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Original Paper

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

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Abstract

Regenerating islet-derived family, member 4 (Reg IV) is a candidate marker for cancer and inflammatory bowel disease. In the present study, immunohistochemical analysis of Reg IV was performed in various human neoplastic ($n = 289$) and non-neoplastic tissues. In the stomach, foveolar epithelium was negative for Reg IV, whereas goblet cells of intestinal metaplasia and neuroendocrine cells at the base of intestinal metaplasia expressed Reg IV. Neuroendocrine cells of the small intestine and colon showed strong expression of Reg IV, whereas goblet cells of the small intestine and colon showed weak or no expression of Reg IV. Insulin-producing beta cells of the endocrine pancreas were positive for Reg IV. Among 143 gastric adenocarcinomas, Reg IV expression was detected in 42 (29.4%) and was associated with both the intestinal mucin phenotype and neuroendocrine differentiation. No association was found between Reg IV expression and clinical characteristics such as tumour stage and patient prognosis. Of 36 colorectal adenocarcinomas, 13 (36.1%) were positive for Reg IV, which was associated with tumour stage ($p = 0.0379$, Fisher's exact test). Expression of Reg IV was detected in 14 (93.3%) of 15 colorectal carcinoid tumours. Reg IV expression was also detected in 5 (21.7%) of 23 ductal adenocarcinomas of the pancreas. In contrast, lung cancers ($n = 30$) and breast cancers ($n = 30$) did not express Reg IV. This is the first immunohistochemical analysis of the expression and distribution of Reg IV protein in human tumours. These data suggest that Reg IV is expressed by gastrointestinal and pancreatic tumours, including adenocarcinomas and carcinoid tumours, and that Reg IV is associated with intestinal and neuroendocrine differentiation of the stomach and gastric carcinoma.

Keywords: Reg IV; phenotypic expression; neuroendocrine; gastric carcinoma; immunohistochemistry; RT-PCR

Introduction

Gastric carcinoma is one of the most common human cancers. Genes encoding transmembrane/secretory proteins expressed specifically in cancers may be ideal diagnostic biomarkers [1,2].

We previously performed serial analysis of gene expression (SAGE) of four primary gastric carcinomas and identified several genes and tags that may be involved in invasion, metastasis, and carcinogenesis [3]. Of these genes, regenerating islet-derived family, member 4 (*REGIV*) is a candidate gene for cancer-specific expression, at least in gastric carcinoma. Quantitative reverse transcription (RT)-polymerase chain reaction (PCR) analysis showed that approximately half of the gastric carcinomas analysed overexpressed the *REGIV* gene [3]. Although various normal tissues, including the stomach, intestine, and pancreas, express *REGIV* [4], levels of *REGIV* expression are much lower in normal tissues than in tumour tissues [3]. Overexpression of *REGIV* mRNA was detected in colorectal carcinoma and adenoma by RT-PCR and *in situ* hybridization, respectively [5,6]. It has been reported that high *REGIV* expression is associated with 5-fluorouracil (5-FU) resistance in colon cancer cell lines [5]. In non-neoplastic tissues, immunofluorescence staining revealed that Reg IV (referred to as RELP) co-localizes with chromogranin A in the

neuroendocrine cells of the duodenal epithelium [5,7]. In colonic mucosa, only weak expression of Reg IV protein is observed in selected cells, whereas Reg IV is expressed at higher levels in the cryptal epithelium of the mucosa from patients with ulcerative colitis [7]. Expression of Reg IV protein is detected in the goblet cells of intestinal metaplasia of the stomach [7].

However, the expression and distribution of Reg IV in human tumours remain unclear. Thus, we examined the expression and distribution of Reg IV immunohistochemically in various neoplastic and non-neoplastic human tissues. In addition, because Reg IV is expressed in intestinal metaplasia of the stomach, we investigated the association between Reg IV expression and mucin phenotype in gastric carcinoma. We also examined the co-localization of Reg IV and chromogranin A.

Materials and methods

Tissue samples

Nine gastric carcinomas, nine colorectal carcinomas, nine lung cancers, and nine breast cancers were used for quantitative RT-PCR. The samples were obtained at the time of surgery at Hiroshima University Hospital and affiliated hospitals. We confirmed microscopically that the tumour specimens consisted mainly (>50%) of carcinoma tissue. Samples were frozen immediately in liquid nitrogen and stored at −80 °C until use. Non-tumour samples of the heart, lung, stomach, small intestine, colon, liver, pancreas, kidney, bone marrow, peripheral leukocytes, spleen, skeletal muscle, brain, and spinal cord were purchased directly from Clontech (Palo Alto, CA, USA).

For immunohistochemical analysis, we used archival formalin-fixed, paraffin-embedded tissues from 289 patients who had undergone surgical excision or removal of the tumour by polypectomy for gastric carcinoma ($n = 143$), colorectal carcinoma ($n = 36$), pancreatic carcinoma ($n = 23$), lung cancer ($n = 30$), breast cancer ($n = 30$), colorectal carcinoid tumour ($n = 15$), colorectal adenoma ($n = 10$), small cell carcinoma of the uterine cervix ($n = 1$), or small cell carcinoma of the extrahepatic bile ducts ($n = 1$) [8]. The 143 gastric adenocarcinomas were histologically classified as well ($n = 85$) or poorly ($n = 58$) differentiated. Thirty-six colorectal adenocarcinomas were histologically classified as well ($n = 15$), moderately ($n = 15$) or poorly ($n = 3$) differentiated, or mucinous ($n = 3$). All 23 pancreatic carcinomas were ductal adenocarcinomas. Of the 30 lung cancers, ten were squamous cell carcinomas, ten were adenocarcinomas, and ten were large cell neuroendocrine carcinomas. All 30 breast cancers were invasive ductal carcinomas. Tumour staging was carried out according to the TNM staging system [9].

Because written informed consent was not obtained, identifying information for all samples was removed before analysis for strict privacy protection; the procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government.

Antibodies

Rabbit polyclonal antibodies were raised against His-tagged recombinant Reg IV produced in bacteria and purified with nickel resin (Qiagen, Valencia, CA, USA). Specificity of the anti-Reg IV antibodies was evaluated by ELISA (data not shown). Immunoreactive sera were affinity-purified with the His-tagged recombinant Reg IV protein. We used four antibodies for phenotypic expression analysis of gastric carcinoma: anti-MUC5AC (Novocastra, Newcastle, UK) as a marker of gastric foveolar epithelial cells; anti-MUC6 (Novocastra) as a marker of pyloric gland cells; anti-MUC2 (Novocastra) as a marker of goblet cells in the small intestine and colorectum; and anti-CD10 (Novocastra) as a marker of the microvilli of absorptive cells in the small intestine and colorectum. Anti-chromogranin A antibody (Novocastra) was used as a neuroendocrine marker. Anti-insulin antibody (Nichirei Biosciences, Tokyo, Japan) and anti-glucagon antibody (Euro-Diagnostica, Sweden) were used for analysis of the endocrine pancreas. Antibody against caudal-related homeobox gene 2 (Cdx2) protein was purchased from BioGenex (San Ramon, CA, USA).

Cell lines, expression vector, and transfection

Seven cell lines derived from human gastric carcinoma were used. The TMK-1 cell line was established in our laboratory [10]. Five gastric carcinoma cell lines of the MKN series were kindly provided by Dr T Suzuki. HSC-39 cell line was kindly provided by Dr K Yanagihara [11]. All cell lines were maintained in RPMI 1640 (Nissui Pharmaceutical Co, Ltd, Tokyo, Japan) containing 10% fetal bovine serum (Whittaker, Walkersville, MD, USA) in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. For constitutive expression of *Reg IV*, cDNA was amplified by PCR and sub-cloned into pcDNA 3.1 (Invitrogen, Carlsbad, CA, USA). Transient transfection was carried out with the FuGENE6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN, USA).

Conventional and quantitative RT-PCR analyses

Total RNA was extracted with an RNeasy Mini Kit (Qiagen), and 1 μg of total RNA was converted to cDNA with a First Strand cDNA Synthesis Kit (Amersham Pharmacia, Little Chalfont, UK). Conventional RT-PCR was performed to investigate *REGIV* mRNA expression in gastric carcinoma cell lines. The amplification products were then separated by 1% agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light. *ACTB*-specific PCR

products served as internal controls. *REGIV* primer sequences were 5′-CCA AAC AGA TTT GCA GAT CAA GGA-3′ (sense) and 5′-TGC AGG AGT TAG CAG AAT CTT GAT-3′ (antisense). To analyse the expression of *REGIV* in human tissue samples, we performed real-time RT-PCR. Primer sequences and annealing temperatures were as described previously [3]. PCR was performed with an SYBR Green PCR Core Reagents kit (Applied Biosystems, Foster City, CA, USA). Real-time detection of the emission intensity of SYBR Green bound to double-stranded DNA was performed with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) as described previously [12]. *ACTB*-specific PCR products were amplified from the same RNA samples and served as internal controls.

Western blot analysis

For western blot analysis, cells and culture medium were lysed as described previously [13]. The culture media were concentrated with the PROTEIN Concentrate Kit (Takara Bio, Inc, Shiga, Japan). The lysates (40 μg) were solubilized in Laemmli sample buffer by boiling and then subjected to 12% SDS-polyacrylamide gel electrophoresis followed by electrotransfer onto a nitrocellulose filter. Peroxidase-conjugated anti-mouse IgG was used in the secondary reaction. The immune complex was visualized with an ECL Western Blot Detection System (Amersham Pharmacia Biotech).

Immunohistochemistry

A modified immunoglobulin enzyme bridge technique (ABC method) was used as described previously [14]. Microwave pretreatment in citrate buffer was performed for 15 min to retrieve antigenicity. Sections were treated consecutively at room temperature with primary antibody for 120 min, biotinylated anti-mouse or anti-rabbit IgG horse serum (diluted 1 : 200) for 30 min, and avidin DH-biotinylated horseradish peroxidase complex (Vectastain ABC Kit; Vector, Burlingame, CA, USA) for 30 min. Peroxidase staining was performed for 10–15 min with a solution of 3,3′-diaminobenzidine tetrahydrochloride in 50 mM Tris–HCl (pH 7.5) containing 0.001% hydrogen peroxide. The sections were counterstained with 0.1% haematoxylin. A result was considered positive if at least 10% of the cells were stained. When fewer than 10% of cancer cells were stained, the immunostaining was considered negative. Specificity of Reg IV staining was confirmed by pre-absorption of the anti-Reg IV antibody with an excess of the appropriate Reg IV protein. Reg IV staining was classified as mucin-like (vesicular) and perinuclear on the basis of alcian blue/periodic acid-Schiff (AB/PAS) staining. When the cells were positive for AB/PAS, we considered the cells to have mucin-like staining of Reg IV. If the cells were not positive for AB/PAS, we considered the cells to have perinuclear staining of Reg IV.

Phenotypic analysis of gastric carcinoma

Gastric carcinomas were classified into four phenotypes: gastric (G) type; intestinal (I) type; gastric and intestinal mixed (GI) type; and unclassified (N) type. The criteria [15] for the classification of G type and I type were as follows. Gastric cancers in which more than 10% of the section consisted of at least one gastric or intestinal epithelial cell phenotype were classified as G-type or I-type cancers, respectively. Those sections that showed both gastric and intestinal phenotypes were classified as GI type, and those that lacked both the gastric and the intestinal phenotypes were classified as N type.

