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Discovery of SLC3A2 Cell Membrane Protein as a Potential Gastric Cancer Biomarker: Implications in Molecular Imaging

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Abstract

Despite decreasing incidence and mortality, gastric cancer remains the second leading cause of cancer-related deaths in the world. Successful management of gastric cancer is hampered by lack of highly sensitive and specific biomarkers especially for early cancer detection. Cell surface proteins that are aberrantly expressed between normal and cancer cells are potentially useful for cancer imaging and therapy due to easy accessibility of these targets. Combining two-phase partition and isobaric tags for relative and absolute quantification methods, we compared the relative expression levels of membrane proteins between noncancer and gastric cancer cells. About 33% of the data set was found to be plasma membrane and associated proteins using this approach (compared to only 11% in whole cell analysis), several of which have never been previously implicated in gastric cancer. Upregulation of SLC3A2 in gastric cancer cells was validated by immunoblotting of a panel of 13 gastric cancer cell lines and immunohistochemistry on tissue microarrays comprising 85 matched pairs of normal and tumor tissues. Immunofluorescence and immunohistochemistry both confirmed the plasma membrane localization of SLC3A2 in gastric cancer cells. The data supported the notion that SLC3A2 is a potential biomarker that could be exploited for molecular imaging-based detection of gastric cancer.

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Supporting Information Additional information as noted in text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Keywords

iTRAQ; gastric cancer; plasma membrane; SLC3A2; biomarker

INTRODUCTION

Gastric cancer is the fourth commonest cancer and the second leading cause of cancer-related deaths killing about 800 000 people annually.¹ It is a highly aggressive malignant disease with the median survival rate of less than 10 months.¹ The major reason for poor outcome is that most patients had advanced stage of gastric cancer at the time of diagnosis when cancer has metastasized and surgery is not a viable option. Early detection of gastric cancer offers the best chance for cure. Unfortunately, tumor biomarkers such as CEA and CA19-9 that are currently utilized for the detection of gastric cancer in clinical practice lack the sensitivity and specificity needed to detect potentially curable lesions and therefore are not suitable for population screening.² Consequently, discovery of the gastric cancer biomarkers remains a worthy task.

Over the past years, many global gene and protein expression studies have been conducted in attempts to identify novel gastric cancer markers. For example, cDNA microarray analysis of 9 gastric cancer cell lines by Kim et. al. revealed CDC20 and MT2A to be a potential biomarker of human gastric cancer.³ Through 2-D gel electrophoresis, Tseng et al. found that 14-3-3 β was upregulated in gastric cancer cells, and elevated serum 14-3-3 β levels highly correlated with the number of lymph node metastases, tumor size and a reduced survival rate.⁴ In another study, Melle et al. analyzed 74 cryostat sections of central gastric tumor, tumor margin, and normal gastric epithelium using ProteinChip Arrays and SELDI-TOF-MS. Pepsinogen C was identified to be significantly down-regulated in tumor tissues.⁵ Other proteomics efforts have focused on biological fluids such as gastric juice as well as blood. Candidates identified from these proximal and distant fluids are potentially valuable biomarkers.⁶⁻⁸

One of the major challenges in biomarker/drug target discovery is the large dynamic range of protein concentrations found in samples. Consequently, many potentially important but low-abundance markers are believed to escape detection in conventional shotgun approaches. To overcome this issue, a variety of methods have been employed to enrich proteins of interests. These include proteins from the intracellular organelles,^{9,10} biological complexes,¹¹ and those that had undergone specific post-translational modifications such as phosphorylation and glycosylation.¹² Cell surface proteins are highly relevant to this modern era of molecular imaging and target-directed therapeutics due to easy accessibility of these targets to imaging probes and therapeutic agents. Her2, c-Met, and EGFR are classical examples of cell surface proteins against which small molecules and biologics have been successfully developed and implemented in the clinic. Application of membrane proteomics will therefore increase the chance of generating candidates that are immediately useful

targets for molecular imaging and therapeutics. Global proteomics of membrane-enriched samples from normal versus gastric cancer cells has not been reported before.

Stable isotope-based quantitative proteomics approach for identification and quantification of proteins has introduced new possibilities in the field of biomarker discovery. These include isotope coded affinity tag (ICAT),¹³ isobaric tag for relative and absolute quantification (iTRAQ),¹⁴ ¹⁸O,¹⁵ and stable isotope labeling with amino acids in cell culture (SILAC).¹⁶ In this study, we used iTRAQ to compare the expression level of plasma membrane proteins between a pair of “normal” and gastric cancer cell lines. The noncancer gastric epithelium cell line, HFE145, was derived from normal human gastric epithelial cells following transformation and immortalization with SV40 Large T-antigen and human telomerase vectors.¹⁷ The gastric cancer cell line used, MKN-7, is a well-differentiated gastric adenocarcinoma cell line. Our data revealed SLC3A2 plasma membrane protein to be a potential biomarker for gastric cancer detection.

MATERIALS AND METHODS

Reagents

IPG DryStrips (pH 3–10 L, 24 cm), IPG buffer (pH 3–10), DryStrip cover fluids, and Urea were purchased from Amersham Biosciences (Stockholm, Sweden). ERBB2, ICAM1, PLCG1, SLC3A2, LAMP2, SLC7A5, and ACTIN polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), while anti-rabbit secondary antibody conjugated to horseradish peroxidase was from Sigma Aldrich. Enhanced chemiluminescence (ECL) detection kit was purchased from General Electric Healthcare, Bio-Sciences (Uppsala, Sweden), prestained molecular weight markers were from Bio-Rad (Hercules, CA), and protease inhibitors cocktail was from Roche (Mannheim, Germany).

Cell Culture

Twelve human gastric cancer cell lines (MKN7, MKN28, AGS, MKN45, SCH, KATO3, SNU1, SNU5, SNU16, IM95, NUGC3, and NUGC4) were purchased from American Type Culture Collection (Manassas, VA) and Japanese Riken Cell Bank (Tsukuba, Japan). The noncancer gastric epithelium HFE145 was kindly provided by Professor Hassan Ashktorab (Howard University, Washington, D.C.). The cells were cultured with RPMI1640 medium containing 10% fetal calf serum (Hyclone, Logan, UT), penicillin, and streptomycin (Invitrogen) and incubated at 37 °C in a humidified atmosphere containing 5% CO₂.

Membrane Protein-Enriched Samples for Labeling with iTRAQ Reagents

Plasma membrane proteins were enriched as previously described.¹⁸ Briefly, approximately 5×10^8 cells were grown to 80% confluence. Cells were washed three times in ice-cold PBS before being scraped and homogenized in 5 mL of solution containing 100 mM HEPES, pH 7.9, with 15 mM MgCl₂ and 100 mM KCl. The nuclear and unbroken cells were removed by centrifugation at 218g for 5 min at 4 °C. The supernatant was collected and centrifuged at 30 000g for another 30 min at 4 °C. The pellet is the total cellular membrane protein (containing proteins from both plasma membrane and cellular organelle membrane). Two grams worth of resuspended membranes then was added to the top of 14g two-phase system, which is a polymer mixture containing 6.6% (w/w) Dextran T500, 6.6% (w/w) poly(ethylene glycol) 3350, and 0.2 M potassium phosphate, pH 7.2. The mixture was shaken vigorously for 40 times at 4 °C. The phases were then separated by centrifugation at 1000g for 5 min. The upper phase that contained enriched plasma membranes was diluted with 1 mM bicarbonate and collected by centrifugation at 30 000g for 30 min at 4 °C. The pellets were dissolved in lysis buffer containing 0.5 M triethylammoniumbicarbonate [TEAB] and 8 M urea. The concentration of proteins was determined using 2D

Quantification kit (Amersham Biosciences, Uppsala, Sweden). From each cell line, 100 μg worth of proteins was acetone-precipitated overnight at $-20\text{ }^{\circ}\text{C}$ and dissolved in the lysis buffer before being denatured and alkylated (to prevent cysteines from being oxidized) as per manufacturer's instruction (Applied Biosystems, Framingham, MA). Each sample was digested with 20 μL of 0.1 $\mu\text{g}/\mu\text{L}$ trypsin (Promega, WI) solution at $37\text{ }^{\circ}\text{C}$ overnight and then labeled with the iTRAQ tags in accordance to manufacturer's instruction.

