Quantitative Proteomic and Genomic Profiling Reveals Metastasis-Related Protein Expression Patterns in Gastric Cancer Cells

Yet-Ran Chen,[†] Hsueh-Fen Juan,[‡] Hsuan-Cheng Huang,[§] Hsin-Hung Huang,[†] Ya-Jung Lee,[†]
Mei-Yueh Liao,^{||} Chien-Wei Tseng,[‡] Li-Ling Lin,[‡] Jeou-Yuan Chen,[⊥] Mei-Jung Wang,[⊥]
Jenn-Han Chen,^{*,○} and Yu-Ju Chen^{*,†,§}

Institute of Chemistry and Genomic Research Center, Academia Sinica, Taipei, Taiwan, Department of Life Science and Institute of Molecular and Cellular Biology, National Taiwan University, Taipei, Taiwan, Institute of Bioinformatics, National Yang-Ming University, Taipei, Taiwan, Institute of Bioscience and Biotechnology, National Taiwan Ocean University, Keelung, Taiwan, Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan, School of Dentistry, National Defense Medical Center, National Defense University, Taipei, Taiwan, and Institute of Molecular Biology, National Chung Hsing University, Taichung, Taiwan

Received May 4, 2006

Gastric cancer is a leading cause of death worldwide, and patients have an overall 5-year survival rate of less than 10%. Using quantitative proteomic techniques together with microarray chips, we have established comprehensive proteome and transcriptome profiles of the metastatic gastric cancer TMC-1 cells and the noninvasive gastric cancer SC-M1 cell. Our qualitative protein profiling strategy offers the first comprehensive analysis of the gastric cancer cell proteome, identifying 926 and 909 proteins from SC-M1 and TMC-1 cells, respectively. Cleavable isotope-coded affinity tagging analysis allows quantitation of a total of 559 proteins (with a protein false-positive rate of <0.005), and 240 proteins were differentially expressed (> 1.3-fold) between the SC-M1 and TMC-1 cells. We identified numerous proteins not previously associated with gastric cancer. Notably, a large subset of differentially expressed proteins was associated with tumor metastasis, including proteins functioning in cell–cell and cell–extracellular matrix (cell–ECM) adhesion, cell motility, proliferation, and tumor immunity. Gene expression profiling by DNA microarray revealed differential expression (of >2-fold) of about 1000 genes. The weak correlation observed between protein and mRNA profiles highlights the important complementarities of DNA microarray and proteomics approaches. These comparative data enabled us to map the disease-perturbed cell–cell and cell–ECM adhesion and Rho GTPase-mediated cytoskeletal pathways. Further validation of a subset of genes suggests the potential use of vimentin and galectin 1 as markers for metastasis. We demonstrate that combining proteomic and genomic approaches not only provides a rapid, robust, and sensitive platform to elucidate the molecular mechanisms underlying gastric cancer metastasis but also may identify candidate diagnostic markers and therapeutic targets.

Keywords: cI-CAT • 2D-LC–MS/MS • Mass Spectrometry • Gastric Cancer • Metastasis

Introduction

Gastric cancer is one of the most common malignancies and a leading cause of cancer-related deaths worldwide.¹ Although the incidence of and mortality due to gastric cancer have

declined over the past 40 years² and advances in early diagnosis provide excellent long-term survival probabilities for early stage patients, the overall 5-year survival rate remains less than 10%.³ The poor prognosis is mainly due to the fact that most patients are diagnosed at an advanced stage. Advanced gastric cancer is accompanied by metastasis to the peritoneum, lymph nodes, or other organs.⁴ However, the molecular mechanisms associated with tumor metastasis are still not fully understood. Tumor metastasis is mediated by highly complex, multistep processes beginning with loss of adhesion of the tumor cells to neighboring cells.⁵ These cells dissolve the extracellular matrix (ECM), and/or travel via the circulation to invade and proliferate at

* To whom correspondence should be addressed. For Y.-J. C.: e-mail, yjchen@chem.sinica.edu.tw; phone, 886-2-2789-8660, fax, 886-2-2783-1237.

[†] Institute of Chemistry and Genomic Research Center, Academia Sinica.

[‡] National Taiwan University.

[§] National Yang-Ming University.

^{||} National Taiwan Ocean University.

[⊥] Institute of Biomedical Sciences, Academia Sinica.

[○] National Defense University.

[#] National Chung Hsing University.

distant new sites. The detailed molecular and cellular mechanisms of carcinogenesis, cancer invasion, and metastasis remain to be elucidated.

Mapping the cellular networks perturbed during cancer progression by systematic, high-throughput, genome-wide expression analysis at both the mRNA and protein levels will facilitate our understanding of these processes.^{6,7} However, it is now generally recognized that mRNA microarray results should be considered preliminary data that require independent follow-up validation. In contrast, a change in protein patterns in a given disease state should reflect the pathologic processes taking place within the cells. The highly complex feedback between the transcriptome and proteome should ideally describe most biochemical processes within and between cells. Thus, a combination of genomic and proteomic analyses is essential.⁷

While substantial efforts have been made to conduct comparative proteomic characterizations of gastric adenocarcinoma,^{8–13} most of the efforts to date have used two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS). Other than the comprehensive account of the proteins thought to have altered expression in gastric cancer, only a limited number of tumor-associated proteins have been observed to be differentially expressed, because of the inherent limitations of detecting extreme pH/mass and low-abundance proteins. Compared to a 2-DE strategy, protein shotgun sequencing based on two-dimensional liquid chromatography tandem mass spectrometry (2D-LC–MS/MS) analysis represents a large-scale, robust, and sensitive technology for protein profiling.^{14–16} An alternative proteome profiling approach, isotope-coded affinity tagging (ICAT), combined with the shotgun profiling method, allows quantitative analysis of proteomic changes in response to cellular perturbations with a wide dynamic range and good quantitative accuracy.^{16,17} More recently, a second-generation cleavable ICAT (cICAT) reagent was developed to eliminate chromatographic isotope effects caused by hydrogen and deuterium.¹⁸ Furthermore, the combination of cICAT technology and 2D-LC–MS/MS has been shown to be capable of detecting a large number of proteins, allowing more comprehensive coverage of the proteome.¹⁹

To study gastric cancer metastasis systematically, we performed a quantitative comparison between the nonmetastatic SC-M1 and metastatic TMC-1 gastric cancer cell lines. Although direct analysis of clinical samples is most appropriate in principle, to provide phenotype information, the heterogeneous genetics of clinical samples requires analysis of a large number of patients to guarantee statistical significance. Therefore, well-characterized cell lines may prove more informative because of advantages in reproducibility, availability of large numbers, and genetic homogeneity. SC-M1 is a well-established human gastric carcinoma cell line, originally cultured from a poorly differentiated adenocarcinoma that invaded through the stomach wall but showed no metastasis to lymph nodes or adjacent organs.²⁰ Another gastric cancer cell line, TMC-1, was derived from the lymph node of an individual with a moderately differentiated adenocarcinoma of the stomach and exhibited highly tumorigenic features.²¹ The comparative mRNA and protein profiling of these two cell lines may allow us to study the cellular processes associated with the development of gastric cancer metastasis, facilitating further investigation by clinical specimens.

As shown in Figure 1, we first performed qualitative profiling to define the protein constituents of SC-M1 and TMC-1 cells

Figure 1. Schematic representation of the experimental design and bioinformatics analysis for comprehensive genomic and proteomic profiling of SC-M1 and TMC-1 cells.

by 2D-LC–S/MS. We further compared protein and mRNA expression profiles between SC-M1 and TMC-1 cells using cICAT technology and Affymetrix DNA microarrays. To our knowledge, our results offer a more comprehensive proteomic analysis of gastric cancer cells than previously reported. By the complementary analysis of differentially expressed proteins and mRNAs in the two cell lines, we aimed to address the following two issues: (1) the elucidation of the molecular mechanism of metastasis of gastric cancer and (2) the identification of potential markers and/or expression signatures for prognosis and drug discovery for future therapeutic intervention. While the current study focuses on gastric cancer, we expect that the potential markers and pathways uncovered here may be relevant to the metastatic phenotypes of cancer in general.

Materials and Methods

Chemicals. RPMI 1640 medium was purchased from HyClone (Logan, UT). Fetal bovine serum (FBS) was purchased from Gibco BRL (Grand Island, NY). Trypsin was purchased from Promega (Madison, WI). Tris (2-carboxyethyl) phosphine (TCEP) was obtained from Fisher Scientific Corp. (Pittsburgh, PA). The BCA protein assay kit was obtained from Pierce (Rockford, IL). Tris, SDS, Triton X-100, trifluoroacetic acid (TFA), formic acid, and acetonitrile were purchased from Sigma-Aldrich (St. Louis, MO). Iodoacetamide was purchased from Amersham Biosciences (England, U.K.).

Cell Culture and Lysis. The human gastric cancer cell lines SC-M1 and TMC-1 were grown in RPMI 1640 medium supplemented with 25 mM HEPES, 26 mM NaHCO₃, 2 mM glutamine, 100 units/mL sodium penicillin G, 100 µg/mL streptomycin sulfate, 0.25 µg amphotericin B (fungizone), and 10% fetal bovine serum (FBS, Invitrogen Corporation, Carlsbad, CA) at 37 °C with 5% CO₂. The cells were harvested, washed, divided into aliquots containing about $\sim 2 \times 10^7$ cells each, and suspended in lysis buffer (50 mM Tris at pH 8.0, 0.1% (w/v) SDS, 0.02% (w/v) Triton X-100). The suspensions were lysed by placement into a sonic water bath for 30 min followed by exposure to three liquid nitrogen freeze–thaw cycles. Total protein concentration of each cell extract was determined using the BCA assay.

Qualitative Protein Profiling by 2D-LC–MS/MS. About 1 mg of total protein from each cell extract was reduced by TCEP,

alkylated by IAA, and digested by trypsin. Strong cation exchange (SCX) chromatography was used to fractionate the tryptic peptides. The tryptic peptides were fractionated using a 2.1×200 mm polysulfoethyl A SCX column (Poly LC, Columbia, MD). Peptides were eluted with a gradient of 0–25% buffer B (5 mM K_2HPO_4 , 25% acetonitrile, and 350 mM, KCl, pH 3.0) over 30 min, followed by a gradient of 25–100% buffer B over 20 min. The SCX fractions (retention time: 20–60 min) were analyzed in duplicate by microcapillary-liquid chromatography MS/MS (μ LC–MS/MS).

Quantitative Protein Profiling by cICAT and 2D-LC–MS/MS. About 400 μ g of total protein extracted from TMC-1 and SC-M1 cells was labeled with heavy and light isotope-label cICAT, respectively. The cICAT labeling reaction was performed following the manufacturer's protocol (Applied Biosystems, Framingham, MA). The labeled samples were combined and subjected to the trypsin digestion. The digested samples were further fractionated by SCX chromatography, and the cICAT-labeled peptides were isolated by avidin-affinity chromatography for subsequent μ LC–MS/MS analysis.

μ LC–MS/MS Analysis. Samples were loaded using an autosampler and injected into a $1.5 \text{ cm} \times 100 \mu\text{m}$ trapping column and $12 \text{ cm} \times 75 \mu\text{m}$ separation column packed in-house (Magic C_{18} ; Michrom BioResources, Auburn, CA) and sequentially analyzed by μ LC–MS/MS. Peptides were eluted with a linear gradient of 5–40% buffer B over 120 min at ~ 200 nL/min (buffer A, 0.1% formic acid in H_2O ; buffer B, 0.1% formic acid in acetonitrile). An HP 1100 solvent delivery system (Hewlett-Packard, Palo Alto, CA) was used with precolumn flow splitting. A quadrupole/time-of-flight mass spectrometer (QSTAR Pulsar i, Applied Biosystems, Foster City, CA) with an in-house built microspray device was used for all analyses. Peptide fragmentation by collision-induced dissociation was performed in an automated fashion using the information-dependent option.

