



Proteomic analysis of a highly metastatic gastric cancer cell line using two-dimensional differential gel electrophoresis

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Abstract. Stomach cancer is still a major cause of death in Asian people despite a complete cure after the resection of early cancers, mainly because peritoneal dissemination is difficult to treat. In the present study, we used two-dimensional differential gel electrophoresis (2-D DIGE) to identify specific proteins differentially expressed between a highly metastatic stomach cancer cell line MKN-45-P and its parental cell line MKN-45. We detected 27 protein spots in at least 2 of 3 experiments which showed statistically significant differences in abundance. All 27 protein spots were identified using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) and database-searching software. A proteomic analysis revealed 13 different proteins with some isoforms sharing different biochemical characteristics, and that 8 proteins were up-regulated, and 5 were down-regulated. The 13 proteins were mainly involved in protein synthesis (transfer RNA synthetase), metabolism (flavoprotein subunit, pyruvate kinase, adenylate kinase), receptor and signal transduction (annexins I and A2), the cytoskeleton (keratin 5, cytokeratin 8) and cell cycling (ts11). These results suggested that a proteomic approach including 2-D DIGE would be an efficient way to identify the proteins responsible for specific biological functions. Moreover, these observations might be novel findings leading to the prediction of postoperative peritoneal recurrence.

Introduction

Gastric cancer is still the leading cause of cancer-related deaths in Japan and other Asian countries. Notably, the treatment of peritoneal dissemination as a cancerous recurrence

after surgical resection is very difficult. However, if specific markers for predicting peritoneal recurrence were to be identified, peritoneal dissemination could be detected early and perhaps even eradicated. The phenomenon of metastasis to the peritoneum is thought to require the involvement of many different genes in multiple steps (1). Some specific genes including those for adhesion molecules and apoptosis-related proteins have been reported to be involved in intra-peritoneal metastasis (2-4), but the precise mechanism of peritoneal dissemination remains to be clarified.

Genetic analyses of peritoneal dissemination-related gastric cancer cell lines or clinical specimens have been performed using DNA microarray technology (5,6). So far, many gene candidates considered to be involved in peritoneal dissemination have been reported. However, at present there are no genuine candidates for molecules playing a critical role in metastasis to the peritoneum because of the high variability in the results depending on the type of sample.

Two-dimensional differential gel electrophoresis (2-D DIGE) coupled with matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) has been applied to the analysis of comparative protein expression in different samples in the search for novel biomarkers (7,8). The 2-D DIGE technology is often used for a direct comparison of changes in protein abundance across multiple samples simultaneously on the same gel with a statistical analysis using the DeCyder software. The proteomic approach with 2-D DIGE is less frequently utilized to study gastric cancer (9,10). Jang *et al* (9) reported changes in protein expression in 18 stomach adenocarcinoma tissues compared with surrounding normal tissues.

In the present study, we focused on the highly metastatic gastric cancer cell line MKN-45-P, derived from MKN-45 cells and compared the protein expression between the metastatic clone and parental cells by using 2D-DIGE coupled with MALDI-TOF M/S. The MKN-45-P cell line was previously cloned *in vivo* by repeating intraperitoneal metastasis and demonstrated to have marked invasive activity *in vitro* (1,4). We eventually identified 13 kinds of proteins as being differentially expressed in the highly metastatic clone, MKN-45-P.

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Materials and methods

Cell culture. The human gastric cancer cell line MKN-45 and a highly metastatic derivative, MKN-45-P, were established previously. Both cell lines were cultured with 5% FBS/RPMI-1640 medium.

Protein extraction. When the cells reached 80-90% confluence, they were harvested using cell dissociation buffer and washed twice with PBS(-). The cell pellets were incubated in a lysis buffer (50 mM Tris-HCl, pH 8.5, 7 M urea, 2 M thiourea, and 4% CHAPS). The samples were sonicated at 4°C and centrifuged at 15,000 rpm for 30 min. Then the supernatant containing the cell proteins was collected, and impurities were removed with a 2-D Clean-Up kit (GE Healthcare Amersham Biosciences, Uppsala, Sweden). The pH of the protein samples was adjusted to 8.0-9.0. The protein concentration was measured with a Quick Start protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and adjusted to 5 mg/ml with lysis buffer.

