

Proteomic Differential Display Analysis for TS-1-resistant and -sensitive Pancreatic Cancer Cells Using Two-dimensional Gel Electrophoresis and Mass Spectrometry

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Abstract. *TS-1 is an oral anticancer agent containing two biochemical modulators for 5-fluorouracil (5-FU) and tegafur (FT), a metabolically activated prodrug of 5-FU. TS-1 has been recognized as an effective anticancer drug using standard therapies for patients with advanced pancreatic cancer along with gemcitabine. However, a high level of inherent and acquired tumor resistance to TS-1 induces difficulty in the treatment. To identify proteins linked to the TS-1-resistance of pancreatic cancer, we profiled protein expression levels in samples of TS-1-resistant and -sensitive pancreatic cancer cell lines by using two-dimensional gel electrophoresis (2-DE) and liquid chromatography–tandem mass spectrometry (LC-MS/MS). The cytotoxicity of a 5-FU/5-chloro-2,4-dihydropyridine (CDHP) combination towards pancreatic cancer cell lines was evaluated by MTS assay. Panc-1, BxPC-3, MiaPaCa-2 and PK59 showed high sensitivity to the 5-FU/CDHP combination (TS-1-sensitive), whereas PK45p and KLM-1 were much less sensitive (TS-1-resistant). Proteomic analysis showed that eleven spots, including T-complex protein 1 subunit beta, ribonuclease inhibitor, elongation factor 1-delta, peroxiredoxin-2 and superoxide dismutase (Cu-Zn), appeared to be down-regulated, and 29 spots, including hypoxia up-regulated protein 1, lamin-A/C, endoplasmic, fascin and annexin A1, appeared to be up-regulated in TS-1-resistant cells compared with -sensitive cells. These results suggest that the identified proteins showing different expression between TS-1-sensitive and -resistant pancreatic*

cancer cells possibly relate to TS-1-sensitivity. These findings could be useful to overcome the TS-1-resistance of pancreatic cancer cells.

Pancreatic cancer is one of the most fatal types of cancer worldwide, and is the fifth leading cause of cancer death in Japan. The 5-year survival rate of this cancer is the lowest among patients with common types of cancer. Since pancreatic cancer invades progressively and metastasizes to liver and lymph nodes during early stages without remarkable symptoms, many patients have locally advanced or metastatic disease on presentation (1).

Gemcitabine and TS-1 are currently drugs of choice to treat patients with advanced pancreatic cancer. TS-1 is an oral fluoropyrimidine formulation that combines tegafur, 5-chloro-2,4-dihydropyridine (CDHP), and potassium oxonate at a molar ratio of 1:0.4:1 (2). The effect of TS-1 is expected to prolong survival of patients with advanced pancreatic cancer (3). However, TS-1-induced drug resistance of pancreatic cancer impacts its therapeutic effect. Thus, a better understanding of the molecular mechanisms of TS-1 resistance is essential to allow TS-1 to be used more effectively.

Proteomics is a powerful tool for identifying proteins whose expressions are different between drug-resistant and drug-sensitive cells. Proteomic differential display is a popular method to analyze protein expression profiling from two groups. Generally, two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) have been used for this differential display method (4). Two-DE is able to separate proteins according to both their charge in isoelectric focusing (IEF) gels and their weight in sodium dodecyl sulfate (SDS) gels. Two-DE has unique advantages for examining the expressions of hundreds of proteins simultaneously and for examining post-translational modifications of the protein spots. Our previous reports used proteomics and identified heat-shock protein 27 (HSP27) as a key molecule playing an important role in gemcitabine resistance (5-7).

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The aim of this study was to identify proteins showing differential expression in TS-1-resistant and -sensitive pancreatic cancer cell lines by using proteomics with 2-DE and liquid chromatography (LC)-MS/MS.

