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Molecules and Cells

Identification of Proteins Differentially Expressed in Gastric Cancer Cells with High Metastatic Potential for Invasion to Lymph Nodes

Do Hee Lee[1,6], Youra Lee[2,6], Joohyun Ryu[2], Sung Goo Park[2], Sayeon Cho[3], Je-Jung Lee[4], Chan Choi[5], and Byoung Chul Park[2,*]

In a search for proteins involved in cancer metastasis, we analyzed proteomes of the human gastric cancer cell OCUM-2M and its metastatic subline OCUM-2MLN. We observed that aspartate aminotransferase (AAT), D-site binding protein (DBP), and anterior gradient protein 2 (AGR2) are differentially expressed in metastatic OCUM-2MLN cells. Measurement of protein expression in clinical samples indicated that DBP and AAT are also down-regulated in metastatic adenocarcinoma. Additionally, urokinase-type tissue plasminogen activator is up-regulated in OCUM-2MLN cells and also in metastatic gastric cancer samples. Collectively, these results raise a possibility that AAT, DBP and AGR2 are functionally implicated in the invasiveness of gastric cancer cells.

INTRODUCTION

Metastasis is a highly selective process favoring the outgrowth and survival of a specific subpopulation that pre-exists within the heterogeneous primary tumor and invades other areas upon acquisition of metastatic phenotypes (Fidler, 2002). Several factors implicated in the metastatic cascade - especially those involved in tumor cell migration and/or adhesion (e.g., cell adhesion molecules, cadherins, and integrins) - have been characterized. In addition, selectins, angiogenic factors (e.g., VEGF and FGF), and proteases (e.g., matrix metalloproteases and membrane-bound serine proteases) are known to play important roles in tumor invasion and neovascularization (Kim et al., 2010; Kopfstein and Christofori, 2006; Meyer and Hart, 1998). Researchers often isolate the subpopulations that exhibit different invasion and metastatic potency from the parental tumor cell line in order to search for metastasis-associated factors. However, such attempts have not completely been successful due to the complexity of the tumor metastatic cascade. More importantly, gene alterations as well as the differential expression of multiple genes contribute to the acquisition of malignant phenotypes (e.g., invasiveness) of cancer cells (Yokota, 2000). Traditional studies have focused on the functional analyses of a single gene or combinations of genes implicated in metastatic processes. During the past decade, numerous studies have applied the proteomic tools for identification of the proteins functionally linked to metastasis. For example, proteins uniquely expressed at the plasma membrane of the highly metastatic mammary carcinoma lines have been identified by this approach (Harvey et al., 2001). Proteomic analysis of cancer sublines has also led to the identification of proteins associated with metastasis of breast cancer (Li et al., 2006).

Scirrhous gastric cancer, a diffusely infiltrating type of poorly differentiated gastric cancer, has the highest mortality rate of all gastric cancers due to a high frequency of metastasis to peritoneum or lymph nodes. Metastasis of scirrhous gastric cancer cells involves different factors in multiple steps for both the tumor and the surrounding stromal cells (Yokozaki et al., 2001). In a previous study, microarray analysis was performed with respect to gene expression in the OCUM-2M scirrhous gastric cancer cell line and its derivative OCUM-2MLN with high potential for metastasis to lymph nodes. The microarray analysis identified differentially-expressed genes and showed that the expression of several genes (e.g., antigen-presenting genes) was altered in OCUM-2MLN cells (Hippo et al., 2001). To date, proteome analyses have led to the identification of putative metastatic factors from chemo-resistant gastric carcinoma cells or tissues obtained from gastric cancer-induced animals (Chen et al., 2004; Ebert et al., 2006; Sinha et al., 2001; Takikawa et al., 2006). For example, by combining gene expression profiling and 2D-tandem mass spectrometry, Chen et al. identified a subset of differentially expressed proteins functioning in cell-to-cell interactions, adhesion, and tumor immunity (Chen et al., 2006).

By using a similar approach, we analyzed the proteomes derived from OCUM-2M and OCUM-2MLN cells and identified a number of proteins differentially expressed in metastatic OCUM-2MLN cells. For the verification, we then measured the levels of

[1]Department of Biotechnology, Seoul Women's University, Seoul 139-774, Korea, [2]Medical Proteomics Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-806, Korea, [3]College of Pharmacy, Chung-Ang University, Seoul 156-756, Korea, [4]Department of Hematology-Oncology, Chonnam National University Medical School, Gwangju 501-746, Korea, [5]Department of Pathology, Chonnam National University Medical School, Gwangju 501-746, Korea, [6]These authors contributed equally to this work.
*Correspondence: parkbc@kribb.re.kr

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these proteins (or their mRNA levels) in tissue samples obtained from cancer patients by quantitative RT-PCR and/or immunohistochemical analyses. Analysis of clinical samples also confirmed the results of the proteomic study and raised the possibility that the proteins identified in this study are functionally linked to metastasis of gastric cancer.

MATERIALS AND METHODS

Cell culture and preparation of cell lysate

OCUM-2M and OCUM-2MLN cell lines (generously provided by Prof. K. Hirakawa, Osaka City University Medical School) were maintained in DMEM containing 10% FBS at 37°C under an atmosphere of 5% CO_2. At 80-90% confluence, cells were harvested and centrifuged at 300 × rpm for 10 min at room temperature. After PBS washes, cells were resuspended in RIPA buffer (50 mM Tris-HCl pH 8.5, 150 mM NaCl, 1% Triton X-100, and 1% deoxycholate) plus a mixture of protease inhibitors and then lysed by sonication. To remove unbroken cells and debris, the lysate was centrifuged at 20,000 × *g* for 20 min at 4°C. The clarified lysate was dialyzed against 40 mM Tris-HCl (pH 7.5) and the protein concentration of lysate was determined by using the Bradford reagent.

