

# Discovery of Colorectal Cancer Biomarker Candidates by Membrane Proteomic Analysis and Subsequent Verification using Selected Reaction Monitoring (SRM) and Tissue Microarray (TMA) Analysis\*

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Recent advances in quantitative proteomic technology have enabled the large-scale validation of biomarkers. We here performed a quantitative proteomic analysis of membrane fractions from colorectal cancer tissue to discover biomarker candidates, and then extensively validated the candidate proteins identified. A total of 5566 proteins were identified in six tissue samples, each of which was obtained from polyps and cancer with and without metastasis. GO cellular component analysis predicted that 3087 of these proteins were membrane proteins, whereas TMHMM algorithm predicted that 1567 proteins had a transmembrane domain. Differences were observed in the expression of 159 membrane proteins and 55 extracellular proteins between polyps and cancer without metastasis, while the expression of 32 membrane proteins and 17 extracellular proteins differed between cancer with and without metastasis. A total of 105 of these biomarker candidates were quantitated using selected (or multiple) reaction monitoring (SRM/MRM) with stable synthetic isotope-labeled peptides as an internal control. The results obtained revealed differences in the

expression of 69 of these proteins, and this was subsequently verified in an independent set of patient samples (polyps ( $n = 10$ ), cancer without metastasis ( $n = 10$ ), cancer with metastasis ( $n = 10$ )). Significant differences were observed in the expression of 44 of these proteins, including ITGA5, GPRC5A, PDGFRB, and TFRC, which have already been shown to be overexpressed in colorectal cancer, as well as proteins with unknown function, such as C8orf55. The expression of C8orf55 was also shown to be high not only in colorectal cancer, but also in several cancer tissues using a multicancer tissue microarray, which included 1150 cores from 14 cancer tissues. This is the largest verification study of biomarker candidate membrane proteins to date; our methods for biomarker discovery and subsequent validation using SRM/MRM will contribute to the identification of useful biomarker candidates for various cancers. Data are available via ProteomeXchange with identifier PXD000851. *Molecular & Cellular Proteomics* 13: 10.1074/mcp.M113.037093, 1471–1484, 2014.

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Received December 13, 2013, and in revised form, March 19, 2014  
Published, MCP Papers in Press, March 31, 2014, DOI 10.1074/mcp.M113.037093

Author contributions: H.K. and T.T. designed research; H.K., S.M., T.K., R.N., and J.F. performed research; R.N., S.W., M.K., Y.K., K.M., J.F., T.M., Y.I., H.M., and F.N. contributed new reagents or analytic tools; H.K., T.K., J.A., S.W., M.K., J.F., and T.T. analyzed data; H.K. and T.T. wrote the paper.

Recent advances in proteomic technology have contributed to the identification of biomarkers for various diseases. Improvements in LC-MS technology have led to an increase in the number of proteins that have been identified. In addition, a stable isotopic labeling method using isobaric tag for relative and absolute quantitation (iTRAQ)<sup>1</sup> and stable isotope labeling by amino acids in cell culture has enabled the quantitative analysis of multiple samples (1, 2). Therefore, a large

<sup>1</sup> The abbreviations used are: iTRAQ, isobaric tag for relative and absolute quantitation; SRM, selected reaction monitoring; MRM, multiple reaction monitoring; PTS, phase-transfer surfactant; SI-peptide, stable isotope-labeled peptide; CID, collision-induced dissociation; HCD, higher energy collision-induced dissociation; IHC, immunohistochemistry; LC-MS/MS, liquid chromatography tandem mass spectrometry; CE, collision energy; LTQ, linear ion trap; FDR, false discovery rate; TMA, tissue microarray.

number of proteins have already been identified as biomarker candidates; however, only a few of these have been used in practical applications because most have not yet progressed to the validation stage, in which potential biomarker candidates are quantified on a large scale. The validation of biomarker candidates is generally accomplished using Western blotting and enzyme-linked immunosorbent assays (ELISA) if specific and well-characterized antibodies for these candidates are available. However, highly specific antibodies are not currently available for most novel biomarker candidate proteins, and it takes a significant amount of time and money to obtain these antibodies and optimize ELISA assay systems for many candidates; therefore, another validation assay system needs to be developed. Selected (or multiple) reaction monitoring (SRM or MRM) was previously shown to be a potentially effective method for the validation of biomarker candidates (3–5). The SRM/MRM assay can measure multiple targets at high sensitivity and throughput without antibodies; hence, it is useful for initial quantitative evaluations and the large-scale validation of biomarker candidates, which defines validation of hundreds of biomarker candidate proteins simultaneously.

In addition to these technical improvements, the fractionation process also plays an important role in proteome analysis for biomarker discovery. This procedure very effectively analyzes the proteomes of specific cellular compartments or organelles in detail, which reduces sample complexity. The preparation of a membrane fraction was previously shown to be useful for identifying membrane proteins that are generally expressed at relatively low levels. Membrane proteins play critical roles in many biological functions, such as signal transduction, cell-cell interactions, and ion transport, account for ~38% of all proteins encoded by the mammalian genome and more than one-third of biomarker candidates, and are also potential targets for drug therapy (6, 7). Therefore, membrane proteome analysis is important for biomarker discovery. However, difficulties have been associated with extracting and solubilizing membrane proteins and subsequent protease digestion. Many procedures have consequently been developed to improve the solubilization and digestion of membrane proteins (8–11), and a protocol using phase transfer surfactant (PTS) was shown to be suitable for membrane proteomics using LC-MS/MS (12, 13).

The selection of a control group for comparisons is also important for identifying potential biomarkers. Tissue samples from cancer patients have been used in many studies to discover biomarker candidates by proteomic analysis. Previous studies, including our own, attempted to compare cancer tissues with matched normal tissue (14–17). However, marked differences have been reported in the histology, genetics, and proteomics of normal and cancer tissues, and many biomarker candidates have been identified, by making it difficult to narrow down more reliable candidates for further validation. Lazebnik recently emphasized that the features of

malignant, but not benign tumors could be used as a hallmark of cancer (18), and also that premalignant lesions were more appropriate controls for cancer tissue than normal tissue for the identification of biomarker candidates involved in cancer progression. Moreover, comparisons of cancer with and without metastasis may also assist in the discovery of biomarker candidates involved in cancer metastasis. Therefore, the identification of biomarker candidates that can be used to diagnose and determine the prognosis of cancer should become more effective by comparing cancer tissues at different stages, including benign tumors.

We performed a shotgun proteomic analysis of membrane fractions prepared from colorectal cancer tissue and benign polyps in the present study to identify biomarker candidates for the diagnosis and treatment of cancer. We identified a large number of biomarker candidate proteins associated with the progression of colon cancer by using membrane protein extraction with PTS followed by iTRAQ labeling. SRM/MRM confirmed the altered expression of these biomarker candidates, and these results were further verified using an independent set of tissue samples. A protein with uncharacterized function, C8orf55, was also validated with a tissue microarray that included various types of cancers.

### EXPERIMENTAL PROCEDURES

*Tissue Samples*—Tissues from 33 cases of primary colorectal cancer were surgically resected. A total of 16 colon polyps were obtained by endoscopic polypectomy. Written informed consent was obtained from each patient before surgery. The Ethics Committee of Chiba University School of Medicine and our institute approved the protocol. The excised samples were obtained from polyp and cancer tissues within one hour of surgery. All excised tissues were immediately placed in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for further analyses.

*Preparation of Membrane Fractions*—Membrane fractions were prepared as previously described (19, 20). Tissue samples were washed twice with ice-cold PBS and then homogenized with a Dounce homogenizer in ice-cold PBS containing a protease inhibitor mixture (Roche Diagnostics, Mannheim, Germany). The homogenate was centrifuged at  $1000 \times g$  for 10 min at  $4^{\circ}\text{C}$ , and the post-nuclear supernatant was centrifuged at  $100,000 \times g$  for 1 h at  $4^{\circ}\text{C}$ . The pellet was suspended in ice-cold  $0.1 \text{ M Na}_2\text{CO}_3$  solution and centrifuged at  $100,000 \times g$  for 1 h at  $4^{\circ}\text{C}$ . After its resuspension and centrifugation, the pellet was collected as the membrane fraction. This fraction was solubilized with MPEX PTS reagent solution (GL Science, Tokyo, Japan) at  $95^{\circ}\text{C}$  for 5 min followed by sonication for 5 min using a Bioruptor sonicator (Cosmo Bio, Tokyo, Japan). After centrifugation at  $100,000 \times g$  for 30 min at  $4^{\circ}\text{C}$ , the supernatant was obtained as a membrane fraction extract and quantified using a DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA). The reference pool was arranged by mixing an equal amount ( $40 \mu\text{g}$ ) of 18 membrane fraction extracts prepared from the tissues of patients.

