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REG IV overexpression in an early stage of colorectal carcinogenesis: An immunohistochemical study

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Summary. To clarify the role of REG IV, a new member of the regenerating gene (REG) family, in tumorigenesis and progression of colorectal carcinoma (CRC), 320 CRC specimens, 123 corresponding adjacent non-cancerous mucosa (ANCMs), 46 corresponding non-adjacent non-cancerous mucosa (NANCMs) and 86 adenomas were investigated immunohistochemically to compare REG IV expression with clinicopathological features. In addition, double immunofluorescence labeling was performed to analyze the localization of REG IV and the intestinal mucin, MUC2. The expression of REG IV in CRCs was significantly lower than in NANCMs, ANCMs or adenomas, and inversely correlated with poor differentiation and venous invasion. In cases of ANCM, REG IV expression was positively correlated with the depth of invasion, lymph node metastasis and Duke's staging of corresponding cases. The expression of REG IV in CRC was significantly linked to that of MUC2 and the EGFR phosphorylated on Tyr^{1068}, but not to that of MUC5AC, EGFR, Akt, or Akt phosphorylated on Ser^{473} or Thr^{308}. The double immunofluorescence revealed coexpression, but independent localization, of REG IV and MUC2 in NANCMs, ANCMs, adenomas and CRCs, except for mucinous carcinomas. Univariate analysis using the Kaplan-Meier method indicated no correlation between REG IV expression and the cumulative survival rate of CRC patients. In conclusion, REG IV expression was upregulated in ANCMs and adenomas, then decreased in CRCs. This indicated that REG IV overexpression may be an early event in CRC carcinogenesis. Its expression in CRCs was positively linked to MUC2 and phosphorylation of the EGFR on Tyr^{1068}, suggesting that REG IV may be a useful marker for intestinal type mucinous carcinoma and a good candidate as a molecular therapeutic target for CRCs.

Key words: Colorectal carcinoma, REG IV, Biological behavior

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Introduction

The regenerating gene (REG) was first isolated from a cDNA identified as playing a role in the regeneration of the islet of Langerhans in rat islet ß cells following a partial pancreatectomy (Terazono et al., 1988). There are four types of REG genes (type I, II III and IV), they belong to the calcium-dependent lectin (C-type lectin) gene superfamily, and have been identified in animal and human materials. The human REG family contains 4 small secretory proteins, including REG Iα, Iß, III and IV (Lasserre et al., 1994; Hartupee et al., 2001). REG Iα, Iß and III map to a contiguous 140 kb region of chromosome 2p12, each with 6 exons and 5 introns, suggesting evolution from a common ancestral gene (Miyashita et al., 1995; Nata et al., 2004). In contrast, REG IV, isolated from a large inflammatory bowel disease library, resides on chromosome 1q12-q21. Its cDNA contains an open reading frame of 474 bp encoding a peptide of 158 amino acids with a predicted molecular mass of 18.2 kDa. REG IV shows protein sequence identity or similarity to REG Iα of 38%, REG Iß of 39% and REG III of 39% (Hartupee et al., 2001). While their physiological function is not fully

elucidated, they may be linked to regeneration (Terazono et al., 1988), inflammation (Broekaert et al., 2002) and tumorigenesis (Dhar et al., 2004). In humans, REG Iα is strongly expressed in inflamed epithelium, dysplasia and cancerous lesions in ulcerative colitis (UC). REG Iα is inducible by cytokines and its gene product may function as a mitogenic and/or an antiapoptotic factor in the UC-CRC sequence (Sekikawa et al., 2005). The function of REG Iß is not fully understood, except that REG Iß overexpression is related to colorectal carcinogenesis in association with REG Iα and REG III (Rechreche et al., 1999). REG III is involved in hepatic and pancreatic regeneration and proliferation and, in association with REG Iα, is a downstream target of the Wnt pathway during hepatic tumorigenesis (Cavard et al., 2006).

REG IV mRNA is strongly expressed in inflamed epithelium, dysplasia and cancerous lesions in UC tissues. The level of REG IV mRNA expression was correlated with that of bFGF and HGF mRNA expression in UC tissues. Moreover, expression of REG IV in a colon cancer cell line was enhanced by stimulation with TGFα, EGF, bFGF and HGF, suggesting that it is inducible by growth factors and might function as a growth promoting and/or an antiapoptotic factor in the pathophysiology of UC (Nanakin et al., 2007). Immunostaining revealed that 29% of CRC cases were positive for REG IV, and CRC cases with metastatic recurrence in the liver more frequently showed REG IV staining than those without. A significantly worse survival rate was indicated in patients with CRC demonstrating positive REG IV labeling than in those without REG IV staining (Oue et al., 2007). Whereas low REG IV expression was noted in drug-sensitive cultured cancer cells (HT-29), REG IV was strongly overexpressed in drug-resistant cultured colon cancer cells derived from a drug-resistant subpopulation of HT-29, as observed by differential display-PCR (Violette et al., 2003). The addition of recombinant REG IV to cultured colon cancer cells led to a dose-dependent increase in cell number similar to that observed after treatment with EGF. Recombinant REG IV treatment also led to rapid phosphorylation of the EGF receptor on Tyr^{992} and Tyr^{1068} and of Akt on Thr^{308} and Ser^{473}, resulting in increased AP-1 transcription factor activity and subsequent overexpression of c-Jun, JunB, JunD and FosB. In addition, treatment with recombinant REG IV increased the expression of Bcl-2, Bcl-XL, survivin, and matrilysin, genes associated with poor prognosis in advanced CRC (Bishnupuri et al., 2006).