Fractionation of Peptides by Isoelectric Focusing (IEF) on Immobilized pH Gradient

iTRAQ-labeled tryptic peptide samples were dissolved in 300 μL of 8 M urea and 1% Pharmalyte (Amersham Biosciences). Samples were used to rehydrate IPG strips (pH 3–10, 18 cm long, Amersham Biosciences) for 14 h at 30 V. Peptides were subsequently focused successively for 1 h at 500 V, 1 h at 1000 V, 1 h at 3000 V and 8.5 h at 8000 V to give a total of 68 kV·h on IPGphor (Amersham Biosciences). The strips were then removed and quickly cut into 36 0.5-cm pieces. Peptides extraction was performed by incubating the gel pieces in 100 μL of 2% acetonitrile, 0.1% formic acid for 1 h. These fractions were lyophilized in vacuum concentrator and subjected to C-18 cleanup using a C18 Discovery DSC-18 SPE column (100 mg capacity, Supelco, Sigma-Aldrich). The cleaned fractions were then lyophilized again and stored at $-20\text{ }^{\circ}\text{C}$ prior to mass spectrometric analysis.

Mass Spectrometry

Each cleaned-up peptide fraction was resuspended in 20 μL of Buffer A (0.1% formic acid in 2% acetonitrile). Ten microliters of sample was injected to the nano-LC–ESI–MS/MS system for each analysis. Mass spectrometry was performed using a QStar Elite Hybrid ESI Quadrupole time-of-flight tandem mass spectrometer (ESI-Q-TOF-MS/MS, Applied Biosystems, Framingham, MA; MDS-Sciex, Concord, Ontario, Canada) coupled to an online capillary liquid chromatography system (Dionex Ultimate 3000, Amsterdam, The Netherlands). The peptide mixture was separated on a PepMap C-18 RP capillary column (Dionex) at 0.3 $\mu\text{L}/\text{min}$. A 125-min gradient was used, in which the gradient started with 4% Buffer B (0.1% formic acid in 98% acetonitrile) and 96% Buffer A for 3 min, followed by 3 ramping gradients of 4–10% Buffer B in 7 min, 10–35% Buffer B for 55 min and 35–100% Buffer B for 25 min. This was then held at 100% Buffer B for 15 min and finally at 96% Buffer A for 20 min. The mass spectrometer was set to perform data acquisition in the positive ion mode, with a selected mass range of 300–1800 m/z . The time of summation of MS/MS events was set to be 2 s. This refers to the amount of time allowed for the machine to accumulate MS/MS events before switching back to MS scan. The two most abundant charged peptides above a 20 count threshold were selected for MS/MS and dynamically excluded for 30 s with ± 50 mDa mass tolerance.

Protein identification and quantification for iTRAQ samples were carried out using ProteinPilot software (version 2.0; Applied Biosystems, MDS-Sciex). Following independent analyses, two data sets from biological replicates were searched as one. The search was performed against International protein index (IPI) human database (version 3.41, date of release: March 2008, 72 155 sequences). Database search was performed by setting cysteine modification by MMTS as a fixed modification. Other parameters include mass tolerance of up to 0.2 Da, maximum of one missed cleavage of trypsin, oxidation of methionine, N-terminal iTRAQ labeling and iTRAQ labeled-lysine. Relative quantification of proteins in the case of iTRAQ is performed on the MS/MS scans and is the ratio of the areas under the peaks at 114 and 116 Da, which were the masses of the tags that correspond to the iTRAQ reagents used to label the samples. Statistical calculation for iTRAQ-based detection and relative quantification was performed using the Paragon Algorithm¹⁹ embedded within the ProteinPilot software. Following data analysis by the ProteinPilot software, the protein summary results were exported into an Excel sheet and manually

inspected and processed. Briefly, for protein identification and quantitative analysis, 95% confidence was used. Protein identification must be based on at least two unique peptides and the *p*-values for the relative quantification by iTRAQ must be <0.05. Protein hits that do not satisfy these criteria are removed.

Western Blot Analysis

Twenty micrograms of proteins was separated by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked 1 h with 1% BSA in Tris-buffered saline with Tween 20 (TBS-T) containing 20 mmol/L Tris, pH 7.6, 100 mmol/L NaCl₂, and 0.5% Tween-20. This was followed by 2 h of incubation with the primary antibodies (1:500 dilution) at room temperature. After washing three times for 5 min with TBS-T buffer, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG at 1:30 000 dilution for 1 h at room temperature. After the membranes were washed three times for 10 min in TBS-T buffer, the blots were exposed to ECL reagents and signals detected on X-ray films. All Western blot analyses were repeated at least three times.

Treatment of Cells with Tunicamycin

HFE145, MKN7, MKN28, and SCH cells were treated with 5 μ g/mL of tunicamycin (Sigma Aldrich) or an equivalent volume of methanol control in complete RPMI1640, and incubated at 37 °C for 24 h. Cells were then harvested and protein samples prepared as per our previous studies²⁰ for Western blot analysis.

Immunofluorescence Analysis by Confocal Microscopy

Cells were grown on coverslips until 60% confluent and then fixed with 100% methanol at -20 °C for 5 min. After blocking with 5% BSA for 1 h, the cells were incubated with anti-SLC3A2 antibody (Santa Cruz) (1:25) overnight at 4 °C. This was followed by secondary antibodies conjugated to Alex Fluor 488 (Invitrogen, Molecular Probes) at 1:1000 dilutions for 1 h. Coverslips containing the cells were then mounted onto glass slides with the addition of Prolong anti-fade reagent (Invitrogen, Molecular Probes) and sealed with Eukitt quick hardening mounting medium (Fluka). Analyses were made using the fluorescence microscope (Zeiss Axioplan 2) with a 40 $\times$ objective.

Immunohistochemistry (IHC) and Tissue Microarray

For expression studies of human SLC3A2 in clinical samples, we used tissue microarrays purchased from Biomax, Inc. [BC01114 (now replaced by ST1004) and ST801 (now replaced by ST801a)] containing cores from a total of 85 different cases of gastric cancer with matched adjacent normal tissues and an additional 10 normal only tissues. Each case is represented by duplicate cores to ensure better analytical accuracy. The characteristics of the samples could be viewed from the Biomax Web site (<http://www.biomax.us/tissue-arrays/Stomach/BC01114> and <http://www.biomax.us/tissue-arrays/Stomach/ST801a>). IHC of tissue arrays was performed as described previously.²¹ Briefly, sections were warmed in a 60 °C oven, dewaxed in three changes of xylene, and passaged through graded ethanol (100, 95, and 70%) before a final wash in double distilled H₂O. After quenching of endogenous peroxidase activity with 3% H₂O₂ for 10 min and blocking with BSA for 30 min, sections were incubated at 4 °C overnight with antibodies against SLC3A2 at a 1:50 dilution. Detection was achieved with the Envision/horseradish peroxidase system (Dako-Cytomation). All slides were counterstained with Gill's Hematoxylin for 1 min, dehydrated, and mounted for light microscopic evaluation. SLC3A2 protein expression was assessed using a previously described semiquantitative scoring consisting of an assessment of both staining intensity (scale 0 to 3) and the percentage of positive cells (0 to 100%), which, when multiplied, generate a score ranging from 0 to 300.²¹ Interpretation of hematoxylin

and eosin sections and analysis/scoring of TMA data were all done by the same certified pathologist to maintain consistency. Statistical analysis was done using SPSS v10.0 for Windows (SPSS, Inc., Chicago, IL). The *t* test was performed at 95% confidence.

