Two-dimensional differential in-gel electrophoresis for identification of gastric cancer-specific protein markers

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Abstract. Gastric cancer is the second most common fatal malignancy in the world. Proteomics studies of clinical tumor samples have led to the identification of specific protein markers of gastric cancer detection and better understanding the carcinogenesis of gastric cancer. Gastric cancer tissue of epithelial origin and adjacent normal mucosa were examined in pair by fluorescence 2-D differential in-gel electrophoresis proteomics analysis utilizing 2-D PAGE protein separation. Intensity changes of 33 spots were detected with statistical significance. Twenty-two out of the 33 spots were identified by MALDI-TOF MS or MS/MS. Of the 9 up-regulated proteins, 7 were identified, including heat shock protein 60 (HSP60), mutant desmin, effector cell proteinase receptor 1 splice form 1b, hypothetical protein, unnamed protein product, and manganese superoxide dismutase (MnSOD), a protein similar to α-actin. Of the 20 down-regulated proteins, 16 were identified, including selenium binding protein 1, fibrinogen γ, HSP27, tubulin α 6, zinc finger protein 160, prostaglandin F synthase, and eukaryotic translation elongation factor 1 α 1. Our results suggest that MnSOD may be a potential serum marker for molecular diagnosis of gastric carcinoma, and DIGE is a useful technique for screening differentially expressed proteins in cancer tissues.

Introduction

Gastric cancer is the second most common fatal malignancy in the world (1). It causes more than 750,000 deaths annually (2). About 800,000 new cases were diagnosed worldwide in 2000, with Asia accounting for 75% of these cases (3). In China, the mean annual mortality is estimated to be as high as 16 per 100,000 population, accounting for the leading cause of deaths among malignant tumors (4). The high mortality rate from gastric cancer is due to delayed detection and surgical resection at advanced stages of the disease (5). As people are not typically screened for gastric cancer, most cases are diagnosed at a time when the disease is well established. Therefore, early detection and better understanding of the gastric cancer are important for proper control of gastric cancer.

Proteomics studies of clinical tumor samples have led to the identification of cancer-specific protein markers, which provide a basis for developing new methods for early diagnosis and detection and clues to understand the molecular characterization of cancer progression (6-8). Several groups have earlier carried out proteomic studies of gastric cancer (9,10). A number of proteins with altered expression levels were identified using 2D electrophoresis followed by protein identification using mass spectrometry (11,12). The state of the art 2D gel system can be loaded with a few milligrams of protein and separate thousands of protein spots (13). Although the technique has been widely used and successfully applied in a variety of biological systems, several technical limitations exist. Subtle changes in experimental conditions would render it impossible to fully duplicate the protein expression patterns on a single 2D gel, and make it difficult to find protein changes between gels and quantify changes in protein expression.

Although a comparison of protein expression profiles from regular 2D gel electrophoresis can be carried out with the assistance of various software programs, it typically requires some computerized justification of 2D gel images so that two images can be superimposed and compared. These difficulties limit the speed and accuracy of quantitation of protein spots in normal 2D gel electrophoresis.

The fluorescence differential in-gel electrophoresis (DIGE) technique recently introduced by Amersham Biosciences, Inc., is aimed at improving reproducibility. The concept of DIGE has been described (14), where reactive dyes are used to label protein samples. Several fluorophores are available, allowing multiplexing of samples from two or three different sources on the same gel (e.g, normal surface mucosa, metaplasia or adenocarcinoma). The gel is scanned to detect each dye separately, and an abundance ratio is calculated for each protein, reflecting differences in its expression in different samples (14,15). AS DIGE eliminates gel-to-gel variability, it provides abundance ratios that are much more accurate than can be obtained by matching and quantifying features on different gels. Recently, this technique was used (16) for proteomics study of mouse liver homogenates to examine the molecular basis of hepatotoxin, N-acetyl-p-aminophenol. It was demonstrated that the DIGE technology had adequate sensitivity and reproducibility and a wide dynamic range.

In this study, we applied the DIGE technique for the identification of gastric cancer-specific protein markers that might develop new therapeutic targets of the disease, to understand the mechanism involved in the process of gastric cancer or to find biomarkers of gastric cancer.

Materials and methods

Sample preparation. Three human stomach tissue samples were replicated from freshly isolated resection materials of gastric cancer patients from Shanghai Changzheng Hospital of the Second Military Medical University, China. Resections were examined by a pathologist and the margin tissue samples were replicated from non-cancerous regions, 5 cm apart from the primary tumor, and verified to be without cancer cells by pathology. The necrotic tissues were excluded as much as possible. All tissues were immediately snap-frozen in liquid nitrogen after being washed with isotonic Na chloride three times. The clinicopathological characteristics are described in Table I. None of the patients received antineoplastic therapy prior to surgery. The human tissues were confirmed to contain no tumor cells by histopathologic evaluation. The experimental protocol was approved by the Health Human Research Ethics Committee of Changzheng Hospital.

Two-hundred milligrams of the sample was ground by liquid nitrogen and solubilized in 300 μl modified lysis buffer (7 M urea, 2 M thiourea and 4% CHAPS) together with 5 μl/ml Halt Protease Inhibitor Cocktail (EDTA-free). The sample was vortexed and incubated for 40 min at room temperature, followed by centrifugation at 10000 x g for 1.5 h at 4˚C. Protein concentration was determined using a commercial Bradford reagent (Bio-Rad) using bovine γ-globulin as the standard, and then the samples were stored at -80˚C until use.

