Immunohistochemical analysis revealed that Reg IV is expressed in gastric cancer, colorectal cancer, and pancreatic cancer, whereas lung cancers and breast cancers do not express Reg IV.[22,25] Reg IV is also expressed in gastric SRCC and colorectal SRCC.[26] Therefore, Reg IV may serve as a marker of digestive organ cancer. We also found that expression of claudin-18 is restricted to normal stomach and duodenum, and is not expressed in other normal tissues.[29] Immunohistochemical analysis of claudin-18 revealed that although expression of claudin-18 is down-regulated in several gastric cancers, claudin-18 is expressed in gastric SRCC, suggesting that claudin-18 may be a marker of cancers derived from stomach or duodenum. However, expression of claudin-18 has not been investigated in tumors other than gastric cancers.

In the present study, we analyzed the immunohistochemical characteristics of SRCCs from various organs using antibodies against Reg IV and claudin-18. We also examined expression of cytokeratin (CK) 7, CK20, MUC2, MUC5AC, caudal-related homeobox gene 2 (CDX2), TTF-1, mammaglobin, gross cystic disease fluid protein-15 (GCDFP15), and estrogen receptor (ER). The combined expression patterns of CK7 and CK20 have recently been extensively studied in various primary and metastatic carcinomas.[8,10] Mucin production such as MUC2 and MUC5AC is the biologic hallmark of SRCC. CDX2 is a homeobox gene that is expressed exclusively in normal intestinal epithelium and its neoplasms.[20] Mammaglobin, a mammary-specific member of the uteroglobin family, is known to be overexpressed in human breast cancer.[37] GCDFP15, which is a predominant secretory protein in various body fluids, including saliva, milk, and seminal plasma, is generally considered a relatively specific and somewhat sensitive marker of breast cancers.[4] ER is also reported to be expressed in 60% to 70% of breast cancers.[1] We found that several markers alone and in combination can differentiate the primary site of SRCCs.

MATERIALS AND METHODS

Tissue Samples

We selected 54 cases of SRCC, including 21 cases of gastric SRCC, 16 of colorectal SRCC, 10 of breast SRCC, and 7 of pulmonary SRCC, from the surgical pathology files of the Hiroshima University Hospital or affiliated hospitals. Cases were selected randomly, and gastric SRCCs and colorectal SRCCs for which depth of invasion did not exceed the submucosal layer were excluded because Krukenberg tumors, which are considered a representative metastatic neoplasm, arise from a late-stage gastrointestinal tract neoplasm, in particular from gastric SRCC except few reports.[15] Cases with a known history of other malignancies were excluded. To qualify as SRCC, more than 50% of the examined tumor cells had to be signet-ring cells. Because written informed consent was not obtained, identifying information was removed from all samples before analysis to protect the privacy of all patients. This procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research of the Japanese Government.

Surgically resected specimens were routinely fixed in 10% buffered formalin and examined macroscopically. All sections contained tumor tissue and surrounding non-neoplastic tissues and were embedded in paraffin. Additional consecutive 5μm sections were cut from a selected tissue block and stained with hematoxylin and eosin. We also examined lung SRCC samples on a tissue microarray (TMA), which was provided by the National Cancer Center Hospital East (Chiba, Japan). TMAs were constructed according to a previously described protocol.[34] The study specimens were routinely oversampled with 2 replicate core samples of tumor (different areas). Each tissue-array block contained 40 cases of pulmonary SRCC.

Immunohistochemistry

A Dako Envision Kit (Dako, Carpinteria, CA) was used for immunohistochemical analysis of all markers. In brief, sections were pretreated by microwaving (500 W) in citrate buffer (pH 6.0) for 15 minutes to retrieve antigenicity. After endogenous peroxidase activity was blocked with 3% H_2O_2-methanol for 10 minutes, sections were incubated with normal goat serum (Dako) for 20 minutes to block nonspecific antibody binding sites. Sections were then incubated with the following primary antibodies (Table 1): anti-Reg IV, anticlaudin-18, anti-MUC2, anti-MUC5AC, anti-CDX2, anti-CK7, anti-CK20, anti-TTF-1, antimammaglobin, anti-GCDFP15, and anti-ER. Suppliers and working dilutions are noted in Table 1. Rabbit polyclonal anti-Reg IV antibody was raised in our laboratory.[25] The specificity of the Reg IV antibody has been characterized in detail.[25] Sections were incubated with primary antibody for 1 hour at 25°C, followed by incubations with peroxidase-labeled antirabbit or mouse IgG for 60 minutes. Staining was completed with a 10-minute incubation with the substrate-chromogen solution. The sections were counterstained with 0.1% hematoxylin. Appropriate positive and negative control samples were used.

TABLE 1. Antibodies Used in the Current Study

Antibody	Clone	Dilution	Pretreatment	Source
Reg IV	Polyclonal	1: 50	MW	*
Claudin-18	Polyclonal	1: 50	MW	Zymed Laboratories
MUC2	Ccp58	1: 50	MW	Novocastra
MUC5AC	CLH2	1: 50	MW	Novocastra
CK7	OV-TL 12/30	1: 50	MW	DAKO
CK20	Ks20	1: 50	MW	DAKO
CDX2	CDX2-88	1: 20	MW	BioGenex
TTF-1	SPT24	1: 50	MW	Novocastra
Mammaglobin	304-1A5	1: 50	MW	DAKO
GCDFP15	23A3	1: 50	MW	Novocastra
ER	6F11	Diluted	MW	VENTANA

*Rabbit polyclonal anti-Reg IV antibody was raised in our laboratory.
MW indicates microwaving (500 W) in citrate buffer (pH 6.0) for 15 min.

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Evaluation of Positive Cases and Cutoff-point Thresholds

Immunostaining was evaluated independently by 2 investigators (K.S., and N.O.), and when the evaluations differed, a decision was made by consensus while investigators reviewed the specimen with a multihead microscope. Neoplastic tissue was evaluated semiquantitatively at magnifications of ×100 and ×400. Cytoplasmic immunoreactivity for CK7, CK20, MUC2, MUC5AC, mammaglobin, GCDFP15, and Reg IV; nuclear immunoreactivity for CDX2, TTF-1, and ER; and membranous reactivity for claudin-18 were assessed.

For surgically resected specimens, immunoreactivity was judged on the basis of the percentage of tumor cells expressing a particular antigen in each specimen. For the TMAs, staining was considered positive if any tumor cells were stained appropriately. The percentage of reactive cells necessary for a positive result reflects the viewpoint and opinion of the authors. There can be significant methodologic differences between studies and aware of the potential effect of these differences on a study's results. The aim of the present study was to differentiate SRCCs arising from various organs. Therefore, the cutoff-point for antibody reactivity necessary to define a result as positive was staining of any (>0%) cells in both surgically resected specimens and TMAs.

RESULTS

Staining Patterns of Gastric SRCCs

Results of immunostaining of 21 gastric SRCCs are detailed in Table 2. Images are shown in Figures 1A to C. All cases (21/21) of gastric SRCCs expressed Reg IV. The percentage of Reg IV-positive tumor cells ranged from 1% to 90%. Cytoplasmic staining of Reg IV was considered positive. Of 21 gastric SRCCs, 18 (86%) showed membranous staining for claudin-18. Approximately 50% to 80% of gastric SRCCs expressed MUC2, MUC5AC, CK7, and CK20. Fourteen (67%) cases showed heterogenous CDX2 staining, and none expressed TTF-1, mammaglobin, GCDFP15, or ER.

Staining Patterns of Colorectal SRCCs

Detailed results for the immunostaining of 16 colorectal SRCCs (14 colorectum, and 2 appendix) are given in Table 2. Images are shown in Figures 1D to I. All 16 cases of colorectal SRCC expressed Reg IV. Like gastric SRCC, membranous immunostaining of claudin-18 was observed in 6 colorectal SRCCs. MUC2, CK20, and CDX2 were expressed in more than 80% of colorectal SRCCs. Six (38%) cases were positive for MUC5AC, and 2 (12%) cases were positive for CK7. No cases expressed TTF-1, mammaglobin, GCDFP15, or ER.

Staining Patterns of Pulmonary SRCCs

Detailed immunostaining results for 47 pulmonary SRCCs (7 surgically resected specimens, and 40 cases on TMA) are given in Tables 2 and 3. Images are shown in Figures 2A to D. None of the pulmonary SRCCs expressed Reg IV or claudin-18. TTF-1 was expressed in 42 (89%) pulmonary SRCCs (all 7 surgically resected specimens, and 35 of 40 TMA specimens). All pulmonary SRCCs were positive for CK7 and negative for CK20. Fewer than 50% of pulmonary SRCCs showed cytoplasmic staining of MUC2 and MUC5AC. CDX2, mammaglobin, GCDFP15, and ER were not expressed by pulmonary SRCCs.

Staining Patterns of Breast SRCCs

Detailed immunohistochemical staining data for 10 breast SRCCs are given in Table 2. Images are shown in Figures 2E to I. Neither Reg IV nor claudin-18 was expressed by breast SRCCs. Cytoplasmic expression of mammaglobin was observed in all 10 breast SRCCs. Nine (90%) of 10 breast SRCCs showed cytoplasmic staining of GCDFP15, and nuclear staining of ER. All 10 cases expressed CK7. Three (30%) of 10 breast SRCCs showed cytoplasmic staining of MUC2, whereas 1 (10%) breast SRCC expressed MUC5AC. None expressed CK20, CDX2, or TTF-1.

Summary of Immunostaining for Reg IV, Claudin-18, MUC2, MUC5AC, CK7, CK20, CDX2, TTF-1, Mammaglobin, GCDFP15, and ER

The aim of the present study was to distinguish SRCCs arising from various organs. Therefore, the cutoff-point for antibody reactivity for a positive result was defined as staining of any cells (>0%) in both surgically resected specimens and TMA specimens. The results of immunostaining are given in Table 4. All 21 gastric SRCCs and 16 colorectal SRCCs expressed Reg IV. The remaining SRCCs were negative for Reg IV. Eighteen (86%) of 21 gastric SRCCs and 6 (38%) of 16 colorectal SRCCs were positive for claudin-18 expression, whereas other SRCCs were negative. Fourteen cases of 21 (67%) gastric SRCCs and 14 (88%) colorectal SRCCs were positive for CDX2. The remaining SRCCs were negative. Forty-two cases of 47 (89%) pulmonary SRCCs were positive for TTF-1, whereas SRCCs derived from other organs did not express TTF-1. All cases of breast SRCCs were positive for mammaglobin, whereas the remaining SRCCs were negative. GCDFP15 was expressed in 9 (90%) of breast SRCCs, and ER staining was observed in 9 (90%) of breast SRCCs. Staining patterns of the other molecules, including MUC2, MUC5AC, CK7, and CK20, varied.