*Peptide Labeling with iTRAQ Reagents*—iTRAQ labeling was performed as previously described (19–22). The membrane fraction extract ( $90 \mu\text{g}$ ) for iTRAQ labeling was reduced with 1/20 volume of  $100 \text{ mM DTT}$  in  $50 \text{ mM NaHCO}_3$  for 30 min at room temperature (RT) after the addition of bovine serum albumin (BSA) ( $0.45 \mu\text{g}$ ) as the internal standard. BSA was spiked into each sample and the iTRAQ ratios, 115:114, 116:114, 117:114, of each experiment were normalized according to the iTRAQ ratios of the BSA added to each sample in order

to correct for experimental errors, such as tryptic digestion efficiency, and instrumental errors. A 1/20 volume of 550 mM iodoacetic acid in 50 mM NaHCO<sub>3</sub> was then used for alkylation for 30 min at RT. The alkylated sample was digested with 1% trypsin overnight at 37 °C and treated using the PTS method (12, 13) to remove the MPEX PTS reagent. This tryptic digest was desalted using C<sub>18</sub> stage Tips (23). DTT, which interfered with iTRAQ labeling, was removed using the PTS method and the next stage involved Tip purification. The desalted peptides were then suspended in 30 μl of iTRAQ dissolution buffer and labeled with iTRAQ reagents (Applied Biosystems, Foster City, CA) for 1 h at RT. The tryptic digests of the reference pool, cancer without metastasis, cancer with metastasis, and polyps were labeled with iTRAQ reagents 114, 115, 116, and 117, respectively (supplemental Table S2). The 115:114, 116:114, and 117:114 ratios indicated the relative abundance of proteins in cancer without metastasis, cancer with metastasis, and polyps, respectively, relative to the common reference pool. Therefore, all samples could be compared, even between different experiments. The labeled samples were then pooled and desalted using C<sub>18</sub> stage Tips. A total of six 4-plex iTRAQ experiments were performed.

**Fractionation with the SCX Column**—iTRAQ-labeled peptides were resuspended in buffer A (10 mM KH<sub>2</sub>PO<sub>4</sub> (pH 3) and 25% acetonitrile) and fractionated using a HPLC system (Shimadzu prominence UFLC) with a SCX column (50 × 2.1 mm, 5 μm, 300 Å, ZORBAX 300SCX; Agilent Technology, Santa Clara, CA). Buffer A and buffer B (10 mM KH<sub>2</sub>PO<sub>4</sub> (pH 3), 25% acetonitrile, 1 M KCl) were used in the mobile phase. The loaded peptides were separated at a flow rate of 200 μl/min with a gradient of 0% B for 30 min, 0% to 10% B in 15 min, 10% to 25% B in 10 min, 25% to 40% B in 5 min, 40% to 100% B in 5 min and 100% B for 10 min. The elution was collected every 1 min and desalted using C<sub>18</sub> stage Tips. iTRAQ-labeled peptides were divided into 80 fractions by SCX column chromatography. We monitored the concentrations of these fractions by UV spectroscopy and then combined low-concentration fractions, which resulted in 36 fractions. SCX-fractionated peptides were desalted using C18 stage Tips and dissolved in 20 μl of 2% acetonitrile and 0.1% trifluoroacetic acid.

**LC-MS/MS**—The SCX-fractionated peptides were analyzed by nano-LC-MS/MS using LTQ-Orbitrap XL (Thermo Fisher Scientific, Bremen, Germany) with a nano-LC interface (AMR, Tokyo, Japan), Paradigm MS2 (Michrom Bioresources, Auburn, CA), and HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland). One-quarter or one-fifth of the volume of each SCX fraction was injected into a trap column (0.3 × 5 mm, L-column ODS; Chemicals Evaluation and Research Institute (CERI), Tokyo, Japan) and separated on an analytical column (0.1 × 200 mm in-house developed Tip Column packed with L-column2 C<sub>18</sub> particles; CERI). Buffer A (2% acetonitrile, 0.1% formic acid) and buffer B (90% acetonitrile, 0.1% formic acid) were used in the mobile phase, and the injected peptides were eluted using a gradient from 5% to 30% buffer B at a flow rate of 500 nL/min in 145 min. A spray voltage of 2000 V was applied. The MS scan range was *m/z* 350–1500. The top three precursor ions in the MS scan by Orbitrap were selected for subsequent MS/MS scans by ion trap (CID) and Orbitrap (HCD) in the automated gain control (AGC) mode in which AGC values of 5.00e + 05, 1.00e + 04, and 2.00e + 04 were set for full MS, CID MS/MS, and HCD MS/MS, respectively.

**Identification and Quantification of Proteins**—Raw data were examined using Proteome Discoverer ver.1.3 (Thermo Fisher Scientific) with Mascot v2.3.1 (Matrix Science, London, UK) against UniProt/SwissProt (release-2010\_05), which contained 20,295 sequences of *Homo sapiens*, following LC-MS/MS analysis. The search parameters were as follows: precursor mass tolerance of 7 ppm, fragment ion mass tolerance of 0.6 Da (CID), and 0.01 Da (HCD), and one missed cleavage was allowed. The carboxymethylation of cysteine, iTRAQ

(K), and iTRAQ (N-terminal) was chosen for the fixed modification. iTRAQ (Y) and oxidation (M) were chosen for variable modifications. The false discovery rate (FDR) was calculated by enabling peptide sequence analysis using Percolator. High-confidence peptide identification was obtained by setting a target FDR threshold of <1.0% at the peptide level. A minimum of two peptides meeting the criteria were required for protein identification. Protein quantification was performed using Proteome Discoverer ver.1.3 and the quantitative value was normalized using that of spiked BSA. Unique BSA peptides were examined using Proteome Discoverer ver.1.1 (Thermo Fisher Scientific) against MSIP1-human version 3.67, which contained BSA sequences.

**SRM/MRM Analysis**—We used SRM/MRM to confirm and further verify the biomarker candidates obtained from iTRAQ. We firstly performed two technical replicates of SRM/MRM for confirmation using the same individual tissue samples as those used in the iTRAQ discovery experiment. Assays were constructed to measure two distinct peptides per-protein and that the individual assays for each of the two peptides are labeled SRM-1 and SRM-2. We then performed two technical replicates of SRM/MRM with one peptide per protein target for verification using a separate tissue sample set from that used in the discovery experiment. Five technical replicates from a tissue sample mixture were used to assess the reproducibility of SRM/MRM. In our experiments, technical replicates were performed as follows; a single sample was fully processed to peptides, and analyzed twice or five times by LC-SRM/MRM method. We did not analyze process replicates, which includes tryptic digestion and other sample handling steps, in this study.

SRM/MRM was performed as previously described (19, 21). Stable synthetic isotope-labeled peptides (SI peptides) with a C-terminal 15N- and 13C-labeled arginine or lysine residue (isotopic purity >99%) were purchased from Greiner Bio One (Frickenhausen, Germany) (crude purity). The peptide sequence was selected from the unique peptide sequences identified in the iTRAQ experiments. Peptides containing a cysteine residue (Cys) were also used if another adequate sequence peptide could not be detected. If the SI peptide contained a Cys, the peptide was reduced, alkylated, and then used. The SI peptides were divided into four groups and then mixed, and the four mixtures were separately used for SRM/MRM.

The SI peptide mixture was analyzed by the above-mentioned LC-MS/MS method using LTQ Orbitrap-XL to acquire MS data. A preliminary SRM/MRM-transition list for SI peptides was created from the MS data acquired using Pinpoint ver.1.0 (Thermo Fisher Scientific, Bremen, Germany). The SI peptide mixture was then analyzed using a TSQ-Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) with a nano-LC interface (AMR, Tokyo, Japan), Paradigm MS2 (Michrom Bioresources, Auburn, CA), and HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland). The data obtained were analyzed using Pinpoint software to optimize parameters such as collision energy and acquire the retention times of each SI peptide. The timed-SRM/MRM method (retention time window of ±2 min) was created using these parameters and then optimized. Finally, four optimal transitions per peptide were selected for quantitation using SRM/MRM.

A membrane fraction extract (2 μg) prepared from tissue samples was alkylated with iodoacetamide and then digested as described above for quantitation using SRM/MRM. The digested peptide was dissolved in 2% acetonitrile and 0.1% trifluoroacetic acid, and analyzed using the above-described optimal timed-SRM/MRM method with TSQ-Vantage. We performed a washing step between each LC MS/MS analysis to minimize carry over. The SI peptide mixture was added to the trypsin-digested sample, and the area ratio of the endogenous peptide to the SI peptide was calculated using the transition peak area measured with Pinpoint software. The amount of

each SI peptide was adjusted to be similar to the endogenous peptide estimated by the peak area obtained from preliminary SRM/MRM of the sample mixture. The average of these ratios of more than two transitions was first calculated, and the average ratio of two technical replicates of an individual sample was then determined as the relative quantitative value of the target peptide. Statistical analysis of the area ratios was performed using the *t* test.

We excluded transition peaks with a signal-to-noise ratio <10, which has been used as empirical LOQ (24), and then compared the profile and proportion of the remaining transition peaks between the SI peptide and endogenous peptide to select appropriate peaks for quantitative analysis. The signal-to-noise ratio was identified using Pinpoint software. Removing the outliers of transitions because of interference or co-elution of nonspecific backgrounds was essential to improve accuracy and reliability. Each transition among the samples had to exhibit a similar peak shape to that with the transition of the SI peptide, which resulted in a minimal CV area ratio (CV<35%) between transitions. We confirmed every transition peak by a manual inspection and removed peaks that did not fulfill the above criteria.

**Data Analysis**—The transmembrane domains of the identified proteins were predicted using the TMHMM program (<http://www.cbs.dtu.dk/services/TMHMM/>). Candidate proteins were analyzed using ProteinCenter for cellular component annotation (Thermo Fisher Scientific).