Table I. Clinicopathological characteristics of 3 stomach cancer patients.
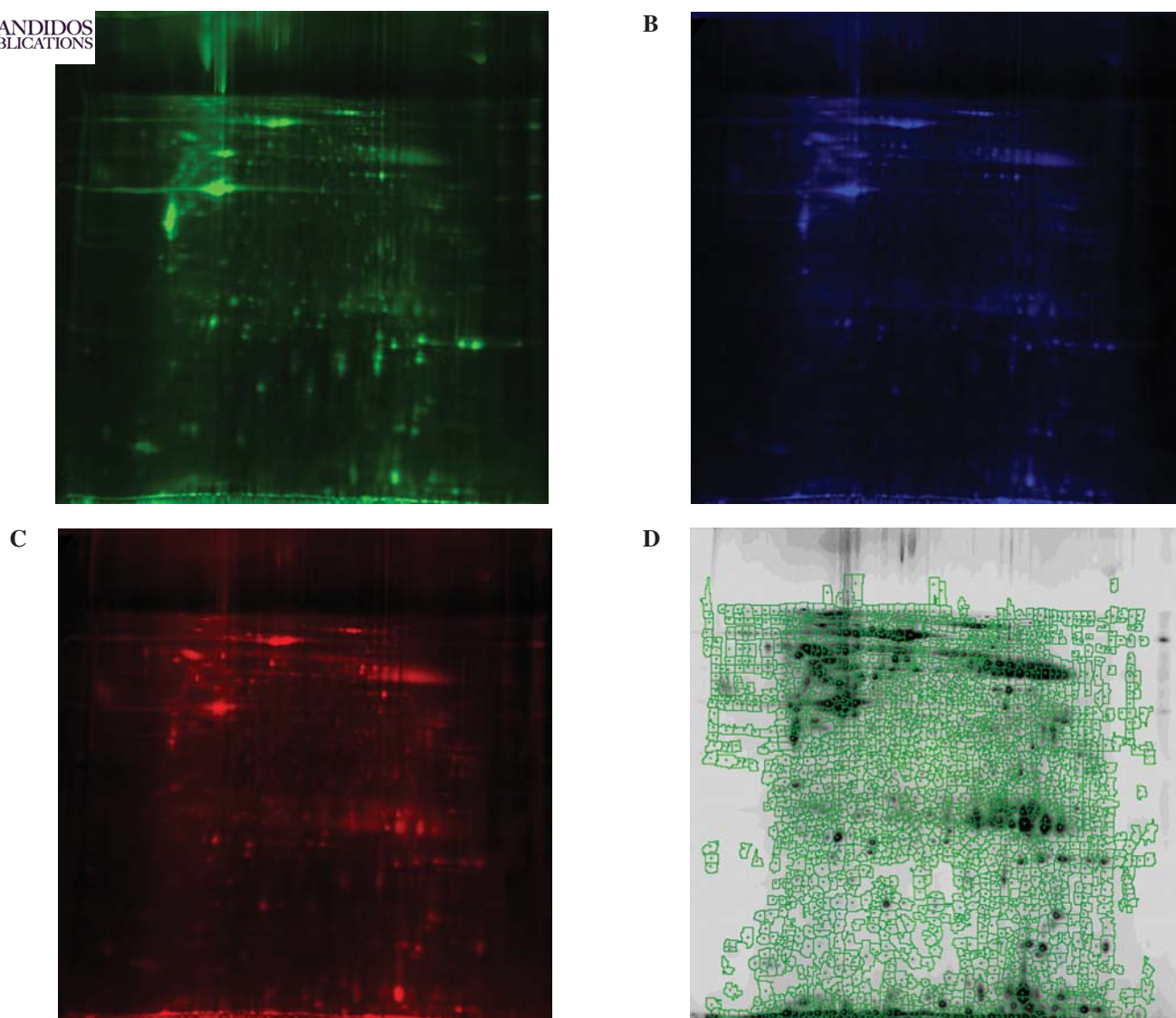
Age (years)	Sex	Cell type	Stage	Borrmann type
80	M	Poor	IIIb	III
71	M	Poor	IIIb	II
50	F	Poor	IIIb	III

Table II. Experiment design of different fluorescent dye labeling for internal standard (Cy2) and samples 1, 2 and 3 (Cy3 or Cy5).

Gel	Cy2	Cy3	Cy5
1	Internal standard	Normal 1	Cancer 2
2	Internal standard	Cancer 1	Normal 3
3	Internal standard	Normal 2	Cancer 3

Sample labeling. The experiment was designed according to the manufacturer's instructions (GE Healthcare) (Table II). An internal standard pool generated by combining equal amounts of extracts from all 3 pairs of tumor and normal mucosa tissues (totally 6 samples) was labeled with Cy2 fluorescent dye. It is able to minimize gel-to-gel variation by allowing the inclusion of an internal standard within each gel. All six samples evenly distributed between CyDye DIGE fluorescence Cy3 and Cy5 to minimize the variation between fluorescence. Fifty micrograms of each tumor and paired normal mucosa protein extracts were minimally labeled with Cy3 or Cy5 fluorescent dyes (400 pmol fluorescent 50 μg protein extracts). Labeling reaction was performed at 4˚C for 30 min and quenched with 10 μl lysine for 10 min on ice in the dark. Equal amounts (50 μg) of quenched Cy3 or Cy5 labeled samples from each patient, together with the aliquoted 50 μg Cy2-labeled internal standard pool (described above), were focused using IPG strips (Ready Strip, Bio-Rad, pH 4-7, 13 cm) in the protein IEF Cell (Bio-Rad), with the addition of DeStreak Reagent (GE Healthcare). The IPG strips were equilibrated with equilibration buffers containing 2% DTT and 2.5% iodoacetamide for 10 min each sequentially. For the first electrophoresis, an electric potential of 30 V was applied for 12 h, 500 V for 1 h, 1000 V for 1 h, 8000 V for 1 h, and 500 V for 4 h. The second 12% SDS-PAGE was then carried out for all 3 gels simultaneously using Ettan DALT electrophoresis system.