Two-dimensional gel electrophoresis

Isoelectric focusing (IEF) was carried out using IPGphore (Amersham Pharmacia Biotech). The protein sample (approximately 420 μg) was diluted to 250 μl with rehydration solution (0.5% pH 3-10 IPG buffer, 8 M urea, 2% CHAPS, 18 mM DTT, and a trace of bromophenol blue) and applied to the IPG strips (pH 3-10 NL). After overnight rehydration, the samples were subjected to IEF - 1,000 V (1 h), 2,000 V (2 h) and 8,000 V (8 h). After IEF, IPG strips were incubated in an equilibration solution (50 mM Tris-HCl pH 8.8, 6 M urea, 2% CHAPS, 30% glycerol, and 1 mM DTT) for 20 min. The strips were then applied to 14% SDS-polyacrylamide gels. After electrophoresis, gels were fixed and the proteins were visualized by using silver staining kit (Amersham Pharmacia Biotech).

In-gel trypsin digestion of proteins

Before performing the trypsin digestion of proteins, silver was removed from the gels with a reducing solution consisting 30 mM potassium ferricyanide and 100 mM sodium thiosulfate (1:1). A volume of 50 μl of reducing solution was added to the gel slices and mixed thoroughly. The gel slices were then incubated with 200 mM ammonium bicarbonate for 20 min. After incubation, the gel slices were cut into small pieces, washed with water, and dehydrated repeatedly with changes of acetonitrile. The gel pieces were dried in a vacuum centrifuge for 30 min and then treated with 10 ng/μl of trypsin in 50 mM ammonium bicarbonate at 37°C overnight. The peptides were extracted from the gels with 20 μl of 5% trifluoroacetic acid in 50% acetonitrile (repeated three times) and concentrated to 4 μl with the same solvent.

Mass spectrometry

Peptide mass fingerprinting was performed using a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystem) operated in the delayed extraction and reflector mode. Peptide mixtures were mixed with a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid. The search engine Aldente from ExPASY (www.expasy.ch) was used for database searches. For protein identification, the search was restricted to human proteins with following criteria: molecular weight range of 20-120 kDa, one missed trypsin cleavage, cysteines unmodified by carbamidomethylation, and mass tolerance within 50 ppm using internal calibration. Mass spectra were calibrated by using the matrix and the tryptic autodigestion ion peaks as internal standards.

Tandem MS analysis was performed using an AutoFlex MALDI-TOF/TOF mass spectrometer with LIFT technology (Bruker Daltonics). Tryptic digests were prepared on an AnchorChip sample plate according to the manufacturer's instructions and MS/MS spectra were acquired using a N_2 laser at a sampling rate of 25-Hz. The data set was submitted to the MASCOT search engine for protein identification.

Immunoblot analysis

To verify the expression level of proteins identified by proteomic analyses, immunoblots were carried out. After gel electrophoresis (14% SDS-PAGE gel; 30 μg of cell proteins), proteins were transferred to PVDF membranes (S&S) and incubated for 1 h at room temperature in 5% nonfat dry milk in TBST (50 mM Tris-HCl pH 7.6, 100 mM NaCl and 0.1% Tween-20). Dilution factors for primary antibodies were as follows; anti-AAT (1:2,000; polyclonal antibody generated in the lab), anti-AGR2 (1:2,000; Abcam) and anti-tubulin (1:10,000; Santa Cruz). After incubation with primary antibodies for approximately 2 to 3 h at room temperature, secondary antibodies conjugated with HRP (1:5,000; Promega) were added and the solution was incubated for 1 h at room temperature. The proteins were visualized using the ECL system (Pierce).

Activity assay of urokinase-type plasminogen activator (uPA) in cell lysate

Enzymatic activity of uPA present in cell lysate was measured using an uPA activity assay kit (CHEMICON). For the assay, 500 μg of cell protein (up to 160 μl) was mixed with 20 μl of assay buffer. The reaction was initiated by adding 20 μl of the chromogenic peptide substrate. After incubation at 37°C for 2 h, absorbance at 405 nm was measured with a microplate reader.

Quantitative real-time reverse transcription-PCR (QT-RT PCR) analysis

Tissue samples and laser-capture microdissection

Fourteen cases of primary gastric cancer tissues and their corresponding normal mucosa were obtained with informed consent from patients who underwent gastrectomy at Chonnam National University Hospital (Korea). The corresponding metastatic cancer tissues of lymph nodes were also obtained in six cases. The tissues were embedded in TissueTek O.C.T. compound (Sakura Finetechnical Co.) and then snap frozen at -80°C. The frozen sections were fixed in 70% ethanol for 30 sec and stained with hematoxylin and eosin (H & E), followed by three steps of dehydration in 70%, 95%, and 99.5% ethanol (repeat 5 times) and a final 5 min dehydration step in xylene. After air-drying, the stained tissues were subjected to laser capture micro-dissection with a PixCell II LCM system (Arcturus Engineering, USA). Normal gastric epithelial cells and carcinoma cells selectively "laser captured" (1×10^4 cells from each sample) were estimated to be > 95% homogeneous as determined by microscopic visualization. Total RNAs were extracted from the laser-captured cells using a PicoPure RNA isolation kit (Arcturus Engineering). Total RNAs were then amplified using a RiboAmp RNA amplification kit (Arcturus Engineering). Two rounds of amplification yielded 8-100 μg of amplified RNA (aRNA) from each sample.

