cancerous tissues (Oue et al., 2005). We reported previously that Reg IV is expressed in GC cells but not stromal cells. Reg IV was expressed in 30% of GC tissues and was associated with both the intestinal mucin phenotype and neuroendocrine differentiation. In CRC, expression of Reg IV was observed in 36% of cases and was associated with tumor stage (Oue et al., 2005). Furthermore, because it is a secreted protein, Reg IV may be a serum biomarker for GC; however, the concentration of Reg IV in serum has not been investigated.

The biologic function of Reg IV is poorly understood. Involvement of *REG4* in drug resistance has been suggested, but the detailed mechanism remains unclear (Violette et al., 2003). A more recent study revealed that Reg IV is a potent activator of the epidermal growth factor receptor (EGFR)/Akt/activator protein-1 (AP-1) signaling pathway and those colon cancer cell lines treated with recombinant Reg IV showed increased expression of Bcl-2, Bcl-xl and survivin, which are proteins associated with inhibition of apoptosis (Bishnupuri et al., 2006). EGFR activation modulates apoptotic susceptibility (reviewed by Kari et al., 2003), and we have shown that EGFR is overexpressed in GC (Yasui et al., 1988). Taken together, Reg IV may be a marker for prediction of resistance to 5-FU-based chemotherapy; however, modulation of apoptotic susceptibility by Reg IV has not been investigated.

In the present study, we show that forced expression of Reg IV inhibits apoptosis induced by 5-FU. Several molecules associated with resistance to 5-FU have been identified (reviewed by Longley et al., 2003). We investigated expression of molecules associated with resistance to 5-FU in Reg IV-overexpressing cells. Because Reg IV activates EGFR, we also performed immunohistochemical analysis of Reg IV and EGFR expression in 161 cases of GC. We measured Reg IV levels in sera from patients with GC by enzyme-linked immunosorbent assay (ELISA) to investigate the potential utility of Reg IV measurements in the diagnosis of GC.

Results

Forced expression of Reg IV inhibits the mitochondrial apoptotic pathway

To investigate the biologic significance of Reg IV, the TMK-1 GC cell line was stably transfected with vector expressing Reg IV. TMK-1 cells were selected because they express low levels of Reg IV (Oue et al., 2005). Clones were selected in G418 and examined for Reg IV expression by Western blot. Two clones, TMK-1-Reg IV-1 and TMK-1-Reg IV-2, expressed Reg IV at significantly higher levels than TMK-1 cells transfected with empty vector (Figure 1a). To determine the effect of Reg IV on 5-FU treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays were performed. Cell growth of TMK-1 cells transfected with empty vector was inhibited by 5-FU in a dose-dependent manner (Figure 1b). This inhibition was partially ameliorated in Reg IV-overexpressing cells (Figure 1b), suggesting that cell proliferation was activated or apoptosis was inhibited in Reg IV-overexpressing cells. We investigated the effect of Reg IV on cell proliferation activity. Reg IV transfectants did not show significant differences in proliferation activity compared with cells transfected with empty vector (data not shown). We next examined the effect of forced Reg IV expression on the apoptotic susceptibility of these cells to 5-FU. As shown in Figure 1c, overexpression of Reg IV in both TMK-1-Reg IV-1 and TMK-1-Reg IV-2 cells significantly inhibited 5-FU-induced apoptosis in comparison with cells transfected with empty vector.

Apoptosis is controlled by two major pathways, the mitochondrial pathway (Green and Reed, 1998) and the membrane death receptor (DR) pathway (Ashkenazi and Dixit, 1999). In the mitochondrial pathway, release of cytochrome *c* by mitochondria into the cytosol is the rate-limiting step for the activation of caspases and endonucleases (Martinou et al., 2000). Cytosolic cytochrome *c* activates procaspase-9 by binding to Apaf1 in the presence of dATP, leading to caspase-9 activation and subsequent activation of downstream effector caspases, including caspase-3, with triggering of apoptosis (Li et al., 1997). Caspase-8 plays an important role in the DR-mediated apoptotic pathway, which is independent of cytochrome *c* release (Ashkenazi and Dixit, 1999). In our previous study, the mitochondrial apoptotic pathway was activated in 5-FU-induced apoptosis in TMK-1 cells (Tahara et al., 2005). To determine the associated pathway inhibited by Reg IV overexpression, we examined expression of cytosolic cytochrome *c* protein in cytosolic extracts of 5-FU-treated and untreated cells by Western blotting. Incubation of cells with 5-FU induced cytochrome *c* expression in empty vector-transfected cells (Figure 1d). Cytochrome *c* release was inhibited in cells overexpressing Reg IV (Figure 1d). Next, we examined the activities of caspase-3, -8 and -9. As shown in Figure 1e, treatment of cells with 5-FU significantly increased caspase-9 and -3 activities; but had no effect on caspase-8 activity. The activities of caspase-9 and -3 were significantly lower in Reg IV-overexpressing cells than in empty vector-transfected cells. The nuclear DNA repair enzyme poly(ADP-ribose)polymerase (PARP) is a target of caspase-3, and its cleavage can serve as a biochemical marker of apoptosis (Kaufmann et al., 1993). We examined whether 5-FU-induced apoptosis is associated with PARP cleavage by Western blotting. Cleaved PARP was detected in 5-FU-treated empty vector-transfected cells; however, cleavage of PARP was reduced in Reg IV-overexpressing cells (Figure 1d). These results indicate that overexpression of Reg IV suppresses 5-FU-induced apoptosis by inhibiting the mitochondrial apoptotic pathway.

Reg IV activates phosphorylation of EGFR

Recombinant human Reg IV has been shown to induce rapid phosphorylation of EGFR at Tyr^{992} and Tyr^{1068}

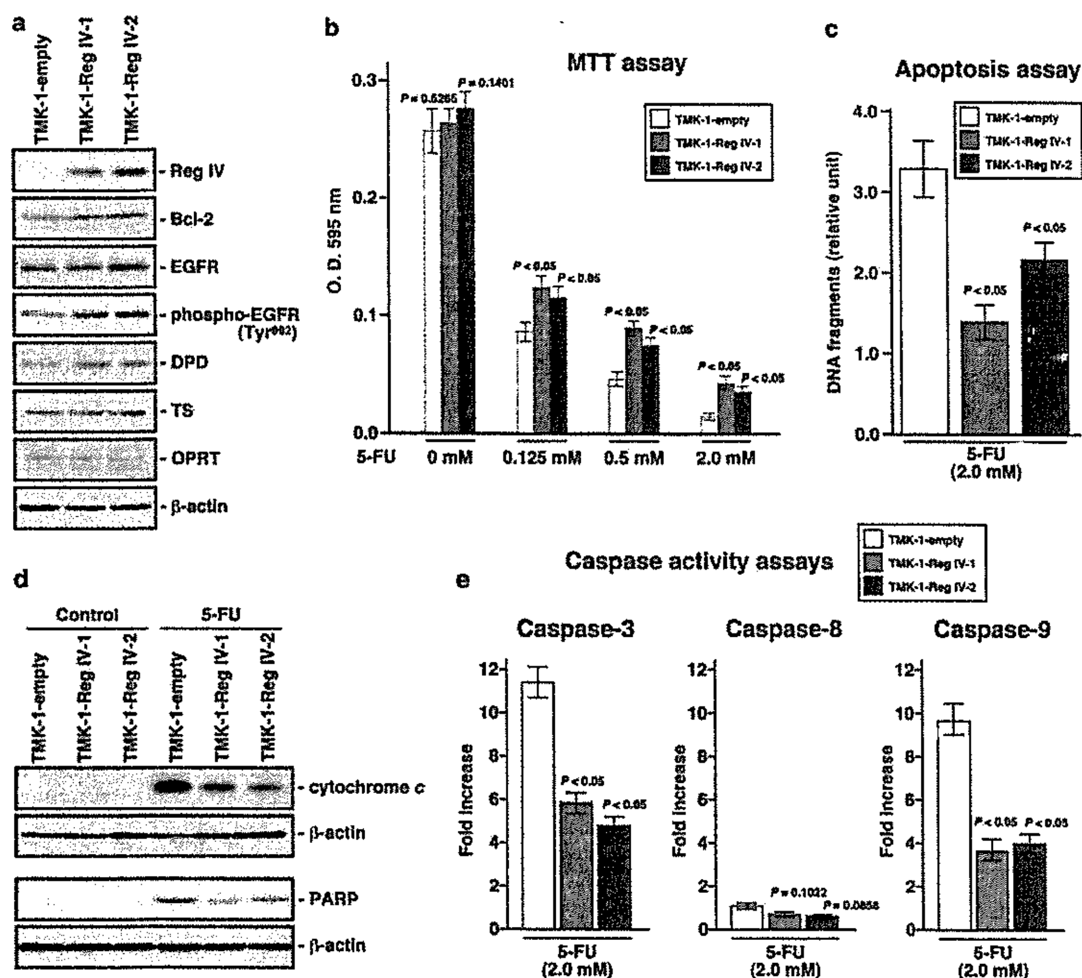

Figure 1 Forced expression of Reg IV inhibits 5-FU-induced apoptosis. (**a**) Western blot analysis of the TMK-1 GC cell line stably transfected with vector expressing Reg IV. Two G418 resistant clones, TMK-1-Reg IV-1 and TMK-1-Reg IV-2, overexpressing Reg IV protein were isolated. (**b**) Effect of forced Reg IV expression on cell growth of TMK-1 cells. Cell growth was assessed by MTT assay at 48 h after 5-FU treatment. Bars and error bars represent mean and s.d., respectively, from three different experiments. *P*-values for all the assays were calculated using Student's *t*-test. (**c**) Forced Reg IV expression inhibits 5-FU-induced apoptosis. Cells were incubated with 2 mM 5-FU for 48 h, and both floating and attached cells were collected. Apoptosis was determined with a Cell Death Detection ELISA[Plus] Kit. Bars and error bars represent mean and s.d., respectively, from three different experiments. *P*-values for all the assays were calculated using Student's *t*-test. (**d**) Forced Reg IV expression inhibits cytochrome *c* release and PARP cleavage induced by 5-FU. Cells were incubated with 2 mM 5-FU for 36 h, and both floating and attached cells were collected for Western blot analysis. (**e**) Forced Reg IV expression inhibits caspase activation by 5-FU. Cells were treated with 5-FU (2 mM) for 36 h, and floating and attached cells were collected. 5-FU induced activation of caspase-9 and -3, and to a lesser extent, caspase-8. Reg IV-overexpressing cells (TMK-1-Reg IV-1 and TMK-1-Reg IV-2) showed significantly lower activation of caspase-9 and -3 in response to 5-FU than empty vector-transfected cells. Bars and error bars represent mean and s.d., respectively, from three different experiments. *P*-values for all the assays were calculated using Student's *t*-test.

and Akt at Thr^{308} and Ser^{473}, resulting in increased AP-1 transcription factor activity (Bishnupuri *et al.*, 2006). In addition, HCT116 and HT29 colon cancer cell lines treated with recombinant Reg IV showed increased expression of Bcl-2 (Bishnupuri *et al.*, 2006). Bcl-2 is an antiapoptotic protein located on mitochondria and expressed at high levels in some tumor cells and tissues (Vander Heiden and Thompson, 1999). In the mitochondrial pathway, antiapoptotic Bcl-2 family proteins prevent mitochondrial membrane permeabilization and thereby inhibit changes in the mitochondrial membrane potential and cytochrome *c* release (Vander Heiden and Thompson, 1999). We examined phosphorylation of EGFR at Tyr^{992} and Tyr^{1068} in Reg IV-overexpressing cells. EGFR was phosphorylated at Tyr^{992} (Figure 1a) but not Tyr^{1068} in our Reg IV-overexpressing cells (data not shown). Expression of Bcl-2 was also examined by Western blotting, and induction of Bcl-2 in Reg IV-overexpressing cells was confirmed (Figure 1a). These results suggest that expression of Bcl-2 contributes to inhibition of the mitochondrial apoptotic pathway in Reg IV-overexpressing cells.