2D DIGE and image analysis. Labeled proteins in each gel were visualized using a Typhoon 9410™ (GE Healthcare) fluorescence scanner at 488/600 nm for Cy2, 532/580 nm for Cy3 and 633/520 nm for Cy5 dyes. Images were analyzed with the help of the DeCyder™ software platform v 6.5 (GE Healthcare, USA). Gel image pairs were processed by the DeCyder™-DIA (Differential In-gel Analysis) software module to co-detect and differentially quantify the protein spots in the images, taking the internal standard

Figures 1. The scan results of one gel and the spot map. Each gel contained the same pooled internal standard sample minimal dye labelled with CyDye DIGE Fluor Cy2 and two protein samples labeled with fluor Cy3 or Cy5 minimal dyes. (A) Identification of differentially expressed proteins, fluorescence at appropriate wavelengths. False color was added to the two-dimensional gel for illustration. (D) Total number of proteins identified following two-dimensional electrophoresis.

sample as a reference to normalize the data, so the rest of the normalized spot maps could be compared among them. At the second stage, the DeCyder™-BVA (Biological Variation Analysis) software module was applied. BVA performs a gel-to-gel matching of the internal standard spot maps from each gel. Comparison between the different experimental groups and the control group was tested by Student's t-test (p≤0.05).

Mass spectrometry analysis and database searching. A replicate gel was made with 1 mg samples, and stained by Coomassie Brilliant Blue and then matched with the DIGE gel maps. The matched proteins spots were excised from gels and cleaved with trypsin by in-gel digestion. The peptide extract from each tryptic digest was crystallized in 0.6 μl matrix solution (50% acetonitrile + 0.1% trifluoroacetic acid + 5 mg/ml CHCA) on the matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) target plate. All MALDI-TOF spectra were externally

and internally calibrated for the correction of masses. The Peptide Mass Fingerprint produced by the MALDI-TOF MS were compared with the published databases of NCBInr 20060526 (3654802 sequences; 1256120150 residues) using the MS-Fit module in Mascot (Matrix Science; http://www.matrixscience.com). A mass tolerance of 100 ppm was used for the peptide search. Protein scores greater than 64 are significant (p<0.05). When searched using MS/MS maps Individual ions scores >36 indicate identity or extensive homology (p<0.05). Two proteins that had the Peptide Mass Fingerprint, but were not matched in the database, were identified by PMF+MS/MS.

Results

2D gel separation of proteins. Three gels were scanned by different lasers, Cy2 dye for blue laser (488 nm), Cy3 dye for green laser (532 nm), and Cy5 dye for red laser (633 nm) as shown in Fig. 1A-C. In total, more than one thousand protein
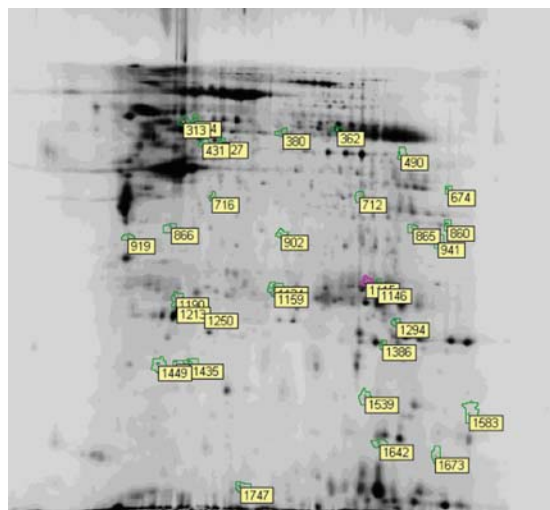
Figure 2. A representative 2-DE map of proteins from gastric cancers and the ID of the up- and down-regulated proteins. Proteins (50 μg) extracted from human gastric cancer tissue were separated on pH 4-7 IPG strip in the first dimension and 12% acrylamide SDS-PAGE in the second dimension. The marked protein spots (green) identified by MALDI-TOF MS and spot 1115, 1386 (red) identified by MS/MS analyses are indicated numerically and shown in Table III.
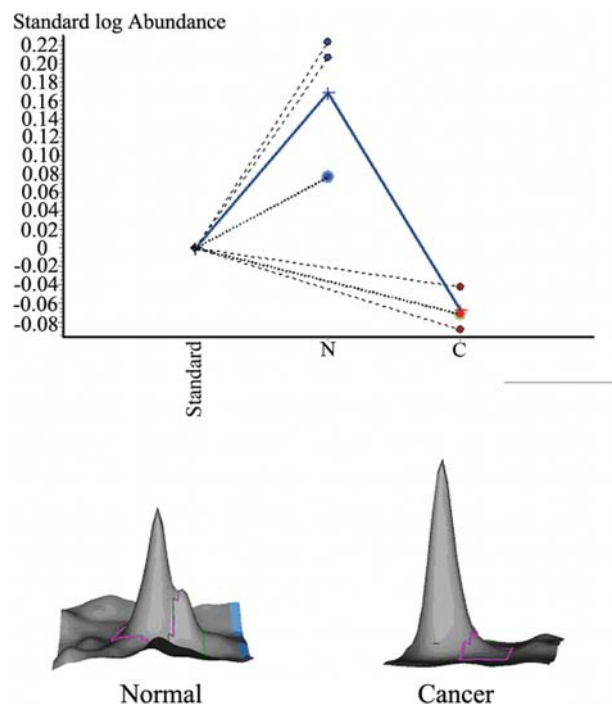

Figure 3. Graph view and 3D simulation view of up- and down-regulated protein spots. The volume of each spot was calculated using Decyder-DIA software and is graphically represented. The spot pairs of up-regulated (A), and down-regulated (B) proteins in cancer tissues are shown. The upper panel shows the graph views of the protein spots (1159), and the lower panel shows the 3D image of the corresponding spots (1386) and their calculated spot volumes. The amount of the protein is proportional to the volume of the protein peak.

spots were detected as shown in Fig. 1D (gel 1, 1898; gel 2, 1824; and gel 3, 1813) by DIGE.

Change of protein expression patterns between Cy3 and Cy5 image gel. After matching with the internal standard (Cy2), statistical analysis was performed to compare the mean ratio of expression from the spots of the cancer and normal tissue in the 2-DE maps. Thirty-three protein spots were chosen after showing a significance level of 1.5 times up- or down-regulation in expression compared to the normal group. A master image showing the statistically relevant spots was therefore obtained (Fig. 2). Of the 33 spots that were differentially expressed in gastric cancer vs normal tissues, 9 spots were up-regulated (spot 304, 313, 490, 866, 902, 919, 1190, 1213, 1386) and 24 down-regulated (spot 362, 380, 427, 431, 674, 712, 716, 860, 865, 941, 1115, 1134, 1146, 1159, 1250, 1294, 1434, 1435, 1449, 1539, 1583, 1642, 1673, 1747). The largest increase was up to 5-fold between the normal group and cancer, while the greatest decrease was down to 10-fold or lower.