Data Processing. Uninterpreted MS/MS spectra were searched against the Celera Discovery System (CDS, Celera Genomics, CA) sequence classification protein database (version date 08/13/2003) using the TurboSEQUEST (v.27-rev.12, ThermoFinnigan, San Jose, CA) and validated by the PeptideProphet.²² The peptides that displayed a PeptideProphet score > 0.7 were analyzed by ProteinProphet²³ to calculate the false-positive score of each identified protein. The proteins or related protein groups were identified with a probability score cutoff of $p > 0.95$. For the qualitative analysis, this value corresponded to a false-positive error rate of 0.005 and 0.004 for SC-M1 and TMC-1, respectively. For the cICAT analysis, this value corresponded to a false-positive error rate of 0.004. The light-to-heavy mass (L/H) ratio resulting from differential expression was calculated and normalized by ASAPRatio.²⁴ All of the cICAT-labeled peptides quantified by ASAPRatio were also verified manually.

Microarray Analysis. Total cellular RNA was extracted from a minimum of 5×10^6 cells using the TRIzol reagent (Invitrogen Corp.). RNA was additionally purified by phenol/chloroform/isoamyl alcohol (25:24:1) extraction, and RNA quality was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Foster City, CA). RNA processing and hybridization to the Affymetrix HG-U133A GeneChip oligonucleotide microarray (Santa Clara, CA) were performed according to the manufacturer's protocol. Initial data analysis was performed using Microarray Suite v 5.0 software (Affymetrix, Santa Clara, CA), setting the scaling of all probe sets to a constant value of 500 for each GeneChip. Additional data analysis was performed

using GeneSpring v 5.1 (Silicon Genetics Inc., Redwood City, CA). Genes with differential expression levels of 2-fold or more between SC-M1 and TMC-1 cells were selected for further analysis.

Functional Classification and Pathway Annotations. Functional classification of the identified proteins was mainly based on the CDS sequence classification protein database (version date 08/13/2003). The proteins without functional annotation in the CDS database were searched against the UniProt (<http://www.pir.uniprot.org/>), NCBI (<http://www.ncbi.nlm.nih.gov/>), PANTHER (<http://www.pantherdb.org/>), and Gene Ontology (GO) (<http://www.geneontology.org/>) consortium databases to obtain their biological functions.

For analysis of the perturbed pathway of each differentially expressed gene and protein, we retrieved 314 BioCarta (<http://www.biocarta.com>) and 155 Kyoto Encyclopedia of Genes and Genome (KEGG, <http://www.genome.ad.jp/kegg/pathway.html>) pathways. The Unigene accession numbers were used as identifiers to compare with the proteomic and microarray data. The proteomic data is also analyzed by the pathway analysis tool in NCI's Cancer Genome Anatomy Project (CGAP, <http://cgap.nci.nih.gov/Pathways>).

Immunocytochemical Staining. For immunocytochemical studies, cells grown on coverslips were fixed in 4% paraformaldehyde for 30 min at room temperature, washed twice with TBS (50 mM Tris-HCl and 150 mM NaCl, pH 7.4), and blocked in TBBS (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, and 3% bovine serum albumin) for 1 h at 37 °C. β -Catenin was detected using anti- β -catenin rabbit polyclonal antibody purchased from Sigma (St. Louis, MO) in conjunction with Alexa Fluor 488-conjugated anti-rabbit IgG secondary antibody purchased from Invitrogen. Cells were also counter-stained with 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI, Vector Laboratories, Inc., Burlingame, CA). Cells were analyzed by a laser scanning confocal system (Radiance-2100; Bio-Rad, Hercules, CA).

Flow Cytometry. Cells were seeded at 1×10^5 cells/well in 6-well microplates and incubated overnight in DMEM containing 10% fetal calf serum. Cells were trypsinized; stained with a monoclonal antibody (mAb) against integrin $\alpha 6$ (CD49f, Serotec, Oxford, U.K.), integrin $\beta 4$ (Serotec), or an isotype IgG; washed with cold PBS three times; labeled with Alexa Fluor 488-conjugated anti-mouse IgG antibody; and subjected to flow cytometry analysis using a FACSCalibur instrument (BD Biosciences). The specific fluorescence index was calculated as the ratio of the mean fluorescence values obtained with the specific Ab and the isotype control Ab.

Alamar Blue Assay for Cell Proliferation and Viability. Cells in a 96-well plate were incubated for 4 h with Alamar Blue dye (Biosource) to measure cellular proliferation and viability. Dye uptake was measured fluorometrically using an ELISA Reader (Molecular Devices Corporation, CA) at an excitation and emission wavelength of 570 and 600 nm, respectively.

Results

Qualitative Protein Profiling of SC-M1 and TMC-1 Cell Lines. As shown in Figure 1, qualitative profiling was performed by 2D-LC–MS/MS to define the protein constituents in SC-M1 and TMC-1 cells prior to quantitative profiling experiments and to facilitate detection of low-abundance proteins and/or cysteine-deficient proteins that cannot be observed by cICAT analysis. Using a stringent identification criterion (protein identification false-positive rate of < 0.005), 926 and 909 protein

Figure 2. Functional classification of (A) SC-M1 and TMC-1 proteins from 2D-LC-MS/MS. (B) Differentially expressed proteins between SC-M1 and TMC-1 cells from cICAT analysis. The numbers indicate the percentage of proteins that were differentially expressed between the two cell lines for each functional classification.

entries from SC-M1 and TMC-1 cells were identified, respectively (Supplemental Tables 1 and 2, Supporting Information). For those proteins identified in the qualitative profiling, 41 and 48 proteins were cysteine-deficient in SC-M1 and TMC-1, respectively.

To obtain an overview of the similarities and differences in protein expression between the cell lines, functional annotation was performed to compare the protein constituents. Figure 2A compares the functional category distribution of SC-M1 and TMC-1 proteins according to biological processes (also see Supplemental Tables 1 and 2, Supporting Information). As expected, the identified proteins in the two cell lines possess similar functional category patterns. Most proteins fit into two main categories: protein metabolism and modification, and nucleic acid metabolism. A majority of the other proteins participate in signal transduction and regulation, cell structure and mobility, cell cycle and proliferation, immunity and defense, and protein traffic and transport. Several proteins were classified to be oncogenesis-associated, including 14-3-3 protein σ , 14-3-3 protein ζ , 14-3-3 protein τ , annexin I, galectin 1, myc box-dependent interacting protein 1, and T-plastin; these proteins have also been previously found to be differentially expressed in cancer cells.^{8,25–27}

Differential Proteomic and Transcriptomic Analyses of SC-M1 and TMC-1 Cell Lines. In cICAT analysis, 1727 cICAT-labeled peptides originated from 615 proteins were identified (with a protein identification false-positive rate of < 0.005), of which 559 could be quantified. From the global mean and standard deviation in this study, we have chosen to highlight proteins that have abundance ratios greater than 1.3-fold. Using this criterion, we identified 240 proteins that displayed more than a 1.3-fold expression difference; 100 proteins were up-regulated and 140 proteins were down-regulated in the TMC-1 cells as compared with the SC-M1 cells. Table 1 lists the summary of these differentially expressed proteins. Figure 2B displays the functional category distribution of differentially expressed proteins. Other than metabolism, prominent among the differential expression findings were proteins involved in

signal transduction and regulation, including down-regulation of the Rho-related GTP-binding protein RhoG, transforming protein RhoA, and transforming protein RhoC in TMC-1 cells. Other significant changes involved proteins related to cell structure and motility. These functional categories showed higher percentage of distributions in comparison with qualitative profiling of SC-M1 and TMC-1 (Figure 2A). Figure 3 shows a differentially expressed protein, β -catenin, quantified by cICAT analysis. The light and heavy cICAT-labeled peptide revealed a 5.9-fold overexpression in TMC-1 cells compared with SC-M1 cells (Figure 3B,C). This observation was confirmed with Western blotting, as shown in Figure 3D.

To examine whether the differential protein expression may be attributable to transcriptional regulation or another regulatory mechanism, mRNA expression profiling was performed on SC-M1 and TMC-1 cells using an Affymetrix HG-U133A GeneChip oligonucleotide microarray. The pathways containing differentially expressed genes are listed in Table 2. Among these pathways, three are involved in cancer transformation and proliferation: inhibition of matrix metalloproteinases, multi-drug resistance (MDR) factors, and thrombospondin-1 (TSP-1)-induced apoptosis. Matrix metalloproteinases are a class of proteases secreted by tumor cells that degrade the proteins of the ECM for subsequent tumor cell invasion and metastasis. There are many MDR genes among the mRNAs differentially expressed in SC-M1 and TMC-1 cells, including members of the MDR/TAP subfamily, cytochrome P450 51A1, and glutathione-S-transferase.²⁸ TSP-1 inhibits angiogenesis and slows tumor growth, apparently by inducing apoptosis of microvascular endothelial cells that line blood vessels.²⁹ Factors that inhibit angiogenesis might act as cancer therapeutic agents by blocking vessel formation in tumors and starving cancer cells. These pathways may be relevant to the differences between SC-M1 and TMC-1 cells.

Transcriptional versus Translational Regulation of Protein Expression. Changes in protein expression levels between the two cancer cell types were further examined for potential correlations with the changes in mRNA levels. Cross-referencing

Table 1. Summary of Differentially Expressed Proteins and Their Functional Classifications