As the major secreted glycoproteins in the gastrointestinal tract, mucins play a role in normal physiological processes and in the neoplastic progression and metastasis of colon cancer cells. MUC2 and MUC5AC belong to the secreted gel-forming mucins and are located on chromosome 11p15.5. The expression of MUC2 is generally decreased in CRC, but preserved in mucinous carcinomas. MUC5AC, a product of normal gastric mucosa, is absent from the normal colon, but frequently present in colorectal adenomas and CRCs (Byrd and Bresalier, 2004). REG IV is also a secreted protein expressed in the gastrointestinal tract. The correlation and co-localization of REG IV and MUC2 have been reported in gastric carcinomas and CRCs (Oue et al., 2005, 2007).

The present study used immunohistochemistry for a large scale investigation of REG IV expression in CRCs, adenomas, ANCMs and NANCMs by tissue microarray, and several whole tissue sections, to explore the role of REG IV in tumorigenesis, progression, and prognosis, and to explore the mechanisms of expression and regulation. Moreover, double immunofluorescence staining was performed to confirm the localization of REG IV and MUC2. The results obtained differ from previous reports.

Materials and methods

Cases

Three hundred and twenty specimens of CRC and 86 of adenoma were collected from the Kouseiren Takaoka Hospital from 1993 to 2002. The adenoma specimens were obtained from endoscopic biopsy and included 76 tubular adenomas, 8 tubulovillous adenomas and 2 villous adenomas. All CRC specimens were surgically resected and 123 specimens of ANCM and 46 of NANCM, at least 3 cm from the cancer lesion, corresponding to each CRC case were obtained. The CRC cases included 176 men and 144 women (mean age, 69.2 years old; range, 18-90 years old). There were 297 well to moderately differentiated adenocarcinoma cases and 23 poorly differentiated or mucinous adenocarcinoma cases. Fifty-six cases were in Duke's Stage A, 134 in Stage B, 79 in Stage C and 51 in Stage D according to Duke's staging system (Zinkin, 1983). One hundred and thirty cases had lymph node metastasis. The histopathological diagnosis was made according to the World Health Organization (WHO) (Hamilton and Aaltonen, 2000). None of the patients underwent chemotherapy or radiotherapy before surgery. All provided consent for the use of tumor tissue for clinical research and the Ethical Committee of Kouseiren Takaoka Hospital and University of Toyama approved the research protocol. Two hundred and seventy-one cases were followed up by consulting case documents and by telephone.

Tissue microarray (TMA)

All specimens were fixed in 10% formalin and embedded in paraffin. The typical lesions of CRC, adenoma, ANCM and NANCM were selected according to hematoxylin and eosin (H&E) staining. Each sample was punched out in 4 mm diameter tissue cores and transferred to a recipient block with a maximum of 24 cores using a Tissue Microarray Instrument (AZUMAYA

KIN-1, Japan). The tissue microarray (TMA) consisted of CRC, adenoma, ANCM and NANCM specimens.

Immunohistochemistry

Four μm sections were consecutively cut from the TMAs and some whole lesion tissues containing cancer and adjacent mucosa. After routine deparaffinization, antigen retrieval was carried out by microwave treatment in the target retrieval solution (DAKO, CA, USA) for 15 min. Subsequently, the sections were immersed in 3% hydrogen peroxide for 5 min to block endogenous peroxidase activity and incubated in 5% bovine serum albumin (BSA) for 30 min to block non-specific antibody binding sites. The immunohistochemistry was performed with intermittent microwave radiation as previously described (Kumada et al., 2004). Primary antibodies, source and working dilution are listed in Table 1. Anti-goat IgG, anti-mouse IgG or anti-rabbit IgG (1:100, DAKO, CA, USA) were used as secondary antibodies to detect the respective primary antibodies. After each treatment, the sections were washed three times with TBST (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20) for 1 min each rinse. Binding was visualized with 3,3'-diaminobenzidine (DAB) and counterstaining with Mayer's hematoxylin was performed to aid orientation. The omission of the primary antibody was used as a negative control.

Immunoreactivity to REG IV, MUC2, MUC5AC, EGFR, Akt and Ser^{473}-phosphorylated Akt was localized in the cytoplasm. However, the Tyr^{1068}-phosphorylated EGFR and Thr^{308}-phosphorylated Akt were expressed in the nuclei. Each section was observed by two independent observers (Y Takano and XH Li) and the percentage of positive cells was graded semi-quantitatively using a four-tier scoring system: negative (-), 0-5%; weakly positive (+), 6-25%; moderately positive (++), 26-50%; and strongly positive (+++), 51-100%.

Double immunofluorescence staining

Double immunofluorescence staining was used to analyze the localization of REG IV and MUC2 in NANCM, ANCM, adenoma, and CRC. Deparaffinized tissue sections were initially treated the same as for immunohistochemical staining. Then, the sections were incubated with mixed primary antibodies REG IV (1:50, R&D Systems, MN, USA) and MUC2 (1:100, Novocastra, UK) at 4°C overnight. The immune complexes were detected by incubating with a mixture of Alexa Fluor 594-conjugated donkey anti-goat IgG and Alexa Fluor 488-conjugated donkey anti-mouse IgG (1:500, Invitrogen Corporation, CA, USA) for 1 hour. After each treatment, the slides were washed with TBST three times for 1 min. Finally, the sections were mounted with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, CA, USA). The omission of the primary antibody was used as a negative control. The staining results were observed by fluorescence microscopy (Olympus AX80, Japan).

Statistical analysis

The statistical evaluation was performed using the Spearman correlation test to analyze the rank data. Kaplan-Meier survival plots were generated and comparisons between survival curves were made with the log-rank statistic. The Cox proportional hazards model was employed for the multivariate analysis. $p<0.05$ was considered to be statistically significant. The SPSS 10.0 software program was employed to analyze all data.