DISCUSSION

Identification of the primary sites of SRCCs, especially in cases of extensive tumor progression, is important for appropriate patient management because the treatment and prognosis of SRCCs from different tissues differ. However, determination of the site of origin is difficult, if not impossible, especially with biopsy material. Occasionally, metastases are the first manifestation of the disease, and this poses a diagnostic problem. Therefore, we examined various SRCC specimens to

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TABLE 2. Staining Distribution for Reg IV, claudin-18, MUC2, MUC5AC, CK7, CK20, CDX2, TTF-1, Mammaglobin, GCDFP15, and ER in SRCC of Various Organs*

Case No.	Primary Site	Reg IV	Claudin-18	MUC2	MUC5AC	CK7	CK20	CDX2	TTF-1	Mammaglobin	GCDFP15	ER
1	Stomach	60	70	25	0	0	20	50	0	0	0	0
2	Stomach	80	3	80	15	10	80	20	0	0	0	0
3	Stomach	80	0	70	0	15	70	60	0	0	0	0
4	Stomach	70	10	90	20	15	70	80	0	0	0	0
5	Stomach	90	3	70	0	0	60	0	0	0	0	0
6	Stomach	10	70	0	0	25	0	20	0	0	0	0
7	Stomach	20	40	20	60	70	0	2	0	0	0	0
8	Stomach	70	30	20	0	20	0	40	0	0	0	0
9	Stomach	1	40	0	0	5	0	5	0	0	0	0
10	Stomach	70	40	0	0	25	10	0	0	0	0	0
11	Stomach	60	20	70	80	80	0	20	0	0	0	0
12	Stomach	80	5	60	0	30	5	5	0	0	0	0
13	Stomach	70	20	80	30	70	15	0	0	0	0	0
14	Stomach	3	0	0	10	60	0	0	0	0	0	0
15	Stomach	20	30	10	0	5	0	0	0	0	0	0
16	Stomach	3	90	0	70	30	0	70	0	0	0	0
17	Stomach	3	70	0	20	60	0	0	0	0	0	0
18	Stomach	5	60	5	20	60	0	0	0	0	0	0
19	Stomach	30	30	40	30	0	15	60	0	0	0	0
20	Stomach	1	0	90	0	0	3	40	0	0	0	0
21	Stomach	60	10	80	0	15	40	15	0	0	0	0
22	Colon	90	0	100	1	0	70	90	0	0	0	0
23	Colon	80	3	70	0	0	5	80	0	0	0	0
24	Colon	75	0	20	15	0	30	30	0	0	0	0
25	Colon	2	10	0	0	0	30	0	0	0	0	0
26	Colon	90	15	90	10	0	40	80	0	0	0	0
27	Colon	90	15	80	0	0	70	70	0	0	0	0
28	Colon	80	0	20	0	0	30	60	0	0	0	0
29	Colon	70	20	60	0	0	15	70	0	0	0	0
30	Colon	70	0	70	10	0	70	20	0	0	0	0
31	Colon	50	0	30	25	20	60	50	0	0	0	0
32	Colon	70	30	70	60	10	30	40	0	0	0	0
33	Colon	40	0	50	0	0	15	30	0	0	0	0
34	Colon	40	0	70	0	0	15	0	0	0	0	0
35	Rectum	80	0	80	0	0	50	60	0	0	0	0
36	Appendix	60	0	90	0	0	3	80	0	0	0	0
37	Appendix	5	0	40	0	0	10	20	0	0	0	0
38	Lung	0	0	1	0	60	0	0	60	0	0	0
39	Lung	0	0	0	0	100	0	0	40	0	0	0
40	Lung	0	0	0	0.5	100	0	0	70	0	0	0
41	Lung	0	0	0	2	100	0	0	40	0	0	0
42	Lung	0	0	0	5	100	0	0	80	0	0	0
43	Lung	0	0	1	80	100	0	0	40	0	0	0
44	Lung	0	0	0	20	100	0	0	90	0	0	0
45	Breast	0	0	80	0	60	0	0	0	3	60	80
46	Breast	0	0	0	0	60	0	0	0	80	30	60
47	Breast	0	0	0	0	100	0	0	0	10	20	5
48	Breast	0	0	0	0	80	0	0	0	5	40	50
49	Breast	0	0	10	0	90	0	0	0	10	10	20
50	Breast	0	0	0	0	80	0	0	0	60	70	70
51	Breast	0	0	0	0	80	0	0	0	5	15	10
52	Breast	0	0	20	60	90	0	0	0	70	40	90
53	Breast	0	0	0	0	90	0	0	0	3	90	0
54	Breast	0	0	0	0	90	0	0	0	80	0	80

*Data are the percentage of reactive cells in each tumor.

address the issue of whether immunohistochemistry could be useful for differential diagnosis of SRCC. In the present study, we found that Reg IV and claudin-18 are immunohistochemical markers of gastrointestinal SRCC. We also examined expression of CK7, CK20, MUC2, MUC5AC, CDX2, TTF-1, mammaglobin, GCDFP15, and ER, and found that mammaglobin is useful for detection of breast SRCC.

In the present study, all gastrointestinal SRCCs expressed Reg IV, whereas SRCCs from other organs, including lung and breast, did not express Reg IV. Therefore, staining for Reg IV is useful to identify SRCCs that originated from gastrointestinal sites. CDX2 is generally used as a marker of gastrointestinal tumors. However, CDX2 is expressed in 90% of gastric SRCCs and 89% of colorectal SRCCs.[9] Therefore, CDX2 is not

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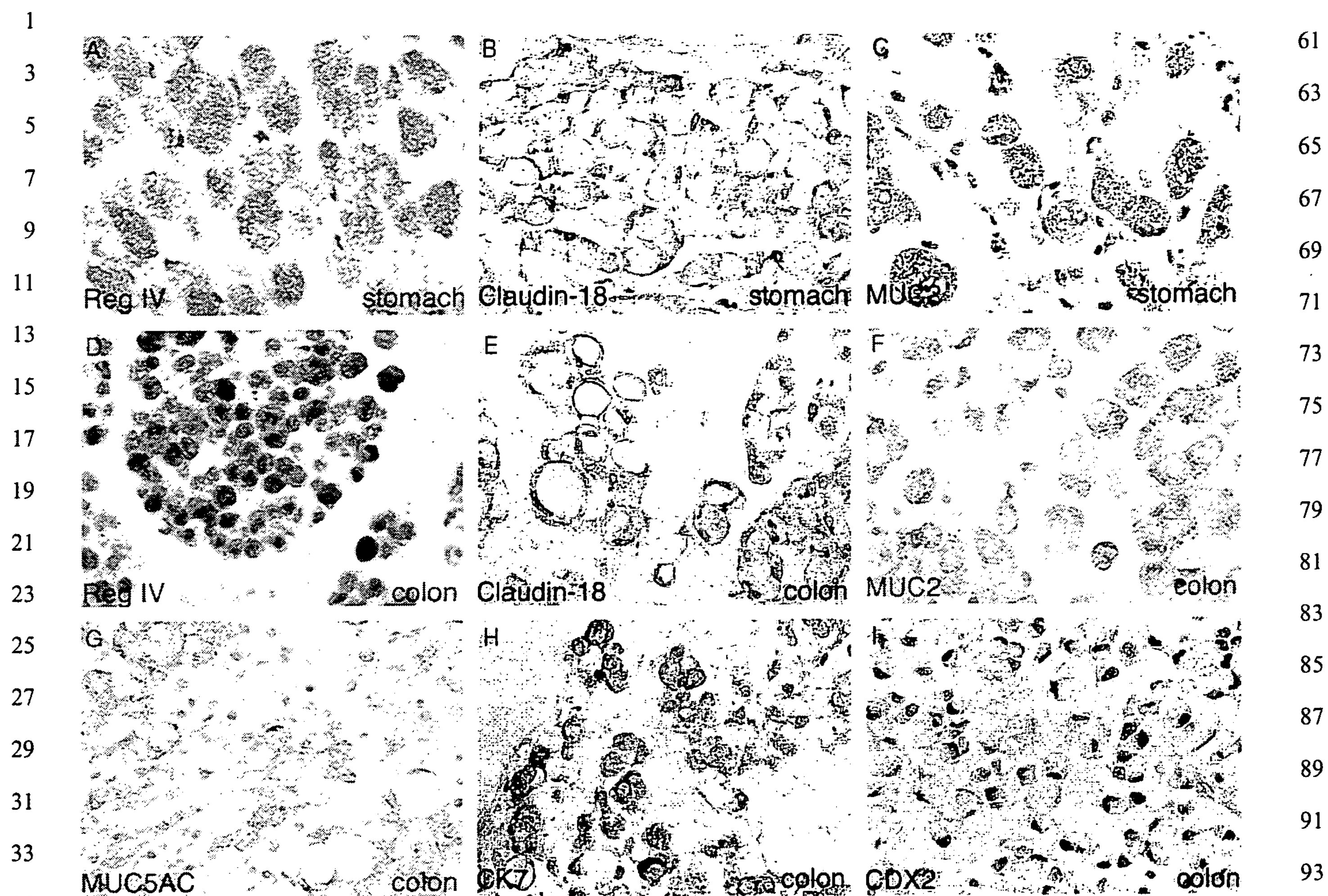

FIGURE 1. Immunohistochemical staining of digestive organ SRCCs [(A–C): gastric SRCC, (D–I): colorectal SRCC]. A and D, Reg IV immunoreactivity in the cytoplasm of SRCC (original magnification ×400). B and E, Membranous claudin-18 immunoreactivity (original magnification ×400). C and F, Cytoplasmic staining specific for MUC2 (original magnification ×400). G, Cytoplasmic staining of MUC5AC (original magnification ×400). H, Cytoplasmic staining of CK7 (original magnification ×400). I, Nuclear staining of CDX2 (original magnification ×400).

TABLE 3. Frequency of Marker Staining in 40 Cases of Pulmonary SRCCs on TMAs*

Antibody	Positivity (%)
Reg IV	0 (0)
Claudin-18	0 (0)
MUC2	3 (8)
MUC5AC	11 (28)
CK7	40 (100)
CK20	0 (0)
CDX2	0 (0)
TTF-1	35 (88)
Mammaglobin	0 (0)
GCDFP15	0 (0)
ER	0 (0)

*Data are number of positive cases (%).

always a reliable marker of gastrointestinal SRCC. In the present study, CDX2 expression was limited to gastrointestinal SRCCs; however, not all tumors were stained. The expression pattern of Reg IV resembles that of CDX2; however, Reg IV is expressed by 100% of gastrointestinal SRCCs.