	UniProt	protein name	peptide number	prob. ^a	cICAT ^b	Affymetrix ^c	molecular function
Amino Acid Metabolism							
1	P31327	Carbamoyl-phosphate synthase	2	1.00	0.16 ± 0.03	NA ^d	Synthase and synthetase
2	O43175	D-3-phosphoglycerate dehydrogenase	6	1.00	0.31 ± 0.07	0.52	Oxidoreductase
3	P26639	Threonyl-tRNA synthetase, cytoplasmic	2	1.00	0.35 ± 0.04	0.92	Synthase
4	P11586	C-1-tetrahydrofolate synthase	2	1.00	0.43 ± 0.17	0.58	Synthase and synthetase
5	P35520	Cystathionine beta-synthase	2	0.99	0.44 ± 0.07	0.45	Synthase
6	Q9BWD1	Acetyl CoA transferase-like protein	2	1.00	0.52 ± 0.03	1.00	Molecular function unclassified
7	P54886	Delta 1-pyrroline-5-carboxylate synthetase	2	1.00	0.55 ± 0.08	0.58	Kinase
8	P30046	D-dopachrome tautomerase	2	1.00	0.56 ± 0.05	0.59	Isomerase
9	O95671	N-acetylserotonin O-methyltransferase-like protein	1	0.96	1.39 ± 0.12	1.25	Transferase
10	Q9BSE5	Agmatinase, mitochondrial precursor	2	1.00	1.54 ± 0.15	NA ^d	Hydrolase
11	Q9UJ54	DTDP-4-keto-6-deoxy-D-glucose 4-reductase	2	1.00	1.63 ± 0.14	1.27	Oxidoreductase
12	Q9Y617	Phosphoserine aminotransferase	1	0.98	1.78 ± 0.41	1.54	Transferase
13	P00505	Aspartate aminotransferase, mitochondrial precursor	1	0.95	1.84 ± 0.13	0.37	Transferase
14	P27708	CAD protein	3	1.00	2.18 ± 0.53	1.44	Synthase and synthetase
Apoptosis							
1	O43293	Prostate apoptosis response protein par-4	1	1.00	0.31 ± 0.03	1.27	kinase
2	Q6FI81	PRO0915	1	0.99	0.59 ± 0.20	NA ^d	Select regulatory molecule
3	O95831	Programmed cell death protein 8, mitochondrial precursor	1	1.00	2.77 ± 0.80	1.70	Oxidoreductase
Carbohydrate Metabolism							
1	P15121	Aldose reductase	2	1.00	0.08 ± 0.04	NA ^d	Reductase
2	P47895	Aldehyde dehydrogenase 6	1	0.99	0.13 ± 0.03	NA ^d	Hydrolase
3	P19367	Hexokinase, type I	3	1.00	0.31 ± 0.08	0.29	Kinase
4	P29401	Transketolase	1	0.99	0.31 ± 0.03	0.45	Transferase
5	P36871	Phosphoglucomutase	1	0.99	0.37 ± 0.34	0.23	Isomerase
6	Q01813	6-phosphofructokinase, type C	3	1.00	0.43 ± 0.09	0.31	Kinase
7	P18669	Phosphoglycerate mutase 1	2	1.00	0.44 ± 0.04	0.47	Isomerase
8	P14786	Pyruvate kinase, M2 isozyme	4	1.00	0.51 ± 0.09	0.52	kinase
9	Q92621	Hypothetical protein KIAA0225	3	1.00	0.51 ± 0.13	0.50	Kinase
10	P00338	L-lactate dehydrogenase A chain	3	1.00	0.65 ± 0.03	0.83	Oxidoreductase
11	P16152	Carbonyl reductase [NADPH] 1	2	1.00	0.65 ± 0.03	NA ^d	Reductase
12	P53396	ATP-citrate synthase	3	1.00	0.69 ± 0.04	0.80	Lyase
13	P48735	Isocitrate dehydrogenase [NADP], mitochondrial precursor	2	1.00	1.33 ± 0.11	0.51	Oxidoreductase
14	P14550	Alcohol dehydrogenase [NADP+]	1	1.00	1.36 ± 0.24	0.86	Oxidoreductase
15	P42330	Aldo-keto reductase family 1 member C3	2	1.00	1.56 ± 0.10	2.68	Oxidoreductase
16	P52789	Hexokinase, type II	3	1.00	1.66 ± 0.16	1.15	Kinase
17	Q02218	2-oxoglutarate dehydrogenase E1 component, mitochondrial precursor	2	1.00	1.67 ± 0.39	2.24	Oxidoreductase
18	P37837	Transaldolase	2	1.00	1.78 ± 0.19	1.29	Transferase
19	Q00796	Sorbitol dehydrogenase	1	1.00	1.90 ± 0.21	3.04	Oxidoreductase
20	P10768	Esterase D	2	1.00	2.21 ± 1.09	1.56	Hydrolase
21	P40925	Malate dehydrogenase, cytoplasmic	2	1.00	2.24 ± 0.12	1.03	Oxidoreductase
22	P40926	Malate dehydrogenase, mitochondrial precursor	11	1.00	4.49 ± 0.60	2.60	Oxidoreductase
23	O60547	GDP-mannose 4,6 dehydratase	1	1.00	6.15 ± 1.02	3.09	Lyase
Cell Cycle, Proliferation							
1	O75369	Beta-filamin	13	1.00	0.20 ± 0.09	0.08	Cytoskeletal protein
2	P58107	Epiplakin	1	1.00	0.21 ± 0.04	NA ^d	Cytoskeletal protein
3	Q15691	Microtubule-associated protein RP/EB family member 1	1	0.98	0.48 ± 0.09	0.98	Signaling molecule
4	Q14980	NuMA protein	2	1.00	0.68 ± 0.18	0.45	Cytoskeletal protein
5	Q14683	Structural maintenance of chromosome 1-like 1 protein	1	1.00	0.68 ± 0.33	0.77	Nucleic acid binding
6	Q9Y2Z0	Suppressor of G2 allele of SKP1 homolog	1	1.00	2.29 ± 0.20	NA ^d	Molecular function unclassified
Cell Structure and Motility							
1	Q04695	Cytoskeletal 17	2	1.00	0.03 ± 0.03	NA ^d	Membrane
2	Q96AC1	Hypothetical protein	1	1.00	0.04 ± 0.00	0.04	protein binding
3	Q9NZM1	Myoferlin	2	1.00	0.17 ± 0.08	0.40	Membrane traffic protein
4	Q9P2E9	Ribosome-binding protein 1	4	1.00	0.38 ± 0.26	NA ^d	Receptor
5	P35579	Myosin heavy chain, nonmuscle type A	7	1.00	0.47 ± 0.05	0.38	Cytoskeletal protein
6	P24572	Myosin light chain alkali, smooth-muscle isoform	1	1.00	0.48 ± 0.03	0.63	Select regulatory molecule
7	P52907	F-actin capping protein alpha-1 subunit	2	1.00	0.54 ± 0.05	0.86	Cytoskeletal protein
8	P07737	Profilin I	3	1.00	0.63 ± 0.09	0.10	Cytoskeletal protein
9	P47756	F-actin capping protein beta subunit	2	1.00	0.63 ± 0.09	0.86	Cytoskeletal protein
10	Q8NAG3	Hypothetical protein FLJ35393	1	1.00	0.63 ± 0.29	NA ^d	Cytoskeletal protein
11	Q9Y3F5	A6 related protein	1	1.00	0.68 ± 0.13	NA ^d	Cytoskeletal protein
12	O75083	WD-repeat protein 1	2	1.00	0.69 ± 0.12	0.58	Cytoskeletal protein
13	O43707	Alpha-actinin 4	1	1.00	1.37 ± 0.07	1.59	Cytoskeletal protein
14	P21333	Filamin A	11	1.00	1.44 ± 0.07	1.87	Cell adhesion molecule
15	P15924	Desmoplakin	3	1.00	1.57 ± 0.12	2.07	Cytoskeletal protein
16	P02545	Lamin A/C	1	0.99	2.11 ± 0.72	0.90	Cytoskeletal protein
17	P27816	Microtubule-associated protein 4	1	0.95	2.29 ± 0.37	0.66	Cytoskeletal protein

Table I (Continued)

	UniProt	protein name	peptide number	prob. ^a	cICAT ^b	Affymetrix ^c	molecular function
Cell Structure and Motility							
18	Q9NQW6	Actin binding protein anillin	1	1.00	2.58 ± 0.45	NA ^d	Cytoskeletal protein
19	P13647	Cytoskeletal 5	1	1.00	3.43 ± 1.10	NA ^d	Cytoskeletal protein
20	P08670	Vimentin	1	0.99	25.67 ± 5.81	NA ^d	Cytoskeletal protein
Developmental Processes							
1	P21291	Cysteine-rich protein 1	1	1.00	0.3 ± 0.04	0.59	Ion binding
2	Q14192	Skeletal muscle LIM-protein 3	1	0.93	0.54 ± 0.09	0.92	Transcription factor
3	Q09666	Neuroblast differentiation associated protein AHNK	2	1.00	0.67 ± 0.13	0.44	Molecular function unclassified
4	Q15668	Epididymal secretory protein E1 precursor	1	1.00	0.63 ± 0.04	0.23	Signaling molecule
5	Q9HDC9	Adipocyte plasma membrane-associated protein	1	1.00	1.36 ± 0.42	0.82	Molecular function unclassified
6	Q16658	Fascin	4	1.00	2.98 ± 0.39	1.07	Cytoskeletal protein
Electron Transport							
1	P23634	Plasma membrane calcium-transporting ATPase 4	1	0.99	0.00 ± 0.00	0.06	Hydrolase
2	P08574	Cytochrome c1, heme protein, mitochondrial precursor	1	1.00	0.68 ± 0.04	0.82	Oxidoreductase
3	Q9NQ11	Hypothetical protein	1	0.99	1.41 ± 0.11	NA ^d	Hydrolase
4	P22695	Ubiquinol-cytochrome C reductase complex core protein 2	2	1.00	1.56 ± 0.24	0.72	Reductase
5	P51970	NADH-ubiquinone oxidoreductase 19 kDa subunit	2	1.00	1.90 ± 0.36	1.34	Oxidoreductase
6	O96000	NADH-ubiquinone oxidoreductase PDSW subunit	1	0.92	2.17 ± 0.47	NA ^d	Oxidoreductase
Immunity Defenses							
1	O14879	Interferon-induced protein with tetratricopeptide repeats 4	3	1.00	0.08 ± 0.05	NA ^d	Defense/immunity protein
2	P09914	Interferon-induced protein with tetratricopeptide repeats 1	2	1.00	0.29 ± 0.14	NA ^d	Molecular function unclassified
3	Q03405	Urokinase plasminogen activator surface receptor precursor	2	1.00	0.42 ± 0.12	0.16	Receptor
4	P30041	Peroxisomal protein 6	2	1.00	0.55 ± 0.07	0.67	Oxidoreductase
5	P01892	HLA class I histocompatibility antigen, A-10 alpha chain (Fragment)	1	1.00	0.64 ± 0.16	NA ^d	Defense/immunity protein
6	P13987	CD59 glycoprotein precursor	1	0.98	0.67 ± 0.56	0.39	Signaling molecule
7	P30044	Peroxisomal protein 5, mitochondrial precursor	1	1.00	1.84 ± 0.89	NA ^d	Oxidoreductase
8	P09382	Galectin-1	4	1.00	2.33 ± 0.72	0.90	Signaling molecule
Protein Traffic							
1	Q96C19	Swiprosin 1	2	1.00	0.34 ± 0.04	0.28	Nucleic acid binding
2	O60664	Cargo selection protein TIP47	1	1.00	0.55 ± 0.13	0.40	Receptor
3	Q9NR31	GTP-binding protein SAR1a	2	1.00	0.59 ± 0.18	0.78	Select regulatory molecule
4	O60684	Importin alpha 7 subunit	1	0.94	0.67 ± 0.09	0.75	Membrane traffic protein
5	P05218	Tubulin beta-5 chain	13	1.00	1.33 ± 0.22	0.50	Cytoskeletal protein
6	Q9BS26	Thioredoxin domain containing protein 4 precursor	5	1.00	1.33 ± 0.21	0.86	Isomerase
7	P55072	Transitional endoplasmic reticulum ATPase	8	1.00	1.35 ± 0.12	0.95	Hydrolase
8	Q14204	Dynein heavy chain, cytosolic	3	1.00	1.44 ± 0.17	1.03	Cytoskeletal protein
9	O00159	Myosin Ic	2	1.00	1.45 ± 0.64	0.57	Cytoskeletal protein
10	P10809	Heat shock protein 60	9	1.00	1.56 ± 0.09	1.13	Chaperon
11	Q13509	Tubulin beta-4 chain	1	0.98	1.77 ± 0.33	0.77	Cytoskeletal protein
12	O43765	Small glutamine-rich tetratricopeptide repeat-containing protein	2	1.00	1.88 ± 0.40	NA ^d	Chaperon
13	P30740	Leukocyte elastase inhibitor	1	0.91	2.41 ± 0.44	3.76	Select regulatory molecule
Lipid, Fatty Acid, and Steroid Metabolism							
1	Q99541	Adipophilin	1	0.99	0.01 ± 0.00	N. A.	Molecular function unclassified
2	Q01581	Hydroxymethylglutaryl-CoA synthase	2	1.00	0.13 ± 0.03	0.18	Synthase and synthetase
3	Q9Y6L9	Titin	1	0.99	0.16 ± 0.01	NA ^d	Cytoskeletal protein-
4	Q9UBJ2	Peroxisomal farnesylated protein	2	1.00	0.43 ± 0.08	0.21	Miscellaneous function
5	P37802	Transgelin 2	2	1.00	0.56 ± 0.01	0.79	Cytoskeletal protein-
6	P67936	Tropomyosin alpha 4 chain	2	1.00	0.61 ± 0.16	0.83	Cytoskeletal protein-
7	Q5T0M7	Fatty acid synthase	2	1.00	0.65 ± 0.17	NA ^d	Synthase
8	P49327	Fatty acid synthase	2	1.00	0.65 ± 0.17	0.48	Synthase and synthetase
9	O00151	PDZ and LIM domain protein 1	1	0.99	0.67 ± 0.78	0.17	Cytoskeletal protein-
10	Q16850	Cytochrome P450 51A1	1	0.92	0.67 ± 0.08	1.24	Oxidoreductase
11	P04083	Annexin 1	4	1.00	0.68 ± 0.07	1.02	Select calcium binding protein
12	P21964	Catechol O-methyltransferase	2	1.00	1.35 ± 0.55	0.81	Transferase
13	Q08257	Quinone oxidoreductase	1	1.00	1.40 ± 0.28	10.73	Oxidoreductase
14	P36969	Phospholipid hydroperoxide glutathione peroxidase	1	0.99	1.56 ± 0.55	1.14	Oxidoreductase
15	Q9H845	Acyl-CoA dehydrogenase family member 9	1	1.00	1.58 ± 0.77	NA ^d	Oxidoreductase
16	Q15738	NAD(P)-dependent steroid dehydrogenase	2	1.00	1.92 ± 0.24	NA ^d	Oxidoreductase
17	Q01469	Fatty acid-binding protein	2	1.00	2.45 ± 0.21	1.67	Transfer/carrier protein
18	Q99714	3-hydroxyacyl-CoA dehydrogenase type II	2	1.00	2.51 ± 0.80	1.90	Oxidoreductase
Nucleic Acid Metabolism							
1	Q7Z2W4	Zinc-finger CCCH type antiviral protein 1	3	1.00	0.27 ± 0.05	2.51	Molecular function unclassified
2	Q13951	Core-binding factor, beta subunit	1	1.00	0.31 ± 0.05	0.40	Transcription factor
3	P48512	Transcription initiation factor IIB	1	1.00	0.39 ± 0.11	1.25	Transcription factor
4	P53384	Nucleotide-binding protein 1	2	1.00	0.44 ± 0.05	0.40	Nucleic acid binding
5	Q8TEK0	FLJ00195 protein (Fragment)	1	0.98	0.46 ± 0.07	0.31	Transcription factor
6	P18583	SON protein	1	0.98	0.47 ± 0.11	0.86	Nucleic acid binding
7	Q8N1F7	Hypothetical protein KIAA0095	1	1.00	0.52 ± 0.16	NA ^d	Nucleic acid binding
8	P78527	DNA-dependent protein kinase catalytic subunit	7	1.00	0.55 ± 0.16	0.41	Kinase