Results

REG IV expression in NANCMs, ANCMs, adenomas, and CRCs

REG IV was mostly observed in the cytoplasm with goblet cell-like staining. Perinuclear expression was found in NANCMs and ANCMs, but seldom in adenomas and CRCs. Twenty-four of 46 NANCM specimens (52.2%) showed weakly to moderately positive staining with REG IV (Fig. 1a). All of the 130 ANCM specimens (100%) demonstrated intense staining with REG IV (Fig. 1b). In adenomas, REG IV staining was recognized in 81 of 86 specimens (95.3%) (Fig. 1c). Only five low-grade tubular adenoma specimens showed no immunoreactivity to REG IV. It was expressed with

Table 1. List of primary antibodies for the immunohistochemical study.

Antibody	Clone	source	Company	Dilution
REG IV	polyclonal	goat	R&D Systems, MN, USA	1:50
MUC2	monoclonal	mouse	Novocastra, UK	1:100
MUC5AC	monoclonal	mouse	Novocastra, UK	1:100
EGFR	polyclonal	rabbit	Santa cruz, CA, USA	1:50
phosphorylated EGFR on Tyr^{1068}	monoclonal	rabbit	Epitomics, CA,USA	1:50
Akt	polyclonal	rabbit	Rockland, PA, USA	1:300
phosphorylated Akt on Thr^{308}	polyclonal	rabbit	Santa cruz, CA, USA	1:100
phosphorylated Akt on Ser^{473}	polyclonal	rabbit	Rockland, PA, USA	1:100

stronger intensity and at a higher rate in ANCMs than in NANCMs or CRCs (p<0.001, Table 2). Ninety-nine of 320 specimens (30.9%) were positive for REG IV in CRCs (Fig. 1d, e). The REG IV expression in CRCs was significantly lower than in NANCMs (p=0.043), ANCMs, or adenomas (p<0.001, Table 2). In addition, the expression of REG IV was detected in some whole sections (Fig. 2a). REG IV expression gradually increased from the mucosa most distant from the cancer lesion (Fig. 2b) to the mucosa closest to the cancer lesion (Fig. 2c) or dysplastic lesion (Fig. 2d), and then decreased in invasive CRC (Fig. 2e).

Table 2. Relationship of REG IV expression in NANCMs, ANCMs, adenomas and CRCs.

Groups	N	REG IV expression				
		-	+	++	+++	PR(%)
NANCMs	46	22	20	4	0	52.2
ANCMs	123	0	10	61	52	100*
Adenomas	86	5	17	33	31	95.29**
CRCs	320	221	50	30	19	30.94***

PR: positive rate *in comparison to NANCMs (p<0.001), adenomas (p=0.029), and carcinomas (p<0.001) **in comparison to NANCMs and carcinomas, p<0.001 ***in comparison to NANCMs (p=0.043), ANCMs, and adenomas, p<0.001.

The relationship between REG IV expression and clinicopathological features

REG IV expression in CRCs was inversely correlated with poor differentiation (p<0.001) and venous invasion (p=0.011), but not with age, sex, tumor size, depth of invasion, lymphatic invasion, lymph node metastasis or Duke's staging (p>0.05). All mucinous adenocarcinomas and signet–ring cell carcinomas showed stronger REG IV expression than other adenocarcinomas (p<0.001, Table 3).

As summarized in Table 4, REG IV expression in ANCMs was significantly correlated with depth of invasion (p=0.034), lymph node metastasis (p=0.013) and Duke's staging (p=0.016), but not with age, sex,

Table 3. Relationship between REG IV expression and clinicopathological features in CRCs.

Clinicopathological features	N	REG IV expression in CRCs					
		-	+	++	+++	PR(%)	*p* value
Age							0.102
<65	119	76	22	13	8	36.13	
≥ 65	201	145	28	17	11	27.86	
Sex							0.608
male	176	120	26	19	11	31.82	
Female	144	101	24	11	8	29.86	
Tumor size (cm)							0.847
≤ 5	180	123	29	20	8	31.67	
>5	140	98	21	10	11	30	
Depth of invasion							0.064
T_{is}~T_1	22	13	4	3	2	40.90	
T_2~T_4	298	208	46	27	17	30.20	
Differentiation							<0.001
well	157	103	25	20	9	34.39	
moderate	140	107	22	5	6	23.57	
poor	12	11	1	0	0	8.33	
Mucinous	11	0	2	5	4	100	
Lymphatic invasion							0.466
-	226	153	36	25	12	32.30	
+	94	68	14	5	7	27.66	
Venous invasion							0.011
-	271	180	44	29	18	33.58	
+	49	41	6	1	1	16.33	
Lymph node metastasis							0.060
-	190	123	33	21	13	35.26	
+	130	98	17	9	6	24.62	
Duke's staging							0.055
A~B	190	122	34	21	13	35.78	
C~D	130	99	16	9	6	23.85	

PR=positive rate; T_{is}: carcinoma in situ; T_1: invades submucosa; T_2: invades muscularis propria; T_3: invades through the muscularis propia into the subserosa.; T_4: directly invades other organs or structures.