Quantitation of protein expression. Part of the graph view and 3D simulation view of up- and down-regulated protein spots are shown in Fig. 3. The 3D peak of a protein spot was generated based on the pixel versus area data from the images obtained by the 2D Master Imager. Because of the low abundance of the proteins and the impact of dyes on the MS identify, a replicate gel with 1 mg protein extracts from cancer and normal tissues respectively was performed after statistical analysis of the DIGE map, and the replicate gel was stained by Coomassie Brilliant Blue. After comparison with the dye images, 27 protein spots were found and excised manually from the replicate 2-DE gel for further mass spectrum identification.

Protein identification by MALDI-TOF MS or MS/MS. The peptides produced by proteolytic digestion of 27 spots excised
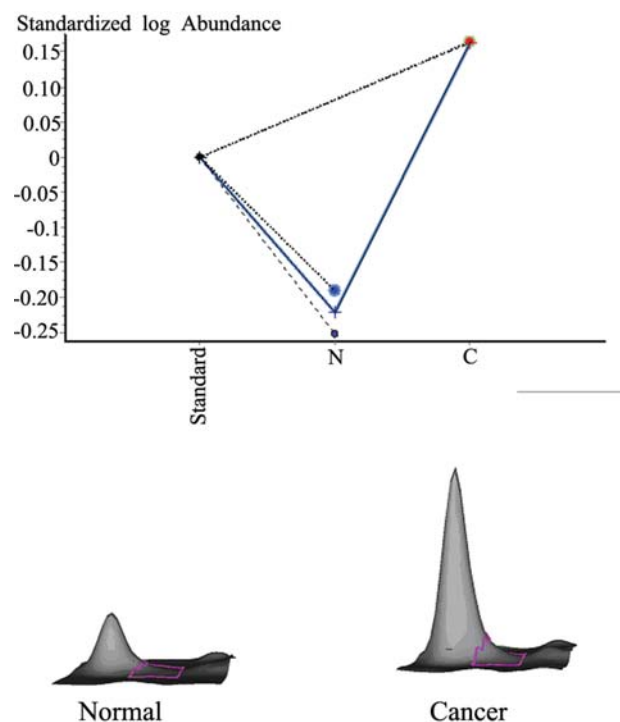
from the replicate 2-DE gel were identified by MALDI-TOF MS or MS/MS. Of them, five proteins with PMF map were not identified in the database, 20 proteins were identified

Identification of spots from the 2-DE maps by MALDI-TOF MS or MALDI-TOF/TOF tandem mass spectrometry.

A. The up-regulated proteins

Spot no.	Protein name	Regulation	Accession no.	Protein scorei	Obs.pI	Obs.Mr (103)	Average ratio	t-test value	Sequence cov. (%)	Protein sequence
304	Chaperonin (HSP60)	Up	gil306890	83	5.70	61157	2.1386	0.003619	17	573 aa
313	Mutant desmin effector	Up	gil19908424	103	5.21	53544	2.0555	0.002593	25	470 aa
	cell proteinase receptor 1 splice form 1b	Up	gil2135059	56	9.27	12659	2.0555	0.002593	51	110 aa
490	Hypothetical protein	Up	gil31873302	68	7.57	47405	1.5531	0.009242	16	434 aa
919	Unnamed protein product	Up	gil10435239	67	4.76	59383	5.0270	0.001426	15	533 aa
1213	Predicted: similar to actin, α, cardiac; α-actin (Pan troglodytes)	Up	gil55641797	116	5.23	42334	1.6482	0.004129	24	377 aa
1386	Manganese superoxide dismutase	Up	gil34707	81	8.35	24866	2.4171	0.006526	9	222

