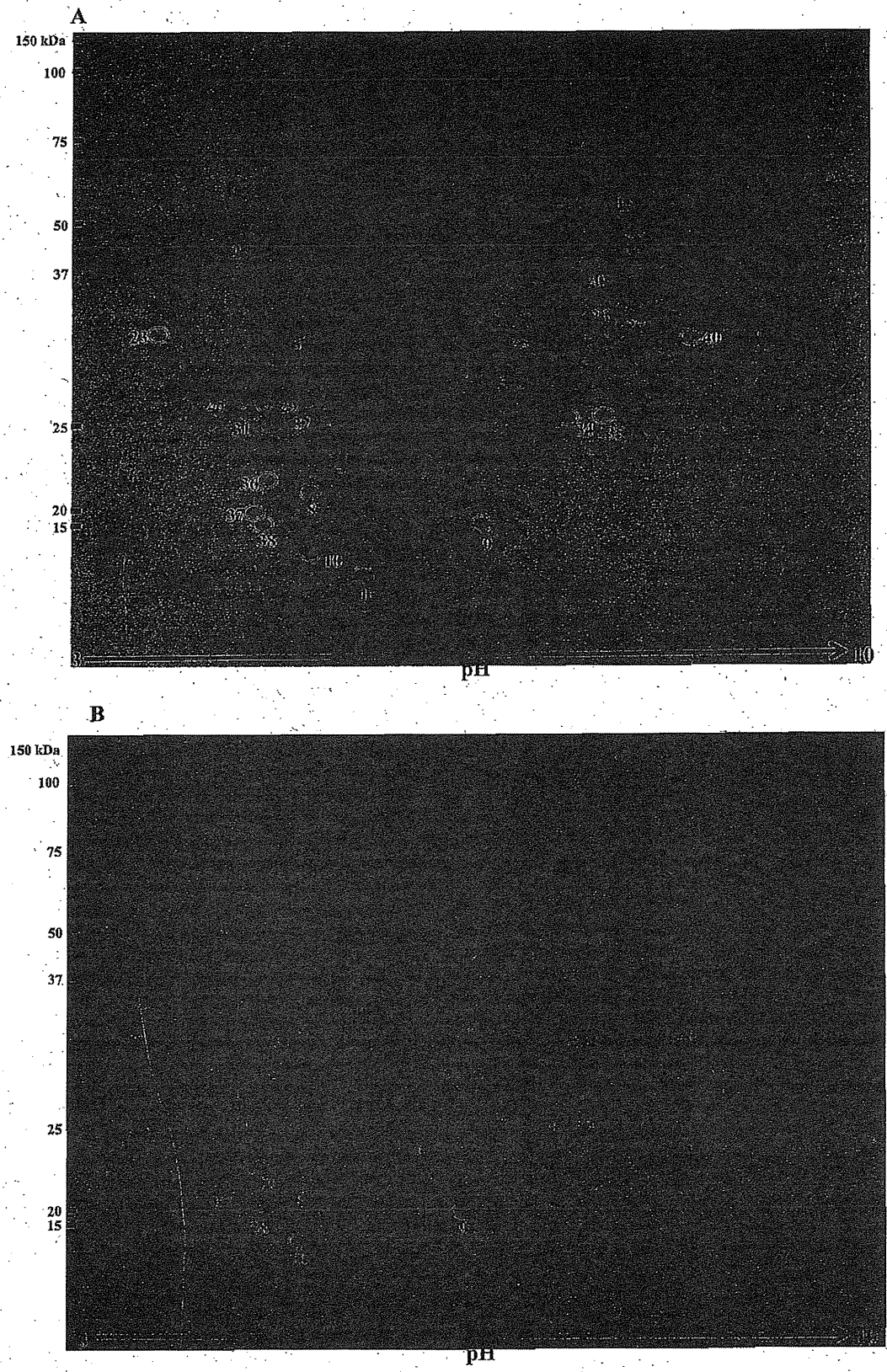

Figure 1. *2-DE gel pattern of A: BxPC-1 (TS-1-sensitive cell line) and B: PK45p (TS-1-resistant cell line) cells. Proteins (80 μg) were separated on precast polyacrylamide gel with a linear concentration gradient of 5-20%.*

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	Endoplasmin	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.

Endoplasmin, 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. Endoplasmin is reported to be overexpressed in aggressive cancer cells (24). Di Michele *et al.* reported that endoplasmin was also up-regulated in paclitaxel-resistant ovarian cancer cells (25). Therefore, there is a possibility that up-regulation of endoplasmin 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. Daunomycin 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 dihydroxy-acetone 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.

References

1 Vital Statistics of Japan 2003, Statistics and Information Dept., Minister's Secretariat, Ministry of Health, Labor and Welfare of Japan.

2 Komoto M, Nakata B, Nishii T, Kawajiri H, Shinto O, Amano R, Yamada N, Yashiro M and Hirakawa K: *In vitro* and *in vivo* evidence that a combination of lapatinib plus S-1 is a promising treatment for pancreatic cancer. Cancer Sci *101*: 468-473, 2010.

3 Nakai Y, Isayama H, Sasaki T, Sasahira N, Ito Y, Kogure H, Togawa O, Matsubara S, Arizumi T, Yagioka H, Yashima Y, Kawakubo K, Mizuno S, Yamamoto K, Hirano K, Tsujino T, Ijichi H, Tateishi K, Toda N, Tada M, Omata M and Koike K: Impact of S-1 on the survival of patients with advanced pancreatic cancer. Pancreas *39*: 989-993, 2010.

4 Kuramitsu Y and Nakamura K: Current progress in proteomic study of hepatitis C virus-related human hepatocellular carcinoma. Expert Rev Proteom 2: 589-601, 2005.

5 Mori-Iwamoto S, Kuramitsu Y, Ryozawa S, Mikuriya K, Fujimoto M, Maehara S, Maehara Y, Okita K, Nakamura K and Sakaida I: Proteomics finding heat-shock protein 27 as a biomarker for resistance of pancreatic cancer cells to gemcitabine. Int J Oncol *31*: 1345-1350, 2007.

6 Mori-Iwamoto S, Kuramitsu Y, Ryozawa S, Taba K, Fujimoto M, Okita K, Nakamura K and Sakaida I: A proteomic profiling of gemcitabine resistance in pancreatic cancer cell lines. Mol Med Rep *1*: 429-434, 2008.

7 Mori-Iwamoto S, Taba K, Kuramitsu Y, Ryozawa S, Tanaka T, Maehara S, Maehara Y, Okita K, Nakamura K and Sakaida I: Interferon-γ down-regulates HSP27 of pancreatic cancer cells, and helps the cytotoxic effect of gemcitabine. Pancreas *38*: 224-226, 2009.

8 Kim WY, Nakata B and Hirakawa K: Alternative pharmacokinetics of S-1 components, 5-fluorouracil, dihydrofluorouracil and alpha-fluoro-beta-alanine after oral administration of S-1 following total gastrectomy. Cancer Sci *98*: 1604-1608, 2007.

9 Kuramitsu Y, Taba K, Ryozawa S, Yoshida K, Tanaka T, Zhang X, Maehara S, Maehara Y, Sakaida I and Nakamura K: Identification of up- and down-regulated proteins in gemcitabine-resistant pancreatic cancer cells using two-dimensional gel electrophoresis and mass spectrometry. Anticancer Res *30*: 3367-3372, 2010.

10 Kuramitsu Y, Miyamoto H, Tanaka T, Zhang X, Fujimoto M, Ueda K, Tanaka T, Hamano K and Nakamura K: Proteomic differential display analysis identified up-regulated astrocytic phosphoprotein PEA-15 in human malignant pleural mesothelioma cell lines. Proteomics *9*: 5078-5089, 2009.

11 Tanaka T, Kuramitsu Y, Fujimoto M, Naito S, Oka M and Nakamura K: Down-regulation of two isoforms of ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCH-L1) correlates with high metastatic potentials of human SN12C renal cell carcinoma cell clones. Electrophoresis *29*: 2651-2659, 2008.

12 Kuramitsu Y, Baron B, Yoshino S, Zhang X, Tanaka T, Yashiro M, Hirakawa K, Oka M and Nakamura K: Proteomic differential display analysis shows up-regulation of 14-3-3 protein sigma in human scirrhous-type gastric carcinoma cells. Anticancer Res *30*: 4459-4465, 2010.

13 Kuramitsu Y, Hayashi E, Okada F, Tanaka T, Zhang X, Ueyama Y and Nakamura K: Proteomic analysis for nuclear proteins related to tumour malignant progression: a comparative proteomic study between malignant progressive cells and regressive cells. Anticancer Res *30*: 2093-2099, 2010.

14 Kuramitsu Y, Hayashi E, Okada F, Zhang X, Tanaka T, Ueyama Y and Nakamura K: Staining with highly sensitive Coomassie Brilliant Blue SeePico™ stain after Flamingo™ fluorescent gel stain is useful for cancer proteomic analysis by means of two-dimensional gel electrophoresis. Anticancer Res *30*: 4001-4005, 2010.

15 Zhang X, Kuramitsu Y, Fujimoto M, Hayashi E, Yuan X and Nakamura K: Proteomic analysis of macrophages stimulated by lipopolysaccharide: Lipopolysaccharide inhibits the cleavage of nucleophosmin. Electrophoresis *27*: 1659-1668, 2006.

16 Kuramitsu Y, Harada T, Takashima M, Yokoyama Y, Hidaka I, Iizuka N, Toda T, Fujimoto M, Zhang X, Sakaida I, Okita K, Oka M and Nakamura K: Increased expression, and phosphorylation of liver glutamine synthetase in well-differentiated hepatocellular carcinoma tissues of patients infected with hepatitis C virus. Electrophoresis *27*: 1651-1658, 2006.

17 Namba T, Hoshino T, Tanaka K, Tsutsumi S, Ishihara T, Mima S, Suzuki K, Ogawa S and Mizushima T: Up-regulation of 150-kDa oxygen-regulated protein by celecoxib in human gastric carcinoma cells. Mol Pharmacol 71: 860-870, 2007.

18 Stojadinovic A, Hooke JA, Shriver CD, Nissan A, Kovatich AJ, Kao TC, Ponniah S, Peoples GE and Moroni M: HYOU1/ORP150 expression in breast cancer. Med Sci Monit *13*: 231-239, 2007.

19 de Graauw M, van Miltenburg MH, Schmidt MK, Pont C, Lalai R, Kartopawiro J, Pardali E, Le Dévédec SE, Smit VT, van der Wal A, Van't Veer LJ, Cleton-Jansen AM, ten Dijke P and van de Water B: Annexin A1 regulates TGF-beta signaling and promotes metastasis formation of basal-like breast cancer cells. Proc Natl Acad Sci USA *107*: 6340-6345, 2010.

20 Keenan J, Murphy L, Henry M, Meleady P and Clynes M: Proteomic analysis of multidrug-resistance mechanisms in adriamycin-resistant variants of DLKP, a squamous lung cancer cell line. Proteomics *9*: 1556-1566, 2009.

21 Wang Y, Serfass L, Roy MO, Wong J, Bonneau AM and Georges E: Annexin-I expression modulates drug resistance in tumor cells. Biochem Biophys Res Commun *314*: 565-570, 2004.

22 Hashimoto Y, Skacel M and Adams JC: Roles of fascin in human carcinoma motility and signaling: prospects for a novel biomarker? Int J Biochem Cell Biol *37*: 1787-1804, 2005.

23 Yamakita Y, Ono S, Matsumura F and Yamashiro S: Phosphorylation of human fascin inhibits its actin binding and bundling activities. J Biol Chem *271*: 12632-12638, 1996.

24 Zheng HC, Takahashi H, Li XH, Hara T, Masuda S, Guan YF and Takano Y: Overexpression of GRP78 and GRP94 are markers for aggressive behavior and poor prognosis in gastric carcinomas. Hum Pathol *39*: 1042-1049, 2008.

