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. 6). 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 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 form 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 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 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 due to 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.

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Figure Legends

Figure 1. Outline of the experimental workflow.

Figure 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 5 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.

Figure 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.

Figure 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 5 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 μm.

Figure 5. Tissue microarray of C8orf55.

C8orf55 immunohistochemistry score (staining intensity) between normal and cancer tissues. Statistical analyses were performed using the Wilcoxon test.

Table 1. Number of predicted membrane proteins

Total identified proteins	5566
	number	%
Number of proteins with transmembrane domains	**1567** [a]	**28.2**
GO-annotated	**5287**	**100**
Membrane	**3087**	**58.4** [b]
Extracellular	**652**	**12.3** [c]

[a] Number of proteins with transmembrane domains predicted by TMHMM algorithm.

[b, c] The ratio of membrane or extracellular proteins to GO-annotated proteins.

Table 2. Number of proteins with significant difference in expression

		C / P		Cm / C		Cm / P	
ratio	p-value	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

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.

Table 3. List of the proteins analyzed by SRM/MRM and their quantitation data using iTRAQ

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	p-value	Cm/C	p-value	Cm/P	p-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
O95832	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
Q8WUY1	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	Transmem protein 41A	TMEM41A	6	mem		2.04	<0.01	0.76	0.060	1.54	<0.01
Q9ULS5	Transmem and coiled-coil domains protein 3	TMCC3	2	mem		2.04	0.040	0.61	0.195	1.25	0.434
Q01628	Interferon-induced transmem 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

Table 3. List of the proteins analyzed by SRM/MRM and their quantitation data using iTRAQ (continued)

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
A8K7I4	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:betaGal beta-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	Progestin and adipoQ receptor family member 4	PAQR4	3	mem		1.82	<0.01	1.23	0.342	2.25	0.019
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

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.

Table 4. SRM/MRM analysis of biomarker candidate proteins

gene name	C / P	p-value	Cm / C	p-value	Cm / P	p-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
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

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.