It was recently reported that AP-1 induces expression of dihydropyrimidine dehydrogenase (DPD) (Ukon

et al., 2005). DPD, an initial and rate-limiting enzyme in 5-FU catabolism, has significance for the pharmacokinetics and toxicity of 5-FU (Harris *et al.*, 1990). Overexpression of DPD in tumor cell lines is associated with resistance to 5-FU (Takebe *et al.*, 2001). Degradation of 5-FU by induction of DPD expression may also inhibit 5-FU-induced apoptosis. We examined expression of DPD in Reg IV-overexpressing cells by Western blotting. Induction of DPD expression was observed in Reg IV-overexpressing cells (Figure 1a). We also examined expression of other enzymes involved in 5-FU metabolism. Expression of thymidylate synthase (TS) and orotate phosphoribosyl transferase (OPRT) was not changed significantly (Figure 1a). These results indicate that degradation of 5-FU by induction of DPD is also involved in inhibition of apoptosis by Reg IV.

Expression and distribution of Reg IV and EGFR in GC tissues

Because forced Reg IV expression induces phosphorylation of EGFR at Tyr^{992} in TMK-1 cells, we examined whether expression of Reg IV activates phosphorylation of EGFR at Tyr^{992} in human GC tissue samples. Immunostaining of Reg IV and EGFR was observed in 61 (37.9%) and 40 (24.8%) of 161 GC cases, respectively. Immunostaining of Tyr^{992} phospho-EGFR was also performed in 40 EGFR-positive GC cases. Interestingly, some, but not all, EGFR-positive cells showed phosphorylation at Tyr^{992}. Immunohistochemical analysis revealed that Reg IV was expressed in almost all EGFR-positive GC cases. Of 40 EGFR-positive GC cases, 37 (92.5%) were positive for Reg IV, whereas of 121 EGFR-negative GC cases, only 24 (19.8%) ($P<0.0001$, Fisher's exact test) were positive for Reg IV. In 37 GC cases expressing both Reg IV and EGFR, Reg IV and EGFR were rarely expressed in the same GC cells; however, Reg IV-positive GC cells were found near EGFR-positive GC cells (Figure 2a–f). Some GC cells were positive for both Reg IV and EGFR. Triple-immunofluorescence staining revealed that GC cells positive for both Reg IV and EGFR did not show phosphorylation of EGFR at Tyr^{992}. In addition, GC cells positive for phosphorylation at Tyr^{992} were located near Reg IV-positive cells (Figure 2g–j). We then analysed the relation of Reg IV and EGFR expression to clinicopathologic characteristics. There was no clear association between Reg IV expression and clinical characteristics (Table 1). In contrast, expression of EGFR was associated with advanced T grade (depth of invasion, $P=0.0004$, Fisher's exact test) and N grade (degree of lymph node metastasis, $P=0.0218$, Fisher's exact test) (Table 1). Moreover, EGFR staining was observed more frequently in stage III/IV cases (27 of 84 cases, 32.1%) than in stage I/II cases (13 of 77 cases, 16.9%, $P=0.0291$, Fisher's exact test) (Table 1). No statistically significant prognostic effect of Reg IV was found in the 101 advanced GC patients ($P=0.9857$, log-rank test) (Figure 2k); however, expression of EGFR was associated with poor survival ($P=0.0006$, log-rank test) (Figure 2k). These results suggest that Reg IV-positive GC cells were different from EGFR-positive GC cells, but GC cases containing EGFR-positive GC cells also contained Reg IV-positive GC cells, resulting in phosphorylation of EGFR at Tyr^{992} in human GC tissues.

Relation between Reg IV expression and response of GC to a combination chemotherapy of low-dose 5-FU and cisplatin

We next examined the relation between Reg IV expression and response of GC to combination chemotherapy of low-dose 5-FU and cisplatin in recurrent GC tissue specimens. Reg IV expression was investigated in primary tumor samples obtained by surgical resection before the initiation of chemotherapy. We did not investigate the Reg IV expression in metastatic lesions because of lack of biopsy materials from the metastatic sites. The overall results are summarized in Table 2. Among the 36 patients treated with the combination chemotherapy, all 14 patients with Reg IV expression showed no change (NC) or a progressive disease (PD) to the combination chemotherapy, whereas eight (36.4%) of 22 patients without Reg IV expression showed a partial response (PR) ($P=0.0132$, Fisher's exact test). There was no association between EGFR expression and response to combination therapy ($P=0.1596$, Fisher's exact test). In these 36 GC cases, Reg IV expression was observed in all EGFR-positive GC cases.

Serum Reg IV concentration in healthy subjects, non-cancer patients and GC patients

We next examined whether Reg IV could be detected by ELISA in sera from patients with GC. Western blot analysis did not detect Reg IV protein in culture media of the MKN-1 and TMK-1 GC cell lines, whereas high levels of Reg IV protein were found in culture media of Reg IV-transfected TMK-1 cells (TMK-1-Reg IV-1 and TMK-1-Reg IV-2) and the MKN-45 GC cell line (Figure 3a). We confirmed by anti-β-actin Western blot that contamination of cells in culture medium was minimal. We used ELISA to test culture media from these cell lines. Reg IV protein was detected in culture media from TMK-1-Reg IV-1, TMK-1-Reg IV-2 and MKN-45 cell lines (Figure 3b), and the levels of Reg IV protein detected by ELISA were similar to those obtained by Western blot analysis (Figure 3a). Reg IV protein was not detected in culture media of MKN-1 and TMK-1 cell lines by ELISA (Figure 3b). Culture media of Reg IV-transfected TMK-1 and MKN-45 cells were preabsorbed with recombinant Reg IV protein before being tested by ELISA. The specificity of Reg IV recognition was confirmed by the marked decrease in the ELISA signals after preabsorption (Figure 3b).

The levels of serum Reg IV in healthy individuals, patients with chronic-active gastritis (*Helicobacter pylori* positive) and patients with GC before surgery are shown in Figure 3c. The serum Reg IV concentration was similar between healthy individuals ($n=101$, mean$\pm$s.e., 0.52 ± 0.05 ng/ml) and patients with

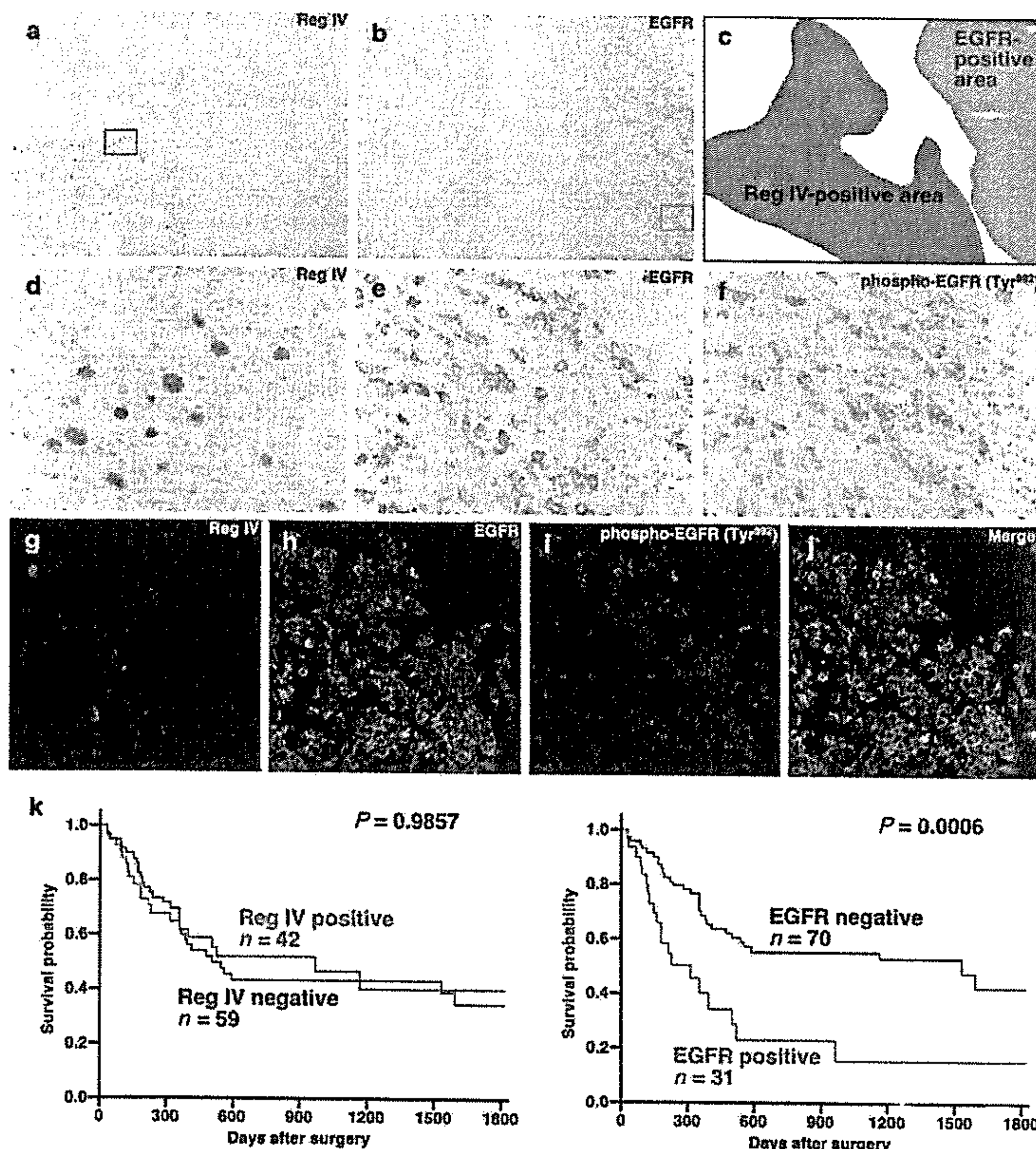

Figure 2 Expression and distribution of Reg IV and EGFR in GC tissues. Immunostaining of Reg IV (a, brown color) and EGFR (b, brown color) in both Reg IV- and EGFR-positive GC case (original magnification, ×40). (c) Schematic representation of Reg IV-positive GC cells (blue) and EGFR-positive GC cells (green). In both Reg IV- and EGFR-positive GC case, Reg IV-positive GC cells were different from EGFR-positive GC cells although Reg IV-positive GC cells were found near EGFR-positive GC cells. (d and e) High magnification images of the fields indicated by boxes in panels a and b (original magnification, ×400). A subset of EGFR-positive GC cells was positive for Tyr^{992} phospho-EGFR (f) (original magnification, ×400). (g–j) Triple-immunofluorescence staining of Reg IV (g), EGFR (h) and Tyr^{992} phospho-EGFR (i). Cells with EGFR with phosphorylation at Tyr^{992} were located near Reg IV-positive GC cells (j) (original magnification, ×400). (k) Prognostic value of Reg IV and EGFR staining.