QT RT-PCR analysis

For amplification of the aRNA, a 10 μl reaction mixture containing 1.5 mM $MgCl_2$, 0.5 μM of each primer, and 1 μl of LightCycler FastStart DNA Master SYBR Green I (containing Taq polymerase, reaction buffer, dNTP mix with dUTP instead of dTTP, and SYBR Green I dye) were mixed with 1 μl of aRNA. Amplification of uPA (NM_002658) was carried out using forward (5′-GATGGTCTGGAGGCTGGTCC-3′; nucleotides 480-499) and reverse (5′-GGTGGGATGTAGTCAAAGCGC-3′; nucleotides 1011-991) primers. Amplification of GAPDH (NM_002046) was performed using forward (5′-CAGTCCATGCCATCACTGC CACCCAG-3′; nucleotides 605-630) and reverse (5′-CAGTGT AGCCCAGGATGCCCTTGAG-3′; nucleotides 907-883) primers. The amplification conditions were as follows; 1) initial denaturation at 95°C for 10 min followed by 42 cycles of 2) denaturation at 95°C for 5 s, 3) annealing at 62°C for uPA and 55°C for GAPDH, and 4) extension at 72°C for 10 s. Quantitative analysis was performed using LightCycler Software (Roche Diagnostics) with a real-time fluorogenic detection system for kinetic analysis. The generation of quantitative data was based on different PCR kinetics of samples with different levels of target gene expression. We used a relative quantification in which the expression levels of the gastric cancer samples were compared to the cerebellum 5′-STRETCH cDNA library of PCR products from human brain (BD Biosciences) in five step 10-fold serial dilutions. The regression coefficient *r* (which is -1) obtained from the linear regression analysis was used to confirm accuracy and reproducibility of the approach. The relative fluorescence intensity of each analyzed cDNA was determined as the mean values of three independent experiments.

Immunohistochemical analysis

Tissue microarray (TMA) construction

Formalin-fixed, paraffin-embedded tissue samples derived from gastric adenocarcinoma were randomly chosen from the archives of the Department of Pathology at Chonnam National University Hospital. The original H & E-stained slides were reviewed by a pathologist. TMA blocks were constructed following a previously described procedure (Kononen et al., 1998) using 3 mm-diameter tissue cores arrayed into paraffin blocks to a density of 60 to 72 cores per block. When available, at least three primary tumors and one matching normal gastric epithelium were targeted for each case.

Cases

Ten arrays encompassing a total of 634 tissue spots from 150 patients with gastric adenocarcinoma were utilized. The patient ages ranged from 30-87 years, (mean age = 61 years). The male to female ratio was 2.75:1. These tumors were grouped according to the degree of invasiveness: 33 T1 tumors were grouped as superficially invasive (lamina propria or submucosa), and the remaining 117 tumors were classified as deeply invasive. The latter group includes 33 T2a tumors that invade muscularis propria, 36 T2b tumors that invade subserosa, 47 T3 tumors penetrate serosa, and 1 T4 tumor invades adjacent structure. These tumors were also grouped based on the degree of metastasis: 68 N0 tumors (no regional lymph node metastasis), 49 N1 tumors (metastasis in 1-6 regional lymph nodes), 25 N2 tumors (metastasis in 7-15 regional lymph nodes), and 8 N3 tumors (metastasis in more than 15 regional lymph nodes). The classified extent of differentiation among members of this group is as follow; 40 well-differentiated, 76 moderately-differentiated, and 34 poorly-differentiated adenocarcinomas. None of the patients had received anticancer therapy prior to the operation.

Immunohistochemical staining

The following dilution factors for primary antibodies were used for the immunohistochemical analysis; anti-DBP (1:3,000) and anti-AAT (1:3,000). The primary antibodies were diluted in PBS supplemented with 5% normal horse serum and 1% BSA. Immunohistochemical staining was carried out using Microprobe Immuno/DNA Stainer (Fisher Scientific). The paraffin sections were immersed in 0.6% hydrogen peroxide for 8 min to block the endogenous peroxidase activity and then incubated with the primary antibodies for 15 min at room temperature. Anti-mouse immunoglobulin G (Sigma) labeled with biotin was used as a secondary antibody for the detection of primary antibodies, and the samples were incubated for 7 min at 45°C. After rinses with universal buffer, the streptavidin-horseradish-peroxidase detection reagent (Research Genetics) was applied for 8 min. Finally, the slides were developed for 20 min by adding the substrate 3-amino-9-ethylcarbazole (Sigma). The slides were counterstained with hematoxylin solution for 1 min (Research Genetics). After dehydration, the tissues were sealed with a universal mount. Positive and negative controls were used in each experiment.

Evaluation of immunoreactivity

Assessment of the staining was performed by two independent pathologists without knowledge of the clinical outcomes, such as tumor stage, lymph node metastasis, and distant metastasis. Consensus scores were assigned to each case by reviewing the slides with discrepancies in scoring. All sections upon which the two observers disagreed were re-evaluated, and after discussion, there was total agreement on the classification. The tumor samples with unsatisfactory or un-interpretable results were eliminated from further consideration. The intensity of immunoreactivity was determined by a 0 to 3+ semiquantitation scoring system. The intensity grading scale ranged from no detectable signal (0) to strong signal seen at low power (3). A grade of 2 indicates a moderate signal seen at low to intermediate power while a grade of 1 indicates a weak signal seen only at intermediate to high power. Tumor samples were classified as positive if they had a score of 2+ or 3+ and as negative if they had a score of 0 or 1+.

Statistical analysis

The Student *t*-test was used to assess the results of 2-DE quantification, western-blot analysis and uPA activity assay. The Mann-Whitney U-test was used to assess the results of quantitative RT-PCR analysis. The χ^2 test was used to assess the data linking the protein expression levels to various clinicopathological parameters. *P* values less than 0.05 were considered statistically significant unless otherwise specified.