Table I (Continued)

	UniProt	protein name	peptide number	prob. ^a	cICAT ^b	Affymetrix ^c	molecular function
Nucleic Acid Metabolism							
9	Q12769	Nuclear pore complex protein Nup160	1	0.94	0.55 ± 0.11	1.02	transporter
10	O75534	UNR protein	2	1.00	0.56 ± 0.16	0.98	Nucleic acid binding
11	P33991	MCM4 minichromosome maintenance deficient 4	2	1.00	0.56 ± 0.07	0.18	Nucleic acid binding
12	P33992	DNA replication licensing factor MCM5	3	1.00	0.58 ± 0.25	0.15	Nucleic acid binding
13	Q96EI0	Similar to KIAA0801 gene product	1	0.99	0.58 ± 0.28	NA ^d	Nucleic acid binding
14	Q16576	Histone acetyltransferase type B subunit 2	1	1.00	0.58 ± 0.11	0.54	Miscellaneous function
15	Q86VP6	TIP120 protein	3	1.00	0.58 ± 0.04	NA ^d	Transcription factor
16	Q92878	RAD50	1	0.96	0.58 ± 0.18	NA ^d	Nucleic acid binding
17	P17812	CTP synthase	4	1.00	0.59 ± 0.03	0.81	Synthase and synthetase
18	O75766	TRIP protein	3	1.00	0.59 ± 0.14	0.92	Transcription factor
19	Q96KR1	Putative zinc finger protein	2	1.00	0.59 ± 0.09	1.04	Nucleic acid binding
20	Q9P0L0	Vesicle-associated membrane protein-associated protein A	2	1.00	0.59 ± 0.17	0.83	Synthase and synthetase
21	Q00839	Heterogeneous nuclear ribonucleoprotein U	6	1.00	0.60 ± 0.20	1.13	Nucleic acid binding
22	Q15097	Polyadenylate-binding protein 2	3	1.00	0.64 ± 0.09	1.33	Nucleic acid binding
23	P11518	60S ribosomal protein L7a	3	1.00	0.67 ± 0.09	1.26	Nucleic acid binding
24	P82979	Nuclear protein Hcc-1	1	0.96	0.67 ± 0.05	NA ^d	Nucleic acid binding
25	Q08945	Structure-specific recognition protein 1	4	1.00	0.68 ± 0.14	1.19	Transcription factor
26	Q9NXD9	Hypothetical protein FLJ20303	2	1.00	0.68 ± 0.29	NA ^d	Nucleic acid binding
27	O43172	U4/U6 small nuclear ribonucleoprotein Prp4	2	1.00	0.69 ± 0.29	0.55	Nucleic acid binding
28	P26358	DNA (cytosine-5)-methyltransferase 1	1	0.99	0.69 ± 0.04	0.70	Nucleic acid binding
29	P26368	Splicing factor U2AF 65 kDa subunit	2	1.00	0.69 ± 0.06	1.26	Nucleic acid binding
30	Q09161	80 kDa nuclear cap binding protein	3	1.00	1.32 ± 0.52	1.73	Nucleic acid binding
31	P14866	Heterogeneous nuclear ribonucleoprotein L	2	1.00	1.33 ± 0.29	1.15	Nucleic acid binding
32	P48634	Hypothetical protein (Fragment)	2	1.00	1.33 ± 0.13	1.19	Nucleic acid binding
33	Q13242	Splicing factor, arginine/serine-rich 9	2	1.00	1.33 ± 0.22	0.75	Nucleic acid binding
34	Q9NQ74	Exosome complex exonuclease RRP46	1	0.97	1.36 ± 0.13	NA ^d	Nucleic acid binding
35	Q9GZT3	DC50 (DC23)	2	1.00	1.41 ± 0.26	NA ^d	Nucleic acid binding
36	P31949	Calgizzarin	2	1.00	1.44 ± 0.19	2.06	Select calcium binding protein
37	P33240	Cleavage stimulation factor, 64 kDa subunit	1	0.90	1.44 ± 0.25	0.73	Nucleic acid binding
38	Q9UKK9	ADP-sugar pyrophosphatase YSA1H	1	1.00	1.44 ± 0.21	NA ^d	Hydrolase
39	P13489	Placental ribonuclease inhibitor	6	1.00	1.45 ± 0.24	1.02	Select regulatory molecule
40	P49321	Nuclear autoantigenic sperm protein	2	1.00	1.50 ± 0.34	0.77	transporter
41	Q96B26	U6 snRNA-associated Sm-like protein LSM7	1	0.99	1.52 ± 0.20	3.41	Nucleic acid binding
42	P33316	Deoxyuridine 5'-triphosphate nucleotidohydrolase	3	1.00	1.54 ± 0.21	1.14	Phosphatase
43	P12956	ATP-dependent DNA helicase II, 70 kDa subunit	2	1.00	1.61 ± 0.20	0.81	Nucleic acid binding
44	Q96P11	NOL1R	1	1.00	1.61 ± 0.32	NA ^d	Nucleic acid binding
45	P55795	Heterogeneous nuclear ribonucleoprotein H'	1	0.99	1.62 ± 0.41	1.12	Nucleic acid binding
46	Q01081	Splicing factor U2AF 35 kDa subunit	1	0.99	1.63 ± 0.24	1.29	Nucleic acid binding
47	P49915	GMP synthase [glutamine-hydrolyzing]	2	0.98	1.71 ± 0.34	1.23	Synthase
48	P05455	Lupus La protein	1	1.00	1.82 ± 0.10	1.51	Nucleic acid binding
49	Q9BV68	Hypothetical protein	2	0.93	1.82 ± 0.28	1.35	Nucleic acid binding
50	Q9Y3A5	Shwachman-Bodian-Diamond syndrome protein	1	1.00	1.83 ± 0.30	NA ^d	Molecular function unclassified
51	P55769	NHP2-like protein 1	2	1.00	1.86 ± 0.69	2.01	Nucleic acid binding
52	P09429	High mobility group protein 1	5	1.00	1.92 ± 0.24	1.25	Transcription factor
53	P18754	Regulator of chromosome condensation	1	0.99	1.92 ± 0.25	0.87	Nucleic acid binding
54	P31939	Bifunctional purine biosynthesis protein PURH	4	1.00	2.07 ± 0.64	2.21	Hydrolase
55	Q9Y265	RuvB-like 1	1	0.99	2.03 ± 0.38	0.70	Nucleic acid binding
56	Q16531	DNA damage binding protein 1	1	0.93	2.26 ± 0.13	1.25	Nucleic acid binding
57	Q15365	Poly(rC)-binding protein 1	5	1.00	2.29 ± 0.34	1.38	Nucleic acid binding
58	P00492	Hypoxanthine-guanine phosphoribosyltransferase	4	1.00	2.67 ± 0.19	1.25	Transferase
59	Q9HCU5	Prolactin regulatory element-binding protein	1	0.98	3.24 ± 0.29	5.10	Nucleic acid binding
60	P36959	GMP reductase 1	1	0.99	33.64 ± 6.76	1.57	Oxidoreductase
Oncogenesis							
1	P08729	Keratin, type II cytoskeletal 7	1	1.00	0.00 ± 0.00	0.02	Select regulatory molecule
2	Q9BYX2	TBC1 domain family member 2	1	0.96	0.13 ± 0.03	NA ^d	Select regulatory molecule
3	Q06136	Follicular variant translocation protein 1 precursor	1	1.00	0.31 ± 0.05	0.43	Oxidoreductase
4	P57764	DFN5-like protein FLJ12150	1	1.00	0.33 ± 0.24	NA ^d	Molecular function unclassified
5	P13797	T-plastin	4	1.00	0.34 ± 0.21	0.33	Cytoskeletal protein
6	P46013	Antigen KI-67	3	1.00	0.52 ± 0.05	0.49	Miscellaneous function
7	P18206	Vinculin	4	1.00	0.54 ± 0.27	0.40	Cytoskeletal protein
8	Q92688	Acidic leucine-rich nuclear phosphoprotein 32 family member B	2	1.00	1.66 ± 0.16	1.01	Select regulatory molecule
9	P39687	Acidic leucine-rich nuclear phosphoprotein 32 family member A	2	0.99	1.67 ± 0.41	1.40	Select regulatory molecule
Other Metabolism							
1	Q16881	Thioredoxin reductase, cytoplasmic precursor	1	0.93	0.6 ± 0.20	0.56	Oxidoreductase
2	Q04760	Lactoylglutathione lyase	2	1.00	1.31 ± 0.20	1.11	Lyase
3	P49189	Aldehyde dehydrogenase, E3 isozyme	1	0.99	1.44 ± 0.86	0.65	Oxidoreductase
4	P10599	Thioredoxin	3	1.00	1.63 ± 0.10	2.11	Oxidoreductase
5	Q8UBL6	Uroporphyrinogen decarboxylase	1	1.00	1.99 ± 0.27	0.98	Lyase
6	Q9BW04	Hypothetical protein FLJ36507	1	1.00	6.72 ± 0.92	NA ^d	Select regulatory molecule

Table I (Continued)