chronic-active gastritis ($n=20$, 0.36 ± 0.09 ng/ml). However, the serum Reg IV concentration in presurgical GC patients ($n=61$, 1.96 ± 0.17 ng/ml) was significantly elevated (healthy individuals vs all GC patients, $P<0.0001$, Mann–Whitney U-test), even at stage I (healthy individuals vs stage I GC patients, $P<0.0001$, Mann–Whitney U-test) (Figure 3c). In Cases 36 and 42, which showed high serum concentrations of Reg IV, strong and extensive Reg IV staining was observed in the primary GC samples. In contrast, in Case 18, in which the serum Reg IV concentration was very low, no Reg IV staining was observed in the primary GC sample (Figure 3d). The Reg IV concentration in serum samples from patients with GC showing Reg IV-positive immunostaining ($n=12$, 2.51 ± 0.40 ng/ml) was statistically significantly higher than that with GC showing Reg IV-negative immunostaining ($n=49$, 1.82 ± 0.18 ng/ml) ($P=0.0251$, Mann–Whitney U-test). When the

cutoff level for Reg IV was set at 2.00 ng/ml, the sensitivity and specificity for detection of GC were 36.1% (22/61) and 99.0% (100/101), respectively.

CEA and CA19-9 levels were also measured in the same serum samples. The sensitivity and specificity of CEA for detection of GC were 11.5% (7/61) and 100.0% (101/101), respectively. The sensitivity and specificity of CA19-9 for detection of GC were 13.1% (8/61) and 100.0% (101/101), respectively. Spearman's rank correlation test revealed only a weak correlation between serum Reg IV and CEA ($r=0.0173$, $P=0.3123$) or CA19-9 ($r=0.0107$, $P=0.4279$) (Figure 3e and f). Of GC patients with normal serum CEA values, 31.5% were found to express Reg IV at 99.0% specificity, and 32.1% of GC patients with normal serum CA19-9 values were found to express Reg IV at 99.0% specificity. The sensitivities of serum Reg IV, CEA and CA19-9 with respect to tumor stage are shown in Table 3. In patients with stage I GC, the sensitivity of serum Reg IV (36.1%) was significantly higher than that of CEA (5.6%, $P=0.0028$, Fisher' exact test) or CA19-9 (8.3%, $P=0.0093$, Fisher' exact test).

Table 1 Association of Reg IV and EGFR expression with clinicopathologic features of gastric cancer

	Reg IV expression			*EGFR expression*		
	Positive	*Negative*	P-*value*ᵃ	*Positive*	*Negative*	P-*value*ᵃ
T grade						
T1	8 (29.6%)	19	0.3895	0 (0.0%)	27	0.0004
T2/3/4	53 (39.6%)	81		40 (29.9%)	94	
N grade						
N0	20 (34.5%)	38	0.6120	8 (13.8%)	50	0.0218
N1/2/3	41 (39.8%)	62		32 (31.1%)	71	
Stage						
I/II	27 (35.1%)	50	0.5180	13 (16.9%)	64	0.0291
III/IV	34 (50.5%)	50		27 (32.1%)	57	

Abbreviations: EGFR, epidermal growth factor receptor; Reg IV, regenerating gene IV. ᵃFisher's exact test.

Discussion

It is generally accepted that apoptosis suppresses oncogenic transformation. The ability of tumor cell populations to expand in number is determined not only by the rate of cell proliferation but also by the rate of cell attrition. Apoptosis represents a major source of this attrition (Hanahan and Weinberg, 2000). Thus, resistance to apoptosis is a hallmark of most and perhaps all types of cancer. In the present study, we showed that overexpression of Reg IV inhibits 5-FU-induced apoptosis. At least two mechanisms are involved in inhibition of apoptosis by Reg IV, induction of Bcl-2 and induction of DPD.

In 5-FU-treated TMK-1 cells, overexpression of Reg IV inhibited the mitochondrial apoptotic pathway that involves cytosolic cytochrome *c* release and subsequent activation of caspase-9 and -3. Increased Bcl-2 by forced Reg IV expression may act to inhibit the mitochondrial apoptotic pathway. Bcl-2 induction by Reg IV is blocked by AG1478, a tyrosine kinase inhibitor specific for EGFR (Bishnupuri *et al.*, 2006), indicating that phosphorylation of EGFR is required for Bcl-2 induction and that EGFR plays an important role in inhibition of apoptosis by Reg IV. In the present study, immunohistochemical analysis of GC tissues revealed that almost all EGFR-positive GC cases (92.5%) also expressed Reg IV and that EGFR was phosphorylated at Tyr^{992} in all EGFR-positive GC cases. Although it is possible that other molecules, such as EGF, induce phosphorylation of EGFR at Tyr^{992}, the present results

Table 2 Association of Reg IV and EGFR expression with response to combination chemotherapy of low-dose 5-FU and cisplatin

	Reg IV expression			*EGFR expression*		
	Positive	*Negative*	P-*value*ᵃ	*Positive*	*Negative*	P-*value*ᵃ
CR and PR	0 (0.0%)	8	0.0132	0 (0.0%)	8	0.1596
NC and PD	14 (50.0%)	14		9 (32.1%)	19	

Abbreviations: CR, complete response; EGFR, epidermal growth factor receptor; NC, no change; PD, progressive disease; PR, partial response; Reg IV, regenerating gene IV. ᵃFisher's exact test.

Figure 3 ELISA of serum samples from patients with GC. (**a**) Western blot analysis of Reg IV. Culture media of Reg IV-transfected TMK-1 cells (TMK-1-Reg IV-1 and TMK-1-Reg IV-2) and MKN-45 GC cell lines contain Reg IV. Extracts of MKN-45 cells served as a positive control. Western blotting with anti-β-actin antibody confirmed that there was minimal contamination of culture medium with cells. (**b**) Detection of Reg IV in culture media by ELISA. Reg IV was detected in culture media of Reg IV-transfected TMK-1 and MKN-45 cells but not MKN-1 and TMK-1 cells. Culture media of Reg IV-transfected TMK-1 and MKN-45 cells were preincubated with recombinant Reg IV and then tested by ELISA. A significant reduction in the signal intensity of the ELISA was observed. *P*-values were calculated using Student's *t*-test. (**c**) Detection of Reg IV protein in serum samples by ELISA. A high concentration (2.00 ng/ml) of Reg IV was detected in 22 serum samples from patients with GC. Yellow bars indicate the cutoff levels defined in this study. Red bars indicate the means±s.e. Differences in the serum concentration of Reg IV between two groups were tested by non-parametric Mann–Whitney *U*-test. (**d**) Immunostaining of Reg IV in primary GC samples. Strong and extensive Reg IV staining was observed in Cases 36 and 42, which also showed high concentrations of Reg IV in serum samples (**c**). In Case 18, no staining of Reg IV was observed, and the serum concentration of Reg IV was low (**c**). (**e**) Relation between serum concentrations of Reg IV and CEA. Correlation was examined using Spearman's rank correlation. (**f**) Relation between serum concentrations of Reg IV and CA19-9. Correlation was examined using Spearman's rank correlation.

suggest that in a subset of EGFR-positive GC, Reg IV affects phosphorylation of EGFR at Tyr[992] and stimulates tumor cell growth by inhibiting apoptosis. It is important to note that in the immunohistochemical analysis, GC cells expressing both Reg IV and EGFR were rare. Triple-immunofluorescence staining revealed

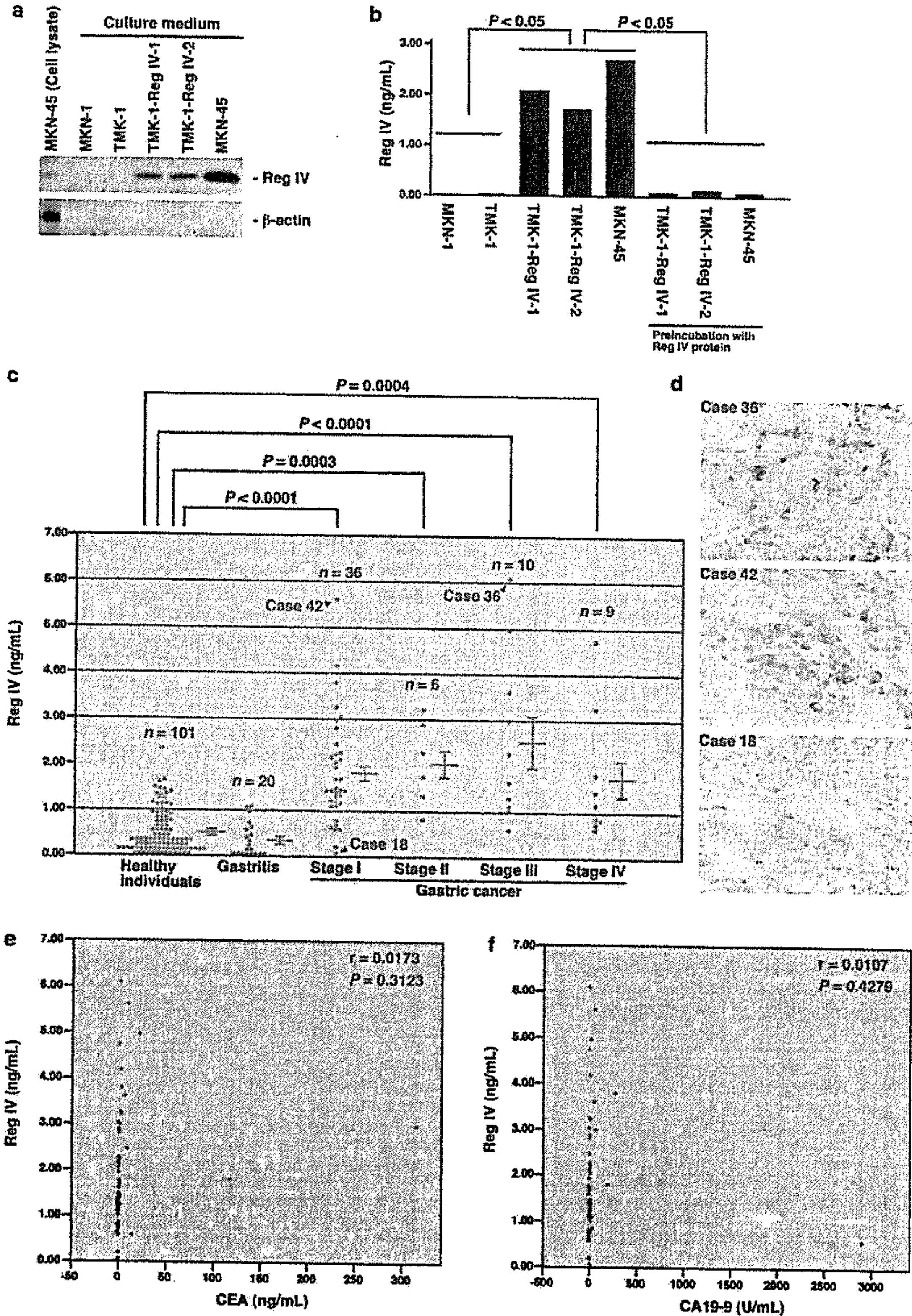

Table 3 Diagnostic sensitivity of serum Reg IV, CEA and CA19-9 with respect to tumor stage