25 Di Michele M, Marcone S, Cicchillitti L, Della Corte A, Ferlini C, Scambia G, Donati MB and Rotilio D: Glycoproteomics of paclitaxel resistance in human epithelial ovarian cancer cell lines: towards the identification of putative biomarkers. J Proteomics *73*: 879-898, 2010.

26 Boyer J, Allen WL, McLean EG, Wilson PM, McCulla A, Moore S, Longley DB, Caldas C and Johnston PG: Pharmacogenomic identification of novel determinants of response to chemotherapy in colon cancer. Cancer Res *66*: 2765-2777, 2006.

27 Bacolod MD, Lin SM, Johnson SP, Bullock NS, Colvin M, Bigner DD and Friedman HS: The gene expression profiles of medulloblastoma cell lines resistant to preactivated cyclophosphamide. Curr Cancer Drug Targets *8*: 172-179, 2008.

28 Lee KW, Ko BC, Jiang Z, Cao D and Chung SS: Overexpression of aldose reductase in liver cancers may contribute to drug resistance. Anticancer Drugs *12*: 129-132, 2001.

Received February 25, 2011
Revised May 2, 2011
Accepted May 4, 2011

Identification of Differentially Expressed Proteins in Tumour Necrosis Factor-alpha-resistant and -sensitive Rat Hepatoma Cells

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Abstract. *Background: Our earlier studies reported that ONO-4007, a synthetic lipid A analogue with low endotoxic activity, had shown much effect on tumour necrosis factor (TNF)-alpha-sensitive rat hepatoma cells, even though it had no effect on TNF-alpha-resistant cells. Materials and Methods: To find biomarkers which relate to the sensitivity of cancer cells to TNF-alpha, proteomic differential display analysis for TNF-alpha-resistant cKDH-8/11 and -sensitive KDH-8/YK rat hepatoma cell lines was carried out using two-dimensional gel electrophoresis and liquid chromatography–tandem mass spectrometry. Results: Two-DE analysis showed 32 spots, whose expression was different between cKDH-8/11 cells and KDH-8/YK cells. Of these, 12 were up-regulated and 20 were down-regulated in cKDH-8/11 cells compared to KDH-8/YK cells. The up-regulated proteins include transitional endoplasmic reticulum ATPase, 78kDa glucose-regulated protein (GRP78), heat-shock cognate 71 kDa protein (HSC71) and protein disulfide-isomerase A6. The down-regulated proteins included alpha-enolase, aldose reductase, glutathione reductase, annexin A1, glutamate dehydrogenase 1 and dihydrolipoyl dehydrogenase. Conclusion: These findings suggest that these differentially regulated proteins could be factors responsible for the resistance of cKDH-8/11 cells to TNF-alpha-induced cell death.*

KDH-8 is a transplantable rat hepatoma cell line. From this cell line, two sublines were established. One is tumour necrosis factor (TNF)-sensitive KDH-8/YK, and the other is TNF-resistant cKDH-8/11. Although these two cell lines proliferated equally, only KDH-8/YK was TNF sensitive (1).

ONO-4007 is a lipid A analogue with low endotoxic activity. *In vivo* treatment with this agent of rats bearing KDH-8/YK cells was significantly effective, in spite of there being no effect on rats bearing cKDH-8/11 (2). Further studies suggested that the therapeutic effects of ONO-4007 depended on the TNF sensitivity of the cells, and the development of ONO-4007 as a new therapeutic agent against TNF-sensitive cancers was expected. In order to ensure that anticancer therapy with ONO-4007 is successful, it is very important to find particular proteins as biomarkers from cancer cells which show TNF resistance (3). Our previous study identified phosphatidylethanol-amine-binding protein (PEBP) as an up-regulated protein in TNF-resistant hepatoma cells compared with TNF-sensitive cells. However, it is still necessary to find other biomarker proteins (4).

Proteomic differential display is a basic method to compare the protein expression profiling among different sample groups. For this method, two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) techniques have been employed in many studies (5, 6). By means of 2-DE, proteins are separated according to their charge in isoelectrofocusing (IEF) gels and to their size in sodium dodecyl sulfate (SDS) gels (7). In this study, we used 2-DE to compare the expression patterns of intracellular proteins in TNF-sensitive KDH-8/YK cells and TNF-resistant cKDH-8/11 cells.

The purpose of present study was to identify biomarkers which may be responsible for TNF resistance and account for ONO-4007-treatment results.

Materials and Methods

Tumour cell lines and culture condition. KDH-8 is a rat hepatoma cell line induced by 3'-methyl-4-dimethylaminoazo-benzene in WKAH rat. It has been maintained *in vivo* by intraperitoneal passage every 5 days (1). KDH-8/YK is a cell line isolated from the primary culture of KDH-8 tumor cells. cKDH-8/11 is a sub-clone

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Key Words: TNF-alpha, hepatoma, proteomics, 2-DE, LC-MS/MS, apoptosis.

0250-7005/2011 $2.00+.40

isolated from the primary culture of KDH-8 tumor cells by limiting dilution (2). These cell lines have properties similar to those of the parent KDH-8 cells *in vivo* and have been maintained in a continuous *in vitro* culture in RPMI 1640 medium supplemented with 10% fetal bovine serum.

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 PMSF, 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 for 2-DE.

Two-dimensional gel electrophoresis. 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, Hercules, CA, USA) at 50 μA/strip. IEF was performed in the following voltage program: rehydration for 10 h (no voltage), a stepwise increase from 0 to 500 V for 4 h, 500 to 1,000 V for 1 h, 1,000 to 8,000 V for 4 h, a linear increase from 8,000 V for 20 min, and a final phase of 500 V from 20,000 to 30,000 Vh. SDS-polyacrylamide gel electrophoresis (PAGE) was performed on a precast polyacrylamide gel with a linear concentration gradient of 5-20% (BIO RAD), run at 200 V (8).

Image analysis and spot picking. After 2-DE, the gels were washed with ultra-pure water three times to remove SDS. After fixing, the gels were stained with highly sensitive Coomassie Brilliant Blue SeePico™ (Benebiosis Co., Ltd, Seoul, Korea) (9), and expression levels of the protein spots were quantified with Progenesis SameSpot software (Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK) (10). The differences in expression between KDH-8/YK and cKDH-8/11 cells were analyzed statistically by ANOVA test. The 2-DE analysis was repeated three times. The spots whose expression was significantly different between KDH-8/YK and cKDH-8/11 cells were selected for further analysis (11).

High performance liquid chromatography (LC)-MS/MS. After in-gel digestion, samples dissolved in 0.1% formic acid were centrifuged at 21,500×*g* for 5 min and the supernatant was used for HPLC-MS/MS analysis (12). An Agilent 1100 LC/MSD Trap XCT instrument (Agilent Technologies, Palo Alto, CA, USA) was used for HPLC and MS/MS. Twenty-five microliters of each sample was applied and separated on a Zorbax 300SB-C18 column, 75 μm, 150 mm; Agilent Technologies) (13). Protein identification was performed in 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). The criteria for positive identification of proteins were as follows: filter by protein score>10.0, and filter peptide by score>8, % scored peak intensity (% SPI) >70. The Spectrum Mill workbench can search MS/MS spectra using an MS/MS ion search (14).

Results

Figure 1A and B show the 2-DE patterns of KDH-8/YK and cKDH-8/11 cells, respectively. More than 600 protein spots were detected. Twelve spots (no. 1-12) appeared to be up-regulated, and 20 spots (no. 13-32) appeared to be down-regulated in cKDH-8/11 compared with KDH-8/YK cells.

The 32 differentially expressed protein spots were picked up and identified by the Agilent 1100 LC/MSD Trap XCT LC-MS/MS system. The results are shown in Table I.

Discussion

The results of the present study showed that 32 protein spots were differentially expressed between TNF-alpha-resistant and -sensitive rat hepatoma cell lines as identified by proteomic differential display analysis employing 2-DE and LC-MS/MS. The up-regulated proteins in TNF-resistant cKDH-8/11 cells were identified as transitional endoplasmic reticulum ATPase, heat-shock 90 kDa protein (HSP90)-alpha, 78 kDa glucose-regulated protein (GRP78), hea-shock cognate 71 kDa protein (HSC71), vimentin, protein disulfide-isomerase A6, heterogeneous nuclear ribonucleoprotein F, suppressor of G2 allele of S-phase kinase-associated protein 1 (SKP1) homolog, elongation factor 1-delta, ubiquitin carboxyl-terminal hydrolase isozyme L3 (UCHL3), peroxiredoxin-2 and myotrophin. The 20 down-regulated proteins in TNF-resistant cKDH-8/11 included endoplasmin and alpha-enolase.

GRP78 and HSC71 belong to the HSP70 family. GRP78 is a molecular chaperone, involved in the unfolded protein response. It facilitates the assembly of multimeric protein complexes inside the endoplasmic reticulum. HSC71 is a constitutively expressed chaperone. It binds to nascent polypeptides to facilitate correct folding. Down-regulation of GRP78 sensitizes prostate cancer cells which are resistant to adenovirus carrying Dickkopf-3-induced apoptosis (15). Castagna *et al.* reported that the basal level of HSC71 in cisplatin-resistant cervix squamous cell carcinoma cells was up-regulated compared to cisplatin-sensitive cells (16). Since TNF is very strong apoptosis inducer, the protectant role of GRP78 and HSC71 from apoptosis induced TNF could be strong in cKDH-8/11 cells.

UCHL3 is a member of the ubiquitin *C*-terminal hydrolase family. This enzyme de-ubiquitinates ubiquitin–protein conjugates in the ubiquitin-proteasome system. Although the function of UCHL3 in apoptosis is not completely understood, from the report of the analysis for UchL3-deficient mice, loss of UCHL3 leads to mitochondrial oxidative stress-related photoreceptor cell apoptosis in a caspase-independent manner (17).

Vimentin is a type III intermediate filament found in various non-epithelial cells, especially mesenchymal cells. Vimentin plays a role in the regulation of cell motility. Although its role in cell apoptosis is still controversial, Maxwell *et al.* reported that vimentin induced resistance against the CHOP chemotherapeutic regimen (cyclophosphamide, doxorubicin, vincristine, prednisone) in diffuse large B-cell lymphoma (18).