CyDye labeling of proteins. The proteins were labeled with fluorescent cyanine dyes developed for 2-D DIGE (GE Healthcare Amersham Biosciences) following the manufacturer's directions. Protein samples (50 µg) were labeled with 400 pmol of amine reactive cyanine dyes. The internal control mixture was labeled with the fluorescent dye Cy2. The other samples were labeled with Cy3 or Cy5. The labeling reaction was conducted at 4 °C in the dark for 30 min. The reaction was quenched with 10 nmol lysine. The samples labeled with each Cy were mixed, and equal volumes of 2x sample buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, and 2% IPG buffer (GE Healthcare Amersham Biosciences), and 2.4% Destreak reagent (GE Healthcare Amersham Biosciences) were added to the mixture. The volume of the labeled protein mixture was adjusted to 450 µl by adding rehydration buffer (GE Healthcare Amersham Biosciences) containing 1% IPG buffer.

Experimental design. The methodology established by Alban *et al* was used. MKN-45-derived proteins (50 µg) were labeled with Cy3, and MKN-45-P-derived proteins (50 µg) were labeled with Cy5. A mixture of the proteins of both cells (MKN-45+MKN-45-P: 25 µg+25 µg) was labeled with Cy2. The sample for the identification of proteins was made separately. A protein mixture (400 µg) of MKN-45 and MIN-45-P was adjusted to 450 µl by adding rehydration buffer (GE Healthcare Amersham Biosciences) containing 1% IPG buffer. All samples were separated by 2-DE as detailed in the next section (labeled samples: n=5, sample for identification: n=1). The experiment was independently repeated three times for reproducibility.

Protein separation by 2-DE. All samples were separated by 2-DE. One-dimensional separation was performed according to pI, and two-dimensional separation was done according to molecular weight. The IPG strips (24 cm length, pI range between 3.0 and 11; GE Healthcare Amersham Biosciences) were rehydrated with the CyDye-labeled protein mixture for 12 h at 20°C and 30 V using the IPGphor system (GE

Healthcare Amersham Biosciences). Isoelectric focusing was performed at a total of 45.5 kVh at 20°C. The cysteine sulphydryls were reduced and carbamidomethylated while the proteins were equilibrated in the two-dimensional loading buffer (glycerol, SDS, urea, trace BPB) supplemented with 1% DTT for 15 min at room temperature, followed by 2.5% iodoacetamide in fresh equilibration buffer for an additional 15 min at room temperature. After equilibration, the IPG gels were applied onto a 24 cm acrylamide gel (10-12.5%). SDS-PAGE was performed at 2.5 W/gel for 30 min and then at 30 W/gel (Max. 100 W) for 4-5 h at 15°C. All electrophoretic procedures were performed in the dark.

Gel imaging. Labeled sample gels were scanned at appropriate wavelengths for Cy2, Cy3 and Cy5 using a Typhoon 9410 (GE Healthcare Amersham Biosciences). Cy2 images were scanned using a 488-nm laser and a 520-nm band pass (BP) 40 emission filter. Cy3 images were scanned using a 532-nm laser and a 580-nm BP30 emission filter. Cy5 images were scanned using a 633-nm laser and 670-nm BP30 emission filter. The gel for protein identification was fixed in 10% methanol, 7% acetic acid for 2 h, and then incubated in Sypro Ruby in the dark overnight. Sypro Ruby was acquired on the same imager using a 457-nm laser and 610-nm BP 30 emission filter. All gels were scanned at a resolution of 100 µm.

Spot detection and statistical analysis. The spots were detected and quantified with the DIA mode in DeCyder software (GE Healthcare Amersham Biosciences). The estimated number of spots for the detection procedure was set at 4,000. As recommended, an exclusion filter was used to remove spots greater than the parameter to reject dust particles etc. The intensity of the spots in the Cy3 and Cy5 image were normalized to that of the Cy2 image in the same gel. Any differences observed between the gels were due to electrophoretic artifacts, and could be compensated for by normalization. Next, the BVA mode in DeCyder software was used to simultaneously match all 15 protein-spot maps from the five gels, and using the Cy3: Cy2 and Cy5: Cy2 DIA ratios, calculated average abundance changes and Student's t-test p-values. Only those spots with over 2-fold changes in volume after normalization between Cy3 and Cy5 were defined as spots of interest.