RESULTS

Identification of proteins differentially-expressed in metastatic gastric cancer cells

To search for proteins implicated in metastasis of scirrhous gastric cancer, we analyzed the soluble proteomes derived from the OCUM-2M cell line and its subline OCUM-2MLN. Using a typical 2-DE system (pH 3-10; 14% SDS-PAGE; 13 cm gel), we resolved more than 600 spots from approximately 420 μg of OCUM cell proteins. Expectedly, the overall proteome profiles of these two isogenic cells were almost identical. However, after analyzing gel images with an aid of PDQuest™ software, we were able to select six protein spots whose expres-

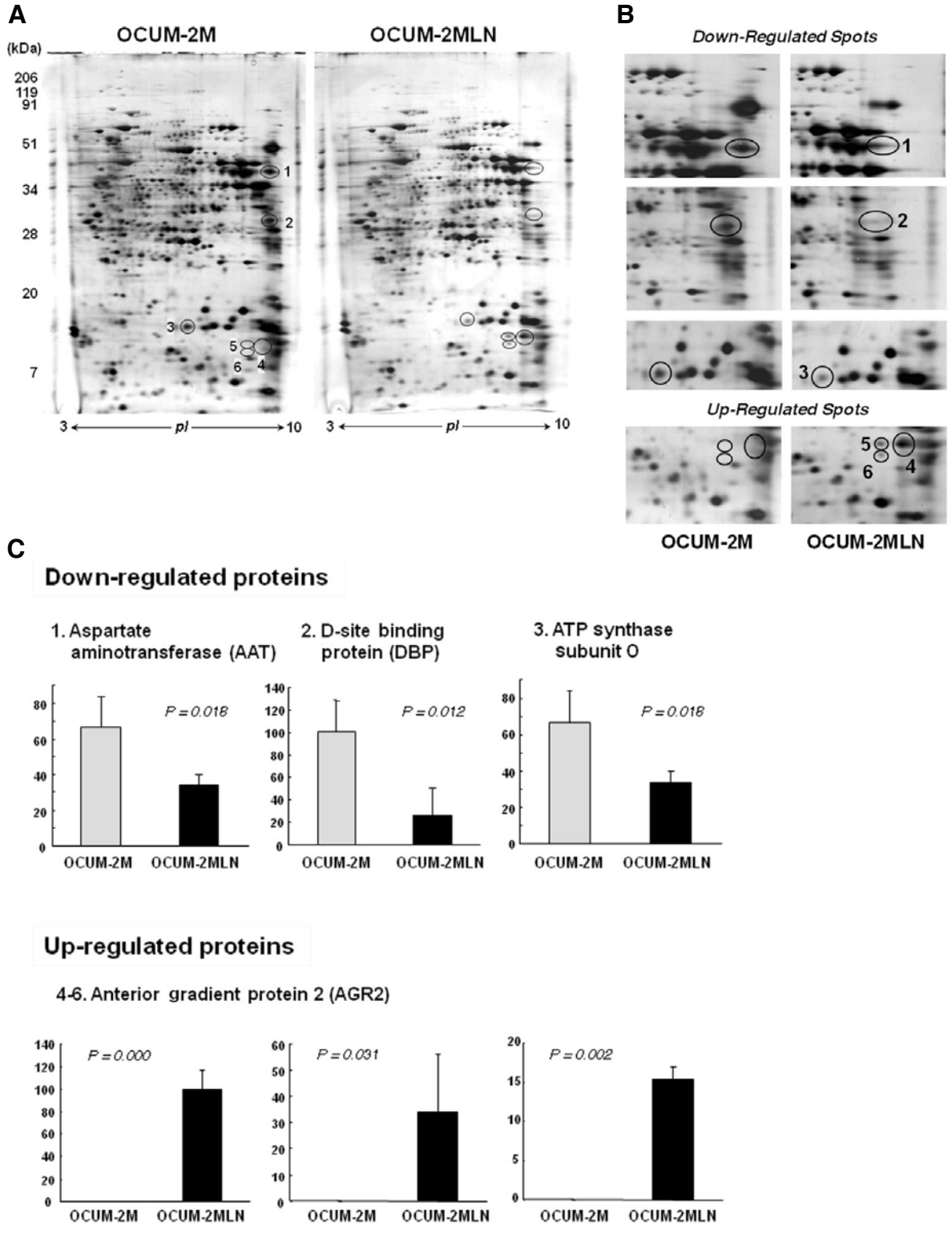


Fig. 1. Identification of differentially-expressed proteins in OCUM-2M and OCUM-2MLN gastric cancer cell lines. (A) Soluble proteins (420 μg) extracted from OCUM-2M and OCUM-2MLN cells were resolved by 2-DE (pH 3-10 NL; 14% SDS-PAGE) and then visualized by silver staining. Protein spots showing more than a 2-fold difference in the expression level between OCUM-2M and OCUM-2M LN cells are marked by circles. For reproducibility, 2-DE was performed three times with freshly prepared samples. (B) Enlarged areas of 2-DE gels containing differentially-expressed proteins (spot 1-3, down-regulated; spot 4-6, up-regulated in OCUM-2MLN cells). For protein identification, the spots were excised from the gel and subjected to MALDI-TOF MS analysis. (C) Quantification of the differentially-expressed proteins. The relative amount of each protein spot in 2-DE gel was determined by using Phoretix™ software and then normalized to the total quantity of protein spots in each gel. Data represent mean ± S.D. ($n = 3$).