B. The down-regulated proteins

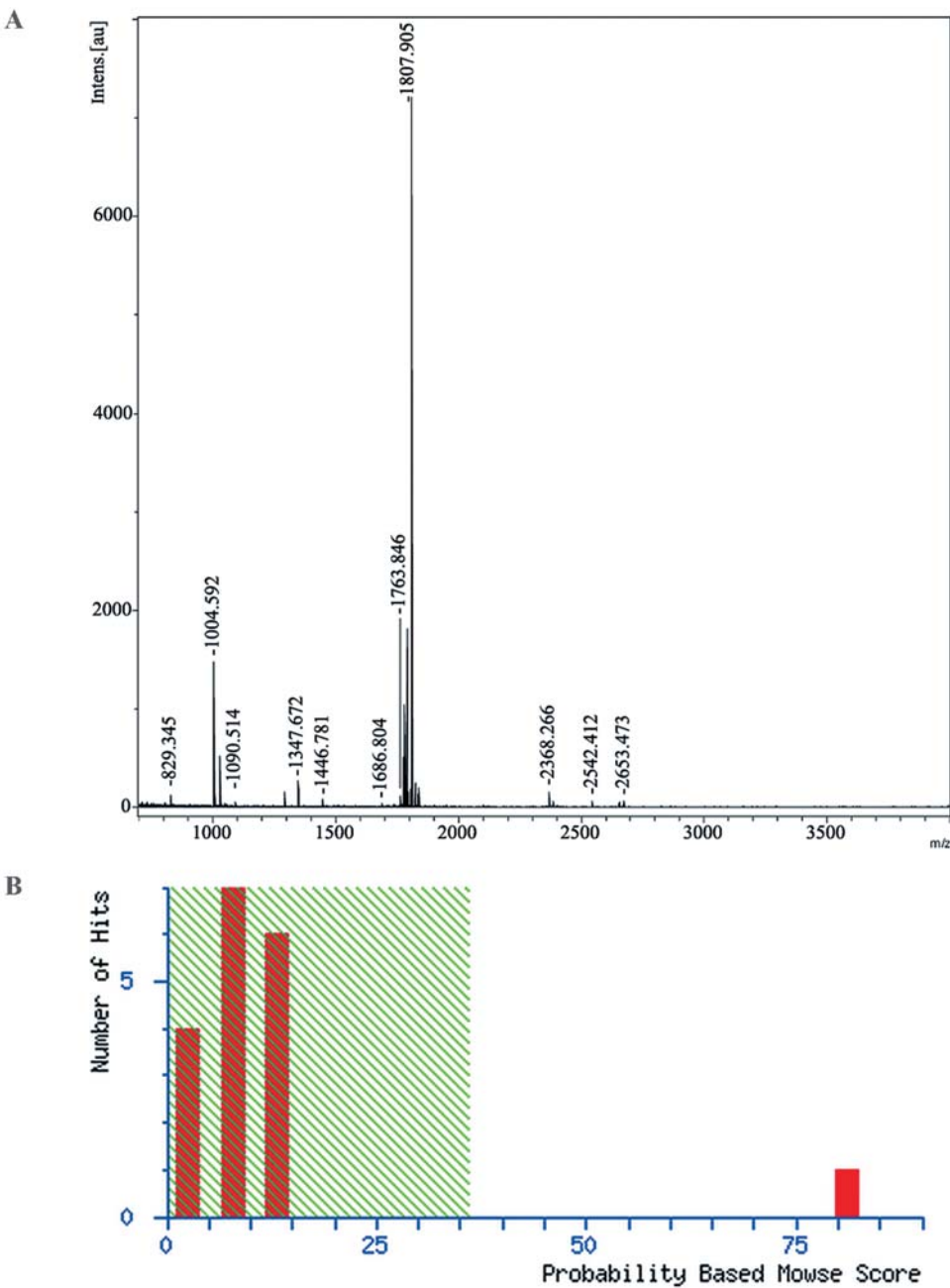| Spot no.	Protein name	Regulation	Accession no.	Protein score	Obs.pI	Obs.Mr (103)	Average ratio	t-test value	Sequence cov. (%)	Protein sequence
380	Selenium binding protein 1	Down	gil16306550	161	5.93	52928	-2.0807	0.007936	37	472 aa
427	Fibrinogen γ	Down	gil223170	70	5.54	46823	-1.9469	0.004727	20	410 aa
431	Fibrinogen γ	Down	gil223170	70	5.54	46823	-2.8840	0.006495	24	410 aa
674	Human Muscle Fructose 1,6-Bisphosphate Aldolase Complexed With Fructose 1,6-Bisphosphate	Down	gil4930291	116		39720	-1.6685	0.001905		
716	Tubulin α 6	Down	gil14389309	84	4.96	50548	-1.7639	0.00983	26	449 aa
860	Eukaryotic translation elongation factor 1 α 1	Down	gil48734966	69	9.10	50433	-1.9516	0.002592	16	462 aa
865	Zinc finger protein 160	Down	gil38788302	67	9.44	96788	-1.8226	0.003333	11	818 aa
1115	Drug-protein interactions: Structure of Sulfonamide Drug Complexed With Human Carbonic Anhydrase I	Down	gil515109	126		28778	-2.0881	0.005951		
1134	Mitochondrial short-chain enoyl-coenzyme A hydratase 1, precursor	Down	gil14286220	71	8.34	31835	-3.0766	0.0013	23	290 aa
1146	Prostaglandin F synthase	Down	gil46389820	74	8.05	37220	-3.8801	0.008038	26	323 aa
1159	Heat shock protein 27	Down	gil662841	79	7.83	22427	-1.7393	0.008047	37	199 aa
1250	Chain C, Crystal Structure of Lipid-Free Human Apolipoprotein A-I	Down	gil90108666	68	5.27	28061	-1.6441	0.006301	22	243 aa
1294	Biliverdin reductase B [flavin reductase (NADPH)]	Down	gil32891807	98	7.13	22219	-1.8057	0.008066	49	206 aa
1435	Chain A, Human Serum Albumin Mutant R218h Complexed With Thyroxine (3,3',5,5'-tetraiodo-L-thyronine) and Myristic Acid (tetradecanoic acid)	Down	gil31615333	76	5.66	68406	-10.9719	0.007126	17	585 aa
1539	Chain A, crystal structure of the Ga module complexed with human serum albumin Ga module	Down	gil55669910	78	5.57	67174	-8.5195	0.007528	15	572 aa
1642	Chan F, Cypa Complexed With Hvgpia;	Down	gil2981764	77	7.82	18098	-2.6387	0.007108	43	164 aa
	Chain H, Monoclinic Form of Human Peroxiredoxin 5	Down	gil16975162	64	6.96	17060	-2.6387	0.007108	31	161 aa

Figure 4. Protein identification by mass spectrometry. (A) Peptide mass fingerprint for trypsin digest of spot 1386. The x-axis represents mass-to-charge ratio (m/z), whereas the y-axis represents relative abundance. Labels correspond to amino acid numbers of each peptide fragment. (B) After searching the NCBInr database, the software identified MnSOD as the top candidate with a sequence coverage of 9%, and the probability based mowse scor of 81. Matched peptides are shown in strong red;

1 MLSRAVCGTS RQLAPALGYL GSRQKHSLPD LPYDYGALEP HINAQIMQLH
51 HSKHHAAYVN NLNVTEEKYQ EALAKGDVTA QTALQPALKF NGGGHINHSI
101 FWTNLSPNGG GEPKGELLEA IKRDFGSFDK FKEKLTAASV GVQGSGWGWL
151 GFNKERGHLQ IAACPNQDPL QGTTGLIPLL GIDVWEHAYY LQYKNVRPDY
201 LKAIWNVINW ENVTERYMAC K

successfully by MALDI-TOF MS, and two proteins were identified successfully by MALDI-TOF MS/MS. The data of the 23 proteins identified from the 22 spots are summarized in Table III. Either spot 313 (mutant desmin; effector cell proteinase receptor 1 splice form 1b) or 1642 (Chain F, Cypa Complexed With Hvgpia; Chain H, Monoclinic Form of Human Peroxiredoxin 5) comprised two proteins, and spot 427 and 431 were identified to be the same protein,

fibrinogen γ. The significantly up- or down-regulated proteins might be the biomarkers of gastric cancer. Fig. 4A shows a typical MALDI-TOF peptide mass fingerprint for one of the proteins that was found to be up-regulated in gastric cancer. Corresponding amino acid residue numbers are indicated on peaks that were matched to the identified protein based on a query of the database NCBInr. The probability based mowse score of the protein is shown in Fig. 4B. The protein

was MnSOD, with a mowse score of 76 (protein reater than 64 are significant, p<0.05), which corresponds with a coverage of 9%.