	Reg IV (%)	*CEA (%)*	P-*value*[a]	*CA19-9 (%)*	P-*value*[b]
Stage I ($n=36$)	36.1	5.6	0.0028	8.3	0.0093
Stage II ($n=6$)	50.0	0.0	0.1818	0.0	0.1818
Stage III ($n=10$)	50.0	40.0	1.0000	30.0	0.6499
Stage IV ($n=9$)	22.2	11.1	1.0000	22.2	1.0000
Specificity	99.0	100.0		100.0	

Abbreviations: CA19-9, carbohydrate antigen; CEA, carcinoembryonic antigen; Reg IV, regenerating gene IV. [a]Fisher's exact test. Reg IV vs CEA. [b]Fisher's exact test. Reg IV vs CA19-9.

that GC cells positive for both Reg IV and EGFR did not show phosphorylation of EGFR at Tyr[992]. We cannot explain completely the discrepancy between GC cell line data and GC tissue data. Because GC cells positive for phosphorylation at Tyr[992] were located near Reg IV-positive cells, Reg IV expression may induce expression and secretion of EGFR ligands, such as EGF, which could lead to EGFR phosphorylation of the adjacent cells. EGF secretion in Reg IV-overexpressing cells should be examined. Furthermore, clinical characteristics of Reg IV-positive GC cases were quite different from those of EGFR-positive GC cases in the present study. Expression of Reg IV was found in both early- and late-stage GC, whereas expression of EGFR was detected mainly in late-stage tumors. These results led us to speculate that continuous expression of Reg IV may be a selective pressure for the development of EGFR-positive GC cells and may confer selective growth advantage to EGFR-positive GC cells. In Reg IV-positive GC, even at an early stage, EGFR-positive GC cells may develop in accordance with tumor progression.

Forced Reg IV expression induced expression of DPD. DPD is a catabolic enzyme of 5-FU (Harris *et al.*, 1990), and several studies have indicated an inverse correlation between expression or activity of DPD and sensitivity to 5-FU-based chemotherapy in GC (Inada *et al.*, 2000; Ishikawa *et al.*, 2000). Degradation of 5-FU by DPD may play an important role in inhibition of 5-FU-induced apoptosis. In the present study, Reg IV expression in primary tumors was associated with response to 5-FU/cisplatin combination chemotherapy in recurrent GC cases. Although we did not evaluate activation of AP-1 in the present study, EGFR-dependent activation of AP-1 by Reg IV has been reported (Bishnupuri *et al.*, 2006). Because DPD is a downstream target gene of AP-1 (Ukon *et al.*, 2005), phosphorylation of EGFR may be required for induction of DPD by Reg IV. As mentioned above, induction of Bcl-2 also depends on phosphorylation of EGFR. Phosphorylation of EGFR may be a crucial event in inhibition of 5-FU-induced apoptosis by Reg IV. Interestingly, EGFR expression in primary tumors was not associated with response to combination chemotherapy in the present study. We confirmed that all EGFR-positive GC cases were positive for phosphorylation of EGFR at Tyr[992]. We investigated both Reg IV and EGFR expression in primary tumors, but the response to chemotherapy was evaluated in metastatic lesions. Several lines of evidence suggest that primary and metastatic lesions bear different biologic properties. DPD gene expression levels were reported to be lower in primary cancers than in liver metastases in several studies (Kuramochi *et al.*, 2006). Because expression of EGFR is a late event in GC progression, metastatic lesions may express EGFR even though EGFR is not expressed by the primary tumor. Because we investigated neither Reg IV nor EGFR expression in metastatic lesions, further studies are needed to clarify the predictive value of Reg IV and EGFR expression analysis.

It is important to note that while TMK-1-Reg IV-2 clone expresses more Reg IV than TMK-1-Reg IV-1, the intensity of the DPD band for TMK-1-Reg IV-1 looks slightly more intense than the intensity of the DPD band for TMK-1-Reg IV-2. In the present study, Western blotting was performed 1 or more months after the TMK-1 cell line had been stably transfected with vector expressing Reg IV. It has been reported that EGFR phosphorylation induced by treatment with recombinant human Reg IV occurred rapidly, with maximum effect at 5 min (Bishnupuri *et al.*, 2006), suggesting that EGFR phosphorylation levels may reach plateau in Reg IV-transfected TMK-1 cells and DPD expression may not increase further with increase in Reg IV expression.

Serum Reg IV is a novel biomarker for GC. Despite the reliability of CEA and CA19-9 as markers for detection of GC, CEA and CA19-9 are unsuitable for detection of early GC. In fact, in the present study, CEA and CA19-9 were found in serum in 5.6 and 8.3%, respectively, of patients with stage I GC. Of 36 serum samples from patients with stage I GC, 13 (36.1%) showed high levels of Reg IV, indicating that Reg IV is a good serum marker for early detection of GC. In addition, because Reg IV levels in serum samples from patients with GC expressing Reg IV were significantly higher than those of Reg IV-negative GC, Reg IV concentration in sera may be a marker for prediction of the response to 5-FU-based chemotherapy. However, all GC cases with high serum concentrations of Reg IV did not necessarily show Reg IV immunostaining of the primary tumor. This discrepancy between immunostaining and ELISA results may be due to methodologic differences. Reg IV immunohistochemistry results were evaluated as the percentage of stained cancer cells; the intensity of immunostaining was not evaluated because we had no suitable internal control for the immunohistochemistry. More detailed quantitative methods for the measurement of Reg IV protein, such as intratumor Reg IV concentration, are needed to clarify the relation between levels of Reg IV protein in sera and levels in primary GC samples.

In conclusion, our present data show that Reg IV can confer resistance to 5-FU-induced apoptosis in GC cells, suggesting that overexpression of Reg IV may represent a novel mechanism of intrinsic drug resistance in human GC. We detected Reg IV in sera from patients with GC. This indicates not only that serum Reg IV is a novel

biomarker for GC but also that serum Reg IV may have some effect on normal organs in patients with GC. Identification of a cell-surface receptor for Reg IV may further improve our understanding of the basic biology of Reg IV.

Materials and methods

Cell line, expression vector, transfection and 5-FU treatment
A human GC-derived cell line, TMK-1, was established in our laboratory (Ochiai *et al.*, 1985). TMK-1 cells were maintained in Roswell's Park Memorial Institute 1640 medium (Nissui Pharmaceutical, 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 the *REG4* gene, cDNA was PCR amplified and subcloned into pcDNA 3.1 (Invitrogen, Carlsbad, CA, USA). The pcDNA-Reg IV expression vector was transfected into TMK-1 cells with FuGENE6 (Roche Diagnostics, Indianapolis, IN, USA), according to the manufacturer's instructions. Stable transfectants were selected after 2 weeks of culture with 100 μg/ml G418 (Invitrogen). The effect of 5-FU (Acros Organics, Fairlawn, NJ, USA) on apoptosis was studied. 5-FU was dissolved in 100% dimethyl sulfoxide (DMSO) and then diluted in cell culture medium for experiments. The final concentration of DMSO was maintained at 0.1%. For apoptosis assay, caspase activation assays and Western blot analysis, both floating and attached cells were collected after 5-FU treatment.

MTT, cell proliferation, apoptosis and caspase activation assays
For MTT assay, the cells were seeded at a density of 2000 cells per well in 96-well plates. The cells were then treated with 5-FU for 48 h. Cell growth was monitored by MTT assay (Alley *et al.*, 1988). Cell proliferation activity was determined with a Cell Proliferation ELISA (Amersham Biosciences, Piscataway, NJ, USA), according to the manufacturer's instructions. For apoptosis assay, cultured cells were treated for 48 h with 2 mM 5-FU, and apoptosis was evaluated with a Cell Death Detection ELISA^{Plus} Kit (Roche Diagnostics), according to the manufacturer's instructions. For caspase activation assays, cultured cells were treated for 36 h with 2 mM 5-FU and the activities of caspase-3, -8 and -9 were determined with caspase-3, -8 and -9 Colorimetric Activity Assay Kits, respectively (Chemicon, Temecula, CA, USA), according to the manufacturer's instructions. *P*-values for all the assays were calculated using Student's *t*-test.

Western blot analysis
Western blot analysis was performed as described previously (Yasui *et al.*, 1993). The filter was incubated for 1 h at room temperature with an anti-Reg IV antibody (rabbit polyclonal antibody raised in our laboratory; Oue *et al.*, 2005), anti-Bcl-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-EGFR antibody (Cell Signaling Technology, Beverly, MA, USA), anti-phospho-EGFR (Tyr^{992}) antibody (Cell Signaling Technology), anti-phospho-EGFR (Tyr^{1068}) antibody (Cell Signaling Technology), anti-DPD antibody (Taiho Pharmaceutical, Tokyo, Japan), anti-TS antibody (Taiho Pharmaceutical), anti-OPRT antibody (Taiho Pharmaceutical), anti-PARP p85 fragment antibody (Promega, Madison, MD, USA) or anti-β-actin antibody (Sigma Chemical, St Louis, MO, USA). To quantify cytochrome *c* release into the cytosol, floating and attached cells were collected after incubation with 2 mM 5-FU for 36 h, and cytochrome *c* in cytosolic extracts was detected with an ApoAlert Cell Fractionation Kit (Takara Bio, Shiga, Japan).

Tissue samples
In all, 161 primary tumors and 61 serum samples were collected from patients diagnosed with GC. Patients were treated at the Hiroshima University Hospital or an affiliated hospital.