RESULTS

Detection and Relative Quantification of Plasma Membrane Proteins in Noncancer and Gastric Cancer Cell Line

As this was meant to be a pilot study to evaluate the effectiveness of 2-phase extraction in enriching plasma membrane proteins compared to whole cell proteomics approach, only 2 cell lines were used, namely, the noncancer HFE145 and MKN7 cancer cells. Samples enriched for plasma membrane proteins were prepared from the cells as described in Materials and Methods. Proteins were trypsin-digested, labeled with iTRAQ reagents, and analyzed using tandem mass spectrometry to profile the protein expression levels between the noncancer HFE145 and MKN7 cancer cells. To increase the coverage of protein identifications and the confidence of the data generated, two biological preparations were labeled with the same pair of iTRAQ reagents, analyzed and mass data searched as one. While this enables the biological variations to be averaged out, it remains important that the relative expression of candidate proteins revealed by iTRAQ–LC/MS–MS approach be validated by other immunological means such as Western blotting. In each experiment, HFE145 and MKN7 were labeled with iTRAQ reagents 114 and 116, respectively. The final 116:114 ratios therefore indicate the relative abundance of proteins in MKN7 with respect to HFE145.

For ProteinPilot-based database searching and identification, the protein threshold [unused protscore (conf)] was set to achieve 95% confidence at 5% FDR (false discovery rate). Although relative quantification analysis by ProteinPilot 2.0 software comes with statistical analysis and since most methods are prone to technical variation, we included an additional 1.3-fold cutoff for all iTRAQ ratios to add stringency when classifying proteins as up- or down-regulated. This value was applied as the technical variation of many of our iTRAQ-based analyses was consistently below 30%.^{7,8,20–25} Therefore, the upper and lower range worked out to be 1.3 (1×1.3) and 0.77 ($1/1.3$), respectively. In other words, proteins with iTRAQ ratios below the lower range were considered to be underexpressed, while those above the higher range were considered overexpressed. A total of 873 proteins were identified with 95% confidence. Of these, 175 proteins were found to have >1.3-fold difference in expression between the noncancer and gastric cancer epithelial cells, while the rest showed no difference. Of these, 74 proteins were underexpressed in gastric cancer, whereas 101 were overexpressed in gastric cancer compared to “normal” cells.

The iTRAQ data for all 175 proteins are found in Supplementary Table 1 but only the top 50 up- and down-regulated plasma membrane (cell surface) proteins in gastric cancer cell line compared to noncancer cell line are shown in Table 1 since we are interested in easily accessible cell surface membrane proteins that can be exploited for molecular imaging. Additional information such as ion scores, error factor and percentage coverage are provided in Supplementary Table 2. As an example, the overall iTRAQ data for SLC3A2 protein, MS/MS spectra for one of its peptides and relative levels of iTRAQ tags extracted from the ProteinPilot software are shown in Figure 1.

Evaluating the Effectiveness of Two-Phase Extraction in Enriching for Plasma Membrane and Membrane-Associated Proteins

The aqueous polymer two-phase partitioning has method been shown to be an attractive and superior alternative for the isolation of plasma membrane from eukaryotic cells.²⁶ Here, this

approach was used to enrich for plasma membrane proteins in gastric epithelial cells. To assess the efficacy of our protocol for the enrichment of plasma membrane proteins and to examine the degree of contamination by other cellular organelles, such as mitochondria and endoplasmic reticulum, we classified the cellular localization of all the detected proteins (Supplementary Table 2) based on Gene Ontology (GO) annotation in GeneCards (www.genecards.org) and other public databases (e.g., Panther classification at www.pantherdb.org). Of these 873 proteins, 362 proteins (about 32.3%) have been assigned as plasma membrane or membrane-associated proteins, and these include Sodium/potassium-transporting ATPase, a known marker for plasma membrane. Of the remaining proteins with subcellular annotation, 266 (23%) were classified as cytoplasmic, 166 (14.8%) as nuclear, 49 (4.4%) as ribosomal, 40 (3.6%) as mitochondrial, 23 (2.0%) as belonging to the endoplasmic reticulum, and 23 (2.0%) as extracellular (Figure 2a).

Next, we determined the proportion of plasma membrane/associated proteins detected in iTRAQ-based mass spectrometric analysis on the whole cell lysates of HFE145 and MKN7. Cells were harvested as per our previous study²¹ and analyzed as described in this study. The raw data and list of proteins showing >1.3-fold difference in expression between the 2 cell lines are shown in Supplementary Table 3. The subcellular localization of all the 1050 proteins detected in the whole cell lysates (under “raw data” tab) were classified as described above. The percent distribution of proteins into various subcellular compartments for the 2-phase partition versus the whole cell lysate data sets are tabulated and compared in Figure 2a. In the whole cell lysate analysis, plasma membrane/plasma membrane-associated proteins comprised only about 10.8% of the total proteins detected. This is in contrast with about 32.3% of plasma membrane and associated proteins in the 2-phase partition method. This is a 3-fold increase in the number of plasma membrane/plasma membrane associated proteins. Not surprisingly, the 2-phase partition method yielded lesser cytosolic and nuclear proteins compared to whole cell lysate analysis. These results are support for the aqueous polymer two-phase partitioning approach enabling a good enrichment of plasma membrane proteins.

Subsequently, we examined the proteins that are common between the data sets generated from whole cell and 2-phase partition analyses and determined the degree of congruence in terms of their relative expressions between normal and gastric cancer cells. As seen in Supplementary Table 4, while 419 proteins are common between the 2 data sets, only 392 proteins have iTRAQ ratios in both data sets and therefore used for analysis. Log transformation values of the available iTRAQ ratios of the common proteins in both the 2-phase partition and whole cell analysis were used for correlation analysis using SPSS v10.0. Pearson correlation coefficient of 0.572 ($p < 0.0001$) was obtained. The data indicates that the degree of congruence is moderate at about 60%. The not-so-excellent congruence between the data sets obtained from the two separate sample preparation methods could be due to the fact that many membrane/associated proteins are also localized to the cytosol (e.g., Calmodulin) and/or nucleus (e.g., DDX5). It further implies that it is useful to determine the relative expression of proteins at the specific subcellular site of function.

Functional Characteristics of the Proteins Detected in the Data Set

To better appreciate the molecular and functional characteristics of the 87 differentially expressed plasma membrane or membrane-associated proteins, these proteins were grouped according to their reported molecular functions and biological process using the PANTHER (Protein Analysis through Evolutionary Relationships) Classification System (www.pantherdb.org/). The total number of 87 differentially expressed proteins was found to represent a total of 8 molecular functions and 14 biological processes (Figure 3). Proteins with receptor, binding and catalytic activities constitute the largest groups of plasma membrane and associated proteins (Figure 3A). The presence of the former 2 groups in the

plasma membrane fraction is not surprising and includes receptors that bind lipoprotein, channels that bind ions, solute carriers, cell adhesion, integrin proteins and ErbB2 (Her2) receptor kinase oncogene well-known to be overexpressed in many human cancers.²⁷ Consistently, transport and cell communication are two of the major biological processes that are associated with these proteins (Figure 3B). Proteins with catalytic activities (Figure 3A) are largely comprised of signaling proteins/enzymes that are associated with the inner side of the plasma membrane such as PLCG1 and the small GTPases. These proteins are usually involved in cellular processes such as growth and migration (Figure 3B).