Discussion

Two-dimensional differential gel electrophoresis is a novel technique for proteomic analysis (16,17). In 2D-DIGE, a relatively small amount of protein extracts from more than two sources is labeled with reactive cyanine dyes that label lysine residues and fluoresce at different wavelengths. The labeled samples are then mixed and analyzed in the same large format 2D gel. Multi-wavelength imaging allows for quantitation of the precise ratio of different proteins in each sample. The unique aspect of this technology allows for migration and analysis of the multi-labeled protein samples on the same gel. Each gel includes an internal standard sample, which is created by pooling an aliquot of all biological samples in the experiment and labeling it with one of the CyDye DIGE Fluor minimal dyes (usually Cy2 for a 3-dye experiment). The samples are evenly labeled with CyDye DIGE Fluors Cy3 and Cy5 to minimize variation between fluors (Table II). This uniquely designed 2-D DIGE technique has a number of advantages: accurate quantification and accurate spot statistics between gels, increased confidence in matching between gels, flexibility of statistical analysis depending on the relationship between samples, and separation of induced biological change from system variation from inherent biological variation. So this technique appears to have advantages of adequate sensitivity, high reproducibility and a wide dynamic range (16).

In this study, we used cyanine dyes that afford labeling of only 1 or 2% of all proteins because of solubility constraints. To supplement the protein used for mass spectrometry, we used a replicate gel with 1 mg samples of unlabeled protein sensitive to enrich each protein spot for MS identification. This analysis identified 9 up-regulated protein spots and 24 down-regulated proteins in gastric cancer tissues. Totally 27 excised proteins spots on the replicate gel were identified by MALDI-TOF MS. Twenty-two spots were identified successfully by MS or MS/MS. Five of the 27 spots presented with spectra, but no novel marker protein for gastric cancer was identified. We obtained seven over-expressed proteins and 16 under-expressed proteins (Table III).

The differentially expressed proteins could be divided into seven groups based on their functions: molecular chaperones, cystoskeleton proteins, metabolic enzymes, proteins associated with cell cycle, proteins associated with cell proliferation differentiation and apoptosis, and proteins associated with lipoid metabolism. HSPs are molecular chaperones which regulate the activity of multiple intracellular signaling intermediates, many of which are intimately involved in the control of the apoptotic signaling pathways. HSPs include anti-apoptotic and pro-apoptotic proteins that interact with a variety of cellular proteins (18). HSP27 and HSP70 are anti-apoptotic, while HSP60 and HSP10 are pro-apoptotic. This suggests that the balance of HSP proteins can determine the fate of stressed cells. HSP60 mainly refolds and prevents the aggregation of denatured proteins (19). In our research, we found that two heat shock proteins (HSP), HSP 60 and

HSP27, were differently expressed in cancer tissues: HSP60 was up-regulated while HSP27 was down-regulated in cancer tissues. He *et al* (20) reported that HSP 60 was up-regulated in gastric tumor tissues. Kamiya *et al* (21) reported that among the various virulence factors of *Helicobacter pylori* (*H. pylori*) the role of its heat shock protein 60 in mucosal inflammation after *H. pylori* infection was examined, and the HSP60 epitope was also detected on the surface of both human gastric cancer cells (MKN45, KATOIII and MKN28) and human gastric biopsy specimens. *H. pylori* is generally accepted as the first known bacterial risk factor for the development of gastric cancer, and therefore HSP 60 may have some association with *H. pylori* infection and with the occurrence and progression of gastric cancer. The role of HSP27 in gastric cancer is not clear. Kapranos *et al* (22) found that HSP27 expression was detected in 68 (79%) out of 86 cases of normal gastric mucosa, and in 54 (62.7%) out of 86 cases of gastric carcinoma. Our DIGE results were consistent with theirs. HSP27 was also found to be associated with lymph node metastasis, suggesting that HSP27 was down-regulated in gastric cancer. On the contrary, Ryu *et al* (10) reported that cytoplasmic proteins (transgelin, prohibitin and HSP27) were up-regulated in gastric cancer tissues. These conflicting results may suggest that shock protein abundance varies in different stages of gastric cancer, which needs further study. Chen *et al* (23) reported that HSP27 was expressed in human gastric cancer, whereas non-tumorous, non-regenerative human foveolar epithelium did not express HSP27. In our research, HSP27 was down-regulated on DIGE. The discrepancy may be either related to the different nature or the different stage of the gastric cancer.

The term cytoskeleton is commonly used to describe the subcellular framework of filamentous contractile and structural proteins that function in motility-related activities and in maintenance of cell shape. We identified that cystoskeleton protein mutant desmin was up-regulated but tubulin α 6 and eukaryotic translation elongation factor 1 α 1 (eEF1A1) were down-regulated in gastric cancer. These cystoskeleton proteins are necessary for mitosis and cell proliferation and are primarily located in the epithelium. The centrosome protein of tubulin, including α- and γ-tubulin, was reported to be overexpressed in premalignant lesions and breast cancer (24,25), Giarnieri *et al* (26) reported that there was a significant difference in α- and ß-tubulin protein expression in polyps and invasive cancer of the rectum, implying a possible role of tubulins in the development of invasive, but not preinvasive cancer. α-tubulin has also been reported to be differentially expressed in the normal colon compared to colon tumors (27) and α-tubulin was down-regulated during differentiation of HT29-D4 cells (28). Eukaryotic translation elongation factor 1 α, which is an abundant member of the actin bundling protein, is the cofactor of eukaryotic protein synthesis responsible for binding aminoacyl-tRNA to ribosome during polypeptide elongation. eEF1A1 is widely expressed, whereas eEF1A2 is normally expressed only in neurons and muscle (29). Tomlinson *et al* (30) reported that eEF1A2 should be considered as a putative oncogene in breast cancer, which may be a useful diagnostic marker and therapeutic target for a high proportion of breast tumors. They also think that oncogenicity of eEF1A2 may be related

to its role in protein synthesis or to its potential non-canonical functions in cytoskeletal remodelling or apoptosis.