Double immunofluorescence staining

Dewaxed tissue sections were immersed in methanol containing 0.03% hydrogen peroxide for 30 min to block endogenous peroxidase activity. Microwave pretreatment in citrate buffer was performed for 15 min to retrieve antigenicity. Sections were then incubated with normal horse serum for 30 min to block non-specific antibody binding sites. Sections were treated consecutively at room temperature with primary antibody for 120 min, and immunocomplexes were detected with Alexa Fluor 546-conjugated goat anti-rabbit IgG and Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA).

Statistical methods

Associations between clinicopathological parameters and Reg IV expression were analysed by Fisher's exact test. Kaplan–Meier survival curves were constructed for Reg IV-positive and Reg IV-negative patients. Survival rates were compared between Reg IV-positive and Reg IV-negative groups. The differences between the survival curves between groups were tested for statistical significance by the log-rank test [16]. p values less than 0.05 were considered statistically significant.

Results

Expression of REGIV mRNA in neoplastic and non-neoplastic tissues

Quantitative RT-PCR was performed to investigate the specificity of *REGIV* expression. As shown in Figure 1, *REGIV* expression was clearly detected in the stomach, small intestine, colon, and pancreas. High levels of *REGIV* were detected in gastric carcinoma and colorectal carcinoma, whereas *REGIV* expression was not detected in lung or breast cancers.

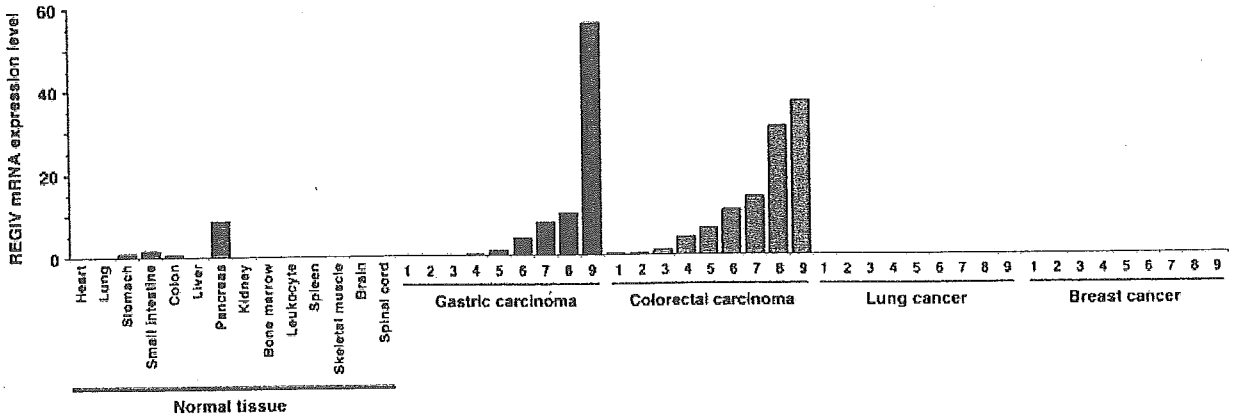

Figure 1. Quantitative RT-PCR analysis of *REGIV* in various human normal and tumour tissues. Clear *REGIV* expression is present in normal stomach, small intestine, colon, and pancreas. High levels of *REGIV* were observed in some gastric carcinomas and colorectal carcinomas. Expression was not observed in lung or breast cancers. The units are arbitrary and *REGIV* expression was calculated by standardization of 1.0 μg of total RNA from normal stomach as 1.0

Western blotting

The polyclonal anti-Reg IV antibody detected an approximately 17 kD band on western blots of both cell extracts and culture media from MKN-45, MKN-74, HSC-39, and KATO-III cells (Figure 2). These results are consistent with those of previous reports [7,17]. We confirmed that the *REGIV* mRNA levels determined by RT-PCR were consistent with the Reg IV protein levels determined by western blotting. Moreover, we performed western blot analysis of cell extracts of MKN-28 cells transiently transfected with pcDNA 3.1 or pcDNA-Reg IV. We detected an approximately 17 kD band corresponding to Reg IV. Furthermore, the 17 kD band disappeared with pre-incubation of the antibody with the appropriate Reg IV protein (data not shown).

Immunohistochemical analysis of Reg IV in non-tumour stomach, small intestine, colon, and pancreas

Quantitative RT-PCR revealed obvious *REGIV* expression in non-tumour stomach, small intestine, colon, and pancreas, although the levels were low. We therefore performed immunohistochemical analysis of

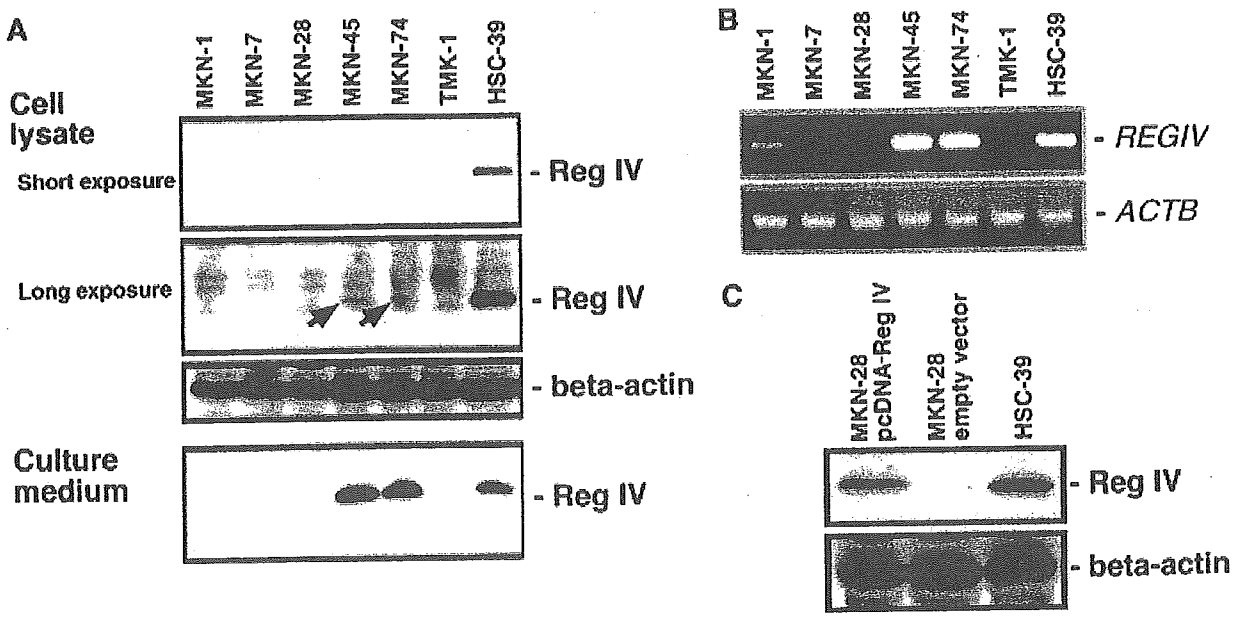

Figure 2. Expression of Reg IV in gastric carcinoma cell lines. (A) Western blot analysis of Reg IV with anti-Reg IV antibody. In cell lysates, an approximately 17 kD band is visible in HSC-39 cells after a short exposure. A longer exposure revealed that Reg IV is expressed in MKN-45 and MKN-74 cells. Culture media of MKN-45, MKN-74, and HSC-39 cells contain Reg IV. (B) RT-PCR analysis showed strong expression of *REGIV* in MKN-45, MKN-74, and HSC-39 cells, which is consistent with the western blot results. (C) Western blot analysis of extracts of MKN-28 cells transiently transfected with pcDNA-Reg IV or pcDNA 3.1 (empty vector). With anti-Reg IV antibody, an approximately 17 kD band corresponding to Reg IV is visible. HSC-39 extract served as a positive control

these tissues (Figure 3). In the stomach, foveolar epithelium was negative for Reg IV, whereas goblet cells in intestinal metaplasia showed strong Reg IV expression in goblet cell vesicles (Figure 3A). In addition, neuroendocrine cells at the base of intestinal metaplasia displayed strong Reg IV staining in the perinuclear region (Figures 3A and 3B). All the cells with Reg IV staining of the perinuclear region were positive for chromogranin A; however, chromogranin A-positive cells were not always positive for Reg IV. In general, among neuroendocrine cells of the stomach, as defined by chromogranin A staining, only neuroendocrine cells at the base of intestinal metaplasia were positive for Reg IV. Expression of Reg IV was not detected in stromal cells, such as inflammatory cells and fibroblasts. As described previously [5,7], in the small intestine and colon, only weak or no expression of Reg IV was observed in epithelial cells, whereas strong expression of Reg IV was detected in neuroendocrine cells (Figures 3D, 3E, 3G, and 3H). Although goblet cells of intestinalized stomach, small intestine, and colon were positive for MUC2, only those of intestinal metaplasia of the stomach were positive for Reg IV, and those of the small intestine and colon were negative or weakly positive for Reg IV (Figures 3A, 3C, 3D, 3F, 3G, and 3I). This suggests that goblet cells of intestinal metaplasia of the stomach have different biological characteristics from those of the intestine. Specific immunostaining of gastric, duodenal, or colonic mucosa was not seen with pre-absorbed anti-Reg IV antibody (data not shown). In the pancreas, the insulin-producing beta cells of the endocrine pancreas were positive for Reg IV (Figures 3J–3L).

Immunohistochemical analysis of Reg IV in human tumours

We performed immunohistochemical analysis of Reg IV in 289 human tumour samples. The overall results are summarized in Table 1.

Adenocarcinoma of the stomach

Representative results of Reg IV immunostaining of gastric carcinoma are shown in Figure 4. Of 143 gastric carcinomas, 42 (29.4%) were positive for Reg IV. Reg IV was detected in goblet cell-like vesicles of cancer cells in well-differentiated adenocarcinomas (Figure 4A). Most signet ring cell carcinoma cells were positive for Reg IV (Figure 4C). In some tumour cells, strong Reg IV staining was observed in the perinuclear region (Figures 4B and 4F). We observed two patterns of Reg IV staining, mucin-like staining and strong perinuclear staining, on the basis of AB/PAS staining results. Mucin-like staining was observed in the goblet cells of intestinal metaplasia, goblet cell-like vesicles of well-differentiated adenocarcinoma, signet ring cell carcinomas, and some poorly differentiated adenocarcinomas. In contrast, strong perinuclear staining was detected in neuroendocrine cells at the base of intestinal metaplasia. In well-differentiated adenocarcinoma, neuroendocrine-like cells were positive for Reg IV. In poorly differentiated adenocarcinoma, small tumour cells with scant cytoplasm were positive for Reg IV. Mucin-like staining was observed in 41 of 143 cases and perinuclear staining was found in 17 of 143 cases. There were several gastric carcinomas in which both staining patterns were observed (Table 2). We also analysed the relationship of Reg IV expression to clinicopathological characteristics. Mucin-like

