Human Pathology (2009) **xx**, xxx–xxx

ELSEVIER

Human PATHOLOGY

www.elsevier.com/locate/humpath

Original contribution

The role of *Reg IV* gene and its encoding product in gastric carcinogenesis☆

Hua-chuan Zheng MD, PhD[a,*], Xiao-yan Xu PhD[a], Miao Yu MD[b], Hiroyuki Takahashi MD, PhD[c], Shinji Masuda MD, PhD[d], Yasuo Takano MD, PhD[c]

[a]*Department of Biochemistry and Molecular Biology, College of Basic Medicine, China Medical University, Shenyang, 110001 China*
[b]*Department of Surgical Oncology, The First Affiliated Hospital of China Medical University, Shenyang, 110001 China*
[c]*Department of Diagnostic Pathology, Graduate School of Medicine and Pharmaceutical Science, University of Toyama, Toyama, 930-0194 Japan*
[d]*Kouseiren Takaoka Hospital, Takaoka, 933-8555 Japan*

Received 2 June 2009; revised 18 June 2009; accepted 19 June 2009

Keywords:
Gastric carcinoma;
Reg IV;
Pathobiologic behavior;
Prognosis

Summary Although the biologic function of Reg IV is poorly understood, it has been reported that Reg IV is a potent activator of the epidermal growth factor receptor/Akt/AP-1 signaling pathway in colon cancer cells and closely linked with the inhibition of apoptosis. To clarify the role of *Reg IV* in gastric carcinogenesis and subsequent progression, we examined its expression by immunohistochemistry and in situ hybridization on tissue microarray containing gastric carcinoma, adjacent nonneoplastic mucosa, adenoma, intestinal metaplasia, or gastritis. Gastric carcinoma cell lines (MKN28, AGS, MKN45, KATO-III, and HGC-27) were studied for Reg IV expression by Western blot and reverse transcriptase–polymerase chain reaction followed by sequencing. Frozen samples of gastric carcinoma and adjacent nonneoplastic mucosa were subjected to Western blot, and patient serum, to enzyme-linked immunosorbent assay for Reg IV. Gastric carcinoma cell lines showed different levels of Reg IV mRNA and its encoding protein. The Reg IV protein expression was gradually decreased from intestinal metaplasia, adenoma, and carcinoma to gastritis ($P < .05$). The positive rate of its mRNA was higher in intestinal metaplasia than carcinoma or nonneoplastic mucosa ($P < .05$). Elevated serum Reg IV level in gastric carcinoma patients was detected in comparison with that in health individuals ($P < .05$). Reg IV expression was significantly correlated with the MUC-2 and MUC-5AC expression ($P < .05$). Among histologic subtypes of the World Health Organization, signet ring cell carcinoma more frequently expressed Reg IV than the others ($P < .05$), whereas it is the converse for the poorly differentiated group ($P < .05$). Our study indicated that Reg IV expression experienced up-regulation in gastric intestinal metaplasia and adenoma and then down-regulation with malignant transformation of gastric epithelial cells. It was suggested that Reg IV expression should be considered as a good biomarker for gastric precancerous lesions and was especially related to the histogenic pathway of signet ring cell carcinoma.

☆ This study was supported by Shenyang Outstanding Talent Foundation of China, Liaoning BaiQianWan Talents Program; Scientific and Technological Projects for Overseas Returning Persons; Grant-in aid for Scientific Research from the Ministry of Education, Culture, Sports, and Technology of Japan (20659109; 21790624); and Smoking Research Foundation.

* Corresponding author.
E-mail address: zheng_huachuan@hotmail.com (H. Zheng).

0046-8177/$ – see front matter © 2009 Published by Elsevier Inc.
doi:10.1016/j.humpath.2009.06.013

1. Introduction

Despite a worldwide decline in incidence and mortality beginning the second half of the 20th century, gastric cancer still ranks as the fourth most common cancer and the second most lethal cancer, accounting for 10.4% of cancer deaths worldwide. It continues to be a major health concern because of the slow decrease in incidence in Asia and high mortality from diagnosed gastric carcinomas in the West, although sophisticated diagnostic and operative techniques are widely applied in clinical practice [1,2]. Tumorigenesis and progression of gastric carcinoma is a multistage process, involving a multifactorial etiology, which mainly results from gene-environmental interactions. Generally, it is believed that cancer develops as a result of multiple genetic and epigenetic alterations. Therefore, increased understanding of the changes in gene expression that occur during carcinogenesis, particularly identification of novel biomarkers for cancer diagnosis and novel targets for treatment, may lead to improvements in cancer diagnosis, treatment and prevention.

Regenerating (*Reg*) gene family belongs to the calcium depending lectin gene super family and encodes 4 multifunctional small-secreted proteins, which can function as acute phase reactants, lectins, or antiapoptotic or growth agents [3]. *Reg IV*, a novel member of the family, was identified by high-throughput sequencing of a cDNA library from ulcerative colitis (UC) tissues, implying its important role in initiating the multistep process of colorectal carcinogenesis. It is located on human chromosome 1q12-q21, whose cDNA contains an open reading frame of 477 bp encoding a peptide of 158 amino acids with a predicted molecular mass of 18 kd [4]. Although the biologic function of Reg IV is poorly understood, it has been reported that Reg IV is a potent activator of the epidermal growth factor receptor/Akt/activator protein 1 signaling pathway in colon cancer cells and increases the expression of Bcl-2, Bcl-xl, and surviving proteins, associated with the inhibition of apoptosis [5]. These findings indicate that Reg IV might function as a tissue mitogen or play a role in the cell growth. The colocalization of Reg IV and Ki-67 expression suggested that Reg IV might be associated with proliferative behavior of epithelial cells in line with the speculation mentioned above [6]. Reg IV also induces the expression of matrix metalloproteinase 7 (also known as matrilysin). The expression of Reg IV may contribute to liver metastasis through induction of matrix metalloproteinase 7 [5].

Recently, Nanakin et al [6] found that *Reg IV* mRNA was strongly expressed in inflamed epithelium, dysplasia, and cancerous lesions of UC tissues, and correlated with basic fibroblast growth factor and hepatocyte growth factor mRNA expression in UC tissues. In colon cancer cell line, Reg IV expression was enhanced by stimulation with transforming growth factor α, epidermal growth factor, basic fibroblast growth factor, and hepatocyte growth factor [7]. Taken together, it is concluded that Reg IV is inducible by growth factors and may function as a growth promoting and/or an antiapoptotic factor in the pathophysiologic process of UC. In addition to gastric, colorectal, and pancreatic, hepatocellular cancers, *Reg IV* mRNA overexpression has been reported in prostate carcinoma (PCa) [5,8-13]. Most primary PCa tumors expressed a low level of *Reg IV* mRNA, whereas most metastatic PCa tumors expressed its high level [13]. It was reported that 29% of colorectal cancer (CRC) cases were positive for Reg IV, and CRC cases with metastatic recurrence to the liver showed more frequently Reg IV staining. Patients with Reg IV-positive CRC had a significantly worse survival than those without Reg IV staining [7]. At the protein level, Reg IV protein is expressed in a few epithelial cells showing neuroendocrine and mucin-producing features [14,15]. However, Reg IV expression is abundantly enhanced, and the distributions of Reg IV and chromogranin A were apparently distinct in UC tissues [6]. Recently, it has been reported that forced expression of Reg IV induces phosphorylation of the epidermal growth factor receptor and inhibits 5-fluorouracil–induced apoptosis in gastric cancer cells [16].