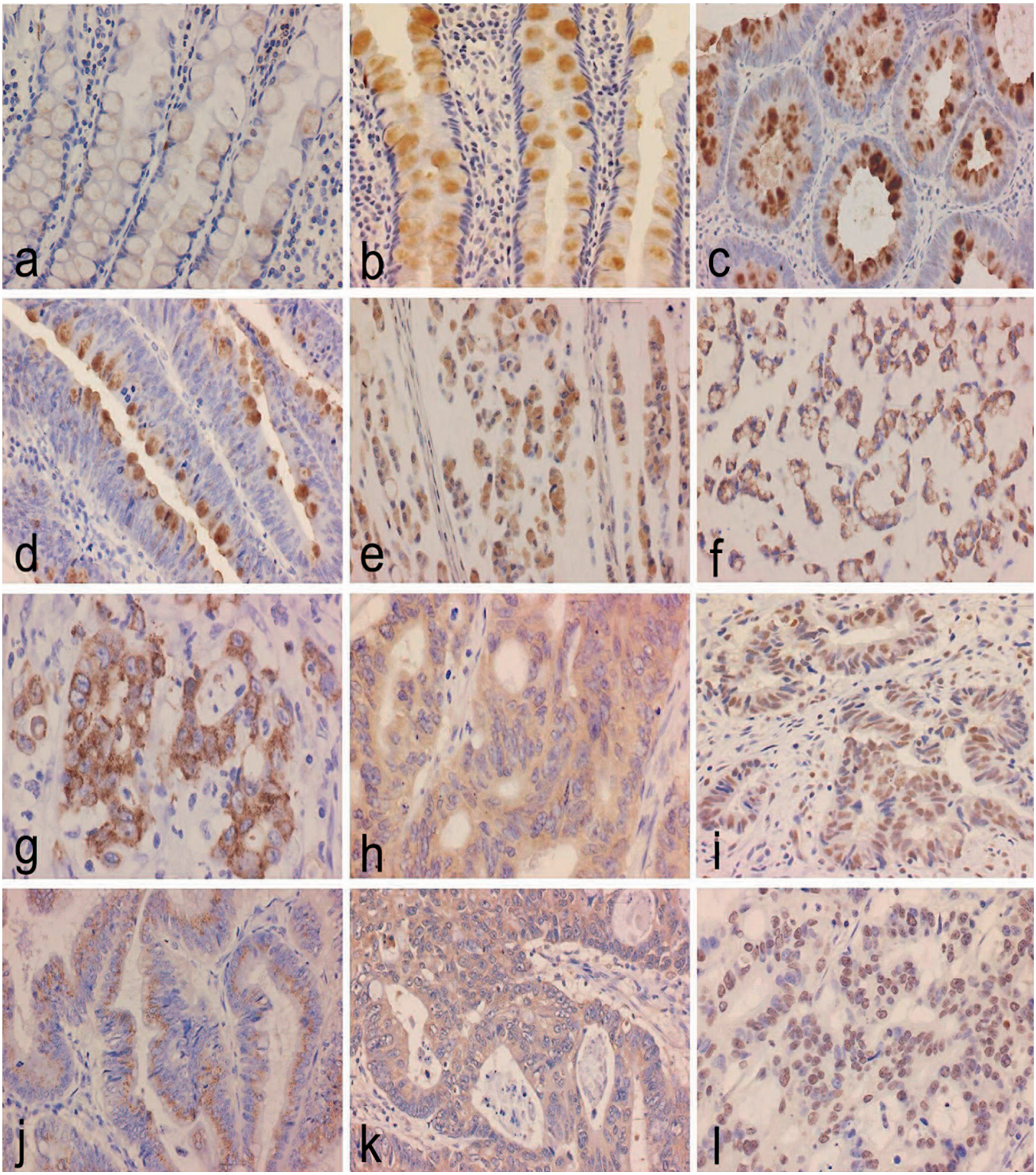

Fig. 1. Immunohistochemical staining of REG IV and other molecular markers. Note: Positive REG IV staining was observed in goblet-like vesicles in NANCM **(a)**, ANCM **(b)**, adenoma **(c)**, well-differentiated adenocarcinoma **(d)**, and signet-ring cell carcinoma **(e)**. The expression of MUC2 **(f)**, MUC5AC **(g)**, EGFR **(h)**, Akt **(j)** and Ser^{473}-phosphorylated Akt **(k)** protein were found in the cytoplasm of tumor cells. However, the EGFR phosphorylated on Tyr^{1068} **(i)** and Akt phosphorylated on Thr^{308} **(l)** were positively expressed in the nucleus.