Table 1. Summary of Reg IV protein expression in human tumours

Organ and histological type	No of cases	No of Reg IV-positive cases		
		Mucin-like	Perinuclear	Total
Stomach				
Adenocarcinoma	143	41 (28.7%)	17 (11.9%)	42 (29.4%)
Colorectum				
Adenocarcinoma	36	13 (36.1%)	0 (0.0%)	13 (36.1%)
Adenoma	10	3 (30.0%)	0 (0.0%)	3 (30.0%)
Carcinoid tumour	15	0 (0.0%)	14 (93.3%)	14 (93.3%)
Pancreas				
Ductal adenocarcinoma	23	5 (21.7%)	0 (0.0%)	5 (21.7%)
Lung				
Squamous cell carcinoma	10	0 (0.0%)	0 (0.0%)	0 (0.0%)
Adenocarcinoma	10	0 (0.0%)	0 (0.0%)	0 (0.0%)
Large cell neuroendocrine carcinoma	10	0 (0.0%)	0 (0.0%)	0 (0.0%)
Breast				
Invasive ductal carcinoma	30	0 (0.0%)	0 (0.0%)	0 (0.0%)
Uterine cervix				
Small cell carcinoma	1	0 (0.0%)	0 (0.0%)	0 (0.0%)
Extrahepatic bile duct				
Small cell carcinoma	1	0 (0.0%)	0 (0.0%)	0 (0.0%)

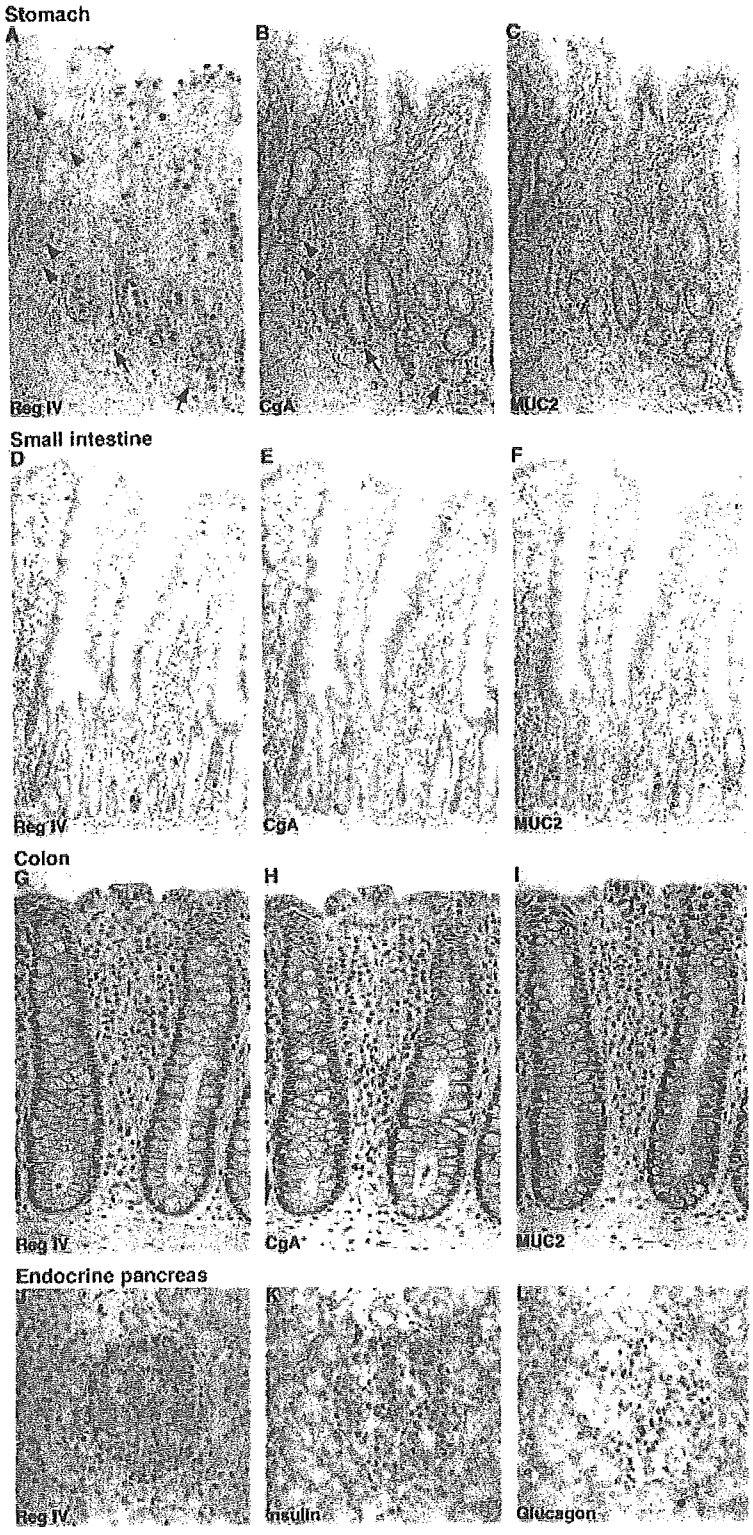

Figure 3. Immunohistochemical analysis of Reg IV in non-neoplastic human tissues. In the stomach, foveolar epithelium does not express Reg IV (A, red arrowheads). Goblet cell vesicles in goblet cells of intestinal metaplasia are positive for both Reg IV (A) and MUC2 (C). The perinuclear region of neuroendocrine cells at the base of intestinal metaplasia (A and B, black arrows) is positive for Reg IV, whereas in other neuroendocrine cells, as determined by chromogranin A expression (B, black arrowheads), Reg IV is not expressed (A, black arrowheads). In the small intestine and colon, weak or no expression of Reg IV is observed in epithelial cells, whereas strong Reg IV expression is detected in neuroendocrine cells (D, G), as defined by chromogranin A expression (E, H). We confirmed that MUC2 is positive in goblet cells in these samples (F, I). In the pancreas, Reg IV is expressed in beta cells of the endocrine pancreas (J) that express insulin (K). Glucagon-positive alpha cells do not express Reg IV (L). Original magnification: (A–F) ×100; (G–I) ×200; (J–L) ×400. CgA = chromogranin A

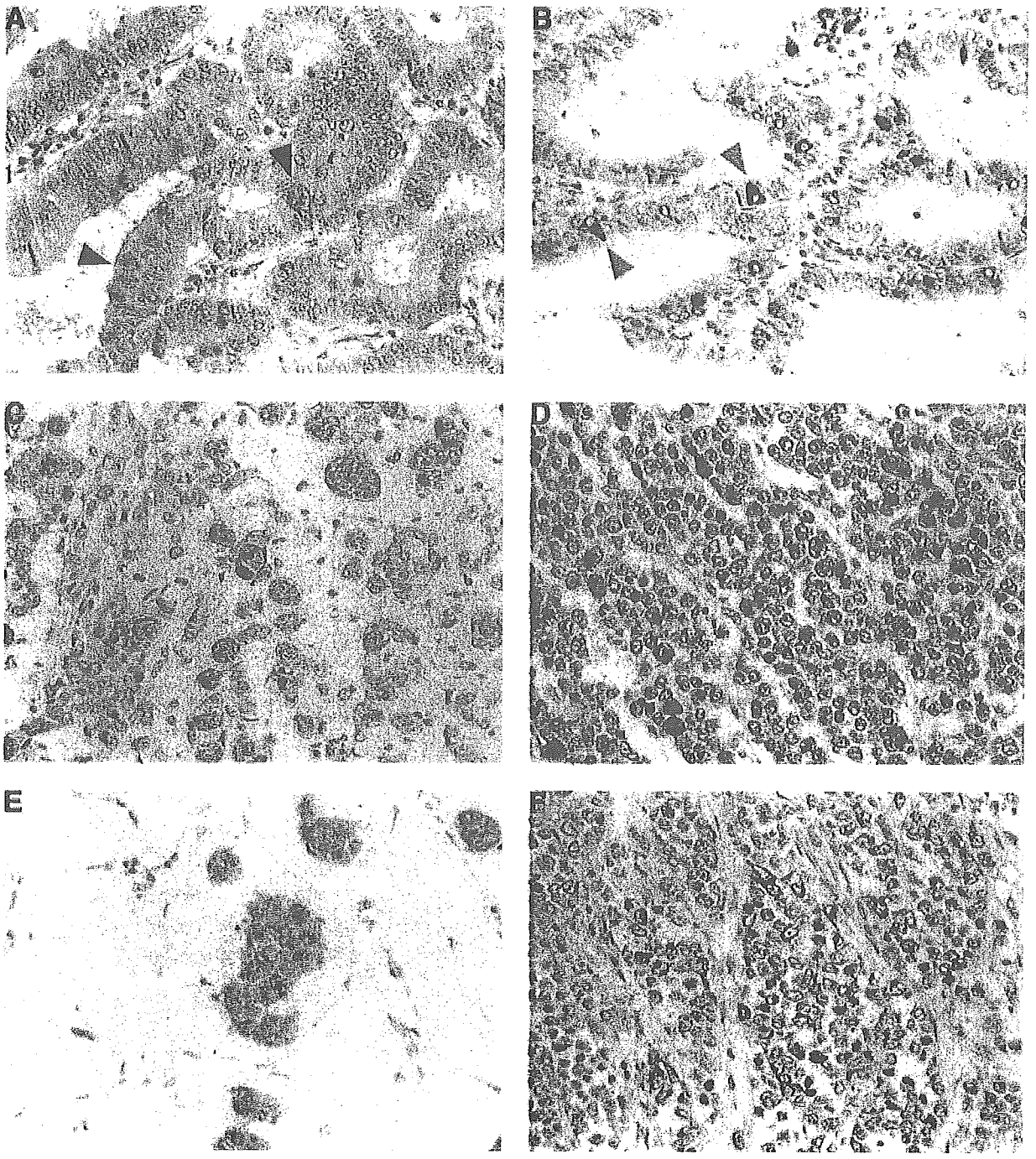

Figure 4. Expression and distribution of Reg IV in adenocarcinoma of the stomach. (A) Well-differentiated adenocarcinoma. Reg IV is expressed in goblet cell-like vesicles (black arrowheads). (B) Well-differentiated adenocarcinoma. Strong perinuclear Reg IV staining is present in neuroendocrine-like cells (red arrowheads). We confirmed that these cells are also positive for chromogranin A. (C) Signet ring cell carcinoma. The cytoplasm of tumour cells is positive for Reg IV. (D) Poorly differentiated adenocarcinoma. The cytoplasm of tumour cells is positive for Reg IV. (E) Mucinous adenocarcinoma. The cytoplasm of tumour cells is positive for Reg IV. These three tumour cell types (C, D, and E) show round morphology and are positive for MUC2. (F) Poorly differentiated adenocarcinoma. Strong perinuclear expression of Reg IV is present in tumour cells. These tumour cells are small with scant cytoplasm. Chromogranin A was detected in these cells. Original magnification: ×400

staining of Reg IV was detected more frequently in poorly differentiated adenocarcinoma (41.4%) than in well-differentiated adenocarcinoma (20.0%, $p = 0.0081$, Fisher's exact test, Table 3). In contrast, no correlation was found between perinuclear Reg IV staining and histological type (Table 3). There was no clear correlation between Reg IV expression and clinical characteristics (Table 3). No statistically significant prognostic impact was found in the group of 52 advanced gastric carcinoma patients or in the group of 24 stage III/IV gastric carcinoma patients (data not shown).