Validation of iTRAQ Data on Selected Candidates

To verify the iTRAQ data, Western blot analyses were performed on selected candidates. These candidates were chosen based on the interests of our lab including the well-known signaling proteins such as ErbB2 (Her2) and PLCG1 oncogenes as well as cell surface markers not known previously reported to be upregulated in gastric cancer [e.g., SLC3A2-(CD98), LAMP-2] since the key objective of this study is to identify potential biomarkers for molecular imaging of cancer. We have also included a couple of “positive controls” ICAM1 and SLC7A5 (LAT1), which have been shown to be implicated in gastric cancer.^{28,29} Figure 4a shows that the up- or down-regulation trend of candidate proteins between noncancer and cancer cell revealed by the Western blot data is congruent with that revealed by iTRAQ. Only 1 representative actin blot was shown to demonstrate equal loadings although each immunoblotting has its own control. This supported the notion that amine-specific isobaric tagging labeling method for the large-scale protein quantification is robust. All the 6 candidates except PLGG1 are upregulated in MKN7 cancer compared to HFE145 noncancer cells. Down-regulation of PLCG1 in gastric cancer cells is unexpected since it is a well-known oncogene.³⁰ It is not entirely clear why this is so, but it is likely due to the molecular heterogeneity of gastric cancers where gastric cancer cells from different patients are not necessarily all “addicted” to the same oncogene. In fact, we do not rule out that PLCG1 oncogene may behave like a tumor suppressor in some gastric cancer cells. Indeed, it is not uncommon to see oncogene behaving like tumor suppressor and vice versa in different cancer types. Runx 3 tumor suppressor is a good example. It is often down-regulated in gastric, breast and colon cancers but was found to be overexpressed in basal cell carcinoma³¹ and ovarian cancer.³²

Although this study was started as a pilot study using only 2 cell lines for evaluating the efficiency of the 2-phase partition method coupled to iTRAQ-LC/MS/MS in enriching plasma proteins, we decided to pursue our findings further since our data provided convincing evidence that SLC3A2 and LAMP-2 are potentially novel gastric cancer associated proteins. SLC3A2 was chosen for further analysis in a larger panel of gastric cancer cell lines because a PubMed search revealed that its role in cancer in general is not as well-known compared to LAMP-2. Hence, in addition to the 2 cell lines used in iTRAQ analysis, we examined the expression of SLC3A2 in 12 other gastric cancer cell lines using immunoblotting to investigate the frequency of its aberrant expression. Remarkably, SLC3A2 was detected to be at higher levels in at least two-thirds of the gastric cancer cell lines compared to HFE145 “normal” cells (Figure 4b). We also observed that while SLC3A2 appeared as a single band in HFE145 “normal” cells, SLC3A2 presented itself as multiple bands in cancer cells. Some cancer cell lines expressed more of the higher molecular weight species (e.g., MKN7, MKN28 and SCH) while others had largely the lower molecular weight species.

SLC3A2 Is Glycosylated in Gastric Cancer Cells

SLC3A2 protein has a predicted molecular weight of 57 kDa; however, it has been reported to be extensively glycosylated.^{33,34} It is well-known that glycosylation could slow the

migration of target proteins in SDS-PAGE by as much as 30–40 kDa. To test the hypothesis that the high molecular weight species was attributed to glycosylation, we treated MKN7, MKN28 and SCH cells with Tunicamycin, an inhibitor which blocks glycosylation of newly synthesized proteins. HFE145 was included as a control. As shown in Figure 4c, following treatment with Tunicamycin and probing with SLC3A2 antibody, the high molecular weight species in MKN7, MKN28 and SCH had largely disappeared with the concomitant appearance of a major band around 60 kDa. The data indicates that SLC3A2 protein in certain gastric cancer cells might be extensively glycosylated. Given that HFE145 “normal” cells do not express the more glycosylated species of SLC3A2, it is conceivable that hyperglycosylation of SLC3A2 might be disease causing. It would be interesting to test this hypothesis for future work since aberrant glycosylation is known to be important for cancer progression.³⁵

SLC3A2 Is Relevant to Clinical Gastric Cancer and a Potential Target for Molecular Imaging

The prerequisite for molecular imaging for diagnostics is that the targets must be detectable when probed with exogenous sensors such as antibodies. Hence, we conducted immunofluorescence of SLC3A2 in MKN7, MKN28 and SCH to determine whether SLC3A2 in these cancer cells could be detected by exogenously added antibodies. As shown in Figure 5, plasma membrane-localized SLC3A2 could be detected in all the gastric cancer cell lines tested. Note that these cells grew in islands/clusters that are characteristic of epithelial cells. Although immunofluorescence is only semiquantitative, it is obvious that the SLC3A2 signals in all 3 gastric cancer cell lines were stronger than that in HFE145 noncancer cells. Collectively, the data suggest that it is feasible to produce novel reagents that can detect SLC3A2 for molecular imaging of cancer.

Since *in vitro* cell lines lack the physiological context, we asked whether the upregulation of SLC3A2 could also be observed in clinical samples. To assess the clinical relevance, we examined the expression of SLC3A2 in tissue microarrays containing 85 matched normal and gastric cancer tissues by immunohistochemistry. The TMA also includes ten additional unmatched normal gastric tissues. The raw IHC scores for SLC3A2 and the clinico-histopathological characteristics of the clinical samples are provided in Supplementary Table 5. The expression levels of SLC3A2 across the clinical samples are presented in a distribution plot (Figure 6a). As the clinical samples included nonmatched normal and tumor samples, two-samples *t* test was used. The statistical analysis revealed that the expression of SLC3A2 in cancer/tumor samples is significantly higher than that of noncancer/normal tissues ($p < 0.01$). The same result, that is, $p < 0.01$, was obtained when pair sample *t* test was conducted on the 85 matched normal and tumor samples. In addition, 41% (35/85) of the matched cases showed higher SLC3A2 expression in the tumor compared to normal tissues while only 19% of the matched cases showed the reverse trend (Figure 6b). Forty percent of the matched cases had no detectable level of SLC3A2. The expression data from clinical samples analysis revealed that the upregulation of SLC3A2 has quite a high penetrance (>40%) in gastric cancer, is consistent with the data from *in vitro* cell lines, and reiterates the molecular heterogeneity of gastric cancers. However, no statistically significant correlations could be observed between SLC3A2 expression and (i) diffuse and intestinal subtypes of gastric cancer, (ii) cancer stages, or (iii) grades. A future study with larger sample size may be necessary to clarify the association of SLC3A2 with these clinical parameters. Representative images of the immunohistochemistry of SLC3A2 in 2 sets of matched normal and gastric cancer tissues are shown in Figure 6b. Consistent with the immunofluorescence data from *in vitro* cell lines, surface expression of SLC3A2 on gastric cancer epithelial cells was confirmed in clinical samples by immunohistochemistry (inset, Figure 6b).

DISCUSSION

Early detection of gastric cancer can improve the outcomes of our patients and save lives. However, this requires biomarkers that will outperform current, clinically implemented markers like CEA. Indeed, many attempts have succeeded in identifying potential biomarkers that have higher specificity and sensitivity than CEA. These include but are not limited to serum 14-3-3 β^4 plasma C9,⁷ and plasma ITIH3.⁸ It would be interesting to see which of these candidates would eventually be implemented in the clinical setting over the next few years. There will be attritions but it is certain that breakthroughs will surface from these efforts.

For cancer diagnostics, minimally invasive methods such as blood test and molecular imaging are preferred because they are better accepted by patients due to their simple procedures and ease of sampling. By combining the 2-phase partition, which has been confirmed by this study to enrich plasma membrane proteins, with mass spectrometry, the present study identified 175 proteins which are differentially expressed between gastric cancer and noncancer cells. Out of these, 50 proteins were assigned as plasma membrane proteins, most of which are potentially accessible target useful for imaging and therapy.

LAMP-2, a lysosome-associated membrane protein-2, is a member of a family of membrane glycoproteins. It has been found to shuttle between the lysosomes, endosomes and plasma membrane (see <http://www.genecards.org/cgi-bin/carddisp.pl?gene=LAMP2>). LAMP molecules have been implicated in tumor cell metastasis through various studies. For example, it was shown that highly metastatic tumor cells express more plasma membrane-bound LAMP molecules than the poorly metastatic ones.³⁶ Sarafian et al. examined the expression of LAMPs and their interactions with galectin-3 in different human tumor cell lines, and strongly suggested that LAMPs may constitute a new family of adhesive glycoproteins that participate in the complex process of tumor invasion and metastasis.³⁷ It has also been detected in our previous study to be elevated in the conditioned media of gastric cancer cells compared to noncancer cells.³⁸ Coupled to the observations made here, upregulation of LAMP-2 is confirmed in gastric cancer cells. It is conceivable that LAMP-2, as in the reported cancer types discussed above, also mediated gastric cancer cell invasion and metastasis, and can serve as a target for antimetastasis therapy in gastric cancer. It would be interesting to test this hypothesis in future studies.