Materials and Methods

Tumor cell lines and culture conditions. All cell lines were obtained from Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University. Two human pancreatic adenocarcinoma cell lines Panc-1 and MiaPaCa-2 were grown in Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose and 10% fetal bovine serum. BxPC-3, KLM-1, PK45p and PK59 cells were grown in RPMI-1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, 1.0 mM sodium pyruvate and 10% FBS. Cells were incubated in a humidified atmosphere containing 5% CO₂ at 37°C (6). All cell lines are not known to be TS-1-resistant or -sensitive.

Compounds. 5-Fluorouracil (5-FU) and a reversible competitive dihydropyrimidine dehydrogenase (DPD) inhibitor, 5-chloro-2,4-dihydrogenase (CDHP) were kindly provided by Taiho Inc. (Tokyo, Japan).

Effect of 5-FU/CDHP on proliferation of pancreatic cancer cells. Concentrations of 5-FU/CDHP which induced 50% cell death (IC₅₀) of each cell line were assessed by using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega Corp. Madison, WI, USA) assay, which is based on the reduction of this tetrazolium salt by viable cells. Briefly, cells (1×10⁴ cells per well) were seeded in complete medium in 96-well plates, and cultured for 24 h. The 5-FU/CDHP mixture was used at a molar ratio of 1:1, which mimics human plasma pharmacokinetics after oral administration of TS-1(8). To determine the growth rate, cells in sextuplicate wells were mixed with 20 µL of MTS solution 72 h after 5-FU/CDHP exposure. After 3 h, the optical density of the dissolved material was measured at 490 nm with a microtiter plate reader (Model 550 Microplate Reader; Bio-Rad, Hercules, CA, USA). Results were derived from at least three independent sets of sextuplicate experiments.

Sample preparation. Cells were homogenized 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, 10 mM 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 used as samples (9).

Two-dimensional gel electrophoresis (2-DE). The 2-DE consisted of IEF and SDS-polyacrylamide gel electrophoresis (SDS-PAGE). IEF was performed in an IPGphor 3 IEF unit (GE Healthcare, Buckinghamshire, UK) on 11 cm, immobilized, pH 3-10 linear gradient strips (Bio-Rad) at 50 µA/strip. SDS-PAGE was performed on a precast polyacrylamide gel with a linear concentration gradient of 5-20% (Bio-Rad), run at 200 V (10, 11).

Fluorescent gel staining. After 2-DE, the gels were washed with Milli-Q water three times, and fixed with 40% ethanol and 10%

acetic acid for 4 h, and then stained with Flamingo™ Fluorescent Gel Stain (Bio-Rad) overnight. Stained gels were washed with Milli-Q water three times (12).

Image analysis and spot selection. Digitized images of the gels were acquired by scanning with a ProEXPRESS 2D Proteomic Imaging System (PerkinElmer Inc., Waltham, MA, USA). Image alignment, spot detection, background removal and expression analysis were performed by Progenesis SameSpot software (Nonlinear Dynamics Ltd. Newcastle upon Tyne, UK) (13). The differences in expression between TS-1-sensitive pancreatic cancer cell lines and TS-1-resistant pancreatic cancer cell lines were analyzed statistically by ANOVA test. 2-DE analysis was performed three times. After statistical analysis, the gels were re-stained with See Pico™ (Benelab Co., Ltd, Seoul, Korea) (14), and the selected spots whose expression was significantly different between TS-1-sensitive pancreatic cancer cell lines and TS-1-resistant pancreatic cancer cell lines were cut and removed for LC-MS/MS analysis.

LC-MS/MS. After in-gel digestion, protein samples were dissolved in 0.1% formic acid, centrifuged at 21,500×g for 5 min and the supernatant was used. An Agilent 1100 LC/MSD Trap XCT (Agilent Technologies, Palo Alto, CA, USA) was used for LC-MS/MS. Twenty-five microliters of each sample were applied and separated on a column (Zorbax 300SB-C18, 75 µm, 150 mm; Agilent Technologies). The Agilent 1100 capillary pump was operated under the following conditions: solvent A: 0.1% formic acid, solvent B: acetonitrile in 0.1% formic acid; column flow: 0.3 µl/min, primary flow: 300 µl/min; gradient: 0-5 min 2% solvent B, 60 min 60% solvent B; stop time: 60 min. Protein identification was performed in an Agilent Spectrum Mill MS proteomics workbench seeking MS/MS spectra using MS/MS ion search (15, 16) with 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). The criteria for positive identification of proteins were set as follows: filter by protein score >10.0, and filter peptide by score >8, % scored peak intensity (% SPI) >70.