Table 1. Identification of proteins differentially expressed in OCUM-2MLN cells

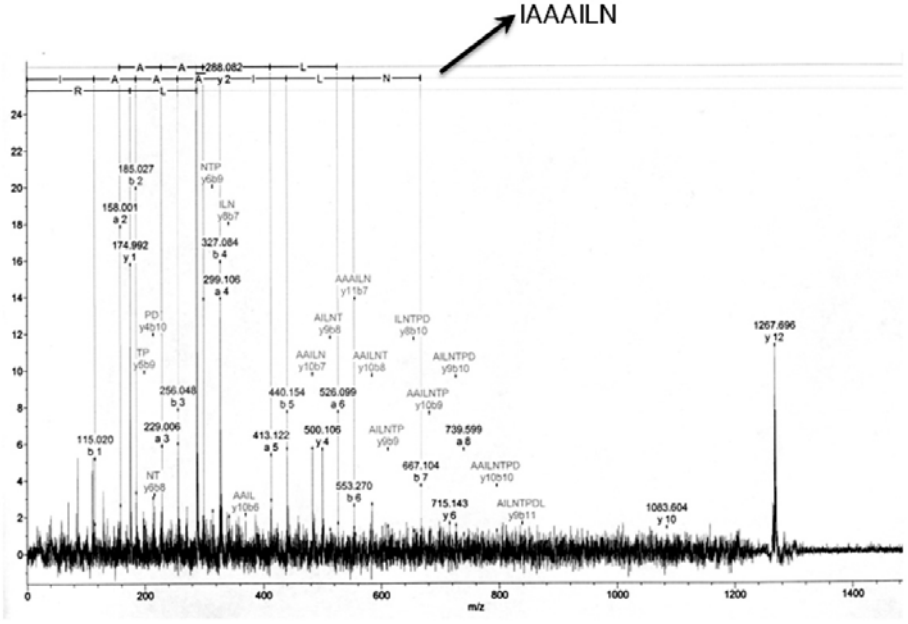
Spot no.	Swiss-prot accession no.	Protein	MW (kDa)	pI	Coverage (%)
Down-Regulated Proteins					
1	P00505	Aspartate aminotransferase, mitochondrial (precursor)	44.6	8.99	24
2	Q10586	D-site binding protein	34.3	9.34	12
3	P48047	ATP synthase subunit O, mitochondrial	20.8	9.82	23
Up-Regulated Proteins					
4-6	O95954	AGR2_human (Anterior gradient protein 2 homolog)	17.8	9.06	30 (# 4) 46 (# 5) 35 (# 6)

sion levels were consistently altered more than 2-fold between these two cell lines (Fig. 1). Incidentally, all of the differentially-expressed proteins were localized in the basic region of gels and MALDI-TOF MS analysis revealed that all of the up-regulated spots represent a single protein whereas the down-regulated spots represent different proteins (Table 1). The functions and sub-cellular localizations of identified proteins were diverse and included; mitochondrial proteins (AAT and ATP synthase subunit O; functioning in metabolic pathways), an endoplasmic reticulum protein (AGR2; a member of the protein disulfide isomerase (PDI) family), as well as a nuclear protein (DBP; a transcription factor). The extent of involvement of these proteins in metastatic processes is not well understood with the exception of AGR2, which has recently been implicated in the metastasis of adenocarcinomas including breast and pancreatic cancers (Liu et al., 2005; Ramachandran et al., 2008).

To verify the results of MALDI-TOF MS analysis, we performed MALDI-TOF/TOF analysis of AAT and demonstrated that a peptide sequence deduced from MS/MS spectra (IAAAILN) was perfectly matched to the sequence present in the open reading frame of AAT (Fig. 2). For further confirmation, we measured the expression of AAT and AGR2 in OCUM-2M and OCUM-2MLN cells by immunoblot analysis. As shown in Fig. 3, Western-blot analysis revealed that AAT was down-regulated while AGR2 was up-regulated in OCUM-2MLN cells. Interestingly, cytoskeletal proteins, glycolytic enzymes and molecular chaperones, a group of proteins whose expression are frequently altered in a number of different types of cancer cell lines or clinical samples from cancer patients, were not detected in this study.

Altered expression of AAT and DBP in gastric adenocarcinoma samples

Although we found that AAT, AGR2 and DBP were differentially-expressed in OCUM-2MLN cells, most of these proteins have not been previously recognized to be associated with metastatic process of gastric cancer. One exception is that over-expression of AGR2 causes proliferation of tumor cells and increases cell migration (Wang et al., 2008). Although it was suggested that AGR2 is functionally linked to the invasion of tumor cells, the involvement of AGR2 in metastasis of gastric cancer has yet to be established. Moreover, the roles of AAT



1 MALLHSGRVL PGIAAAFHPG LAAAASARAS SWWTHVEMGP PDPILGVTEA FKRDTNSKKM
61 NLGVGAYRDD NGKPYVLPSV RKAEAQIAAK NLDKEYLPIG GLAEFCKASA ELALGENSEV
121 LKSGRFVTVQ TISGTGALRI GASFLQRFFK FSRDVFLPKP TWGNHTPIFR DAGMQLQGYR
181 YYDPKTCGFD FTGAVEDISK IPEQSVLLLH ACAHNPTGVD PRPEQWKEIA TVVKKRNLFA
241 FFDMAYQGFA SGDGDKDAWA VRHFIEQGIN VCLCQSYAKN MGLYGERVGA FTMVCKDADE
301 AKRVESQLKI LIRPMYSNPP LNGARIAAAI LNTPDLRKQW LQEVKGMADR IIGMRTQLVS
361 NLKKEGSTHN WQHITDQIGM FCFTGLKPEQ VERLIKEFSI YMTKDGRISV AGVTSSNVGY
421 LAHAIHQVTK

Fig. 2. Identification of aspartate aminotransferase (AAT) by MALDI-TOF/TOF mass spectrometry. For the confirmation of results obtained from MALDI-TOF MS analysis, TOF/TOF analysis of AAT was performed. The MS/MS spectra show the presence of peptide peaks corresponding to the sequence (IAAAILN) present in the open reading frame of AAT (amino acids 336-342).

and DBP in the metastatic process of gastric cancer are still unknown. To examine if expression levels of DBP and AAT are also altered in clinical samples, we performed immunohistochemical analyses of tissues obtained from 150 patients with gastric adenocarcinoma. In agreement with the results of proteome analysis, we found that AAT and DBP were frequently down-regulated (reduced expression) in tissues derived from tumors exhibiting various characteristics of metastasis including invasion to adjacent tissues, high rates of lymph node metastasis and poor differentiation state (Table 2). Moreover, the immunoreactivities of AAT and DBP were consistently lower in undifferentiated tumors than in differentiated samples (Fig. 4). These findings confirmed that these proteins are down-regulated in tissues manifesting metastatic phenotypes.