Claudin-18 was reported to be detected in gastric carcinoma, and is expressed in gastric SRCC.[29] Our current results show that claudin-18 expression is limited to gastrointestinal SRCC. Although claudin-18 was not expressed in 100% of gastrointestinal SRCCs, claudin-18 in combination with Reg IV may be a useful marker for

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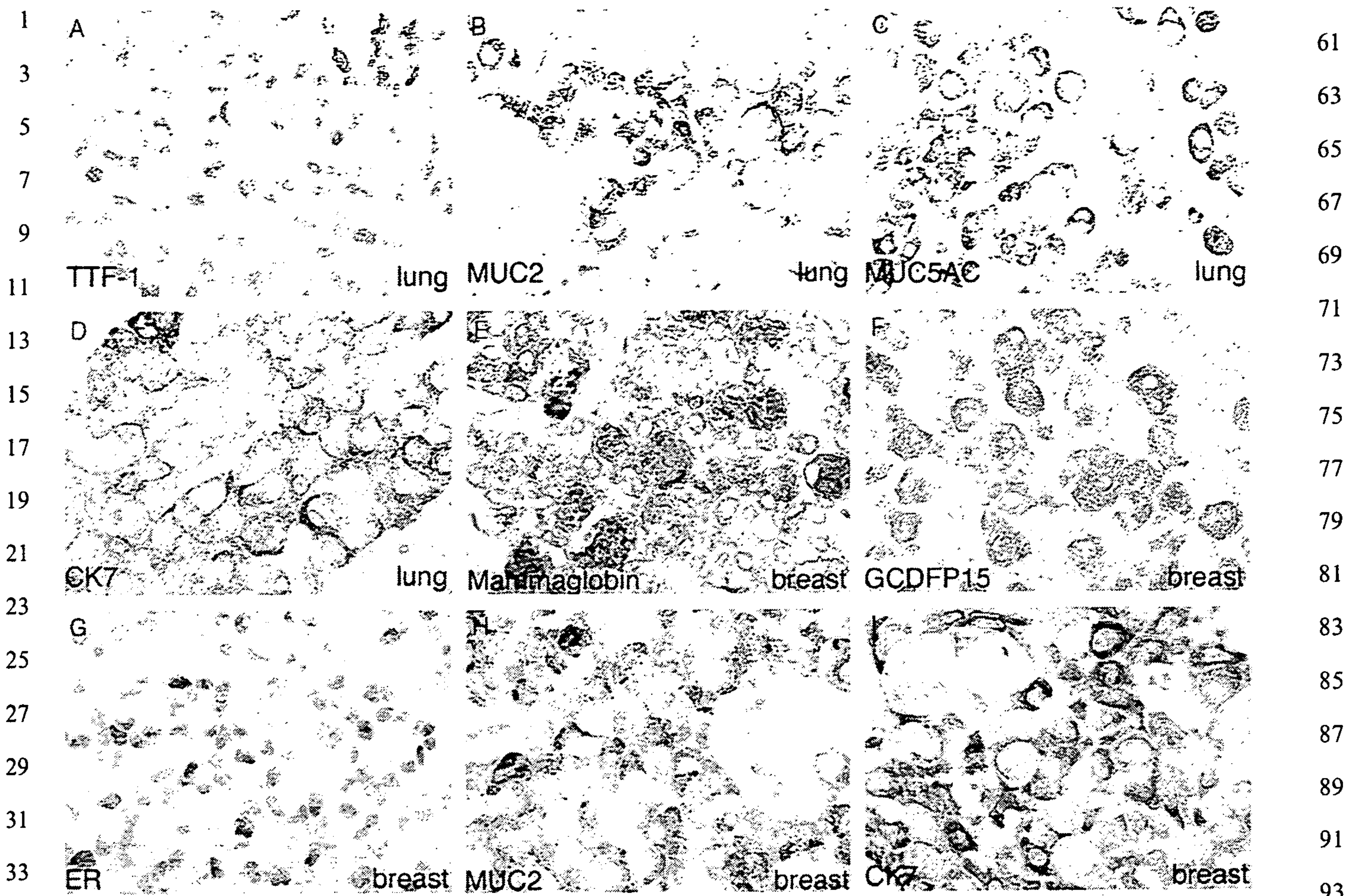

FIGURE 2. Immunohistochemical staining of extradigestive organ SRCCs [(A–D): pulmonary SRCC, (E–I): breast SRCC]. A, Tumor cells in a pulmonary SRCC show nuclear staining of TTF-1 (original magnification ×400). B and H, Cytoplasmic staining of MUC2 in a lung or breast (original magnification ×400). C, Cytoplasmic staining of MUC5AC in a lung SRCC (original magnification ×400). D and I, Cytoplasmic staining of CK7 in a lung or breast SRCC (original magnification ×400). E, Tumor cells in a breast SRCC show cytoplasmic staining of mammaglobin (original magnification ×400). F, Cytoplasmic staining of GCDFP15 in a breast SRCC (original magnification ×400). G, Nuclear staining of ER (original magnification ×400).

detecting gastrointestinal SRCC and excluding other types of SRCC.

TTF-1 is generally considered to be a marker of differentiated alveolar lining cells. Pulmonary adenocarcinoma cells tend to express TTF-1, and studies have reported expression in more than 70% of cases.[19] TTF-1 expression rates of 81%[34] to 100%[7] have been reported for SRCC of the lung. In the present study, TTF-1 was expressed in 89% of pulmonary SRCCs, and all other SRCCs were negative. Therefore, TTF-1–positive cases of SRCC are likely of pulmonary origin.

Mammaglobin is expressed in mammary tissue and primary breast tumors[36]; however, it is also reported to be expressed in carcinomas other than breast cancers[13] and in the secretory coil of the eccrine sweat glands of human skin.[30] Mammaglobin expression was detected immunohistochemically in 81 of 100 breast tumors independent of stage and histologic type.[37] To date, the pattern of expression of mammaglobin in SRCCs had not been studied, and this is the first report of immunohistochemical analysis of mammaglobin in SRCC. In the present study, mammaglobin was expressed in all cases of breast SRCC, but not in other SRCCs. Therefore, mammaglobin may be a useful marker to differentiate breast SRCC from other SRCCs.

GCDFP15 expression rates in breast cancer have been reported to range from 43% to 77%.[5,12,16,31] GCDFP15 was reported to be expressed in 80% of breast SRCC.[27] In the present study, GCDFP15 was expressed in 90% of breast SRCC, and all other SRCCs were

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1 **TABLE 4.** Summary of Positive Staining for Reg IV, Claudin-18, MUC2, MUC5AC, CK7, CK20, CDX2, TTF-1, Mammaglobin, GCDFP15, and ER in Various SRCCs*

Antibody	Stomach (n = 21)	Colorectum (n = 16)	Lung (n = 47)	Breast (n = 10)
Reg IV	21 (100)	16 (100)	0 (0)	0 (0)
Claudin-18	18 (86)	6 (38)	0 (0)	0 (0)
MUC2	15 (71)	15 (94)	5 (11)	3 (30)
MUC5AC	10 (48)	6 (38)	16 (34)	1 (10)
CK7+/CK20+	7 (33)	2 (12)	0 (0)	0 (0)
CK7+/CK20−	10 (48)	0 (0)	47 (100)	10 (100)
CK7−/CK20+	4 (19)	14 (88)	0 (0)	0 (0)
CK7−/CK20−	0 (0)	0 (0)	0 (0)	0 (0)
CDX2	14 (67)	14 (88)	0 (0)	0 (0)
TTF-1	0 (0)	0 (0)	42 (89)	0 (0)
Mammaglobin	0 (0)	0 (0)	0 (0)	10 (100)
GCDFP15	0 (0)	0 (0)	0 (0)	9 (90)
ER	0 (0)	0 (0)	0 (0)	9 (90)

*Data are number of positive cases (%).

negative. GCDFP15 was reported to be a more specific marker than mammaglobin for breast cancer, but it does not come up to the sensitivity of mammaglobin.[4]

In the present study, ER was expressed in 90% of breast SRCC, and all other SRCCs were negative. Although ER is expressed exclusively in breast carcinoma, approximately 20% of breast SRCC is negative for ER,[32] whereas some studies have found that up to 30% of gastrointestinal adenocarcinomas were positive for ER.[6,39] Thus, it is possible that immunohistochemical analysis for ER alone might not distinguish breast SRCC from gastrointestinal SRCC.

The expression patterns of CK7, CK20, MUC2, and MUC5AC were not sufficiently unique to differentiate completely SRCC primary sites. The combined expression patterns of CK7 and CK20 have been studied extensively in various primary and metastatic carcinomas.[8,10] In the present study, the CK7-negative, CK20-positive expression pattern was observed in most of colorectal SRCCs, whereas gastric SRCCs had no fixed pattern. Our results revealed that breast SRCCs were CK7-positive and CK20-negative. Tsuta et al[34] found that pulmonary SRCCs are CK7-positive and CK20-negative. In the present study, all pulmonary SRCCs expressed CK7 but not CK20, similar to breast SRCC. These results suggest that the combined CK7 and CK20 expression pattern is useful to predict some SRCC primary sites; however, the specificity is low. MUC2 is an intestinal mucin and MUC5AC is a gastric mucin. Both are secreted. MUC2 and MUC5AC can be used as markers of gastric and colorectal SRCCs.[9,23] In the present study, MUC2 and MUC5AC were expressed with variable proportions of each tumor. The specificity of MUC2 and MUC5AC expression pattern was low.

In conclusion, we found that Reg IV staining and claudin-18 staining can aid in the diagnosis of gastrointestinal SRCC. Larger trials are needed to confirm these results. To differentiate SRCC primary sites, we propose that immunohistochemical panels that include Reg IV, claudin-18, TTF-1, and mammaglobin should be used. Staining for Reg IV is useful to identify SRCCs originating from the gastrointestinal tract. Claudin-18 can differentiate gastrointestinal SRCCs, TTF-1 can differentiate pulmonary SRCCs, and mammaglobin can distinguish mammary SRCCs.