	UniProt	protein name	peptide number	prob. ^a	cICAT ^b	Affymetrix ^c	molecular function
Protein Metabolism and Modification							
1	P21980	Protein-glutamine gamma-glutamyltransferase	1	0.97	0.00 ± 0.00	NA ^d	Transferase
2	Q9UK22	F-box only protein 2	2	1.00	0.14 ± 0.04	1.26	Miscellaneous function
3	Q86YM9	Calpastatin (Fragment)	1	1.00	0.25 ± 0.22	0.78	Select regulatory molecule
4	P48594	Squamous cell carcinoma antigen 2	1	1.00	0.27 ± 0.03	NA ^d	Select regulatory molecule
5	P63208	S-phase kinase-associated protein 1A	1	0.97	0.35 ± 0.05	0.90	Nucleic acid binding
6	Q15418	Ribosomal protein S6 kinase alpha 1	2	1.00	0.39 ± 0.09	0.27	Kinase
7	P15170	G1 to S phase transition protein 1 homologue	2	1.00	0.41 ± 0.27	0.60	Nucleic acid binding
8	O43776	Asparaginyl-tRNA synthetase, cytoplasmic	2	1.00	0.48 ± 0.13	0.96	Nucleic acid binding
9	P18282	Destrin	5	0.91	0.50 ± 0.13	0.93	Cytoskeletal protein
10	Q13564	Amyloid protein-binding protein 1	2	1.00	0.50 ± 0.05	0.43	Ligase
11	P36578	60S ribosomal protein L4	2	1.00	0.51 ± 0.05	1.86	Nucleic acid binding
12	Q16763	Ubiquitin-conjugating enzyme E2-24 kDa	1	0.98	0.54 ± 0.07	1.17	ligase
13	P13862	Casein kinase II beta chain	2	1.00	0.55 ± 0.09	1.84	Kinase
14	P04643	40S ribosomal protein S11	3	1.00	0.59 ± 0.05	1.57	Nucleic acid binding
15	P04645	60S ribosomal protein L30	2	1.00	0.60 ± 0.03	1.34	Nucleic acid binding
16	Q12765	Y193_HUMAN Hypothetical protein KIAA0193	1	1.00	0.60 ± 0.26	1.13	Select regulatory molecule
17	P02433	60S ribosomal protein L32	2	1.00	0.63 ± 0.18	1.11	Nucleic acid binding
18	P50914	60S ribosomal protein L14	4	1.00	0.64 ± 0.08	2.35	Nucleic acid binding
19	P28838	Cytosol aminopeptidase	3	1.00	0.64 ± 0.25	0.95	Protease
20	Q92524	26S protease regulatory subunit S10B	2	1.00	0.65 ± 0.11	1.91	Hydrolase
21	P31689	DnaJ homologue subfamily A member 1	3	1.00	0.67 ± 0.52	0.56	Chaperone
22	Q15070	Cytochrome oxidase biogenesis protein OXA1, mitochondrial precursor	2	1.00	0.67 ± 0.09	0.88	Transporter
23	Q93009	Ubiquitin carboxyl-terminal hydrolase 7	1	1.00	0.67 ± 0.13	0.82	Protease
24	Q9Y5M8	Signal recognition particle receptor beta subunit	2	1.00	0.67 ± 0.05	0.96	signaling molecule
25	P08238	Heat shock protein 90 beta	4	1.00	0.68 ± 0.21	0.89	Chaperone
26	P49721	Proteasome subunit beta type 2	3	1.00	0.68 ± 0.11	1.08	Protease
27	Q96AE4	FUSE binding protein	1	1.00	0.69 ± 0.12	0.79	Nucleic acid binding
28	Q8N425	Likely ortholog of mouse ubiquitin-conjugating enzyme E2-230K	2	1.00	0.69 ± 0.07	NA ^d	ligase
29	Q99543	Zuotin related factor-1	1	0.96	1.39 ± 0.29	3.39	Chaperone
30	P30405	Peptidyl-prolyl cis-trans isomerase, mitochondrial precursor	1	1.00	1.40 ± 0.29	1.62	Isomerase
31	Q02790	FK506-binding protein 4	2	1.00	1.46 ± 0.15	1.17	Isomerase
32	Q9NR50	Translation initiation factor eIF-2B gamma subunit	1	0.99	1.48 ± 0.35	0.90	Nucleic acid binding
33	P49591	Seryl-tRNA synthetase	1	0.99	1.49 ± 0.41	2.87	Nucleic acid binding
34	P36776	Lon protease homolog, mitochondrial precursor	1	1.00	1.57 ± 0.41	1.25	protease
35	Q92598	Heat-shock protein 105 kDa	7	1.00	1.60 ± 0.17	1.33	Chaperone
36	P07858	Cathepsin B	3	1.00	1.61 ± 0.14	2.16	Protease
37	P53634	Cathepsin C	1	1.00	1.62 ± 0.36	0.28	Protease
38	P34932	Heat shock 70 kDa protein 4	3	1.00	1.67 ± 0.26	2.24	Chaperone
39	P35998	26S protease regulatory subunit 7	2	1.00	1.69 ± 0.51	2.37	Hydrolase
40	P14868	Aspartyl-tRNA synthetase	1	0.96	1.73 ± 1.30	2.36	Nucleic acid binding
41	P62276	40S ribosomal protein S29	1	1.00	1.74 ± 0.32	1.59	Nucleic acid binding
42	Q13404	DNA-binding protein	1	0.94	1.74 ± 0.06	NA ^d	Ligase
43	Q99538	Legumain precursor	1	0.97	1.77 ± 0.30	NA ^d	Protease
44	Q9UBT2	ANTHRACYCLINE-associated resistance ARX	3	1.00	1.86 ± 0.23	2.19	Ligase
45	Q15765	Prefoldin subunit 3	2	1.00	1.87 ± 0.43	3.08	transporter
46	P27924	Ubiquitin-conjugating enzyme E2-25 kDa	1	0.96	1.95 ± 0.23	1.60	Ligase
47	Q92507	ES1 protein homologue, mitochondrial precursor	1	1.00	2.28 ± 0.24	110.10	Miscellaneous function
48	P25685	DnaJ homologue subfamily B member 1	2	1.00	2.46 ± 0.41	1.58	Chaperone
49	P29144	Tripeptidyl-peptidase II	4	1.00	3.01 ± 1.10	2.92	Hydrolase
50	P41250	Glycyl-tRNA synthetase	1	1.00	6.8 ± 2.66	2.79	Synthase and synthetase
Signal Transduction and Regulation							
1	Q86ST5	Similar to centaurin, gamma 3	1	0.99	0.00 ± 0.00	NA ^d	GTP binding
2	Q9UBI6	Guanine nucleotide-binding protein G(I)/G(S)/G(O) gamma-12 subunit	2	1.00	0.20 ± 0.04	0.58	Select regulatory molecule
3	P35613	Basigin precursor	2	1.00	0.30 ± 0.03	0.56	Signaling molecule
4	P06749	Transforming protein RhoA	4	1.00	0.35 ± 0.22	1.13	Select regulatory molecule
5	P05362	Intercellular adhesion molecule-1 precursor	2	1.00	0.38 ± 0.05	NA ^d	Cell adhesion molecule
6	Q13578	DNA repair protein	1	1.00	0.41 ± 0.07	NA ^d	Phosphatase
7	P30086	Phosphatidylethanolamine-binding protein	1	0.98	0.48 ± 0.09	0.62	Select regulatory molecule
8	P08134	Transforming protein RhoC	2	0.99	0.54 ± 0.13	0.58	Select regulatory molecule
9	P84097	Rho-related GTP-binding protein RhoG	1	1.00	0.55 ± 0.13	0.83	Select regulatory molecule
10	Q9Y572	Receptor-interacting serine/threonine protein kinase 3	1	0.91	0.58 ± 0.05	NA ^d	Kinase
11	P43490	Pre-B cell enhancing factor precursor	2	1.00	0.65 ± 0.09	1.46	Signaling molecule
12	P60953	Cell division control protein 42 homologue	3	1.00	0.67 ± 0.20	0.88	Select regulatory molecule
13	P04901	Guanine nucleotide-binding protein G(I)/G(S)/G(T) beta subunit 1	1	1.00	0.68 ± 0.05	0.70	Select regulatory molecule
14	P04899	Guanine nucleotide-binding protein G(i), alpha-2 subunit	1	1.00	1.32 ± 0.24	0.44	Select regulatory molecule
15	Q9HCY8	S100 calcium-binding protein A14	2	1.00	1.41 ± 0.19	0.36	Select calcium binding protein
16	P29312	14-3-3 protein zeta/delta	3	1.00	1.48 ± 0.12	1.40	Select regulatory molecule

Table I (Continued)

	UniProt	protein name	peptide number	prob. ^a	cICAT ^b	Affymetrix ^c	molecular function
Signal Transduction and Regulation							
17	Q12797	Aspartyl/asparaginyl beta-hydroxylase	1	0.91	1.69 ± 0.34	1.09	Oxidoreductase
18	O60716	Catenin, 120ctn	1	1.00	1.78 ± 0.34	1.16	Cell junction protein
19	P16144	Integrin beta-4	2	1.00	1.96 ± 0.96	1.91	Cell adhesion molecule
20	P01116	Transforming protein p21A	1	0.98	2.07 ± 0.49	2.78	Select regulatory molecule
21	P16422	Tumor-associated calcium signal transducer 1 precursor	1	0.99	2.12 ± 0.43	1.49	Signaling molecule
22	Q86VB7	Similar to CD163 antigen	1	0.95	2.18 ± 0.46	NA ^d	Receptor
23	P31947	14–3–3 protein sigma	4	1.00	2.45 ± 0.17	1.79	Select regulatory molecule
24	P27348	14–3–3 protein tau	3	1.00	2.51 ± 0.24	1.56	Select regulatory molecule
25	P23229	Integrin alpha-6	1	0.99	3.34 ± 1.06	1.81	Cell adhesion molecule
26	P35222	Catenin, beta	4	1.00	5.89 ± 1.3	1.91	Signaling molecule
27	O95996	APCL protein	1	0.99	8.37 ± 0.35	NA ^d	Cytoskeletal protein
28	Q8IXW0	Hypothetical protein	1	0.94	9999 ± 0.00	NA ^d	Signaling molecule
Transport							
1	P16615	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	2	1.00	0.64 ± 0.16	0.50	Transporter
2	P35658	Nuclear pore complex protein Nup214	2	1.00	0.69 ± 0.08	NA ^d	Transporter
3	P45880	Voltage-dependent anion-selective channel protein 2	1	1.00	0.69 ± 0.13	1.34	Ion channel
4	O00299	Chloride intracellular channel protein 1	4	1.00	2.38 ± 0.63	1.99	Ion channel
Biological Process Unclassified							
1	Q9H690	Hypothetical protein FLJ22484	2	1.00	0.00 ± 0.00	NA ^d	Molecular function unclassified
2	Q9BSE0	Hypothetical protein	1	0.94	0.13 ± 0.01	NA ^d	Molecular function unclassified
3	Q8N8W6	Hypothetical protein FLJ38753	2	1.00	0.24 ± 0.08	NA ^d	Molecular function unclassified
4	Q96G34	DC12 protein	1	0.91	0.37 ± 0.09	NA ^d	Molecular function unclassified
5	Q9BTE3	Hypothetical protein FLJ13081	2	1.00	0.50 ± 0.05	NA ^d	Molecular function unclassified
6	Q96FT1	Hypothetical protein (Fragment)	1	0.98	0.50 ± 0.08	NA ^d	Nucleic acid binding
7	Q86U38	Similar to RIKEN cDNA 2610027L16 gene	1	1.00	0.56 ± 0.07	NA ^d	Molecular function unclassified
8	Q9BQ69	Protein LRP16	2	1.00	0.56 ± 0.22	NA ^d	transporter
9	Q9Y2L0	KIAA1007 protein (Fragment)	2	1.00	0.59 ± 0.08	NA ^d	Molecular function unclassified
10	Q9Y4W6	AFG3-like protein 2	4	1.00	0.63 ± 0.12	0.81	protease
11	Q14166	Hypothetical protein KIAA0153	2	0.98	0.65 ± 0.04	1.30	Molecular function unclassified
12	Q13826	Autoantigen	2	1.00	0.69 ± 0.14	NA ^d	protein binding
13	P42704	130 kDa leucine-rich protein	2	1.00	1.54 ± 0.17	1.32	Nucleic acid binding
14	Q8N3B3	Hypothetical protein (Fragment)	2	1.00	1.57 ± 0.25	NA ^d	Molecular function unclassified
15	Q9H3K6	My016 protein	1	0.95	1.91 ± 0.41	3.44	Molecular function unclassified
16	O75223	Hypothetical protein	2	1.00	2.2 ± 0.23	2.60	Molecular function unclassified
17	Q9H4L5	Oxysterol binding protein-related protein 3	1	1.00	3.06 ± 5.19	3.73	Protein binding
18	Q810P0	similar to testis expressed sequence 13A	1	1.00	7.51 ± 9.10	NA ^d	Molecular function unclassified
19	Q9H6W2	Hypothetical protein FLJ21810	1	0.98	11.04 ± 2.88	NA ^d	Molecular function unclassified

^a ProteinProphet score. ^b The fold change in the protein ratio of TMC-1/SC-M1 cells. ^c The fold change in the mRNA ratio of TMC-1/SC-M1 cells. ^d ND, not available.

of microarray and proteomic results were performed by their corresponding gene names. Among the 240 differentially expressed proteins, 226 of the proteins found by cICAT analysis could be matched to their corresponding mRNA expression levels. In the correlation scattergram shown in Figure 4, proteins for which mRNA fold changes have a positive correlation with protein level changes appear on or near the 45° diagonal. The majority of differentially expressed mRNAs and cognate proteins correlated positively with a statistically significant correlation coefficient of 0.29. In TMC-1 cells, 29 of the 226 proteins with elevated mRNA levels were found to be at least overexpressed by 1.3-fold at the protein level. Likewise, 38 TMC-1 cell proteins showed a correlation between reduced protein levels and reduced mRNA levels. However, the result also revealed that some proteins are not transcriptionally regulated or show opposite regulation patterns. As shown in Figure 4, seven proteins showed a negative correlation between protein and mRNA expression levels. This is in accordance with the conclusion that noncell-structure-related proteins show poor correlation between mRNA and protein levels.³⁰

Validation of Candidate Genes That Are Differentially Expressed in SC-M1 and TMC-1 Cells. To further evaluate the results obtained from the global comparative expression studies, we examined the expression status of several candidate

genes identified by comparative proteomics in the SC-M1 and TMC-1 cells. β -Catenin, galectin 1, IQ motif-containing GTPase activating protein (IQGAP1), vimentin, integrin α 6, and integrin β 4 were chosen because they have been previously shown to be involved in the pathogenic development of tumor formation and progression, and in particular, some are implicated in the process of acquiring metastatic capability in various cancers (Table 3). Semiquantitative RT-PCR analysis was performed to examine the transcript levels of these genes. In agreement with the proteomic and genomic findings, higher levels of β -catenin, galectin 1, vimentin, integrin α 6, and integrin β 4 transcripts were observed in the metastatic TMC-1 cells, whereas IQGAP1 was expressed at a lower level in TMC-1 cells (Figure 5A). Integrins are the major cell surface receptors involved in cell–cell attachment and cell migration. The flow cytometry analysis showed significantly higher levels of integrin α 6 and β 4 were localized at the cell surface of metastatic TMC-1 cells than SC-M1 cells (Figure 5B). β -Catenin plays dual roles in adhesion and transducer/transcriptional regulator.³¹ When the Wnt signal is activated, β -catenin is not phosphorylated and accumulates in the cytoplasm, leading to the subsequent nuclear translocation.³² Therefore, we examined the expression status of β -catenin by immunocytochemical staining. As shown in Figure 5C, β -catenin was abundantly expressed and accumulated in the

Figure 3. Quantitation and confirmation of differential expression of β -catenin in SC-M1 and TMC-1 cells. The MS/MS spectrum (A) and MS spectrum (B) of the light (TMC-1) and heavy (SC-M1) cLCAT isotope-labeled peptide AGDREDITEPAICALR. (C) The corresponding extracted ion chromatogram revealed a 5.9-fold overexpression of β -catenin in TMC-1 cells. (D) Western blotting analysis confirmed that β -catenin was overexpressed in TMC-1 cells.