For immunohistochemical analysis, we used archival formalin-fixed, paraffin-embedded tissues from 161 patients who had undergone surgical excision of GC. Of the 161 patients, 27 had early GC and 134 had advanced GC. Early GC is limited to the mucosa or the mucosa and submucosa regardless of nodal status. Advanced GC is a tumor that has invaded beyond the muscularis propria (Hohenberger and Gretschel, 2003). Information on patient survival was available for 101 of the 134 advanced GC cases.

Of the 161 patients, 36 had recurrent GC and were treated with a combination of low-dose 5-FU and cisplatin (Kim *et al.*, 1999). All 36 patients with recurrent GC provided a medical history and underwent physical examination, including evaluation of performance status, complete blood cell count, serum chemistry profile, chest X-ray and computed tomography (CT) and/or magnetic resonance imaging, at the time of enrollment. Tumor markers, including CEA and CA19-9, were checked monthly. The responses of metastatic lesions to treatment were assessed according to the World Health Organization criteria. Metastatic lesions were evaluated by CT, ultrasonography and other radiographic examinations. Complete response (CR) was defined as disappearance of all evidence of cancer for more than 4 weeks. PR was defined as at least 50% reduction in the sum of the products of the perpendicular diameters of all the lesions for more than 4 weeks without any evidence of new lesions or progression of existing lesions. NC was defined as less than 50% reduction or less than 25% increase in the sum of the products of the perpendicular diameters of all lesions without any evidence of new lesions. PD was defined as more than a 25% increase in more than one lesion or the appearance of new lesions.

Among the 161 GC cases used for immunohistochemical analysis, serum samples were available for ELISA from 61 cases (36 men and 25 women; age range, 35–88 years; mean, 67.5 years). Serum samples were collected before surgery and before initiation of therapy, and were stored at −80°C until analysis. Serum samples from 20 patients with chronic active gastritis with *H. pylori* infection (13 men and 7 women; age range, 57–85 years; mean, 68.8 years) were also collected. Control serum samples were obtained from 101 healthy individuals (75 men and 26 women; age range, 32–79 years; mean, 59.4 years).

Tumor staging was according to the TNM classification system (Sobin and Wittekind, 2002). Because written informed consent was not obtained, for strict privacy protection, identifying information for all samples was removed before analysis. This procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research of the Japanese Government.

Immunohistochemistry
For immunostaining of EGFR and phospho-EGFR (Tyr^{992}), a Dako LSAB Kit (Dako, Carpinteria, CA, USA) was used as described previously (Oue *et al.*, 2005). Sections were incubated with the following antibodies: goat anti-Reg IV (diluted 1:50, R&D Systems, Abingdon, UK), mouse anti-EGFR (1:20, Novocastra, Newcastle, UK) and rabbit anti-phospho-EGFR (Tyr^{992}) antibody (1:20, Cell Signaling

Technology). For immunostaining of Reg IV, peroxidase-conjugated anti-goat IgG was used as the secondary antibody. Staining was completed with a 10-min incubation with the substrate-chromogen solution. The sections were counter-stained with 0.1% hematoxylin. The percentage of stained cancer cells was evaluated for each antibody. A result was considered positive if at least 10% of cells were stained. When fewer than 10% of cancer cells were stained, the immunostaining was considered negative.

For triple-immunofluorescence staining, Alexa Fluor 405-conjugated anti-goat IgG (Molecular Probes, Eugene, OR, USA), Alexa Fluor 488-conjugated anti-mouse IgG (Molecular Probes) and Alexa Fluor 546-conjugated anti-rabbit IgG (Molecular Probes) were used as secondary antibodies.

ELISA

For measurement of the serum concentration of Reg IV, a sandwich ELISA was developed. First, polystyrene microtiter plates were coated with mouse monoclonal anti-Reg IV antibody (R&D Systems) by overnight incubation of 50 μl/125 ng/well antibody diluted in Tris buffer (pH 7.4). The plates were then washed three times with washing buffer. After the plates were blocked with 1% milk in phosphate-buffered saline, 50 μl of recombinant Reg IV standard or sample was added to each well and incubated overnight at 4°C. After three washes, 50 μl of biotinylated goat polyclonal anti-Reg IV antibody (R&D Systems) in assay buffer (1% bovine serum albumin (BSA), Tris buffer (pH 7.4), 0.05% normal goat serum) was added to each well (75 ng antibody/well). The mixture was then incubated for 1 h with shaking at 37°C and washed three times with washing buffer. The plates were incubated with 50 μl/well alkaline phosphatase-conjugated streptavidin (Dako) diluted 2000-fold in diluent containing 1% BSA and Tris buffer (pH 7.4) for 1 h at 37°C and washed three times. Color development was performed with the addition of pNPP chromogenic substrate (Sigma) followed by incubation at 37°C for 1 h. Absorbance at 405 nm was measured with an ELISA plate reader. As a reference standard, known concentrations of human recombinant Reg IV (Oue *et al.*, 2005) from 0 to 30 ng/ml were tested in triplicate.

Measurement of CEA and CA19-9

CEA and CA19-9 were measured with a commercially available automated immunoassay method (Modular Analytics, Roche Diagnostics). The upper limits of normal for this method are 5.0 ng/ml for CEA and 37 U/ml for CA19-9.

Statistical methods

Associations between clinicopathologic parameters and Reg IV or EGFR expression were analysed by Fisher's exact test. Kaplan–Meier survival curves were constructed for Reg IV- or EGFR-positive and Reg IV- or EGFR-negative patients. Survival rates were compared between Reg IV- or EGFR-positive and Reg IV- or EGFR-negative groups. Differences between survival curves were tested for statistical significance by log-rank test (Mantel, 1966). Differences in the serum concentration of Reg IV between two groups were tested by non-parametric Mann–Whitney *U*-test. Correlation between the serum concentration of Reg IV and that of CEA or CA19-9 was assessed by Spearman's rank correlation. A *P*-value of less than 0.05 was considered statistically significant.

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DNA demethylation of *Vascular endothelial growth factor-C* is associated with gene expression and its possible involvement of lymphangiogenesis in gastric cancer

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Previous studies have indicated that lymphangiogenesis in solid tumors is associated with lymphatic metastasis. Overexpression of Vascular endothelial growth factor (VEGF)-C plays a major role in lymphangiogenesis in cancers. In the present study, DNA methylation and expression of the *VEGF-C* gene was investigated in gastric cancer (GC). Four GC cell lines (MKN-45, MKN-74, HSC-39 and HSC-43) showed no expression of *VEGF-C*, and the *VEGF-C* gene was found to be methylated in these cells. In contrast, 7 GC cell lines (MKN-1, MKN-7, MKN-28, TMK-1, KATO-III, SH101-P4 and HSC-44PE) expressed *VEGF-C*, and the *VEGF-C* gene was found to be unmethylated in these cell lines. In addition, expression of *VEGF-C* mRNA was retrieved by treatment with a demethylating agent, Aza-2′-deoxycytidine. In GC tissue samples, bisulfite DNA sequencing analysis revealed that *VEGF-C* was not methylated in 9 (29.0%) of 31 GC samples, whereas demethylation was not observed in corresponding non-neoplastic mucosa samples. Overexpression of *VEGF-C* mRNA was observed in 16 (51.6%) of 31 GC samples by quantitative reverse transcription-polymerase chain reaction. Of the 9 GC cases with *VEGF-C* demethylation, 8 (88.9%) overexpressed *VEGF-C*. In contrast, of the 22 GC cases without *VEGF-C* demethylation, 8 (36.4%) overexpressed *VEGF-C* ($p = 0.0155$). Furthermore, lymphatic vessel density determined by immunostaining of podoplanin in GC tissues was associated with overexpression of *VEGF-C* ($p < 0.0001$). These results suggest that demethylation and activation of the *VEGF-C* gene is likely involved in lymphangiogenesis in GC.
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Key words: DNA methylation; DNA demethylation; *VEGF-C*; lymphangiogenesis; gastric cancer

According to the World Health Organization, gastric cancer (GC) is the fourth most common malignancy in the world, with ~870,000 new cases every year, and mortality from GC is second only to that from lung cancer.[1] Despite improvements in diagnostic and therapeutic methods, the prognosis of advanced GC with extensive invasion and metastasis remains poor. Several molecules associated with invasion and metastasis have been identified[2,3]; however, all the mechanisms underlying metastasis remains unclear.

We previously reported that hypoacetylation of histone H4 is associated with tumor progression and lymph node metastasis.[4] Genes with expression regulated by histone acetylation may be involved in tumor progression or metastasis. In GC, expression of $p21^{WAF1/CIP1}$[5] and *PINX1*[6] are regulated by histone acetylation, but expression of these genes is not associated with tumor progression or metastasis. Histone deacetylation also plays an important role in CpG island methylation-associated gene inactivation.[7] DNA methylation of CpG islands is detected commonly in human cancers including GC.[8–11] Hypermethylation of CpG islands is associated with silencing of several genes,[12,13] especially defective tumor-related genes, and has been proposed as an alternative way to inactivate tumor-related genes in human cancers.[14,15]

Several genes whose expression is activated by DNA demethylation have been reported. Demethylation of both *MAGE*[16] and *synuclein* γ[17] are correlated with tumor progression and lymph node metastasis in GC. Activation of *matrix metalloproteinase* genes by DNA demethylation has been observed in pancreatic cancer cell lines.[18] Taken together, the currently available data suggest that certain genes activated by DNA demethylation may be involved in tumor progression and lymph node metastasis.

It has been shown a close association between vascular endothelial growth factor (VEGF) family members and tumor metastasis.[19] VEGF has been established as a primary angiogenic molecule involved in development, adult physiology and pathology. VEGF-C and VEGF-D are primarily lymphangiogenic factors, but they can also induce angiogenesis under some conditions. Overexpression of VEGF-C has been detected in a variety of cancers.[20] However, the role of demethylation of *VEGF-C* in human cancer has not been examined.

In the present study, we examined whether DNA demethylation may be associated with the overexpression of the *VEGF-C* in GC. We show that the overexpression of *VEGF-C* is associated with DNA demethylation and can be restored in GC cell lines after aza-2′-deoxycytidine (Aza-dC)-induced demethylation. We further investigated DNA demethylation of *VEGF-C* and its possible involvement in lymphangiogenesis by immunostaining of podoplanin, a marker of lymphatic endothelial cell, in GC.

Material and methods

GC cell lines and drug treatment

Eleven cell lines derived from human GC were used. The TMK-1 cell line was established in our laboratory from a poorly differentiated adenocarcinoma.[21] Five gastric carcinoma cell lines of the MKN series (MKN-1, adenosquamous cell carcinoma; MKN-7; MKN-28; MKN-74, well differentiated adenocarcinoma; and MKN-45, poorly differentiated adenocarcinoma) were kindly provided by Dr. Toshimitsu Suzuki. KATO-III cell lines were kindly provided by Dr. Morimasa Sekiguchi. SH101-P4, HSC-39, HSC-43 and HSC-44PE cell lines were kindly provided by Dr. Kazuyoshi Yanagihara.[22–25] All cell lines were maintained in RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (BioWhittaker, Walkersville, MD) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. To analyze transcriptional activation of *VEGF-C*, MKN-1, MKN-45 and MKN-74 cells were incubated for 5 days with 1 μM Aza-dC (Sigma Chemical, St. Louis, MO, USA) or for 24 h with 300 nM TSA (Wako, Tokyo, Japan).