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Overexpression of RegIV in Peritoneal Dissemination of Gastric Cancer and Its Potential as A Novel marker for the Detection of Peritoneal Micrometastasis

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Abstract. *Background: Regenerating gene type IV (RegIV) is a candidate marker for cancer and inflammatory bowel disease. In this study, its potential as a novel marker for the detection of gastric cancer peritoneal micrometastases was examined. Materials and Methods: RegIV mRNA levels in the peritoneal washes of 95 gastric cancer patients and 22 with benign disease were quantified by real-time RT-PCR. To examine whether expression of RegIV enhance tumorigenicity or not, thirty two mice were injected intraperitoneally or subcutaneously with RegIV transfectants of TMK-1 cells, parental TMK-1 cells, or neomycin control transfectants. Results: RegIV expression was markedly higher in patients with peritoneal metastases compared to those without. The level of RegIV mRNA in gastric cancer patients was related to the extent of wall penetration. A cut-off value for RegIV-positive expression was based on an analysis of negative control patients with benign disease, and gastric cancer patients above the cut-off value constituted the micrometastasis (MM+) group. Based on this criteria, 3 out of 43 T1 or T2 cases were MM+ (93% specificity). Among 15 patients with peritoneal dissemination (7 out of 15 cases were positive by cytology), 14 cases were positive for RegIV expression (93% sensitivity), while analysis of carcinoembryonic antigen (CEA) mRNA failed to detect micrometastases in 4 cases (73% sensitivity). Combined analysis of CEA and RegIV improved the accuracy of diagnosis to 100%. The prognosis of RegIV-positive cases was significantly worse than that of RegIV-negative cases. Multivariate analysis using the Cox proportional hazards model suggested that RegIV may be an independent prognostic factor. Stable expression of RegIV significantly enhanced peritoneal metastasis in an animal model of gastric cancer. Conclusion: These findings suggest that RegIV mRNA expression has the potential to serve as a novel marker for detecting peritoneal dissemination in gastric cancer.*

*Both authors contributed equally to this study.

Abbreviations: RT-PCR: Reverse transcriptase-polymerase chain reaction; CY: cytology; MM: micrometastasis; RegIV: Regenerating gene type IV.

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Key Words: RegIV, gastric cancer, peritoneal metastasis, RT-PCR.

Gastric cancer is the most common malignancy of the gastrointestinal tract in Japan and in certain Southeast Asian populations, and the second most common cause of cancer-related deaths in the world (1). The prognosis of patients with gastric cancer that has invaded the gastric serosa is poor, with a 5-year survival rate of less than 35% (2). In such cases, peritoneal dissemination is reported to be the most frequent type of recurrence after curative resection (3, 4).

Free cancer cells derived from serosal invasion may be an indicator of early peritoneal seeding with subsequent formation of metastatic colonies. Thus, their detection represents a potentially valuable predictor of outcome for patients with advanced gastric cancers (5, 6). Cytological examination of peritoneal washes from laparotomies to detect free metastatic cancer cells has been used to evaluate the risk of recurrent disease (6, 7). Conventional cytology, however, lacks sufficient sensitivity, as some patients with negative cytology results have presented with recurrence in the form of peritoneal dissemination (5). Carcinoembryonic antigen (*CEA*)-specific RT-PCR has been used to detect cancer cells in peritoneal fluids (8); however, the results indicate that *CEA* expression is not 100% accurate as a marker, suggesting that more reliable markers are needed.

Previously, we examined global differential gene expression in gastric cancer cell lines established from a primary tumor and from metastases to the peritoneal cavity (9). Using a high-density cDNA microarray, we analyzed the expression of approximately 21,168 genes. The results of this study revealed that 24 genes were up-regulated and 17 genes were down-regulated in gastric cancer cell lines established from metastases to the peritoneal cavity. One of the up-regulated genes was *RegIV*. *RegIV* is a member of the regenerating gene (Reg) family, which is part of the larger calcium-dependent lectin (C-type) gene superfamily (10). Reg family members are a group of small secretory proteins which can function as acute phase reactants, lectins, antiapototic factors, or growth factors for pancreatic β cells, neural cells and epithelial cells in the digestive tract (11). The Reg family proteins also play an important role in the injury response in the gastrointestinal mucosa. *RegIV* expression is up-regulated in response to mucosal injury in active Crohn's disease and ulcerative colitis, and is increased in most colorectal cancers compared to normal tissue (10, 12-14). Recently, *RegIV* expression in gastric cancer was reported and was found to be closely related to the infiltrating potential of the carcinoma (15-17). In one study, RT-PCR analysis was used to show a high level of *RegIV* expression in gastric cancer (16). However, while overexpression of *RegIV* in gastric cancer has been reported, the role of RegIV in gastric cancer peritoneal dissemination has not been investigated. In this study, amplification of *RegIV* by quantitative RT-PCR from peritoneal lavage cells was used to develop a highly sensitive method for detecting micrometastases of cancer cells. This detection method has the potential to predict peritoneal recurrence in gastric cancer patients with a higher level of accuracy than previous methods.

Materials and Methods

Cell culture. The gastric cancer cell lines SNU-1, SNU-5, SNU-16, and SNU-719 were established previously by Park *et al.* (18). The mesothelial cell line Met5A was established by Duncan *et al.* (19, 20). The gastric cancer cell lines KATO-III and GT3TKB, and the acute myeloid leukemia cell line HL60 were purchased from Riken Cell Bank (Tsukuba, Japan). Another gastric cancer cell line, TMK-1 was kindly donated by Professor Tahara, of Hiroshima University. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in high-glucose RPMI-1640 (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum, penicillin and streptomycin.

Clinical samples and peritoneal washes. Patients underwent surgery at Kyoto Prefectural University of Medicine between 1999 and 2004 and underwent regular postoperative surveillance for at least 2 years or until death. One patient who died within 30 days of surgery as a result of perioperative complications was excluded. The current study population consisted of 95 patients with gastric cancer and 22 with benign disease, such as cholelithiasis. Of the 95 patients with resectable cancer, 77 underwent potentially curative R0 resection; the remaining 18 were treated with palliative resection. The 95 cases included 21 patients with T1 tumors (tumor confined to the mucosa or invading as far as the submucosa), 22 with T2 tumors (invasion beyond the submucosa but not as far as the serosa), 37 with T3 tumors (serosal invasion), and 15 with T4 tumors (invasion to adjacent tissues). The population included 15 patients with synchronous peritoneal metastasis.

The peritoneal wash was collected from the Douglas cavity at laparotomy. Written informed consent was obtained from each patient prior to tissue acquisition. In the absence of ascites, 150 ml of saline was introduced into the Douglas cavity at the beginning of the operation and aspirated after general stirring. The washes were centrifuged at 2000 rpm for 10 minutes to collect intact cells, which were then rinsed with phosphate-buffered saline (PBS).

RNA preparation. Total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). The mRNA from each cell line was extracted using the FAST Track Kit Ver.2 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Northern blot analysis. Northern blot analysis was performed as described elsewhere (21-23). In brief, mRNA was prepared from each cell line, then fractionated on 1% agarose/2.2 M formaldehyde gels. Probes were labeled with ^{32}P by random priming. Each blot was hybridized with the *RegIV* probe and a β-actin probe as a control. Hybridization signals were analyzed with a BAS 2000 image analyzer (Fuji, Tokyo, Japan) and calculated the degree of overexpression in comparison to the β-actin control.

Real-time quantitative RT-PCR. cDNA was produced from 2 μg of total RNA using the Superscript Preamplification System (BRL, Bethesda, MD, USA), according to the manufacturer's instructions. Briefly, RNA was heated to 70°C for 10 min in 14 μl of diethylpyrocarbonate-treated water containing 0.5 μg of oligo (dT) primer. Synthesis buffer (10x, 500 mM Tris-HCl, pH 8.3, 750 mM KCl, 30 mM $MgCl_2$), 2 μl of 10 mM dNTP mixture, 2 μl of 0.1 M DTT and reverse transcriptase (Superscript RT; 200U/μL, Gibco BRL, Gaithersburg, MD, USA) were added to the sample. The reaction mixture was incubated at 42°C for 50 min, and the reaction was terminated by incubation at 90°C for 5 min.

Quantitative PCR was performed using real time Taqman TM technology, as described by Nakanishi *et al.* (24). Results were analyzed on a Model 5700 Sequence Detector (Applied Biosystems Corp., Foster City, CA, USA).