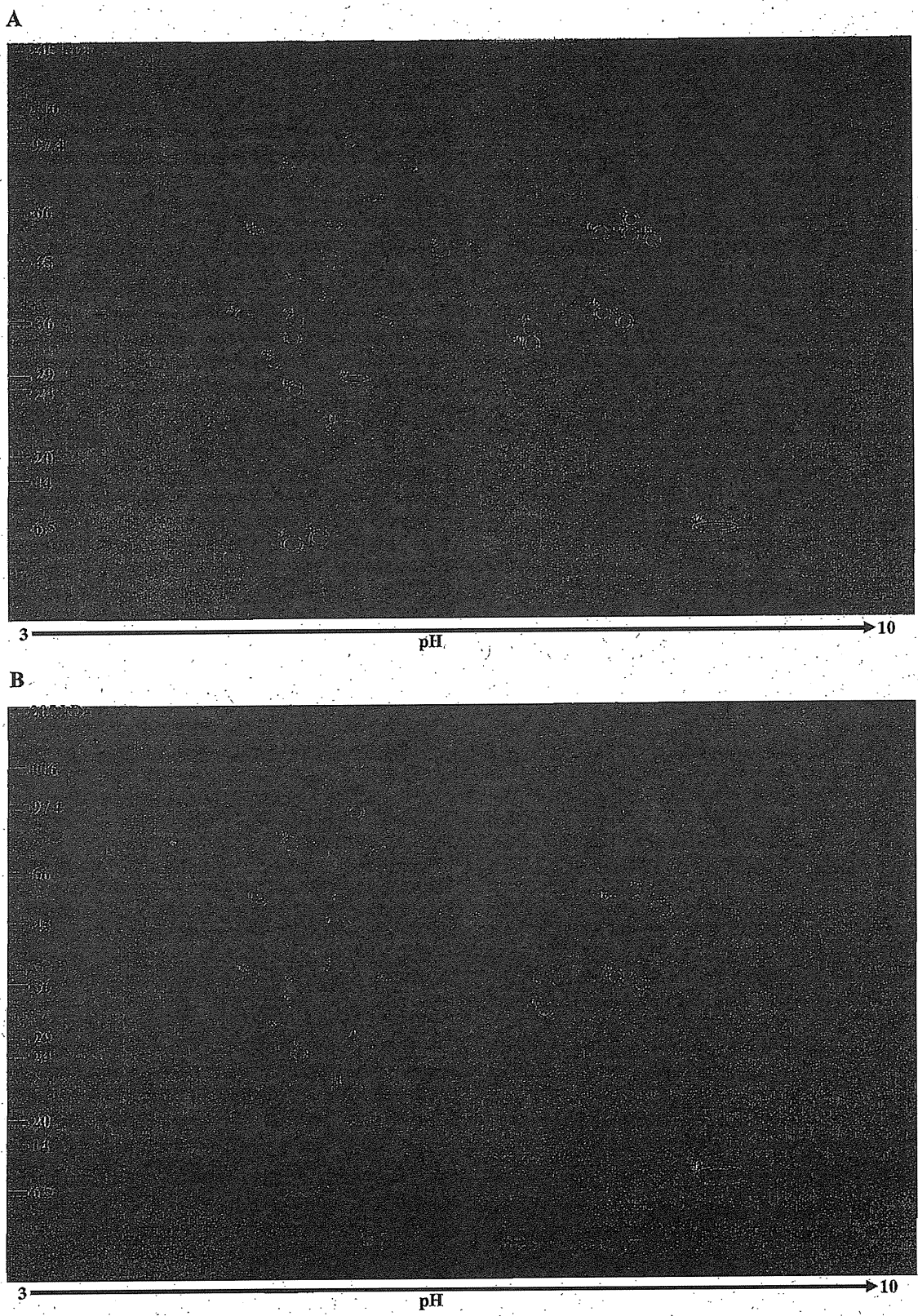

Figure 1. *2-DE gel pattern of A: cKDH-8/11 (TNF-alpha-resistant cell line), and B: KDH-8/YK (TNF-alpha-sensitive cell line) cells. Proteins (80 μg) were separated on precast polyacrylamide gel with a linear concentration gradient of 5-20%. After fixing, the gels were stained with highly sensitive Coomassie Brilliant Blue SeePico™.*

Table I. *Identification of proteins up- and down-regulated in cKDH-8/11 cells compared to KDH-8/YK cells.*

Spot no.	Protein name	Accession no.	Theoretical p*I*	Theoretical Mr	Distinct peptides	Sequence coverage (%)	MS/MS search score	Change in cKDH-8/11 (fold)
1	Transitional endoplasmic reticulum ATPase	P46462	5.14	89,349.3	24	36	319.78	6.0
2	Heat-shock 90 kDa protein-alpha	P82995	4.93	84,815.3	1	1	16.41	1.6
3	78 kDa Glucose-regulated protein	P06761	5.07	72,347.3	24	42	363.86	1.6
4	Heat-shock cognate 71 kDa protein	P63018	5.38	70,871.4	31	49	459.13	3.7
5	Vimentin	P31000	5.06	53,733.0	4	11	49.48	2.1
6	Protein disulfide-isomerase A6	Q63081	4.99	48,173.7	13	26	199.72	1.6
7	Heterogeneous nuclear ribonucleoprotein F	Q794E4	5.31	45,730.1	7	19	98.57	1.5
8	Suppressor of G2 allele of SKP1 homolog	B0BN85	5.17	38,091.1	4	17	47.15	2.2
9	Elongation factor 1-delta	Q68FR9	4.95	31,330.3	3	16	34.86	1.5
10	Ubiquitin carboxyl-terminal hydrolase isozyme L3	Q91Y78	5.01	26,123.8	3	10	32.70	1.8
11	Peroxiredoxin-2	P35704	5.34	21,783.8	6	29	81.24	2.0
12	Myotrophin	P62775	5.28	12,860.8	3	43	54.11	2.5
13	Heat-shock 70 kDa protein 4	O88600	5.13	94,057.0	3	5	29.19	−1.7
14	Endoplasmin	Q66HD0	4.72	92,771.4	3	4	32.99	−1.5
15	Pyruvate kinase isozymes M1/M2	P11980	6.63	57,818.1	4	8	54.16	−2.4
16	Dihydrolipoyl dehydrogenase, mitochondrial	Q6P6R2	7.96	54,038.4	3	7	35.18	−1.6
17	Glutamate dehydrogenase 1, mitochondrial	P10860	8.05	61,416.3	3	6	43.37	−2.1
18	Glutathione reductase (fragment)	P70619	8.06	46,301.4	1	2	18.12	−1.7
19	Alpha-enolase	P04764	6.16	47,128.1	15	28	209.10	−2.1
20	Alpha-enolase	P04764	6.16	47,128.1	23	56	328.48	−1.8
21	Actin, cytoplasmic 2	P63259	5.31	41,793.1	4	14	54.02	−1.5
22	Tropomyosin beta chain	P58775	4.66	32,836.9	5	15	70.17	−1.8
23	Cell surface glycoprotein CD200 receptor 1	Q9ES58	5.45	35,533.7	1	9	13.35	−1.7
24	Eukaryotic translation initiation factor 3 subunit 1	B0BNA7	5.38	36,461.0	2	7	33.21	−1.4
25	Aldose reductase	P07943	6.26	35,797.5	9	34	127.36	−1.7
26	Annexin A1	P07150	6.96	38,829.7	6	17	78.56	−1.5
27	Glyceraldehyde-3-phosphate dehydrogenase	P04797	8.14	35,828.2	1	3	20.75	−2.0
28	Inositol monophosphatase 1	P97697	5.18	30,511.5	2	8	31.86	−1.8
29	Actin, cytoplasmic 2	P63259	5.31	41,793.1	6	20	91.39	−1.5
30	Aldose reductase	P07943	6.26	35,797.5	5	16	68.21	−1.5
31	Protein S100-A4	P05942	5.04	11,776.5	5	28	62.07	−2.0
32	Fatty acid-binding protein, adipocyte	P70623	7.72	14,708.1	4	34	51.59	−3.3

Spot numbers refer to those shown in Figure 1. These spots were cut out and 32 spots were subsequently identified by MS.

Up-regulation of vimentin might play a protectant role against apoptosis induced not only by chemotherapy, but also by TNF.

Peroxiredoxin-2 is induced by various oxidative stimuli and plays an important role in eliminating peroxides generated during metabolism to protect from oxidative damage by hydrogen peroxide. Some reports showed that up-regulation of peroxiredoxin-2 inhibited cisplatin-induced apoptosis and that peroxiredoxin-2 inhibited H_2O_2-induced cell death (19, 20). Like the other identified up-regulated proteins, up-regulated peroxiredoxin-2 may also protect cKDH-8/11 cells from TNF-induced apoptosis.

Elongation factor (EF) 1-delta is a part of the EF 1 protein complex which is active in the elongation step of protein synthesis. Sinha *et al.* reported that EF 1-delta was up-regulated in chemoresistant melanoma cell lines (21). Although the role of EF 1-delta in TNF-induced apoptosis is not clear, its up-regulation may protect cKDH-8/11 cells from apoptosis.

Twenty down-regulated proteins were identified in cKDH-8/11 cells compared with KDH-8/YK cells. Unfortunately from the literature describing them, down-regulation of these proteins seemed not to play important roles in the resistance to apoptosis induced by TNF. Up-regulation of proteins related to protection from cell apoptosis may therefore play an important role in TNF resistance.

Some of the proteins identified by proteomic differential display analysis for the rat hepatoma cell lines studied here may be involved in the mechanism of resistance to TNF-induced apoptosis, and could also be indicators of the response to ONO-4007 therapy.

References

1 Kuramitsu Y, Nishibe M, Ohiro Y, Matsushita K, Yuan L, Obara M, Kobayashi M and Hosokawa M: A new synthetic lipid A analog, ONO-4007, stimulates the production of tumor necrosis factor-alpha in tumor tissues, resulting in the rejection of transplanted rat hepatoma cells. Anticancer Drugs *8*: 500-508, 1997.

2 Kuramitsu Y, Matsushita K, Ohiro Y, Obara M, Kobayashi M and Hosokawa M: Therapeutic effects of a new synthetic lipid A analog, ONO-4007, on rat hepatoma KDH-8 depend on tumor necrosis factor-sensitivity of the tumor cells. Anticancer Drugs *8*: 898-901, 1997.

3 Kuramitsu Y, Ohiro Y, Matsushita K, Obara M, Kobayashi M and Hosokawa M: The mechanism of locally enhanced production of tumor necrosis factor-alpha in tumor tissues by the administration of a new synthetic lipid A analog, ONO-4007, in hepatoma-bearing rats. Anticancer Drugs *8*: 886-893, 1997.

4 Kuramitsu Y, Fujimoto M, Tanaka T, Ohata J and Nakamura K: Differential expression of phosphatidylethanol-amine-binding protein in rat hepatoma cell lines: analyses of tumor necrosis factor-alpha-resistant cKDH-8/11 and -sensitive KDH-8/YK cells by two-dimensional gel electrophoresis. Electrophoresis *21*: 660-664, 2000.

5 Kuramitsu Y, Miyamoto H, Tanaka T, Zhang X, Fujimoto M, Ueda K, Tanaka T, Hamano K and Nakamura K: Proteomic differential display analysis identified up-regulated astrocytic phsphoprotein PEA-15 in human malignant pleural mesothelioma cell lines. Proteomics *9*: 5078-5089, 2009.

6 Kuramitsu Y, Baron B, Yoshino S, Zhang X, Tanaka T, Yashiro M, Hirakawa K, Oka M and Nakamura K: Proteomic differential display analysis shows up-regulation of 14-3-3 protein sigma in human scirrhous-type gastric carcinoma cells. Anticancer Res *30*: 4459-4465, 2010.

7 Kuramitsu Y and Nakamura K: Proteomic analysis of cancer tissues: shedding light on carcinogenesis and possible biomarkers. Proteomics *6*: 5650-5661, 2006.

8 Kuramitsu Y, Taba K, Ryozawa S, Yoshida K, Tanaka T, Zhang X, Maehara S, Maehara Y, Sakaida I and Nakamura K: Identification of up- and down-regulated proteins in gemcitabine-resistant pancreatic cancer cells using two-dimensional gel electrophoresis and mass spectrometry. Anticancer Res *30*: 3367-3372, 2010.

9 Kuramitsu Y, Hayashi E, Okada F, Zhang X, Tanaka T, Ueyama Y and Nakamura K: Staining with highly sensitive Coomassie Brilliant Blue SeePico™ stain after Flamingo™ fluorescent gel stain is useful for cancer proteomic analysis by means of two-dimensional gel electrophoresis. Anticancer Res *30*: 4001-4005, 2010.

10 Tanaka T, Kuramitsu Y, Fujimoto M, Naito S, Oka M and Nakamura K: Down-regulation of two isoforms of ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCH-L1) correlates with high metastatic potentials of human SN12C renal cell carcinoma cell clones. Electrophoresis *29*: 2651-2659, 2008.

11 Kuramitsu Y, Hayashi E, Okada F, Tanaka T, Zhang X, Ueyama Y and Nakamura K: Proteomic analysis for nuclear proteins related to tumour malignant progression: a comparative proteomic study between malignant progressive cells and regressive cells. Anticancer Res *30*: 2093-2099, 2010.

12 Yuan X, Kuramitsu Y, Furumoto H, Zhang X, Hayashi E, Fujimoto M and Nakamura K: Nuclear protein profiling of Jurkat cells during heat stress-induced apoptosis by two-dimensional gel electrophoresis and tandem mass spectrometry. Electrophoresis *28*: 2018-2026, 2007.

13 Takashima M, Kuramitsu Y, Yokoyama Y, Iizuka N, Fujimoto M, NIshisaka T, Sakaida I, Okita K, Oka M and Nakamura, K: Overexpression of alpha enolase in hepatitis C virus-related hepatocellular carcinoma: association with tumor progression as determined by proteomic analysis. Proteomics *5*: 1686-1692, 2005.