Abbreviations: VEGF, vascular endothelial growth factor; GC, gastric cancer; Aza-dC, aza-2′-deoxycytidine; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; LVD, lymphatic vessel density; T, GC tissue; N, corresponding non-neoplastic mucosa; TAM, tumor-associated macrophage.

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TABLE I – PRIMER SEQUENCES FOR BISULFITE DNA SEQUENCING, CONVENTIONAL RT-PCR AND QUANTITATIVE RT-PCR

Primer sequence	Annealing temperature (°C)	Size (bp)
Bisulfite DNA sequencing (Region 1)		
F: 5′-TTTTTTCGGTATTGGTTGGG-3′	53	181
R: 5′-CCGCTAACGAAAACAAAAAT-3′		
Bisulfite DNA sequencing (Region 2)		
F: 5′-GCGGGGTGTTTTGGTGTTTT-3′	54	187
R: 5′-ACGCCCTCCCAACCAATACC-3′		
Conventional RT-PCR (*VEGF-C*)		
F: 5′-TCGCGACAAACACCTTCTTT-3′	56	610
R: 5′-CTGGGGCAGGTTCTTTTACA-3′		
Conventional RT-PCR (*ACTB*)		
F: 5′-CTGTCTGGCGGCACCACCAT-3′	55	254
R: 5′-GCAACTAAGTCATAGTCCGC-3′		
Quantitative RT-PCR (*VEGF-C*)		
F: 5′-TGCCGATGCATGTCTAAACT-3′	55	251
R: 5′-TGAACAGGTCTCTTCATCCAGC-3′		
Probe: 5′-FAM CAGCAACACTACCACAGTGTCAGGCA TAMRA-3′		
Quantitative RT-PCR (*ACTB*)		
F: 5′-TCACCGAGCGCGGCT-3′	55	60
R: 5′-TAATGTCACGCACGATTTCCC-3′		

Tissue samples

Frozen tissue samples were collected from 31 patients (age range, 41–86 years; mean, 67.8 years) with GC who underwent surgery between 1998 and 2001 at the Department of Surgical Oncology, Hiroshima University Hospital (Hiroshima, Japan). All patients underwent curative resection, and all GC samples were advanced GC. These 31 GC tissue specimens and 5 corresponding non-neoplastic mucosa samples from the 5 GC patients (age range, 57–75 years; mean, 68.3 years) were analyzed for methylation of the *VEGF-C* gene. Total RNA was available for 31 pairs of tumor and corresponding non-neoplastic mucosa. GC and corresponding non-neoplastic mucosa were removed surgically, frozen immediately in liquid nitrogen and stored at −80°C until use. We confirmed microscopically that the tumor specimens consisted mainly of carcinoma tissue (>50%, on a nuclear basis) and that specimens of non-neoplastic mucosa did not show tumor cell invasion or significant inflammatory involvement. Tumor staging was carried out according to the TNM staging system.[26] We also examined levels of *VEGF-C* mRNA in 10 samples of normal gastric mucosa obtained endoscopically from 10 healthy young individuals (age range, 22–35 years; mean, 26.4 years) and evaluated methylation status of the *VEGF-C* gene in 2 samples from normal healthy young individuals. These healthy volunteers were confirmed to be free of malignancy by gastrointestinal endoscopy and biopsy. 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 Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government.

Conventional and quantitative reverse transcription-polymerase chain reaction analyses

Total RNA was extracted with an RNeasy Mini Kit (Qiagen, Valencia, CA), and 1 μg of total RNA was converted to cDNA with a First Strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ). Conventional RT-PCR was performed to investigate *VEGF*, *VEGF-B*, *VEGF-C* and *VEGF-D* expression in GC cell lines. 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. Primer sequences and additional PCR conditions are available upon request.

Quantitation of *VEGF-C* mRNA levels in human tissue samples was done by real-time fluorescence detection as described previously.[27] Primer sequences and annealing temperatures are shown in Table I. PCR was performed with a TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Real-time detection of the emission intensity of fluorescent reporter dye was performed with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) as described previously.[28] *ACTB*-specific PCR products were amplified from the same RNA samples and served as internal controls. We calculated the ratio of *VEGF-C* mRNA levels in GC tissue (T) to those in corresponding non-neoplastic mucosa (N). T/N ratios >2-fold were considered to represent overexpression.

Genomic DNA extraction and bisulfite genomic DNA sequencing

Genomic DNAs were extracted with a Genomic DNA Purification Kit (Promega, Madison, WI). To examine DNA methylation patterns, genomic DNA was treated with 3 M sodium bisulfite as described previously.[29] For analysis of DNA methylation of *VEGF-C*, we performed bisulfite genomic DNA sequencing analysis. Two sets of primers were used to assess the different regions (Regions 1 and 2) of the *VEGF-C* gene (Fig. 1*c*). Except for primer complementary sequences, Region 1 contains 18 CpG sites, and Region 2 contains 20 CpG sites. Two-microliter aliquots were used as templates for PCR reactions. Primer sequences and annealing temperatures are shown in Table I. Each target sequence was amplified in a 50-μl reaction containing 0.2 μM dNTPs, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.3 μM each primer and 0.75 units of AmpliTaq Gold (Applied Biosystems). PCR amplification consisted of 35 cycles after the initial AmpliTaq Gold activation step.

PCR products were purified and cloned into pCR2.1 (Invitrogen, Carlsbad, CA). The cloned PCR fragments obtained from each sample were sequenced with M13 forward primer and a PRISM AmpliTaq DNA Polymerase FS Ready Reaction Dye Terminator Sequencing Kit (Applied Biosystems). Reamplified DNA fragments were purified with Centri-Sep Columns (Applied Biosystems) and sequenced with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Immunostaining of lymphatic vessels and determination of lymphatic vessel density

Consecutive 4-μm sections of formalin-fixed, paraffin-embedded tissue were cut onto glass slides from each study block. Sections were immunostained for podoplanin. Podoplanin, a 38-kDa membrane glycoprotein originally identified on podocytes, is expressed on the endothelium of lymphatic capillaries but not in quiescent or proliferating blood vascular endothelium.[30] Immunostaining was done with the Histofine Simplestain MAX-PO (MULTI) (Nichirei Biosciences, Tokyo, Japan) immunoperoxi-

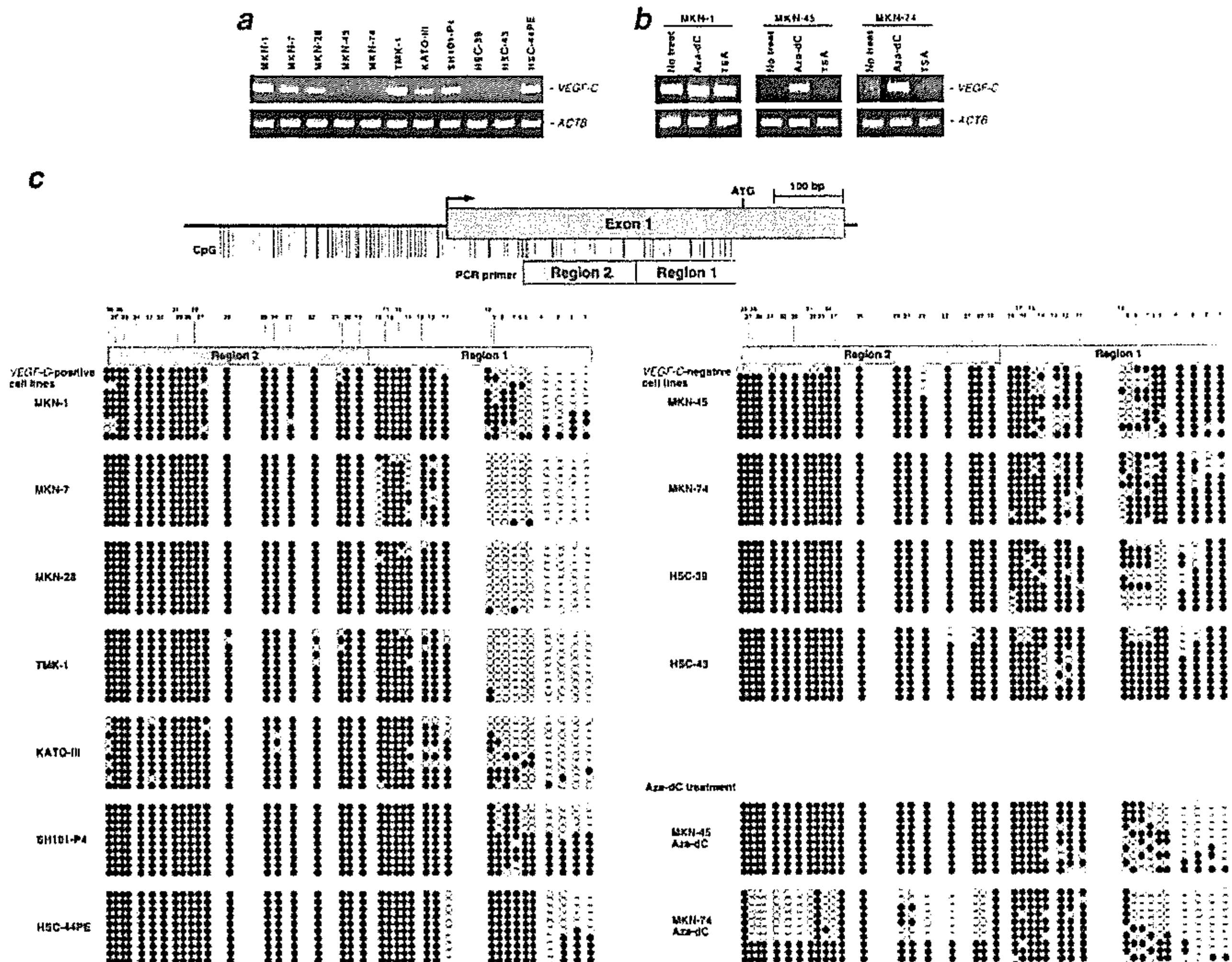

FIGURE 1 – RT-PCR analysis and bisulfite genomic DNA sequencing analysis of *VEGF-C* in GC cell lines. (*a*) Conventional RT-PCR of *VEGF-C*. *VEGF-C* was not expressed in the MKN-45, MKN-74, HSC-39 and HSC-43 cell lines. (*b*) Treatment of MKN-45 and MKN-74 cells with Aza-dC activated *VEGF-C* expression. Treatment of these cells with TSA did not induce *VEGF-C* expression. Treatment with Aza-dC and TSA did not significantly affect *VEGF-C* expression in MKN-1 cells. (*c*) Bisulfite genomic DNA sequencing analysis of *VEGF-C* in GC cell lines. Vertical lines indicate CpG sites. Ten cloned bisulfite PCR products of *VEGF-C* were analyzed. Each row of circles represents a single clone, and each circle represents a single CpG site (open circle, nonmethylated cytosine; filled circle, methylated cytosine). The numbering in this scheme corresponds to the position relative to the translation initiation site.

dase technique. Primary antibody was a mouse antipodoplanin monoclonal antibody (1:200, AngioBio, Del Mar, CA) and was incubated on the sections for 3 h at room temperature. Negative controls were done with nonspecific IgG as the primary antibody. Sections were counterstained with hematoxylin. LVD was evaluated by 2 independent investigators (S.M., N.O.), who were blind to the clinical course of the patients and the *VEGF-C* expression status of the tumors. In brief, after scanning an immunostained section at low magnification (×100), the area of tissue with the greatest number of distinctly highlighted lymphatic vessels ("hot spot") at the border of invasive cancer or inside the tumor was selected. LVD was then determined by counting all antipodoplanin immunostained lymphatic vessels at ×200 in an examination area. Only vessels with typical morphology (lumen) were considered lymphatic microvessels. After the 6 areas of highest neovascularization were identified, lymphatic vessels were counted and the average count was determined.