**Protein Extraction and Western Blotting**—Frozen tissue samples were solubilized in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, protease inhibitor mixture; Roche Diagnostics, Mannheim, Germany) using a Bioruptor sonicator (Cosmo Bio, Tokyo, Japan) following centrifugation at  $100,000 \times g$  for 30 min at 4 °C. The supernatant proteins were separated by electrophoresis on 5% to 20% precast gradient gels (DRC Co., Ltd., Tokyo, Japan). Proteins were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA), and the membranes were then blocked with ImmunoBlock (DS Pharma Biomedical, Osaka, Japan). An anti-ITGA5 antibody (R&D Systems; 1:1000) and anti-C8orf55 antibody (Sigma-Aldrich; 1:1000) were used as primary antibodies. Antigens on the membrane were detected with enhanced chemiluminescence detection reagents (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

**Immunohistochemistry**—Tissues were fixed on slide glasses with 4% paraformaldehyde for 10 min at 4 °C. After three washes with PBS, the specimens were treated with 0.5% Triton X-100 in PBS followed by blocking with 3% bovine serum albumin in PBS containing 0.1% Tween-20 (PBST) for 1 h. Samples were then incubated with anti-C8orf55 (1:1000) for 1 h. After washing three times with PBST, samples were treated with horseradish peroxidase-conjugated anti-rabbit IgG (GE Healthcare) for 1 h. After another three washes with PBST, the DAKO EnVision/HRP kit (DAKO Japan, Kyoto, Japan) was used to visualize tissue antigens according to the manufacturer's instructions. Tissue sections were counterstained with hematoxylin for 30 s, dehydrated with 100% ethanol and xylene, and coverslips were mounted with Malinol (Mito Pure Chemicals, Tokyo, Japan).

The tissue microarray used in this study (TMA1150) had 1150 cores from 14 common cancer types (100 cases each of lung (squamous cell carcinoma), lung (adenocarcinoma), breast, kidney, biliary tract, thyroid, liver, colon, and stomach cancer; and 50 cases each of prostate, pancreas, bladder, ovary, and uterine body cancer) (25). The normal tissue array used here contained 280 cores from 13 normal tissues (20 or 40 cases each of lung, breast, kidney, biliary tract, thyroid, liver, colon, stomach, prostate, pancreas, bladder, ovary, and uterine body cancer). These tissue arrays were purchased from Pathology Institute Corp. (Toyama, Japan). After sections were deparaffinized and hydrated, antigen retrieval was performed using a pressure chamber (Pascal; DAKO Japan) in which tissues were heated to 125 °C, maintained at this temperature for 1 min, and then cooled to

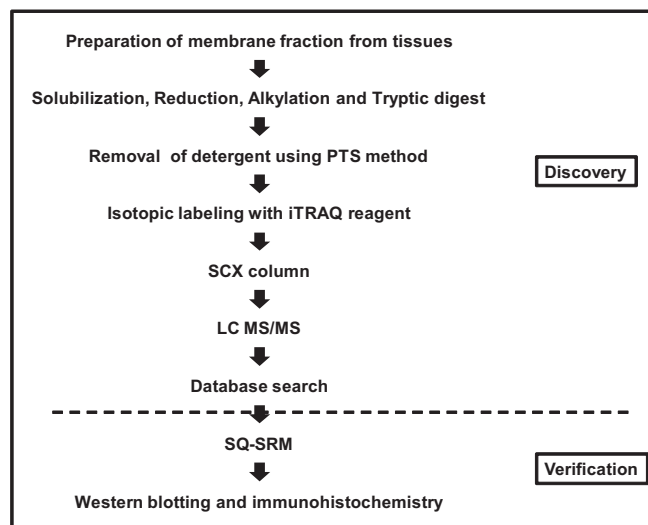


FIG. 1. Outline of the experimental workflow.

90 °C. After rinsing, slides were placed in an Autostainer (DAKO Japan) and an Envision+ detection system was used as suggested by the manufacturer's protocol (DAKO Japan). The cores stained with anti-C8orf55 were examined by three of the authors. Staining intensity was recorded using the following scale: 0, no staining, or cytoplasm staining in <10% of tumor cells; 1, faint/barely perceptible cytoplasm staining in >10% of tumor cells (cells exhibited incomplete cytoplasm staining); 2, weak or moderate cytoplasm staining in >10% of tumor cells or strong cytoplasm staining in <30%; and 3, strong cytoplasm staining in >30% of tumor cells.

## RESULTS

**iTRAQ Analysis of Membrane Proteins Prepared from Colorectal Cancer Tissue and Polyps**—We performed shotgun proteomics of colorectal cancer tissue and premalignant lesions using iTRAQ to identify biomarker candidate proteins for colorectal cancer. Six tissues each were collected from patients with colorectal polyps and cancer with and without metastasis to examine the changes in protein expression associated with cancer progression (Supplemental Table 1). We were particularly interested in changes in membrane proteins; therefore, the membrane fraction prepared from these specimens was dissolved in PTS solution and digested with trypsin, followed by the removal of detergents (Fig. 1). Portions of the extracts of all samples were mixed in equal amounts and treated in the same manner to obtain a reference pool. The trypsin-digested reference pool, cancer without metastasis, cancer with metastasis, and polyps were labeled with iTRAQ reagents 114, 115, 116, and 117, respectively (supplemental Table S2). The iTRAQ-labeled peptides were merged in each experimental set, fractionated by SCX chromatography, and analyzed using LC-MS/MS. The ratios 115:114, 116:114, and 117:114 indicated the higher abundance of proteins in cancer without metastasis, cancer with metastasis, and polyps, respectively, than in the same reference pool. The iTRAQ ratios 115:114, 116:114, and 117:114 of each experiment were normalized using the iTRAQ

ratios of the BSA added to each sample in order to correct for experimental errors, such as tryptic digestion efficiency, and instrumental errors (supplemental Table S3).

The reproducibility of the sample preparation was demonstrated by labeling membrane fractions from the same tissue (supplemental Fig. S1). The iTRAQ ratios 116:114 of the BSA added were all close to 1, which indicated minimal technical errors including the digestion of proteins with trypsin (supplemental Fig. S1). A total of 5566 unique proteins were identified using six iTRAQ analysis sets (4195–4633 unique proteins in each experiment; supplemental Tables S2–S4). However, data for cancer without metastasis in the 6th iTRAQ set were removed from this list because we could not obtain adequate data for this group, and this was attributed to a failure in iTRAQ labeling. A total of 1567 proteins (28.2%) were predicted to have a transmembrane domain by the TMHMM program (Table I). In addition, 5287 of the 5566 identified proteins were annotated by GO cellular component analysis: 3087 (58.4%) and 652 (12.3%) were predicted to be membrane proteins and extracellular proteins, respectively (Table I). A total of 4747 proteins were quantified with iTRAQ in at least two of the six analysis sets (supplemental Table S3); thus, we investigated changes in the expression of these 4747 proteins with cancer progression. Differences were observed in the expression of 159, 32, or 99 membrane proteins between polyps and cancer without metastasis, cancer with and without metastasis, or polyps and cancer with metastasis, respectively (ratio >2.0,  $p$  value <0.1; ratio <0.5,  $p$  value <0.1) (Table II). Differences were also noted in the expression of 55, 17, or 37 extracellular proteins between polyps and cancer without metastasis, cancer with and

without metastasis, or polyps and cancer with metastasis, respectively. We then focused on extracellular proteins because they are secreted or shed from cancer cells and may be useful markers.

**Confirmation of Biomarker Candidates by SRM/MRM**—Many biomarker candidate proteins have been identified using proteomic analysis; however, most were not validated for the following reasons: (a) the number of candidate proteins was large, (b) specific and well-characterized antibodies for most of these candidates were unavailable for verification by Western blotting, immunostaining, and ELISA, (c) it took too much time and money to optimize these assays, and (d) only a small amount of protein was available to validate biomarker candidates when the protein was prepared from patient tissue, especially the membrane fraction. These difficulties were recently overcome with the SRM/MRM assay, which was shown to be useful for the validation of biomarker candidates because multiple target proteins in a small sample could be analyzed in a single run (3–5). Thus, we used the SRM/MRM method to confirm the results obtained in the iTRAQ experiments and prioritized further validation studies.

In the present study, we selected 105 proteins of the biomarker candidates identified based on the following criteria (Table III): (a) The candidate proteins were quantified in at least two of six iTRAQ experiments. (b) The proteins were predicted to be membrane or extracellular proteins (Human leukocyte antigens were excluded from the candidate list because the proteins were expressed systemically. Proteins such as nuclear or mitochondrial proteins were also excluded.), (c) Differences were observed in the expression of the candidates (ratio >2.0,  $p$  value <0.1; ratio <0.5,  $p$  value <0.1) between polyps and cancer without metastasis, cancer with and without metastasis, or polyps and cancer with metastasis. Of the selected candidates, 66 proteins were more strongly expressed in nonmetastatic cancer than in polyps, whereas 10 proteins were more strongly expressed in metastatic cancer than in nonmetastatic cancer (Tables 3A and B). Thirteen proteins were more weakly expressed in nonmetastatic cancer than in polyps, whereas six proteins were more weakly expressed in metastatic cancer than in nonmetastatic cancer (Tables 3C and D). Ten proteins were more strongly expressed in metastatic cancer than in polyps (Table 3E).