Immunohistochemical analysis revealed that Reg IV is expressed in gastric, colorectal, and pancreatic cancer, but not in lung and breast cancer [15]. Therefore, Reg IV may serve as a marker of digestive organ cancer. In the present study, Reg IV expression was for the first time examined in a large number of gastric samples including carcinoma, adjacent nonneoplastic mucosa (NNM), adenoma, intestinal metaplasia (IM), or gastritis in combination of immunohistochemistry (IHC), in situ hybridization (ISH), tissue microarray (TMA) and compared with the clinicopathologic parameters of carcinomas, as well as prognosis to explore the clinicopathologic significance and molecular roles of Reg IV expression in stepwise development of gastric carcinoma. In addition, gastric carcinoma cell lines and frozen carcinoma tissues were subjected to Western blot or reverse transcriptase–polymerase chain reaction (RT-PCR). We also assessed the serum Reg IV level in patients with gastric carcinoma by enzyme-linked immunosorbent assay (ELISA) to determine its potential diagnostic utility.

2. Materials and methods

2.1. Subjects

Gastric adenocarcinomas (n = 372) and adjacent nonneoplastic mucosa (NNM; n = 44) were collected from surgical resection and gastric intestinal metaplasia (n = 63), adenoma (n = 42), and gastritis (n = 93) from endoscopic biopsy or polypectomy in Takaoka Citizen Hospital, Kouserein Takaoka Hospital, or the Affiliated Hospital, University of Toyama, between 1993 and 2007. The IM or gastritis was sometime present in the NNM adjacent to carcinoma and included in ISH analysis but not in IHC. The

endoscopic biopsy samples were derived from the patients without gastric carcinoma. The patients with gastric carcinoma were 258 men and 114 women (29-91years, mean = 64.8 years). Among them, 144 cases have tumors accompanied with lymph node metastasis. Eight cases of gastric carcinoma and adjacent NNM were obtained from the Affiliated Shengjing Hospital of China Medical University and frozen in −80°C until protein extraction by homogenization in lysis buffer (50 mmol/L Tris-HCl [pH 7.5], 150 mmol/L NaCl, 5 mmol/L EDTA, 0.5% Nonidet P-40, 5 mmol/L dithiothreitol, 10 mmol/L NaF, protease inhibitor cocktail (Nacalai, Tokyo, Japan). The serum samples were collected before surgery or initiation of therapy from 24 carcinoma patients with gastric carcinoma and 16 healthy individuals in the First Affiliated Hospital of China Medical University and stored in −80°C until concentration determination. None of the patients underwent chemotherapy or radiotherapy before surgery. They all provided consent for use of tumor tissue for clinical research and our University Ethical Committee approved the research protocol. We followed up the patients by consulting their case documents and through telephone.

2.2. Pathology

All tissues were fixed in 10% neutral formalin, embedded in paraffin, and incised into 4-μm sections. These sections were stained by hematoxylin and eosin (HE) to confirm their histologic diagnosis and other microscopic characteristics. The TNM staging for each gastric carcinoma was evaluated according to the Union Internationale Contre le Cancer system for the extent of tumor spread [17]. Histologic architecture of gastric carcinoma was expressed in terms of Lauren's [18,19] and the World Health Organization (WHO) classification [20]. Furthermore, tumor size, depth of invasion, and lymphatic and venous invasion were determined.

2.3. TMA

Representative areas of solid tumors were identified in HE-stained sections of the selected tumor cases and a 2-mm-diameter tissue core per donor block was punched out and transferred to a recipient block with a maximum of 48 cores using a Tissue Microarrayer (AZUMAYA KIN-1, Tokyo, Japan). Four-micrometer-thick sections were consecutively incised from the recipient block and transferred to poly-lysine-coated glass slides. HE staining was performed on TMA for confirmation of tumor tissue.

2.4. Cell lines and culture

Gastric carcinoma cell lines, MKN28 (well-differentiated adenocarcinoma), AGS (moderately-differentiated adenocarcinoma), MKN45 (poorly differentiated adenocarcinoma), KATO-III (poorly differentiated adenocarcinoma), and HGC-27 (undifferentiated adenocarcinoma) come from Japanese Physical and Chemical Institute. They were maintained in RPMI 1640 (MKN28, MKN45, and KATO-III), MEM (HGC-27), and Ham F12 (AGS) medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin, in a humidified atmosphere of 5% CO_2 at 37°C. Total protein was prepared from all cells by cell disruption buffer according to PARIS manual (Arctiris Bioscience, Basel, Switzerland). All cells were collected by centrifugation, rinsed with phosphate-buffered saline, fixed by 10% formalin, and finally embedded in paraffin as routinely processed.

2.5. Western blot

One hundred or 70 μg denatured protein was separated on an SDS-polycrylamide gel and transferred to Hybond membrane (Amersham, Amersham, Germany), which was then blocked overnight in 5% skim milk in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20). For immunoblotting, the membrane was incubated for 15 minutes with the antibody against Reg IV (RD Systems Inc, Minneapolis, MN, USA; 1:500) or MUC-5AC (Santa Cruz CA, USA; 1:200). Then, it was rinsed by TBST (10 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.1% Tween 20) and incubated with antigoat IgG conjugated to horseradish peroxidase (DAKO, Carpinteria, CA, 1:1000) or antimouse IgG conjugated to horseradish peroxidase (DAKO, 1:1000) for 15 minutes. All the incubations were performed in a microwave oven to allow intermittent irradiation as recommended by Li et al [21]. Bands were visualized with X-ray film (Fujifilm, Japan) or LAS4000 (Fujifilm, Japan) by ECL-Plus detection reagents (Santa Cruz). After that, membrane was washed with WB Stripping Solution (pH 2-3, Nacalai) for 1 hour and treated as described above except mouse anti–β-actin antibody (Sigma, St. Louis, MO; USA; 1:5000) as an internal control.

2.6. RT-PCR

Total RNA was extracted from 3 gastric carcinoma cell lines using QIAGEN RNase mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Two micrograms of total RNA was subjected to cDNA synthesis using the AMV transcriptase and random primer (Takara, Japan). According to the Genebank (GI:36054181), oligonucleotide primers for PCR were designed as follows: forward: 5′-AAAGAAGCGC-TAGTAAGGTC-3′ and reverse: 5′-TACTTGCACAG-GAAGTGTTG-3′ (204-733, 530 bp) for *Reg IV* A1; forward: 5′-GCAAGTCCATGGGTGGGAA-3′ and reverse: 5′-GTACTTGCACAGGAAGT GTTGG-3′(625-734, 110 bp) for *Reg IV* A2; forward: 5′-TAACTTGGAGCAGCAAC-GAATG -3′ and reverse: 5′-GGCTAGCAGAAAGGAA-GAGGA-3′(682-803, 122 bp); forward: 5′-CCTTTCCACAGTATCCTTCTTCCCT-3′ and reverse: 5′-

TATGGCCAAA GAC CCAGCT GTT-3′(953-1056, 104 bp) for *Reg IV* A4; and forward: 5′-TCTACACCCTTCTGCCCT CTCT-3′ and reverse: 5′-GGAATGTATGGCCCACAT-CAACCT-3′ (1120-1250, 131 bp) for *Reg IV* A5. The primers for the internal control β-actin were forward: 5′-CGGGACCTGA CTGACTAC-3′ and reverse: 5′-GAAG-GAAGGCTGGAAGAG-3′ (252 bp). PCR amplification of cDNA was performed in 25-μL mixtures containing 0.125-μL Pfu (Statagene West Cedar Creek, TX, USA), 2.0-mmol/L $MgCl_2$, 2.5 μL ×10 PCR buffer, 2 μL 2.5 mmol/L dNTP mixture, 1 μmol/L of each primer set, and 100ng of template cDNA. PCR conditions were denatured at 95°C for 10 minutes, followed by 25 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 45 seconds. As a termination step, the extension time of the last cycle was increased to 7 minutes. After that, the secondary amplification was carried out as the first PCR using 2% (vol/vol) of the first PCR product as template DNA. Finally, the amplicons were electrophoresized in 2% agarose gel for 30 minutes.