14 Takashima M, Kuramitsu Y, Yokoyama Y, Iizuka N, Fujimoto M, Sakaida I, Okita K, Oka M and Nakamura K: Proteomic analysis of autoantibodies in patients with hepatocellular carcinoma. Proteomics *6*: 3894-3900, 2006.

15 Tanimoto R, Sakaguchi M, Abarzua F, Kataoka K, Kurose K, Murata H, Nasu Y, Kumon H, Huh NH: Down-regulation of BiP/GRP78 sensitizes resistant prostate cancer cells to gene-therapeutic overexpression of REIC/Dkk-3. Int J Cancer *126*: 1562-1569, 2010.

16 Castagna A, Antonioli P, Astner H, Hamdan M, Righetti SC, Perego P, Zunino F and Righetti PG: A proteomic approach to cisplatin resistance in the cervix squamous cell carcinoma cell line A431. Proteomics *4*: 3246-3267, 2004.

17 Sano Y, Furuta A, Setsuie R, Kikuchi H, Wang YL, Sakurai M, Kwon J, Noda M: and Wada K: Photoreceptor cell apoptosis in the retinal degeneration of Uchl3-deficient mice. Am J Pathol 169: 132-141, 2006.

18 Maxwell SA, Cherry EM and Bayless KJ: Akt, 14-3-3ζ, and vimentin mediate a drug-resistant invasive phenotype in diffuse large B-cell lymphoma. Leuk Lymphoma *52*: 849-864, 2011.

19 Bae JY, Ahn SJ, Han W and Noh DY: Peroxiredoxin I and II inhibit H_2O_2-induced cell death in MCF-7 cell lines. J Cell Biochem *101*: 1038-1045, 2007.

20 Chung YM, Yoo YD, Park JK, Kim YT and Kim HJ: Increased expression of peroxiredoxin II confers resistance to cisplatin. Anticancer Res *21*: 1129-1133, 2001.

21 Sinha P, Kohl S, Fischer J, Hütter G, Kern M, Köttgen E, Dietel M, Lage H, Schnölzer M and Schadendorf D: Identification of novel proteins associated with the development of chemoresistance in malignant melanoma using two-dimensional electrophoresis. Electrophoresis *21*: 3048-3057, 2000.

Received March 8, 2011
Revised May 2, 2011
Accepted May 4, 2011

Screening for serological biomarkers of pancreatic cancer by two-dimensional electrophoresis and liquid chromatography-tandem mass spectrometry

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Received January 25, 2011; Accepted March 17, 2011

DOI: 10.3892/or.2011.1278

Abstract. Pancreatic cancer (PC) is one of the most lethal malignant tumors because of late diagnosis and the lack of response to various therapies. To identify potential biomarkers in cancerous serum from early stage PC patients, we carried out two-dimensional gel electrophoresis (2-DE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) to compare the serum proteomic profiles from 45 patients with PC and 20 healthy volunteers. Seven spots showed differential expression on 2-DE gels and two up-regulated protein spots were identified by LC-MS/MS as α-1-antitrypsin (AAT). These protein spots were also confirmed by Western blotting. This is the first time that AAT isoforms have been identified as potential serum biomarkers for PC. The serum isoforms of AAT may be clinically useful for PC diagnosis and monitoring.

Introduction

Pancreatic cancer (PC) is one of the most lethal malignant tumors in human diseases. Median survival is <12 months and overall 5-year survival is <5% after aggressive multi-modality treatment. Because of rapid aggressiveness and low probability of diagnosis at an early stage, most cases are diagnosed after metastatic spread (1). Therefore, detection of PC at an early disease stage is critical for successful clinical therapy. Although the serum tumor marker CA19-9 has been widely used for PC, it lacks sufficiently sensitive and accurate, especially in early diagnosis (2). There is, therefore, an urgent need to discover more sensitive and specific biomarkers to improve diagnosis and also prognostic monitor for patients of PC.

The combination of two-dimensional gel electrophoresis (2-DE) with mass spectrometry (MS) is a powerful tool for identification of novel biomarkers or therapeutic targets from cancer-associated samples (3). In previous proteome studies, this technique has been applied successfully to identify various proteins in cancer cell lines, cancer tissues and sera from cancer patients (4,5) and has been used to detect biomarkers of colorectal cancer (6), ovarian cancer (7), breast cancer (8), prostate cancer (9,10), bladder cancer (11) and other cancers. The detection for biomarkers is clinically useful, especially for screening or diagnosis.

In recent years, many efforts have been made to identify biomarkers by these proteomic methods. Bloomston *et al* identified fibrinogen γ as a potential tumor marker for pancreatic cancer (12). Yu *et al* confirmed that apolipoprotein E, α-1-antichymotrypsin and inter-α-trypsin inhibitor increased in pancreatic cancer serum (13). Sun *et al* discovered that cyclin I and GDI2 may be potential molecular targets for pancreatic cancer diagnostics and therapeutics (14). Thus, we will continue applying them to validate the candidate tumor markers and also improve the current proteomic method.

In the present study, we utilized proteomic differential display analysis using 2-DE with LC-MS/MS to examine the difference in serum proteins between normal and PC patients. We discuss the usefulness of electrophoretic techniques to find serum tumor biomarker candidates which might be a promising target for diagnosis of PC in early stage.

Materials and methods

Serum specimens and sample preparation. A total of 65 serum samples were used, with 45 samples randomly selected from patients with pancreatic cancer, who had undergone surgical resection at the Department of Surgery II, Yamaguchi University Hospital and 20 samples obtained from healthy donors as controls. Written informed consent was obtained from all patients before surgery. All serum samples were stored at -80°C until use.

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Key words: two-dimensional gel electrophoresis, liquid chromatography-tandem mass spectrometry, pancreatic cancer

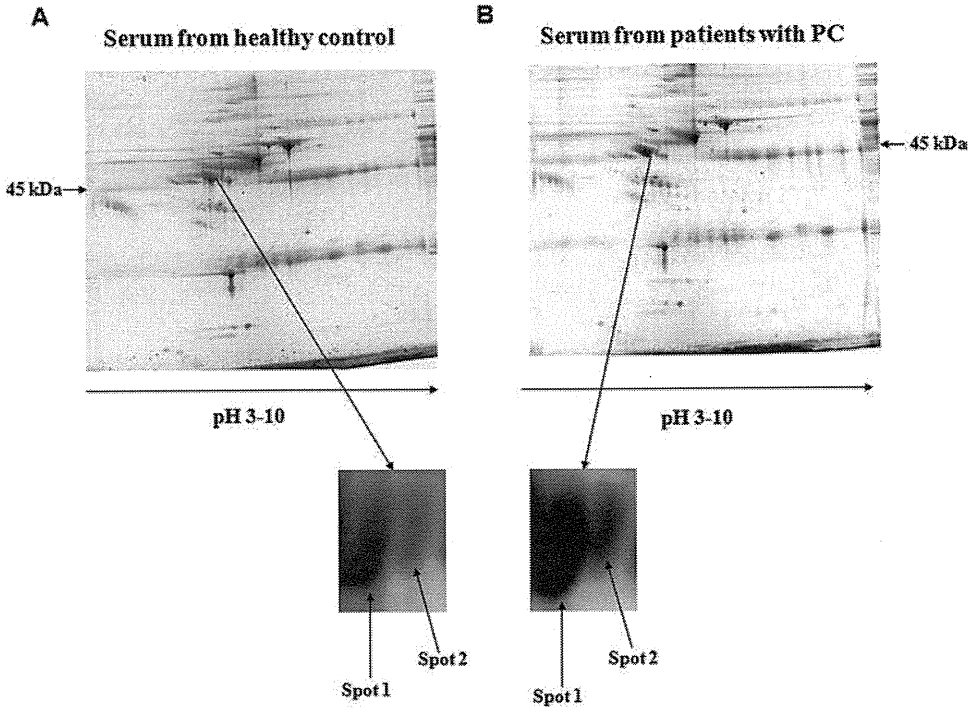

Figure 1. Protein patterns obtained by 2-DE of serum from patients with PC (A) and healthy controls (B). Proteins were separated on pH 3-10 linear, immobilized pH gradient strips and then by SDS-PAGE with a linear concentration gradient of 5-20%. Gels were stained with Flamingo Gel Stain™. Two spots were up-regulated in cancerous serum and numbered 1 and 2.

Table I. Up-regulated α-1-antitrypsin (AAT) isoforms in PC sera.

Isoform[a]	Protein name	Accession no.[b]	p*I*[c]	Molecular weight (Da)[c]	Distinct peptides[d]	MS/MS search score	Sequence coverage
1	α-1-antitrypsin	P01009	5.37	46736.8	14	190.84	32%
2	α-1-antitrypsin	P01009	5.37	46736.8	5	56.20	10%

[a]Protein spot numbers on 2-DE gel. [b]Accession number derived from the protein database. [c]Theoretical p*I* and molecular weight (Da) from the protein database. [d]Number of matched peptides.

Albumin and IgG were removed by using ProteoPrep™ Blue Albumin depletion kit (Sigma, St. Louis, MO, USA) from serum samples following product information of this kit. Separated samples were obtained and stored at -80˚C until analysis.

2-DE. Isoelectric focusing (IEF) was performed on 11 cm, immobilized pH gradient strips with a linear gradient pH 3.0-10.0 (Bio-Rad, Hercules, CA, USA) at 50 A/strip. Protein (100 μg) was used for each 2-DE. Samples were mixed with 200 μl of rehydration buffer [8 M urea, 2% CHAPS, 0.01% bromophenol blue, 1.2% Destreak reagent (GE Healthcare, Uppsala, Sweden)] and 0.5% IPG buffer (GE Healthcare) and loaded into the IPGphor strip holder (GE Healthcare). IEF was performed in six steps which were: rehydration for 10 h (no voltage), 0 to 500 V for 4 h, 500 to 1000 V for 1 h, 1000 to 8000 V for 4 h, 8000 V for 20 min, and the final phase of 500 V from 20000 to 30000 Vh. The IPG strips were equilibrated as described previously and then transferred onto the gels, run at 200 V. SDS-PAGE was performed on a precast polyacrylamide gel with a linear concentration gradient of 5-20% (Bio-Rad) (15).

Fluorescence staining. The SDS-PAGE gels were fixed with the 40% ethanol and 10% acetic acid for 2.5 h and then stained with a fluorescent gel staining, Flamingo™ Fluorescent Gel Stain (Bio-Rad) for 18 h. The stained gels were washed with Milli-Q water 3 times for 5 min. All experimental procedures were carried out on a shaker.

Image analysis and spot picking. The gels were scanned by using the ProXpress 2D Proteomic Imaging System (PerkinElmer, Waltham, MA, USA) in order to record the positions of the protein spots on the gel. Image analysis was performed with Progenesis Samespots software (Nonlinear, Newcastle, Upon Tyne, UK) following the user manual. After image analysis, the gels were stained with See Pico™ (Benebiosis Co, Ltd., Seoul, Korea) over night. The selected protein spots that showed at different intensities were excised from the gels and stored in 100 μl Milli-Q water at -80˚C until analysis.