One or two peptide sequences corresponding to the 105 candidate proteins were selected as target sequences for

TABLE I  
Number of predicted membrane proteins

	Total identified proteins	
	number	%
Number of proteins with transmembrane domains	1567 <sup>a</sup>	28.2
GO-annotated	5287	100
Membrane	3087	58.4 <sup>b</sup>
Extracellular	652	12.3 <sup>c</sup>

<sup>a</sup> Number of proteins with transmembrane domains predicted by TMHMM algorithm.

<sup>b,c</sup> The ratio of membrane or extracellular proteins to GO-annotated proteins.

TABLE II  
Number of proteins with significant difference in expression. C/P, ratio of cancer without metastasis to polyps. Cm/C, ratio of cancer with metastasis to cancer without metastasis. Cm/P, ratio of cancer with metastasis to polyps. TM + mem, number of proteins with predicted transmembrane domain or annotated as membrane protein. Extra, number of proteins annotated as extracellular protein

ratio	$p$ value	C/P		Cm/C		Cm/P	
		TM + mem	Extra	TM + mem	Extra	TM + mem	Extra
> 2.0	<0.1	108	34	21	8	79	21
< 0.5	<0.1	51	21	11	9	20	16
total		159	55	32	17	99	37

## Discovery and Validation of Membrane Protein Tumor Markers

TABLE III

List of the proteins analyzed by SRM/MRM and their quantitation data using iTRAQ. *p* values were calculated by *t*-test. TM, number of transmembrane domain. C/P, average ratio of cancer without metastasis to polyps. Cm/C, average ratio of cancer with metastasis to cancer without metastasis. Cm/P, average ratio of cancer with metastasis to polyps

A. The list of proteins increased in expression between polyps and cancer without metastasis (*n* = 66)

Accession	Protein name	Gene name	TM	GO (mem)	GO (extra)	C/P	<i>p</i> value	Cm/C	<i>p</i> value	Cm/P	<i>p</i> value
Q12884	Seprase	FAP	1	mem		5.98	<0.01	0.67	0.190	4.03	0.029
P32926	Desmoglein-3	DSG3	0	mem		4.54	<0.01	0.41	0.083	1.87	0.323
Q6P5W5	Zinc transporter ZIP4	SLC39A4	7	mem		4.35	0.075	0.42	0.189	1.84	0.217
Q8NFJ5	Retinoic acid-induced protein 3	GPRC5A	7	mem		3.99	<0.01	0.77	0.359	3.06	0.012
P40199	Carcinoembryonic antigen-related cell adhesion molecule 6	CEACAM6	0	mem		3.69	0.029	0.85	0.690	3.12	0.031
Q95832	Claudin-1	CLDN1	4	mem		3.47	0.054	0.51	0.180	1.77	0.127
Q8TF66	Leucine-rich repeat-containing protein 15	LRRC15	1	mem		3.40	0.032	0.58	0.193	1.96	0.060
P24158	Myeloblastin	PRTN3	0	mem	extra	3.35	0.098	0.38	0.134	1.28	0.526
P50150	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-4	GNG4	0	mem		3.31	0.074	0.77	0.570	2.56	0.051
P80511	Protein S100-A12	S100A12	0	mem	extra	3.28	0.068	1.06	0.857	3.46	0.070
P06731	Carcinoembryonic antigen-related cell adhesion molecule 5	CEACAM5	0	mem		3.27	<0.01	0.79	0.275	2.57	<0.01
Q9UKX5	Integrin alpha-11	ITGA11	1	mem		3.23	<0.01	0.62	0.081	2.00	0.016
Q10588	ADP-ribosyl cyclase 2	BST1	1	mem		3.16	0.023	0.55	0.102	1.75	0.033
P08253	72 kDa type IV collagenase	MMP2	0	mem	extra	3.02	<0.01	0.39	<0.01	1.19	0.599
Q9H6X2	Anthrax toxin receptor 1	ANTXR1	1	mem		2.98	<0.01	0.70	0.027	2.09	<0.01
Q9Y6I8	Peroxisomal membrane protein 4	PXMP4	0	mem		2.97	<0.01	0.74	0.084	2.19	<0.01
Q12805	EGF-containing fibulin-like extracellular matrix protein 1	EFEMP1	0	mem	extra	2.97	<0.01	0.57	0.037	1.71	0.040
Q14767	Latent-transforming growth factor beta-binding protein 2	LTBP2	0	mem	extra	2.91	<0.01	0.69	0.182	2.01	0.141
P16444	Dipeptidase 1	DPEP1	0	mem		2.85	0.033	1.06	0.854	3.02	<0.01
P84157	Matrix-remodeling-associated protein 7	MXRA7	1	mem		2.82	0.069	0.45	0.112	1.26	0.569
P11169	Solute carrier family 2, facilitated glucose transporter member 3	SLC2A3	10	mem		2.74	0.051	0.58	0.181	1.60	0.064
P08648	Integrin alpha-5	ITGA5	1	mem		2.59	<0.01	0.66	0.056	1.70	0.044
P55001	Microfibrillar-associated protein 2	MFAP2	0		extra	2.56	<0.01	0.46	<0.01	1.16	0.322
Q9ULK5	Vang-like protein 2	VANGL2	4	mem		2.55	0.098	0.39	0.086	1.00	0.989
Q5BJF2	Transmembrane protein 97	TMEM97	4	mem		2.54	<0.01	0.73	0.250	1.85	0.040
Q07075	Glutamyl aminopeptidase	ENPEP	1	mem		2.53	<0.01	0.70	0.201	1.77	0.104
Q9UGT4	Sushi domain-containing protein 2	SUSD2	1	mem		2.46	0.013	0.58	0.066	1.43	0.062
Q8N6Q3	CD177 antigen	CD177	0	mem		2.45	0.031	0.50	0.055	1.23	0.378
P07093	Glia-derived nexin	SERPINE2	0	mem	extra	2.43	0.059	0.85	0.699	2.06	0.132
Q96KR6	Transmembrane protein C20orf108	C20orf108	3	mem		2.39	0.020	0.73	0.287	1.75	0.041
P09619	Beta-type platelet-derived growth factor receptor	PDGFRB	1	mem		2.38	<0.01	0.85	0.423	2.01	0.014
Q7L4E1	Protein FAM73B	FAM73B	0	mem		2.34	<0.01	0.50	<0.01	1.17	0.289
O75954	Tetraspanin-9	TSPAN9	4	mem		2.31	<0.01	0.70	0.088	1.61	<0.01
Q9Y625	Glypican-6	GPC6	0	mem	extra	2.31	<0.01	0.63	0.055	1.45	0.179
Q8IUS5	Epoxide hydrolase 4	EPHX4	1	mem		2.29	0.043	1.13	0.614	2.59	<0.01
P36269	Gamma-glutamyltransferase 5	GGT5	1	mem		2.28	<0.01	0.71	0.172	1.63	0.047
Q8IWU6	Extracellular sulfatase Sulf-1	SULF1	0		extra	2.28	<0.01	0.82	0.445	1.88	0.074
Q6ZMP0	Thrombospondin type-1 domain-containing protein 4	THSD4	0		extra	2.26	0.042	0.59	0.278	1.35	0.656
P21730	C5a anaphylatoxin chemotactic receptor	C5AR1	7	mem		2.22	0.090	0.42	0.065	0.93	0.776
P35555	Fibrillin-1	FBN1	0	mem	extra	2.22	0.039	0.38	0.022	0.84	0.363
P98095	Fibulin-2	FBLN2	0		extra	2.20	<0.01	0.68	0.206	1.49	0.300
P31997	Carcinoembryonic antigen-related cell adhesion molecule 8	CEACAM8	0	mem	extra	2.20	0.090	0.51	0.118	1.11	0.592
Q14766	Latent-transforming growth factor beta-binding protein 1	LTBP1	0	mem	extra	2.19	<0.01	0.62	0.015	1.35	0.087
Q99720	Sigma non-opioid intracellular receptor 1	SIGMAR1	1	mem		2.19	<0.01	0.86	0.439	1.88	<0.01
P50281	Matrix metalloproteinase-14	MMP14	1	mem	extra	2.19	<0.01	0.70	0.096	1.53	0.078
P02786	Transferrin receptor protein 1	TFRC	1	mem	extra	2.18	<0.01	1.09	0.579	2.38	<0.01
P31431	Syndecan-4	SDC4	1	mem	extra	2.16	0.082	0.55	0.143	1.20	0.172
Q9UBG0	C-type mannose receptor 2	MRC2	1	mem		2.15	<0.01	0.68	0.078	1.47	0.230
Q9P121	Neurotrimin	NTM	0	mem		2.15	0.058	0.56	0.082	1.20	0.368
P09486	SPARC	SPARC	0		extra	2.14	<0.01	0.85	0.321	1.81	0.025
P05106	Integrin beta-3	ITGB3	1	mem		2.13	0.023	0.70	0.245	1.49	0.165
P04792	Heat shock protein beta-1	HSPB1	0	mem		2.11	<0.01	1.34	0.421	2.83	0.035
Q9NVM1	Protein FAM176B	FAM176B	1	mem		2.08	0.046	1.03	0.951	2.13	0.276
P08514	Integrin alpha-IIb	ITGA2B	1	mem		2.08	0.083	0.88	0.759	1.83	0.130