Table 2. Heterogeneity of intracellular localization of Reg IV protein in individual gastric carcinomas

		Mucin-like staining		
		Positive	Negative	p value
Perinuclear Staining	Positive	16 (94.1%)	1	0.0001
	Negative	25 (19.8%)	101	

We next investigated the association between Reg IV expression and mucin phenotype, because Reg IV was detected in goblet cells of intestinal metaplasia of the stomach. Gastric and intestinal markers were detected in 57 of 143 (39.9%) cases for MUC5AC, 12 (8.4%) cases for MUC6, 52 (36.4%) cases for MUC2, and 17 (11.9%) cases for CD10. Both MUC2 and Reg IV were positive in goblet cell-like vesicles in tumour cells (Figures 5A and 5B), whereas tumour cells with a MUC2-positive perinuclear region were negative for Reg IV (Figures 5C and 5D). We confirmed that tumour cells showing mucin-like staining of Reg IV were positive for MUC2 by double immunofluorescence staining (Figures 5E–5G). Tumour cells with a Reg IV-positive perinuclear region were negative for MUC2 (Figures 5H and 5J). In total, both mucin-like staining and perinuclear Reg IV staining were observed more frequently in MUC2-positive cases (76.9% and 28.8%, respectively) than in MUC2-negative cases (1.1% and 2.2%, $p = 0.0001$ and $p = 0.0001$, respectively, Fisher's exact test, Table 4). There was no clear relationship between expression of Reg IV and other markers (Table 4). On the basis of the expression of these four markers, we classified the 143 gastric carcinomas phenotypically as 32 (22.4%) G-type, 37 (25.9%) I-type, 28 (19.6%) GI-type, and 46 (32.2%) N-type carcinomas. As expected, mucin-like Reg IV staining was observed only in I- (20/37, 54.1%) and GI-type (21/28, 75.0%) gastric carcinomas. Perinuclear Reg IV staining was observed in I- (11/37, 29.7%) and GI-type (6/28, 21.4%) gastric carcinomas. Because it has been reported that Cdx2 interacts with the *MUC2* promoter and activates *MUC2* transcription [18], the relationship between Cdx2 and Reg IV was examined. Of 143 gastric carcinomas, 53 (37.1%) were positive for Cdx2. Cdx2 nuclear staining was detected in I- and GI-type carcinomas, but not in G- and N-type carcinomas. Both mucin-like staining and perinuclear staining of Reg IV were observed more frequently in Cdx2-positive cases (35/53, 66.0% and 16/53, 30.2%, respectively) than in Cdx2-negative cases (6/90, 6.7% and 1/90, 1.1%, $p = 0.0001$ and $p = 0.0001$, respectively, Fisher's exact test, Table 4).

We next examined the relationship between Reg IV and chromogranin A expression in gastric carcinomas. Twenty-seven cases (18.9%) showed chromogranin A staining. Tumour cells with a Reg IV-positive perinuclear region always showed chromogranin A staining, whereas chromogranin A-positive tumour cells were not always positive for Reg IV (Figures 5H and 5I). In total, the frequency of Reg IV-positive (both mucin-like and perinuclear staining) cases was significantly higher among chromogranin A-positive cases (63.0% and 44.4%, respectively) than among chromogranin A-negative cases (20.7% and 4.3%, $p = 0.0001$ and $p = 0.0001$, respectively, Fisher's exact test, Table 4).

Adenocarcinoma and adenoma of the colorectum

In adenocarcinoma of the colorectum, Reg IV was seen in goblet cell-like vesicles of tumour cells (Figure 6A). In mucinous adenocarcinoma cells and poorly differentiated adenocarcinoma cells, the cytoplasm was also positive for Reg IV (Figures 6B and 6C). In total, 13 (36.1%) of 36 adenocarcinomas of the colorectum were positive for Reg IV, and AB/PAS staining revealed that all of these cases

Table 3. Relationship between Reg IV protein expression and clinicopathological characteristics in gastric carcinoma

	Reg IV staining								
	Mucin-like			Perinuclear			Total		
	+	−	p value	+	−	p value	+	−	p value
T status									
T1	20 (33.9%)	39	NS	6 (10.2%)	53	NS	20 (33.9%)	39	NS
T2/3/4	21 (26.2%)	63		11 (13.1%)	73		22 (26.2%)	62	
N status									
N0	28 (35.0%)	52	NS	11 (13.8%)	69	NS	28 (35.0%)	52	NS
N1/2/3	13 (20.6%)	50		6 (9.5%)	57		14 (22.2%)	49	
Stage									
I/II	33 (32.7%)	68	NS	14 (15.9%)	74	NS	33 (32.7%)	68	NS
III/IV	8 (19.0%)	34		3 (5.5%)	52		9 (21.4%)	33	
Histological type									
Well differentiated	17 (20.0%)	68	0.0081	10 (11.8%)	75	NS	17 (20.0%)	68	0.0047
Poorly differentiated	24 (41.4%)	34		7 (12.1%)	51		25 (43.1%)	33	

NS = not significant.

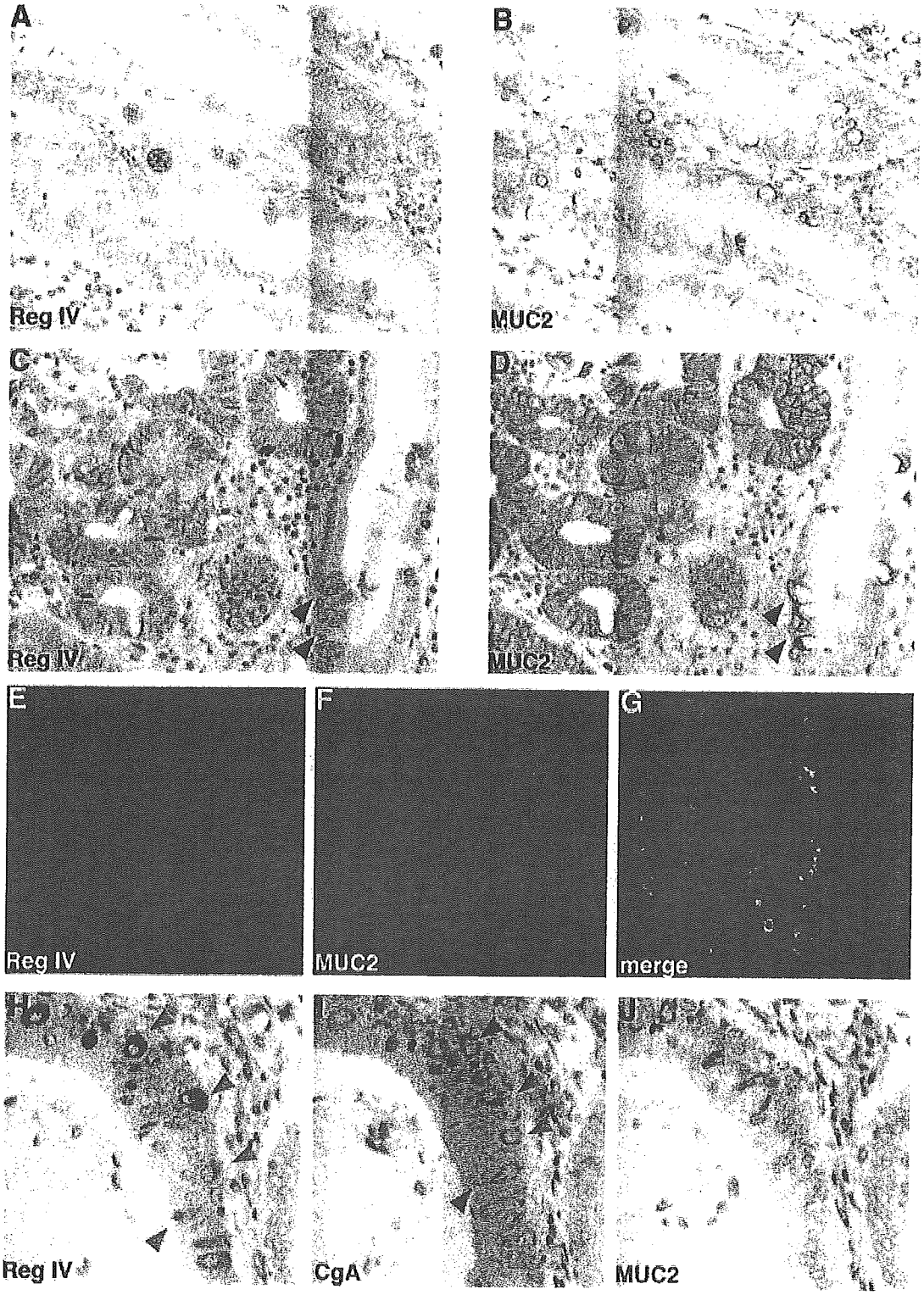

Figure 5. Co-localization of Reg IV, MUC2, and chromogranin A in gastric carcinoma. Goblet cell-like vesicles of cancer cells are positive for both Reg IV (A) and MUC2 (B). Cancer cells with a MUC2-positive perinuclear region (D) do not express Reg IV (C), although both molecules are present in goblet cell vesicles in intestinal metaplasia (black arrowheads). Double immunofluorescence staining shows that Reg IV (E) is co-expressed with MUC2 (F), but some tumour cells express only MUC2 (G). Perinuclear Reg IV staining co-localizes with chromogranin A staining (H, I, red arrowheads), but not with MUC2 (J). Original magnification: (A–D, H–J) ×400; (E–G) ×200. CgA = chromogranin A

showed mucin-like staining. In contrast to gastric carcinoma, expression of Reg IV by colorectal carcinoma was associated with lymph node metastasis ($p = 0.0379$, Fisher's exact test, Table 5). Moreover, Reg IV staining was observed more frequently in stage III/IV cases (9/16, 56.3%) than in stage I/II cases

Table 4. Relationship between Reg IV protein expression and gastric, intestinal, and neuroendocrine markers in gastric carcinoma

	Reg IV staining								
	Mucin-like			Perinuclear			Total		
	+	−	*p* value	+	−	*p* value	+	−	*p* value
MUC5AC									
Positive	20 (35.1%)	37	NS	5 (8.8%)	52	NS	20 (35.1%)	37	NS
Negative	21 (24.4%)	65		12 (14.0%)	74		22 (25.6%)	64	
MUC6									
Positive	5 (41.7%)	7	NS	3 (25.0%)	9	NS	5 (41.7%)	7	NS
Negative	36 (27.5%)	95		14 (10.7%)	117		37 (28.2%)	94	
MUC2									
Positive	40 (76.9%)	12	0.0001	15 (28.8%)	37	0.0001	40 (76.9%)	12	0.0001
Negative	1 (1.1%)	90		2 (2.2%)	89		2 (2.2%)	89	
CD10									
Positive	5 (29.4%)	12	NS	4 (23.5%)	13	NS	6 (35.3%)	11	NS
Negative	36 (28.6%)	90		13 (10.3%)	113		36 (28.6%)	90	
Cdx2									
Positive	35 (66.0%)	18	0.0001	16 (30.2%)	37	0.0001	36 (67.9%)	17	0.0001
Negative	6 (6.7%)	84		1 (1.1%)	89		6 (6.7%)	84	
Chromogranin A									
Positive	17 (63.0%)	10	0.0001	12 (44.4%)	15	0.0001	18 (66.7%)	9	0.0001
Negative	24 (20.7%)	92		5 (4.3%)	111		24 (20.7%)	92	

NS = not significant.

(4/20, 20.0%, $p = 0.0379$, Fisher's exact test). Reg IV was detected in three (30.0%) of ten colorectal adenomas. Goblet cell-like vesicles of adenoma cells were stained with Reg IV (Figure 6D).