Results

Selection of pancreatic cancer cell lines by TS-1 exposure.

To evaluate the cytotoxicity of TS-1 in the pancreatic cancer cell lines, MiaPaCa-2, Panc-1, BxPC-3, PK45p and PK59 cells were exposed to different concentrations of the drug for 72 h. The 5-FU/CDHP mixture was used at a molar ratio of 1:1, which mimics human plasma pharmacokinetics after oral administration of TS-1. Based on the measurements obtained, the IC₅₀ value was defined as the concentration of 5-FU/CDHP mixture causing 50% inhibition of growth. Four cell lines, Panc-1, BxPC-3, MiaPaCa-2 and PK59 showed a high sensitivity to 5-FU/CDHP mixture, with IC₅₀ values of 33.4, 217.9, 250.8 and 330.5 µM, respectively. In contrast, PK45p and KLM-1 cells were much less sensitive; their IC₅₀ values were 2087.0 and 3343.7 µM, respectively. The cell lines were thus classified into two groups: TS-1-sensitive cells (Panc-1, BxPC-3, MiaPaCa-2 and PK59) and TS-1-resistant cells (PK45p and KLM-1).

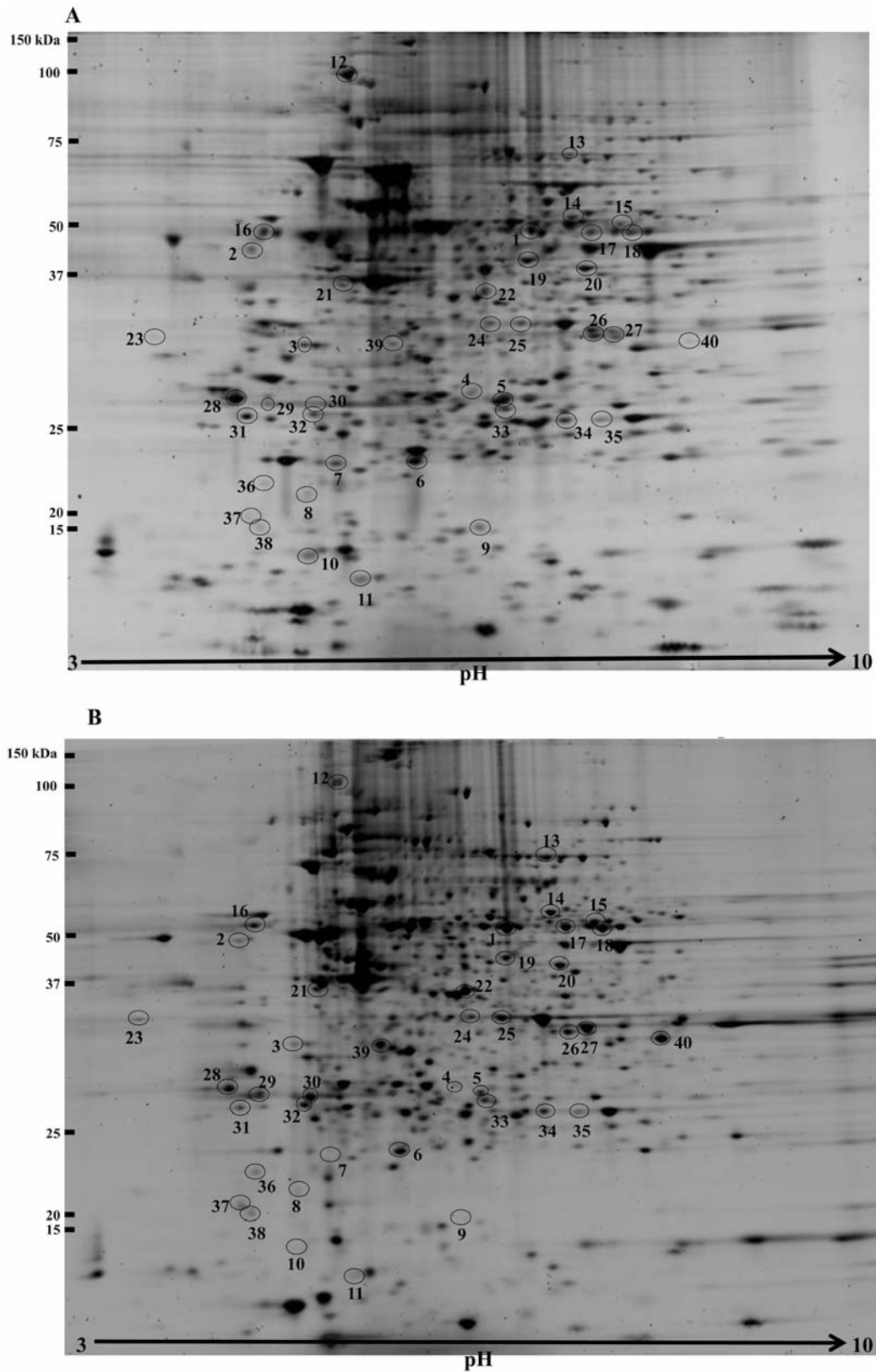


Figure 1. 2-DE gel pattern of A: BxPC-1 (TS-1-sensitive cell line) and B: PK45p (TS-1-resistant cell line) cells. Proteins (80 μ g) were separated on precast polyacrylamide gel with a linear concentration gradient of 5-20%.

Table I. Identification of up- and down-regulated proteins in TS-1-resistant cells compared to TS-1-sensitive cells.