Statistical methods

Differences were analyzed statistically by Fisher's exact and Mann–Whitney *U* tests. *p* values less than 0.05 were considered statistically significant.

Results

Expression of VEGF, VEGF-B, VEGF-C, *and* VEGF-D *mRNAs in GC cell lines*

To examine expression of *VEGF* genes, we performed conventional RT-PCR analysis of 11 GC cell lines. Expression of *VEGF*, *VEGF-B* and *VEGF-D* was detected in all GC cell lines (data not shown). Four cell lines, MKN-45, MKN-74, HSC-39, and HSC-43, showed no expression of *VEGF-C* (Fig. 1*a*). We hypothesized that loss of *VEGF-C* expression might be caused by DNA methylation or histone hypoacetylation. To test this hypothesis, MKN-1, MKN-45 and MKN-74 cells were treated with Aza-dC or TSA and then subjected to RT-PCR analysis (Fig. 1*b*). Treatment with Aza-dC induced *VEGF-C* expression in MKN-45 and MKN-74 cells, whereas treatment with TSA did not. Treatment with Aza-dC or TSA did not significantly change *VEGF-C* expression in MKN-1 cells. These results suggest that DNA methylation may suppress *VEGF-C* expression.

Analysis of VEGF-C *methylation in GC cell lines*

To evaluate the extent of *VEGF-C* methylation, we performed bisulfite DNA sequencing of genomic DNAs from 7 *VEGF-C*-

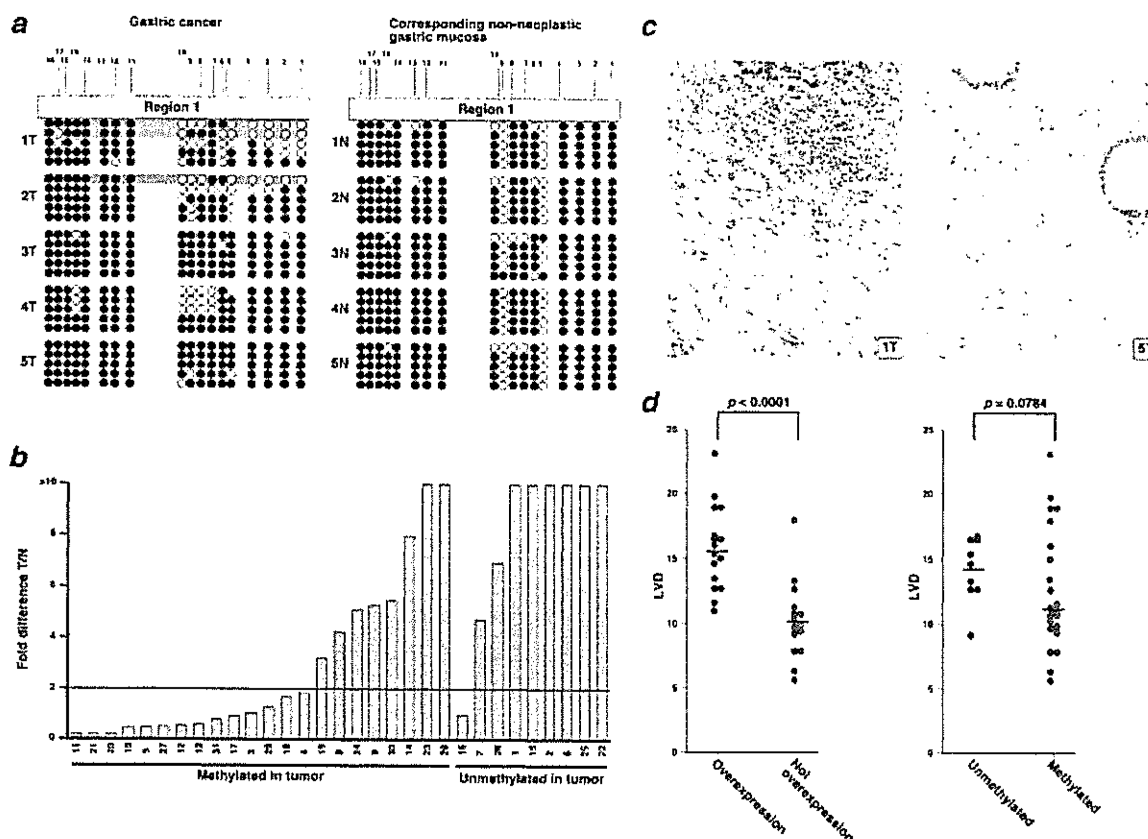

FIGURE 2 – DNA methylation and expression of *VEGF-C* and LVD analysis in GC tissue. (*a*) Representative results of bisulfite genomic DNA sequencing for GC tissues. In cases 1T and 2T, the unmethylated allele (allele contains unmethylated CpG sites 1, 2, 3 and 4, red box) was detected. In corresponding non-neoplastic mucosa, the unmethylated allele was not found. (*b*) Quantitative RT-PCR of *VEGF-C* in GC tissues. Fold-change indicates the ratio of target gene mRNA level in GC (T) to that in corresponding non-neoplastic mucosa (N) (T/N ratio). Overexpression (T/N > 2) of *VEGF-C* was detected in 16 (51.6%) of the 31 GC samples. Of 9 GC cases showing *VEGF-C* demethylation, 8 (88.9%) cases, including Cases 1 and 2, showed overexpression of this gene. (*c*) Immunostaining of lymphatic vessels in GC. In case 1T, which overexpresses *VEGF-C*, podoplanin-positive lymphatic vessels are present at the tumor margin. (original magnification, ×200). In case 5T, in which overexpression of *VEGF-C* was not observed, LVD was low (original magnification, ×200). (*d*) Summary of LVD. Overexpression of *VEGF-C* was associated with high LVD ($p < 0.0001$, Mann–Whitney *U* test), whereas DNA methylation status of *VEGF-C* was not associated with LVD ($p = 0.0784$, Mann–Whitney *U* test).

positive (MKN-1, MKN-7, MKN-28, TMK-1, KATO-III, SH101-P4 and HSC-44PE) and 4 *VEGF-C*-negative (MKN-45, MKN-74, HSC-39 and HSC-43) cell lines. Results of bisulfite genomic DNA sequencing are shown in Figure 1*c*. In *VEGF-C*-positive cells, all 4 CpG sites (sites 1, 2, 3 and 4) of the 3′ region of *VEGF-C* were unmethylated in 6 of 10 MKN-1 clones, all MKN-7 clones, all MKN-28 clones, all TMK-1 clones, 7 of 10 KATO-III clones, 4 of 10 SH101-P4 clones and 5 of 10 HSC-44PE clones. In contrast, no *VEGF-C*-negative cell clones showed unmethylation in all 4 CpG sites in the 3′ region of *VEGF-C*. In addition, in Aza-dC-treated MKN-45 and MKN-74 cells, all 4 CpG sites in the 3′ region were demethylated. Thus, DNA methylation of the 4 CpG sites plays an important role in transcriptional inactivation of *VEGF-C* gene, at least in the MKN-45 and MKN-74 cell lines.

DNA methylation status of VEGF-C *gene in GC tissue samples*

Because overexpression of *VEGF-C* has been reported in GC,[31,32] we examined whether DNA demethylation is involved in overexpression of *VEGF-C* in GC tissue samples. Bisulfite DNA sequencing was performed on genomic DNAs from 31 GC samples. Because bisulfite sequencing analyses of the GC cell lines revealed that DNA methylation of 4 CpG sites in the 3′ region (sites 1, 2, 3 and 4) was associated with *VEGF-C* expression, we analyzed the DNA methylation status of Region 1. Five clones from each GC sample were sequenced. Representative results of bisulfite genomic DNA sequencing analysis are shown in Figure 2*a*. In general, many CpG sites were methylated; however, the 4 CpG sites of the 3′ region of Region 1 were unmethylated in several GC samples. In most cases, the CpG sites of the 5′ region of Region 1 were densely methylated. On the basis of the data obtained from the GC cell lines, it was considered "unmethylated clone" if all 4 CpG sites 1, 2, 3 and 4 were unmethylated. We regarded the *VEGF-C* methylation status of a case as "unmethylated" if that case contains at least 1 unmethylated clone. *VEGF-C* was unmethylated in 9 (29.0%) of 31 GC samples. No association was detected between methylation status of *VEGF-C* and T grade ($p = 0.7043$), N grade ($p = 1.0000$) or tumor stage ($p = 0.4564$, Table II).

We then examined DNA methylation status of *VEGF-C* in 5 samples of corresponding non-neoplastic mucosa. In contrast to the variations in DNA methylation patterns observed in the GC samples, the DNA methylation pattern in non-neoplastic gastric mucosa samples was fairly consistent. With the exception of 2 CpG sites (sites 5 and 9), 5′ and 3′ CpG sites were densely methylated. Importantly, in contrast to GC samples, all 4 CpG sites (sites 1, 2, 3 and 4) were methylated in non-neoplastic samples, suggesting that demethylation of these 4 CpG sites is a cancer-specific event.

Relation between VEGF-C *DNA methylation status, mRNA expression and LVD*

We measured levels of *VEGF-C* mRNA by quantitative RT-PCR to investigate whether methylation of *VEGF-C* was associ-

TABLE II – ASSOCIATION BETWEEN *VEGF-C* METHYLATION STATUS AND CLINICAL FEATURES

	VEGF-C methylation status		p Value[1]
	Unmethylated	Methylated	
T grade			
T1/2	5 (33.3%)	10	0.7043
T3/4	4 (25.0%)	12	
N grade			
N0	3 (33.3%)	6	1.0000
N1/2/3	6 (27.3%)	16	
Stage			
Stage I/II	6 (35.3%)	11	0.4564
Stage III/IV	3 (21.4%)	11	

[1]Fisher's exact test.