2.7. DNA direct sequencing

Amplicons were subjected to electrophoresis in 2% agarose gel and purified with QIAquick gel extraction kit (QIAGEN). After extraction, the DNA was quantified by Nanodrop ND-1000 Spetrophotometer (Laboratory and Medical Supplies, Tokyo, Japan) and then sequenced using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems Foster City, CA, USA) as the recommendation's protocol. The sequence data were compared with the human *Reg IV* cDNA sequence (GI: 36054181) using BLAST.

2.8. Immunohistochemistry

Consecutive sections were deparaffinized with xylene, dehydrated with alcohol, and subjected to antigen retrieval by irradiating in target retrieval solution (DAKO) for 15 minutes with microwave oven (Oriental rotor Ltd. Co, Tokyo, Japan). Five percent bovine serum albumin was then applied for 5 minutes to prevent nonspecific binding. The sections were incubated with the goat anti-human Reg IV antibody (1:50), mouse antihuman MUC-2 antibody (Novocastra, New Castle Upon Tyne, UK; 1:100), mouse antihuman MUC-5AC antibody (Novocastra, UK; 1:100) or mouse antihuman MUC-6 antibody (Novocastra; 1:100) for 15 minutes, then treated with the antigoat or antimouse conjugated to horseradish peroxidase (DAKO; 1:100) antibodies for 15 minutes. All the incubations were performed in a microwave oven to allow intermittent irradiation as described previously [22]. After each treatment, the slides were washed with TBST 3 times for 1 minutes. Binding sites were visualized with 3,3′-diaminobenzidine. After counterstaining with Mayer's hematoxylin, the sections were dehydrated, cleared, and mounted. Omission of the primary antibody was used as a negative control.

As indicated in Fig. 3A, all the markers were positively localized in the cytoplasm. One hundred cells were randomly selected and counted from 5 representative fields of each section blindly by 2 independent observers (Takano Y and Zheng HC). The positive percentage of counted cells was graded semiquantitatively according to a 4-tier scoring system: negative (−), 0% to 5%; weakly positive (+), 6% to 25%; moderately positive (++), 26% to 50%; and strongly positive (+++).

2.9. In situ hybridization

To perform RNA-DNA in situ hybridization for *Reg IV*, a digoxygenin-labeled *Reg IV* probe was made in 35-cycle PCR using A3 primer sets, template DNA of 25-cycle A3 products from the MKN45 cDNA and Pfu polymerase (Statagene). Four-micrometer-thick sections were deparaffinized and digested with 20 μg/mL proteinase K in 50 mmol/L Tris-HCl at 37°C for 10 minutes. Then 100 μL of a 1:20 probe dilution in hybridization buffer (22 mmol/L Tris-HCl, pH 7.4, 2.75 mmol/L EDTA, 660 mmol/L NaCl, 1× Denhardt solution, 5.5% dextran sulfate, 0.33% dimethyl sulfoxide, 0.55% ethoquad 18/25, and 44% deionized formamide) was added to each slide. After cover-slipping, heating at 95°C for 5 minutes, and incubation overnight in a humidified chamber at 37°C, sections were rinsed for 10 minutes in TBST and incubated with anti-digoxygenin antibody conjugated with alkaline phosphatase (Roche Diagnostics, Mannheim, Germany) for 20 minutes at 37°C. The slides were then washed for 5 minutes and immersed in solution II (100 mmol/L Tris-HCl [pH 9.5], 100 mmol/L NaCl, and 50 mmol/L $MgCl_2$) for 15 minutes and followed by exposure to NBT (nitro-blue tetrazolium chloride)/BCIP (5-Bromo-4-Chloro-3′-Indolylphosphatase p-Toluidine salt) as a chromogen. Finally, counterstaining was performed using methyl green for 2 minutes. Omission of the probe or RNase digestion was used as a negative control.

2.10. Double fluorescence IHC

To clarify the distribution and correlation of Reg IV and mucin expression in gastric carcinoma, double fluorescent immunostaining was performed. Consecutive sections were deparaffinized with xylene, dehydrated with alcohol, and subjected to antigen retrieval by irradiating in target retrieval solution (DAKO) for 15 minutes with microwave oven (Oriental rotor Ltd, Co, Tokyo, Japan). Five percent bovine serum albumin was then applied for 1 minutes to prevent nonspecific binding. The sections were incubated with both antibodies against Reg IV and MUC-2 (or MUC-5AC) at 4°C overnight, then treated with Alexa Fluor 488 (green) donkey antimouse and Alexa Fluor 568 (red) antigoat IgG (Invitrogen; Carlsbad, CA, USA, 1:500) for 1 hour. After each treatment, the slides were washed with TBST 3 times for 1 minutes. The sections were mounted with VECTASHIELD

Mounting Medium with DAPI (Vector Laboratories, Tokyo, Japan). Finally, the microphotography was performed under the Bio-zero fluorescence microscopy (BZ-8000, KEYENCE, Osaka, Japan).

2.11. Enzyme-linked immunosorbent assay

To measure the serum Reg IV concentration, a sandwich ELISA was carried out using Reg-IV ELISA kit (Cusabio, Wuhan, China). Firstly, anti–Reg IV antibody–coated polystyrene microtiter plates were incubated with 100 μL standard or serum sample at 4°C by overnight. After the liquid was removed, we added 100 μL biotin-antibody working solution was added to each well, followed by incubation at 37°C for 2 hours. The liquid was aspirated from each well. After 3 washes with 350 μL wash buffer, 100 μL HRP (horseradish peroxidase)-avidin working solution was added to each well and the plates were subjected to incubation for 1 hour at 37°C. After washing for 3 times, the plates were incubated with 90 μL TMB (3,3′,5,5′-tetramethylbenzidine) substrate for 30 minutes at 37°C. And then, 50 μL stopping solution was dispensed to each well. Absorbance was measured at 405 nm with a reference standard of human recombinant Reg IV (0.312-20 ng/mL). All the standard and serum samples were tested in triplicate.

2.12. Statistical analysis

Statistical evaluation was performed using Fisher exact possibility to differentiate the positive rate, Spearman correlation test to analyze the rank data, and Mann-Whitney U to differentiate the means of different groups. Kaplan-Meier survival plots were generated, and comparisons between survival curves were made with the log-rank statistics. $P < .05$ was considered as statistically significant. SPSS 10.0 software (SPSS Inc, Chicago, IL) was used to analyze all data.

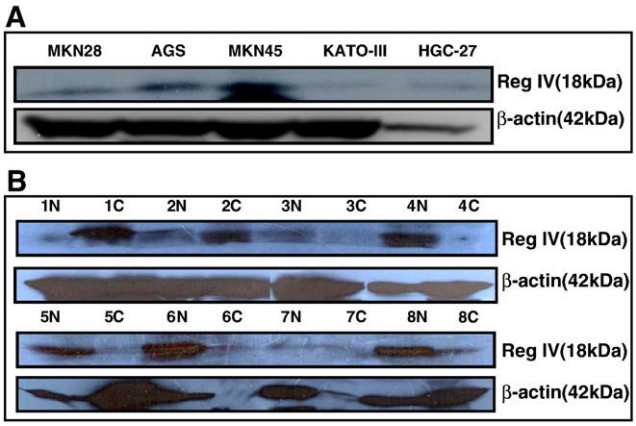


Fig. 1. Western blot analysis of Reg IV protein in gastric carcinoma cell lines and tissues. A, Cell lysate (100 μg) was loaded and probed with anti-Reg IV (panel 1, 18 kDa) antibody with β-actin (panel 2, 42 kDa) as an internal control. Lane 1, MKN28; 2, AGS; 3, MKN45; 4, KATO-III; 5, HGC-27. B, Tissue lysate (70 μg) was loaded and probed with anti–Reg IV (Panel 1, 18 kDa) with β-actin (Panel 2, 42 kDa) as an internal control. Abbreviations: N, paring nonneoplastic mucosa; C, cancer.