Several metabolic enzymes were found up- or down-regulated in gastric cancer. Which include prostaglandin (PG) F synthase, fructose 1,6-bisphosphate aldolase (FBP-Ald), carbonic anhydrase I (CA I), manganese superoxide dismutase, mitochondrial short-chain enoyl-coenzyme A hydratase 1, precursor, biliverdin reductase B [flavin reductase (NADPH)], Chain F Cypa and peroxiredoxin 5 (Prdx 5). Prostaglandin (PG) F synthase is one of the major prostanoids produced by the kidneys. Recent studies (31) suggest that up-regulation of PGF synthase, especially cyclooxygenase 2 (cox-2) plays an important role in carcinogenesis of gastro-intestinal cancer. Carbonic anhydrase (CA) is a zinc-containing metalloenzyme that catalyzes reversible hydration of CO_2. The role of the enzyme has been well established, the main function of which is to produce HCO_3^- for the intermediate metabolism and to maintain pH, water, and ion equilibrium in the body (32). CA I protein is associated with cell growth. It is likely expressed by rapidly proliferating tumor cells or cells that are about to enter the proliferative state, because the CA domain and other elements of the molecule take part in the regulation of cell growth in certain tumor cell types (33). Wang *et al* (9) found that it was overexpressed in gastric carcinoma. Manganese superoxide dismutase (MnSOD) is a tetrameric enzyme with four identical subunits each harboring an Mn^{+3} atom, which catalyzes the dismutation of two mole-cules of superoxide anion into water and hydrogen peroxide. Chen *et al* (34) reported that MnSOD can mediate growth inhibition of gastric cancer cell line SGC7901. Czeczot *et al* (35) also found that MnSOD in gastric cancer was higher than that in healthy stomach tissues. There are studies showing that enhanced expression of MnSOD in progressive gastric cancer was related to postoperative 5-year survival, and to sensitivity to chemotherapy (36,37) Yoshihara *et al* (38) reported similar results in Japanese patients. Thus, MnSOD is a potential biomarker of gastric cancer.

Proteins associated with cell cycle, proliferation, dif-ferentiation and apoptosis as well as lipoid metabolism were found to be differently expressed in gastric cancer. He *et al* (21) found that, like fibrinogen γ, zinc finger protein 160 and apolipoprotein A1 (ApoA1), SeBP 1 was also down-regulated in gastric cancer tissues. There are also an unnamed protein product and a hypothetical protein that were up-regulated in cancer tissues. They may have a specific pathological role in gastric carcinogenesis. Further comprehensive evaluation considering all the altered factors may result in the discovery of a biomarker index for effective assessment of the disease and may provide in-depth information for better under-standing the pathogenesis of gastric cancer.

In summary, we analyzed primary tumor biopsies and normal tissues in the same patients, and observed the up- and down-regulated proteins in human gastric cancer tissue. It is hoped that these results would correlate with clinical data in future studies to define clinically useful biomarkers. The approach we described in this study has shown that the high-throughput 2D-DIGE analysis might be a valuable tool for proteomic study and biomarkers screening method. The identification of the function of protein found in proteomic studies is essential to provide a more effective therapy for

patients suffering from gastric carcinoma. A primary tumor biopsy may contain several different cell types other than carcinoma cells, so the exact definition for major cellular changes during the conversion of normal to stomach malignancy is limited.

The result also showed that carcinogenesis of gastric cancer is a complex process involving multiple factors such as enzyme metabolism, lipoid metabolism, and cell proliferation, differentiation and apoptosis.

Acknowledgements

References

1. Ohgaki H and Matsukura N: Stomach cancer. In: World Cancer Report. Stewart BW and Kleihues P (eds). IARC Press, Lyon, p197, 2003.
2. Murray CJ and Lopez AD: Mortality by cause for eight regions of the world: global burden of disease study. Lancet 349: 1269-1276, 1997.
3. Ferlay J, Bray F, Pisani P and Parkin DM: Globocan 2000: Cancer Incidence, Mortality and Prevalence Worldwide. Version 1. 0. IARC Cancer Base No. 5. IARC Press, Lyon, 2001.
4. Xue FB, Xu YY, Wan Y, Pan BR, Ren J and Fan DM: Association of *H. pylori* infection with gastric carcinoma: a meta-analysis. World J Gastroenterol 7: 801-804, 2001.
5. Doglietto GB, Pacelli F, Caprino P, Sgadari A and Crucitti F: Surgery: independent prognostic factor in curable and far advanced gastric cancer. World J Surg 24: 459-463, 2000.
6. Hanash SM: Biomedical applications of two-dimensional electrophoresis using immobilized pH gradients: current status. Electrophoresis 21: 1202-1209, 2000.
7. Emmert-Buck MR, Gillespie JW, Paweletz CP, *et al*: An approach to proteomic analysis of human tumors. Mol Carcinog 27: 158-165, 2000.
8. Bi X, Lin Q, Foo TW, *et al*: Proteomic analysis of colorectal cancer reveals alterations in metabolic pathways: mechanism of tumorigenesis. Mol Cell Proteomics 5: 1119-1130, 2006.
9. Wang KJ, Wang RT and Zhang JZ: Identification of tumor markers using two-dimensional electrophoresis in gastric carcinoma. World J Gastroenterol 10: 2179-2183, 2004.
10. Ryu JW, Kim HJ, Lee YS, *et al*: The proteomics approach to find biomarkers in gastric cancer. J Korean Med Sci 18: 505-509, 2003.
11. Dongre AR, Eng JK and Yates JR III: Emerging tandem-mass-spectrometry techniques for the rapid identification of proteins. Trends Biotechnol 15: 418-425, 1997.
12. Gygi SP, Han DK, Gingras AC, Sonenberg N and Aebersold R: Protein analysis by mass spectrometry and sequence database searching: tools for cancer research in the post-genomic era. Electrophoresis 20: 310-319, 1999.
13. Gorg A, Obermaier C, Boguth G, *et al*: The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 21: 1037-1053, 2000.
14. Unlu M, Morgan ME and Minden JS: Difference gel electro-phoresis: a single gel method for detecting changes in protein extracts. Electrophoresis 18: 2071-2077, 1997.
15. Tonge R, Shaw J, Middleton B, *et al*: Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology. Proteomics 1: 377-396, 2001.
16. Zhou G, Li H, De Camps D, *et al*: 2D differential in-gel electro-phoresis for the identification of esophageal scans cell cancer-specific protein markers. Mol Cell Proteomics 1: 117-124, 2002.