Ductal adenocarcinoma of the pancreas

In ductal adenocarcinoma of the pancreas, Reg IV was detected in goblet cell-like vesicles or the cytoplasm of cancer cells (Figures 6E and 6F). In total, 5 (21.7%) of 23 ductal adenocarcinomas of the pancreas were positive for Reg IV. These cells were stained with AB/PAS and all of these cases showed mucin-like staining. There was no clear correlation between Reg IV expression and clinicopathological characteristics such as T grade, N grade, and histological differentiation.

Table 5. Relationship between Reg IV protein expression and clinicopathological characteristics in colorectal carcinoma

	Reg IV expression		
	Positive	Negative	*p* value
T status			
T1/2	2 (22.2%)	7	0.4379
T3/4	11 (40.7%)	16	
N status			
N0	4 (20.0%)	16	0.0379
N1/2	9 (56.3%)	7	
Stage			
Stage I/II	4 (20.0%)	16	0.0379
Stage III/IV	9 (56.3%)	7	
Histology			
Well/moderately differentiated	8 (26.7%)	22	0.0015*
Poorly differentiated	2 (66.7%)	1	
Mucinous	3 (100.0%)	0	

* Well/moderately vs poorly and mucinous.

Carcinoid tumour (well-differentiated endocrine neoplasm) of the colorectum

Of 15 colorectal carcinoid tumours, 14 (93.3%) were positive for Reg IV. Among these, diffuse strong staining of Reg IV was seen in two (Figure 7A). In the remaining 12, expression of Reg IV was observed focally (data not shown). Co-expression of Reg IV and chromogranin A was observed in some carcinoid tumour cells by double immunofluorescence staining (Figures 7C–7E).

Lung and breast cancer

Ten squamous cell carcinomas and ten adenocarcinomas of the lung were negative for Reg IV. Ten large cell neuroendocrine carcinomas were also negative for Reg IV despite positive chromogranin A staining (Figures 7F and 7G). All 30 invasive ductal carcinomas of the breast were negative for Reg IV (data not shown).

Small cell carcinoma (poorly differentiated endocrine neoplasm) of the uterine cervix and the extrahepatic bile duct

Two small cell carcinomas (uterine cervix and extrahepatic bile duct) were studied. Both were Reg IV-negative but chromogranin A-positive (Figure 7H).

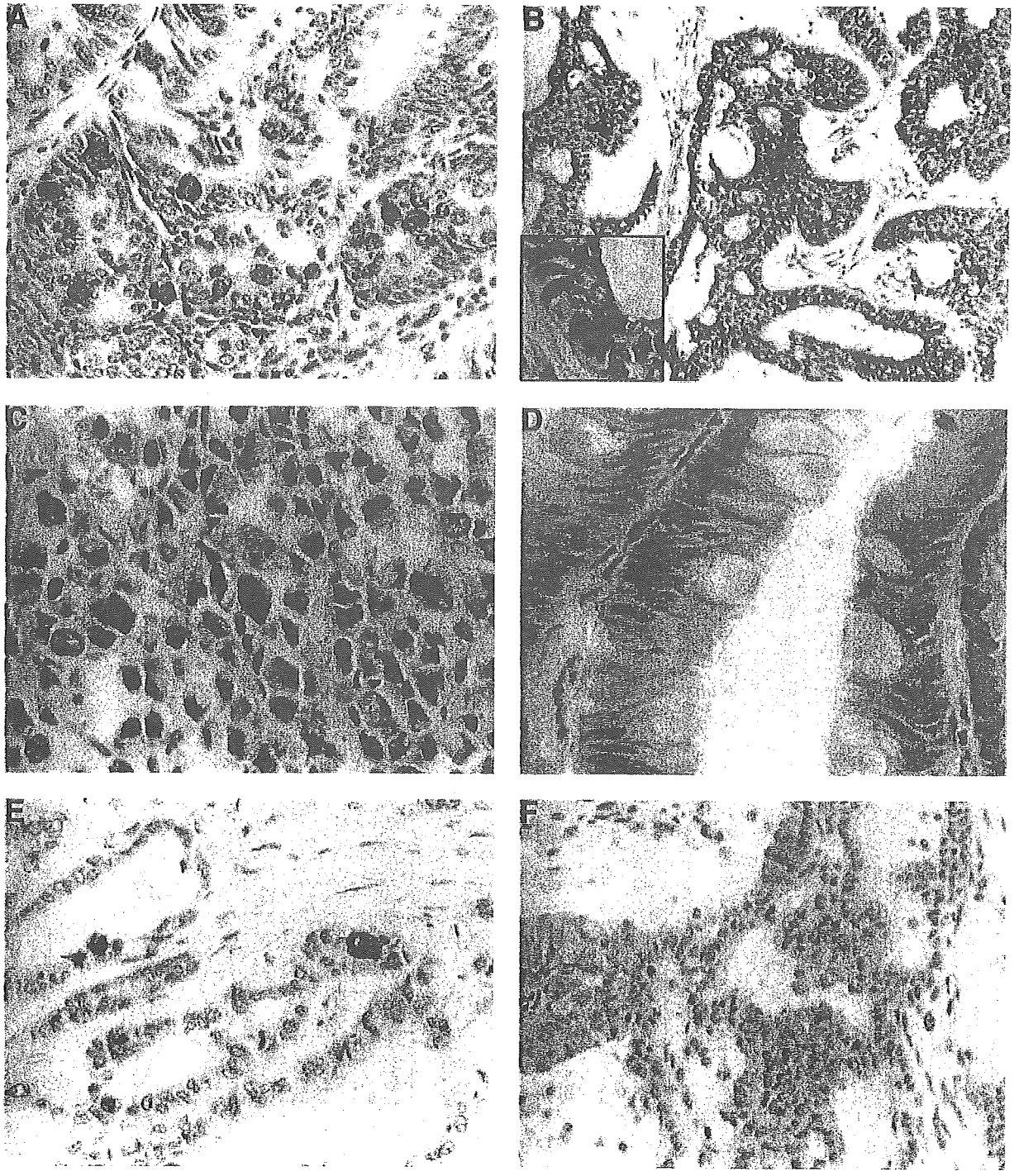

Figure 6. Expression and distribution of Reg IV in adenocarcinoma and adenoma of the colorectum (A–D), and ductal adenocarcinoma of the pancreas (E, F). (A) Well-differentiated adenocarcinoma of the colorectum. Goblet cell-like vesicles of tumour cells express Reg IV. (B) Mucinous adenocarcinoma of the colorectum. The cytoplasm of tumour cells is positive for Reg IV. (C) Poorly differentiated adenocarcinoma of the colorectum. Reg IV is expressed in the cytoplasm. (D) Adenoma of the colorectum. Reg IV is observed in goblet vesicle-like structures in tumour cells. (E, F) Ductal adenocarcinoma of the pancreas. Reg IV is observed in goblet vesicle-like structures or the cytoplasm of tumour cells. Original magnification: (B) ×200; (A, E, F) ×400; (B inset, C, D) ×1000

Discussion

In the present study, we performed immunohistochemical analysis of Reg IV in human tissues. We found that expression of Reg IV is associated with the intestinal mucin phenotype and neuroendocrine differentiation in the stomach and in gastric carcinomas. It is known that Cdx2, a mammalian caudal-related intestinal transcription factor, is important for the maintenance of intestinal epithelial cells

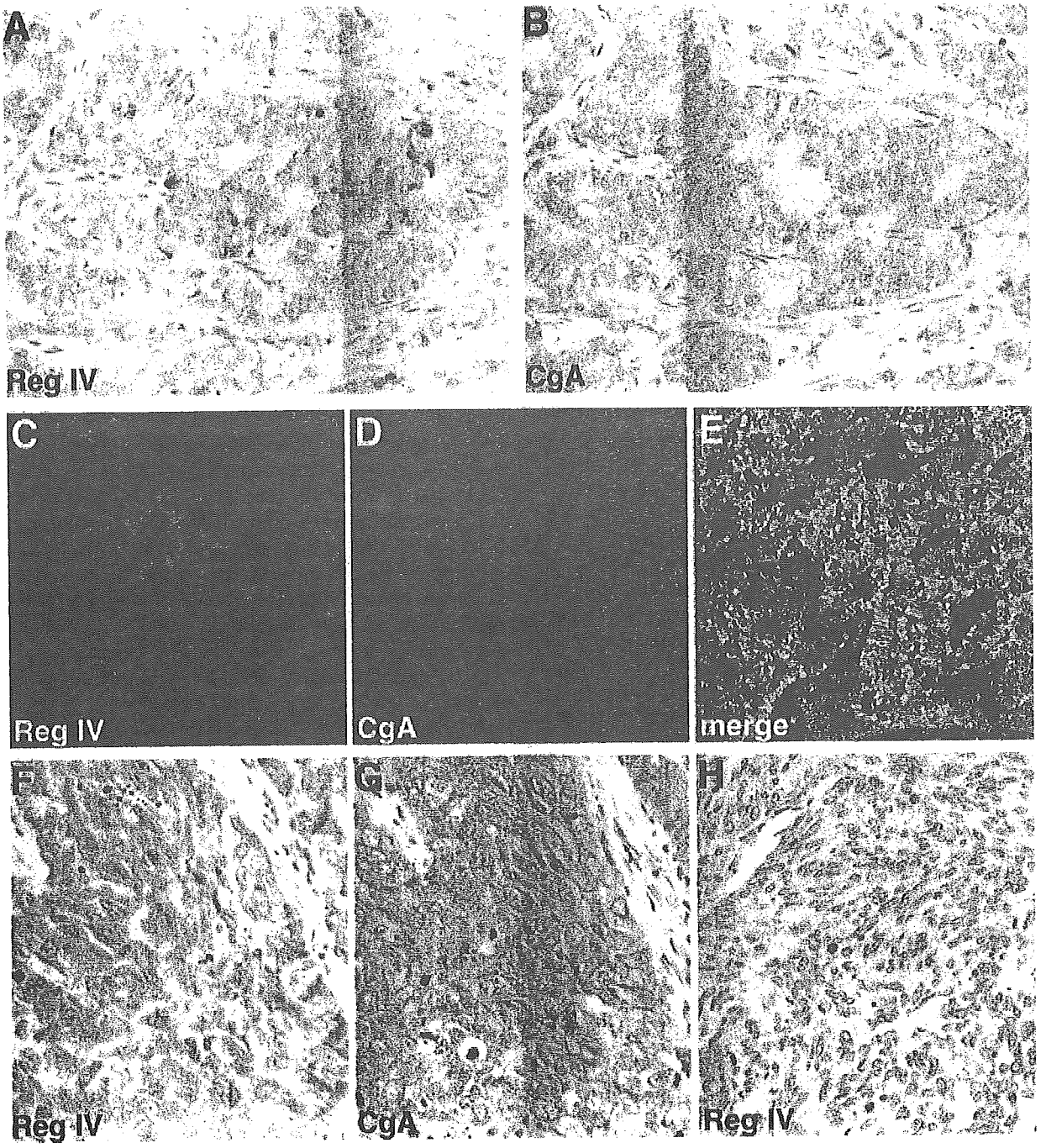

Figure 7. Immunohistochemical analysis of Reg IV in neuroendocrine neoplasms. Diffuse Reg IV staining is visible in carcinoid tumour of the colorectum (A, B). Double immunofluorescence staining shows that Reg IV (C) is co-expressed with chromogranin A (D), but some tumour cells express only chromogranin A. In large cell neuroendocrine carcinoma of the lung (F, G) and small cell carcinoma of the extrahepatic bile duct (H), expression of Reg IV is not detected (F, H). Chromogranin A is positive in all of these tumours (B, G). Original magnification: (C–E) ×200; (A, B, F–H) ×400. CgA = chromogranin A

[19,20]. In addition, several lines of evidence have suggested that intestinal metaplasia of the stomach and gastric adenocarcinoma with the intestinal mucin phenotype are associated with ectopic Cdx2 expression [21–24]. Because it has been reported that Cdx2 interacts with the *MUC2* promoter and activates *MUC2* transcription [18], Cdx2 may regulate transcription of the *REGIV* gene. In the present study, expression of Reg IV correlated with Cdx2 expression in gastric carcinomas, suggesting that Cdx2 is associated with Reg IV expression. However, ectopic expression of Cdx2 alone cannot explain the expression of Reg IV in intestinal metaplasia of the stomach and in gastric carcinoma with the intestinal mucin phenotype, because expression of Reg IV was weak or absent in the small intestine and colon, both of which express Cdx2. Furthermore, gastric carcinoma cells with a MUC2-positive perinuclear region were negative for Reg IV.