3. Results

3.1. Reg IV expression in gastric carcinoma cell lines or samples and its serum level in carcinoma patients

As shown in Fig. 1A, Reg IV protein strongly existed in MKN45, AGS, and HGC-27 but weakly in MKN28 and KATO-III. In contrast, no positive immunoreactivity to Reg IV was observed in 5 carcinoma cell lines by IHC (data not shown). To check its mRNA expression, we performed RT-

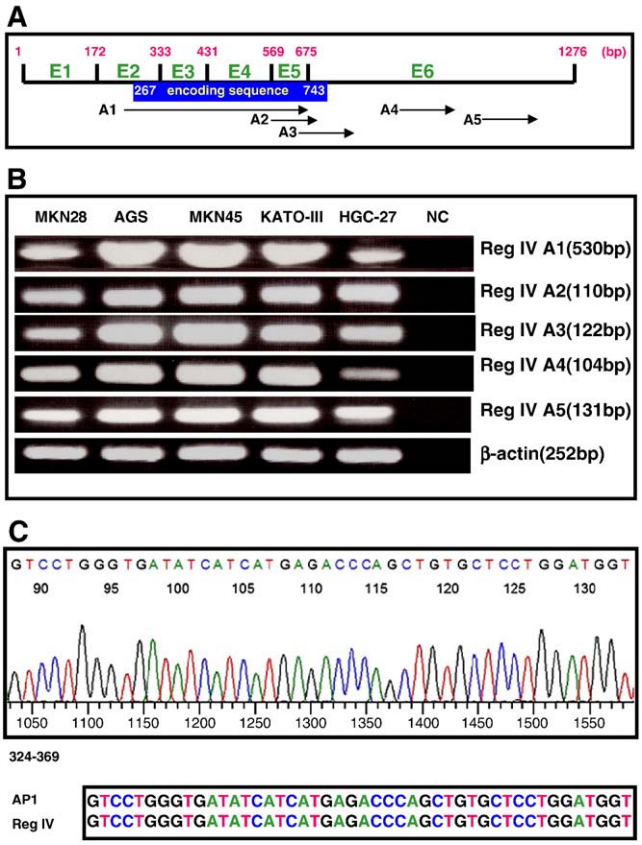
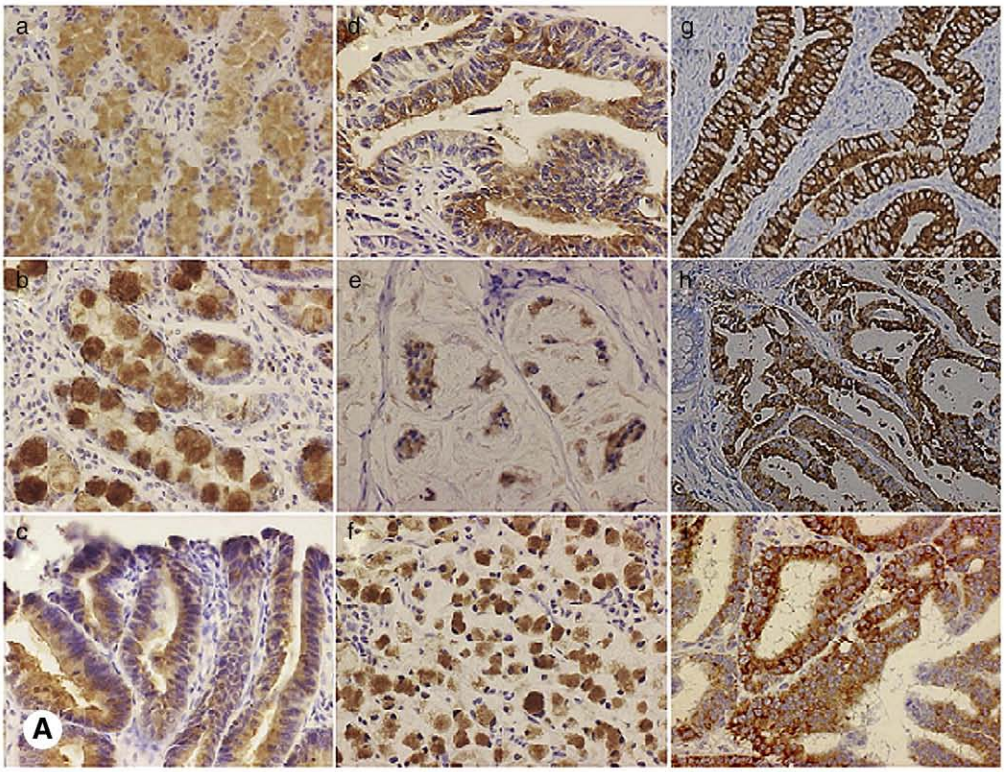
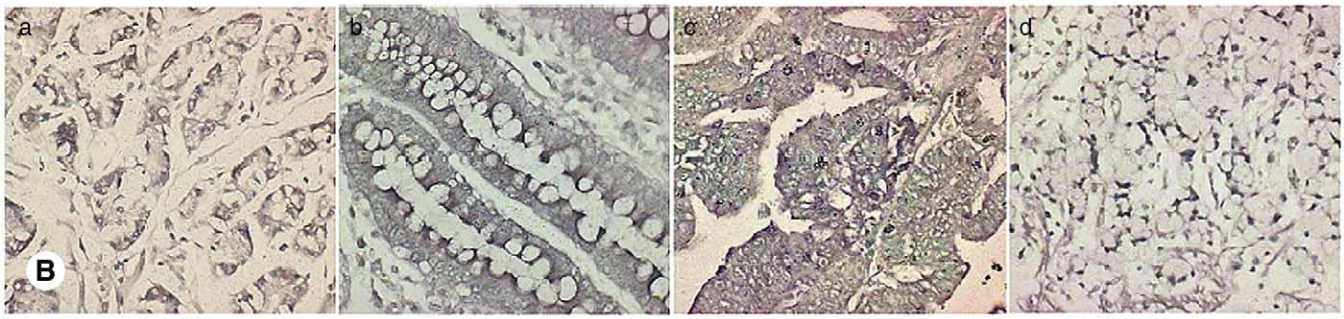


Fig. 2. RT-PCR analysis of *Reg IV* gene followed by direct sequencing in gastric carcinoma cell lines. A, The schematic representation for Reg IV cDNA (GI: 36054181) and our primers' design. Reg IV contains 6 exons, and its encoding sequence ranges 267 to 743 of the gene. The serial amplicons produced by our 5 pairs of primers distributed to A1-5. B, Different amplicons of Reg IV mRNA were detected and showed consistent density in all gastric carcinoma cell lines with an internal control of β-actin. A little weak expression of Reg IV was observed in MKN28 and HGC-27 cell lines. Lane 1, MKN28; 2, AGS; 3, MKN45; 4, KATO-III; 5, HGC-27. C, The sequence of Reg IV AP1 is located in 324-369 of Reg IV cDNA (GI: 36054181).