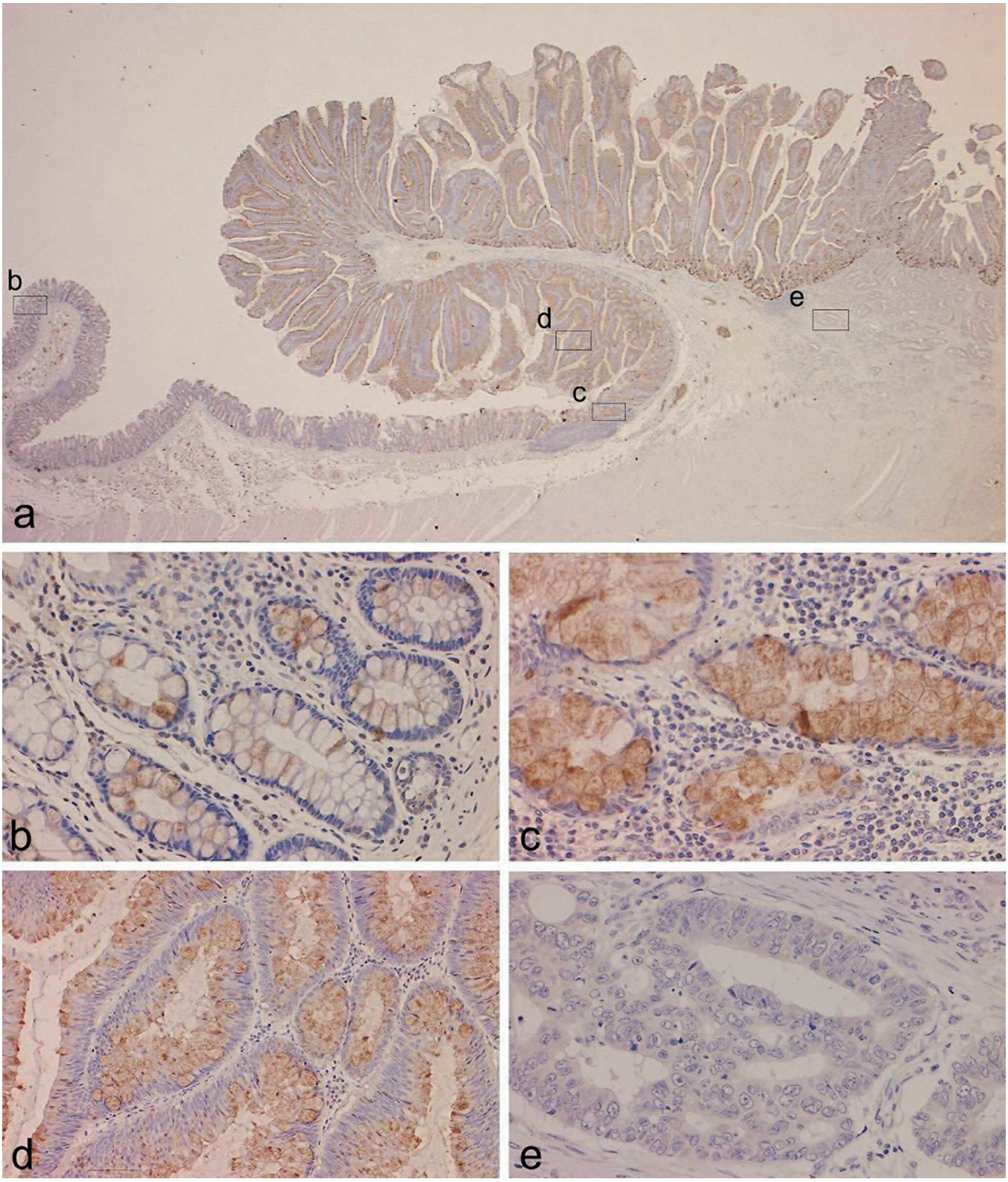

Fig. 2. Immunohistochemical staining of REG IV in whole tissue sections Note: The immunohistochemical staining of REG IV in whole tissue sections under low magnification **(a)**. High-magnification images of the fields indicated by boxes showed that the expression of REG IV gradually increased from the mucosa most distant from the cancer lesion **(b)** to the mucosa close to the cancer lesion **(c)** and dysplastic lesion **(d)**, then decreased in invasive CRC **(e)**.

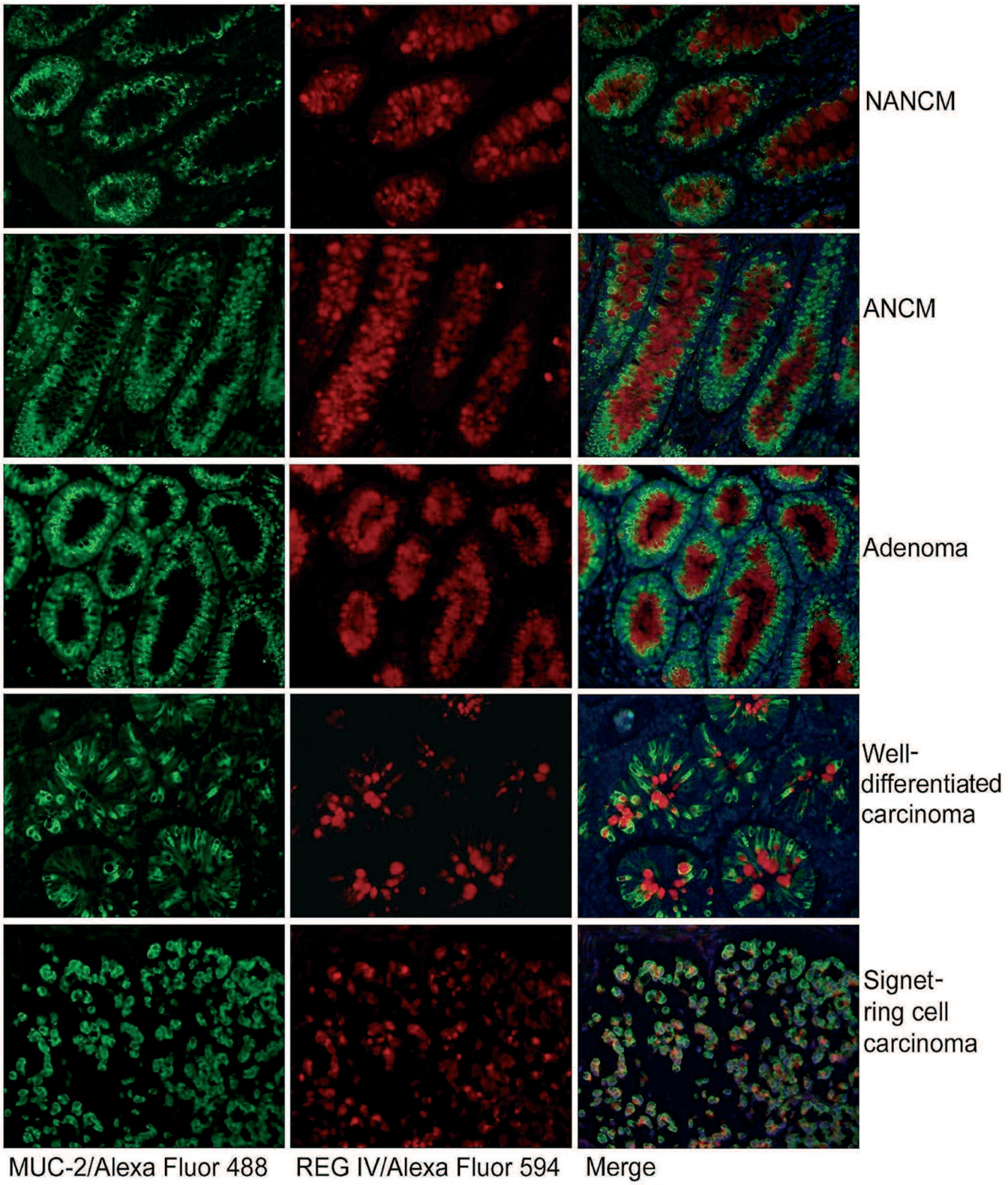

Fig. 3. Double fluorescence immunohistological staining REG IV and MUC2 in NANCM, ANCM, adenoma, well differentiated carcinoma and signet-ring cell carcinoma. Note: The REG IV protein was stained with red and MUC-2 with green. After the images were merged, the co-localization of both proteins was observed in orange.