While LAMP-2 is interesting, this study focused instead on another novel gastric cancer-associated protein discovered, SLC3A2, because its expression is more restricted to the plasma membrane and therefore a better candidate biomarker for molecular imaging. Unlike LAMP-2, SLC3A2 was not detected in the secretome of gastric cancer cells that we studied previously.³⁸ Here, the levels of the solute carrier family of proteins, such as SLC7A5 and SLC3A2, in the gastric cancer cells MKN7 were found to be up-regulated compared to noncancer cells. Abnormal expression of SLC3A2 has been also confirmed in clinical gastric cancers in this study. CD98 is a glycoprotein that is a heterodimer composed of SLC7A5 (CD98 light chain) and SLC3A2 (CD98 heavy chain) that form the L-type amino acid transporters, and transports large neutral amino acids such as phenylalanine, tyrosine, leucine, arginine and tryptophan.^{39,40} SLC3A2 is highly expressed on proliferating lymphocytes and on other rapidly growing cells,⁴¹ and the overexpression of SLC3A2 has also been described in human melanoma,⁴² squamous cell carcinoma of the larynx,⁴³ adenocarcinoma of the lung,⁴⁴ and renal cell cancer.⁴⁵ Therefore, aberrant expression of SLC3A2 in cancer must have some bearings on cancer cell metabolism. However, it is not evident from the above studies that the species of SLC3A2 examined was glycosylated. In contrast, our work has explicitly established that the overexpressed SLC3A2 in gastric cancer cell lines is glycosylated. Previous studies have demonstrated the functional roles of

SLC3A2 in cellular processes. Overexpression of SLC3A2, which interacts with integrin β 1A subunit, led to anchorage-independent CHO cells growth⁴⁶ and tumorigenesis in NIH 3T3 fibroblasts cells.⁴⁷ SLC3A2 also contributes to integrin-dependent cell spreading, cell migration, and protection from apoptosis.⁴⁸ While it remains to be proven, it is conceivable that the interaction of SLC3A2 with integrin requires SLC3A2 glycosylation and this may in turn influence cellular transformation. Further experimentations would be required to test this hypothesis. Furthermore, embryonic stem cells null for SLC3A2 lost their tumorigenic potential in vivo.⁴⁸ Collectively, the evidence strongly indicate that SLC3A2 is an oncogene for various cancer types.

For the first time, the association of SLC3A2 in gastric cancer has been established in our study. Our results demonstrated that almost 50% of the gastric cancer cell lines and clinical gastric cancer samples possessed elevated amounts of SLC3A2, suggesting that SLC3A2 is a promising candidate for future biomarker development for gastric cancer. Future studies involving a larger sample size and determination of sensitivity/specificity performed as per our previous studies^{7,8} should clarify the role of SLC3A2 as a biomarker. In addition, SLC3A2 is localized to the plasma membrane, positioning it an attractive target for imaging and antgastric cancer therapy. Our data, coupled to existing literature, hinted at a potential role for SLC3A2 in tumorigenesis of gastric cancer and/or maintenance of the tumor phenotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

iTRAQ	isotope tagging for relative and absolute quantification
SCX	strong cation exchange
ESI	electrospray ionization
LC-MS/MS	liquid chromatography–tandem mass spectrometry
MMTS	methyl methanethiosulfonate

REFERENCES

- (1). Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int. J. Cancer*. 2010; 127(12):2893–917. [PubMed: 21351269]
- (2). Akdogan M, Sasmaz N, Kayhan B, Biyikoglu I, Disibeyaz S, Sahin B. Extraordinarily elevated CA19-9 in benign conditions: a case report and review of the literature. *Tumori*. 2001; 87(5): 337–339. [PubMed: 11765186]
- (3). Kim JM, Sohn HY, Yoon SY, Oh JH, Yang JO, Kim JH, Song KS, Rho SM, Yoo HS, Kim YS, Kim JG, Kim NS. 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(2 Pt 1):473–482. [PubMed: 15701830]
- (4). Tseng CW, Yang JC, Chen CN, Huang HC, Chuang KN, Lin CC, Lai HS, Lee PH, Chang KJ, Juan HF. Identification of 14-3-3 β in human gastric cancer cells and its potency as a

diagnostic and prognostic biomarker. *Proteomics*. 2011; 11(12):2423–2439. [PubMed: 21598387]

- (5). Melle C, Ernst G, Schimmel B, Bleul A, Kaufmann R, Hommann M, Richter KK, Daffner W, Settmacher U, Claussen U, von Eggeling F. Characterization of pepsinogen C as a potential biomarker for gastric cancer using a histo-proteomic approach. *J. Proteome Res.* 2005; 4(5): 1799–1804. [PubMed: 16212435]
- (6). Lee K, Kye M, Jang JS, Lee OJ, Kim T, Lim D. Proteomic analysis revealed a strong association of a high level of alpha1-antitrypsin in gastric juice with gastric cancer. *Proteomics*. 2004; 4(11): 3343–3352. [PubMed: 15378737]
- (7). Chong PK, Lee H, Loh MC, Choong LY, Lin Q, So JB, Lim KH, Soo RA, Yong WP, Chan SP, Smoot DT, Ashktorab H, Yeoh KG, Lim YP. Upregulation of plasma C9 protein in gastric cancer patients. *Proteomics*. 2010; 10(18):3210–3221. [PubMed: 20707004]
- (8). Chong PK, Lee H, Zhou J, Liu SC, Loh MC, Wang TT, Chan SP, Smoot DT, Ashktorab H, So JB, Lim KH, Yeoh KG, Lim YP. ITIH3 is a potential biomarker for early detection of gastric cancer. *J. Proteome Res.* 2010; 9(7):3671–3679. [PubMed: 20515073]
- (9). Huber LA, Pfaller K, Vietor I. Organelle proteomics—Implications for subcellular fractionation in proteomics. *Circ. Res.* 2003; 92(9):962–968. [PubMed: 12750306]
- (10). Dreger M. Subcellular proteomics. *Mass Spectrom. Rev.* 2003; 22(1):27–56. [PubMed: 12768603]
- (11). Ho Y, Gruhler A, Heilbut A, Bader GD, Moore L, Adams SL, Millar A, Taylor P, Bennett K, Boutilier K, Yang LY, Wolting C, Donaldson I, Schandorff S, Shewnarane J, Vo M, Taggart J, Goudreaux M, Muskut B, Alfarano C, Dewar D, Lin Z, Michalickova K, Willems AR, Sassi H, Nielsen PA, Rasmussen KJ, Andersen JR, Johansen LE, Hansen LH, Jespersen H, Podtelejnikov A, Nielsen E, Crawford J, Poulsen V, Sorensen BD, Matthiesen J, Hendrickson RC, Gleeson F, Pawson T, Moran MF, Durocher D, Mann M, Hogue CWV, Figeys D, Tyers M. Systematic identification of protein complexes in *Saccharomyces cerevisiae* by mass spectrometry. *Nature*. 2002; 415(6868):180–183. [PubMed: 11805837]
- (12). Oda Y, Nagasu T, Chait BT. Enrichment analysis of phosphorylated proteins as a tool for probing the phosphoproteome. *Nat. Biotechnol.* 2001; 19(4):379–382. [PubMed: 11283599]
- (13). Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat. Biotechnol.* 1999; 17(10):994–999. [PubMed: 10504701]
- (14). Ross PL, Huang YLN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, Purkayastha S, Juhasz P, Martin S, Bartlett-Jones M, He F, Jacobson A, Pappin DJ. Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. *Mol. Cell. Proteomics*. 2004; 3(12):1154–1169. [PubMed: 15385600]
- (15). Mirgorodskaya OA, Kozmin YP, Titov MI, Korner R, Sonksen CP, Roepstorff P. Quantitation of peptides and proteins by matrix-assisted laser desorption/ionization mass spectrometry using (18)O-labeled internal standards. *Rapid Commun. Mass Spectrom.* 2000; 14(14):1226–1232. [PubMed: 10918372]
- (16). Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, Mann M. Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol. Cell. Proteomics*. 2002; 1(5):376–386. [PubMed: 12118079]
- (17). Marlink KL, Bacon KD, Sheppard BC, Ashktorab H, Smoot DT, Cover TL, Deveney CW, Rutten MJ. Effects of *Helicobacter pylori* on intracellular Ca²⁺ signaling in normal human gastric mucous epithelial cells. *Am. J. Physiol.: Gastrointest. Liver Physiol.* 2003; 285(1):G163–G176. [PubMed: 12606301]
- (18). Morre DM, Morre DJ. Aqueous two-phase partition applied to the isolation of plasma membranes and Golgi apparatus from cultured mammalian cells. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* 2000; 743(1–2):377–387.
- (19). Shilov IV, Seymour SL, Patel AA, Loboda A, Tang WH, Keating SP, Hunter CL, Nuwaysir LM, Schaeffer DA. The Paragon Algorithm, a next generation search engine that uses sequence temperature values and feature probabilities to identify peptides from tandem mass spectra. *Mol. Cell. Proteomics*. 2007; 6(9):1638–1655. [PubMed: 17533153]