These results suggest that goblet cells of intestinal metaplasia of the stomach may have different biological characteristics from those of the small intestine and colon. In fact, the proliferative activity of goblet cells in intestinal metaplasia of the stomach is higher than that of goblet cells in normal small intestine [25]. Up-regulation of Reg IV in goblet cells of the colon has been reported in inflammatory bowel disease [4,7]. Cdx2 and other factors are needed to express Reg IV. Therefore, in intestinal metaplasia of the stomach and gastric carcinoma with the intestinal mucin phenotype, mechanisms in addition to expression of Cdx2 may be involved. Additional investigation will clarify whether Cdx2 induces Reg IV expression.

In the present study, only neuroendocrine cells at the base of intestinal metaplasia in non-neoplastic stomach were positive for Reg IV. A possible link between intestinal metaplasia of the stomach and neuroendocrine cells has been observed in *neurogenin 3* knockout mice [26]. In these mice, intestinal metaplasia occurs in the stomach, and glucagon-secreting A-cells, somatostatin-secreting D-cells, and gastrin-secreting G-cells are absent, whereas the number of serotonin-expressing enterochromaffin cells is decreased but present. These data suggest that the neuroendocrine cells at the base of intestinal metaplasia may have origins different from those of other neuroendocrine cells. Reg IV-positive neuroendocrine cells may be positive for serotonin.

We observed two Reg IV staining patterns, namely mucin-like staining and strong perinuclear staining. Mucin-like staining was observed in goblet cells, signet ring cell carcinomas, some poorly differentiated gastric adenocarcinomas, and some adenocarcinomas of the colorectum and pancreas. These cells were positive for MUC2 and AB/PAS. In contrast, strong perinuclear staining was detected in neuroendocrine cells at the base of intestinal metaplasia of the stomach, small intestine, and colon. In well-differentiated adenocarcinoma of the stomach, neuroendocrine-like cells were positive for Reg IV. In poorly differentiated adenocarcinoma of the stomach, small tumour cells with scant cytoplasm were positive for Reg IV. Carcinoid tumour cells were also positive for Reg IV. Chromogranin A, but not MUC2, was expressed by these cells. The significance of the difference between the Reg IV staining patterns is not clear. However, there were several gastric carcinomas in which both staining patterns were observed and we presume that these staining patterns are not independent.

There was substantial variability in the *REGIV* mRNA levels between different samples taken from gastric carcinomas and colorectal carcinomas. *REGIV* is expressed in both normal stomach and colon tissues, but expression was not detected in four samples of gastric carcinoma and was very low in two colorectal carcinoma samples. Because bulk tissues were used for quantitative RT-PCR analysis, the resulting data may not reflect the expression levels of Reg IV in tumour cells alone. Reg IV-positive cells were observed in intestinal metaplastic glands, suggesting that a sample of normal stomach used for quantitative RT-PCR may contain foci of intestinal metaplasia. In the present study, several cancer samples showed no Reg IV staining, whereas several cancer samples showed extensive Reg IV staining, resulting in the large variability in *REGIV* mRNA levels determined by quantitative RT-PCR.

The biological function of Reg IV is poorly understood. Because it is associated with 5-FU resistance, Reg IV may be involved in apoptosis resistance. I- and GI-type gastric carcinomas are reported to show high 5-FU resistance [27]. Reg IV is also thought to be a candidate marker of stem cells in the intestine [17]. Although Regenerating gene I (Reg I) is expressed in foveolar neck cells [28], some of which are thought to be stem cells of the stomach, we did not observe expression of Reg IV in foveolar neck cells in the present study. Thus, Reg IV is not a marker of stem cells, at least in the stomach. In colorectal adenomas and carcinomas, mucin-like staining of Reg IV protein was observed. The adenoma–carcinoma sequence describes the development of sporadic colorectal carcinoma from pre-cancerous adenomatous mucosal lesions [29]. Dysregulation of Reg IV expression may be associated with an early step in the adenoma–carcinoma sequence.

Is the detection of Reg IV expression of clinical benefit? Previously, we identified *REGIV* as a cancer-specific (at least gastric cancer-specific) gene by SAGE and quantitative RT-PCR analysis of bulk gastric carcinoma tissues [3]. In the present study, we did not detect expression of Reg IV in normal foveolar epithelium of the stomach; however, Reg IV-positive cells were observed in non-neoplastic tissues. Thus, Reg IV does not appear to be a true cancer-specific marker. However, expression of Reg IV was associated with both lymph node metastasis and tumour stage in colorectal carcinoma. Moreover, carcinoid tumours of the colorectum were positive for Reg IV, whereas large cell neuroendocrine carcinomas of the lung and small cell carcinomas of the uterine cervix and the extrahepatic bile duct were all negative for Reg IV. Reg IV was not expressed by squamous cell carcinomas and adenocarcinomas of the lung, or by invasive ductal carcinomas of the breast. Therefore, Reg IV may be a good marker for gastrointestinal and pancreatic tumours.

In conclusion, our present study provides detailed information about the expression and distribution of Reg IV in various neoplastic and non-neoplastic human tissues. Reg IV expression is associated with gastrointestinal and pancreatic carcinoma and carcinoid tumours. Because there are many types of tumour, further investigation is needed to establish the specificity of Reg IV for gastrointestinal and pancreatic tumours. The difference between Reg IV expression in goblet cells of intestinal metaplasia of the stomach and those of the intestine may possibly explain

the differences between metaplastic and normal cells, even though the histological appearance is similar.

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DNA methylation of genes linked to retinoid signaling in squamous cell carcinoma of the esophagus: DNA methylation of *CRBP1* and *TIG1* is associated with tumor stage

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Hypermethylation of CpG islands is associated with the silencing of various tumor suppressor genes. *Retinoic acid receptor-beta* (*RAR-beta*), *cellular retinol-binding protein 1* (*CRBP1*), and *tazarotene-induced gene 1* (*TIG1*) have been linked to retinoic acid signaling. Little is known about the involvement of these three genes in esophageal squamous cell carcinoma (ESCC). In this study, we investigated the methylation status of these genes and analyzed the role of methylation of their DNA in ESCC. Methylation-specific polymerase chain reaction (PCR) was performed to study the methylation of CpG islands in 28 ESCC (stages I, II, and III) and 10 samples of corresponding non-neoplastic mucosa. The mRNA expression levels of the three genes were measured by quantitative reverse transcription-PCR. DNA hypermethylation of *RAR-beta* was found in seven (25.0%) of the 28 ESCC, of *CRBP1* in five (17.9%), and of *TIG1* in five (17.9%). DNA methylation of *RAR-beta* was identified in one of 10 samples of corresponding non-neoplastic mucosa (10.0%), whereas no DNA methylation of *CRBP1* or *TIG1* was detected. In total, at least one of the three genes was hypermethylated in 12 (42.9%) ESCC. Reduced expression of *RAR-beta*, *CRBP1*, and *TIG1* was found in 14 (50.0%), 15 (53.6%), and 13 (46.4%) ESCC, respectively. DNA methylation of each gene was significantly associated with reduced expression of the respective mRNA. No correlation was found between the DNA methylation status of *RAR-beta* and clinicopathological factors such as depth of invasion, lymph node metastasis, or tumor stage. In contrast, DNA methylation of both *CRBP1* and *TIG1* was observed only in stage III ESCC. These results show that inactivation of the retinoic acid signaling-associated genes *RAR-beta*, *CRBP1*, and *TIG1* by DNA methylation occurs frequently in ESCC. (*Cancer Sci* 2005; *96*: 571–577)

Esophageal squamous cell carcinoma (ESCC) is one of the most common cancers worldwide, but the prognosis for patients with this condition is extremely poor because of difficulties with early diagnosis and a lack of effective treatments.[1] The development of ESCC is a multi-step and progressive process, an early indicator of which is the increased proliferation of epithelial cells, including basal cell hyperplasia and dysplasia, which are regarded as precancerous lesions. Multiple genetic alterations are involved, including amplification/overexpression of the *epidermal growth factor* (*EGF*)/*EGF receptor* (*EGFR*)[2,3] and *cyclin D1/hst-1/int-2* genes,[4] abnormal retention of intron 9 in the *CD44* gene,[5] loss of heterozygosity (LOH) at multiple chromosomal loci,[6,7] microsatellite instability,[8] and mutation of the *TP53* gene.[9]

In addition to genetic alterations, epigenetic alterations, such as hypermethylation of CpG islands, are commonly observed in human cancers. Hypermethylation of CpG islands is associated with the silencing of several tumor-related genes, and has been proposed as an alternative way to inactivate tumor suppressor genes in cancer.[10,11] The expression of some tumor suppressor genes, such as $p16^{INK4a}$, *FHIT*, *CDH1*, *ECRG4*, *MGMT*, and *LRP1B*[12–17] is commonly downregulated by CpG island hypermethylation in ESCC. However, despite recent advances in DNA methylation studies of esophageal adenocarcinoma,[18,19] gastric cancer,[20,21] and colorectal cancer,[22,23] the extent of DNA methylation in ESCC is poorly understood.

Several lines of evidence suggest that retinoids suppress carcinogenesis and prevent the development of cancer. Retinoids regulate the growth, differentiation, and apoptosis of normal cells during embryonic development, and of premalignant and malignant cells during carcinogenesis. The effects of retinoids are mediated predominantly by retinoic acid receptors (RAR-alpha, -beta, and -gamma), which act as retinoic acid-dependent transcriptional activators in their heterodimeric forms with retinoid X receptors (RXR-alpha, -beta, and -gamma).[24,25] Among RAR and RXR, RAR-beta is thought to function as a tumor suppressor. Previous studies have shown that overexpression of RAR-beta induces growth arrest and apoptosis in several cancer cells.[26,27] In addition, the *RAR-beta* gene is hypermethylated in cancers of the stomach,[27] breast,[28] lung,[29] and head and neck.[30] Although diminished expression of RAR-beta in ESCC has been reported,[31] the DNA methylation status of *RAR-beta* in ESCC is unclear.