TABLE III—continued

Accession	Protein name	Gene name	TM	GO (mem)	GO (extra)	C/P	p value	Cm/C	p value	Cm/P	p value
Q8WUJ1	UPF0670 protein C8orf55	C8orf55	1		extra	2.07	<0.01	1.40	0.158	2.90	<0.01
P12314	High affinity immunoglobulin gamma Fc receptor I	FCGR1A	1	mem		2.07	<0.01	0.68	0.105	1.41	0.149
P04216	Thy-1 mem glycoprotein	THY1	0	mem		2.06	<0.01	0.77	0.092	1.59	0.023
P08174	Complement decay-accelerating factor	CD55	0	mem	extra	2.05	<0.01	1.04	0.879	2.13	0.020
Q96HV5	Transmembrane protein 41A	TMEM41A	6	mem		2.04	<0.01	0.76	0.060	1.54	<0.01
Q9ULS5	Transmembrane and coiled-coil domains protein 3	TMCC3	2	mem		2.04	0.040	0.61	0.195	1.25	0.434
Q01628	Interferon-induced transmembrane protein 3	IFITM3	2	mem		2.04	0.021	1.07	0.770	2.18	<0.01
P04920	Anion exchange protein 2	SLC4A2	11	mem		2.04	0.044	0.82	0.441	1.66	<0.01
Q9Y289	Sodium-dependent multivitamin transporter	SLC5A6	14	mem		2.03	<0.01	0.65	0.030	1.31	0.086
P30273	High affinity immunoglobulin epsilon receptor subunit gamma	FCER1G	1	mem		2.02	<0.01	0.71	0.140	1.43	0.141
P08473	Neprilysin	MME	1	mem		2.01	0.097	0.87	0.626	1.74	0.030
P13688	Carcinoembryonic antigen-related cell adhesion molecule 1	CEACAM1	1	mem	extra	2.00	0.014	0.74	0.169	1.48	0.173

B. The list of proteins increased in expression between cancer without and with metastasis (n = 10)

Accession	Protein name	Gene name	TM	GO (mem)	GO (extra)	C/P	p value	Cm/C	p value	Cm/P	p value
Q96HR9	Receptor expression-enhancing protein 6	REEP6	2	mem		1.13	0.651	3.18	0.070	3.61	0.035
P05451	Lithostathine-1-alpha	REG1A	0		extra	0.20	0.164	3.08	<0.01	0.60	0.379
Q8N323	Protein FAM55A	FAM55A	1		extra	0.22	0.102	2.98	0.057	0.65	0.416
O95395	Beta-1,3-galactosyl-O-glycosyl-glycoprotein beta-1,6-N-acetylglucosaminyltransferase 3	GCNT3	1	mem		0.82	0.595	2.85	0.086	2.33	0.089
O95994	Anterior gradient protein 2 homolog	AGR2	0		extra	0.44	0.012	2.56	0.094	1.12	0.727
Q9NRD8	Dual oxidase 2	DUOX2	6	mem		0.43	0.081	2.51	0.045	1.07	0.843
Q8TD06	Anterior gradient protein 3 homolog	AGR3	0		extra	0.51	0.028	2.49	0.017	1.26	0.301
Q09327	Beta-1,4-mannosyl-glycoprotein 4-beta-N-acetylglucosaminyltransferase	MGAT3	1	mem		0.89	0.694	2.24	0.046	1.99	0.024
Q9Y5L3	Ectonucleoside triphosphate diphosphohydrolase 2	ENTPD2	2	mem	extra	1.36	0.369	2.09	0.028	2.83	<0.01
Q8NCC5	Sugar phosphate exchanger 3	SLC37A3	12	mem		0.94	0.838	2.06	0.016	1.92	0.030

C. The list of proteins decreased in expression between polyps and cancer without metastasis (n = 13)

Accession	Protein name	Gene name	TM	GO (mem)	GO (extra)	C/P	p Value	Cm/C	p Value	Cm/P	p Value
A8K714	Calcium-activated chloride channel regulator 1	CLCA1	0	mem	extra	0.14	<0.01	0.85	0.673	0.12	<0.01
Q01524	Defensin-6	DEFA6	0		extra	0.18	0.053	0.69	0.428	0.12	0.043
Q9Y6R7	IgGfC-binding protein	FCGBP	0	mem	extra	0.23	<0.01	1.12	0.830	0.25	<0.01
Q6ZMB0	UDP-GlcNAc:betaGalbeta-1,3-N-acetylglucosaminyltransferase 6	B3GNT6	1	mem		0.26	<0.01	1.10	0.713	0.29	<0.01
Q02817	Mucin-2	MUC2	0		extra	0.26	<0.01	1.31	0.537	0.34	<0.01
Q07654	Trefoil factor 3	TFF3	0		extra	0.32	<0.01	1.14	0.651	0.37	<0.01
Q9HC84	Mucin-5B	MUC5B	0		extra	0.36	0.038	1.29	0.352	0.47	0.042
P27216	Annexin A13	ANXA13	0	mem		0.37	<0.01	1.24	0.574	0.46	0.018
P24588	A-kinase anchor protein 5	AKAP5	0	mem		0.43	0.017	1.12	0.606	0.48	0.011
Q7Z3J2	UPF0505 protein C16orf62	C16orf62	0	mem		0.46	<0.01	1.39	0.072	0.65	<0.01
P13727	Bone marrow proteoglycan	PRG2	0		extra	0.48	0.045	0.89	0.695	0.43	0.021
Q6UXG2	UPF0577 protein KIAA1324	KIAA1324	1	mem		0.49	0.051	1.25	0.390	0.61	0.085
Q9Y2J2	Band 4.1-like protein 3	EPB41L3	0	mem		0.49	0.062	1.35	0.086	0.66	0.167

D. The list of proteins decreased in expression between cancer without and cancer with metastasis (n = 6)

Accession	Protein name	Gene name	TM	GO (mem)	GO (extra)	C/P	p Value	Cm/C	p Value	Cm/P	p Value
P08123	Collagen alpha-2(I) chain	COL1A2	0	mem		1.87	0.086	0.34	0.010	0.63	0.213
O75015	Low affinity immunoglobulin gamma Fc region receptor III-B	FCGR3B	1	mem		2.14	0.236	0.34	0.057	0.73	0.673
P02452	Collagen alpha-1(I) chain	COL1A1	0	mem	extra	1.86	0.109	0.36	0.025	0.66	0.252
P02461	Collagen alpha-1(III) chain	COL3A1	0	mem		1.70	0.182	0.39	0.039	0.66	0.152
Q15063	Periostin	POSTN	0	mem		1.57	0.214	0.43	<0.01	0.67	0.406
O43934	UNC93-like protein MFSD11	MFSD11	10	mem		1.27	0.434	0.45	0.067	0.58	0.110

E. The list of proteins increased in expression between polyps and cancer with metastasis (n = 10)

Accession	Protein name	Gene name	TM	GO (mem)	GO (extra)	C/P	p Value	Cm/C	p Value	Cm/P	p Value
P21589	5'-nucleotidase	NT5E	2	mem		1.69	0.104	1.41	0.447	2.39	0.082
Q92968	Peroxisomal membrane protein PEX13	PEX13	0	mem		1.35	0.012	1.73	0.031	2.34	0.012
O43291	Kunitz-type protease inhibitor 2	SPINT2	1	mem	extra	1.63	<0.01	1.39	0.419	2.27	0.087
Q8N4S7	Progesterin and adipoQ receptor family member 4	PAQR4	3	mem		1.82	<0.01	1.23	0.342	2.25	0.019

TABLE III—continued

Accession	Protein name	Gene name	TM	GO (mem)	GO (extra)	C/P	<i>p</i> value	Cm/C	<i>p</i> value	Cm/P	<i>p</i> value
Q8NBM4	Ubiquitin-associated domain-containing protein 2	UBAC2	4	mem		1.95	<0.01	1.14	0.441	2.22	<0.01
Q96CP7	TLC domain-containing protein 1	TLCD1	5	mem		1.33	0.288	1.67	0.226	2.21	0.100
P05546	Heparin cofactor 2	SERPIND1	0		extra	2.15	0.124	0.99	0.978	2.13	0.024
P11166	Solute carrier family 2, facilitated glucose transporter member 1	SLC2A1	12	mem		1.92	<0.01	1.10	0.716	2.11	0.031
Q9BQD7	Protein FAM173A	FAM173A	1	mem		1.55	<0.01	1.36	0.236	2.11	0.050
Q96B21	Transmembrane protein 45B	TMEM45B	5	mem		1.29	0.132	1.61	0.100	2.07	0.018

SRM/MRM. We performed two technical replicates for each analysis. SI peptides were synthesized (supplemental Table S5) and spiked into the sample as an internal standard in SRM/MRM. Four transitions per peptide were selected based on precursor and product ion intensities, and parameters such as collision energy were optimized (supplemental Table S6).