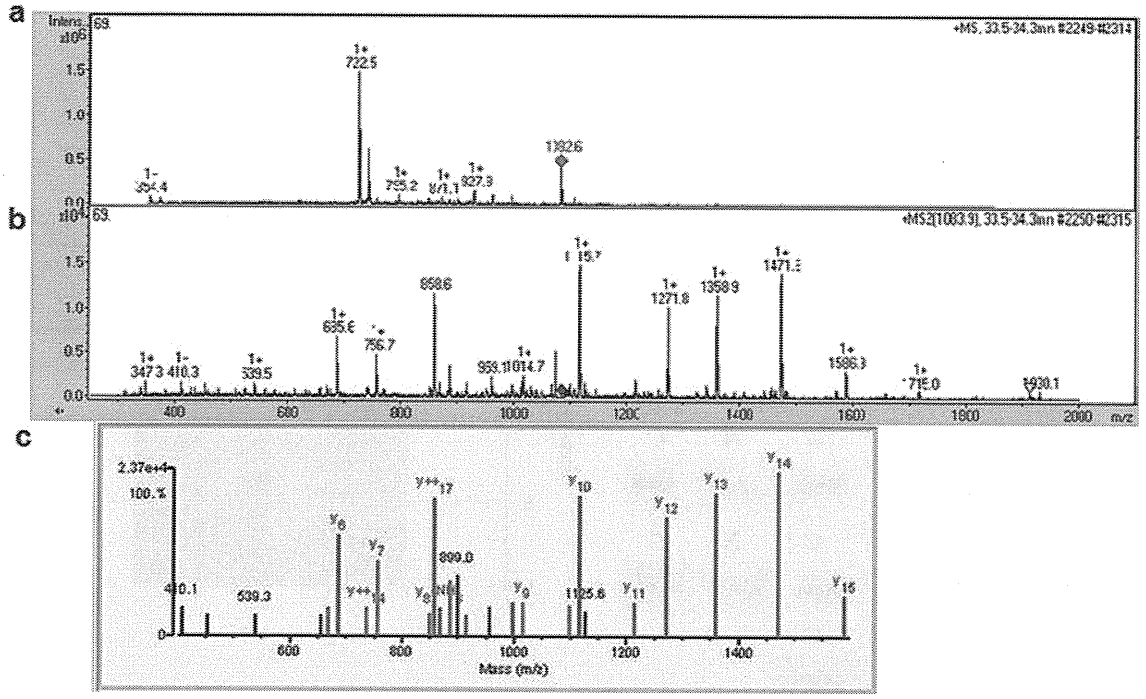

Figure 2. MS and MS/MS spectra of trypsin-digested spot 1. (a) LC-MS spectra of trypsin-digested of spot 1; α-1-antitrypsin; precursor ion m/z is 1082.6. (b and c) LC-MS/MS spectrum of a precursor ion with m/z 1082.6 marked by a lozenge in (a). The MS/MS spectrum is identified as the partial tryptic peptide VFSNGADLSGVTEEAPLKLSK from α-1-antitrypsin processed with a Spectrum Mill workbench.

In-gel digestion. The gel pieces were rinsed 3 times in 60% methanol, 0.05 M ammonium bicarbonate, and 0.005 M DTT for 15 min. The sample in the gel piece was reduced twice in 50% methanol, 0.05 M ammonium bicarbonate, and 0.005 M DTT for 10 min. The gel pieces were dehydrated twice in 100% ACN for 30 min. Enzyme digestion was performed with an in-gel digestion reagent containing 10 μg/ml sequencing-grade-modified trypsin (Promega, Madison, WI, USA) in 30% ACN, 0.05 M ammonium bicarbonate, and 0.005 M DTT at 30˚C for 16 h. The samples were lyophilized over night with the use of Labconco Lyph-lock 1L Model 77400 (Labconco, Kansas, MO, USA) (16). Lyophilized samples were dissolved in 0.1% formic acid after 30 sec were centrifuged at 21500 x g for 5 min and the supernatant was stored at -80˚C as samples for MS until use.

LC-MS/MS analysis. LC-MS/MS was performed by using Agilent 1100 LC-MSD Trap XCT (Agilent Technologies, Palo Alto, CA, USA). Each sample (15 μl) was 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, ACN in 0.1% formic acid. Column flow, 0.3 μl/min; primary flow 300 μl/min. Gradient, 0-5 min 2% B, 60 min 60% B. Stop time: 60 min. Protein identification was performed in 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). The criteria for positive identification of proteins were set as follows: filter by protein score >10.0, and filter peptide by score >8, percentage scored peak intensity. The Spectrum Mill workbench can search MS/MS spectra using an MS/MS ion search (17,18).

Western blotting. Total protein was used for electrophoresis with SDS-PAGE gels and transfer onto PVDF membranes at 90 mA for 78 min. Protein (1 μg) was used for each 2-DE. After that, the membranes were blocked over night at 4˚C with TBS containing 5% milk. Membranes were incubated with the primary antibody against α-1-antitrypsin (polyclonal rabbit anti-human α-1-antitrypsin, DakoCytomation, Glostrup, Denmark. 1:500) for 1 h at room temperature. Membranes were washed three times with TBS containing Tween-20 and once with TBS, and then incubated with the HRP-conjugated secondary antibody (1:10,000) for 1 h at room temperature. After washing, membranes were treated with a chemiflourescence reagent (ECL Plus Western Blotting Detection Reagents, GE Healthcare) and detected by using the ProXpress 2D Proteomic Imaging System (19).

Results

Differential spots between pancreatic cancer sera and healthy sera. After protein spots on the 2-DE gels were visualized by fluorescence staining, differences in the spot intensities between sera from patients with pancreatic cancer and healthy volunteers were analyzed with Progenesis Samespots software. At least 230 protein spots were matched on each 2-DE gel. Two spots were up-regulated in cancerous serum samples by >1.5-fold higher intensity (Fig. 1).

MS/MS analysis. The LC-MS/MS system identified the two up-regulated protein spots as α-1-antitrypsin isoform 1 and 2. MS and MS/MS spectra of trypsin-digested spot 1

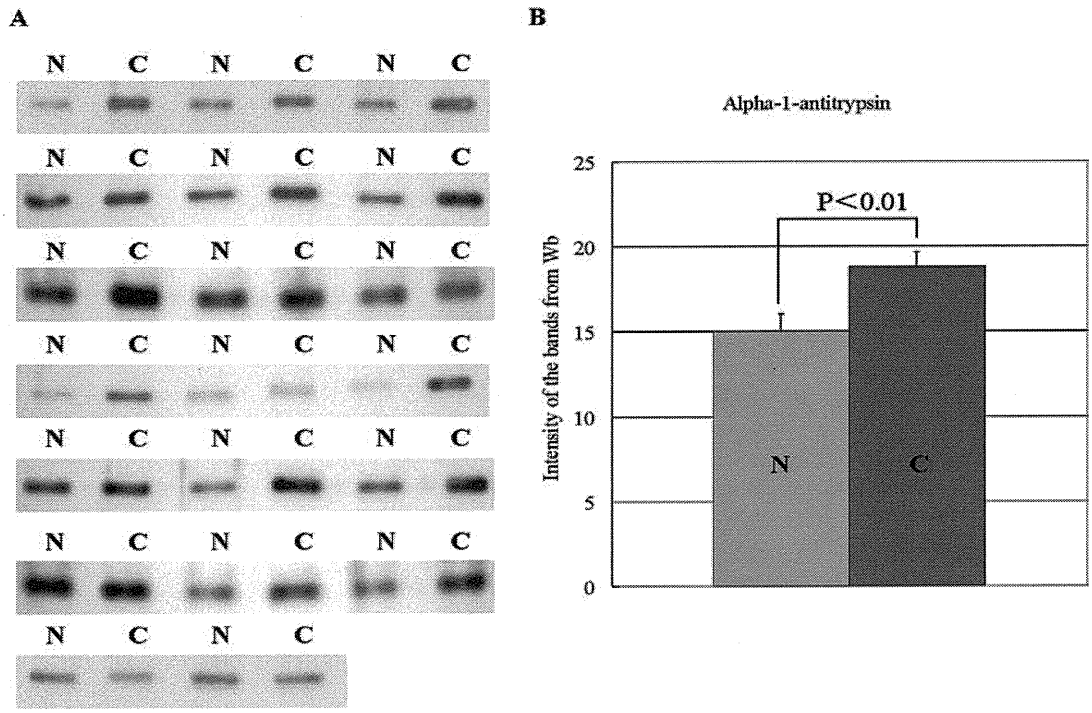

Figure 3. Western blot analysis of α-1-antitrypsin (AAT) in pancreatic cancerous serum. (A) Sera from 20 patients with pancreatic cancer (C) and 20 normal donors (N) were used for anti-AAT antibody. The expression of AAT was increased in pancreatic cancerous serum (70%). (B) Comparison of the intensity of Western blot bands between cancerous serum and normal serum by Student's t-test (n=20, P<0.01). The relative standard errors of cancerous serum samples and normal serum samples were 0.860 and 1.037, respectively.

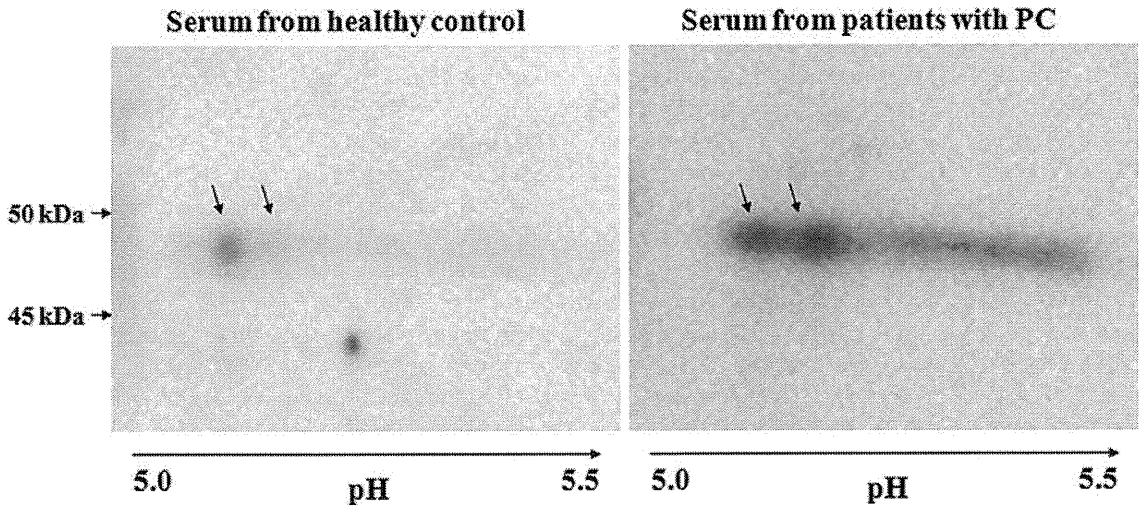

Figure 4. 2-D immunoblot analysis of α-1-antitrypsin (AAT) in pancreatic cancerous serum. We performed 2-D immunoblotting with a pair of samples on pH 5.0-8.0 linear and confirmed the location sites of AAT isoforms on immunoblot membranes. Compared with non-cancerous serum, the up-regulated spots of AAT in cancerous serum were observed in the pH 5.0-5.5, 45-50 kDa area.

are shown in Fig. 2 and MS/MS data for the two isoforms are summarized in Table I.

Western blot analysis of α-1-antitrypsin (AAT). As seen in Fig. 3A, compared with sera from healthy volunteers, up-regulated ATT in sera from PC patients was detected by Western blot analysis, and the average intensity of bands of α-1-antitrypsin is shown in Fig. 3B. Location of ATT isoforms on the 2-DE gel was displayed by 2-D Western blot analysis (Fig. 4).

Discussion

In this study, we detected two up-regulated protein spots in sera from patients with PC compared with healthy volunteers. The two protein spots were identified as α-1-antitrypsin (AAT) by LC-MS/MS. AAT belongs to acute phase protein (APP) whose expression is significantly increased in inflammation, trauma, infection and tumorigenesis. Many studies have suggested that serum levels of APP have a high clinical value in various cancer diseases including colorectal cancer

(20), lung cancer (21), hepatoma (22), breast cancer (23) and others (24).

Protein level of serum AAT was significantly increased during inflammation trauma, infection, and tumorigenesis (20), especially in lung cancer (21) and liver cancer (25). AAT represents a diagnostic index of tumor diseases, highly sensitive but with low specificity (26). Up-regulation of AAT in some pathological conditions, such as malignant proliferation, is believed to be a part of protective physiological response (27). An increase of serum ATT levels is reported to be associated with acute malignancy and shorter survival (28). Therefore, AAT may be a valuable marker for prognosis in PC. Although circulating AAT is supplied primarily by hepatocytes and mononuclear phagocytes, AAT is also present in a variety of tissues and cells, including tumor cells (29,30). More aggressive tumor growth associated with reduced local AAT expression (31). AAT with specific forms have multiple effects on tumor cell viability and promote tumorigenesis (30). Therefore, there is a possibility of production of AAT in pancreatic cancerous tissues. Presumably destruction of pancreatic cancerous tissues leads to abundant AAT release into the blood, resulting in increasing concentration in sera of PC patients.