17. ...i S, Gaffney P, Yang A, *et al*: Evaluation of two-...sional differential gel electrophoresis for proteomic expression analysis of a model breast cancer cell system. Mol Cell Proteomics 1: 91-98, 2002.

18. Sinha P, Poland J, Schnolzer M, Celis JE and Lage H: Characterization of the differential protein expression associated with thermoresistance in human gastric carcinoma cell lines. Electrophoresis 22: 2990-3000, 2001.

19. Bakau B and Horwich AL: The Hsp70 and Hsp60 chaperone machines. Cell 92: 351-366, 1998.

20. He QY, Cheung YH, Leung SY, Yuen ST, Chu KM and Chiu JF: Diverse proteomic alterations in gastric adenocarcinoma. Proteomics 4: 3276-3287, 2004.

21. Kamiya S, Yamaguchi H, Osaki T and Taguchi H: A virulence factor of *Helicobacter pylori*: role of heat shock protein in mucosal inflammation after *H. pylori* infection. J Clin Gastroenterol 27 (Suppl. 1): S35-S39, 1998.

22. Kapranos N, Kominea A, Konstantinopoulos PA, *et al*: Expression of the 27-kDa heat shock protein (HSP27) in gastric carcinomas and adjacent normal, metaplastic, and dysplastic gastric mucosa, and its prognostic significance. J Cancer Res Clin Oncol 128: 426-432, 2002.

23. Chen J, Kahne T, Rocken C, Gotze T, Yu J, Sung JJY, Chen M, Hu P, Malfertheiner P and Ebert MPA: Proteome analysis of gastric cancer metastasis by two-dimensional gel electrophoresis and matrix assisted laser desorption/ionization-mass spectrometry for identification of metastasis-related proteins. J Proteome Res 3: 1009-1016, 2004.

24. Burns RG and Farrell KW: Getting to the heart of beta-tubulin. Trends Cell Biol 6: 297-303, 1996.

25. Niu Y, Wang Y, Yu Y, Ding XM, Lu SH and Xiao XQ: Expression of alpha-tubulin and gamma-tubulin in premalignant lesion and carcinoma of breast and the significance thereof. Zhonghua Yi Xue Za Zhi 86: 56-60, 2006.

26. Giarnieri E, De Francesco GP, Carico E, *et al*: Alpha- and beta-tubulin expression in rectal cancer development. Anticancer Res 25: 3237-3241, 2005.

27. Van Belzen N, Dinjens WN, Eussen BH and Bosman FT: Expression of differentiation-related genes in colorectal cancer: possible implications for prognosis. Histol Histopathol 13: 1233-1242, 1998.

28. Van Belzen N, Diesveld MP, van der Made AC, *et al*: Identification of mRNAs that show modulated expression during colon carcinoma cell differentiation. Eur J Biochem 234: 843-848, 1995.

29. Chambers DM, Peters J and Abbott CM: The lethal mutation of the mouse wasted (wst) is a deletion that abolishes expression of a tissue-specific isoform of translation elongation factor 1alpha, encoded by the Eef1a2 gene. Proc Natl Acad Sci USA 95: 4463-4468, 1998.

30. Tomlinson VA, Newbery HJ, Wray NR, *et al*: Translation elongation factor eEF1A2 is a potential oncoprotein that is overexpressed in two-thirds of breast tumours. BMC Cancer 5: 113, 2005.

31. Lim HY, Joo HJ, Choi JH, *et al*: Increased expression of cyclooxygenase-2 protein in human gastric carcinoma. Clin Cancer Res 6: 519-525, 2000.

32. Ivanov S, Liao SY, Ivanova A, *et al*: Expression of hypoxia-inducible cell-surface transmembrane carbonic anhydrases in human cancer. Am J Pathol 158: 905-919, 2001.

33. Nogradi A: The role of carbonic anhydrases in tumors. Am J Pathol 153: 1-4, 1998.

34. Chen J, Lin GJ, Cheng J, Tang F, Zhu HG and Liu SL: Mechanism of manganese superoxide dismutase mediated growth inhibition of gastric cancer cell line SGC7901. World Chin J Digestol 13: 1386-1389, 2005.

35. Czeczot H, Scibior D, Skrzycki M and Podsiad M: Antioxidant barrier in patients with gastric cancer - preliminary study. Pol Merkuriusz Lek 19: 521-525, 2005.

36. Kim JJ, Chae SW, Hur GC, Cho SJ, Kim MK, Choi J, Nam SY, Kim WH, Yang HK and Lee BL: Manganese superoxide dismutase expression correlates with a poor prognosis in gastric cancer. Pathobiology 70: 353-360, 2003.

37. Hur GC, Cho SJ, Kim CH, Kim MK, Bae SI, Nam SY, Park JW, Kim WH and Lee BL: Manganese superoxide dismutase expression correlates with chemosensitivity in human gastric cancer cell lines. Clin Cancer Res 9: 5768-5775, 2003.

38. Yoshihara T, Kadota Y, Yoshimura Y, Tatano Y, Takeuchi N, Okitsu H, Umemoto A, Yamauchi T and Itoh K: Proteomic alteration in gastic adenocarcinomas from Japanese patients. Molecular Cancer 25: 75-86, 2006.