Table 2. Pathways Containing Genes Differentially Expressed between TMC-1 and SC-M1 Cells from Microarray Gene Expression Measurements

pathway	N^a	T^b	% differentially expressed genes
Proepithelin Conversion to Epithelin and Wound Repair Control	6	6	100
Inhibition of Matrix Metalloproteinases	8	10	80
IL-18 Signaling Pathway	7	9	78
OX40 Signaling Pathway	5	7	71
Multidrug Resistance Factors	5	7	71
Neuroregulin Receptor Degradation Protein-1 Controls ErbB3 Receptor Recycling	9	13	69
Antigen-dependent B Cell Activation	11	16	69
GATA3 Activation of the Th2 Cytokine Genes Expression	10	15	67
CDK Regulation of DNA Replication	6	9	67
Nitric Oxide Signaling Pathway	6	9	67
Bystander B Cell Activation	7	11	64
Platelet Amyloid Precursor Protein Pathway	7	11	64
Sonic Hedgehog (Shh) Pathway	7	11	64
TSP-1-Induced Apoptosis in Microvascular Endothelial Cells	5	8	63
Vit-C in Brain	5	8	63
METS Effect on Macrophage Differentiation	12	20	60
FOSB Gene Expression and Drug Abuse	3	5	60

^a N = number of differentially expressed genes in the pathway. ^b T = the total number of probed genes in the pathway.

cytoplasm of TMC-1 cells, whereas it had a more restricted distribution in SC-M1 cells, mostly localized to the plasma membranes.

Because apoptosis dysfunction in metastases has been suggested to participate in their poor response to conventional anticancer treatments³³ and because of our finding of dif-

Figure 4. Scatter plot showing the protein ratios obtained by the cICAT analysis, and 2D-LC-MS/MS and mRNA ratios obtained from the Affymetrix DNA microarray for SC-M1 and TMC-1 cells. The labeled proteins showed a significant negative correlation between protein and mRNA expression levels. Both expression ratios are plotted in logarithmic scale.

ferential expression of proteins in the TSP-1-induced apoptosis pathway, we also examined whether SC-M1 and TMC-1 cells displayed different degrees of resistance toward chemotherapeutic drug-induced apoptosis. Cell viabilities were measured after treatment with increasing concentrations of 5-fluorouracil. As shown in Figure 6, 5-fluorouracil increased cell death in a time- and dose-dependent manner in SC-M1 cells. By comparison, TMC-1 cells were more resistant to 5-fluorouracil-induced cell death.

Discussion

Comparison of Genomic and Proteomic Profiling. In this study, we compared the global protein and mRNA patterns of nonmetastatic SC-M1 and metastatic TMC-1 gastric cancer cells to elucidate the molecular changes associated with carcinogenesis and metastasis. As shown in Tables 1 and 2, the functional classification of the differentially expressed proteins and mRNAs indicates that the DNA microarray and cICAT technique have different functional category detection preferences. The limited correlation ($r^2 = 0.2872$, Figure 4) between the steady-state levels of mRNA and protein abundance is not surprising, because they are individually controlled by their own rates of synthesis and turnover, determined by, for example, gene-specific chromatin structure, sequence content, and binding of accessory proteins.³⁴ Alternatively, some observed examples of negative correlation may suggest unexpected biological uncoupling between the expression levels of mRNA and the corresponding proteins.

DNA microarrays are generally considered to have better sensitivity and to provide more comprehensive identification and quantification of transcriptional changes. However, about 67 proteins (30% of the differentially expressed proteins) that were reproducibly identified and quantified by cICAT were not detected by DNA microarray analysis. Many of these orphan proteins were identified with multiple peptide hits; therefore, we are confident of their identification. Most prominent among these proteins are transcription initiation factor IIB (in the nucleic acid metabolism category), microtubule-associated

protein RP/EB family member 1, cytochrome P450 51A1, Innexin 1, and GMP reductase 1 (Table 1). These observations highlight the advantage of the addition of the cICAT strategy to a genomics analysis to reveal subtle changes in the proteome not observed in the transcriptome.

Proteins Differentially Expressed between SC-M1 and TMC-1 Cells. Although the changes in protein and mRNA levels did not show a strong linear correlation, the proteome and transcriptome analysis identified gene products from functionally similar categories. Of particular interest, a significant number of gene products that were up-regulated in TMC-1 cells are involved in cancer metastasis, such as cell-cell adhesion signaling (catenin-120nt, α -catenin, β -catenin, integrin $\alpha 6$, integrin $\beta 4$, RhoG, and IQGAP1), cell metastasis and motility (cytokeratin, myosin, S100 calcium binding protein (S100) family, and vimentin), cell cycle and proliferation (cdc42 and cell division protein kinase 6), tumor immunity and defense (galectin 1 and high mobility group protein 1), and protein degradation (cathepsin B, C). The protein expression levels and a comparison of the results of this study with previous reports are shown in Table 3. The different expression levels of these proteins may reflect biological differences leading to the distinct phenotype of the metastatic TMC-1 cell line.

Identification of accurate prognostic factors helps in therapeutic decision-making, in particular in patients with advanced stage cancer. To explore the possibility that the identified proteins may also serve as markers of metastasis for other types of human cancers, we examined the expression of a subset of proteins, β -catenin, galectin 1, vimentin, integrin $\alpha 6$, and integrin $\beta 4$ by semiquantitative RT-PCR analysis in various cancer cells with differing invasion potential as assessed by the Boyden chamber assay. Our results showed that higher levels of vimentin and galectin 1 were readily detected in all of the cell lines with demonstrated invasive capability, whereas much lower or insignificant levels of expression were observed in cell lines with low invasiveness (unpublished data, Supplemental Figure 1, Supporting Information). Galectins are a family of structurally related carbohydrate-binding proteins and con-

Table 3. Summary of Differentially Expressed Proteins Related to Metastasis

gene name	UniProt ID	protein name	prob. ^a	molecular function	this study		reprotd expression change ^d	cancer type ^f
					cICAT ^b	Affymetrix ^c		
Cell to Cell Adhesion Signaling								
CTNND1	O60716	Catenin, 120ctn	1.00	Interacts with cadherin to regulate cell-adhesion properties	1.78	1.21	Up	Fibroblasts and Epithelial Cells (1)
CTNNA1	P35221	Catenin, alpha-1	1.00	Actin cross linking at adherens junctions	0.97	0.90	Down	Lung Carcinomas (2), Prostate Cancer (3)
CTNNB1	P35222	Catenin, beta	1.00	Modulates of cytoskeletal dynamics and cell proliferation	5.89	1.91	Down	Breast Cancer (4), Colorectal Cancer (5)(6)
ITGA6	P23229	Integrin alpha 6	0.99	Cell–matrix adhesion,cell-substrate junction assembly, cell adhesion	3.34	1.81	Up	Breast Carcinoma Cells (7)
ITGB4	P16144	Integrin beta 4	1.00	Cell–matrix adhesion, cell adhesion	1.96	1.80	Up	Breast Carcinoma Cells (7)
PRDX5	P30044	Peroxiredoxin 5	1.00	Contains high antioxidant efficiency to effect cell differentiation and apoptosis	1.84	NA ^e	Up	Gastric Cancer (8)
PBP	P30086	Raf kinase inhibitor protein	0.98	Suppresses metastasis through decreasing angiogenesis and vascular invasion	0.48	0.62	Down	Osteosarcoma (9)
RHOG	P84097	Rho-related GTP-binding protein RhoG	1.00	Small GTPase-mediated signal transduction, positive regulation of cell proliferation, actin cytoskeleton organization	0.55	0.83	Up	Breast Cancer (10)
IQGAP1	P46940	Ras GTPase-activating-like protein IQGAP1	1.00	Involves in actin cytoskeleton assembly and E-cadherin-mediated cell adhesion	0.35	0.19	Up	Gatric Cancer (11)
VCL	P18206	Vinculin	1.00	Mediates the interactions between integrins and the actin cytoskeleton	0.54	0.40	Down	Basal and Squamous Cell Tumors (12)
Cell Metastasis and Migration								
ACTN4	O43707	Alpha-actinin 4	1.00	Anchors actin to a variety of intracellular structures	1.37	1.59	Up	Colorectal Cancer (13)
ANXA1	P04083	Annexin I	1.00	Regulates of cell proliferation, promotes membrane fusion	0.68	1.02	Up/down	Hepatocellular Carcinoma (14), Lung Cancer (15), Head/Neck Cancer (16), Breast Carcinoma (17)
KRT17	Q04695	Cytoskeletal 17	1.00	Marker of basal cell differentiation in complex epithelia	0.03	NA ^e	Up	Breast Cancer (18)
KRT5	P13647	Cytoskeletal 5	1.00	Epidermis development, cytoskeleton organization and biogenesis, cellular morphogenesis	3.43	NA ^e	Up	Breast Cancer (18)
TPM3	Q8NAG3	Tropomyosin 3	1.00	Stabilizes of actin filaments	0.48	NA ^e	Down	Prostate Cancer (19), Breast Cancer (20)
TPM4	P67936	Tropomyosin 4	1.00	Binds to actin filaments in muscle and nonmuscle cells	0.61	0.82	Up/down	Lung Cancer (21)
VIM	P08670	Vimentin	0.99	Intermediate filament-based process in mesenchymal cells related to migration	25.67	NA ^e	Up	Breast Carcinoma (17), Hepatocellular Carcinoma (22), Propstate Cancer (23)
S100A11	P31949	S100 calcium-binding protein A11	1.00	Actin filament bundle formation and cell motility	1.34	NA ^e	Up	Hepatocellular Carcinoma (14)
COMT	P21964	Catechol O-methyltransferase	1.00	Involved in cancer progression and lymph node metastasis	1.35	0.81	Up	Breast Cancer (24)
Cell Cycle and Cell Proliferation Regulation								
SKP1A	P63208	S-phase kinase-associated protein 1A	0.97	Mediates the ubiquitination of proteins involved in cell cycle progression, signal transduction and transcription	0.35	0.80	Up	Colorectal Carcinoma (25), Hepatocellular Carcinoma (26)
CDC42	P60953	Cell division control protein 42 homologue	1.00	Regulates cadherin-mediated cell–cell adhesion	0.67	0.88	Up	Breast Cancer (27)
CDK6	Q00534	Cell division kinase 6	1.00	Related to cell proliferation, tumor heterogeneity, invasion and metastasis	1.72	NA ^e	Up	Retinoblastoma (28)Immunity and Defense
Immunity and Defense								
HMGB1	P09429	High mobility group protein 1	1.00	Interacts with transcription factors and regulates of transcription related to tumor growth and invasion	1.92	1.24	Up	Gastrointestinal Stromal Tumors (29)
CD44	Q16208	CD44	0.96	Mediates cell–cell and cell–matrix interactions through high affinity for hyaluronic acid	1.01	0.58	Up	Melanoma (30), Gastric Carcinoma (31)
LGALS1	P09382	Galectin-1	1.00	Promotes cancer cell invasion and metastasis	2.33	0.90	Up	Breast Carcinoma (32), Astrocytoma, Melanoma, Prostate, Colon, Bladder, Ovary (33)
HSPD1	P10809	Heat shock protein 60	1.00	Facilitates the correct folding of imported proteins	1.5	1.12	Down	Lung Cancer (15)
HSPCB	P08238	Heat shock protein 90 beta	1.00	Promotes the maturation of MMP2 involved in invasive cancer	0.68	0.87	Up	Murine Fibrosarcoma (34)
HLAA	P10892	MHC class I antigen	1.00	Inhibits evasion of the immune system and enhances tumor growth	0.64	NA ^e	Up	Gastric Cancer (35)