Spot no.	Protein name	Accession no.	Theoretical pI	Theoretical Mr	Distinct peptides	Sequence coverage (%)	MS/MS search score	Change in QRsP-11 (fold)
1	T-Complex protein 1 subunit beta	P78371	6.01	57,488.5	17	47	249.64	-2.7
2	Ribonuclease inhibitor	P13489	4.71	49,973.8	3	7	43.12	-1.6
3	Elongation factor 1-delta	P29692	4.90	31,122.0	10	46	134.49	-1.5
4	Charged multivesicular body protein 2a	O43633	5.88	25,104.1	4	18	63.17	-1.6
5	Glutathione S-transferase omega-1	P78417	6.24	27,566.0	10	26	133.76	-1.4
6	Peroxiredoxin-2	P32119	5.66	21,892.0	12	43	158.37	-1.5
7	Lactoylglutathione lyase	Q04760	5.12	20,777.8	6	29	80.64	-2.5
8	Deoxyribonucleoside 5'-monophosphate N-glycosidase	O43598	4.97	19,108.5	4	28	53.63	-1.4
9	Transcription factor BTF3 homolog 4	Q96K17	5.95	17,270.6	1	17	19.52	-2.2
10	Superoxide dismutase (Cu-Zn)	P00441	5.70	15,935.8	8	72	140.10	-2.3
11	Tubulin-specific chaperone A	O75347	5.25	12,854.9	5	35	72.97	-1.9
12	Hypoxia up-regulated protein 1	Q9Y4L1	5.16	111,335.9	26	31	369.56	1.4
13	Lamin-A/C	P02545	6.57	74,139.8	23	39	303.47	1.7
14	Glucose-6-phosphate 1-dehydrogenase	P11413	6.39	59,257.1	29	66	407.07	1.6
15	Dihydrolipoyl dehydrogenase, mitochondrial	P09622	7.59	54,150.5	19	45	245.82	1.5
16	Endoplasmic	P14625	4.76	92,469.3	9	13	133.89	1.7
17	Fascin	Q16658	6.84	54,530.3	19	51	265.42	1.6
18	Fascin	Q16658	6.84	54,530.3	26	60	365.43	1.7
19	Proliferation-associated protein 2G4	Q9UQ80	6.13	43,787.1	14	41	187.37	1.2
20	Elongation factor Tu, mitochondrial	P49411	7.26	49,541.8	11	26	147.48	1.3
21	Actin, cytoplasmic 2	P63261	5.31	41,793.1	4	18	61.62	1.7
22	Leukocyte elastase inhibitor	P30740	5.90	42,742.0	22	60	338.63	1.4
23	Protein SET	Q01105	4.23	33,489.0	4	14	53.46	1.4
24	Annexin A1	P04083	6.57	38,714.5	12	42	185.62	1.3
25	Annexin A1	P04083	6.57	38,714.5	19	56	287.85	1.7
26	LIM and SH3 domain protein 1	Q14847	6.61	29,717.3	13	51	179.70	1.4
27	Aldose reductase	P15121	6.52	35,853.6	12	50	167.80	1.8
28	14-3-3 protein sigma	P31947	4.68	27,774.2	17	70	248.09	1.7
29	14-3-3 protein gamma	P61981	4.80	28,302.7	10	38	142.66	1.4
30	Calretinin	P22676	5.06	31,540.1	11	40	164.95	1.4
31	Proteasome subunit alpha type-5	P28066	4.74	26,411.2	5	27	79.56	1.6
32	Calpain small subunit 1	P04632	5.05	28,315.9	9	41	140.14	1.4
33	Endoplasmic reticulum protein ERp29	P30040	6.77	28,993.6	4	23	62.01	1.4
