# 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. Patients 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

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

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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.

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 cancerrelated 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

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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 highdensity 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 downregulated 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.

# **Patients 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  $37^{\circ}\mathrm{C}$  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<sub>2</sub>), 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).

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

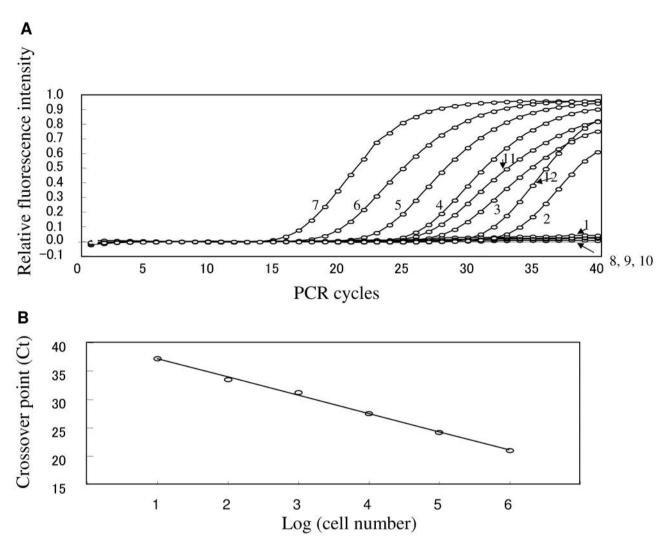


Figure 1. Representative real-time RT-PCR result and calibration curve for estimating RegIV mRNA expression. A, Relative fluorescence vs. number of PCR cycles. Six external standards (lines 1-6) and two patient samples (lines 10 and 11) of unknown concentration were amplified with real-time 'Taqman TM' technology and analyzed with a Model 5700 Sequence Detector. Line 1=1, line 2=10, line 3=10², line 4=10³, line 5=10⁴, line 6=10⁵, and line 7=10⁶ 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.

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  $\beta$ -actin mRNA was analyzed as an internal reference. The values from target RNAs were then normalized to  $\beta$ -actin mRNA. The primers and the probe for  $\beta$ -actin were purchased from Applied Biosystems. Normalized results are expressed as the ratio of number of copies of target gene to  $\beta$ -actin.

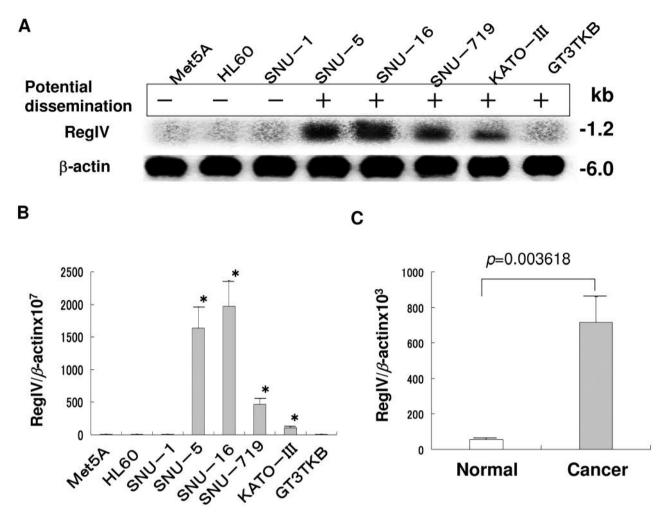
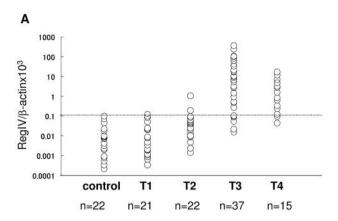


Figure 2. RegIV mRNA expression in gastric cancer cell lines and primary gastric cancer specimens. A) RegIV mRNA expression in eight gastric cancer cell lines analyzed by Northern blot. RegIV was up-regulated in gastric cancer cells derived from malignant ascites compared to cells derived from a primary lesion.  $\beta$ -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  $\beta$ -actin. Note that RegIV expression in gastric cancer tissue was significantly higher than in gastric mucosa (p=0.003168).

The ratio of  $RegIV/\beta$ -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 Bg/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<sup>7</sup> 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 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.