Table 3. (Continued)

gene name	UniProt ID	protein name	prob. ^a	molecular function	this study		reputed expression change ^d	cancer type ^f
					cICAT ^b	Affymetrix ^c		
Protein Degradation								
CTSB	P07858	Cathepsin B	1.00	Protein targeting, proteolysis and peptidolysis	1.61	2.16	Up	Melanoma (30), Breast Cancer (4), Lung cancer (31)
CTSC	P53634	Cathepsin C	1.00	Intracellular protein degradation and turnover	1.62	0.28	Up	Nonsmall Cell Lung Cancer (36)
CCT2	P78371	T-complex protein beta subunit	1.00	Related to p53 and activates DNA damage checkpoints	1.23	NA ^e	Down	Breast Carcinoma (17)

^a ProteinProphet Score. ^b The fold change in the protein ratio of TMC-1/SC-M1 cells. ^c The fold change in the mRNA ratio of TMC-1/SC-M1 cells. ^d Literature report(s) of change in protein level in metastatic specimens. ^e NA, not available. ^f References were provided as Supporting Information.

Figure 5. Expression of candidate genes in SC-M1 and TMC-1 cell lines. (A) RT-PCR analysis of β -catenin, galectin 1, vimentin, integrin $\alpha 6$, and integrin $\beta 4$ transcripts. The TMC-1/SC-M1 cell expression ratios obtained by Affymetrix DNA microarray and cICAT analysis are also shown for comparison. (B) Cell surface expression of integrins 6 and 4 as measured by flow cytometry. The specific fluorescence index was calculated as the ratio of the mean fluorescence values obtained with the specific Ab and the isotype control Ab. (C) The immunocytochemical images of β -catenin in SC-M1 and TMC-1 cells obtained by confocal laser microscopy.

tribute to tumor transformation, cell cycle regulation, apoptosis, cell adhesion, migration, and inflammation.^{9,10} Their expression correlates with tumor aggressiveness and acquisition of a metastatic phenotype in different tumor types.³⁵ Vimentin is a cytoskeletal protein that is often expressed when epithelial cells are stimulated to proliferate by growth factors.³⁶ In cancer, vimentin expression is associated with a dedifferentiated malignant phenotype, increased motility and invasiveness, and poor clinical prognosis.³⁶ The significant overexpression of galectin 1 and vimentin we observed in TMC-1 cells is consistent with the reported correlation between cellular adhesion and tumor aggregation³⁷ and may be possible markers for metastasis.³⁸

Although expression level changes in all of the proteins listed in Table 3 have been shown to be closely related to cancer metastasis, to the best of our knowledge, most have not been linked to gastric cancer. Previous studies have shown that the members of S100 family, S100A4, S100A6, and S100A11, are involved in cell motility, proliferation, differentiation, and cancer cell progression in breast cancer, colorectal cancer,³⁹

gall bladder cancer, and hepatocellular carcinomas.⁴⁰ We also found overexpression of the S100A11 protein in TMC-1 cells. Annexin I is reportedly up-regulated in highly metastatic lung carcinoma cell lines⁴¹ and down-regulated in a nonmetastatic type of breast cancer.⁴² Increased expression of S100A11 may be correlated with observed down-regulation of Annexin I, the binding target of S100A11.

In addition, cathepsin B and cathepsin C were up-regulated in metastatic TMC-1 cells compared to the nonmetastatic SC-M1 cells. Elevated expression of these lysosomal proteases, cathepsins B, L, and D, and diminished levels of their inhibitors have been observed in breast and prostate cancer, especially in aggressive cancer cells.⁴³ Evidence suggests that cathepsin B has an important function in matrix degradation and invasion, facilitating the growth capability of invasive carcinomas through loss of calcium-dependent cell–cell adhesion and proteolysis of E-cadherin, which is a tumor suppressor and effector of adherens junction function.⁴⁴ Our results suggest that cathepsin C may also be involved in the metastasis of gastric cancer cells.

Figure 6. Inhibition of cell viability of the SC-M1 and TMC-1 cell lines by 5-fluorouracil. The cell lines were incubated with (A) 10 μ M or (B) 50 μ M 5-fluorouracil for 24, 48, and 72 h. Cells were then assayed by Alamar Blue dye uptake.

Metastasis-Perturbed Pathway. Furthermore, the differentially expressed proteins identified in the current comparative approach enabled us to study their correlations in multiple cellular pathways. Using the pathway analysis tools available at the BioCarta, KEGG, PANTHER, and PathwayAssist, we found differences in expression of proteins in the pathways related to cytoskeleton rearrangement, cell–cell adhesion, and cell–ECM attachment between the metastatic TMC-1 and nonmetastatic SC-M1 cells. Figure 7 depicts a summary of these proteins and pathways, including the integrin signaling pathway, the

Rho cell motility signaling pathway, and pathways associated with cell–cell adhesion and adherens junctions.

Recently, increasing evidence suggests that epithelial–mesenchymal transitions (EMT) play a specific role in the migration of cells from primary tumors into the circulation.⁴⁵ The defining event for the epithelial–mesenchymal transition is disruption of E-cadherin-mediated cell–cell adhesion, which results in loss of epithelial morphology and acquisition of a motile, mesenchymal phenotype.⁴⁶ Cancer cells that undergo the EMT, therefore, lose their ability to adhere to their neighboring cells, resulting in weakened cell–cell adhesion and subsequent tumor cell migration. Much evidence indicates that loss or reduction of E-cadherin-mediated adhesion, thus, allowing cell migration, is required for the metastatic conversion of carcinomas. Cadherins are calcium-dependent adhesion molecules responsible for the establishment of tight cell–cell contact. Anchorage to the actin cytoskeleton occurs through the binding of the cytoplasmic domain of cadherins to β -catenin or γ -catenin, which concurrently binds to α -catenin.^{47,48} β -Catenin plays a central role in linking the membrane-bound E-cadherin to the cytoskeleton, and the interaction of β -catenin and IQGAP1 negatively regulates E-cadherin-mediated cell–cell adhesion.⁴⁹ Fukata et al. have proposed that β -catenin exists in a dynamic equilibrium between the E-cadherin– β -catenin– α -catenin complex and the E-cadherin– β -catenin–IQGAP1 complex at sites of cell–cell contact. Weakening cell–cell adhesion shifts the balance of β -catenin from the cadherin-bound pool to the cytoplasmic pool. In this study, immunocytochemical staining showed more cytoplasmic localization of β -catenin in the metastatic TMC-1 cells (Figure 5). A newly discovered β -catenin interaction partner, 14-3-3 ζ ,⁵⁰ enhances the transcription of β -catenin target promoters, leading to elevated

Figure 7. Summary of differentially expressed proteins in multiple pathways involved in cell–cell adhesion and cell–ECM interactions. The fold changes and standard deviation of the cCAT TMC-1/SC-M1 cell expression ratios are indicated. The solid lines indicate pathways derived from the BioCarta, KEGG, and PANTHER databases, and dashed lines indicate those found in the literature (using PathwayAssist).

steady-state levels of β -catenin in the cytoplasm. The accumulation of free cytosolic β -catenin has been reported to lead to translocation of β -catenin to the nucleus, which results in sustained transcriptional activation of oncogenes and causes tumor growth.⁵¹ Although we did not observe β -catenin in the nucleus, up-regulation of both 14-3-3 ζ and β -catenin in TMC-1 cells was observed, which suggests activation of the Wnt signaling pathway.⁵⁰

In addition, anchoring junction dynamics can be regulated by intricate interactions of the cadherin–catenin complex with Cdc42, a member of the Rho GTPase subfamily known for its role in regulating cell division.⁵² Rac1 and Cdc42 might also regulate the cleavage and endocytosis of E-cadherin, thereby modifying E-cadherin-mediated cell–cell adhesion. Meanwhile, Cdc42 and Rac1 also regulate cell–cell adhesion through IQGAP1 by the interaction of IQGAP1 with β -catenin, leading to stabilization of the E-cadherin–catenin complex.^{53,54} The decreased levels of IQGAP1 and Cdc42 we observed seem to imply that the Rac1–Cdc42–IQGAP1 complex is involved in the weak intercellular adhesion of TMC-1 cells.

The dysregulation of ECM adhesion, which is mediated mainly by integrins through GTPases, is another crucial determinant for tumor progression.⁵⁵ E-cadherin-mediated adhesion may also be regulated by integrin $\alpha_6\beta_4$ signaling through phosphatidylinositol 3 kinase to Rac, or alternatively, the transcription of E-cadherin can be suppressed through integrin-linked kinase (ILK) by up-regulating the transcriptional suppression of E-cadherin by repressors SNAIL/SLUG.⁵⁵ In addition, the p120ctn in the cytosol and in the cadherin-based complex provides one mechanism for communication between cadherin-mediated cell–cell junctions and the motile machinery of cells.⁵⁶ The observed up-regulation of p120ctn and down-regulation of RhoA suggest that p120ctn may negatively modulate RhoA to affect other downstream effectors. Active RhoA increases the stability of actin-based focal adhesions. Therefore, the decreased level of RhoA and its downstream effectors, vinculin and myosin heavy/light chain (MHC), that we observed in the cICAT analysis of TMC-1 cells also implies a weakened capability to adhere to the ECM.

In general, advanced gastric cancer is refractory to chemotherapy, leading to poor prognosis. To search for novel targets for screening and therapeutic intervention, it is increasingly important to recognize that discovery of disease-perturbed pathways is more fruitful than identification of individual gene products.⁵⁷ In summary, our results that both the cell–cell adhesion and the cell–ECM attachment pathways are disrupted provide identification of several differentially expressed pathways. Their associated proteins may be potential biomarkers of metastatic gastric tumor cells and of invasiveness. The regulation of cell–cell adhesion or dissociation is extremely complicated, and the plasticity the cells exhibit likely requires real-time control by transcriptional networks. Further investigation on the temporal profiling of these complex signaling cascades will provide insight into the invasive behavior and the metastatic potential of cancer cells.

Abbreviations: cICAT, cleavable isotope-coded affinity tag; TCEP, Tris-(2-carboxyethyl)phosphine; SCX, strong cation exchange; DAPI, 4',6-diamidino-2-phenylindole-dihydrochloride; ECM, extracellular matrix.

Acknowledgment. The authors acknowledge financial support from Academia Sinica and National Science Council in Taiwan. We are also grateful to Prof. Ruedi Aebersold, Prof.

David R. Goodlett, and Drs. Eugene C. Yi, and Hookeun Lee at Institute for Systems Biology, Seattle WA, for helpful advice of cICAT analysis.