In-gel digestion and peptide purification. DeCyder software was used to record the positions of the spots of interest as a text file, and an automated spot collector, SpotPicker (GE Healthcare Amersham Biosciences), was used to pick the spots. In-gel digestion of the chosen spots was carried out to generate tryptic peptides. Gel pieces were washed three times with 100 mM ammonium bicarbonate (NH₄HCO₃) for 20 min, dehydrated with acetonitrile (AN) for 10 min, and dried up thoroughly. Mass spectrometry grade trypsin (Promega, Madison, WI, USA) of 125 ng in 0.1% RapiGest (Waters, Milford, MA, USA)/100 mM NH₄HCO₃ was added to the gel pieces, and incubated at 37°C for 1 h. HCl was added to the protein digested solution, to resolve the RapiGest remaining, and incubated at 37°C for 45 min. The supernatants were removed and transferred to separate tubes, and gel pieces were re-extracted twice with 80% AN/1% trifluoroacetic acid

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 Proteins identified in the 2-D DIGE analysis.

No.	Proteins	Av. Ratio	T-test	Score	Mr	pI
1	Interferon-induced Mx protein	3.72	9.80E-11	123	75530	5.65
2	Interferon-induced Mx protein	4.55	1.50E-08	140	75530	5.65
3	Glycyl-tRNA synthetase	2.30	2.10E-09	178	77463	5.88
4	Flavoprotein subunit of complex II	2.23	4.10E-07	141	72645	7.06
5	Ts11 cell cycle protein	4.99	4.60E-07	228	62291	6.71
6	Pyruvate kinase	-2.05	2.10E-04	174	57841	7.58
7	Pyruvate kinase	-2.02	1.30E-07	176	57841	7.58
8	Tyrosyl-tRNA synthetase	2.58	1.50E-09	198	59106	6.61
9	Keratin 5	2.04	2.40E-06	255	62340	-
10	Transfer RNA-Trp synthetase	3.04	1.50E-07	78	53054	5.73
11	Cytokeratin 8 (279 AA)	-2.08	1.20E-07	274	30840	4.91
12	Cytokeratin 8	-2.11	1.10E-07	127	30840	4.91
13	Cytokeratin 8 (279 AA)	-2.31	3.90E-06	234	30840	4.91
14	Cytokeratin 8 (279 AA)	-2.04	1.90E-06	177	30840	4.91
15	Cytokeratin 8 (279 AA)	-2.66	3.70E-09	205	30840	4.91
16	Dihydrodiol dehydrogenase isoform DD1	-2.13	2.09E-06	122	34771	8.1
17	Annexin I	-2.01	3.60E-04	69	35018	7.77
18	Annexin A2, isoform 2	-2.02	7.70E-04	239	38552	7.57
19	Annexin A2, isoform 2	-2.51	2.30E-05	277	38552	7.57
20	Annexin A2, isoform 2	-2.05	1.60E-05	95	38552	7.57
21	Annexin A2, isoform 2	-2.01	1.90E-10	244	38594	7.57
22	Annexin A2, isoform 2	-2.63	2.80E-06	240	38552	7.57
23	Annexin A2, isoform 2	-2.17	8.20E-09	274	38552	7.57
24	Annexin A2, isoform 2	-2.14	1.60E-05	224	38552	7.57
25	Adenylate kinase 2	2.05	2.00E-10	138	25598	-
26	Carbonic anhydrase II	-2.10	8.20E-08	161	29200	6.87
27	Cytokeratin 8 (279 AA)	-3.21	4.10E-06	257	30840	4.91

The numbers in the table correspond with spot nos. in Fig. 3. Ratio, the difference in protein expression between MKN-45-P and MKN-45; Mr and pI, molecular weight and isoelectric point, respectively.