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Another key component of retinoid signaling is cellular retinol-binding protein 1 (CRBP1). Retinoic acid is present in the circulation, but most tissues rely on the uptake and cytosolic metabolism of retinoic acid to activate RAR and RXR. CRBP1 possesses high-affinity binding for retinoic acid, possibly functioning as a chaperone-like protein to regulate this prenuclear phase of retinoic acid signaling.[32] The *CRBP1* gene is known to be hypermethylated in various human cancers.[33] However, there are no reports on the role of CRBP1 in ESCC.

Tazarotene-induced gene 1 (*TIG1*) is one of the genes induced by tazarotene, a synthetic retinoid that binds RAR-beta and RAR-gamma.[34] TIG1 may function as a cell adhesion protein, and its expression on the cell surface may lead to increased cell–cell contact and reduced proliferation.[35] The *TIG1* gene is also known to be hypermethylated in various human cancers.[36,37] However, little is known about the role of *TIG1* in ESCC.

In the present study, we examined the methylation status of the *RAR-beta*, *CRBP1*, and *TIG1* genes, and the expression levels of these genes in 28 primary ESCC samples, as well as in samples of corresponding non-neoplastic mucosa. To determine whether hypermethylation causes transcriptional inactivation, we compared the methylation status with the mRNA expression levels of these genes. We also studied the relationship between the *RAR-beta*, *CRBP1*, and *TIG1* genes with respect to methylation status.

Materials and Methods

Tissue samples

Twenty-eight ESCC tissue specimens from 28 patients were analyzed for methylation of the *RAR-beta*, *CRBP1*, and *TIG1* genes. Ten samples of corresponding non-neoplastic mucosa were also analyzed. Total RNA was available for the 28 pairs of cancer tissues and corresponding non-neoplastic mucosa to study expression of these genes. Cancers and corresponding non-neoplastic samples were surgically removed, immediately frozen in liquid nitrogen, and stored at −80°C until use. We confirmed microscopically that the tumor specimens consisted mainly (> 50%) of cancer tissue and that the non-neoplastic samples did not exhibit any tumor cell invasion or significant inflammatory involvement. Tumors were evaluated according to the TNM staging system.[38] Because written informed consent was not obtained, for strict privacy protection, identifying information for all samples was removed before analysis; the procedure was in accordance with the Japanese Government's Ethical Guidelines for Human Genome/Gene Research.

Genomic DNA extraction and methylation analysis

To examine the DNA methylation patterns of the *RAR-beta*, *CRBP1*, and *TIG1* genes, we extracted genomic DNA with a Genomic DNA Purification Kit (Promega, Madison, WI, USA) and performed methylation-specific PCR (MSP).[39] In brief, 2 μg of genomic DNA was denatured by treatment with 2 M NaOH and modified with 3 M sodium bisulfite for 16 h. DNA samples were purified with Wizard DNA Purification Resin (Promega), treated with 3 M NaOH, precipitated with ethanol, and resuspended in 25 μL water. Two-microliter aliquots were used as templates for PCR reactions. The sequences of primers and the annealing temperature for *RAR-beta*, *CRBP1*, and *TIG1* MSP were as described previously (Table 1).[33,36,40] We determined the number of PCR cycles according to the correlation between the mRNA expression and DNA methylation of each gene in gastric cancer cell lines.[41] Hot-start PCR with a total cycle number of 30 was used in all MSP DNA amplifications.

Table 1. Primer sequences for methylation-specific polymerase chain reaction (MSP) and quantitative reverse transcription–polymerase chain reaction (RT-PCR)

Primer sequence	Annealing temperature (°C)
MSP (*RAR-beta*, methylated) F: 5′-GGT TAG TAG TTC GGG TAG GGT TTA TC-3′ R: 5′-CCG AAT CCT ACC CCG ACG-3′	64
MSP (*RAR-beta*, unmethylated) F: 5′-TTA GTA GTT TGG GTA GGG TTT ATT-3′ R: 5′-CCA AAT CCT ACC CCA ACA-3′	55
MSP (*CRBP1*, methylated) F: 5′-TTG GGA ATT TAG TTG TCG TCG TTT C-3′ R: 5′-AAA CAA CGA CTA CCG ATA CTA CGC G-3′	70
MSP (*CRBP1*, unmethylated) F: 5′-GTG TTG GGA ATT TAG TTG TTG TTG TTTT-3′ R: 5′-ACT ACC AAA ACA ACA ACT ACC AAT ACT ACA-3′	67
MSP (*TIG1*, methylated) F: 5′-GCG GGG TTC GGG GAT TTC-3′ R: 5′-GTA CGC GAA CAA ACA AAC G-3′	56
MSP (*TIG1*, unmethylated) F: 5′-GTG GGG TTT GGG GAT TTT GAT-3′ R: 5′-ATA CAC AAA CAA ACA AAC ACA-3′	55
Quantitative RT-PCR (*RAR-beta*) F: 5′-ACC ACT GGA CCA TGT AAC TCT AGT GT-3′ R: 5′-GGC ATC AAG AAG GGC TGG A-3′	60
Quantitative RT-PCR (*CRBP1*) F: 5′-CAA CAG TGA GCT GGG ACG G-3′ R: 5′-GCC ACG CCC CTC CTT C-3′	60
Quantitative RT-PCR (*TIG1*) F: 5′-GGC CGC GCG TGG AT-3′ R: 5′-GGT TGT AGC GCT CTG TGC TG-3′	60
Quantitative RT-PCR (*ACTB*) F: 5′-TCA CCG AGC GCG GCT-3′ R: 5′-TAA TGT CAC GCA CGA TTT CCC-3′	60

F, forward; R, reverse.

Quantitative reverse transcription-PCR analysis

Total RNA was extracted with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and 1 μg of total RNA was converted to cDNA with a First Strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ, USA). PCR was performed with a SYBR Green PCR Core Reagent Kit (Applied Biosystems, Foster City, CA, USA). Real-time detection of the emission intensity of SYBR green bound to double-stranded DNA was performed with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems), as described previously.[42] Primer sequences are listed in Table 1. We calculated the ratio of target gene mRNA expression levels between ESCC tissue (T) and corresponding non-neoplastic mucosa (N). We considered T/N < 0.5 to represent reduced expression. *ACTB*-specific PCR products were amplified from the same RNA samples and served as internal controls.

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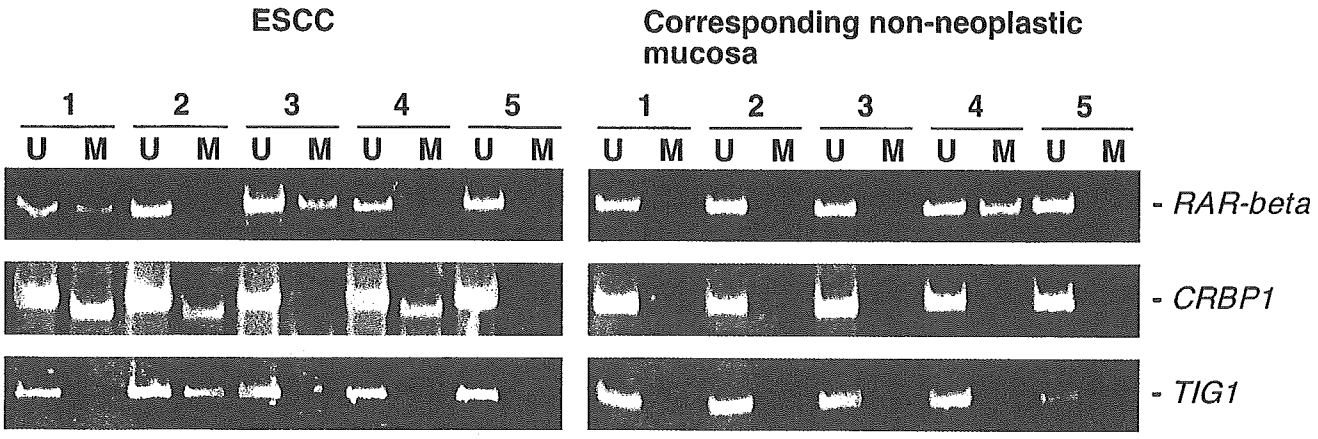

Fig. 1. Methylation-specific polymerase chain reaction (PCR) analysis of *RAR-beta*, *CRBP1*, and *TIG1* genes in esophageal squamous cell carcinoma and corresponding non-neoplastic mucosa. U, unmethylated PCR product; M, methylated PCR product. A methylated allele of the *RAR-beta* gene was detected in samples 1T and 3T. A methylated allele of the *CRBP1* gene was detected in samples 1T, 2T, and 4T. A methylated allele of the *TIG1* gene was detected in sample 2T. In corresponding non-neoplastic mucosa, a methylated allele of the *RAR-beta* gene was detected in sample 4N. Methylated alleles of *CRBP1* and *TIG1* were not detected.

Statistical methods

Statistical analysis was performed by using Fisher's exact test. *P*-values of less than 0.05 were regarded as statistically significant.

Results

Frequencies of *RAR-beta*, *CRBP1*, and *TIG1* methylation in ESCC and corresponding non-neoplastic esophageal mucosa

Representative MSP results for the *RAR-beta*, *CRBP1*, and *TIG1* genes in ESCC tissues and corresponding non-neoplastic samples are shown in Figure 1. Among the 28 ESCC, DNA hypermethylation was detected in seven (25.0%) for *RAR-beta*, five (17.9%) for *CRBP1*, and five (17.9%) for *TIG1*. The overall results are shown in Figure 2. Concordant hypermethylation of *RAR-beta* and *CRBP1* was found in only one ESCC sample, and concordant hypermethylation of *RAR-beta* and *TIG1* was found in only one ESCC sample. There was a tendency toward concordant methylation of *CRBP1* and *TIG1* ($P = 0.0269$, Fisher's exact test; Table 2). Among the 28 ESCC, at least one of the three genes was hypermethylated in 12 (42.9%). We analyzed the relationship between the methylation status of each gene and the clinicopathological factors. There was no correlation between the DNA methylation of *RAR-beta* and clinicopathological data (Table 3). However, DNA methylation of both *CRBP1* and *TIG1* was detected only in ESCC of advanced T grade, N grade, and tumor stage (Tables 4,5). The frequency of DNA methylation of *CRBP1* was significantly higher in stage III ESCC (five of 10, 50.0%) than in stage I/II ESCC (0 of 18, $P = 0.0026$, Fisher's exact test). The frequency of DNA methylation of *TIG1* was also significantly higher in stage III ESCC (five of 10, 50.0%) than in stage I/II ESCC (0 of 18, $P = 0.0026$, Fisher's exact test).