Table 2. Expression levels of DBP and AAT and the clinicopathological parameters in gastric adenocarcinoma

Parameters	Preserved[1]	Reduced[1]	*p*	Preserved	Reduced	*p*
Primary tumor[2]						
T1, T2a	57	9	*0.007*	49	17	*0.003*
T2b, T3, T4	55	27		52	31	
Lymph node metastasis						
No	61	7	*0.000*	55	31	*0.002*
Yes	51	29		46	35	
Differentiation[3]						
WD	28	5	*0.164*	26	8	*0.217*
MD or PD	84	31		75	40	

[1]Preserved, preserved expression; reduced, reduced expression
[2]T1, tumor invades lamina propria or submucosa; T2a, tumor invades muscularis propria; T2b, tumor invades subserosa; T3, tumor penetrates serosa; T4, tumor invades adjacent structures
[3]WD, well-differentiated tumor; MD, moderately-differentiated tumor; PD, poorly-differentiated tumor

Up-regulation of urokinase-type plasminogen activator (uPA) in OCUM-2MLN cells and metastatic gastric adenocarcinoma

Despite the observations that AGR2, AAT and DBP were differentially-regulated in OCUM-2MLN cells as well as in the clinical samples, it was still necessary to further understand the metastatic characteristics of OCUM-2M cell lines. Urokinase-type plasminogen activator (uPA) is a factor that catalyzes the conversion of plasminogen to active plasmin, which in turn promotes degradation of extracellular matrix. This proteolytic enzyme plays an important role in neo-vascularization, invasion and metastasis of many types of solid tumors (Andreasen et al., 2000; Mazar, 2008). To determine if this metastatic factor is also up-regulated in OCUM-2MLN cells, we first measured the

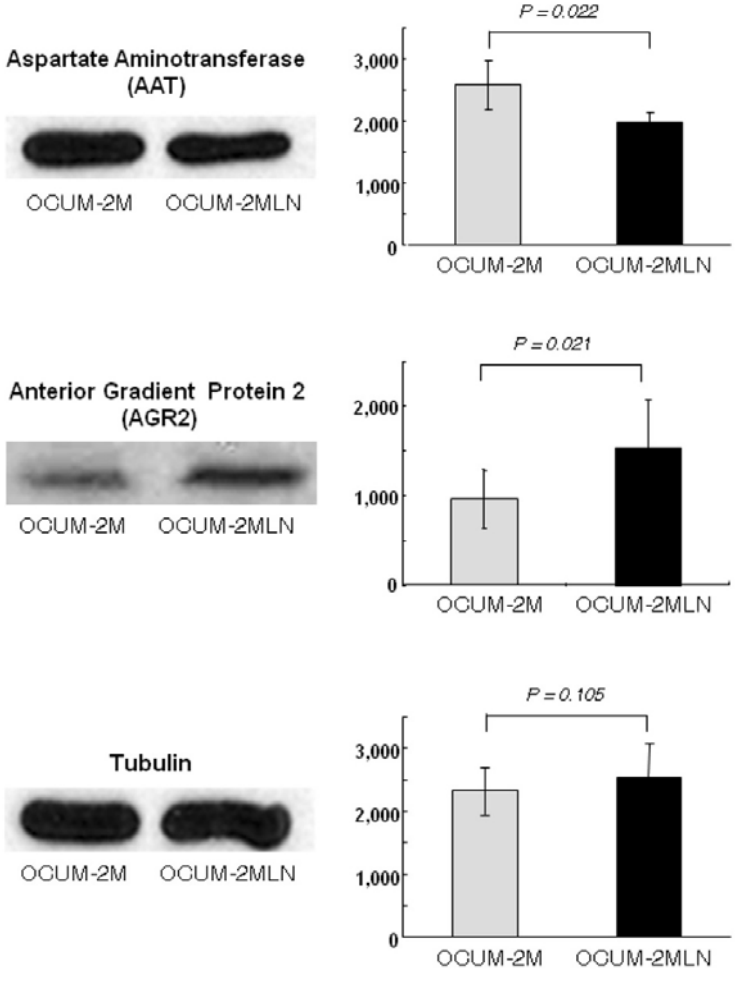


Fig. 3. Immunoblot analysis of AAT and AGR2. To verify the results of MALDI-TOF MS analysis, 30 μg of cell proteins extracted from OCUM-2M and OCUM-2MLN cells were subjected to 12% SDS-PAGE followed by immunoblot analysis with antibodies against AAT and AGR2. For a loading control, immunoblot analysis of tubulin was performed. Data represent mean ± S.D. (n = 3).

