32). It has been reported, however, to be linked to metastization of pancreatic cancer *via* interaction with β-catenin (33, 34).

14-3-3σ expression is known to be up-regulated in pancreatic adenocarcinoma cells (35-37) and contribute to the resistance of pancreatic cancer cells toward γ-irradiation, as well as anticancer drugs (cisplatin, GEM, adriamycin and mitoxantrone) by causing resistance to treatment-induced apoptosis (together with a decrease in caspase-3 activation and poly (ADP-ribose) polymerase (PARP) cleavage) and G_2/M arrest (36-38) This over-expression was further correlated with the degree of differentiation, metastasis and poor prognosis (37, 39, 40). However, this goes against the findings of Guweidhi *et al*. (35) that the degree of 14-3-3σ expression was not important in the maintenance of the G_2/M checkpoint or induction of apoptosis. Based on the use of various inhibitors, it has been hypothesised that high levels of 14-3-3σ act with PI3K, MAPK and Src to increase pancreatic tumor progression, invasiveness and metastasis (38, 41). Interestingly pancreatic cancer cell lines were found to secrete 14-3-3σ into the medium, although the significance of this is not yet understood (38).

Conclusion

Mimicing nutrient-related stress due to a restriction in serum and the included growth factors within the pancreatic tumor (KLM1) micro-environment leads to the formation of a stress-resistant sub-population of pancreatic cancer cells (KLM1-S), which present a distinct morphological change, as well as a shift in biochemical processes, as reflected by the change in protein profile obtained through comparative proteomic analysis. This, in turn, leads to an increased resistance of this sub-population to chemotherapy. The sub-set of dysregulated proteins analyzed by western blotting seems to reflect modifications at different levels along a common complex pathway both mitigating stress and preventing cell death *via* apoptosis and autophagy.

These results suggest that with each cell division, a sub-set of the cells produced within a specific tumor region are at different levels from a stress-related threshold and have the potential to activate different stress-mitigation mechanisms. However, not all cells produced will be able to survive the stress as was observed for the senescent-type polynucleated cells and the percentage of dead cells at each passage. The advantage of producing cells, which do not activate stress-mitigating mechanisms, is that, once the stress period is over, such cells can replicate faster and easily repopulate the tumor micro-environment (or culture flask). Thus, it can be concluded that in KLM1 cultures, different phenotypes are a result of cellular plasticity (depending on the stressor) rather than a committed, irreversible transformation.

Acknowledgements

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

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