In samples of corresponding non-neoplastic mucosa, DNA methylation of *RAR-beta* was detected in one (10.0%) of 10 samples. On the other hand, the corresponding tumor sample

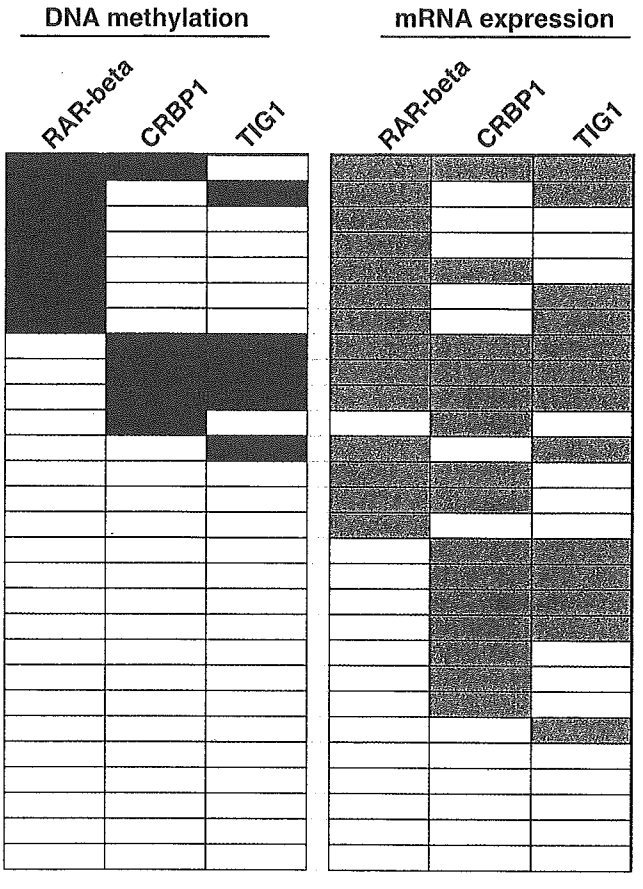

Fig. 2. Summary of DNA methylation and mRNA expression of *RAR-beta*, *CRBP1*, and *TIG1* in esophageal squamous cell carcinoma (ESCC) tissues. DNA methylation of each gene was associated with low expression of the respective mRNA. Concordant hypermethylation of *CRBP1* and *TIG1* was noted. Concordant hypermethylation of *RAR-beta* and *CRBP1* was found in only one ESCC sample, and concordant hypermethylation of *RAR-beta* and *TIG1* was found in only one ESCC sample. Black boxes represent samples with DNA methylation. Gray boxes represent samples with reduced expression.

Table 2. DNA methylation status of retinoic acid signaling-associated genes

		RAR-beta methylation status		*P*-value*
		Methylated	Unmethylated	
CRBP1 methylation status	Methylated	1 (20.0%)	4	1.000
	Unmethylated	6 (26.1%)	17	
TIG1 methylation status	Methylated	1 (20.0%)	4	1.000
	Unmethylated	6 (26.1%)	17	
		CRBP1 methylation status		
		Methylated	Unmethylated	
TIG1 methylation status	Methylated	3 (60.0%)	2	0.0269
	Unmethylated	2 (8.7%)	21	

*Fisher's exact test.

Table 3. Association between DNA methylation and mRNA expression of *RAR-beta* and clinicopathological parameters

		DNA methylation		*P*-value*	mRNA expression		*P*-value*
		M	U		Reduced†	Not reduced	
T grade	T1/2	3 (27.3%)	8	NS	6 (54.5%)	5	NS
	T3	4 (23.5%)	13		8 (47.1%)	9	
N grade	N0	2 (20.0%)	8	NS	2 (20.0%)	8	0.0461
	N1	5 (27.8%)	13		12 (66.7%)	6	
Stage	I/II	4 (22.2%)	14	NS	7 (38.9%)	11	NS
	III	3 (30.0%)	7		7 (70.0%)	3	
Differentiation§	W/M	4 (19.0%)	17	NS	10 (47.6%)	11	NS
	P	3 (42.9%)	4		4 (57.1%)	3	
DNA methylation	M	–	–		7 (100%)	0	0.0058
	U	–	–		7 (33.3%)	14	

*Fisher's exact test. †We considered T (tumor)/N (normal) < 0.5 to represent reduced expression. NS, not significant. §W, well-differentiated; M, moderately differentiated; P, poorly differentiated.

Table 4. Association between DNA methylation and mRNA expression of *CRBP1* and clinicopathological parameters

		DNA methylation		*P*-value*	mRNA expression		*P*-value*
		M	U		Reduced†	Not reduced	
T grade	T1/2	0 (0.0%)	11	NS	3 (27.3%)	8	NS
	T3	5 (29.4%)	12		12 (70.6%)	5	
N grade	N0	0 (0.0%)	10	NS	6 (60.0%)	4	NS
	N1	5 (27.8%)	13		9 (50.0%)	9	
Stage	I/II	0 (0.0%)	18	0.0026	9 (50.0%)	9	NS
	III	5 (50.0%)	5		6 (60.0%)	4	
Differentiation§	W/M	4 (19.0%)	17	NS	13 (61.9%)	8	NS
	P	1 (14.3%)	6		2 (28.6%)	5	
DNA methylation	M	–	–		5 (100%)	0	0.0437
	U	–	–		10 (43.5%)	13	

*Fisher's exact test. †We considered T (tumor)/N (normal) < 0.5 to represent reduced expression. NS, not significant. §W, well-differentiated; M, moderately differentiated; P, poorly differentiated.

(case no. 4) did not show *RAR-beta* methylation. Thus, the origin of this tumor may not be non-neoplastic mucosa with DNA methylation of *RAR-beta*. It is possible that tumor cells may be heterogeneous with regard to aberrant methylation, resulting in a lack of DNA methylation. DNA methylation of *CRBP1* or *TIG1* was not detected (Fig. 1).

MRNA expression of *RAR-beta*, *CRBP1*, and *TIG1* in ESCC

We used quantitative reverse transcription (RT)–PCR analysis to determine whether DNA methylation of the *RAR-beta*, *CRBP1*, and *TIG1* genes affects the expression of the their respective mRNA. Overall results are shown in Figure 2. Reduced expression of *RAR-beta*, *CRBP1*, and *TIG1* was found in 14 (50.0%), 15 (53.6%), and 13 (46.4%) of the 28 ESCC, respectively. Among the 14 ESCC with reduced expression of *RAR-beta*, seven (50.0%) had DNA methylation of *RAR-beta*, whereas of the 14 ESCC without reduced expression of *RAR-beta*, no *RAR-beta* methylation was detected ($P = 0.0058$, Fisher's exact test; Table 3). Reduced expression of *CRBP1* and of *TIG1* mRNAs was also associated with hypermethylation

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Table 5. Association between DNA methylation and mRNA expression of *TIG1* and clinicopathological parameters

		DNA methylation		*P*-value*	mRNA expression		*P*-value*
		M	U		Reduced†	Not reduced	
T grade	T1/2	0 (0.0%)	11	NS	3 (27.3%)	8	NS
	T3	5 (29.4%)	12		10 (58.8%)	7	
N grade	N0	0 (0.0%)	10	NS	5 (50.0%)	5	NS
	N1	5 (27.8%)	13		8 (44.4%)	10	
Stage	I/II	0 (0.0%)	18	0.0026	7 (38.9%)	11	NS
	III	5 (50.0%)	5		6 (60.0%)	4	
Differentiation§	W/M	4 (19.0%)	17	NS	11 (52.4%)	10	NS
	P	1 (14.3%)	6		2 (28.6%)	5	
DNA methylation	M	–	–		5 (100%)	0	0.0131
	U	–	–		8 (34.8%)	15	

*Fisher's exact test. †We considered T (tumor)/N (normal) < 0.5 to represent reduced expression. NS, not significant. §W, well-differentiated; M, moderately differentiated; P, poorly differentiated.

of respective genes ($P = 0.0437$ for *CRBP1*, $P = 0.0131$ for *TIG1*, Fisher's exact test; Tables 4 and 5). Among the 14 ESCC with reduced expression of *RAR-beta*, 12 (85.7%) were positive for lymph node metastasis ($P = 0.0461$, Fisher's exact test). There was no statistically significant association between clinicopathological factors and mRNA expression of *CRBP1* or *TIG1*.

Discussion

In this study, we analyzed the DNA methylation and mRNA expression status of three genes associated with retinoid signaling. DNA methylation of these genes was significantly associated with reduced gene expression, suggesting that DNA methylation plays an important role in transcriptional inactivation of these genes in ESCC. It is important to note that several samples showed reduced mRNA expression in the absence of DNA methylation. Alternative gene-inactivating mechanisms, such as hemizygous deletion or alteration of transcription factors, may account for the reduced gene expression in these samples. The *RAR-beta* gene is located on chromosome 3p24, the *CRBP1* gene is located on chromosome 3q23, and the *TIG1* gene is located on chromosome 3q25. LOH in chromosomes 3p and 3q has been reported in 35% and 30% of ESCC, respectively.[7] Previously, lack of correlation between expression of *RAR-beta* and LOH on 3p24 in ESCC has been reported,[31] thus only LOH on 3p24 does not cause the reduced gene expression of *RAR-beta*. In the present study, because the mRNA expression levels of the *RAR-beta* gene in tumor tissues were correlated with DNA methylation, it is possible that the *RAR-beta* gene may have monoallelic methylation in non-neoplastic tissue and biallelic methylation or monoallelic methylation plus LOH in tumors. High-level gains at 3q25–29 have been reported in ESCC by comparative genomic hybridization.[43]

Reduced expression of *RAR-beta* was detected in 50.0% of ESCC, and half of these cases showed DNA methylation of *RAR-beta*. Although DNA methylation of *RAR-beta* was detected in the corresponding non-neoplastic samples (10.0%), the frequency of methylation in ESCC (25.0%) was higher, suggesting that methylation of the *RAR-beta* gene may contribute to esophageal carcinogenesis. DNA methylation occurs in premalignant and histologically normal squamous epithelium of the esophagus.[19,44] The frequency of *RAR-beta* methylation did not differ significantly between early-stage and late-stage ESCC in this study. However, among the 14 ESCC with reduced expression of *RAR-beta*, 12 (85.7%) were positive for lymph nodes metastasis. A previous study indicated that retinoic acid induces the expression of *nm23-H1*,[45] which is known to reduce cell motility.[46,47] Reduced expression of *RAR-beta* followed by reduced expression of *nm23-H1* may occur frequently in ESCC with lymph node metastasis.

DNA methylation of *CRBP1* and *TIG1* was detected only in late-stage ESCC, and no methylation was detected in corresponding non-neoplastic mucosa, indicating that DNA methylation of these two genes may contribute not to carcinogenesis but to tumor progression. However, reduced expression of both *CRBP1* and *TIG1* was not associated with tumor stage. Therefore, the correlation between DNA methylation of these two genes and tumor stage may be a secondary effect of global changes in chromatin structure. In breast cancer, it has been reported that global DNA hypomethylation occurs during tumor progression.[48] Nevertheless, DNA methylation of both *CRBP1* and *TIG1* may be a marker of tumor progression.

Although concordant hypermethylation of *RAR-beta* and *CRBP1*[33] and of *RAR-beta* and *TIG1*[36] has been reported, there was no such tendency in ESCC in our study. Approximately half of the ESCC in our study had methylated DNA for at least one of the three genes, indicating that alterations of retinoic acid signaling are widely involved in ESCC and that inactivation of *RAR-beta* and *CRBP1* as well as of *TIG1* may not occur synergistically, but rather are random events. In contrast, concordant hypermethylation of *CRBP1* and *TIG1* was observed. Because both *CRBP1* and *TIG1* genes are located on chromosome 3q, it is possible that global DNA methylation effected this change.

In conclusion, our results show that inactivation of the retinoic acid signaling-associated genes *RAR-beta*, *CRBP1*, and *TIG1* due to DNA methylation occurs frequently in ESCC. Because methylated DNA can be induced by demethylating agents,[37] these three genes may be good molecular targets for effective therapeutic strategies for ESCC.