a
d
g
b
e
h
c
f
A


a
b
c
d
B

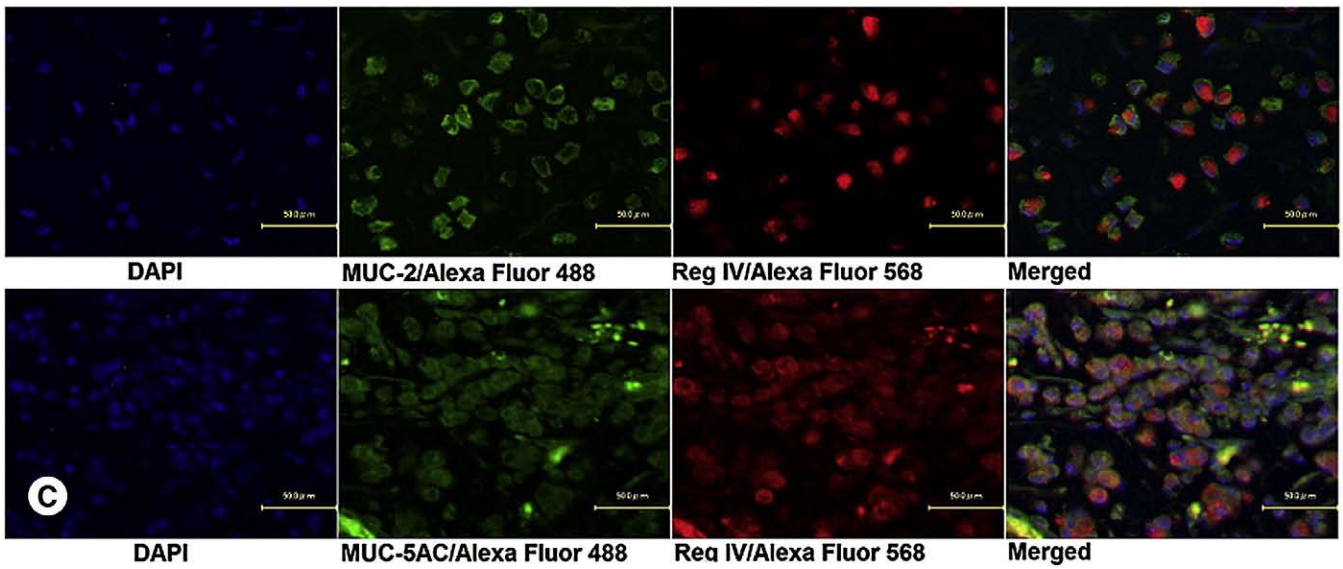

DAPI
MUC-2/Alexa Fluor 488
Reg IV/Alexa Fluor 568
Merged
C
DAPI
MUC-5AC/Alexa Fluor 488
Reg IV/Alexa Fluor 568
Merged

Table 1 Reg IV expression in gastric carcinogenesis

Groups	n	Reg IV expression				
		−	+	++	+++	PR (%)
Gastritis	93	66	14	7	6	29.0
Intestinal metaplasia	63	0	0	0	63	100.0 [a]
Adenoma	42	6	10	19	7	85.7 [b]
Carcinoma	372	204	123	34	11	45.2 [c]

Abbreviation: PR, positive rate.
[a] Compared with gastritis, adenoma and carcinoma, $P < .001$.
[b] Compared with gastritis and carcinoma, $P < .001$.
[c] Compared with gastritis, $P = .028$.

PCR and observed a little weak expression of *Reg IV* mRNA in MKN28 and HGC-27 cell lines in comparison with the others (Fig. 2B). In addition, all the amplicons were subjected to direct DNA sequencing and proved correct (Fig. 2C).

Among 8 cases of frozen gastric samples, 18 kDa Reg IV was detected in all samples of gastric carcinoma or the adjacent NNM. Its expression was greater in 3 cases of carcinoma than adjacent NNM and lower in 3 cases, whereas no difference was observed in 2 cases (Fig. 1B). Generally, the adjacent IM to carcinoma showed Reg IV overexpression, whereas the expression level became lost or weaker in carcinoma (data not shown). As indicated in Fig. 3A, Reg IV was strongly expressed in gastric deep gland, IM and adenomas but only in some of the gastric carcinomas. Overall, Reg IV expression was detected respectively in 29.0% of gastritis (93 cases), all IM (63 cases), 85.7% of adenoma (42 cases), and 45.2% of carcinoma (372 cases). Statistically, gradually decreased Reg IV expression was seen from IM, adenoma, carcinoma to gastritis ($P < .05$, Table 1).

ISH was performed on the TMA of gastric carcinoma and adjacent NNM to confirm *Reg IV* mRNA expression. *Reg IV* mRNA was detectable in gastric deep IM or carcinoma (Fig. 3B). The Reg IV-positive carcinoma cases were well differentiated, or signet ring cell (SRC) carcinoma where *Reg IV* mRNA signal was mainly localized in the perinucleus of SRC carcinoma because of too more mucin production. There was only one adjacent adenoma involved in our study and showing strong *Reg IV* mRNA expression (data not shown). Statistically, its positive rate was higher in IM (40/42, 95.2%) than adjacent NNM (17/44, 38.6%) or carcinoma (14/69, 20.3%; $P < .05$).

To check the diagnostic significance, ELISA was used, and it was found that the mean level of serum Reg IV in healthy individuals was 0.6341 ± 0.0841 ng/mL (n = 16), significantly lower than the gastric carcinoma patients (1.6993 ± 2.5000, n = 24; $P < .05$).

Table 2 Relationship between Reg IV expression and clinico-pathologic features of gastric carcinomas

Clinicopathologic features	n	Reg IV expression					
		−	+	++	+++	PR (%)	*P* value
Age (y)							.307
<65	153	86	55	11	1	43.8	
≥65	219	118	68	23	10	46.1	
Sex							.093
Male	258	146	86	21	5	43.4	
Female	114	58	37	13	6	49.1	
Tumor size (cm)							.109
<4	191	96	71	19	5	49.7	
≥ 4	181	108	52	15	6	40.3	
Depth of invasion							.118
T_{is}, T_1	182	91	68	19	4	50.0	
$T_{2\text{-}4}$	190	113	55	15	7	40.5	
Lymphatic invasion							.516
−	239	128	81	26	4	46.4	
+	133	76	42	8	7	42.9	
Venous invasion							.197
−	317	170	106	31	10	46.4	
+	55	34	17	3	1	38.8	
Lymph node metastasis							.832
−	228	124	74	25	5	45.6	
+	144	80	49	9	6	44.4	
TNM staging							.152
0-I	196	100	70	22	4	49.0	
II-IV	176	104	53	12	7	35.2	
Lauren's classification							.948
Intestinal type	212	116	71	21	4	45.3	
Diffuse type	160	88	52	13	7	45.0	
MUC-2 expression							<.001
−	243	154	69	15	5	36.6	
+ to +++	129	50	54	19	6	61.2	
MUC-5AC expression							.001
−	181	118	43	13	7	34.8	
+ to +++	191	86	80	21	4	55.0	
MUC-6 expression							
−	225	128	67	20	10	43.1	.587
+ to +++	147	76	56	14	1	48.3	

Abbreviations: T_{is}, carcinoma in situ; T_1, lamina propria and submucosa; T_2, muscularis propria and subserosa; T_3, exposure to serosa; T_4, invasion into serosa.