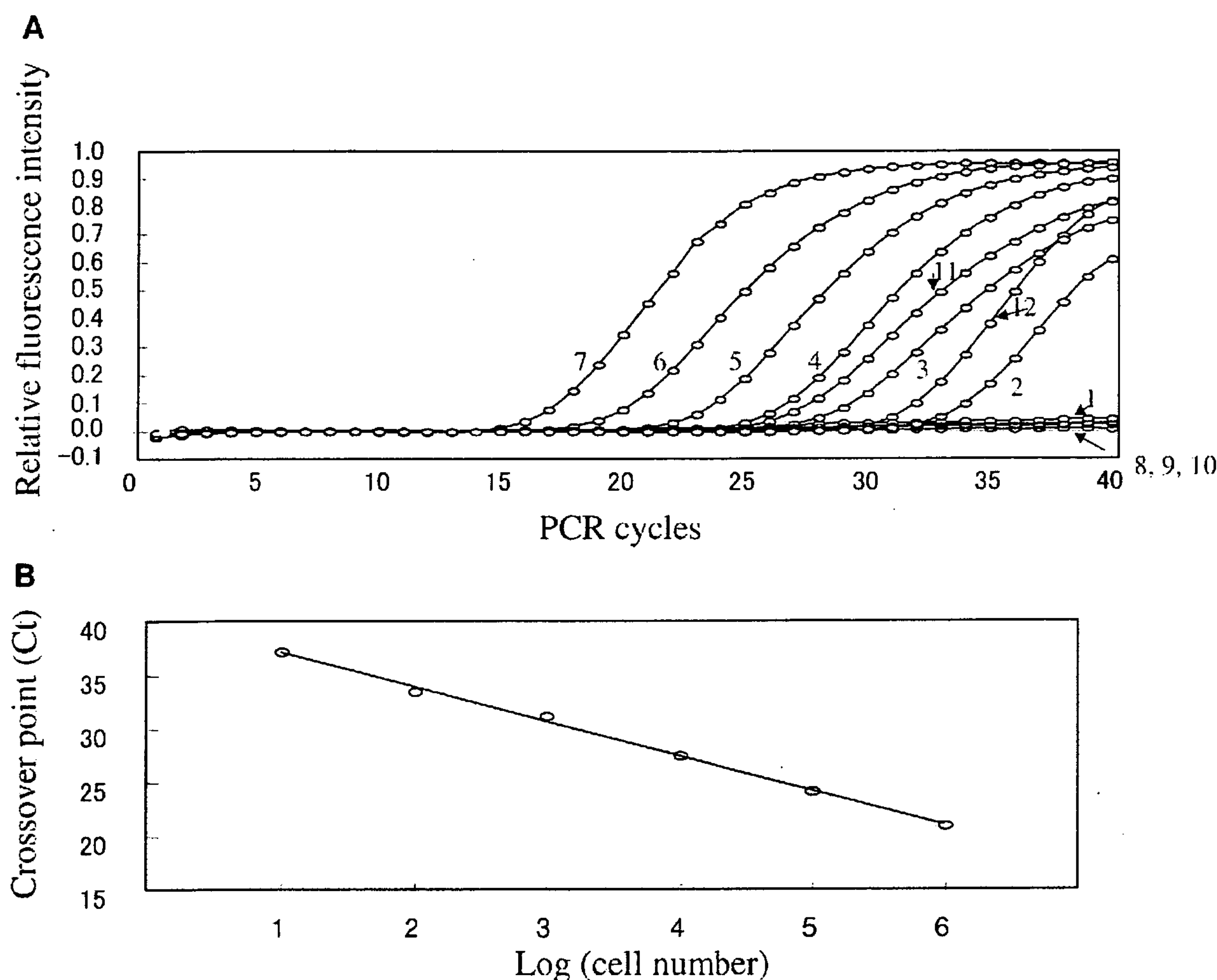

Figure 1. *Representative real-time RT-PCR result and calibration curve for estimating RegIV mRNA expression. A, Relative fluorescence vs. number of PCR cycles. Six external standards (lines 1-6) and two patient samples (lines 10 and 11) of unknown concentration were amplified with real-time 'Taqman TM' technology and analyzed with a Model 5700 Sequence Detector. Line 1=1, line 2=10, line 3=10^2, line 4=10^3, line 5=10^4, line 6=10^5, and line 7=10^6 SNU-16 gastric cancer cell equivalents of cDNA; line 8=Met 5A cells; line 9=HL60 cells; line 10=negative peritoneal wash; lines 11 and 12=positive peritoneal washes. B, Calibration curve for estimating RegIV mRNA expression. Curve was generated using data from the external controls in A by plotting the crossover points (Ct) against log SNU-16 cell number. Relative RegIV mRNA values in patient samples were calculated using this curve.*

The *RegIV* RT-PCR primers used were 5'-TCCTTGCAC TAGCTACATCC-3' and 5'-GGAATGTATGGCCCACATCA-3'. The *CEA* RT-PCR primers used were 5'-TCTGGAACT TCTCCTGGTCTCTCAGCTGG-3' and 5'-TGAAGCTGTTGCA AATGCTTTAAGGAAGAAGC-3'. Hybridization probes for detecting PCR products were labeled with a reporter dye (FAM), at the 5' end and a quenching dye (TAMRA), on the 3'end. The sequence of the *CEA* hybridization probe was 5'-(FAM) CATCTGGAACTTCTCCTGGTCTCTCAGC(TAMRA)-3'; the identification number for the hybridization probe for *RegIV* is Hs00230746 (Applied Biosystems Corp.).

For RT-PCR, the reaction mixture contained 1.25 units of Amp-Taq DNA polymerase, 1xPCR reaction buffer, 180 ng of each primer, 200 mM dNTP, 400 mM dUTP, 100 nM Taqman probe and 0.5 U Amplirase (Applied Biosystems Corp.). Serial dilutions of control cDNA were analyzed for each target gene. Crossover point (Ct) values were determined corresponding to the cycle number at which fluorescence emission reached a threshold standard deviation of ten above the mean baseline emission derived from 40 cycles. *CEA*- and *RegIV*-specific primers were used to generate standard curves from which the rate of change in the Ct value was determined for each patient sample (as shown in Figure 1). The cycling parameters were as follows: 2 min at 50°C, 10 min at 95°C, then 40 cycles of 15s at 95°C and 1 min at 60°C. To minimize errors arising from variations in the amount of starting RNA among the samples, amplification of β-actin mRNA was analyzed as an internal reference. The values from target RNAs were then normalized to β-actin mRNA. The primers and

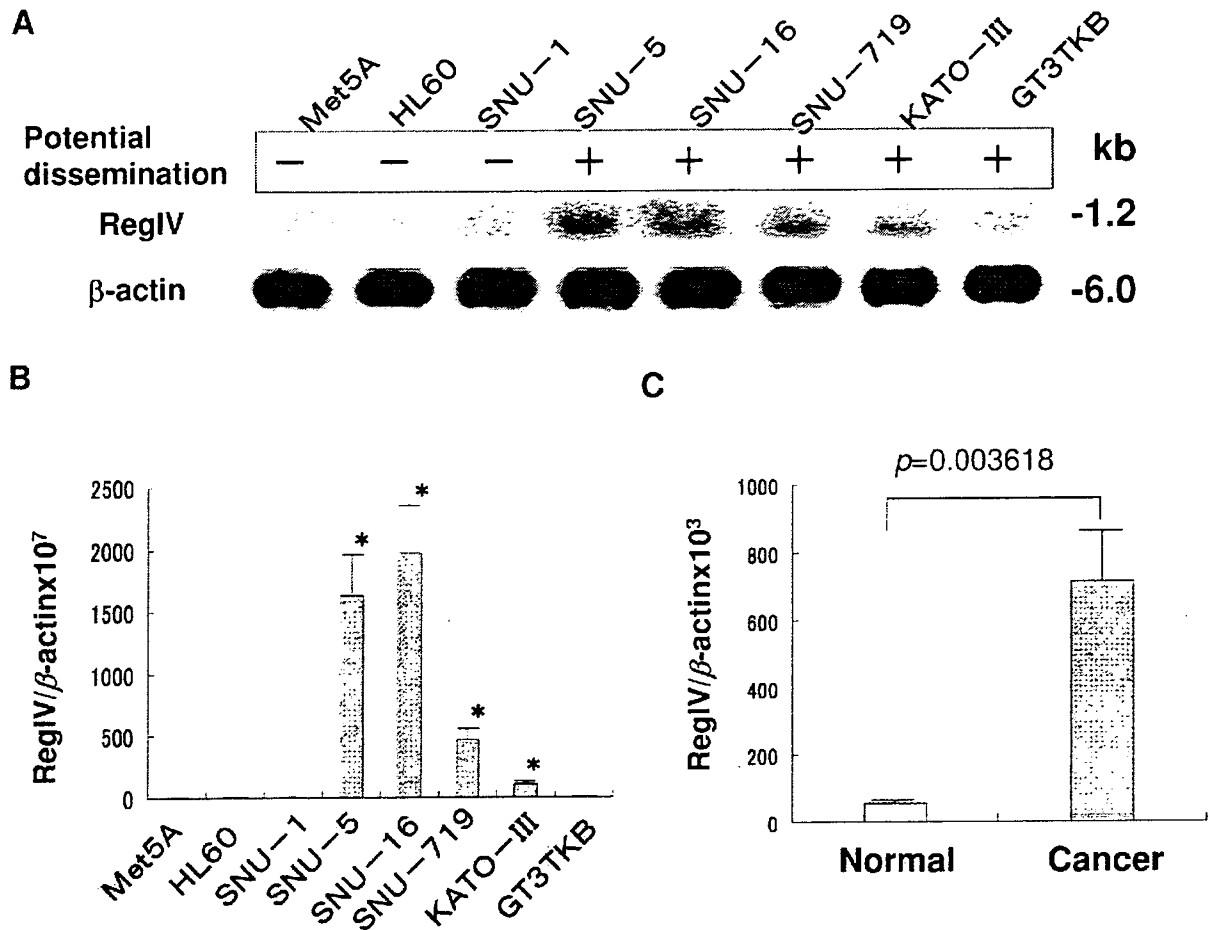

Figure 2. *RegIV mRNA expression in gastric cancer cell lines and primary gastric cancer specimens. A) RegIV mRNA expression in eight gastric cancer cell lines analyzed by Northern blot. RegIV was up-regulated in gastric cancer cells derived from malignant ascites compared to cells derived from a primary lesion. β-Actin was probed as a control for loading variations in each lane. (+) and (-): peritoneal dissemination potential. B) RegIV mRNA expression in primary gastric cancer cells, mesothelial cells, leukemia cell, and gastric cancer cells from malignant ascites was analyzed by quantitative RT-PCR, as described in Material and Methods. RegIV expression in gastric cancer cells from malignant ascites was significantly higher than in primary gastric cancer cells, mesothelial cells, leukemia cell (*p<0.01). C) RegIV mRNA expression in primary gastric cancers and normal gastric mucosa was analyzed by quantitative RT-PCR. Expression levels of RegIV were normalized to β-actin. Note that RegIV expression in gastric cancer tissue was significantly higher than in gastric mucosa (p=0.003168).*

the probe for β-actin were purchased from Applied Biosystems. Normalized results are expressed as the ratio of number of copies of target gene to β-actin.

The ratio of *RegIV/β-actin* mRNA derived from negative control patients (patients with benign disease undergoing surgery) was determined and the highest value was adopted as the cut-off value (Figure 3A, broken line). Samples with ratios that were greater than this limit were regarded as positive (*RegIV* +).

Plasmids and transfection. To obtain stable RegIV-expressing TMK-1 transfectants, pEF-BOS-RegIV was transfected into TMK-1 cells using LipofectAMINE, according to the manufacturer's instructions (Life Technologies, Carlsbad, CA, USA). G418 (600 μg/mL)-resistant colonies were selected and subcultured as described elsewhere (23). Independent clonal cell lines that strongly expressed RegIV were identified by Northern blot analysis. TMK-1 cells transfected with pEF-BOS-neo and treated as described above were obtained as controls.