34	Triosephosphate isomerase	P60174	6.45	26,669.6	13	66	184.18	1.4
35	Triosephosphate isomerase	P60174	6.45	26,669.6	9	48	121.00	1.5
36	Proteasome subunit beta type-9	P28065	4.93	23,264.4	7	43	107.42	1.9
37	Myosin regulatory light chain 12A	P19105	4.67	19,794.2	5	33	72.43	1.7
38	Myosin regulatory light chain 12B	P14950	4.71	19,779.3	5	45	82.83	1.9
39	Regulation of nuclear pre-mRNA domain-containing protein 1B	Q9NQG5	5.73	36,899.9	4	23	55.89	2.1
40	Glyceraldehyde-3-phosphate dehydrogenase	P04406	8.57	36,053.4	11	38	168.17	2.7

Spot numbers refer to those shown in Figure 1. These spots were cut out and 40 spots were subsequently identified by MS.

Detection of protein spots with different expression between TS-1-sensitive and TS-1-resistant pancreatic cancer cell lines on 2-DE gels. Protein expression was assessed in three samples each from TS-1-sensitive and TS-1-resistant pancreatic cancer cells. More than 600 spots were visualized on the 2-DE gels, and differences in intensity between the TS-1-sensitive and TS-1-resistant pancreatic cancer cells were compared and analyzed with Progenesis SameSpot software for each gel. Of a total of 40 differentially expressed protein

spots, 11 appeared to be down-regulated, and 29 spots appeared to be up-regulated in TS-1-sensitive cells (Figure 1A) compared with TS-1-resistant cells (Figure 1B). The spots from TS-1-sensitive cells whose expression level significantly increased or decreased ($p < 0.05$) compared with that of TS-1-resistant cells are indicated by circles in Figure 1. The identification of these 40 protein spots with different expression levels was accomplished by measuring tryptic peptide masses using the Agilent 1100 LC-MS/MS Trap XCT

system in the positive ion mode and carrying out a database search in the Agilent Spectrum Mill MS proteomics workbench against the Swiss-Prot protein database search engine and the MASCOT MS/MS Ions Search engine, and the resulting data are summarized in Table I. Each sample provided good spectra of amino acid sequences.

Discussion

The results of the present study showed that 40 differentially expressed protein spots between TS-1-resistant and -sensitive pancreatic cancer cell lines were identified by using 2-DE and LC-MS/MS. There were up-regulated protein spots in TS-1-resistant cell lines 29 and 11 down-regulated protein spots.