Herein, we showed up-regulated AAT in sera of PC patients. However, since AAT is highly sensitive, it could be useful in early diagnosis of PC. Although it is difficult to diagnose the patients with PC in early stage, it is important to clarify if AAT is highly sensitive in early stage of PC. In order to confirm whether AAT is useful for developing new diagnostic or prognostic marker for PC, a large number of clinical investigations are needed in further study.

Tountas *et al* have reported AAT in PC patient sera (32). It is commonly believed that the specificity of ATT is low (33), but our data indicate that its serum isoforms might be more specific for diagnosis of PC. Electrophoretic techniques of 2-DE and Western blotting combined with LC-MS/MS in this study indicate AAT to be a candidate protein for biomarker in PC patient sera and also provide more visualized and detailed information for diagnosis or prognosis of PC. AAT can be separated into seven different isoforms in plasma with linear gradient pH 4.0-7.0 strips by 2-DE (34). Therefore, we will also depend on proteomics for clarifying the relationship between expression of those isoform levels of serum AAT and early PC in the future.

References

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J and Thun MJ: Cancer statistics, 2009. CA Cancer J Clin 59: 225-249, 2009.
2. Goggins M, Canto M and Hruban R: Can we screen high-risk individuals to detect early pancreatic carcinoma? J Surg Oncol 74: 243-248, 2000.
3. Wasinger VC, Cordwell SJ, Cerpa-Poljak A, Yan JX, Gooley AA, Wilkins MR, Duncan MW, Harris R, Williams KL and Humphery-Smith I: Progress with gene-product mapping of the Mollicutes: Mycoplasma genitalium. Electrophoresis 16: 1090-1094, 1995.
4. Kuramitsu Y and Nakamura K: Proteomic analysis of cancer tissues: shedding light on carcinogenesis and possible biomarkers. Proteomics 6: 5650-5661, 2006.
5. Kuramitsu Y and Nakamura K: Current progress in proteomic study of hepatitis C virus-related human hepatocellular carcinoma. Expert Rev Proteomics 2: 589-601, 2005.
6. Ma YL, Peng JY, Zhang P, Huang L, Liu WJ, Shen TY, Chen HQ, Zhou YK, Zhang M, Chu ZX and Qin HL: Heterogeneous nuclear ribonucleoprotein A1 is identified as a potential biomarker for colorectal cancer based on differential proteomics technology. J Proteome Res 8: 4525-4535, 2009.
7. Petricoin EF, Ardekani AM, Hitt BA, Levine PJ, Fusaro VA, Steinberg SM, Mills GB, Simone C, Fishman DA, Kohn EC and Liotta LA: Use of proteomic patterns in serum to identify ovarian cancer. Lancet 359: 572-577, 2002.
8. Li J, Zhang Z, Rosenzweig J, Wang YY and Chan DW: Proteomics and bioinformatics approaches for identification of serum biomarkers to detect breast cancer. Clin Chem 48: 1296-1304, 2002.
9. Bañez LL, Prasanna P, Sun L, Ali A, Zou Z, Adam BL, McLeod DG, Moul JW and Srivastava S: Diagnostic potential of serum proteomic patterns in prostate cancer. J Urol 170: 442-446, 2003.
10. Adam BL, Qu Y, Davis JW, Ward MD, Clements MA, Cazares LH, Semmes OJ, Schellhammer PF, Yasui Y, Feng Z and Wright GL Jr: Serum protein fingerprinting coupled with a pattern-matching algorithm distinguishes prostate cancer from benign prostate hyperplasia and healthy men. Cancer Res 62: 3609-3614, 2002.
11. Vlahou A, Schellhammer PF, Mendrinos S, Patel K, Kondylis FI, Gong L, Nasim S and Wright GL Jr: Development of a novel proteomic approach for the detection of transitional cell carcinoma of the bladder in urine. Am J Pathol 158: 1491-1502, 2001.
12. Bloomston M, Zhou JX, Rosemurgy AS, Frankel W, Muro-Cacho CA and Yeatman TJ: Fibrinogen gamma overexpression in pancreatic cancer identified by large-scale proteomic analysis of serum samples. Cancer Res 66: 2592-2599, 2006.
13. Yu KH, Rustgi AK and Blair IA: Characterization of proteins in human pancreatic cancer serum using differential gel electrophoresis and tandem mass spectrometry. J Proteome Res 4: 1742-1751, 2005.
14. Sun ZL, Zhu Y, Wang FQ, Chen R, Peng T, Fan ZN, Xu ZK and Miao Y: Serum proteomic-based analysis of pancreatic carcinoma for the identification of potential cancer biomarkers. Biochim Biophys Acta 1774: 764-771, 2007.
15. Tanaka T, Kuramitsu Y, Fujimoto M, Naito S, Oka M and Nakamura K: Downregulation of two isoforms of ubiquitin carboxyl-terminal hydrolase isozyme L1 correlates with high metastatic potentials of human SN12C renal cell carcinoma cell clones. Electrophoresis 29: 2651-2659, 2008.
16. Kuramitsu Y, Miyamoto H, Tanaka T, Zhang X, Fujimoto M, Ueda K, Tanaka T, Hamano K and Nakamura K: Proteomic differential display analysis identified upregulated astrocytic phosphoprotein PEA-15 in human malignant pleural mesothelioma cell lines. Proteomics 9: 5078-5089, 2009.
17. Takashima M, Kuramitsu Y, Yokoyama Y, Iizuka N, Harada T, Fujimoto M, Sakaida I, Okita K, Oka M and Nakamura K: Proteomic analysis of autoantibodies in patients with hepatocellular carcinoma. Proteomics 6: 3894-3900, 2006.
18. Takashima M, Kuramitsu Y, Yokoyama Y, Iizuka N, Fujimoto M, Nishisaka T, Okita K, Oka M and Nakamura K: Overexpression of alpha enolase in hepatitis C virus-related hepatocellular carcinoma: association with tumor progression as determined by proteomic analysis. Proteomics 5: 1686-1692, 2005.
19. Kuramitsu Y, Harada T, Takashima M, Yokoyama Y, Hidaka I, Iizuka N, Toda T, Fujimoto M, Zhang X, Sakaida I, Okita K, Oka M and Nakamura K: Increased expression and phosphorylation of liver glutamine synthetase in well-differentiated hepatocellular carcinoma tissues from patients infected with hepatitis C virus. Electrophoresis 27: 1651-1658, 2006.
20. Stamatiadis AP, St Toumanidou M, Vyssoulis GP, Manouras AJ and Apostolidis NS: Value of serum acute-phase reactant proteins and carcinoembryonic antigen in the preoperative staging of colorectal cancer. A multivariate analysis. Cancer 65: 2055-2057, 1990.
21. Kasprzyk M, Dyszkiewicz W, Zwaruń D, Leśniewska K and Wiktorowicz K: The assessment of acute phase proteins as prognostic factors in patients surgically treated for non-small cell lung cancer. Pneumonol Alergol Pol 76: 321-326, 2008.
22. Kang X, Sun L, Guo K, Shu H, Yao J, Qin X and Liu Y: Serum protein biomarkers screening in HCC patients with liver cirrhosis by ICAT-LC-MS/MS. J Cancer Res Clin Oncol 136: 1151-1159, 2010.
23. Thompson DK, Haddow JE, Smith DE and Ritchie RF: Elevated serum acute phase protein levels as predictors of disseminated breast cancer. Cancer 51: 2100-2104, 1983.

24. Juan HF, Chen JH, Hsu WT, Huang SC, Chen ST, Yi-Chung Lin J, Chang YW, Chiang CY, Wen LL, Chan DC, Liu YC and Chen YJ: Identification of tumor-associated plasma biomarkers using proteomic techniques: from mouse to human. Proteomics 4: 2766-2775, 2004.
25. Hong WS and Hong SI: Clinical usefulness of alpha-1-antitrypsin in the diagnosis of hepatocellular carcinoma. J Korean Med Sci 6: 206-213, 1991.
26. Schena M, Quadri A, Ragazzoni G and Corti P: Alpha 1-antitrypsin as a tumor marker. Quad Sclavo Diagn 21: 87-96, 1985.
27. Marchandise FX, Mathieu B, Francis C and Sibille Y: Local increase of antiprotease and neutrophil elastase-alpha 1-proteinase inhibitor complexes in lung cancer. Eur Respir J 2: 623-629, 1989.
28. Tahara E, Ito H, Taniyama K, Yokozaki H and Hata J: Alpha 1-antitrypsin, alpha 1-antichymotrypsin, and alpha 2-macroglobulin in human gastric carcinomas. a retrospective immunohistochemical study. Hum Pathol 15: 957-964, 1984.
29. Carlson JA, Rogers BB, Sifers RN, Hawkins HK, Finegold MJ and Woo SL: Multiple tissues express alpha 1-antitrypsin in transgenic mice and man. J Clin Invest 82: 26-36, 1988.
30. Zelvyte I, Sjögren HO and Janciauskiene S: Effects of native and cleaved forms of alpha1-antitrypsin on ME 1477 tumor cell functional activity. Cancer Detect Prev 26: 256-265, 2002.
31. Huang H, Campbell SC, Nelius T, Bedford DF, Veliceasa D, Bouck NP and Volpert OV: Alpha1-antitrypsin inhibits angiogenesis and tumor growth. Int J Cancer 112: 1042-1048, 2004.
32. Tountas Y, Sparos L, Theodoropoulos C and Trichopoulos D: Alpha 1-antitrypsin and cancer of the pancreas. Digestion 31: 37-40, 1985.
33. Irigoyen Oyarzabal AM, Amiguet García JA, López Vivanco G, Genollá Subirats J, Muñoz Villafranca MC, Ojembarrena Martínez E and Liso Irurzun P: Tumoral markers and acute-phase reactants in the diagnosis of pancreatic cancer. Gastroenterol Hepatol 26: 624-629, 2003.
34. Mateos-Cáceres PJ, García-Méndez A, López Farré A, Macaya C, Núñez A, Gómez J, Alonso-Orgaz S, Carrasco C, Burgos ME, de Andrés R, Granizo JJ, Farré J and Rico LA: Proteomic analysis of plasma from patients during an acute coronary syndrome. J Am Coll Cardiol 44: 1578-1583, 2004.

ANTICANCER RESEARCH *31*: 1259-1264 (2011)

Two-dimensional Gel Electrophoresis Using Immobilized pH Gradient Strips and Flamingo™ Fluorescent Gel Stain Identified Non-nuclear Proteins Possibly Related to Malignant Tumour Progression

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Abstract. *The understanding of tumour progression is one of the most important strategies to conquer tumour. QR-32 is a regressive murine fibrosarcoma cell line, and QRsP-11 is a progressive malignant tumour cell clone derived from QR-32. Ina recently published study a differential display analysis for the cytoplasmic proteins was shown by using two-dimensional gel electrophoresis (2-DE) making full use of isoelectric focusing capillary gels and Coomassie brilliant blue R-250 staining. Furthermore, a differential display analysis of the nuclear proteome for QR-32 and QRsP-11 was performed. The present study shows a non-nuclear proteomic differential display analysis, using 2-DE making full use of immobilized pH gradient strips and Flamingo™ fluorescent gel stain, between QR-32 and QRsP-11 to identify particular proteins which may be involved in malignant progression. In QRsP-11 25 proteins were up-regulated, including hypoxia up-regulated protein 1, and 6 were down-regulated compared with QR-32. These results suggest that the identified non-nuclear proteins showing different expression between QR-32 and QRsP-11 possibly related to malignant tumour progression.*

Tumour development and progression are the most crucial features of malignant tumours for patients who are expected to recover from cancer. Progressive tumour cells show rapid growth, invasiveness and metastatic capacity compared with regressive benign tumour cells. Progressive and regressive tumour models of murine fibrosarcoma cells (QR-32 clone and QRsP-11 clone) have been established (1). QR-32 is weakly tumourigenic and non-metastatic. QRsP-11 is a progressive malignant tumour cell clone derived from QR-32.