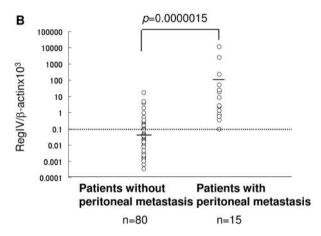


Figure 3. RegIV/ $\beta$ -actin mRNA ratio in peritoneal washes from gastric cancer patients. A) RegIV/ $\beta$ -actin mRNA ratios in peritoneal washes from gastric cancer patients, grouped according to the depth of invasion (pT category). The broken line on the graph indicates the cut-off value for 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).

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  $RegIV/\beta$ -actin mRNA ratios were calculated using the Mann-Whitney U-test.  $RegIV/\beta$ -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<sup>6</sup> RegIV-expressing gastric cancer SNU-16 cells per 10<sup>7</sup> 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  $\beta$ -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 according to T score, the average RegIV/β-actin mRNA ratios (ratio x 10³) 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 (x10³) from all patients, grouped according to T classification. When cases were further classified into cases positive (T3, T4) and

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

	RegIV			
Variable	Positive	Negative	P-value*	
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.

negative (T1, T2) for invasion of the serosa, there was a correlation between  $RegIV/\beta$ -actin mRNA ratio and the degree of wall invasiveness: The  $RegIV/\beta$ -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).

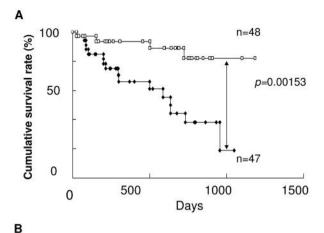
Comparison of the sensitivity and specificity of RegIV and CEA expression as markers for peritoneal micro-metastasis of gastric cancer.  $CEA/\beta$ -actin mRNA ratios were also

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

	Stage*		Markers		3
No.		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		73%	93%	100%
	detection of MM		(11/15	)(14/15)	(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.

measured in all clinical samples. The average values of the  $CEA/\beta$ -actin mRNA ratios (x10<sup>3</sup>) 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 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).



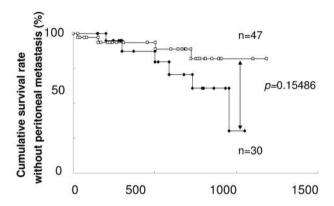


Figure 4. Survival curves of gastric cancer patients. A) Survival curves of all 95 study patients with gastric carcinoma, grouped according to RegIV mRNA expression. RegIV+ cases (closed symbols) had a significantly worse prognosis than RegIV-negative cases (open symbols) (p=0.00153). B) Survival curves 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.

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 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

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

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  $RegIV/\beta$ -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-1neomycin 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 animal (n=6 for each group, p<0.01). All mice that were injected with parental TMK-1 cells or TMK-1neomycin 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

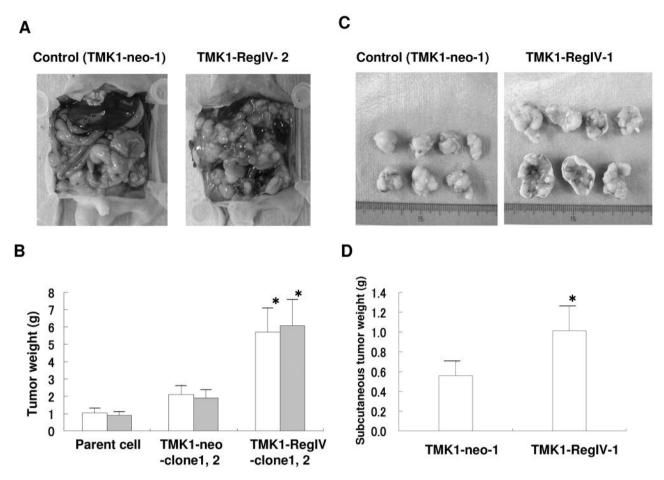


Figure 5. Acceleration of peritoneal metastases of gastric cancer by RegIV expression. A) Macroscopic appearance of peritoneal disseminated metastatic nodules derived from TMK-1-RegIV-2 transfectants and control TMK-1 transfectants. B) Quantification of metastatic nodules in the mouse peritoneal cavity. 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).

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 falsepositive 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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