(TFA) for 30 min. The extracted solution was vacuum dried in a Speedvac (TOMY, Tokyo, Japan), and peptides were resuspended in 5 μ l of 0.1% TFA. C18 silica desalting/concentration microcolumns (ZipTip; Millipore, Billerica, MA, USA) were used to purify and concentrate peptides before mass spectrometry.

Protein identification. The purified peptides were analyzed by MALDI-TOF MS using a 4700 proteomics analyzer (Applied Biosystems). The peptides were mixed with α -cyano-4-hydroxy-trans-cinnamic acid (Sigma, St. Louis, MO, USA) and spotted onto target plates. Peptide mass maps were acquired in the reflectron mode. The background and trypsin-derived peaks were subtracted by using the control gel. Ions specific for each sample were then used to interrogate human sequences entered in the NCBI database using the MASCOT (www.matrixscience.com) database search algorithms. Searches were performed without constraining protein molecular weight or the isoelectric point, and allowed

for the carbamidomethylation of cysteine, partial oxidation of methionine residues, and one missed trypsin cleavage. Highest confidence identification has statistically significant search scores.

Results

Protein expression was compared between a gastric cancer cell line (MKN-45) and a highly metastatic cell line (MKN-45-P) using 2D-DIGE technology with a mixed-sample internal standard (Fig. 1). For gel analysis, MKN-45-derived protein and the MKN-45-P-derived protein were labeled with Cy3 and Cy5 respectively, and an equal mixture of the protein of MKN-45 and MKN-45-P was labeled with Cy2 (internal control). All fluorescence-labeled proteins were mixed. After 2D gel electrophoresis (2DE), the Cy2, Cy3, and Cy5 channels were individually imaged from five gels using mutually exclusive excitation and emission wavelengths. A two-color merged representative gel image is shown in

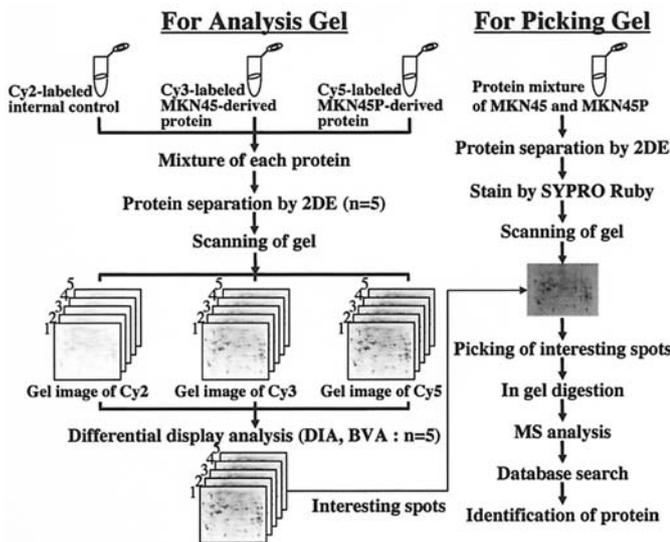


Figure 1. Methods of 2D-DIGE. A mixture of each fluorescent labeled sample was separated by 2DE, and then the gel was scanned at appropriate wavelengths for statistical analysis. As a result of differential display analysis, gel spots of interest were chosen from the picking gel, and proteins were identified using mass spectrometry.

Fig. 2A (Cy3 and Cy5). The green spots indicate that the protein expression was greater in MKN-45 than MKN-45-P. The red spots indicate that the protein expression was less in MKN-45 than MKN-45-P. Next, the images were analyzed using DeCyder software. The number of spots in the five gels was detected with the DIA mode, and those with differential expression were detected with the BVA mode statistically as described above ($n=5$, $p<0.01$). Each pair of protein spots generated with Cy3 and Cy5 were converted into 3-D representations and displayed to show the relative peak volumes, height and area of each spot. A 3-D simulation allowed an objective inspection of spots corresponding to proteins from Cy3 and Cy5. In spot no. 8, the protein expression of MKN-45-P was 2.58-fold that of MKN-45 (Fig. 2B). In spot no. 26, the protein expression of MKN-45-P was 1/2.1 that of MKN-45 (Fig. 2C). The number of spot that changed more than 2-fold was 28 in the first trial, 42 in the second trial, and 42 in the third trial. There were 27 proteins detected more than twice in three attempts.