Proteomics is the comprehensive analysis of the total protein complement of a genome. The combination of two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) is a popular method of proteomics. The technique of 2-DE makes it possible to separate proteins according to both their charges in isoelectric focusing (IEF) gels and their weight in sodium dodecyl sulfate (SDS) gels. The 2-DE technique has unique advantages for examining the expressions of thousands of proteins simultaneously (2). We have reported many proteomic studies of QR-32 and QRsP-11 cells by using 2-DE. A differential display analysis for the expression of nuclear proteins between QR-32 and QRsP-11 showed 8 nuclear proteins, differentially regulated, including zing finger protein ZXDC in QRsP-11 compared with QR-32. However, from nuclear proteomic analysis, it was difficult to elucidate the whole mechanism of tumour progression (3). The proteomic differential display analysis for the expression of cytoplasmic proteins in QR-32 and QRsP-11 showed 11 protein spots, differentially regulated, including heat shock protein (HSP)-90 in QRsP-11 compared with QR-32 (4). However, since the identification of more proteins related to tumour progression is necessary, in the present study, immobilized pH gradient (IPG) strips and Flamingo™ fluorescent gel stain were used in place of IEF capillary gels and Coomassie brilliant blue R-250 staining to identify weakly expressed non-nuclear proteins.

Materials and Methods

Tumour cell lines and culture condition. QR-32 and QRsP-11 are murine fibrosarcoma cell lines, which were established at Hokkaido University, the origin and characteristics of which have been

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Key Words: Progression, inflammation-associated carcinogenesis, fibrosarcoma, non-nuclear protein.

0250-7005/2011 $2.00+.40

described previously (4-6). Briefly, QR-32 cells are unable to grow when injected subcutaneously in normal C57Bl/6 mice and they spontaneously regress in normal syngeneic mice. QRsP-11 cells were obtained from the tumours which arose in mice after subcutaneous co-implantation of QR-32 cells with gelatin sponge, and showed strong tumorigenicity. They were cultured in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, sodium pyruvate, non-essential amino acids and L-glutamine, at 37°C, in a humidified 5% carbon dioxide-95% air mixture. We used these cell lines passaged less than 15 times culture after the cells had been sent to our laboratory.

Sample preparation. The non-nuclear proteins from QR-32 and QRsP-11 were extracted by means of NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instruction (6).

Two-dimensional gel electrophoresis (2-DE). Isoelectric focusing (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, Hercules, CA, USA) at 50 μA/strip, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a precast polyacrylamide gel with a linear concentration gradient of 5-20% (BIO RAD), run at 200 V (7, 8).

Fluorescent gel staining. After 2-DE and fixing the gels were stained with a fluorescent gel staining, Flamingo™ Fluorescent Gel Stain (BIO RAD), overnight. Stained gels were washed with ultra-pure water (Wako Pure Chemical Industries, Osaka, Japan) three times (9, 10).

Image analysis and spot picking. Expression levels of the protein spots were quantified with Progenesis SameSpot software (Nonlinear Dynamics Ltd, Newcastle upon Tyne, UK) (7, 9), and the differences in expression between QR-32 and QRsP-11 were analysed statistically by ANOVA test. 2-DE analysis was repeated three times. After statistical analysis, the gels were re-stained with See Pico™ (Benebiosis Co., Ltd, Seoul, Korea) (11), and the selected spots whose expression was significantly different between QRsP-11 and QR-32 were picked up for the mass spectrometry (MS) analysis.

High-performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS). An Agilent 1100 LC/MSD Trap XCT (Agilent Technologies, Palo Alto, CA, USA) was used for HPLC and MS/MS. Protein identification was performed in 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). The criteria for positive identification of proteins were as follows: filter by protein score≥10.0, and filter peptide by score≥8, % scored peak intensity (% SPI) >70. The Spectrum Mill workbench can search MS/MS spectra using an MS/MS ion search (12-14).

Results

Detection of up- and down-regulated non-nuclear protein spots in 2-DE gels. The cytoplasmic protein marker glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected, but the nuclear protein marker lamin B1 could not be detected in the non-nuclear fraction of either QR-32 or QRsP-11 (data not shown). Figure 1A and B show 2-DE patterns of non-nuclear fractions from QR-32 and QRsP11, respectively. More than 600 protein spots were detected. Expression of 25 proteins appeared to be up-regulated, and that of 6 appeared to be down-regulated in QRsP-11 compared with QR-32.

Identification of up- and down-regulated protein spots. These 31 spots were picked up and identified by using Agilent 1100 LC/MSD Trap XCT LC-MS/MS system. The results are shown in Table I.

Discussion

This proteomic differential display analysis by MS and 2-DE using IPG strips and Flamingo™ fluorescent gel stain in place of IEF capillary gels and Coomassie brilliant blue R-250 staining for non-nuclear proteins from regressive murine fibrosarcoma cell clone QR-32 and its derived malignant progressive cell clone QRsP-11 showed 6 down-regulated and 25 up-regulated non-nuclear proteins in QRsP-11 compared with QR-32 cells.

Hypoxia up-regulated protein 1, an endoplasmic reticulum chaperone, is essential for the maintenance of cellular viability under hypoxia and is reported to be overexpressed in tumour cells. Namba *et al.* reported that up-regulation of this protein reduces the antitumor activity of celecoxib, a cyclooxygenase-2 (COX-2)-selective non-steroidal anti-inflammatory drugs (NSAID), by inhibiting apoptosis (15). Stojadinovic *et al.* reported that it was up-regulated in some invasive breast tumours and its up-regulation appeared to be associated with indicators of poor prognosis and metastasis (16). This protein was up-regulated in QRsP-11 cells here.

Heat-shock 70 kDa (HSP70) protein 4 is one of several heat-shock 70 kDa proteins (17). Some reports showed the relation of HSP70 proteins to cancer progression. Garg *et al.* reported that HSP70-2 expression was associated with early spread and progression of urothelial carcinoma of bladder cancer and that HSP70-2 may be a potential therapeutic target for bladder urothelial carcinoma (18). Ramp *et al.* reported that HSP70-mediated inhibition of apoptosis seems to be of minor importance for carcinogenesis and tumor progression in renal cell carcinoma cells (19). This protein was also up-regulated in QRsP-11 cells here.

It has also been reported that HSP90 is thought to be a very important molecule for cancer progression (10, 20). Some inhibitors have been reported and therapies using them are also being carried out (21). HSP90 was also up-regulated in QRsP-11. These reports from other groups support our results here.

Alpha-enolase is an isoenzyme of enolase, a key protein catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate in the glycolytic pathway. Takashima *et al.* reported that expression of alpha-enolase correlated

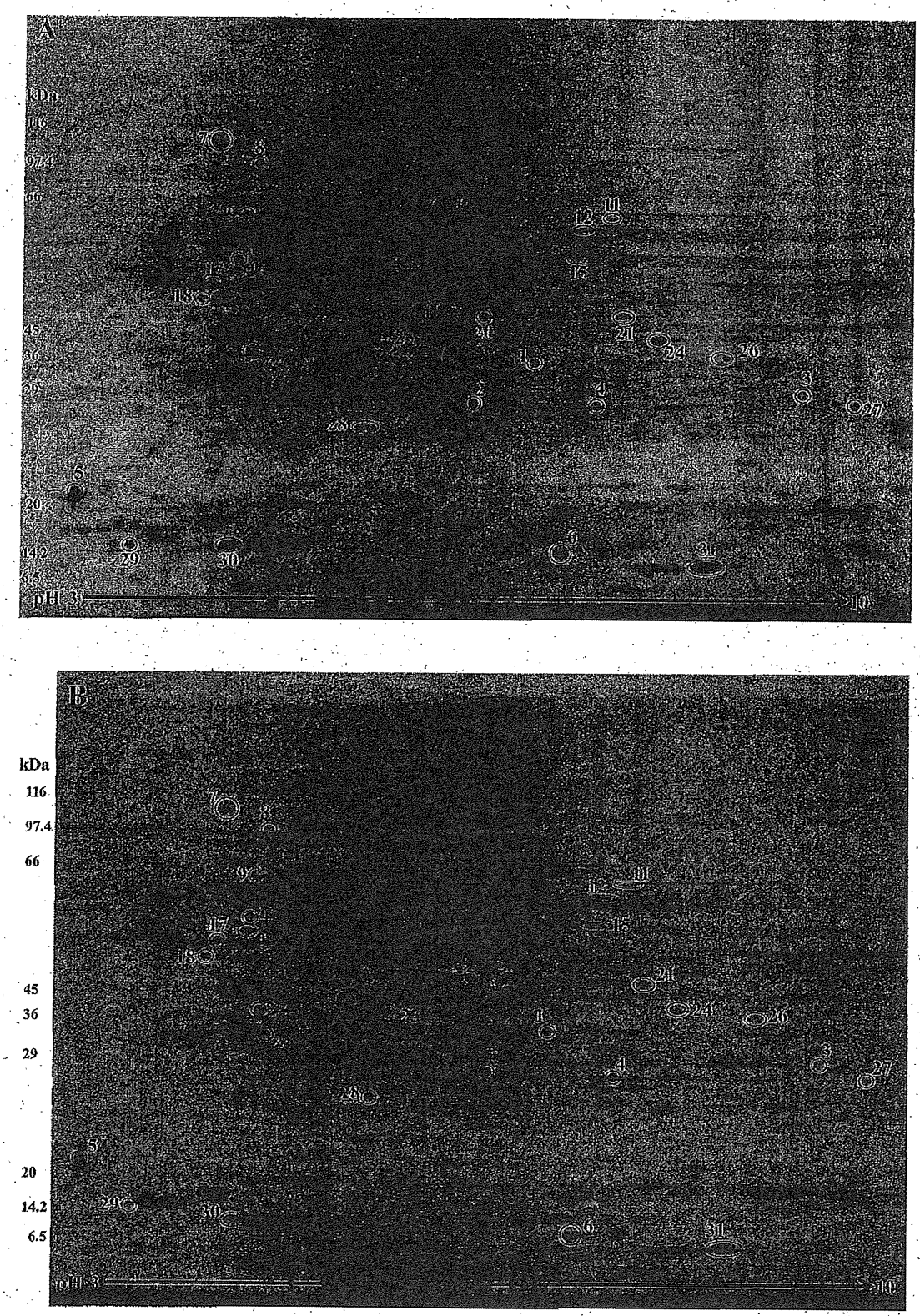

Figure 1. *2-DE patterns of non-nuclear proteins. Panels (A) and (B) show the 2-DE patterns of the non-nuclear fraction of QRsP-11 and QR-32, respectively. Proteins (80 μg) were separated by 2-DE using a pH 3-10 gradient in the first dimension, followed by second-dimensional separation on a precast polyacrylamide gel with a linear concentration gradient of 5-20% stained with Flamingo™ Gel Stain. The intensity of spots 1-6 decreased, while spots 7-31 increased in QRsP-11.*

Table I. *Identification of up- and down-regulated non-nuclear proteins in QRsP-11 compared to QR32. Spot numbers refer to those shown in Figure 1. These spots were cut out and were subsequently identified by MS.*