Fig. 3. Morphological examination of gastric tissue samples. A, Immunohistochemical staining of gastric tissue samples. Note: Reg IV, MUC-2, MUC-5AC, and MUC-6 were positively observed in the cytoplasm. Strong Reg IV expression was detected in gastric deep propria gland (a), intestinal metaplasia (b), adenoma (c), well-differentiated (d), mucinous (e), and SRC carcinoma (f). MUC-2 (g), MUC-5AC (h), and MUC-6 (i) were also expressed in the cytoplasm of gastric adenocarcinomas. B, *Reg IV* mRNA expression in gastric carcinoma and adjacent mucosa by ISH. The signal was observed in the gastric deep gland (a), intestinal metaplasia (b), well-differentiated (c), and SRC carcinoma (d). C, Double fluorescence immunostaining of gastric carcinoma. Note: Reg IV protein was stained with red color and MUC-2/ MUC-5AC with green color.

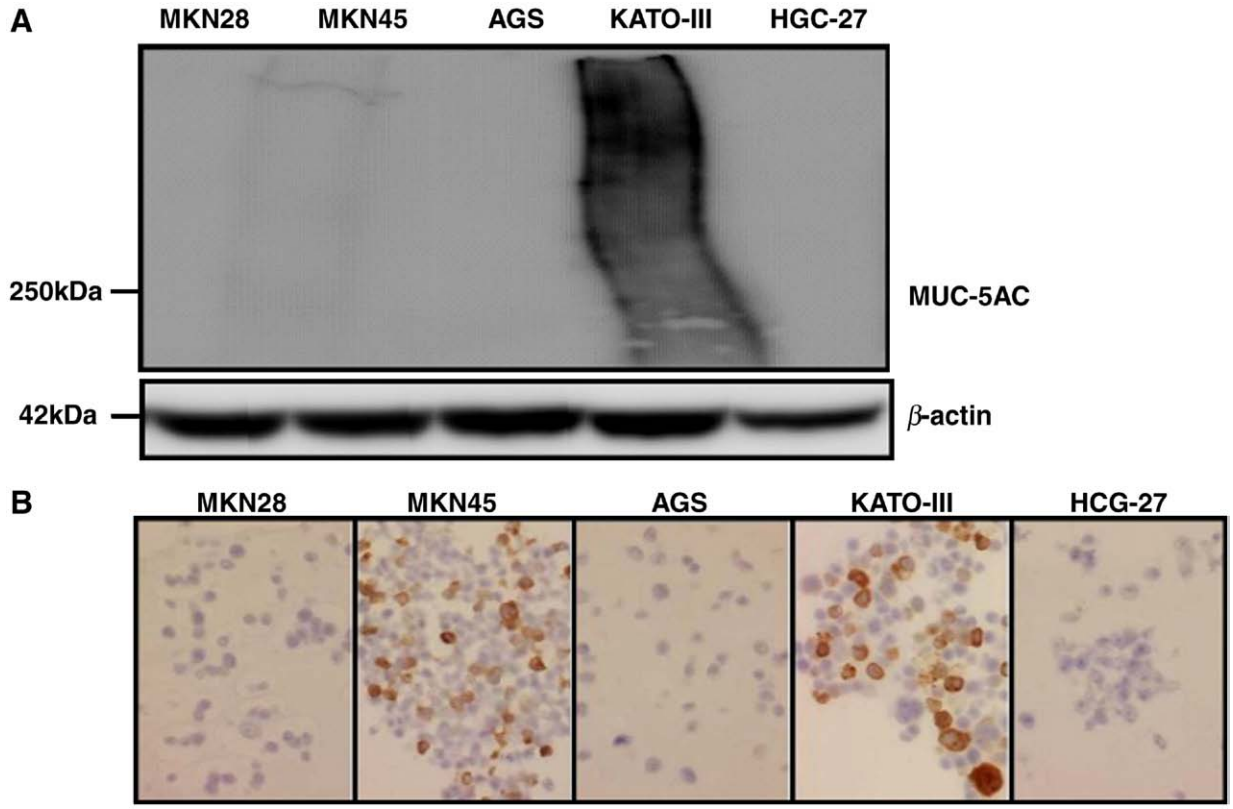


Fig. 4. MUC-5AC expression in gastric carcinoma cell lines. A, Cell lysate (100 μg) was loaded and probed with anti-MUC-5AC (Panel 1, 170-350 kDa) antibody with β-actin (Panel 2, 42 kDa) as an internal control. Lane 1, MKN28; 2, MKN45; 3, AGS; 4, KATO-III; 5, HGC-27. B, Immunohistochemical staining of MUC-5AC in carcinoma cell lines. Note: MUC-5AC was localized in the cytoplasm of carcinoma cells.

3.2. The relationship between Reg IV protein expression and clinicopathologic or prognostic parameters of gastric carcinoma

As summarized in Table 2, Reg IV expression was positively correlated with the expression of MUC-2 and MUC-5AC ($P < .05$), but not with patient age or sex, tumor size, depth of invasion, lymphatic or venous invasion, lymph node metastasis, TNM staging or MUC-6 expression ($P > .05$). The double fluorescence staining indicated the Reg IV and MUC-2 (or MUC-5AC) were colocalized in gastric carcinoma cells (Fig. 3C). However, there was no expression

Table 3 Reg IV expression in the different subtypes of gastric adenocarcinoma according to WHO classification

Histologic subtypes of WHO	n	Reg IV expression				
		−	+	++	+++	PR(%)
Papillary	2	1	1	0	0	50.0
Well-differentiated	162	87	54	18	3	46.3
Moderately differentiated	46	28	15	3	0	39.1
Poorly differentiated	117	85	30	1	1	27.4 [a]
Mucinous	2	0	1	0	1	100.0
Signet ring cell	43	3	22	12	6	95.3 [b]
Total	372	204	123	34	11	45.2

[a] Compared with well-differentiated, moderately differentiated, or SRC carcinoma, $P < .001$.
[b] Compared with well-, moderately, or poorly differentiated carcinoma, $P < .001$.

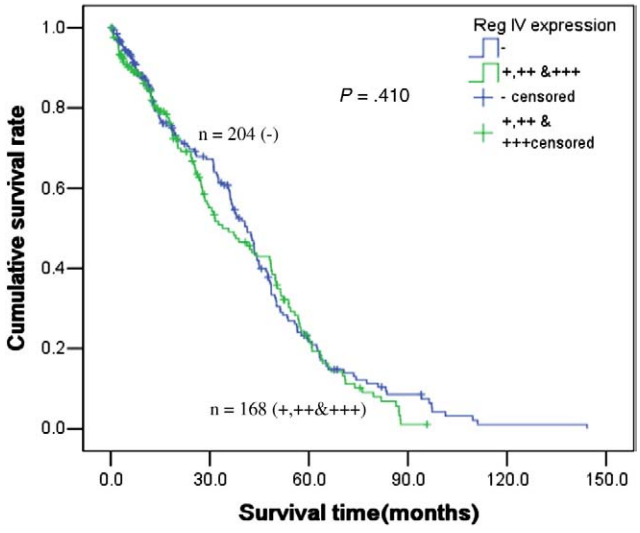


Fig. 5. Correlation between the status of Reg IV expression and prognosis of the gastric carcinoma patients. Kaplan-Meier curves for cumulative survival rate of patients with gastric carcinomas according to the Reg IV expression in gastric carcinomas.

of MUC-2 or MUC-6 protein in gastric carcinoma cell lines (data not shown) even if Reg IV expression was detectable in these carcinoma cell lines. MUC-5AC protein was detectable in the MKN45 and KATO-III cells by IHC and Western blot (Fig. 4). There was no difference in Reg IV expression between intestinal and diffuse-type carcinomas ($P > .05$). Among histologic subtypes of WHO, SRC carcinoma more frequently expressed Reg IV than well, moderately, or poorly differentiated carcinoma ($P < .05$). The poorly differentiated subtype displayed the lower Reg IV expression than the well or moderately differentiated carcinoma ($P < .05$, Table 3).