Experimental model of gastric cancer in nude mice. Four-week-old male C3H nude mice (Clea Japan, Inc., Osaka, Japan) were inoculated with $2x10^7$ TMK-1 gastric cancer cells intraperitoneally in 0.5 mL PBS, or subcutaneously in 0.3 mL PBS. Mice were injected with *RegIV* transfectants of TMK-1 cells, parental TMK-1 cells, or neomycin control transfectants in PBS. Six mice were injected intraperitoneally, and seven were injected subcutaneously. Five weeks after injection, the presence of disseminated foci or ascites was determined. Six weeks after inoculation intraperitoneally and five weeks after inoculation subcutaneously, the mice were sacrificed and examined. All animal experiments

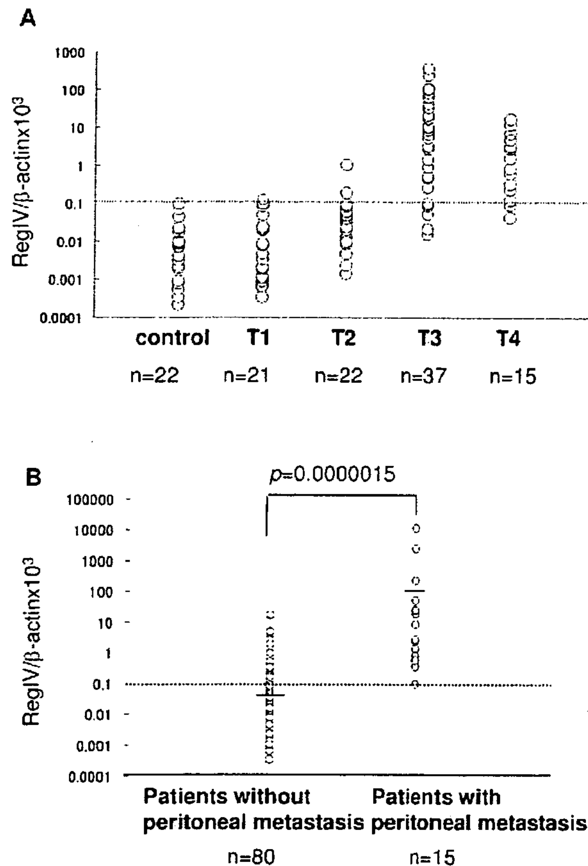

Figure 3. *RegIV/β-actin mRNA ratio in peritoneal washes from gastric cancer patients. A) RegIV/β-actin mRNA ratios in peritoneal washes from gastric cancer patients, grouped according to the depth of invasion (pT category). The broken line on the graph indicates the cut-off value for identifying RegIV+ samples, and was determined using data from negative control patients with benign disease. RegIV mRNA values correlated statistically with the depth of cancer invasion (p<0.01). B) RegIV mRNA values for peritoneal washes from gastric cancer patients with or without synchronous peritoneal metastasis. RegIV mRNA values for metastasis-positive patients were significantly higher than for metastasis-negative patients (p=0.0000015).*

were conducted in accordance with our institutional guidelines for animal welfare. Representative whole mount specimens of tumors from the abdominal cavity and in the subcutaneous tissue in animals that received stable *RegIV* transfectants or control neomycin transfectants of TMK-1 cells were used to calculate tumor weight.

Statistical analysis. Survival was analyzed with Kaplan-Meier curves, using death and clinical diagnosis of peritoneal carcinomatosis as the endpoints. For the analysis of survival with peritoneal metastasis as the endpoint, deaths resulting from other types of metastasis in the absence of clinical signs of peritoneal carcinomatosis were treated as censored cases.

The statistical significance of intergroup differences in *RegIV/β-actin* mRNA ratios were calculated using the Mann-Whitney *U*-test. *RegIV/β-actin* mRNA ratios of different groups, classified on the basis of their pT categories, were compared using the Kruskal-Wallis test. *P*-values of less than 0.05 were considered statistically significant. To identify independent prognostic factors, all 95 patients were analyzed by multivariate analysis using the Cox proportional hazards model.

Results

Quantitative RT-PCR using the Gene Amp 5700 sequence detection system. Real-time fluorescence RT-PCR using the Gene Amp 5700 sequence detection system allowed rapid, sensitive detection of *RegIV* mRNA from patient samples. With this method, 10 to 10^6 *RegIV*-expressing gastric cancer SNU-16 cells per 107 mesothelial cells were quantified (Figure 1A). No significant level of *RegIV* mRNA was detected in peripheral blood lymphocytes or mesothelial cells from healthy volunteers.

mRNA levels were quantified using Ct, which was the PCR reaction cycle when the fluorescence of a given sample rose above the background level to yield the maximal slope with respect to log-linear amplification. Figure 1B illustrates a standard curve generated by plotting on a log scale the number of SNU-16 cells (serial 10-fold dilutions) against their respective Cts. *RegIV* mRNA values for patient samples of unknown concentration were calculated using this calibration curve as a reference.

Expression of RegIV mRNA in gastric cancer cell lines, a mesothelial cell line, normal gastric mucosa, and cancerous tissues. Northern blot analysis showed a high level of expression of *RegIV* in cells with a high potential for peritoneal dissemination, and a low level of expression in cells with a low potential. Intense bands were observed in SNU-5, SNU-16, SNU-719 and KATO-III cells (5.8-, 8.1-, 2.3- and 1.5-fold, respectively compared to control Met5A and HL60 cells) (Figure 2A). The level of β-actin was probed as a control for loading variations. Quantitative RT-PCR yielded a similar pattern of expression of *RegIV* mRNA (Figure 2B).

RegIV expression was also detected in both normal gastric mucosa and clinical specimens of gastric cancer. In cancerous tissues, *RegIV* expression was significantly higher than in the normal mucosa (Figure 2C).

RegIV/β-actin mRNA ratio in peritoneal washes of gastric cancer patients varies with the degree of wall invasiveness. To normalize the amount of RNA in each patient sample, β-actin mRNA was used as an internal control. The value for *RegIV* mRNA expression level was then determined as the ratio of *RegIV* mRNA to β-actin mRNA (*RegIV*/β-actin mRNA). When gastric cancer patients were grouped

Table I. *Expression of RegIV mRNA and clinicopathological factors in gastric cancer patients.*

Variable	RegIV mRNA		P-value*
	Positive	Negative	
Gender			
Male	31	34	0.636174
Female	16	14	
Differentiation			
Differentiated	16	29	0.017843**
Undifferentiated	31	19	
Depth of invasion			
T1, T2	3	40	0.000001**
T3, T4	44	8	
Lymphatic invasion			
Negative	5	28	0.000023**
Positive	42	20	
Vascular invasion			
Negative	16	32	0.004893**
Positive	31	16	
Lymph node metastasis			
Negative	6	34	0.000001**
Positive	41	14	
Peritoneal dissemination			
Negative	33	47	0.000176**
Positive	14	1	

T classification: T1, mucosa to submucosa; T2, muscularis propria to subserosa; T3, serosa-exposed; T4, serosa-infiltrating. *Mann-Whitney test, **statistically significant.

Table II. *Clinicopathological features of recurrent gastric cancer patients with peritoneal dissemination and malignant ascites.*

Case No.	Stage*	Markers			
		Histology**	CEA	RegIV	CEA and RegIV
1	P1H0N2T3CY1 Stage IV	Por	+	+	+
2	P0H0N2T3CY0 Stage IIIB	Sig	-	+	+
3	P0H0N1T3CY1 Stage IV	Tub	+	+	+
4	P0H0N1T3CY0 Stage IV	Por	-	+	+
5	P1H0N2T3CY0 Stage IIIA	Sig	+	+	+
6	P0H1N1T4CY1 Stage IV	Sig	+	+	+
7	P0H1N3T3CY0 Stage IIIB	Tub	+	+	+
8	P1H1N2T3CY0 Stage IV	Por	-	+	+
9	P0H0N2T3CY1 Stage IV	Por	+	+	+
10	P1H0N1T4CY1 Stage IV	Sig	+	+	+
11	P0H0N2T3CY0 Stage IIIA	Tub	+	-	+
12	P0H0N3T3CY0 Stage IV	Por	+	+	+
13	P0H0N1T3CY0 Stage IIIB	Sig	-	+	+
14	P0H0N2T3CY1 Stage IV	Por	+	+	+
15	P0H0N2T3CY1 Stage IV	Por	+	+	+
	Sensitiviy for the detection of MM		73% (11/15)	93% (14/15)	100% (15/15)

*Clinical stage according to the Japanese Gastric Cancer Classification; **Histology of the primary lesion according to Japanese Gastric Cancer Classification. Por: poorly differentiated adenocarcinoma; Sig: signet ring cell carcinoma; Tub: tubular adenocarcinoma.

according to T score, the average *RegIV*/β-actin mRNA ratios (ratiox10^3) in peritoneal washes were as follows (average±standard deviation): control, 0.014906±0.024916; T1, 0.027919±0.038021; T2, 0.086225±0.071765; T3, 38.01328±15.08207; T4, 3.286277±4.87313. Figure 3A shows a plot of *RegIV*/β-actin mRNA ratios (x10^3) from all patients, grouped according to T classification. When cases were further classified into cases positive (T3, T4) and negative (T1, T2) for invasion of the serosa, there was a correlation between *RegIV*/β-actin mRNA ratio and the degree of wall invasiveness: The *RegIV*/β-actin mRNA ratio was significantly higher in cases that were positive for serosal invasion compared to those that were negative. *RegIV* mRNA values were also significantly higher in washes from metastasis-positive patients than in metastasis-negative patients (p=0.0000015, Figure 3B).

RegIV mRNA expression and clinicopathological factors. Among the 95 cases examined, 47 were *RegIV+*. Fifteen patients had positive cytology (CY+) or were observed to have peritoneal metastases. Fourteen out of these 15 patients with peritoneal metastases had *RegIV* values that were above the cut-off value (93% sensitivity). Of note, 3 of 43 T1 or T2 patients were *RegIV+* (93% specificity). Differentiation, depth of invasion, lymphatic invasion, vascular invasion, lymph node invasion, and peritoneal dissemination showed statistically significant differences with respect to expression of *RegIV* (Table I).