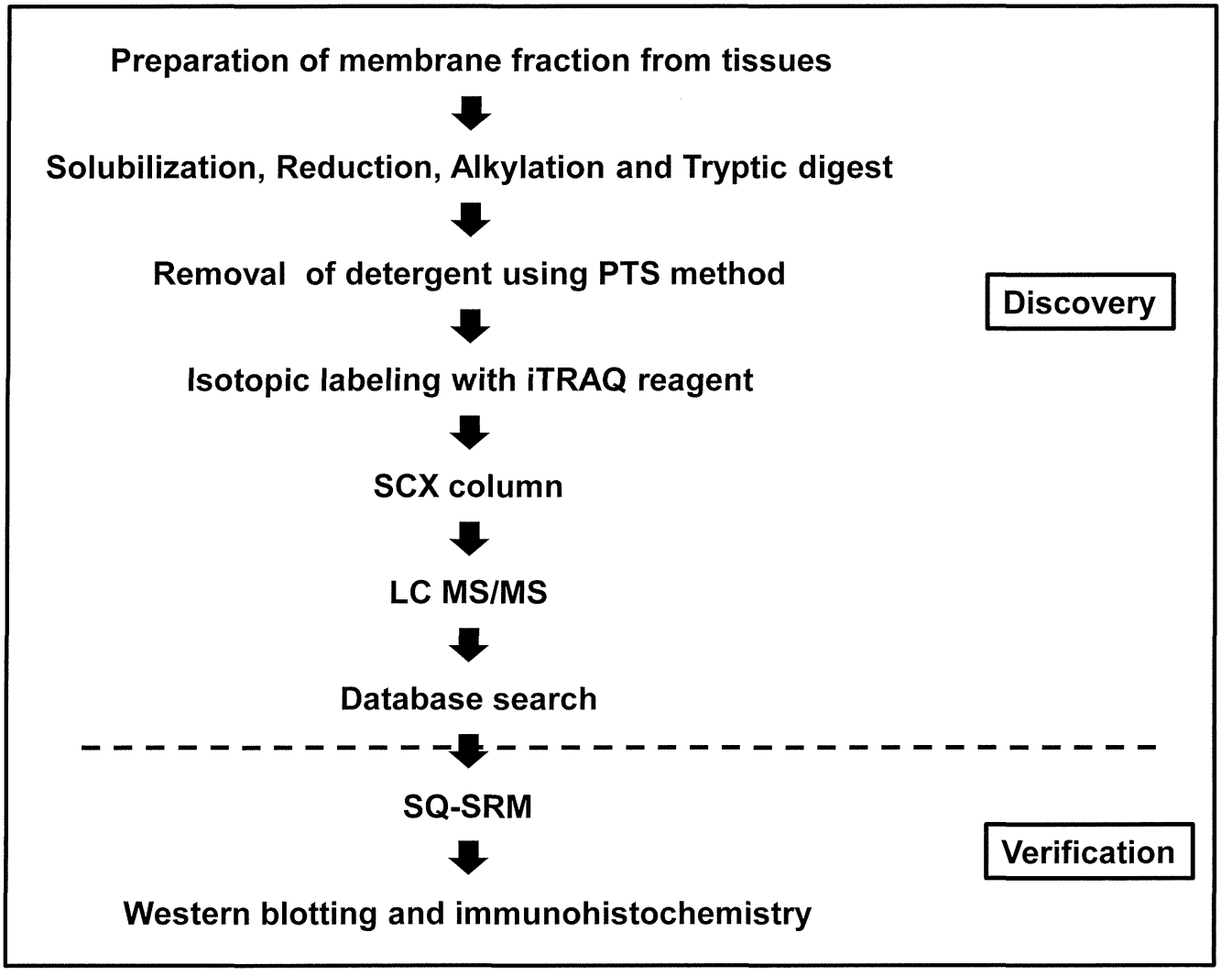
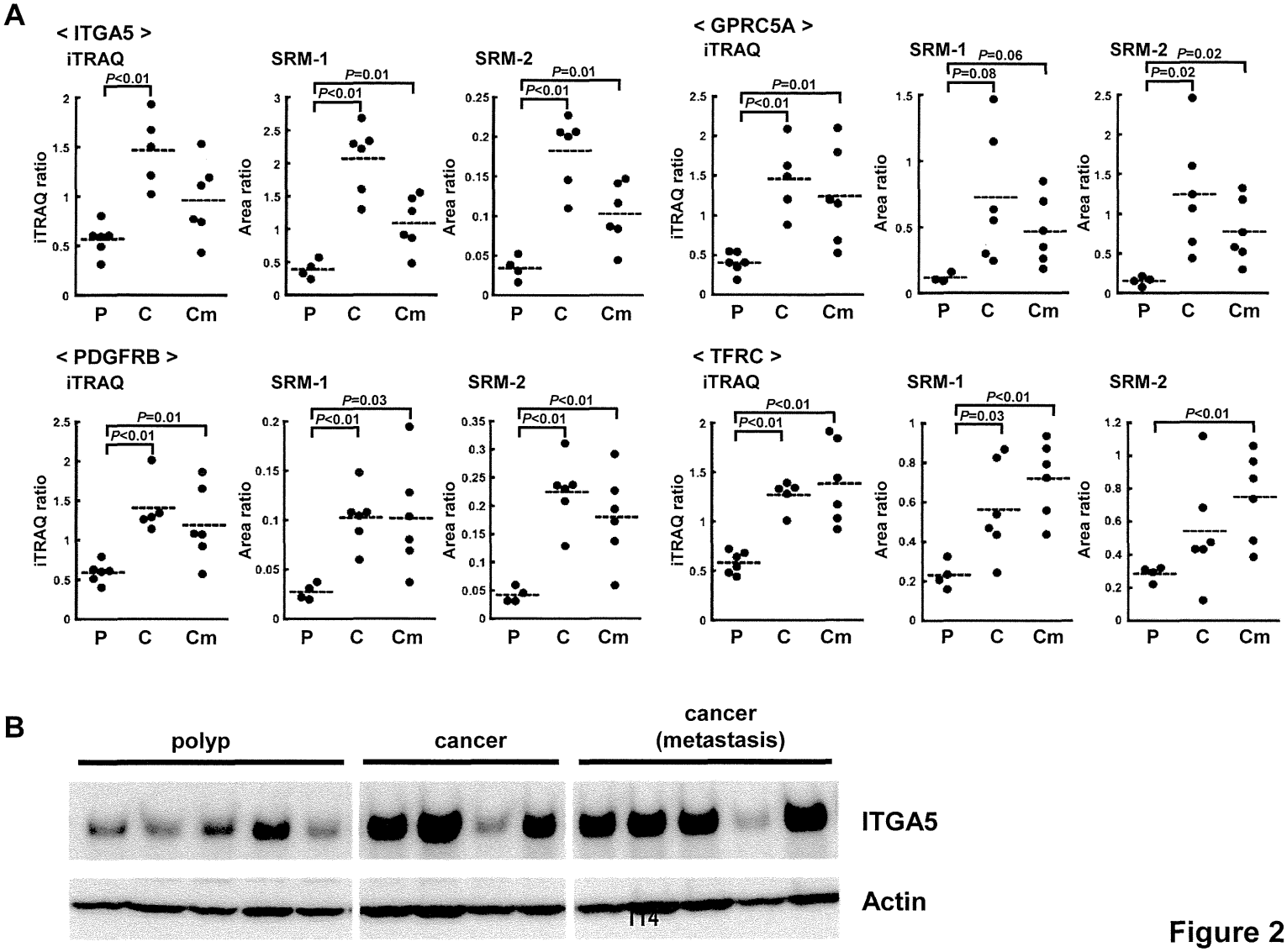

Preparation of membrane fraction from tissues
Solubilization, Reduction, Alkylation and Tryptic digest
Removal of detergent using PTS method
Isotopic labeling with iTRAQ reagent
SCX column
LC MS/MS
Database search
Discovery
SQ-SRM
Western blotting and immunohistochemistry
Verification

Figure 1


A
< ITGA5 >
iTRAQ
SRM-1
SRM-2
< GPRC5A >
< PDGFRB >
< TFRC >
iTRAQ ratio
Area ratio
P<0.01
P=0.01
P=0.03
P=0.06
P=0.08
P=0.02
P
C
Cm

B
polyp
cancer
cancer
(metastasis)
ITGA5
Actin

Figure 2