Table 4. Relationship between REG IV expression in ANCMs and clinicopathological features of CRCs .

Clinicopathological features	N	REG IV expression in ANCMs				
		-	+	++	+++	*p* value
Age						0.648
<65	49	0	3	27	19	
≥ 65	72	0	7	32	33	
Sex						0.089
male	67	0	7	32	28	
Female	56	0	3	29	24	
Tumor size (cm)						0.535
≤ 5	63	0	4	31	28	
>5	59	0	5	30	24	
Depth of invasion						0.034
T_{is}~T_1	16	0	2	11	3	
T_2~T_4	105	0	7	49	49	
Differentiation						0.277
well	57	0	5	31	21	
moderate	54	0	4	25	25	
poor	7	0	1	3	3	
Mucinous	5	0	0	2	3	
Lymphatic invasion						0.217
-	83	0	7	38	38	
+	40	0	3	23	14	
Venous invasion						0.588
-	101	0	7	51	43	
+	22	0	3	10	9	
Lymph node metastasis						0.013
-	69	0	8	38	23	
+	54	0	2	23	29	
Duke's staging						0.016
A~B	69	0	8	37	24	
C~D	54	0	2	24	28	

T_{is}: carcinoma in situ; T_1: invades submucosa; T_2: invades muscularis propria; T_3: invades through the muscularis propia into the subserosa. T_4: directly invades other organs or structures.

tumor size, differentiation, lymphatic invasion or venous invasion ($p>0.05$).

Correlation of expression between REG IV and MUC2, MUC5AC, Akt and the EGFR

Expression of MUC2, MUC5AC, EGFR, Akt, and Akt phosphorylated on Ser^{473} was observed in the cytoplasm of tumor cells (Fig. 1f-h,j,k). The EGFR phosphorylated on Tyr^{1068} and Akt phosphorylated on Thr^{308} were demonstrated in the nuclei of tumor cells (Fig. 1i,l). REG IV expression was positively correlated with MUC2 ($p<0.001$) and the Tyr^{1068}-phosphorylated EGFR ($p=0.025$), but not with MUC5AC, EGFR, Akt, or Akt phosphorylated on Ser^{473} or Thr^{308} (Table 5). In addition, all molecular markers were positively expressed with different intensity in ANCMs, except MUC5AC. No MUC5AC immunoreactivity was observed in any ANCMs specimen. However, the expression of REG IV in ANCMs was not statistically correlated with the expression of any molecular marker (Table 6).

Double immunofluorescence labeling of REG IV and MUC2

Double immunofluorescence labeling revealed the independent localization of REG IV and MUC2 in ANCMs, NANCMs, adenomas and CRCs, but not in signet-ring cell carcinomas (Fig. 3). Although co-expressed in the cytoplasm, REG IV was mainly

Table 5. Relationship between REG IV expression and MUC2, MUC5AC, Akt and the EGFR in CRCs.

Clinicopathological markers	N	REG IV expression in CRCs					
		-	+	++	+++	PR(%)	*p* value
MUC2							<0.001
-	126	108	10	5	3	14.28	
+~+++	190	110	40	24	16	42.10	
MUC5AC							0.884
-	263	183	42	23	15	30.42	
+~+++	52	35	8	6	3	32.70	
EGFR							0.568
-	57	39	6	5	7	31.58	
+~+++	262	182	44	25	11	30.53	
phosphorylated EGFR on Tyr^{1068}							0.025
-	161	122	18	10	11	24.22	
+~+++	158	99	32	20	7	37.34	
Akt							0.275
-	116	78	13	13	12	32.76	
+~+++	200	141	37	16	6	29.5	
phosphorylated Akt on Ser^{473}							0.207
-	87	57	13	8	9	34.48	
+~+++	229	161	37	22	9	29.69	
phosphorylated Akt on Thr^{308}							0.566
-	9	7	1	1	0	22.22	
+~+++	309	214	48	29	18	30.74	

PR: positive rate

localized in the cytoplasmic periphery, while MUC2 was localized to the perinuclear region. The localization of these molecules overlapped only in signet-ring cell carcinomas.

Univariate and multivariate survival analysis

Follow-up information was available for 271 CRC patients for periods ranging from 0.9 months to 12.1 years (mean=66.8 months). Fig. 4 showed survival curves according to REG IV expression in the CRCs and corresponding ANCMs. Univariate analysis using the Kaplan-Meier method indicated no statistical significance between REG IV expression and the cumulative survival rate of patients. Multivariate analysis using Cox's proportional hazard model suggested that lymphatic invasion, venous invasion, and lymph node metastasis were independent prognostic factors for overall CRCs ($p<0.05$), while age, sex, tumor size, depth of invasion, Duke's staging, and the expression of REG IV were not (Table 7).

Table 6. Relationship between expression of REG IV and MUC2, Akt and the EGFR in ANCMs.

Clinicopathological markers	N	REG IV expression in ANCMs				
		-	+	++	+++	*p* value
MUC2						0.758
-	18	0	2	8	8	
+~+++	105	0	8	53	44	
EGFR						0.334
-	80	0	7	42	31	
+~+++	43	0	3	19	21	
phosphorylated EGFR on Tyr^{1068}						0.460
-	75	0	8	37	30	
+~+++	48	0	2	24	22	
Akt						0.489
-	69	0	7	40	22	
+~+++	54	0	3	21	30	
phosphorylated Akt on Ser^{473}						0.486
-	98	0	9	48	41	
+~+++	25	0	1	13	11	
phosphorylated Akt on Thr^{308}						0.306
-	38	0	2	18	18	
+~+++	85	0	8	43	34	

Table 7. Multivariate analysis of clinicopathological features for survival with CRCs.