Hypoxia up-regulated protein 1, also named oxygen-regulated protein (ORP150), was found to be up-regulated in resistant cells here. This protein is an endoplasmic reticulum chaperone. Under hypoxia, a common feature of the tumour cell environment, ORP150 is essential for the maintenance of cellular viability. In tumor cells, ORP150 was up-regulated, and up-regulation of ORP150 was found to reduce the antitumor activity of celecoxib, a COX-2-selective NSAID, by inhibiting apoptosis (17). Up-regulation of ORP150 was also reported in some invasive breast tumors, and appeared to be associated with indicators of poor prognosis and metastasis (18). Our finding of up-regulation of ORP150 in the TS-1-resistant cell line compared with TS-1-sensitive cell line supports these results.

Two up-regulated protein spots in TS-1-resistant cells compared with TS-1-sensitive cells were identified as annexin A1. Annexin A1 is a calcium/phospholipid-binding protein belonging to the annexin superfamily. Annexin A1 plays a role in membrane fusion and exocytosis. This protein regulates phospholipase A2 activity. A link between the up-regulation of annexin A1 and progression of breast cancer has been reported (19). Furthermore, some papers have reported a relation between the up-regulation of annexin A1 and drug resistance in cancer cells (20, 21).

From the 2-DE gels, two up-regulated protein spots in TS-1-resistant cells compared with TS-1-sensitive cells were identified as fascin. Fascin is an actin crosslinker protein in filopodia and its up-regulation in cancer cells and its relation to tumour progression has been reported (22). Yamakita *et al.* reported that the serine residue 39 of fascin was phosphorylated, and phosphorylated fascin inhibited actin binding and bundling activities (23). Although it is not clear whether inhibition of actin binding and bundling activities by phosphorylated fascin plays a role in TS-1-resistance or not, phosphorylated fascin possibly relate to TS-1-resistance.

Endoplasmic, a molecular chaperone, functions in the processing and transport of secreted proteins. We found it to be up-regulated in TS-1-resistant pancreatic cancer cells.

Endoplasmic is reported to be overexpressed in aggressive cancer cells (24). Di Michele *et al.* reported that endoplasmic was also up-regulated in paclitaxel-resistant ovarian cancer cells (25). Therefore, there is a possibility that up-regulation of endoplasmic relates to TS-1-resistance.

Calretinin is a cytosolic calcium-modulating protein. Boyer *et al.* reported that calretinin was up-regulated in oxaliplatin-resistant colorectal cancer cells (26). Oxaliplatin is a platinum-based anti-cancer drug showing mechanism of action like alkylating agent. Although oxaliplatin has a different mechanism of action from TS-1, the up-regulation of calretinin in TS-1-resistant cells is interesting.

Aldose reductase is an NADPH-dependent enzyme which reduces glucose to sorbitol. Aldose reductase is known to be overexpressed in hepatoma cells. Bacolod *et al.* performed gene expression profiling for the preactivated form of cyclophosphamide-resistant medulloblastoma cells, and the results showed a 20-fold increase in the expression of the aldose reductase gene in the resistant cells (27). Lee *et al.* reported that overexpression of aldose reductase made the cells more resistant to daunorubicin (28). Cyclophosphamide is an alkylating agent. Daunorubicin intercalates to DNA and inhibits DNA replication. Albeit both cyclophosphamide and daunorubicin show different mechanisms of action from TS-1, the up-regulation of aldose reductase in TS-1-resistant cells is also interesting.

Triosephosphate isomerase catalyzes the reversible interconversion of the triose phosphate isomers dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. Di Michele *et al.* reported that triosephosphate isomerase was up-regulated in paclitaxel-resistant ovarian cancer cells (25). Paclitaxel is an inhibitor of cell division, and its mechanism of action is also different from that of TS-1. However, up-regulation of triosephosphate isomerase seems interesting.

Proteomic analysis was shown here to be useful for detecting intracellular proteins with differential expression in pancreatic adenocarcinoma cell lines that were sensitive or resistant to TS-1. Such proteins may be involved in the mechanism of resistance to chemotherapy.

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