For the picking gel, an equal mixture of the protein from both cells was separated by 2DE. Then the gel was stained with Sypro Ruby, and scanned at the appropriate wavelength. After gel scanning, the picking gel was matched with the analysis gel. Matched-spots of interest (27 spots) are shown in Fig. 3. Of the 27 spots, 9 were increased and 18 decreased. After the 27 spots were picked by a SpotPicker machine, in-gel digestion was carried out. The extracted peptides were purified by ZipTip and analyzed by mass spectrometry. Mass spectra of spots no. 8 and 26 are shown in Fig. 4A and B.

Discussion

Peritoneal dissemination is suspected to be triggered by multiple genes involved in many aspects of cancer cell invasion and metastasis. However, a proteomic approach

investigating the mechanism of peritoneal dissemination using a specific metastatic model has yet to be reported. Our group previously established a highly metastatic cell line, MKN-45-P, with an almost 100% incidence of peritoneal dissemination, through a serial intraperitoneal passage (1). In the present study, we focused on identifying the proteins expressed differentially in the highly metastatic cell line compared with the parental MKN-45 cell line, in order to analyze the specific parameters which can predict the possibility of peritoneal dissemination. The proteomic analysis revealed 13 different proteins identified from 27 protein spots, 8 of which were up-regulated, and 5 down-regulated.

The up-regulated proteins include the IFN-induced Mx protein, Gly-, Tyr-, Trp-tRNA synthetase, the flavoprotein subunit of complex II, the ts11 cell cycle protein, keratin 5, and adenylate kinase. The detection of interferon (IFN)-induced Mx protein (p78) and tryptophanyl-tRNA synthetase (IFP53) implied that the MKN-45-P cell line was exposed to IFN-like cytokines and regulated to express metastatic biological behavior. With regard to the IFN-inducible protein family, p21 and p27 were reported to be significant prognostic factors in gastric carcinoma patients (11,12). IFP53 was demonstrated to mediate the binding of alpha-IFN activation factors to the gamma-IFN activation site (GAS) (13). There is no significant association of IFN signal activation with a metastatic feature of the MKN-45-P cell line, however, a mechanism stimulating gastric cancer cell adhesion to peritoneal cells might exist, as we reported previously that inflammatory cytokines like IL-8 or other cytokines enhanced the attachment of MKN-45-P cells (4). There is accumulating evidence that IL-8 is the cytokine involved in tumor cell invasion and metastasis in many types of solid tumors (14,15), and even in gastric cancer patients, IL-8 and VEGF expression in the tumor are good candidates for indicators of poor prognosis (16). Considering that MKN-45-P cells produced more IL-8 in the supernatant than MKN-45 cells (4), a contribution of IL-8 to the metastatic phenotype of MKN-45-P cells is possible. The correlation of IL-8 to the invasive and metastatic activity of MKN-45-P should be investigated. The expressions of human tyrosyl- and tryptophanyl-tRNA synthetase were upregulated more than 2-fold. Fragments of the closely related human tyrosyl- and tryptophanyl-tRNA synthetases were found to be active in angiogenesis signaling pathways, i.e. the fragments of tyrosyl-tRNA synthetase stimulate angiogenesis, whereas those of tryptophanyl-tRNA synthetase inhibit angiogenesis (17,18). There might be a coordinated balance of opposing activities with angiogenesis between the two tRNA synthetases. Unfortunately, the link between the angiogenic activity and metastatic potential of the MKN-45-P cell line is not clarified. Cell cycle control protein ts11 encoding asparagine synthetase was found to be up-regulated 5-fold, which is reasonable in terms of accelerating G1 progression resulting in growth stimulation. Additionally, relapsed leukemic cells tend to express high levels of asparagine synthetase and show drug-resistance, especially L-asparaginase-resistance and metastatic characteristics (19). There is accumulating evidence that keratin and cytokeratin are specifically involved in peritoneal recurrence or lymph