Clinicopathological parameters	Relative risk (95%CI)	*p* value
Age (≥ 65years)	0.808 (0.329-1.982)	0.242
Sex	0.868 (0.395-1.906)	0.596
Tumor size (>5)	1.064 (0.458-2.472)	0.485
Depth of invasion (into muscularis propria)	1.968 (0.233-16.628)	0.296
Differentiation (poor and mucinous)	1.218 (0.767-1.934)	0.566
Lymphatic invasion (+)	2.446 (1.081-5.535)	0.032
Venous invasion (+)	3.079 (1.270-7.466)	0.013
Lymph node metastasis (+)	3.191 (1.436-7.091)	0.004
Duke's staging (C~D)	1.291 (0.288-5.786)	0.137
REG IV expression in CRCs (+~+++)	0.723 (0.311-1.680)	0.667

Discussion

In the present study, REG IV was expressed in 99 of 320 CRC specimens (30.9%) and its expression was inversely related to poor differentiation and venous invasion. A previous study noted a positive REG IV rate of 23/80 (29%) in CRCs, with no correlation between REG IV staining, depth of invasion, lymph node

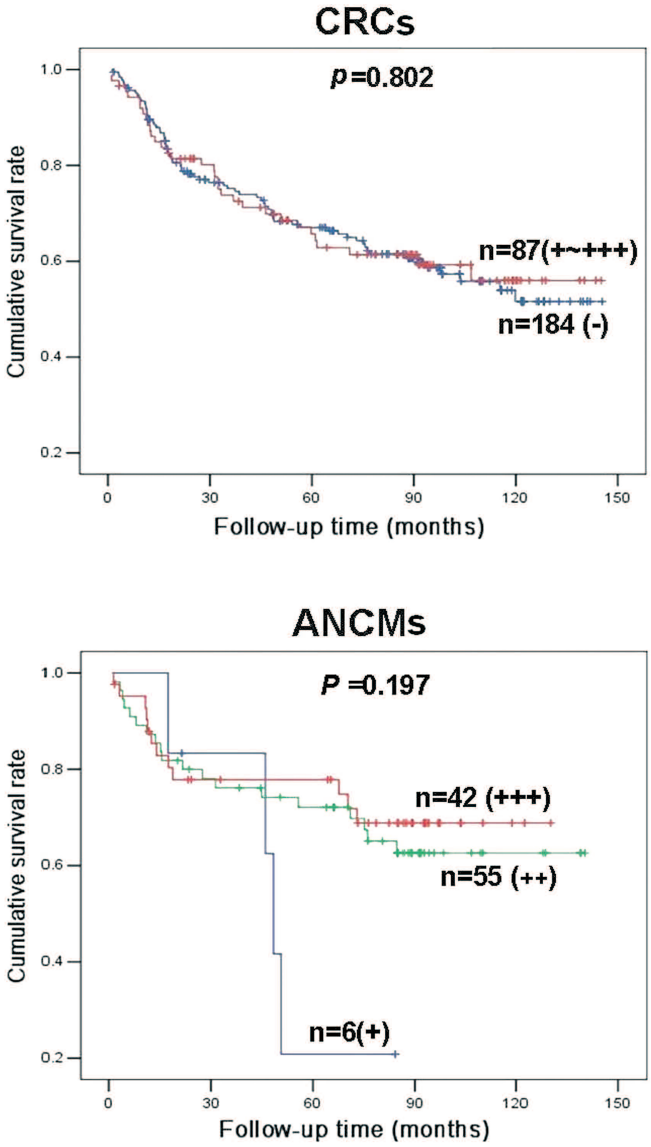

Fig. 4. Correlation between the expression of REG IV and prognosis of the CRC patients. Kaplan-Meier curves for cumulative survival rate of patients according to REG IV expression in whole CRCs and ANCMs.

metastasis, or tumor stage (Oue et al., 2007). A commercial goat antibody for REG IV (R&D systems; 1:50) was used as the primary antibody at the same concentration in both the current and previous study. The positive rate and staining pattern of REG IV was similar in both studies; however, the correlation between positive REG IV labeling and clinicopathological variables was quite different. In the present study, positive staining was defined as >5% of cancer cells being stained, while the prior study used 10% for the cutoff value in CRCs. Furthermore, although mucinous adenocarcinomas and signet-ring cell carcinomas are considered poorly differentiated by convention, we found that REG IV was positively expressed in all mucinous carcinomas and signet-ring cell carcinomas, but seldom expressed in other poorly differentiated carcinomas. This means that the rate of positive REG IV labeling in poorly differentiated carcinomas is decided by the proportion of the former two types of carcinoma. When divided into two groups, we found that REG IV expression was inversely correlated with tumor differentiation. Because REG IV is a secreted protein, we postulated that the decrease of REG IV expression could be a sign that the tumor cell has lost the ability of differentiation. In the previous study, follow up was possible in 30 cases, including stages II and III (equal to Duke B and C), and the patients demonstrating positive expression of REG IV had significantly worse survival than those that were REG IV negative (Oue et al., 2007). The present study followed up on 271 cases that ranged from Duke stage A to D, but no statistical correlation was found between positive REG IV labeling and poor prognosis. Therefore, it is suggested that REG IV is not an appropriate marker for the prognosis of CRC. It is well known that the immunostaining signal is not usually homogeneous in cancerous tissues. Even though the TMA is a very powerful method for examining a large number of tissues simultaneously, it is very important to select a representative lesion to ensure reliable results. With this in mind, we used typical lesions without haemorrhage or necrosis according to the histopathological diagnosis to evaluate the expression of REG IV.