Comparison of the sensitivity and specificity of RegIV and CEA expression as markers for peritoneal micro-metastasis of gastric cancer. CEA/β-actin mRNA ratios were also measured in all clinical samples. The average values of the *CEA*/β-actin mRNA ratios (x10^3) according to T classification were as follows (average±standard deviation): control, 3.4±1.7; T1, 5.3±3.5; T2, 8.4±6.8; T3, 71.1±22.8; and T4, 643.9±378.3. There was a correlation between the degree of wall invasion and *CEA*/β-actin mRNA ratio. When patients were classified according to serosal invasion as positive (T3, T4) or negative (T1, T2), we observed a significant difference between the two groups with respect to *CEA* expression with patients who were CEA-positive exhibiting significantly higher CEA expression than CEA-negative patients. As with *RegIV* mRNA, *CEA*/β-actin mRNA ratios from negative control patients (patients with benign disease) were determined and the highest value was

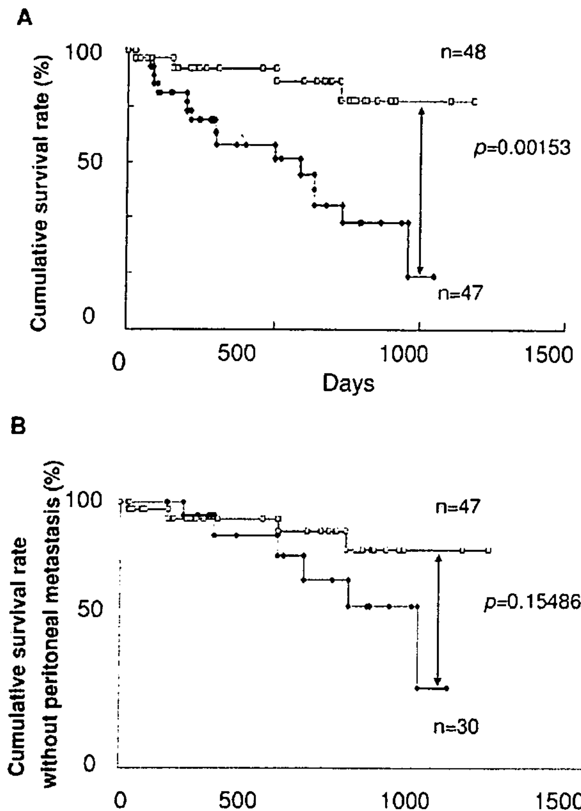

Figure 4. *Survival curves of gastric cancer patients. A) Survival curves of all 95 study patients with gastric carcinoma, grouped according to RegIV mRNA expression. RegIV+ cases (closed symbols) had a significantly worse prognosis than RegIV-negative cases (open symbols) (p=0.00153). B) Survival curves of 77 patients who underwent R0 resection, grouped according to RegIV mRNA expression. There was no significant difference in prognosis between RegIV+ (closed symbols) and RegIV-negative (open symbols) patients.*

set as the cut-off value. Cases with a value greater than the cut-off were regarded as *CEA*-positive. Four of the 43 T1 or T2 patients were *CEA*-positive (91% specificity, data not shown). Table II contains a list of patients who suffered from recurrent gastric cancer with peritoneal dissemination and ascites after surgery. Of the 15 who were CY+ or were observed intraoperatively to have peritoneal metastasis, 11 were *CEA*-positive. However, 4 *CEA*-negative patients had metastasis (Table II, 73% sensitivity).

Although the 4 CEA-negative patients (cases 2, 4, 8 and 13 in Table II) had poorly differentiated adenocarcinoma, two of them had peritoneal metastases at an early stage. Three of these patients were RegIV+ (Table II). As shown in Table I, RegIV+ cases were more frequently observed in undifferentiated adenocarcinoma. These results suggested that *RegIV* may be a novel marker for more extensive

Table III. *Multivariate analysis of RegIV mRNA and other known prognostic factors for 95 patients with gastric cancers.*

Covariate	Hazard ratio	95% Confidence interval	*P*-value
RegIV (cut-off value 0.1)			
Negative	1		
Positive	2.033659	1.059-1.132	0.0151
Vascular invasion			
Negative	1		
Positive	4.149176	1.461-14.940	0.0062
Lymph node metastasis			
Negative	1		
Positive	9.820896	1.660-190.220	0.0080

disease even when a sample is negative for *CEA*. As shown in Table II, combining *CEA* and *RegIV* analysis improved the accuracy of diagnosis to 100%.

RegIV as an independent prognostic factor. Survival analysis was performed for all 95 gastric cancer patients. Univariate analysis of prognosis factors showed that RegIV+ cases (47 of 95) were significantly fewer than RegIV- cases (48 of 95, p=0.00153) (Figure 4A). We also performed survival analysis of 77 patients who underwent R0 resection. Eighteen patients treated with palliative resection, including 15 patients with synchronous peritoneal metastases, died within 490 days with peritoneal metastases. As shown in Figure 4B, there was no significant difference between the survival rate of *RegIV+* cases and *RegIV-* cases in this group of patients.

Multivariate analysis using the Cox proportional hazards model showed that a *RegIV*/β-actin mRNA ratio above a cut-off value of 0.1 was a significant independent factor, along with histological findings of lymph node metastasis and vascular invasion (Table III). In cases of R0 resection, we found a correlation between *RegIV* expression and prognosis; however, the results were not statistically significant (data not shown).

RegIV expression and tumorigenesis in nude mice. Following inoculation of nude mice with either TMK-1-neomycin or TMK-1-RegIV stable transfectants, we found far more peritoneal-disseminated metastatic lesions in TMK-1-RegIV-innoculated mice, as compared to the controls (Figure 5A). The metastatic nodules were found in the mesenterium as well as at the peritoneal wall. There was also an increase in ascites fluid in the peritoneal cavity. In addition to the number, the size of peritoneal metastases increased in TMK-1-RegIV-inoculated mice. Figure 5B shows the aggregate intraperitoneal tumor weight per

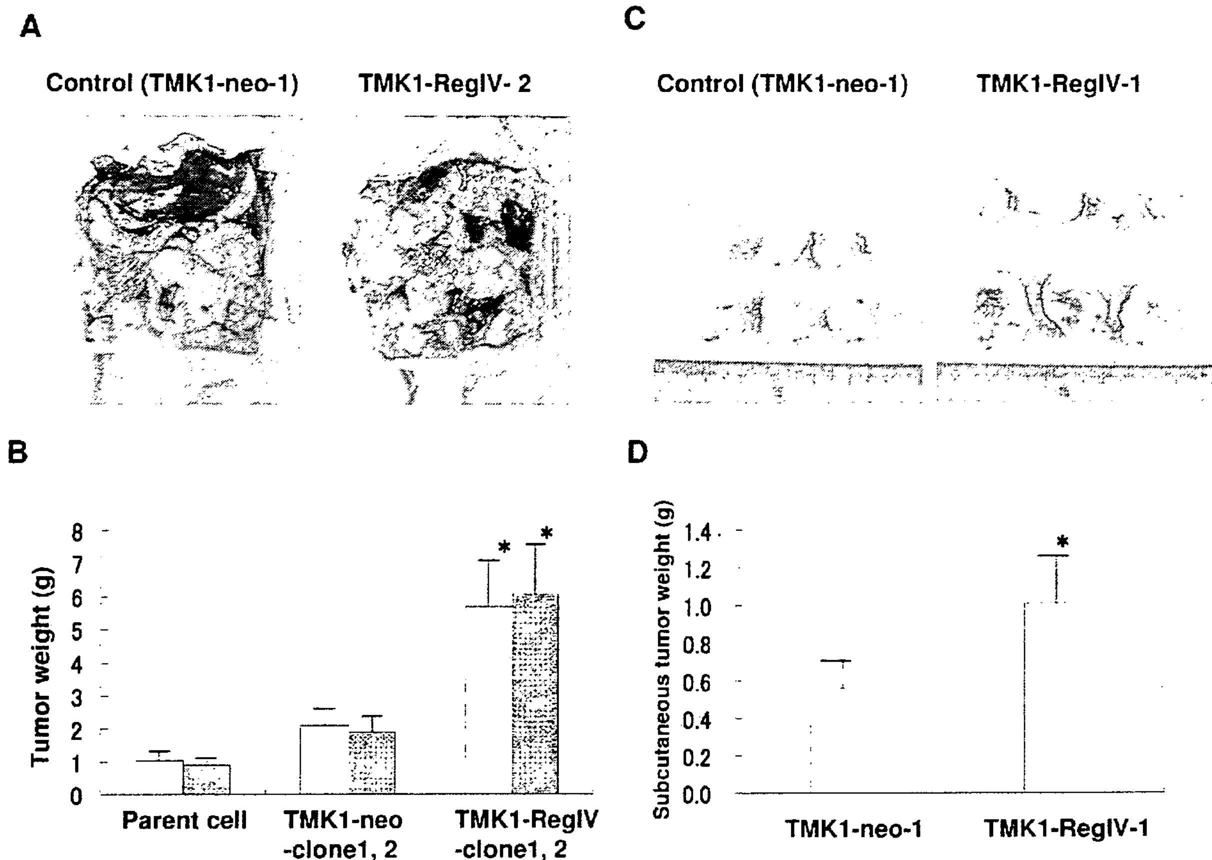

Figure 5. *Acceleration of peritoneal metastases of gastric cancer by RegIV expression. A) Macroscopic appearance of peritoneal disseminated metastatic nodules derived from TMK-1-RegIV-2 transfectants and control TMK-1 transfectants. B) Quantification of metastatic nodules in the mouse peritoneal cavity. Weight of tumors derived from RegIV transfectants were significantly higher than those derived from control cells (*p<0.01). C) Tumors obtained after subcutaneous injection of RegIV and control transfectants. Cells were injected subcutaneously to examine the growth stimulatory activity of RegIV transfectants and control cells. D. Subcutanous tumor weight in TMK-1-RegIV-1 and TMK-1-neomycin-inoculated mice. Subcutanous tumor weight increased in TMK-1-RegIV-1 inoculated mice (p<0.05).*

animal (n=6 for each group, $p<0.01$). All mice that were injected with parental TMK-1 cells or TMK-1-neomycin transfectants (mock transfectants) died within 16 weeks (average lifespan 84 days). In contrast, all mice injected with TMK-1-RegIV transfectants died within 70 days (average lifespan 44 days). Figure 5C shows the tumors obtained from mice that were injected subcutaneously with the stable cell line TMK-1-RegIV-1, and Figure 5D shows the subcutanous tumor weight. The subcutaneous tumor weight was significantly higher in TMK-1-RegIV-1-inoculated mice compared to the neomycin controls ($p<0.05$).

Discussion

Peritoneal dissemination is the most important factor affecting the prognosis of individuals with gastric cancer (4). Previous reports have indicated that intraperitoneal chemotherapy improves the survival of these patients, but it can also be life-threatening because of the side-effects of the chemotherapeutic drugs. Recently we developed a novel technique to administer the anticancer agent mitomycin-C, in which the drug was adsorbed to activated carbon particles (MMC-CH) (25-28). Because MMC-CH particles are not actually absorbed through the capillary wall, a large amount of the anticancer agent was delivered through this route of adminstration. Particles are retained in the cavity, which remains closed for a long period of time; thus a high concentration of the anticancer agent is maintained. We previously reported on the efficacy of MMC-CH in the treatment of peritonitis carcinomatosis in gastric cancer (29). However, this therapeutic approach also had side-effects, such as ileus, fever and leukocytopenia, suggesting that this therapy should be limited to those most likely to benefit from it, in order to minimize these side-effects.