We excluded transition peaks with a signal-to-noise ratio <10, which has been used as empirical LOQ (24), and then compared the profile and proportion of the remaining transition peaks between the SI peptide and endogenous peptide to select appropriate peaks for quantitative analysis. Removing the outliers of transitions because of interference or co-eluting nonspecific backgrounds was essential to improve accuracy and reliability. Each transition among the samples had to exhibit a similar peak shape to that with the transition of the SI peptide, which resulted in a minimal CV area ratio (CV<35%) between transitions. We confirmed every transition peak by a manual inspection and removed the peaks that did not conform to the above criteria, which led to accurate and significant quantitation (supplemental Fig. S2).

We obtained the average of these ratios of more than two transitions as the relative quantitative value of the target peptide. Statistical analysis of the area ratios was performed using the *t* test. In addition, if the expression of one of the two peptides of proteins was significantly different between the sample groups, we considered the protein to be differentially expressed. Using the SRM/MRM method, 172 peptides from 98 proteins were quantified in more than three samples from polyps and cancer with or without metastasis (supplemental Table S7). Significant differences (ratio >2.0, *p* value <0.1; ratio <0.5, *p* value <0.1) in at least one of the targeted peptides were detected in 69 proteins (supplemental Fig. S3, supplemental Table S7).

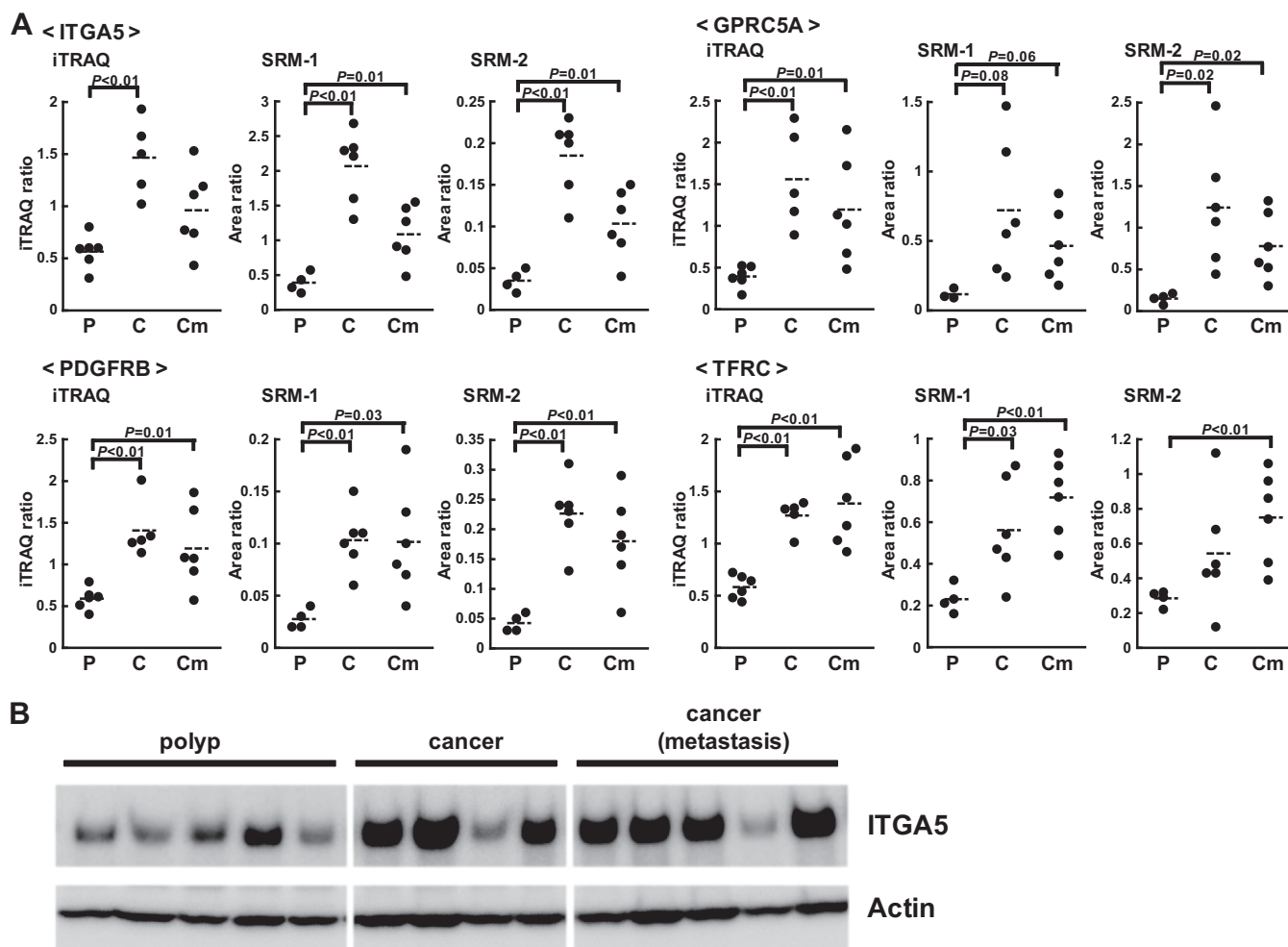
The expression of ITGA5, GPRC5A, PDGFRB, and TFRC was shown to be different in colorectal or other cancer tissues (26–29). The results of iTRAQ and SRM/MRM on these proteins are shown in Fig. 2A. The expression of these proteins showed very similar patterns on iTRAQ and SRM/MRM (supplemental Fig. S4). Furthermore, changes in the expression of ITGA5 were confirmed by Western blotting (Fig. 2B). The similar results obtained by SRM/MRM and iTRAQ were further verified by Western blotting, which indicated that the SRM/

MRM assay can be used to confirm the candidates identified in the discovery phase.

**Verification of Biomarker Candidates by SRM/MRM**—We verified 69 confirmed proteins in an independent set of patient samples (polyps (*n* = 10), cancer without metastasis (*n* = 10), and cancer with metastasis (*n* = 10)) (Table IV, supplemental Table S1, S9, supplemental Fig. S5). We performed five technical replicates using sample mixtures prepared from patient tissue samples to evaluate the reproducibility of our SRM/MRM assay, and obtained high reproducibility (CV<11%) (supplemental Table S8). We did not analyze process replicates, therefore the actual experimental variability is likely higher than shown by the technical replicate performance owing to variability in digestion and other sample handling steps. The expression levels of a total of 20 proteins: GPRC5A, PRTN3, CEACAM5, ANTXR1, PXMP4, SLC2A3, ENPEP, PDGFRB, GGT5, MMP14, TFRC, MRC2, SPARC, HSPB1, FCGR1A, THY1, TMEM41A, SLC4A2, FCER1G, and CEACAM1, were significantly higher in cancer without metastasis than in polyps (ratio >2.0, *p* value <0.05). In addition, the expression levels of 10 proteins: ITGA11, BST1, LTBP2, ITGA5, TMEM97, TSPAN9, SIGMAR1, C8orf55, UBAC2, and SERPIND1, were significantly higher in cancer without or with metastasis than in polyps (ratio >1.7, *p* value <0.05). The expression levels of another five proteins: CEACAM6, LRRRC15, GPC6, C5AR1, and TLCD1, were markedly higher in cancer tissues than in polyps. The expression levels of eight proteins: CLCA1, FCGBP, B3GNT6, MUC2, ANXA13, AKAP5, PRG2, and KIAA1324, were lower in cancer with and without metastasis than in polyps (ratio >0.5, *p* value <0.05). The expression of EPB41L3 was also shown to be lower in cancer tissues than in polyps. This verification step as well as the discovery step revealed that the expression levels of ITGA5, GPRC5A, PDGFRB, and TFRC were markedly higher in cancer tissues than in polyps (Fig. 3). Overall, the expression patterns of 47 out of 69 confirmed proteins were similar between the confirmation and verification analyses.

**Further Validation of C8orf55 by Western Blotting and Immunohistochemistry**—We focused on C8orf55 among the biomarker candidates that displayed significant differences in SRM/MRM because it has not been previously reported as a biomarker candidate for cancer and a specific antibody against this protein was available. C8orf55 (also called





**FIG. 2. Representative results obtained with iTRAQ and SRM/MRM in the discovery and confirmation steps.** A, The iTRAQ and SRM/MRM data for ITGA5, GPRC5A, PDGFRB, and TFRC are shown. P, polyp. C, cancer without metastasis. Cm, cancer with metastasis. Area ratio, the ratio of the peak area of the endogenous peptide to that of the SI peptide. Assays were constructed to measure two distinct peptides per-protein listed in supplemental Table S5 and that the individual assays for each of the two peptides are labeled SRM-1 and SRM-2. B, Western blotting analysis of polyps and cancer with and without metastasis using an anti-ITGA5 antibody.

THEM6) is a 208-amino acid protein that has one predicted transmembrane domain in the N-terminal region; however, its function is unknown. iTRAQ and subsequent confirmation using the SRM/MRM assay revealed that the expression of C8orf55 was up-regulated with cancer progression (Fig. 4A). Furthermore, in the verification step, the expression of this protein was higher in cancer without metastasis than in polyps (ratio = 1.92,  $p$  value < 0.01). Western blotting was also performed to verify these changes in expression levels (Fig. 4B). Immunohistochemical analysis of colorectal cancer tissue showed that the expression of C8orf55 was high in cancer cells, but was negligible in normal cells (Fig. 4C). These results indicated that the expression of C8orf55 increased in a step-wise fashion with cancer progression.