- (20). Chen Y, Choong LY, Lin Q, Philp R, Wong CH, Ang BK, Tan YL, Loh MC, Hew CL, Shah N, Druker BJ, Chong PK, Lim YP. Differential expression of novel tyrosine kinase substrates during breast cancer development. *Mol. Cell. Proteomics*. 2007; 6(12):2072–2087. [PubMed: 17855441]
- (21). Ho J, Kong JW, Choong LY, Loh MC, Toy W, Chong PK, Wong CH, Wong CY, Shah N, Lim YP. Novel breast cancer metastasis-associated proteins. *J. Proteome Res*. 2009; 8(2):583–594. [PubMed: 19086899]
- (22). Lim S, Choong LY, Chong PK, Yunhao C, Lim YP. Regulation of macrophage inhibitory factor (MIF) by epidermal growth factor receptor (EGFR) in the MCF10AT model of breast cancer progression. *J. Proteome Res*. 2009; 8(8):4062–4076. [PubMed: 19530702]
- (23). Yang Y, Lim SK, Choong LY, Lee H, Chen Y, Chong PK, Ashktorab H, Wang TT, Salto-Tellez M, Yeoh KG, Lim YP. Cathepsin S mediates gastric cancer cell migration and invasion via a putative network of metastasis-associated proteins. *J. Proteome Res*. 2010; 9(12):6801.
- (24). Chong PK, Lee H, Zhou J, Liu SC, Loh MC, So JB, Lim KH, Yeoh KG, Lim YP. Reduced plasma APOA1 level is associated with gastric tumor growth in MKN45 mouse xenograft model. *J. Proteomics*. 2010; 73(8):1632–1640. [PubMed: 20399916]
- (25). Choong LY, Lim S, Chong PK, Wong CY, Shah N, Lim YP. Proteome-wide profiling of the MCF10AT breast cancer progression model. *PLoS One*. 2010; 5(6):e11030. [PubMed: 20543960]
- (26). Schindler J, Nothwang HG. Aqueous polymer two-phase systems: Effective tools for plasma membrane proteomics. *Proteomics*. 2006; 6(20):5409–5417. [PubMed: 16972286]
- (27). Bang YJ, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A, Lordick F, Ohtsu A, Omuro Y, Satoh T, Aprile G, Kulikov E, Hill J, Lehle M, Ruschhoff J, Kang YK, To GATI. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. *Lancet*. 2010; 376(9742):687–697. [PubMed: 20728210]
- (28). Maruo Y, Gochi A, Kaihara A, Shimamura H, Yamada T, Tanaka N, Orita K. ICAM-1 expression and the soluble ICAM-1 level for evaluating the metastatic potential of gastric cancer. *Int. J. Cancer*. 2002; 100(4):486–490. [PubMed: 12115535]
- (29). Ichinoe M, Mikami T, Yoshida T, Igawa I, Tsuruta T, Nakada N, Anzai N, Suzuki Y, Endou H, Okayasu I. High expression of L-type amino-acid transporter 1 (LAT1) in gastric carcinomas: comparison with non-cancerous lesions. *Pathol. Int*. 2011; 61(5):281–289. [PubMed: 21501294]
- (30). Wells A, Grandis JR. Phospholipase C-gamma1 in tumor progression. *Clin. Exp. Metastasis*. 2003; 20(4):285–290. [PubMed: 12856715]
- (31). Salto-Tellez M, Peh BK, Ito K, Tan SH, Chong PY, Han HC, Tada K, Ong WY, Soong R, Voon DC, Ito Y. RUNX3 protein is overexpressed in human basal cell carcinomas. *Oncogene*. 2006; 25(58):7646–7649. [PubMed: 16767156]
- (32). Lee CW, Chuang LS, Kimura S, Lai SK, Ong CW, Yan B, Salto-Tellez M, Choolani M, Ito Y. RUNX3 functions as an oncogene in ovarian cancer. *Gynecol. Oncol*. 2011; 122(2):410–417. [PubMed: 21612813]
- (33). Yanagida O, Kanai Y, Chairoungdua A, Kim DK, Segawa H, Nii T, Cha SH, Matsuo H, Fukushima J, Fukasawa Y, Tani Y, Taketani Y, Uchino H, Kim JY, Inatomi J, Okayasu I, Miyamoto K, Takeda E, Goya T, Endou H. Human L-type amino acid transporter 1 (LAT1): characterization of function and expression in tumor cell lines. *Biochim. Biophys. Acta, Biomembr*. 2001; 1514(2):291–302.
- (34). Chillaron J, Roca R, Valencia A, Zorzano A, Palacin M. Heteromeric amino acid transporters: biochemistry, genetics, and physiology. *Am. J. Physiol.: Renal Physiol*. 2001; 281(6):F995–F1018. [PubMed: 11704550]
- (35). Miyagi T, Takahashi K, Hata K, Shiozaki K, Yamaguchi K. Sialidase significance for cancer progression. *Glycoconjugate J*. 2012; 29(8–9):567–577.
- (36). Laferte S, Dennis JW. Purification of two glycoproteins expressing beta 1–6 branched Asn-linked oligosaccharides from metastatic tumour cells. *Biochem. J*. 1989; 259(2):569–576. [PubMed: 2719668]