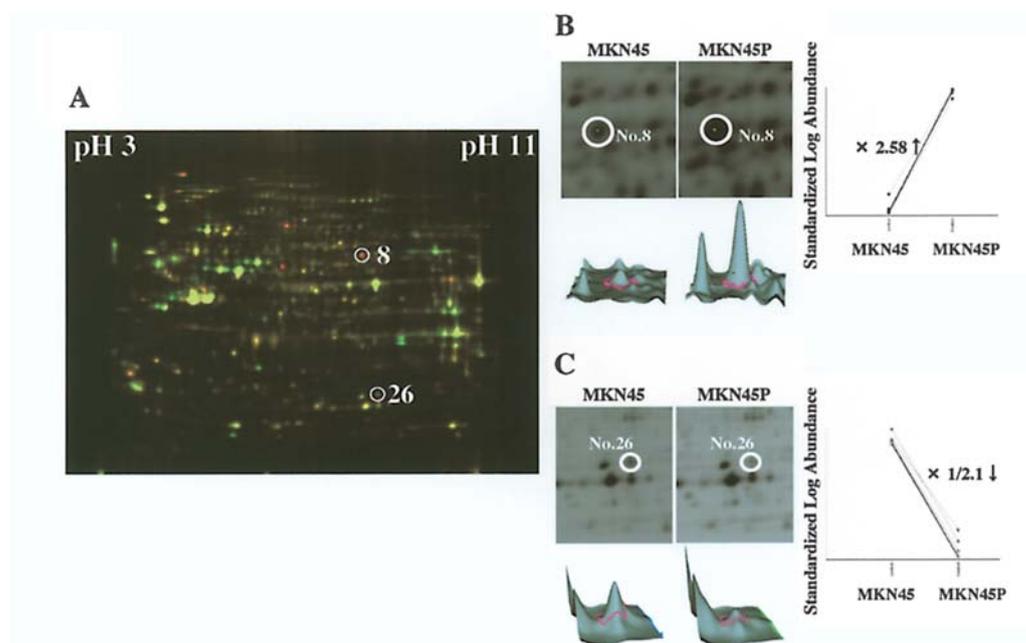


Figure 2. 2D-DIGE gel image and 3D display. (A) Two-color merged 2D-DIGE gel image. The green spots show that the protein expression of MKN-45 was greater than that of MKN-45-P. The red spots show that the protein expression of MKN-45 was less than that of MKN-45-P. The pI ranges from 3 to 11 (left to right). (B and C) The spot where the volume change was extreme between MKN-45 and MKN-45-P was removed, and a graph of volume ratio and a 3D image are shown. Spot no. 8 is shown in B, and spot no. 18 is shown in C.