REG IV expression was up-regulated in ANCMs and adenomas, but reduced in CRCs in the present study. Moreover, positive REG IV labeling in ANCMs was linked to cancer progression. The expression of REG IV was detected in some whole tissue sections, including mucosa distant from the cancer lesion, mucosa near the cancer lesion, dysplastic lesion, and invasive CRC. All displayed the same result. In the previous study, extensive positive REG IV staining was also observed in all pericancerous mucosa samples of CRCs and REG IV, and it gradually decreased with increasing distance from the cancer lesion (Oue et al., 2007). Violette et al. reported that normal colon specimens contained less REG IV mRNA than most cancers, as detected by northern blot and real-time PCR analysis (Violette et al., 2003). In aberrant crypt foci (ACF), one of the earliest identifiable preneoplastic colonic lesions, the overexpression of the REG IV gene was evaluated by array hybridization and real-time RT-PCR versus normal mucosa (Cipolletta et al., 2009). REG IV mRNA overexpression was observed in colon adenomas by semi-quantitative RT-PCR and in situ hybridization (Zhang et al., 2003). In the present study, normal mucosa was not available because of the difficulty in obtaining normal mucosa, since the clinical samples were usually taken because of disease and/or symptoms. Neither ANCMs nor NANCMs are precisely normal. The fact that positive REG IV expression in ANCMs was closely linked to cancer progression is a novel finding in the present study. However, overexpression of REG IV in ANCMs was not statistically correlated with expression of the other suspicious molecular markers. Thus far, no scientific evidence has provided a logical explanation for this phenomenon. The expression of REG IV may reflect the potential proliferative ability as the background of the cancer lesion. ANCMs show promise as a new research subject for tumorigenesis and progression of CRC. Taken together, REG IV expression is an early event in colorectal carcinogenesis, similar to its expression in ulcerative colitis (Nanakin et al., 2007).

The expression of REG IV in CRC correlated with expression of MUC2, but not MUC5AC. Double immunofluorescence labeling confirmed that REG IV and MUC2 were coexpressed but independently localized in NANCMs, ANCMs, adenomas, and CRCs other than mucinous carcinomas. A previous study also reported coexpression of REG IV and MUC2 in non-cancerous cells and cancer cells (Oue et al., 2007). MUC2 is referred to as the intestinal type because its expression is normally limited to goblet cells, where it contributes to the protective barrier function of these cells (Levi et al., 2004). MUC2 is strongly expressed in normal colonic mucosa but its expression is decreased in CRCs, though MUC2 is expressed in mucinous carcinoma (Byrd and Bresalier, 2004). The mechanism of REG IV and MUC2 coexpression is not clear. Mucinous CRC is associated with microsatellite instability (MSI). MSI may directly influence mucus production by altering the genes involved in mucin synthesis or degradation (Messerini et al., 1997). In gastric carcinomas, the expression of REG IV correlated with Cdx2, a mammalian caudal-related intestinal transcription factor, suggests that Cdx2 may regulate transcription of the REG IV gene (Oue et al., 2005). It has been reported that Cdx2 interacts with the MUC2 promoter and activates MUC2 transcription (Yamamoto et al., 2003). Cdx2 may be involved in the regulation of REG IV and MUC2 expression. The coexpression of REG IV and MUC2 indicated that the expression of REG IV was associated with the intestinal mucin phenotype and may be a good marker for mucinous carcinoma.

Although the biological function of REG IV is poorly understood, REG IV is a potent activator of the epidermal growth factor receptor (EGFR)/Akt/ activator

protein-1 (AP-1) signaling pathway in colon cancer cells and increases the expression of Bcl-2, Bcl-xl and survivin proteins associated with the inhibition of apoptosis (Bishnupuri et al., 2006). The present study used immunohistochemistry to examine the correlation between expression of REG IV and Akt, Akt phosphorylated on Ser^{473} or Thr^{308}, EGFR, and the EGFR phosphorylated on Tyr^{1068}, according to the results of the previous reports. REG IV expression was significantly correlated with Tyr^{1068}-phosphorylated EGFR, but not with other molecular markers. The results may differ because of varying intratumoral or intracellular conditions. The cells in the tumor tissue are often exposed to various extratumoral stress factors, such as the potency of the immunological response of the host, hypoxia or cytotoxic treatment, which may effect the regulation of REG IV. The EGFR plays an important role in regulating epithelial proliferation, differentiation and survival. It is also considered to be a major target for treatment of CRC. The EGFR phosphorylated on Tyr^{1068} may be a better tool than the non-phosphorylated EGFR to select patients for targeted therapies (Piazzi et al., 2006). Based on a systematic study across low passage pancreatic cancer cell lines and mice carrying pancreatic cancer xenografts, Harsha et al. proposed that activated EGFR phosphorylated on Tyr^{1068} was an attractive candidate for targeted therapy in a subset of pancreatic cancers (Harsha et al., 2008). Recently, the correlation between REG IV expression and EGFR phosphorylated on Tyr^{1068} was also verified in prostate cancer by immunohistochemical staining and western blotting (Ohara et al., 2008). It was suggested that REG IV expression could activate phosphorylation of the EGFR on Tyr^{1068} in CRC, therefore, REG IV may be a new potential candidate molecular therapeutic target for CRC.

In conclusion, REG IV expression was up-regulated in ANCMs, adenomas and decreased in CRCs, indicating that its expression may be an early event in CRC carcinogenesis. Moreover, the expression of REG IV in ANCMs was correlated with cancer progression. REG IV expression was linked to MUC2 and phosphorylation of the EGFR at Tyr^{1068} in CRCs, suggesting that REG IV may be a useful marker for intestinal type mucinous carcinoma and a good candidate for a molecular therapeutic target for CRCs.

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