Supporting Information Available: Figure showing the semiquantitative analysis of *galectin 1*, *vimentin*, *IQGAP1*, and *β -catenin* transcripts in different cancer cell lines, including breast, colon, ovary, bladder, and lung cancer; tables showing the protein identification summaries of TMC-1 and SC-M1 cells and of the cICAT analysis. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References

- (1) Parkin, D. M.; Pisani, P.; Ferlay, J. Estimates of the worldwide incidence of 25 major cancers in 1990. *Int. J. Cancer* **1999**, *80*, 827–41.
- (2) Stadtlander, C. T.; Waterbor, J. W. Molecular epidemiology, pathogenesis and prevention of gastric cancer. *Carcinogenesis* **1999**, *20*, 2195–208.
- (3) Peddanna, N.; Holt, S.; Verma, R. S. Genetics of gastric cancer. *Anticancer Res.* **1995**, *15*, 2055–64.
- (4) Hippo, Y.; Taniguchi, H.; Tsutsumi, S.; et al. Global gene expression analysis of gastric cancer by oligonucleotide microarrays. *Cancer Res.* **2002**, *62*, 233–40.
- (5) Cai, Z.; Chiu, J. F.; He, Q. Y. Application of proteomics in the study of tumor metastasis. *Genomics, Proteomics Bioinf.* **2004**, *2*, 152–66.
- (6) Wang, W.; Goswami, S.; Sahai, E.; Wyckoff, J. B.; Segall, J. E.; Condeelis, J. S. Tumor cells caught in the act of invading, their strategy for enhanced cell motility. *Trends Cell Biol.* **2005**, *15*, 138–45.
- (7) Lin, B.; White, J. T.; Lu, W.; et al. Evidence for the presence of disease-perturbed networks in prostate cancer cells by genomic and proteomic analyses: a systems approach to disease. *Cancer Res.* **2005**, *65*, 3081–91.
- (8) Sinha, P.; Poland, J.; Schnolzer, M.; Celis, J. E.; Lage, H. Characterization of the differential protein expression associated with thermoresistance in human gastric carcinoma cell lines. *Electrophoresis* **2001**, *22*, 2990–3000.
- (9) Choi, Y. R.; Kim, H.; Kang, H. J.; et al. Overexpression of high mobility group box 1 in gastrointestinal stromal tumors with KIT mutation. *Cancer Res.* **2003**, *63*, 2188–93.
- (10) Chen, J.; Kahne, T.; Rocken, C.; et al. 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.* **2004**, *3*, 1009–16.
- (11) He, Q. Y.; Cheung, Y. H.; Leung, S. Y.; Yuen, S. T.; Chu, K. M.; Chiu, J. F. Diverse proteomic alterations in gastric adenocarcinoma. *Proteomics* **2004**, *4*, 3276–87.
- (12) Das, S.; Sierra, J. C.; Soman, K. V.; et al. Differential protein expression profiles of gastric epithelial cells following *Helicobacter pylori* infection using ProteinChips. *J. Proteome Res.* **2005**, *4*, 920–30.
- (13) Juan, H. F.; Chen, J. H.; Hsu, W. T.; et al. Identification of tumor-associated plasma biomarkers using proteomic techniques: from mouse to human. *Proteomics* **2004**, *4*, 2766–75.
- (14) Washburn, M. P.; Wolters, D.; Yates, J. R., III. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat. Biotechnol.* **2001**, *19*, 242–7.
- (15) Washburn, M. P.; Ulaszek, R.; Deciu, C.; Schieltz, D. M.; Yates, J. R., III. Analysis of quantitative proteomic data generated via multidimensional protein identification technology. *Anal. Chem.* **2002**, *74*, 1650–7.
- (16) Aebersold, R.; Mann, M. Mass spectrometry-based proteomics. *Nature* **2003**, *422*, 198–207.
- (17) Gygi, S. P.; Rist, B.; Gerber, S. A.; Turecek, F.; Gelb, M. H.; Aebersold, R. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat. Biotechnol.* **1999**, *17*, 994–9.
- (18) Li, J.; Steen, H.; Gygi, S. P. Protein profiling with cleavable isotope-coded affinity tag (cICAT) reagents: the yeast salinity stress response. *Mol. Cell. Proteomics* **2003**, *2*, 1198–204.
- (19) Li, C.; Hong, Y.; Tan, Y. X.; et al. Accurate qualitative and quantitative proteomic analysis of clinical hepatocellular carcinoma using laser capture microdissection coupled with isotope-coded affinity tag and two-dimensional liquid chromatography mass spectrometry. *Mol. Cell. Proteomics* **2004**, *3*, 399–409.

- (20) Tzeng, C. C.; Meng, C. L.; Jin, L.; Hsieh, H. F. Cytogenetic studies of gastric adenocarcinoma. *Cancer Genet. Cytogenet.* **1991**, *55*, 67–71.
- (21) Shyu, R. Y.; Jiang, S. Y.; Jong, Y. J.; et al. Establishment and characterization of a human gastric carcinoma cell line TMC-1. *Cells Tissues Organs* **2004**, *177*, 37–46.
- (22) Keller, A.; Nesvizhskii, A. I.; Kolker, E.; Aebersold, R. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal. Chem.* **2002**, *74*, 5383–92.
- (23) Nesvizhskii, A. I.; Keller, A.; Kolker, E.; Aebersold, R. A statistical model for identifying proteins by tandem mass spectrometry. *Anal. Chem.* **2003**, *75*, 4646–58.
- (24) Li, X. J.; Zhang, H.; Ranish, J. A.; Aebersold, R. Automated statistical analysis of protein abundance ratios from data generated by stable-isotope dilution and tandem mass spectrometry. *Anal. Chem.* **2003**, *75*, 6648–57.
- (25) Kim, J. M.; Sohn, H. Y.; Yoon, S. Y.; et al. Identification of gastric cancer-related genes using a cDNA microarray containing novel expressed sequence tags expressed in gastric cancer cells. *Clin. Cancer Res.* **2005**, *11*, 473–82.
- (26) Wechsler-Reya, R. J.; Elliott, K. J.; Prendergast, G. C. A role for the putative tumor suppressor Bin1 in muscle cell differentiation. *Mol. Cell. Biol.* **1998**, *18*, 566–75.
- (27) Su, M. W.; Dorocicz, I.; Dragowska, W. H.; et al. Aberrant expression of T-plastin in Sezary cells. *Cancer Res.* **2003**, *63*, 7122–7.
- (28) Gerk, P. M.; Vore, M. Regulation of expression of the multidrug resistance-associated protein 2 (MRP2) and its role in drug disposition. *J. Pharmacol. Exp. Ther.* **2002**, *302*, 407–15.
- (29) Reiher, F. K.; Volpert, O. V.; Jimenez, B.; et al. Inhibition of tumor growth by systemic treatment with thrombospondin-1 peptide mimetics. *Int. J. Cancer* **2002**, *98*, 682–9.
- (30) Nishizuka, S.; Charboneau, L.; Young, L.; et al. Proteomic profiling of the NCI-60 cancer cell lines using new high-density reverse-phase lysate microarrays. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 14229–34.
- (31) Mulholland, D. J.; Dedhar, S.; Coetzee, G. A.; Nelson, C. C. Interaction of nuclear receptors with the Wnt/beta-catenin/Tcf signaling axis: Wnt you like to know? *Endocr. Rev.* **2005**, *26*, 898–915.
- (32) Feng, Y.; Lee, N.; Fearon, E. R. TIP49 regulates beta-catenin-mediated neoplastic transformation and T-cell factor target gene induction via effects on chromatin remodeling. *Cancer Res.* **2003**, *63*, 8726–34.
- (33) Oliver, L.; Cordel, S.; Barbieux, I.; et al. Resistance to apoptosis is increased during metastatic dissemination of colon cancer. *Clin. Exp. Metastasis* **2002**, *19*, 175–80.
- (34) Decherer, K. J. The transcriptome's drugable frequenters. *Drug Discovery Today* **2005**, *10*, 857–64.
- (35) Rabinovich, G. A. Galectin-1 as a potential cancer target. *Br. J. Cancer* **2005**, *92*, 1188–92.
- (36) Yoon, W. H.; Song, I. S.; Lee, B. H.; et al. Differential regulation of vimentin mRNA by 12-O-tetradecanoylphorbol 13-acetate and all-trans-retinoic acid correlates with motility of Hep 3B human hepatocellular carcinoma cells. *Cancer Lett.* **2004**, *203*, 99–105.
- (37) Kasamatsu, A.; Uzawa, K.; Nakashima, D.; et al. Galectin-9 as a regulator of cellular adhesion in human oral squamous cell carcinoma cell lines. *Int. J. Mol. Med.* **2005**, *16*, 269–73.
- (38) Irie, A.; Yamauchi, A.; Kontani, K.; et al. Galectin-9 as a prognostic factor with antimetastatic potential in breast cancer. *Clin. Cancer Res.* **2005**, *11*, 2962–8.
- (39) Komatsu, K.; Kobune-Fujiwara, Y.; Andoh, A.; et al. Increased expression of S100A6 at the invading fronts of the primary lesion and liver metastasis in patients with colorectal adenocarcinoma. *Br. J. Cancer* **2000**, *83*, 769–74.
- (40) Cui, J. F.; Liu, Y. K.; Pan, B. S.; et al. Differential proteomic analysis of human hepatocellular carcinoma cell line metastasis-associated proteins. *J. Cancer Res. Clin. Oncol.* **2004**, *130*, 615–22.
- (41) Jiang, D.; Ying, W.; Lu, Y.; et al. Identification of metastasis-associated proteins by proteomic analysis and functional exploration of interleukin-18 in metastasis. *Proteomics* **2003**, *3*, 724–37.
- (42) Kreunin, P.; Urquidí, V.; Lubman, D. M.; Goodison, S. Identification of metastasis-associated proteins in a human tumor metastasis model using the mass-mapping technique. *Proteomics* **2004**, *4*, 2754–65.
- (43) Nomura, T.; Katunuma, N. Involvement of cathepsins in the invasion, metastasis and proliferation of cancer cells. *J. Med. Invest.* **2005**, *52*, 1–9.
- (44) Jawhari, A. U.; Noda, M.; Farthing, M. J.; Pignatelli, M. Abnormal expression and function of the E-cadherin-catenin complex in gastric carcinoma cell lines. *Br. J. Cancer* **1999**, *80*, 322–30.
- (45) Huber, M. A.; Kraut, N.; Beug, H. Molecular requirements for epithelial-mesenchymal transition during tumor progression. *Curr. Opin. Cell Biol.* **2005**, *17*, 548–58.
- (46) Rosivatz, E.; Becker, I.; Specht, K.; et al. Differential expression of the epithelial-mesenchymal transition regulators snail, SIP1, and twist in gastric cancer. *Am. J. Pathol.* **2002**, *161*, 1881–91.
- (47) Beavon, I. R. The E-cadherin-catenin complex in tumour metastasis: structure, function and regulation. *Eur. J. Cancer* **2000**, *36*, 1607–20.
- (48) Fukata, M.; Nakagawa, M.; Itoh, N.; et al. Involvement of IQGAP1, an effector of Rac1 and Cdc42 GTPases, in cell–cell dissociation during cell scattering. *Mol. Cell. Biol.* **2001**, *21*, 2165–83.
- (49) Fukata, M.; Kaibuchi, K. Rho-family GTPases in cadherin-mediated cell–cell adhesion. *Nat. Rev. Mol. Cell Biol.* **2001**, *2*, 887–97.
- (50) Tian, Q.; Feetham, M. C.; Tao, W. A.; et al. Proteomic analysis identifies that 14–3–3zeta interacts with beta-catenin and facilitates its activation by Akt. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 15370–5.
- (51) Sparks, A. B.; Morin, P. J.; Vogelstein, B.; Kinzler, K. W. Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectal cancer. *Cancer Res.* **1998**, *58*, 1130–4.
- (52) Lui, W. Y.; Mruk, D. D.; Cheng, C. Y. Interactions among IQGAP1, Cdc42, and the cadherin/catenin protein complex regulate Sertoli-germ cell adherens junction dynamics in the testis. *J. Cell. Physiol.* **2005**, *202*, 49–66.
- (53) Kuroda, S.; Fukata, M.; Nakagawa, M.; et al. Role of IQGAP1, a target of the small GTPases Cdc42 and Rac1, in regulation of E-cadherin-mediated cell–cell adhesion. *Science* **1998**, *281*, 832–5.
- (54) Fukata, M.; Kuroda, S.; Nakagawa, M.; et al. Cdc42 and Rac1 regulate the interaction of IQGAP1 with beta-catenin. *J. Biol. Chem.* **1999**, *274*, 26044–50.
- (55) Guo, W.; Giancotti, F. G. Integrin signalling during tumour progression. *Nat. Rev. Mol. Cell Biol.* **2004**, *5*, 816–26.
- (56) Noren, N. K.; Liu, B. P.; Burridge, K.; Kreft, B. p120 catenin regulates the actin cytoskeleton via Rho family GTPases. *J. Cell. Biol.* **2000**, *150*, 567–80.
- (57) Kramer, R.; Cohen, D. Functional genomics to new drug targets. *Nat. Rev. Drug Discovery* **2004**, *3*, 965–72.

PR060212G