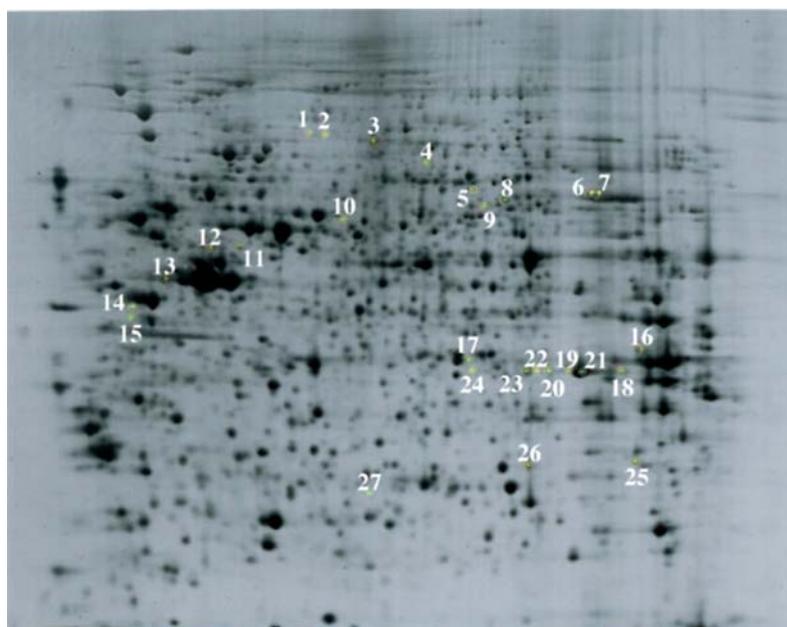


Figure 3. Gel image of Sypro Ruby staining. All annotated spots of the Sypro Ruby staining gel indicated protein spots of interest. There are 27 spots which have >2-fold difference between MKN-45 and MKN-45-P ($p < 0.01$, three times by $n=5$). Nine spots had an increased protein expression in MKN-45-P compared with MKN-45 (Nos. 1-5, 8-10, 25). Eighteen spots had a decreased protein expression in MKN-45-P compared with MKN-45 (Nos. 6, 7, 11-24, 26, 27).

node metastasis in gastric carcinoma patients. Sakakura *et al* reported that gene expression analysis using a gastric cancer cell line established from malignant ascites revealed up-regulation of the keratin 7, 8 and 14 genes (6). Additionally, a proteomic approach analyzing protein differential expression between human stomach adenocarcinoma tissue and paired surrounding normal tissue showed that keratin was one of the differentially up-regulated proteins in stomach cancer tissue (9,10). Elevated levels of keratin were considered to be

involved in the promotion of adhesion of free cancer cells to the peritoneal epithelium and the formation of cancer nests. Meanwhile, our study revealed that cytokeratin 8 was down-regulated in MKN-45-P cells. It was reported that CEA and cytokeratin 20 may be sensitive markers for the detection of micrometastasis to the peritoneum and the prediction of early peritoneal dissemination (20). The association of the down-regulation of cytokeratin 8 with metastatic ability is not clear at present.