Spot	Protein name	Accession No.	Theoretical		Distinct peptides	Sequence coverage (%)	MS/MS search score	Change in QRsP-11 (fold)
			*p*I	Mr				
								Down-regulated
1	Pyrroline-5-carboxylate reductase 1, mitochondrial	Q922W5	6.36	32,373.6	10	40	142.76	2.0
2	Proteasome subunit alpha	Q9QUM9	6.35	27,372.6	1	5	14.78	2.1
3	Proline synthetase co-transcribed bacterial homolog protein	Q9Z2Y8	8.37	30,048.8	2	10	26.16	1.9
4	Triosephosphate isomerase	P17751	6.90	26,712.8	9	46	141.83	1.6
5	Calmodulin	P62204	4.09	16,837.7	5	54	72.65	1.5
6	Cystatin-B	Q62426	6.82	11,045.7	3	26	41.48	1.9
								Up-regulated
7	Hypoxia up-regulated protein 1	Q9JKR6	5.12	111,181.6	1	1	14.00	2.9
8	Heat-shock 70 kDa protein 4	Q61316	5.15	94,133.1	2	3	22.94	2.6
9	Heat-shock protein 90-beta 1	P08113	4.74	92,476.2	3	4	43.86	2.0
10	T-complex protein 1 subunit alpha B	P11983	5.82	60,449.0	10	24	140.95	2.0
11	Pyruvate kinase isozymes M1/M2	P52480	7.17	57,845.2	3	9	45.71	1.8
12	Pyruvate kinase isozymes M1/M2	P52480	7.17	57,845.2	10	28	134.26	1.6
13	Cytoskeleton-associated protein 4	Q8BMK4	5.46	63,692.4	4	8	55.47	1.8
14	Alpha-enolase	P17182	6.37	47,141.1	11	37	170.94	2.2
15	Alpha-enolase	P17182	6.37	47,141.1	7	20	100.46	1.5
16	Vimentin	P20152	5.06	53,687.9	4	10	55.08	2.3
17	Heat-shock protein 90-alpha	P07901	4.93	84,788.3	2	3	29.96	2.3
18	Reticulocalbin-1	Q05186	4.70	38,113.2	3	10	32.15	1.8
19	Pyruvate kinase isozymes M1/M2	P52480	7.17	57,845.2	5	12	76.40	2.1
20	Pyruvate kinase isozymes M1/M2	P52480	7.17	57,845.2	5	12	80.20	1.7
21	Annexin A1	P10107	6.97	38,734.5	3	12	44.54	1.5
22	Annexin A5	P48036	4.83	35,752.6	8	28	108.00	1.8
23	Transitional endoplasmic reticulum ATPase	Q01853	5.14	89,322.3	5	7	70.18	1.8
24	Glyceraldehyde-3-phosphate dehydrogenase	P16858	8.43	35,810.2	10	40	157.23	1.9
25	60 kDa Heat-shock protein, mitochondrial	P63038	5.91	60,955.8	12	26	155.28	5.0
26	Voltage-dependent anion-selective channel protein 2	Q60930	7.44	31,733.0	3	12	36.34	1.7
27	Glutathione S-transferase Mu 1	P10649	7.72	25,970.2	2	12	30.58	2.4
28	Adenylate kinase isoenzyme 1	Q9R0Y5	5.67	21,539.7	3	16	45.01	3.7
29	60S Acidic ribosomal protein P2	P99027	4.42	11,651.0	3	63	43.33	2.3
30	Thioredoxin	P10639	4.80	11,675.5	4	31	48.08	1.6
31	10 kDa Heat-shock protein, mitochondrial	Q64433	7.91	10,962.8	6	74	73.80	1.6

positively with tumor size and venous invasion (16). In response to up-regulated alpha enolase expression, the fibrinolytic system might be inordinately accelerated, and increased local fibrinolysis may contribute to cancer cell invasion and metastasis.

Vimentin is a type III intermediate filament in cells of mesenchymal origin, and acts as a scaffolding protein to stabilise connective tissues and cells. Many reports have shown that vimentin plays important roles in the epithelial-to-mesenchymal transition, invasion and metastasis (22, 23).

Reticulocalbin-1 is a member of the family of Ca^{2+}-binding proteins. This molecule is localized in the endoplasmic reticulum and functions in the secretory pathway of cells. Liu *et al.* reported that reticulocalbin was up-regulated in highly invasive breast cancer cell line compared with poorly invasive cell line (24). QRsP-11 cells are very invasive and metastatic. The result of up-regulation of alpha-enolase, vimentin and reticulocalbin-1 in QRsP-11 cells here supports their specific features.

Annexin A1 is a member of the annexin superfamily. It plays a role in membrane fusion and exocytosis. Links between the up-regulation of annexin A1 and progression of breast cancer have been reported (25).

Although these results demonstrated that some of proteins identified in present study may have a functional potentiality of tumor progression, further studies still necessary.

References

1 Okada F, Hosokawa M, Hamada JI, Hasegawa J, Kato M, Mizutani M, Ren J, Takeichi N and Kobayashi H: Malignant progression of a mouse fibrosarcoma by host cells reactive to a foreign body (gelatin sponge). Br J Cancer *66*: 635-639, 1992.

2 Kuramistu Y and Nakamura K: Proteomic analysis in cancer patients. Humana Press (USA); Cancer Drug Discovery and Development™ Genomics and Pharmacogenomics in Anticancer Drug Development and Clinical Response Innocenti F (ed.). pp. 33-45, 2008.

3 Kuramitsu Y, Hayashi E, Okada F, Tanaka T, Zhang X, Ueyama Y and Nakamura K: Proteomic analysis for nuclear proteins related to tumour malignant progression: a comparative proteomic study between malignant progressive cells and regressive cells. Anticancer Res *30*: 2093-2099, 2010.

4 Hayashi E, Kuramitsu Y, Okada F, Fujimoto M, Zhang X, Kobayashi M, Iizuka N, Ueyama Y and Nakamura K: Proteomic profiling for cancer progression: Differential display analysis for the expression of intracellular proteins between regressive and progressive cancer cell lines. Proteomics *5*: 1024-1032, 2005.

5 Ishikawa M, Hosokawa M, Oh-hara N, Niho Y and Kobayashi H: Marked granulocytosis in C57BL/6 mice bearing a transplanted BMT-11 fibrosarcoma. J Natl Cancer Inst *78*: 567-571, 1987.

6 Kuramitsu Y, Hayashi E, Okada F, Tanaka T, Zhang X, Ueyama Y and Nakamura K: Proteomic analysis for nuclear proteins related to tumour malignant progression: a comparative proteomic study between malignant progressive cells and regressive cells. Anticancer Res *30*: 2093-2099, 2010.

7 Kuramitsu Y, Miyamoto H, Tanaka T, Zhang X, Fujimoto M, Ueda K, Tanaka T, Hamano K and Nakamura K: Proteomic differential display analysis identified up-regulated astrocytic phsphoprotein PEA-15 in human malignant pleural mesothelioma cell lines. Proteomics *9*: 5078-5089, 2009.

8 Tanaka T, Kuramitsu Y, Fujimoto M, Naito S, Oka M and Nakamura K: Down-regulation of two isoforms of ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCH-L1) correlates with high metastatic potentials of human SN12C renal cell carcinoma cell clones. Electrophoresis *29*: 2651-2659, 2008.

9 Kuramitsu Y, Baron B, Yoshino S, Zhang X, Tanaka T, Yashiro M, Hirakawa K, Oka M and Nakamura K: Proteomic differential display analysis shows up-regulation of 14-3-3 protein sigma in human scirrhous-type gastric carcinoma cells. Anticancer Res *30*: 4459-4465, 2010.

10 Kuramitsu Y, Taba K, Ryozawa S, Yoshida K, Tanaka T, Zhang X, Maehara S, Maehara Y, Sakaida I and Nakamura K: Identification of up- and down-regulated proteins in gemcitabine-resistant pancreatic cancer cells using two-dimensional gel electrophoresis and mass spectrometry. Anticancer Res *30*: 3367-3372, 2010.

11 Kuramitsu Y, Hayashi E, Okada F, Zhang X, Tanaka T, Ueyama Y and Nakamura K: Staining with highly sensitive Coomassie brilliant blue SeePico™ stain after Flamingo™ fluorescent gel stain is useful for cancer proteomic analysis by means of two-dimensional gel electrophoresis. Anticancer Res *30*: 4001-4005, 2010.

12 Yuan X, Kuramitsu Y, Furumoto H, Zhang X, Hayashi E, Fujimoto M and Nakamura K: Nuclear protein profiling of Jurkat cells during heat stress-induced apoptosis by two-dimensional gel electrophoresis and tandem mass spectrometry. Electrophoresis *28*: 2018-2026, 2007.

13 Takashima M, Kuramitsu Y, Yokoyama Y, Iizuka N, Fujimoto M, Sakaida I, Okita K, Oka M and Nakamura K: Proteomic analysis of autoantibodies in patients with hepatocellular carcinoma. Proteomics *6*: 3894-3900, 2006.

14 Takashima M, Kuramitsu Y, Yokoyama Y, Iizuka N, Fujimoto M, NIshisaka T, Sakaida I, Okita K, Oka M and Nakamura, K: Overexpression of alpha enolase in hepatitis C virus-related hepatocellular carcinoma: association with tumor progression as determined by proteomic analysis. Proteomics *5*: 1686-1692, 2005.

15 Namba T, Hoshino T, Tanaka K, Tsutsumi S, Ishihara T, Mima S, Suzuki K, Ogawa S and Mizushima T: Up-regulation of 150-kDa oxygen-regulated protein by celecoxib in human gastric carcinoma cells. Mol Pharmacol *71*: 860-870, 2007.

16 Stojadinovic A, Hooke JA, Shriver CD, Nissan A, Kovatich AJ, Kao TC, Ponniah S, Peoples GE and Moroni M: HYOU1/Orp150 expression in breast cancer. Med Sci Monit *13*: 231-239, 2007.

17 Fathallah DM, Cherif D, Dellagi K and Arnaout MA: Molecular cloning of a novel human HSP70 from a B-cell line and its assignment to chromosome 5. J Immunol *151*: 810-813, 1993.

18 Garg M, Kanojia D, Seth A, Kumar R, Gupta A, Surolia A and Suri A: Heat-shock protein 70-2 (HSP70-2) expression in bladder urothelial carcinoma is associated with tumour progression and promotes migration and invasion. Eur J Cancer *46*: 207-215, 2010.

19 Ramp U, Mahotka C, Heikaus S, Shibata T, Grimm MO, Willers R and Gabbert HE: Expression of heat-shock protein 70 in renal cell carcinoma and its relation to tumor progression and prognosis. Histol Histopathol *22*: 1099-1107, 2007.

20 McCarthy MM, Pick E, Kluger Y, Gould-Rothberg B, Lazova R, Camp RL, Rimm DL and Kluger HM: HSP90 as a marker of progression in melanoma. Ann Oncol *19*: 590-594, 2008.

21 Moser C, Lang SA and Stoeltzing O: Heat-shock protein 90 (HSP90) as a molecular target for therapy of gastrointestinal cancer. Anticancer Res *29*: 2031-2042, 2009.

22 Vuoriluoto K, Haugen H, Kiviluoto S, Mpindi JP, Nevo J, Gjerdrum C, Tiron C, Lorens JB and Ivaska J: Vimentin regulates EMT induction by Slug and oncogenic H-Ras and migration by governing Axl expression in breast cancer. Oncogene 2010 Nov 8. [Epub ahead of print]

23 Zhu QS, Rosenblatt K, Huang KL, Lahat G, Brobey R, Bolshakov S, Nguyen T, Ding Z, Belousov R, Bill K, Luo X, Lazar A, Dicker A, Mills GB, Hung MC and Lev D: Vimentin is a novel AKT1 target mediating motility and invasion. Oncogene *30*: 457-470, 2011.

24 Liu Z, Brattain MG and Appert H: Differential display of reticulocalbin in the highly invasive cell line, MDA-MB-435, versus the poorly invasive cell line, MCF-7. Biochem Biophys Res Commun *231*: 283-289, 1997.

25 de Graauw M, van Miltenburg MH, Schmidt MK, Pont C, Lalai R, Kartopawiro J, Pardali E, Le Dévédec SE, Smit VT, van der Wal A, Van't Veer LJ, Cleton-Jansen AM, ten Dijke P and van de Water B: Annexin A1 regulates TGF-beta signaling and promotes metastasis formation of basal-like breast cancer cells. Proc Natl Acad Sci USA *107*: 6340-6345, 2010.

Received February 8, 2011
Revised March 31, 2011
Accepted March 31, 2011