- (37). Sarafian V, Jadot M, Foidart JM, Letesson JJ, van den Brule F, Castronovo V, Wattiaux R, Wattiaux-De Coninck S. Expression of Lamp-1 and Lamp-2 and their interactions with galectin-3 in human tumor cells. *Int. J. Cancer*. 1998; 75(1):105–111. [PubMed: 9426697]
- (38). Yang YX, Kiat LS, Yee CL, Lee HY, Chen YH, Kuan CP, Hassan A, Ting WT, Manuel ST, Guan YK, Pin LY, Cathepsin S. Mediates gastric cancer cell migration and invasion via a putative network of metastasis-associated proteins. *J. Proteome Res*. 2010; 9(9):4767–4778. [PubMed: 20812763]
- (39). Kanai Y, Endou H. Heterodimeric amino acid transporters: Molecular biology and pathological and pharmacological relevance. *Curr. Drug Metab*. 2001; 2(4):339–354. [PubMed: 11766986]
- (40). Palacin M, Nunes V, Font-Llitjos M, Jimenez-Vidal M, Fort J, Gasol E, Pineda M, Feliubadalo L, Chillaron J, Zorzano A. The genetics of heteromeric amino acid transporters. *Physiology*. 2005; 20:112–124. [PubMed: 15772300]
- (41). Verrey F, Closs EI, Wagner CA, Palacin M, Endou H, Kanai Y. CATs and HATs: The SLC7 family of amino acid transporters. *Pfluegers Arch*. 2004; 447(5):532–542. [PubMed: 14770310]
- (42). Dixon WT, Sikora LKJ, Demetrick DJ, Jerry LM. Isolation and characterization of a heterodimeric surface-antigen on human-melanoma cells and evidence that it is the 4f2 cell activation proliferation molecule. *Int. J. Cancer*. 1990; 45(1):59–68. [PubMed: 2404879]
- (43). Esteban F, Ruizcabello F, Concha A, Ayala MP, Delgado M, Garrido F. Relationship of 4f2-antigen with local growth and metastatic potential of squamous-cell carcinoma of the larynx. *Cancer*. 1990; 66(7):1493–1498. [PubMed: 2208000]
- (44). Garber ME, Troyanskaya OG, Schluens K, Petersen S, Thaesler Z, Pacyna-Gengelbach M, van de Rijn M, Rosen GD, Perou CM, Whyte RI, Altman RB, Brown PO, Botstein D, Petersen I. Diversity of gene expression in adenocarcinoma of the lung. *Proc. Natl. Acad. Sci. U.S.A.* 2001; 98(24):13784–13789. [PubMed: 11707590]
- (45). Prager GW, Poettler M, Schmidinger M, Mazal PR, Susani M, Zielinski CC, Haitel A. CD98hc (SLC3A2), a novel marker in renal cell cancer. *Eur. J. Clin. Invest*. 2009; 39(4):304–310. [PubMed: 19292886]
- (46). Henderson NC, Collis EA, Mackinnon AC, Simpson KJ, Haslett C, Zent R, Ginsberg M, Sethi T. CD98hc (SLC3A2) interaction with beta(1) Integrins is required for transformation. *J. Biol. Chem*. 2004; 279(52):54731–54741. [PubMed: 15485886]
- (47). Hara K, Kudoh H, Enomoto T, Hashimoto Y, Masuko T. Malignant transformation of NIH3T3 cells by overexpression of early lymphocyte activation antigen CD98. *Biochem. Biophys. Res. Commun*. 1999; 262(3):720–725. [PubMed: 10471392]
- (48). Feral CC, Nishiya N, Fenczik CA, Stuhlmann H, Slepak M, Ginsberg MH. CD98hc (SLC3A2) mediates integrin signaling. *Proc. Natl. Acad. Sci. U.S.A.* 2005; 102(2):355–360. [PubMed: 15625115]

Figure 1. Relative levels of SLC3A2 in MKN7 (iTRAQ 116) with respect to HFE145 (iTRAQ 114) cells as determined by electrospray ionization tandem mass spectrometry. The upper panel shows a representative tandem mass spectrum of a SLC3A2 peptide (GENSWFSTQVDTVATK) while the lower panel shows the relative peak heights of the iTRAQ labels.

Figure 2.

Comparison of whole cell versus 2-phase partition analysis. (a) Comparison of the distribution of all the proteins detected in the membrane and whole cell proteomics data sets across various subcellular compartments. Classification of proteins into subcellular compartments is done via Gene Ontology. (b) Determination of the congruence of iTRAQ ratios between the entire data sets generated from whole cell and membrane analyses in terms of their relative expression between normal and gastric cancer cells. Correlation analysis was performed using the log transformation values of the available iTRAQ ratios from 392 common proteins using SPSS v10.0. Pearson correlation coefficient between whole cell lysate and membrane sample was 0.572 ($p < 0.0001$).

Figure 3. Classification of proteins identified through membrane proteomics into their molecular functions and biological processes. This is done via PANTHER (Protein Analysis through Evolutionary Relationships) Classification System (www.pantherdb.org/).

Figure 4.

(a) Western blot analyses of the expression of selected candidates (ERBB2, ICAM1, PLCG1, SLC3A2, LAMP2, and SLC7A5) in HFE145 noncancer and MKN7 gastric cancer cells. (b) Immunoblotting of SLC3A2 in the HFE145 noncancer gastric cell versus a panel of 13 gastric cancer cell lines. (c) Immunoblotting of SLC3A2 in HFE145 noncancer and 3 gastric cancer cell lines following pretreatment with Tunicamycin.

Figure 5. Immunofluorescence of SLC3A2 in the HFE145 noncancer gastric cell, and MKN7, MKN28 and SCH cancer cells. Images were all captured at 40 $\times$ magnification. SLC3A2 was observed to be localized mainly to the plasma membrane (indicated by arrows pointing to signals along the edges or borders of cells) and overexpressed in gastric cancer cell lines compared with noncancer cells.

Figure 6.

Immunohistochemistry of SLC3A2 in tissue microarrays of clinical gastric samples. A total of 85 matched normal and cancer tissues plus addition 10 normal tissues were analyzed. (a) Distribution plot of the IHC scores of SLC3A2 in individual normal and gastric cancer samples. Statistically significant higher expression of SLC3A2 was observed in gastric cancer samples compared to normal gastric tissues ($p < 0.01$, independent sample t test). (b) Representative IHC images (10 $\times$ magnification) of SLC3A2 in 2 matched gastric cancer and normal tissues as well as a table showing the frequency of upregulation and down-regulation of SLC3A2 in clinical gastric specimens. Insets contain magnified images (40 $\times$) that show clear cell surface expression of SLC3A2 in gastric cancer cells.

Table 1

Partial List of Proteins Identified To Be Expressed at Different Levels between Noncancer and Gastric Cancer Cell Lines by iTRAQ Analysis^a

<i>n</i>	accession	gene symbol	name	MKN7:HFE145	<i>p</i> -value MKN7:HFE145	unique iTRAQ peptide	molecular function based on GO
1	IPI:IPI00221224.6	ANPEP	Aminopeptidase N	0.13	1.91×10^{-5}	6	angiogenesis, proteolysis
2	IPI:IPI00020557.1	LRP1	Prolow-density lipoprotein receptor-related protein 1 precursor	0.17	1.83×10^{-3}	6	receptor-mediated endocytosis, activation of protein kinase C activity by G-protein coupled receptor protein signaling pathway
3	IPI:IPI00015148.3	RAP1B	Ras-related protein Rap-1b precursor	0.25	1.29×10^{-4}	5	energy reserve metabolic process, GTP catabolic process
4	IPI:IPI00748145.2	GNAI2	Isoform 1 of Guanine nucleotide-binding protein G(i), alpha-2 subunit	0.28	1.34×10^{-2}	13	activation of MAPKK activity, adenosine receptor signaling pathway
5	IPI:IPI00221226.7	ANXA6	Annexin A6	0.31	3.16×10^{-4}	8	calcium ion transport, regulation of muscle contraction
6	IPI:IPI00790571.2	CLTA	Isoform 3 of Clathrin light chain A	0.32	1.96×10^{-3}	2	intracellular protein transport, post-Golgi vesicle-mediated transport
7	IPI:IPI00027505.2	ITGAV	Isoform 1 of Integrin alpha-V precursor	0.34	1.12×10^{-2}	3	angiogenesis, blood vessel development
8	IPI:IPI00878254.1	COMT	COMT 24 kDa protein	0.39	2.81×10^{-4}	2	catecholamine metabolic process, xenobiotic metabolic process
9	IPI:IPI00299024.9	BASP1	Brain acid soluble protein 1	0.4	4.61×10^{-4}	6	thorax and anterior abdomen determination, gonad development
10	IPI:IPI00219682.6	STOM	Erythrocyte band 7 integral membrane protein	0.42	3.18×10^{-2}	3	protein homooligomerization
11	IPI:IPI00465248.5	ENO1	Isoform alpha-enolase of Alpha-enolase	0.42	3.55×10^{-5}	18	negative regulation of transcription from RNA polymerase II promoter, carbohydrate metabolic process
12	IPI:IPI00413958.4	FLNC	Isoform 2 of Filamin-C	0.42	1.23×10^{-2}	11	cell junction assembly, muscle fiber development
13	IPI:IPI00383849.5	PLCG1	phospholipase C gamma 1 isoform a	0.44	3.96×10^{-2}	2	signal transduction, epidermal growth factor receptor signaling pathway
14	IPI:IPI00095891.2	GNAS	Isoform XLas-1 of Guanine nucleotide-binding protein G(s) subunit alpha isoforms XLas	0.46	1.03×10^{-2}	4	skeletal system development, tissue homeostasis
15	IPI:IPI00796942.1	SNAP23	SNAP23 12 kDa protein	0.48	1.32×10^{-2}	4	exocytosis, cellular membrane fusion
16	IPI:IPI00019906.1	BSG	Isoform 2 of Basigin precursor	0.55	3.28×10^{-3}	7	pyruvate metabolic process, cell surface receptor signaling pathway
17	IPI:IPI00221232.9	GNG12	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-12 precursor	0.61	7.47×10^{-3}	2	energy reserve metabolic process, signal transduction
18	IPI:IPI00216308.5	VDAC1	Voltage-dependent anion-selective channel protein 1	0.64	2.22×10^{-3}	9	behavioral fear response, anion transport
19	IPI:IPI00647915.1	TAGLN2	24 kDa protein	0.64	2.51×10^{-4}	3	muscle organ development
20	IPI:IPI00010796.1	P4HB	Protein disulfide-isomerase precursor	0.73	2.69×10^{-2}	10	lipid metabolic process, glycerol ether metabolic process