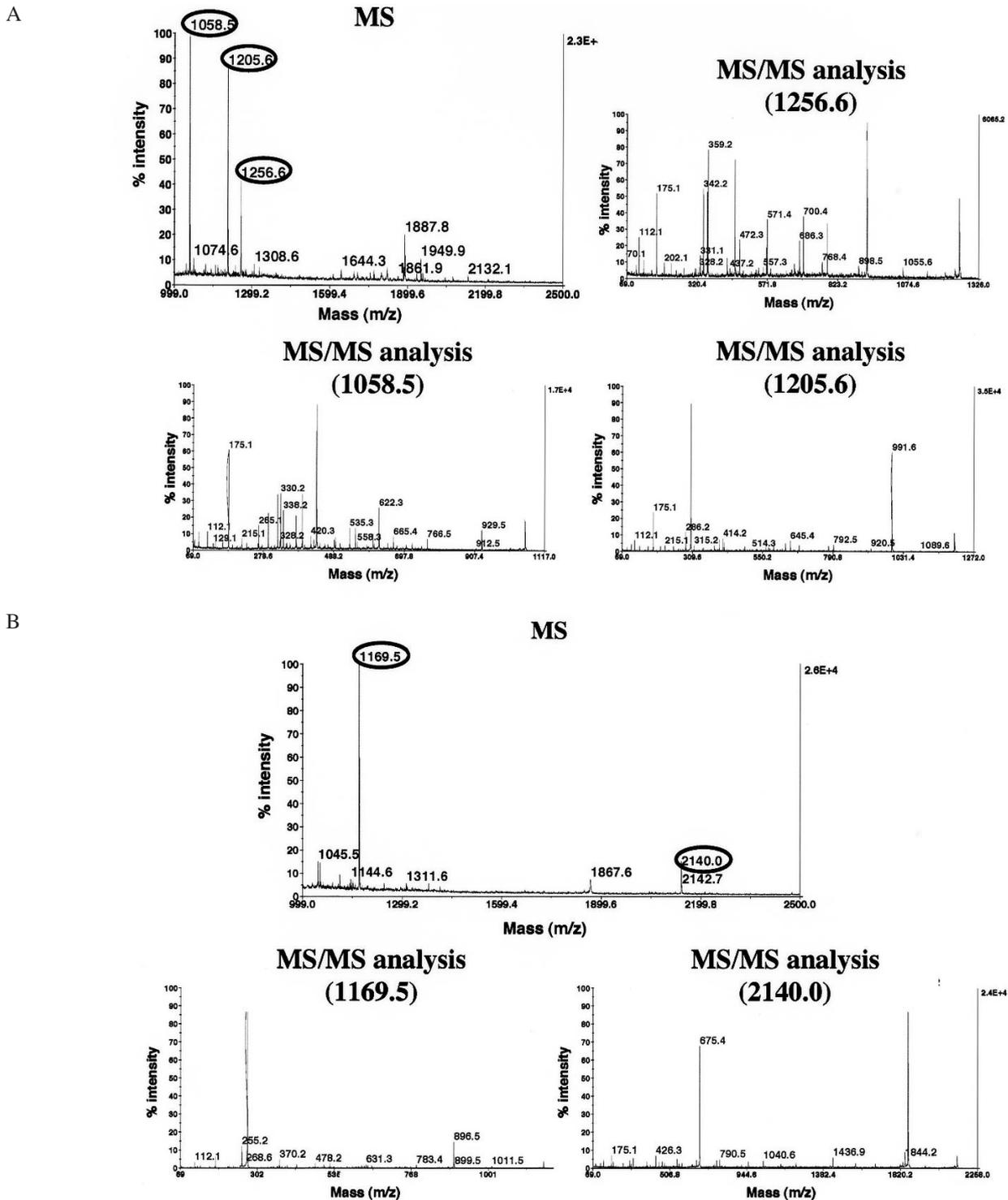


Figure 4. Mass spectrometry of the peptides derived from spots no. 8 and no. 26. Each spot was removed from the gel, and in-gel digestion was performed using trypsin. The digested peptides were purified and concentrated by ZipTip, and then analyzed by mass spectrometry using a 4700 proteomics analyzer. The results of MS and MS/MS analysis are shown. (A) The spectrum of spot no. 8. (B) The spectrum of spot no. 26.

The down-regulated proteins include pyruvate kinase, cytokeratin 8, didyrodioil dehydrogenase, annexin I, and carbonic anhydrase II. Pyruvate kinase M2 (PK-M2) is a glycolytic isoenzyme predominantly found in proliferating tumor cells. PK-M2 has been utilized as a novel biomarker in gastric cancer, colorectal cancer and melanoma (21). In our study, PK-M2 was found to be down-regulated in MKN-45-P cells. Yoo *et al* (22) reported that PK-M2 expression is

clearly down-regulated in cisplatin-resistant human gastric cancer cell lines, which means that the MKN-45-P cell line shows more malignant and drug-resistant features. Annexins belong to a family of calcium-dependent phospholipids-binding proteins. The increased expression of annexin I and II is known to be associated with poor prognosis in cases of gastric or pancreatic cancer (23). However, a genetic analysis using DNA microarray demonstrated that annexin A1

SPANDIDOS intracellularly is down-regulated in breast cancer and neck cancer, which suggests that annexin A1 is an effective differentiation marker for pathological grading (24). Our study showed that annexin A2 was down-regulated in the MKN-45-P cell line. The biological relation of annexin A2 down-regulation with the susceptibility to metastasis to the peritoneum should be investigated. Carbonic anhydrase type II (CAII) is considered to be down-regulated in non-small cell lung cancer and advanced colon cancers and might promote tumor cell invasion or metastasis (25,26). The up-regulated expression of CAII is also demonstrated to be involved in resistance to anticancer drugs (27). It is reasonable that the CAII expression was down-regulated in MKN-45-P cells, however, the specific relation of the down-regulation with the metastatic features of the cells is not clear.

Summarizing the profiling of differentially regulated protein expression according to proteomic analysis of the MKN-45-P cell line, 2 categories of protein profiling exist. One category is the effect of inflammatory cytokines on gastric cancer cells; some cytokines such as interferons or IL-8 stimulated cancer cells, which were promoted to adhere to peritoneal cells and to produce proangiogenic factors resulting in metastatic capability. The other is the mechanism for a more malignant phenotype of gastric cancer cells; down-regulation of CAII and PK, and up-regulation of ts11 cell cycle protein, which make gastric cancer cells more invasive and metastatic. In the near future, aiming at the confirmation of these observations, further proteomic analysis using many gastric cancer tissue specimens will be needed.

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