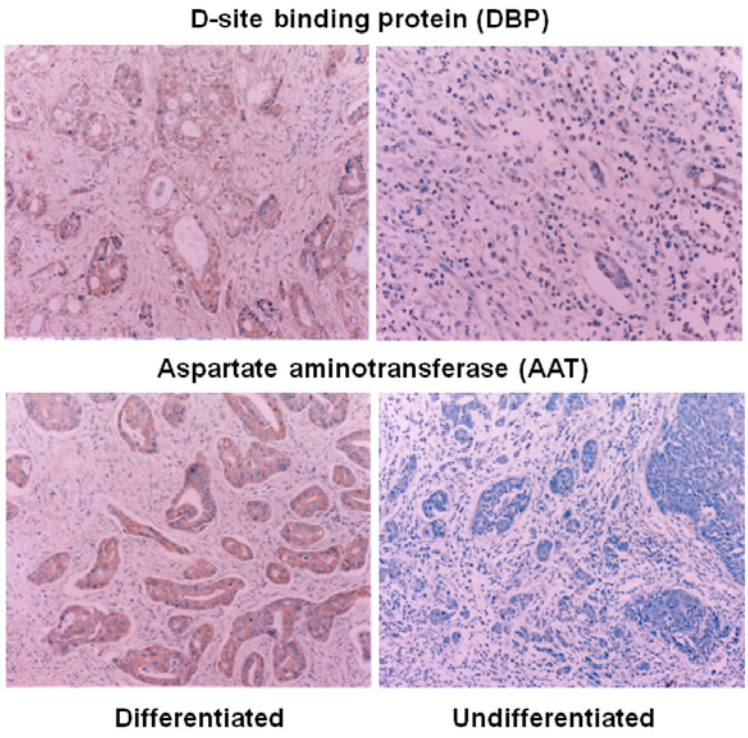


Fig. 4. Immunohistochemical analysis of DBP and AAT. Paraffin-embedded tissue samples prepared from differentiated (left panels) or undifferentiated (right panels) gastric adenocarcinoma were randomly chosen from the archives and immunohistochemical staining with polyclonal antibodies against DBP (upper panels) and AAT (lower panels) was carried out (magnification = 150 ×).

expression level of uPA by immunoblot analysis. The level of uPA protein was consistently up-regulated in OCUM-2MLN cells. This finding was also confirmed by an enzymatic activity assay of uPA (Figs. 5A and 5B). Finally, by employing quantitative RT-PCR analysis, we measured the expression of uPA mRNA in clinical samples (i.e., human gastric cancer cells). While the expression of the uPA protein was found to be slightly up-regulated in gastric adenocarcinoma cells, the increased expression levels of uPA mRNA in adenocarcinoma cells metastasized to lymph node was found to be much greater than that of non-metastatic adenocarcinoma cells relative to normal gastric mucosa (Fig. 5C). These results, together with the previous observations that the uPA system is up-regulated in gastric cancer cells (Kaneko et al., 2003; Shin et al., 2003), suggest that the enhanced expression of uPA seems to be important for the invasive nature of metastatic gastric cancer cells including OCUM-2MLN cells.

DISCUSSION

By using transcriptomic and/or proteomic tools, numerous attempts have already been made to identify proteins associated with metastasis of gastric cancer in rat and human tissues or metastatic human cancer cell lines derived from parental non-metastatic tumor cells (Chen et al., 2004; Ebert et al., 2006; Sinha et al., 2001). A recent investigation using 2-dimensional differential gel electrophoresis (2D-DIGE) revealed that several proteins including annexins, cytokeratins, tRNA synthetases and metabolic proteins are differentially regulated in a highly metastatic gastric cancer cell line (Takikawa et al., 2006). Similarly, the profiling of differentially-expressed proteins in metastatic cancer cells enabled identification of more than 200 proteins including catenins, integrin, and cathepsins (Chen et al., 2006). However, most of the proteins identified in these studies tend to be non-overlapping and vary greatly in a manner dependent upon the nature of samples analyzed (i.e., human tissue samples vs. cancer cell lines). More importantly, the functional relevance of the identified proteins with respect to metastatic processes remains to be established. In the present study, using a similar approach, we identified several proteins differentially expressed in highly metastatic OCUM-2MLN cells and found that most of these proteins, with the exception of uPA, have not been previously implicated in the metastasis of gastric cancer.

The previous microarray study reported that MHC class II molecules or MHC class II accessory molecules are up-regulated and the squamous cell differentiation markers (e.g., SPRR2A), annexin A1, Integrin β4, and E-cadherin are down-regulated in highly metastatic OCUM-2MLN cells (Hippo et al., 2001). However, none of these proteins was found to be differentially expressed in this study, at least at the proteome level. It is worth noting that such a negative correlation between the results of a transcriptome analysis and a proteome analysis is not uncommon. Apparently the rates of synthesis/turnover of mRNA and proteins in cells are controlled differently and thus the steady-state levels of mRNA and proteins are presumed to be maintained individually. Moreover, the majority of proteins identified in the microarray analysis are membrane or extracellular proteins (e.g., MHC class II, invariant chain or integrin), which cannot be readily detected in this study since we analyzed the proteome predominantly derived from the soluble fraction. In future studies, subcellular fractionation and analysis of proteomes in different compartments should be performed to determine if the proteins detected by the microarray analysis are also differentially expressed at the proteome level.

uPA - one of the proteins identified in this study - is well-known to play a role in invasion of several different types of tumor cells including gastric cancer (Kaneko et al., 2003; Shin et al., 2003). The more interesting and potentially important finding is the identification of up-regulation of AGR2 in OCUM-