Peritoneal dissemination is observed in patients with negative cytological results, indicating that conventional cytological analysis lacks appropriate sensitivity. In contrast, RT-PCR has been shown to be of sufficient sensitivity to diagnose micrometastases on the basis of specific mRNA expression in tumor cells derived from the peripheral blood, bone marrow, lymph nodes and cerebrospinal fluid. Quantitative, rapid RT-PCR-based screening methods for the detection of micrometastasis from clinical specimens has now become more widely used as a diagnostic tool (30-36). *CEA*, keratin 19 and alpha-fetoprotein (*AFP*) represent some of the conventional molecular markers that have been used to detect peritoneal micrometastases in RT-PCR-based assays of peritoneal washes from patients with gastric cancer (8, 35). Yonemura *et al.* increased the sensitivity of detection to 62% by using a combination of cytology and RT-PCR-based detection of matrix metalloproteinase (*MMP*)-7 mRNA (36). Using RT-PCR, Schuhmacher *et al.* demonstrated a relationship between the expression of an E-cadherin mutation and metastasis to the peritoneum (37). However, any assay of peritoneal washes is inferior in sensitivity and specificity to real-time RT-PCR in detecting *CEA* mRNA, as described by Nakanishi *et al.* (24). Because of this, *CEA* is currently the standard molecular marker for the detection of gastric cancer micrometastases. However, it is not always expressed in peritoneal metastases and is very weakly expressed in mesothelial cells, making it difficult to exclude completely both false-positive and false-negative results using *CEA* as a marker. To reduce the frequency of missed diagnosis, markers with greater sensitivity and specificity are needed.

When choosing a genetic marker for peritoneal dissemination, genes expressed more highly in cancer cells than in mesothelial cells should be chosen to to minimize false-positive or false-negative results. Previously, using cDNA microarray analysis of gastric cancer cell lines derived from either a primary tumor or from metastatic lesions in the peritoneal cavity, we identified *RegIV* as a candidate marker of peritoneal dissemination of gastric cancer. Thus, *RegIV* satisfies the conditions stated above. In the current study, using fluorescence-based, real-time RT-PCR, we examined *RegIV* mRNA expression in peritoneal washes from 95 patients with gastric cancer and compared it to *CEA* mRNA expression, as a diagnostic tool for predicting peritoneal recurrence. We demonstrated that *RegIV* and *CEA* expression correlated with wall penetration. Using data derived from negative control patients with benign disease to set a cut-off value for expression, we identified a group of MM+ patients and showed that the specificity of *RegIV* and *CEA* expression in this group was 93% and 91%, respectively. Among 15 patients with peritoneal dissemination, 7 of whom were CY+, 14 cases were *RegIV*-positive (93% sensitivity), while 4 cases appeared negative for CEA expression (73% sensitivity). *CEA*-specific RT-PCR failed to detect peritoneal dissemination of poorly differentiated adenocarcinoma, while *RegIV*-specific RT-PCR successfully detected these cancers (Table II). Taken together, quantitative RT-PCR of peritoneal washes to detect the novel marker *RegIV* yielded higher sensitivity and specificity than did similar analysis of *CEA*, particularly in patients with poorly differentiated adenocarcinoma. Our results also indicated that the combination of *CEA*- and *RegIV*-specific RT-PCR may improve the accuracy of diagnosis of peritoneal dissemination.

According to the survival analysis of patients with gastric cancer, *RegIV*-positive cases had a significantly worse prognosis than *RegIV*-negative cases. Moreover, multivariate analysis suggested that *RegIV* is an independent prognostic factor of survival. In view of the correlation between the results from RT-PCR analysis and prognosis, *RegIV* represents a potentially useful and effective marker of peritoneal recurrence of gastric cancer. A large-scale, long-term follow-up study is currently under way in our department to determine the rate of peritoneal recurrence in cytology-negative, PCR-positive patients, and to determine whether these patients remain disease-free.

Expression of *RegIV* in gastric cancer cells established from malignant ascites accelerated the rate of peritoneal metastases in a nude mouse model of gastric cancer. In addition, the tumorigenicity of the *RegIV*-expressing cells, when injected into the peritoneum, was significantly higher than either parental or mock-transfected cells. Given that Reg family members are involved in liver, pancreatic, gastric and intestinal cell proliferation or differentiation (14), our results suggest that *RegIV* is involved in gastric cancer cell proliferation and peritoneal metastasis.

In conclusion, we have presented evidence that *RegIV*-specific RT-PCR analysis of peritoneal washes may be more sensitive than conventional cytology or *CEA*-specific RT-PCR for predicting peritoneal recurrence in gastric cancer. While *RegIV* is overexpressed in gastric cancer peritoneal dissemination, the role of *RegIV* in this process remains the subject of future studies.

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Serum Concentration of Reg IV in Patients with Colorectal Cancer: Overexpression and High Serum Levels of Reg IV Are Associated with Liver Metastasis

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Key Words
Colorectal cancer · Reg IV · Serial analysis of gene expression · Serum tumor marker

Abstract
Objective: Regenerating islet-derived family, member 4 (regenerating gene type IV, Reg IV) is overexpressed in colorectal cancer (CRC). The aim of this study was to investigate the diagnostic utility of Reg IV determination in sera from patients with CRC. ***Methods:*** We examined the expression and distribution of Reg IV in CRC by immunohistochemistry and determined Reg IV levels in sera from patients with CRC by enzyme-linked immunosorbent assay. ***Results:*** Immunostaining revealed that 23 of 80 (29%) CRC cases were positive for Reg IV. CRC cases with metastatic recurrence in the liver showed more frequently Reg IV staining than those without (p = 0.0102). Patients with CRC showing Reg IV staining had a significantly worse survival than those without Reg IV staining (p = 0.0117). Preoperatively, serum Reg IV concentrations were not elevated in CRC patients at stage 0–III, being in contrast to the significantly increased preoperative levels in stage IV CRC patients with liver metastasis. ***Conclusion:*** These results suggest that Reg IV is a prognosticator for poor survival. Serum Reg IV concentration may predict CRC recurrence in the liver.
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Introduction

It is generally accepted that cancer develops as a result of multiple genetic and epigenetic alterations. Better knowledge of changes in gene expression that occur during carcinogenesis may lead to improvements in the diagnosis, treatment and prevention. Identification of novel biomarkers for cancer diagnosis and novel targets for treatment is a major goal [1]. Genes encoding transmem-

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brane/secretory proteins expressed specifically in cancers may be ideal biomarkers for cancer diagnosis [2]. In addition, if the function of the gene product is involved in the neoplastic process, this gene may constitute a therapeutic target. To identify potential molecular markers for cancer and to better understand the development of cancer at the molecular level, comprehensive gene expression analysis may be useful. Among the comprehensive methods used to analyze transcript expression levels, array-based hybridization [3] and serial analysis of gene expression (SAGE) [4] are currently the most common approaches. We previously performed SAGE in four primary gastric cancers (GCs) [5] and identified several GC-specific genes [6]. Of these genes, *Regenerating islet-derived family, member 4* (*REG4*, which encodes Reg IV) is a candidate gene for cancer-specific expression, at least in patients with GC. *REG4* was originally identified by high-throughput sequencing of a cDNA library derived from inflammatory bowel disease patients [7]. Reg IV is a member of the Reg gene family, which includes three other genes (Reg Iα, Reg Iβ and Reg III) [7]. In GC, expression of Reg Iα has been reported [8, 9]. Reg Iα expression is enhanced in advanced T grade GCs and in GCs that were not well differentiated. Overall and disease-free survival are poor for patients with Reg Iα-positive GCs. Quantitative reverse transcription (RT)-polymerase chain reaction (PCR) analysis revealed that approximately 50% of GCs overexpress the *REG4* gene [5]. Although various normal tissues express *REG4*, expression levels are much lower in normal tissues than in cancerous tissues [5]. Our previous immunohistochemical analysis revealed that Reg IV was expressed in 30% of GC tissues and was associated with both the intestinal mucin phenotype and neuroendocrine differentiation [10]. In addition, Reg IV is a secreted protein, and we showed that serum Reg IV represents a novel biomarker for GC [11]. The diagnostic sensitivity of serum Reg IV was superior to that of serum carcinoembryonic antigen (CEA) or carbohydrate antigen 19-9 (CA19-9).

Overexpression of Reg IV has been reported in colorectal cancer (CRC) [10, 12, 13]. In the normal colon, little or no Reg IV immunostaining is observed in epithelial cells, whereas strong Reg IV immunostaining is detected in neuroendocrine cells [10, 12, 14]. In our previous immunohistochemical analysis, Reg IV staining was identified in 36% of CRC cases [10]. Real-time RT-PCR analysis revealed that although more than 70% of human CRC samples showed increased *REG4* expression [12, 13], the other REG mRNAs are present at very low or undetectable levels in most samples [12]. Therefore, Reg IV may be a serum biomarker for CRC; however, serum Reg IV concentrations have not been investigated in CRC patients.

In the present study, we examined the expression and distribution of Reg IV in human CRC by immunohistochemistry, and the relationship between Reg IV staining and clinicopathological characteristics. We have reported two Reg IV staining patterns (mucin-like staining and strong perinuclear staining) [10]. Mucin-like staining is observed in goblet cells and goblet cell-like vesicles of cancer cells. These cells are positive for MUC2 (a marker of goblet cells). In contrast, strong perinuclear staining is detected in neuroendocrine cells. These cells are positive for chromogranin A (a marker of neuroendocrine cells). Therefore, we examined the coexpression of Reg IV and chromogranin A or MUC2 by double-immunofluorescence staining. We also assessed serum Reg IV levels in CRC patients by enzyme-linked immunosorbent assay (ELISA) to investigate the potential diagnostic utility of Reg IV determination.

Patients and Methods

Tissue Samples

Primary tumor samples were collected from 80 patients with CRC (35 women and 45 men; age range, 46–93 years; mean, 68 years) and serum samples from 78 patients with CRC (28 women and 50 men; age range, 46–84 years; mean, 67 years). Patients were treated at the Hiroshima University Hospital or an affiliated hospital. For immunohistochemical analysis, we used archival formalin-fixed, paraffin-embedded tissues from 80 patients with CRC who underwent surgical excision. Information on patient survival was available for 30 of the 80 CRC cases and was not available for the remaining 50 CRC cases. Among the serum samples from the 78 patients with CRC, primary CRC tissue samples were available for immunohistochemical analysis from 50 cases. The remaining 28 primary CRC tissue samples were not available because of lack of tumor tissue samples. Serum samples from 78 patients with CRC were obtained before surgery and before initiation of therapy. Control serum samples were obtained from 50 healthy individuals (14 women and 36 men; age range, 61–86 years; mean, 71 years). Serum samples were stored at –80°C until analysis. Tumor staging was performed according to the TNM classification system [15]. Because written informed consent was not obtained, for strict privacy protection, identifying information was removed from all samples before analysis. This procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research of the Japanese Government.

Immunohistochemistry

Formalin-fixed, paraffin-embedded samples were sectioned, deparaffinized and stained with hematoxylin and eosin to ensure that the sectioned block contained tumor cells. Adjacent sections