**Examination of C8orf55 Expression in Various Cancer Tissues using Tissue Microarrays**—The expression of the tumor markers used in clinical practice, such as CEA and CA19–9, was shown to be higher in multiple cancer types. Therefore, we

investigated whether C8orf55 was expressed in various cancer tissues using tissue microarrays (TMA), which contained 1150 cores from 14 common cancer tissues and 280 cores from corresponding normal tissues (supplemental Fig. S6). TMA revealed that the expression of C8orf55 was high in many of the cores prepared from colon cancer tissue, but was negligible in those from normal colon tissues (Fig. 5). TMA also showed that the expression of C8orf55 was significantly higher in colon cancer tissue than in normal tissue. Immunostaining for C8orf55 was stronger in cancer tissues such as those from the stomach and breast than in normal tissues (Fig. 5). These results demonstrated that C8orf55 may be a potential biomarker for colorectal, stomach, and breast cancer.

#### DISCUSSION

A number of large-scale proteomic analyses of cancer tissues for biomarker discovery have been reported to date (30–32); however, few studies have validated the candidate

TABLE IV

SRM/MRM analysis of biomarker candidate proteins. *p* values were calculated by *t*-test. C/P, average ratio of cancer without metastasis to polyps. Cm/C, average ratio of cancer with metastasis to cancer without metastasis. Cm/P, average ratio of cancer with metastasis to polyps

Gene name	C/P	<i>p</i> Value	Cm/C	<i>p</i> Value	Cm/P	<i>p</i> Value
FAP	1.59	0.515	2.21	0.052	3.52	0.198
GPRC5A	4.31	0.040	1.30	0.514	5.59	<0.01
CEACAM6	13.41	0.096	0.87	0.822	11.61	<0.01
LRRC15	2.51	0.084	1.83	0.237	4.59	0.037
PRTN3	2.68	0.014	1.67	0.098	4.47	<0.01
CEACAM5	7.29	0.044	0.85	0.737	6.22	<0.01
ITGA11	1.90	0.019	0.82	0.408	1.55	0.066
BST1	1.93	0.012	1.84	0.064	3.55	<0.01
MMP2	1.18	0.601	1.11	0.761	1.30	0.396
ANTXR1	3.23	<0.01	1.08	0.818	3.48	<0.01
PXMP4	2.29	<0.01	0.80	0.385	1.82	0.025
EFEMP1	1.30	0.478	1.04	0.900	1.35	0.358
LTBP2	1.83	0.036	1.10	0.676	2.02	<0.01
SLC2A3	3.56	0.030	0.92	0.817	3.28	<0.01
ITGA5	1.83	<0.01	1.79	0.162	3.28	0.031
MFAP2	1.41	0.394	1.17	0.496	1.65	0.128
TMEM97	2.00	<0.01	0.83	0.411	1.67	0.064
ENPEP	3.83	<0.01	1.13	0.445	4.32	<0.01
CD177	1.17	0.581	1.42	0.224	1.66	0.144
C20orf108	1.23	0.368	0.94	0.823	1.16	0.560
PDGFRB	2.22	<0.01	1.00	0.995	2.22	<0.01
FAM73B	1.22	0.207	0.51	<0.01	0.62	0.013
TSPAN9	1.75	<0.01	0.99	0.968	1.74	<0.01
GPC6	1.89	0.072	1.20	0.614	2.26	0.044
GGT5	2.06	0.034	1.24	0.432	2.56	<0.01
C5AR1	1.48	0.120	1.49	0.167	2.21	0.016
FBN1	1.37	0.443	1.34	0.257	1.84	0.072
FBLN2	1.75	0.102	1.02	0.946	1.79	0.087
SIGMAR1	1.74	<0.01	0.98	0.914	1.71	0.013
MMP14	2.43	<0.01	1.00	0.988	2.42	<0.01
TFRC	2.32	0.018	1.01	0.973	2.35	0.027
MRC2	2.09	<0.01	1.13	0.631	2.36	<0.01
SPARC	2.49	<0.01	0.82	0.317	2.03	0.027
HSPB1	2.73	0.016	1.50	0.231	4.10	<0.01
C8orf55	1.92	<0.01	0.74	0.123	1.42	0.024
FCGR1A	2.47	<0.01	1.40	0.277	3.45	<0.01
THY1	2.14	<0.01	1.00	0.983	2.15	<0.01
TMEM41A	2.04	<0.01	0.90	0.593	1.84	<0.01
SLC4A2	2.41	<0.01	0.92	0.746	2.21	0.014
FCER1G	2.23	<0.01	0.97	0.888	2.17	<0.01
MME	5.21	0.058	0.97	0.959	5.05	0.058
CEACAM1	5.95	0.025	0.83	0.646	4.92	<0.01
REEP6	1.21	0.509	0.97	0.934	1.18	0.608
GCNT3	1.75	0.078	1.46	0.306	2.55	0.063
AGR3	0.20	<0.01	1.89	0.073	0.38	0.021
ENTPD2	1.11	0.800	0.88	0.778	0.98	0.942
CLCA1	0.17	0.022	1.32	0.739	0.22	0.019
FCGBP	0.22	<0.01	1.15	0.782	0.25	<0.01
B3GNT6	0.32	<0.01	1.48	0.359	0.48	0.036
MUC2	0.14	<0.01	2.02	0.279	0.29	0.013
TFF3	0.33		2.80		0.93	
ANXA13	0.23	<0.01	1.41	0.259	0.32	<0.01
AKAP5	0.19	0.016	0.83	0.487	0.16	0.013
C16orf62	0.76	0.442	0.59		0.45	
PRG2	0.34	0.018	1.10	0.744	0.38	0.021
KIAA1324	0.32	<0.01	1.13	0.657	0.36	<0.01
EPB41L3	0.55	0.060	0.67	0.142	0.37	<0.01
COL1A2	1.55	0.438	1.22	0.650	1.90	0.031
COL1A1	1.39	0.590	1.19	0.737	1.65	0.093

TABLE IV—continued

Gene name	C/P	p Value	Cm/C	p Value	Cm/P	p Value
COL3A1	1.24	0.642	1.34	0.517	1.67	0.212
POSTN	0.90	0.687	1.94	0.018	1.75	0.033
NT5E	1.05	0.802	1.21	0.473	1.27	0.329
PEX13	1.73	<0.01	0.81	0.224	1.40	0.025
UBAC2	1.87	<0.01	0.95	0.834	1.78	0.044
TLCD1	2.13	<0.01	0.76	0.335	1.63	0.113
SERPIND1	1.57	0.018	1.21	0.371	1.90	<0.01
SLC2A1	2.57	0.175	1.18	0.758	3.03	0.051
FAM173A	1.18	0.433	0.81	0.259	0.96	0.860
TMEM45B	1.35	0.255	0.97	0.918	1.30	0.400

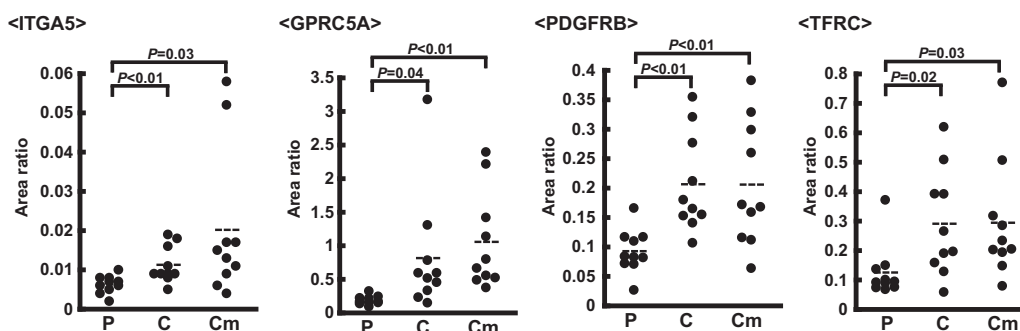
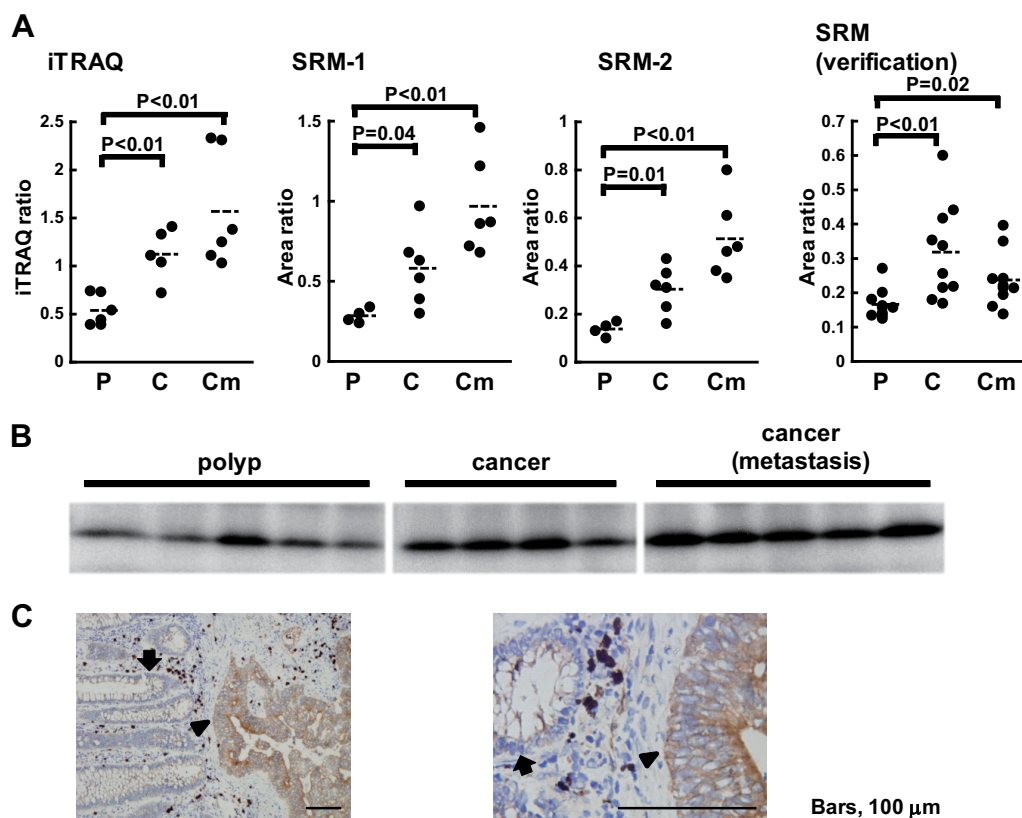