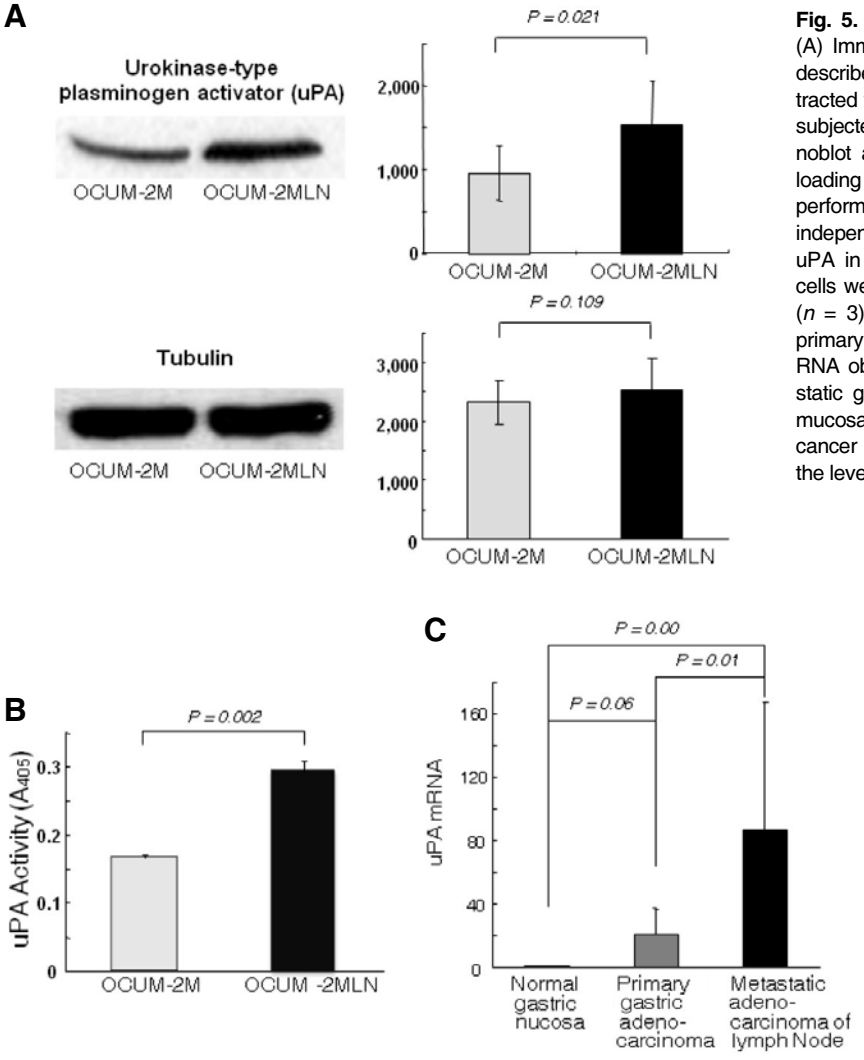


Fig. 5. Up-regulation of uPA in OCUM-2MLN cells. (A) Immunoblot analysis of uPA was performed as described in Fig. 3. The 30 μg of cell proteins extracted from OCUM-2M and OCUM-2MLN cells were subjected to 10% SDS-PAGE followed by immunoblot analysis with antibodies against uPA. For a loading control, immunoblot analysis of tubulin was performed. Data represent mean ± S.D. from three independent experiments. (B) Enzymatic activities of uPA in the lysate of OCUM-2M and OCUM-2MLN cells were measured using an uPA activity assay kit (n = 3). (C) Quantification of uPA mRNA levels in primary and metastatic gastric cancer tissues. Total RNA obtained from 14 cases of primary, non-metastatic gastric cancer and the corresponding normal mucosa tissues and 6 cases of metastatic gastric cancer tissues were amplified by RT-PCR and then the levels of uPA transcripts were measured.

2MLN cells. AGR2 is the human ortholog of a *Xenopus laevis* secretory protein, XAG-2, which was initially reported to be implicated in neural development (Aberger et al., 1998). In humans, it was first identified as an over-expressed gene in estrogen receptor-positive breast cancers (Thompson and Weigel, 1998). Elevated expression of AGR2 was later observed in adenocarcinomas of pancreas, breast and prostate (Fletcher et al., 2003; Liu et al., 2005; Ramachandran et al., 2008; Zhang et al., 2005). Although the molecular function of AGR2 remains uncertain, this protein has been suggested to be a member of protein disulfide isomerase (PDI) family, the ER residential proteins involved in protein folding in this compartment (Ramachandran et al., 2008). Recent studies have demonstrated that over-expression of AGR2 causes proliferation of tumor cells and increases cell migration implicating that this protein may be important for tumorigenesis (Wang et al., 2008). Our findings strongly suggest that this so-called "adenocarcinoma-associated antigen" may also be involved in metastatic processes in gastric cancer.

Involvement of down-regulated proteins in metastatic process is much less clear. DBP is a member of the proline- and acid-rich (PAR) domain subfamily of basic/leucine zipper proteins and is involved in transcriptional regulation in the liver. This 43-kDa protein has been shown to undergo a robust circadian rhythm and transactivates several target genes including cholesterol 7α-hydroxylase and alcohol dehydrogenase (Lamprecht and Mueller, 1999). Recently, increased expression of DBP (together with hepatic leukemia factor) was found to be associated with bFGF-regulated anchorage-independent growth response in mouse epidermal cells (Walters et al., 2009). However, more evidence is needed to determine whether the expression of DBP is correlated with the growth/metastasis of cancer cells. Despite the observations that mitochondrial AAT (also known as transaminase A) is down-regulated in the OCUM-2MLN cell line as well as in gastric adenocarcinoma samples with high rates of metastasis, we know little about the roles of AAT in metastasis. As an enzyme in glycolysis, AAT, in tandem with malate dehydrogenase (MDH), catalyzes the transfer of electrons from NADH across the inner membrane of mitochondria. Given the necessity for a higher rate of glucose uptake and energy utilization for primary and metastatic cancer cells, one can postulate that the glycolytic proteins are usually up-regulated in rapidly growing cancer cells. Indeed, contrary to our results, AAT was found to be up-regulated in metastatic colon cancer cells as well as in the metastatic gastric cancer cell line TMC-1 (Chen et al., 2006; Otsuka et al., 2001). Moreover, AAT expression is also required for the proliferation of breast cancer cells (Thornburg et al., 2008). Perhaps additional

experiments to compare the changes in AAT expression occurring during metastasis of OCUM-2MLN cancer cells to those observed in other metastatic cancer cells (e.g, breast or other gastric cancer cells) are needed to clarify this discrepancy. Functional studies of AAT (including RNA interference experiments) could also provide more insight into the roles of this mitochondrial protein in metastasis of gastric cancer cells.

In conclusion, we identified a number of proteins differentially expressed in highly metastatic gastric cancer cells and confirmed that the expression levels of several of the identified proteins are also altered in gastric adenocarcinoma samples. Importantly AGR2 and uPA, which have already been known to play roles in tumorigenesis of other types of cancer, were found to be over-expressed in metastatic gastric cancer cells. This clearly suggests that these two proteins may be functionally linked to metastasis of gastric cancer cells. Further studies on the biological functions of the up-regulated proteins, especially AGR2, should facilitate our understanding of the metastatic processes of gastric cancer cells.

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