Follow-up information was available on 372 gastric carcinoma patients for periods ranging from 0.2 months to 12.1 years (median, 66.8 months). Univariate analysis using the Kaplan-Meier method indicated there was no correlation of the immunohistochemical expression of Reg IV protein with cumulative survival rate of patients ($P > .05$, Fig. 5) even stratified according to the depth of invasion (dada not shown).

4. Discussion

In the present study, we found that Reg IV expression underwent up-regulation and then down-regulation from gastritis to carcinoma through precancerous lesions like IM and adenoma in line with other reports [9,15]. Our ISH results also showed strong *Reg IV* mRNA expression in adjacent IM in comparison with adjacent NNM or carcinoma. In contrast, there was no significant difference in Reg IV protein and mRNA expression between gastric carcinoma and adjacent NNM according to the results of ISH and Western blot. IM is believed to be an adaptive condition for gastric epithelium with injury and inflammation and could develop into globoid dysplasia, which is closely linked to SRC carcinomas, evidenced by morphological appearance and biologic characters [2]. Here, it was found that SRC carcinoma expressed Reg IV more frequently than other histologic subtypes, indicating that Reg IV might be involved in the molecular mechanisms of gastric carcinogenesis from IM and globoid dysplasia to SRC carcinomas.

Sonic hedgehog (Shh) is a morphogen involved in many aspects of patterning of the gut during embryogenesis and in gastric fundic gland homeostasis [23-25]. Intestinal metaplasia is associated with the loss of Shh expression, and mice that lack Shh expression show intestinal transformation of the gastric mucosa [23,24]. Shh expression is significantly reduced in *Helicobacter pylori*–associated gastritis of Mongolian gerbils, resulting in impaired differentiation of the fundic gland cells, increased expression of trefoil factor family 2, and the formation of spasmolytic polypeptide-expressing metaplasia [25]. Reduced Shh expression was also examined in TFF2-overexpressing lesions of the gastric fundus under hypochlorhydric conditions. Interestingly, our data demonstrated up-regulated Reg IV expression in gastric IM. The opposite expression of both proteins in gastric IM needs further investigation in the future.

Furthermore, Reg IV overexpression was observed in gastric adenoma in line with a previous report in colon [9]. As for ISH, only 1 case of adenoma was involved in our investigation and showed strong *Reg IV* mRNA expression. It was suggested that Reg IV could be regarded as a good biomarker for gastric adenoma. In the APC^{min} transgenic mice model for sequential adenoma-adenocarcinoma process of gut carcinogenesis, up-regulated Reg IV expression was observed to precede the second mutation, but adenomas developed only after the second mutation occurs at the third month [26]. Taken together, it might be concluded that Reg IV played an important role in adenoma formation, development and expansion, which was possibly attributable to its antiapoptotic function because Reg IV overexpression could inhibit the mitochondrial apoptotic pathway that involves cytosolic cytochrome c release and subsequent activation of caspase-9 and caspase-3 [16].

In addition, no correlation of Reg IV expression was evident with the any aggressive behaviors of gastric carcinoma sin the present study. However, Miyagawa et al [27,28] reported that Reg IV mRNA expression level in surgically resected specimens were closely related with wall penetration of gastric carcinoma, and *Reg IV* mRNA expression level in the peritoneal wash was strongly higher in peritoneal metastasis compared to those without peritoneal metastasis. In vitro evidences demonstrated that forced Reg IV expression could accelerate peritoneal metastasis of gastric cancer in nude mice [29]. These data indicate that Reg IV may be involved in peritoneal dissemination of gastric cancers and Reg IV would be a potential novel marker for peritoneal dissemination of gastric cancers. Furthermore, Reg IV expression was associated with both lymph node metastasis and tumor stage in colorectal carcinoma [15]. The discrepancy might be due to differences in immunohistochemical, sampling, statistica, and specimen's fixing methods. For example, we used microwave intermittent irradiation for antibody incubation, which is known to increased sensitivity and specificity of IHC [22]. For statistics, Spearman correlation analysis was used for the present rank data, making full use of our semiquantitative results. Regarding fixation, our specimens were fixed in freshly prepared with 10% formalin within 4 days, which facilitates antigen retrieval. In our study, 372 cases of Japanese gastric carcinoma were randomly selected to ensure the reliability of the data.

Oue et al [15] reported that Reg IV expression was associated with the intestinal mucin phenotype and neuroendocrine differentiation in gastric carcinomas. In this investigation, Reg IV protein was frequently immunoreactive in both mucinous and SRC carcinomas with abundant intracellular mucin accumulation, compatible with other data [14,15]. The positive correlation of Reg IV with MUC-2 and MUC-5AC expression was seen by both statistical analysis and double immunostaining in line with similar distribution pattern of MUC-5AC, MUC-2, and Reg IV in neoplastic

goblet cells of appendiceal mucinous cystadenoma and pseudomyxoma peritonei [30]. Mucin-like Reg IV protein was reportedly immunostained in goblet cells, SRC carcinomas and mucinous carcinomas. Reg IV overexpersssion in SRC carcinoma can partially demonstrate its significant association with these mucins and indicates that Reg IV protein was a good biomarker for gastric mucin-producing carcinomas. It is well known that both MUC-2 and MUC-5AC belong to secreted types of mucin, but MUC-6, to membrane-associated types [31,32]. A study noted that Reg IV expression was strongly detected in the cytosol of SRC carcinoma cell line, HSC-39, but comparatively little secreted into the conditioned medium [15]. In combination with these findings, it is speculated that codistribution of secreted Reg IV with MUC-2 and MUC-5AC might be attributable to the dysfunction of protein and synthesis and secretion. On the other hand, Reg IV expression was higher in well and moderately differentiated adenocarcinoma than the poorly differentiated one, suggesting that Reg IV expression might have impact on the differentiation of gastric carcinoma. More Reg IV expression in well-differentiated and SRC carcinoma can account for no difference in Reg IV expression in intestinal and diffuse-type carcinoma because SRC belongs to the former and well-differentiated group to the latter [18,19].

Serum Reg IV was reported to be a novel biomarker for epithelial malignancies. It was significantly higher in PCa patients than that in control individuals [33]. Preoperative CRC patients did not showed elevated serum Reg IV level in at stage 0 to III, being in contrast to the significantly increased in stage IV CRC patients with liver metastasis [7]. Another report implied that the serum Reg IV concentration in presurgical gastric cancer patients was significantly elevated in stage I to IV, although there was no difference between healthy individuals and patients with chronic-active gastritis [16]. Our data also showed that the elevated Reg IV concentration in the patients with gastric cancer, in line with other reports [7,6,33]. As for the Reg IV source, it might be produced by the carcinoma or inducible adjacent mucosa. Combined with these findings, it was concluded that increased serum Reg IV might be employed as a good marker to indicate the carcinogenesis or following progression.

Although Reg IV was documented significantly to correlate with aggressive biologic behaviors of malignancies [7,15], the results about he prognostic relevance of Reg IV expression were controversial. Mitani et al [16] reported that there was no relationship between Reg IV expression and survival probability of 101 gastric cancer patients. However, Ohara et al [13] revealed that Reg IV was significantly associated with longer relapse-free survival of clinically localized PCa patients as an independent prognostic factor. In this study, we analyzed the relation of Reg IV expression to postsurgical survival of 372 patients with gastric carcinoma and found no link between Reg IV's positivity and the accumulative survival time of cancer patients even stratified according to the depth of invasion, which remarkably determines the prognosis [19,31]. It might be due to no association of Reg IV expression with aggressive parameters of gastric carcinoma, including invasion, metastasis, or TNM staging.