FIG. 3. Representative results of SRM/MRM in the verification step. The SRM/MRM data of ITGA5, GPRC5A, PDGFRB, and TFRC are shown. P, polyp. C, cancer without metastasis. Cm, cancer with metastasis. Area ratio, the ratio of the peak area of the endogenous peptide to that of the SI peptide.

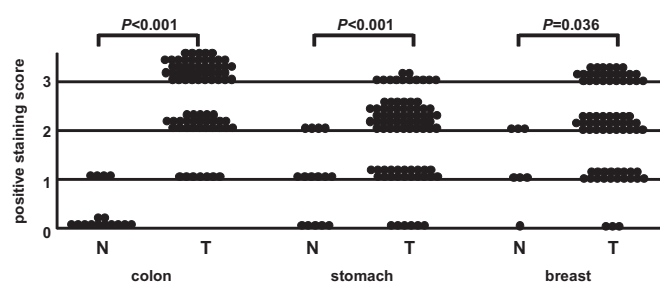
proteins identified because of the absence of an appropriate validation method. SRM/MRM was recently shown to be an efficient validation method (3–5) and several studies, including our own, reported the identification of biomarker candidates by quantitative shotgun proteomics using the iTRAQ labeling method and verification by SRM/MRM (19, 21, 33). In the present study, we performed a proteomic analysis of membrane fractions prepared from colorectal cancer tissue to identify novel biomarker candidates for diagnosis and/or therapeutic targets. We identified membrane proteins, the expression levels of which were altered with the development and progression of colorectal cancer, using comprehensive quantitative analysis with iTRAQ. The most significant achievement of this study was the SRM/MRM-based confirmation and simultaneous large-scale verification using an independent set of tissue samples. Of the 105 biomarker candidate proteins identified by iTRAQ, changes in the expression of 69 proteins were confirmed by SRM/MRM, with significant differences being verified in 44 proteins between groups. This discovery-confirmation-verification workflow should be able to identify more reliable biomarkers for the clinical diagnosis of colon cancer. To the best of our knowledge, we have performed the largest verification of biomarker candidate membrane proteins to date. This verification process using SRM/MRM enabled us to select more potential candidates and prioritize the subsequent validation, and may represent a rapid and effective method to identify novel biomarkers.

We were able to identify 5566 proteins in the membrane fraction in the present study, 3087 (58.4%) of which were predicted to be membrane proteins. This number was markedly higher than that previously reported (34–38); however, non-membrane proteins were also identified in addition to membrane proteins, and this was attributed to the preparation of crude membrane fractions using a simple method. One of the reasons for the increased rate of membrane protein identification was the PTS method-based isolation of membrane proteins (12, 13). The PTS method enables the efficient isolation of membrane proteins and allows the use of a high detergent concentration to achieve the efficient solubilization of very hydrophobic membrane proteins in the cleavage procedure of membrane proteins. Thus, this method may provide deeper proteome coverage for the identification of tissue membrane proteins.

We focused on membrane proteins in this study because membrane proteins are not only involved in the regulation of cell signaling and cell-cell interactions, but are also suitable therapeutic targets for cancers (39). One of the greatest advances in the treatment of cancer in recent years has been the discovery of molecular-targeted drugs, which has resulted in the development of many antibody drugs. Membrane proteins are clearly the best targets for antibody drugs. In this study, we identified a number of previously unreported membrane proteins, the expression of which changed with the development and progression of colorectal cancer. These membrane



**FIG. 4. Validation of the biomarker candidate C8orf55.** *A*, The iTRAQ and SRM/MRM data of C8orf55. P, polyp. C, cancer without metastasis. Cm, cancer with metastasis. Area ratio, the ratio of the peak area of the endogenous peptide to that of the SI peptide. Assays were constructed to measure two distinct peptides per-protein listed in [supplemental Table S5](#) and that the individual assays for each of the two peptides are labeled SRM-1 and SRM-2. *B*, Western blotting analysis of polyps and cancer with and without metastasis using an anti-C8orf55 antibody. *C*, Immunohistochemical staining of colorectal cancer tissue using an anti-C8orf55 antibody. *Left* panel: lower magnification. *Right* panel: higher magnification. The arrowhead shows areas of stained tumor cells and the arrow shows normal colon epithelial glands. Bar, 100  $\mu$ m.



**FIG. 5. Tissue microarray of C8orf55.** C8orf55 immunohistochemistry score (staining intensity) between normal and cancer tissues. Statistical analyses were performed using the Wilcoxon test.

proteins may be novel therapeutic targets for antibody drug discovery.

Membrane proteins are also suitable biomarkers for the screening and diagnosis of various cancers. Diagnostic biomarkers are ideally detected and quantified in biological fluids such as the plasma and/or urine; however, soluble proteins derived from tissue leakage are often very difficult to detect because there are very few and they are unstable. In contrast, membrane proteins and extracellular proteins are potentially shed and secreted from cells into the circulation; some are

actively secreted as microvesicles, such as exosomes, which are very stable and may be potential biomarkers. Several previous studies reported the potential for diagnosing malignant tumors, such as colorectal cancer, melanoma, and glioblastoma, by analyzing exosomal proteins (40–42). Thus, the membrane proteins identified in this study may be promising biomarker candidates for the diagnosis of colorectal cancer.

We observed variations in the quantitative results obtained from iTRAQ and SRM. The samples used for iTRAQ were fractionated with a SCX column, while those for SRM were not. Therefore, variations may have occurred in the quantitative results obtained from iTRAQ and SRM because of differences in the complexities of the samples analyzed. Splicing isoforms or post-translational modifications may also have been involved in these variations because iTRAQ ratios were calculated as the average of all contributing peptide iTRAQ measurements and SRM ratios were obtained by measuring a target peptide.

We investigated differences in the expression levels of proteins between polyps and cancer tissues without metastasis in the present study using proteomic analysis to identify characteristic expression profiles in cancer. Although a number of

previous biomarker studies identified hundreds of candidate proteins by comparing cancer tissues with matched normal tissues, many proteins unrelated to malignant properties may also have been included because cancer is generally not directly derived from normal tissues. Thus, the best negative control would be benign tumors, ideally premalignant lesions. In this regard, colorectal polyps are considered to be the best control for colorectal cancer. Moreover, a comparison between different stages of cancer tissues, including benign tumors, is the optimal procedure to identify more useful biomarker candidates.

In our study, C8orf55 was confirmed by SRM/MRM and Western blotting, the findings of which were further verified by multiple cancer tissue microarrays (TMA1150). TMA1150 had 1150 cores from 50 or 100 cases of 14 cancer types and was previously shown to be useful for evaluating changes in protein expression in multiple cancers (25). TMA1150 can also be used to examine the expression of target proteins in various cancer tissues as well as in dozens of cases of colorectal cancer. The extensive validation of the expression of identified candidates in various types of cancer tissues is important in order to determine their usefulness as biomarkers for diverse cancers. In this regard, multi-cancer TMA is a very effective method that can be used to rapidly and simply evaluate the expression patterns of various cancers. TMA1150 revealed that the expression of C8orf55 was higher not only in colon cancer tissue, but also in other cancer tissues, which suggested that these proteins have the potential to be biomarkers for stomach and breast cancer as well as colon cancer.

In conclusion, we successfully performed a SRM/MRM-based large-scale verification of biomarker candidate membrane proteins for colorectal cancer tissues. The methods described here can be readily applied to any type of cancer tissue and can contribute to the identification of novel biomarkers for the diagnosis and therapeutic targets of diseases.

**Acknowledgments**—The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository (43) with the dataset identifier PXD000851.

\* This work was supported by a Grant-in-Aid, Research on Biological Markers for New Drug Development H20–0005, to T.T. from the Ministry of Health, Labor and Welfare of Japan. It was also supported by Grants-in-Aid 21390354 to T.T and 22590545 to H.K. from the Ministry of Education, Science, Sports and Culture of Japan.

☐ This article contains [supplemental Figs. S1 to S6 and Tables S1 to S9](#).

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