<i>n</i>	accession	gene symbol	name	MKN7:HFE145	<i>p</i> -value MKN7:HFE145	unique iTRAQ peptide	molecular function based on GO
21	IPI:IPI00016513.5	RAB10	Ras-related protein Rab-10	1.33	3.29×10^{-3}	2	GTP catabolic process, intracellular protein transport
22	IPI:IPI00455383.4	CLTC	Isoform 2 of Clathrin heavy chain 1	1.35	1.49×10^{-3}	33	intracellular protein transport, post-Golgi vesicle-mediated transport
23	IPI:IPI00007682.2	ATP6 V1A	Vacuolar ATP synthase catalytic subunit A	1.38	1.20×10^{-2}	3	transport, ion transport
24	IPI:IPI00003527.5	SLC9A3R1	Ezrin-radixin-moesin-binding phosphoprotein 50	1.48	4.06×10^{-2}	3	renal sodium ion transport, protein complex assembly
25	IPI:IPI00017184.2	EHD1	EH domain-containing protein 1	1.53	1.47×10^{-2}	2	intracellular protein transport, endocytosis
26	IPI:IPI00402231.1	DNAJC5	Isoform 1 of DnaJ homologue subfamily C member 5	1.67	3.70×10^{-2}	2	protein folding, synaptic transmission
27	IPI:IPI00848265.1	CTNND1	catenin, delta 1 isoform 1B	1.68	4.73×10^{-3}	8	morphogenesis of a polarized epithelium, cell adhesion
28	IPI:IPI00473136.3	CTNNA1	Isoform 2 of Catenin alpha-1	1.89	7.10×10^{-5}	13	cell adhesion, establishment or maintenance of cell polarity
29	IPI:IPI00007812.1	ATP6	V1B2 Vacuolar ATP synthase subunit B, brain isoform	1.96	1.53×10^{-2}	4	ion transport, cellular iron ion homeostasis
30	IPI:IPI00872375.2	SLC2A1	Uncharacterized protein SLC2A1 (Fragment)	1.97	9.02×10^{-4}	3	carbohydrate metabolic process, energy reserve metabolic process
31	IPI:IPI00384280.5	PCYOX1	Prenylcysteine oxidase 1 precursor	2.07	3.06×10^{-2}	4	chloride transport, prenylated protein catabolic process
32	IPI:IPI00015973.1	EPB41L2	Band 4.1-like protein 2	2.08	8.22×10^{-4}	5	cortical actin cytoskeleton organization
33	IPI:IPI00556590.2	RALA	Ras-related protein Ral-A precursor	2.25	5.57×10^{-3}	5	cytokinesis, intracellular protein transport
34	IPI:IPI00871221.1	ATP1B1	Isoform 2 of Sodium/potassium-transporting ATPase subunit beta-1	2.95	3.46×10^{-2}	2	transport
35	IPI:IPI00549413.2	ANXA1	Annexin A1	3.07	2.42×10^{-3}	3	negative regulation of acute inflammatory response, lipid metabolic process
36	IPI:IPI00027422.1	ITGB4	Isoform Beta-4C of Integrin beta-4 precursor	3.69	1.34×10^{-2}	5	cell communication, cell adhesion
37	IPI:IPI00382926.2	CD55	Decay-accelerating factor splicing variant 5	3.71	4.90×10^{-2}	2	complement activation, classical pathway, innate immune response
38	IPI:IPI00556655.1	LAMP1	LAMP1 protein variant (Fragment)	4.01	4.36×10^{-2}	2	autophagic cell death
39	IPI:IPI00008494.4	ICAM1	Intercellular adhesion molecule 1 precursor	4.27	9.47×10^{-5}	8	response to hypoxia, cell adhesion
40	IPI:IPI00016334.2	MCAM	Isoform 1 of Cell surface glycoprotein MUC18 precursor	4.42	9.34×10^{-6}	6	cell adhesion
41	IPI:IPI00871932.1	SPTBN1	SPTBN1 276 kDa protein	4.42	3.49×10^{-6}	9	common-partner SMAD protein phosphorylation, SMAD protein import into nucleus
42	IPI:IPI00008986.1	SLC7A5	Large neutral amino acids transporter small subunit 1	4.64	8.82×10^{-3}	2	amino acid transmembrane transport, cellular amino acid metabolic process
43	IPI:IPI00017367.6	RDX	Radixin isoform b	4.68	1.72×10^{-8}	9	microvillus assembly, apical protein localization
44	IPI:IPI00554611.1	SLC3A2	solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2 isoform b	4.95	6.05×10^{-6}	5	amino acid transmembrane transport, cellular amino acid metabolic

<i>n</i>	accession	gene symbol	name	MKN7:HFE145	<i>p</i> -value MKN7:HFE145	unique iTRAQ peptide	molecular function based on GO
							process
45	IPI:IPI00216172.2	LAMP2	Lysosomal-associated membrane protein 2C	5.14	1.17×10^{-2}	2	platelet degranulation, blood coagulation
46	IPI:IPI00013744.1	ITGA2	Integrin alpha-2 precursor	6.07	5.00×10^{-3}	5	cell adhesion, cell-matrix adhesion
47	IPI:IPI00879959.1	ALPP	Alkaline phosphatase, placental type precursor	7.41	2.39×10^{-8}	15	metabolic process
48	IPI:IPI00010338.1	F3	Tissue factor precursor	7.61	6.27×10^{-8}	9	positive regulation of endothelial cell proliferation, activation of plasma proteins involved in acute inflammatory response
49	IPI:IPI00296215.2	TACSTD1	Tumor-associated calcium signal transducer 1 precursor	10.91	1.40×10^{-3}	5	positive regulation of cell proliferation
50	IPI:IPI00845478.1	ERBB2	erbB-2 isoform b	11.51	1.57×10^{-2}	4	protein phosphorylation, signal transduction

^aOnly the 50 up- and down-regulated plasma membrane proteins in gastric cancer cells are shown as they represent candidates that may be exploited for molecular imaging. The rest are listed in Supplementary Table 1. MKN7:HFE145 refers to relative level of protein expression in MKN7 gastric cancer cells with respect to noncancer HFE145 cells. Statistical calculation for iTRAQ-based detection and relative quantification were calculated using the Paragon Algorithm in the ProteinPilot software.