In summary, Reg IV expression experiences up-regulation in gastric precancerous lesions and then slight down-regulation with malignant transformation of gastric epithelial cells. Reg IV should be considered as a good biomarker for IM and SRC carcinoma in clinicopathologic practice. It is essential to explore the regulatory mechanisms of Reg IV protein expression in the further investigation.

Acknowledgments

The authors thank Tokimasa Kumada and Hideki Hatta for their technical help and Xing-hua Luan for her manuscript's revision.

References

[1] Kelley JR, Duggan JM. Gastric cancer epidemiology and risk factors. J Clin Epidemiol 2003;56:1-9.
[2] Zheng HC, Zheng YS, Li XH, et al. Arp2/3 overexpression contributed to pathogenesis, growth and invasion of gastric carcinoma. Anticancer Res 2008;28:2225-32.
[3] Zhang YW, Ding LS, Lai MD. *Reg* gene family and human diseases. World J Gastroenterol 2003;9:2635-41.
[4] Hartupee JC, Zhang H, Bonaldo MF, Soares MB, Dieckgraefe BK. Isolation and characterization of a cDNA encoding a novel member of the human regenerating protein family: Reg IV. Biochim Biophys Acta 2001;1518:287-93.
[5] Bishnupuri KS, Luo Q, Murmu N, Houchen CW, Anant S, Dieckgraefe BK. Reg IV activates the epidermal growth factor receptor/Akt/AP-1 signaling pathway in colon adenocarcinomas. Gastroenterology 2006;130:137-49.
[6] Nanakin A, Fukui H, Fujii S, et al. Expression of the *Reg IV* gene in ulcerative colitis. Lab Invest 2007;87:304-14.
[7] Oue N, Kuniyasu H, Noguchi T, et al. Serum concentration of Reg IV in patients with colorectal cancer: overexpression and high serum levels of Reg IV are associated with liver metastasis. Oncology 2007;72:371-80.
[8] Violette S, Festor E, Pandrea-Vasile I, et al. Reg IV, a new member of the regenerating gene family, is overexpressed in colorectal carcinomas. Int J Cancer 2003;103:185-93.
[9] Zhang Y, Lai M, Lv B, et al. Overexpression of Reg IV in colorectal adenoma. Cancer Lett 2003;200:69-76.
[10] Lasserre C, Colnot C, Bréchot C, Poirier F. *HIP/PAP* gene, encoding a C-type lectin overexpressed in primary liver cancer, is expressed in nervous system as well as in intestine and pancreas of the postimplantation mouse embryo. Am J Pathol 1999;154:1601-10.
[11] Lasserre C, Christa L, Simon MT, Vernier P, Bréchot C. A novel gene (*HIP*) activated in human primary liver cancer. Cancer Res 1992;52:5089-95.
[12] Kimura N, Yonekura H, Okamoto H, Nagura H. Expression of human regenerating gene mRNA and its product in normal and neoplastic human pancreas. Cancer 1992;70:1857-63.
[13] Ohara S, Oue N, Matsubara A, et al. Reg IV is an independent prognostic factor for relapse in patients with clinically localized prostate cancer. Cancer Sci 2008;99:1570-7.

[14] Sentani K, Oue N, Tashiro T, et al. Immunohistochemical staining of Reg IV and claudin-18 is useful in the diagnosis of gastrointestinal signet ring cell carcinoma. Am J Surg Pathol 2008;32:1182-9.

[15] Oue N, Mitani Y, Aung PP, et al. Expression and localization of Reg IV in human neoplastic and non-neoplastic tissues: Reg IV expression is associated with intestinal and neuroendocrine differentiation in gastric adenocarcinoma. J Pathol 2005;207:185-98.

[16] Mitani Y, Oue N, Matsumura S, et al. Reg IV is a serum biomarker for gastric cancer patients and predicts response to 5-fluorouracil–based chemotherapy. Oncogene 2007;26:4383-93.

[17] Sobin LH, Wittekind CH. TNM classification of malignant tumours. 6th ed. Hoboken (NJ): John Wiley & Sons; 2002.

[18] Zheng HC, Li XH, Hara T, et al. Mixed-type gastric carcinomas exhibit more aggressive features and indicate the histogenesis of carcinomas. Virchows Arch 2008;452:525-34.

[19] Zheng H, Takahashi H, Murai Y, et al. Pathobiological characteristics of intestinal and diffuse-type gastric carcinoma in Japan: an immunostaining study on the tissue microarray. J Clin Pathol 2007;60:273-7.

[20] Hamilton SR, Aaltonen LA. WHO classification of tumors: pathology and genetics of tumors of the digestive system. lyon, France: IARC press; 2000.

[21] Li W, Murai Y, Okada E, et al. Modified and simplified western blotting protocol: use of intermittent microwave irradiation (IMWI) and 5% skim milk to improve binding specificity. Pathol Int 2002;52: 234-8.

[22] Kumada T, Tsuneyama K, Hatta H, Ishizawa S, Takano Y. Improved 1-h rapid immunostaining method using intermittent microwave irradiation: practicability based on 5 years application in Toyama Medical and Pharmaceutical University Hospital. Mod Pathol 2004;17:1141-9.

[23] van den Brink GR, Hardwick JC, Nielsen C, et al. Sonic hedgehog expression correlates with fundic gland differentiation in the adult gastrointestinal tract. Gut 2002;51:628-33.

[24] Suzuki H, Minegishi Y, Nomoto Y, et al. Down-regulation of a morphogen (sonic hedgehog) gradient in the gastric epithelium of *Helicobacter pylori*-infected Mongolian gerbils. J Pathol 2005;206: 186-97.

[25] Minegishi Y, Suzuki H, Arakawa M, et al. Reduced Shh expression in TFF2-overexpressing lesions of the gastric fundus under hypochlorhydric conditions. J Pathol 2007;213:161-9.

[26] Bishnupuri KS, Luo Q, Korzenik JR, et al. Dysregulation of *Reg* gene expression occurs early in gastrointestinal tumorigenesis and regulates anti-apoptotic genes. Cancer Biol Ther 2006;5:1714-20.

[27] Miyagawa K, Sakakura C, Kin S, et al. Over expression of Reg IV in peritoneal dissemination of gastric cancer. Gan To Kagaku Ryoho 2004;31:1909-11.

[28] Miyagawa K, Sakakura C, Nakashima S, et al. Analysis of Reg IV expression in peritoneal dissemination of gastric cancer using real-time RT-PCR. Gan To Kagaku Ryoho 2005;32:1707-8.

[29] Kuniyasu H, Oue N, Sasahira T, et al. Reg IV enhances peritoneal metastasis in gastric carcinomas. Cell Prolif 2009;42:110-21.

[30] Heiskala K, Giles-Komar J, Heiskala M, Andersson LC. High expression of RELP (Reg IV) in neoplastic goblet cells of appendiceal mucinous cystadenoma and pseudomyxoma peritonei. Virchows Arch 2006;448:295-300.

[31] Zheng H, Takahashi H, Nakajima T, et al. MUC6 down-regulation correlates with gastric carcinoma progression and a poor prognosis: an immunohistochemical study with tissue microarrays. J Cancer Res Clin Oncol 2006;132:817-23.

[32] Li XH, Zheng HC, Wang ZG, et al. The clinicopathological and prognostic significance of MUC-1 expression in Japanese gastric carcinomas: an immunohisto-chemical study of tissue microarrays. Anticancer Res 2008;28:1061-7.

[33] Hayashi T, Matsubara A, Ohara S, et al. Immunohisto-chemical analysis of Reg IV in urogenital organs: frequent expression of Reg IV in prostate cancer and potential utility as serum tumor marker. Oncol Rep 2009;21:95-100.