TABLE III – ASSOCIATION BETWEEN DNA METHYLATION STATUS AND MRNA EXPRESSION OF *VEGF-C*

	VEGF-C overexpression		p Value[1]
	Positive	Negative	
Methylation status			
Unmethylated	8 (88.9%)	1	0.0155
Methylated	8 (36.4%)	14	

[1]Fisher's exact test.

ated with gene expression. Overexpression of *VEGF-C* mRNA (T/N > 2) was observed in 16 (51.6%) of 31 GC samples (Fig. 2*b*). Of 9 GC cases with unmethylated *VEGF-C*, 8 (88.9%) showed overexpression of *VEGF-C*. In contrast, only 8 of 22 (36.4%) GC cases with methylated *VEGF-C* showed overexpression (p = 0.0155, Fisher's exact test, Table III).

To investigate whether an association exists between LVD and methylation status or mRNA expression of *VEGF-C*, we evaluated LVD of 31 GC cases by immunostaining for podoplanin (Fig. 2*c*). Most podoplanin-positive lymphatic vessels were present at the tumor margin, whereas intratumoral lymphatics were rare as previously reported in other tumors.[33,34] LVD was significantly higher in GC cases with overexpressing *VEGF-C* than in those without overexpression ($p < 0.0001$, Fig. 2*d*). No association was noted between DNA methylation status of *VEGF-C* and LVD (p = 0.0784, Fig. 2*d*).

If lack of methylation of *VEGF-C* is cancer-specific and associated with gene expression, non-neoplastic tissues should not express *VEGF-C* mRNA. To investigate this hypothesis, we performed quantitative RT-PCR of 10 normal gastric mucosa samples from healthy young individuals. Expression of *VEGF-C* was not detected in 10 normal gastric mucosa samples from healthy young individuals (Fig. 3*a*). Bisulfite genomic DNA sequencing was performed for 2 of these 10 samples, and we confirmed that all the 4 CpG sites (sites 1, 2, 3 and 4) were methylated (Fig. 3*b*). In contrast, expression of *VEGF-C* was detected in several corresponding non-neoplastic gastric mucosa samples (Fig. 3*a*). However, the *VEGF-C* mRNA levels in corresponding non-neoplastic gastric mucosa samples were very low compared with those in GC tissues with *VEGF-C* unmethylation. As shown in Figure 2*a*, in samples 1N, 2N, 3N, 4N and 5N, *VEGF-C* was methylated, but expression of *VEGF-C* was detected in samples 1N, 2N, 3N and 5N.

Discussion

VEGF-C is a ligand for VEGF receptor-3, which is expressed on endothelial cells of lymphatic vessels.[35] Expression of *VEGF-C* is associated with the development of lymphatic vessels. The prognostic value of VEGF-C overexpression in GC has been reported; overexpression of VEGF-C is associated with lymph node metastasis and poor prognosis.[31,36] However, the mechanism that underlies overexpression of VEGF-C in cancers remains unclear. In the present study, we demonstrated that demethylation

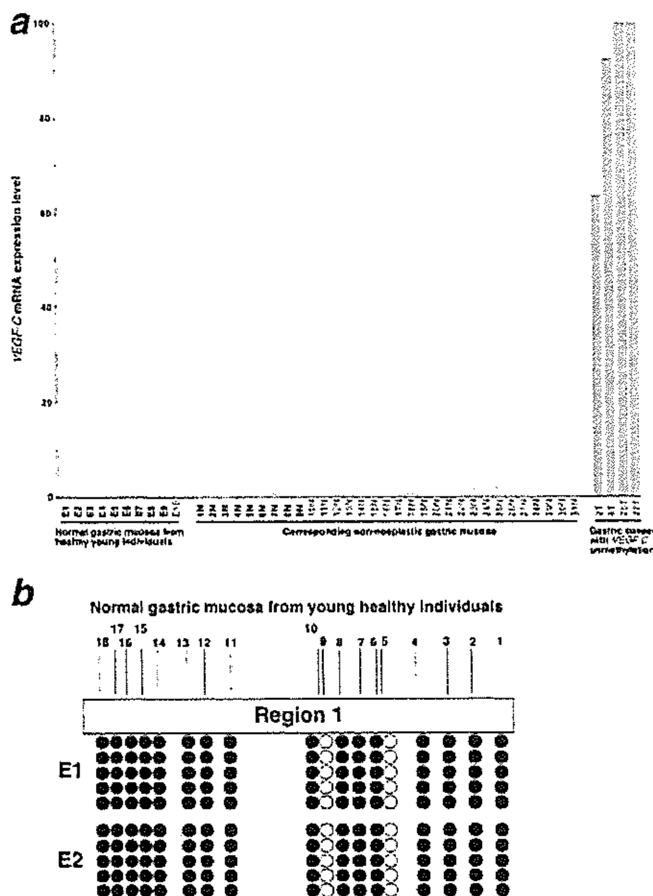

FIGURE 3 – mRNA expression and DNA methylation of *VEGF-C* in non-neoplastic tissue. (*a*) Quantitative RT-PCR analysis of *VEGF-C* in normal gastric mucosa, corresponding non-neoplastic mucosa, and GC. Units are arbitrary, and we calculated *VEGF-C* mRNA levels by standardization against 1 µg total RNA from corresponding non-neoplastic gastric mucosa from Case 1, which was taken as 1.0. (*b*) Bisulfite genomic DNA sequence of *VEGF-C* in normal gastric mucosa samples from healthy young individuals. Unmethylated allele was not detected.

of the *VEGF-C* gene is associated with expression in GC cell lines and that demethylation of *VEGF-C* by Aza-dC can activate expression of *VEGF-C* mRNA although it has been suggested that genes activated by Aza-dC may not result from the direct inhibition of DNA methylation.[37] Our results suggest that demethylation of the *VEGF-C* gene plays an important role in transcriptional activation of *VEGF-C* in GC. In addition, we found that the *VEGF-C* gene is frequently unmethylated in GC tissues and that this lack of methylation is associated with overexpression of *VEGF-C*. We confirmed that *VEGF-C* is methylated in non-neoplastic gastric mucosa samples from patients with GC, indicating that the lack of *VEGF-C* methylation in GC samples is due to demethylation of the gene. It is important to note that the source of VEGF-C in GC tissues can be from the GC cells themselves or from stromal cells such as tumor-associated macrophages (TAMs), because in squamous carcinoma of the uterine cervix, a subfraction of TAMs are a major source of VEGF-C.[38] Our findings cannot determine in which cell population changes in demethylation occur. Our previous immunohistochemical study has indicated that VEGF-C is expressed in GC cells but not in stromal cells.[39,40] Therefore, we presume that demethylation of the *VEGF-C* gene might occur in GC cells.

In the present study, several GC samples showed overexpression of *VEGF-C* mRNA in the absence of DNA demethylation and some GC cases showed partial methylation in the 4 CpG sites

(sites 1, 2, 3 and 4). In normal gastric mucosa samples from healthy young individuals, no expression of *VEGF-C* was observed, but in some non-neoplastic gastric mucosa samples from patients with GC, slight expression of *VEGF-C* was observed without DNA demethylation. These findings indicate that DNA methylation of the region we analyzed does not completely inactivate *VEGF-C* expression in some conditions. Alternative activating pathways, such as alteration of transcription factors, may account for the overexpression of *VEGF-C* in these samples. Although demethylation of *VEGF-C* by Aza-dC treatment activated expression of *VEGF-C* mRNA, our findings cannot rule out that Aza-dC treatment indirectly affects *VEGF-C* expression, for example by demethylation of a transcription factor gene required for *VEGF-C* expression. Recent studies suggest that Foxc2 can regulate *VEGF-C* expression (reviewed in Ref. 41). It is known that only methylation of a small region within a promoter CpG islands can repress gene transcription.[42] Methylation of Exon 1 or a far upstream region can be associated with loss of transcription, but usually does not have a causal role in transcriptional repression. Because slight expression of *VEGF-C* was observed without DNA demethylation in non-neoplastic gastric mucosa samples from patients with GC, DNA methylation of the region we analyzed may not have a causal role in transcriptional repression. At least however, our present data indicate that DNA demethylation is important for *VEGF-C* overexpression because most GC samples (88.9%) showing DNA demethylation overexpressed *VEGF-C* mRNA.

In the present study, high LVD was associated with overexpression of *VEGF-C* but not DNA demethylation, suggesting that overexpression of *VEGF-C* caused by DNA demethylation participate partly in lymphangiogenesis in GC. In addition to DNA demethylation, another mechanism may be involved in lymphangiogenesis. Furthermore, there was no association between DNA methylation status of *VEGF-C* and clinical features, such as lymph node metastasis. Many studies have indicated that *VEGF-C* levels in primary tumors are correlated with lymph node metastasis in thyroid, prostate, gastric, colorectal, lung and esophageal cancers.[43] To produce a metastasis, tumor cells must complete a multistep progression through a series of sequential and selective events,[44] and several molecules associated with metastasis have been reported. Therefore, other molecules may affect lymph node metastasis in the present study.

In conclusion, our data clarify one of the mechanisms involved in overexpression of *VEGF-C* in human GC. Clinical trials of DNA methylation inhibitors as cancer therapeutics are underway.[45,46] Although we did not investigate the potential effect of DNA methylation inhibitors on tumor lymphangiogenesis *in vivo*, DNA methylation inhibitors could stimulate the tumor cell metastasis by activation of *VEGF-C* gene expression at least in GC with DNA methylation of *VEGF-C*. Our data suggest that combinations of DNA methylation inhibitors and VEGF-C inhibitors[47] may be more effective anticancer therapeutics.

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DNA methylation of the *RIZ1* gene is associated with nuclear accumulation of p53 in prostate cancer

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The retinoblastoma protein-interacting zinc finger gene, *RIZ1*, is thought to be a tumor suppressor gene. *RIZ1* is inactivated by mutation, deletion and DNA methylation in several human cancers. In the present study, the relationship between DNA methylation of *RIZ1* and mutation of *p53* was investigated in prostate cancer (PCa). In total, 47 cases of node-negative PCa (stages I–III) were analyzed. DNA methylation of the *RIZ1* gene was detected in 20 (42.6%) of the 47 PCa tissues by methylation-specific polymerase chain reaction. DNA methylation of the *RIZ1* gene was not associated with clinicopathological features. DNA methylation of *RIZ1* tended to be present more frequently in PCa specimens with a high Gleason score (16/30, 53.3%) than in those with a low Gleason score (4/17, 23.5%); however, this tendency was not statistically significant ($P = 0.0675$). Nuclear accumulation of p53 was observed in four (8.5%) of 47 PCa specimens by immunostaining. All four PCa specimens with nuclear accumulation of p53 were stage III disease and showed DNA methylation of *RIZ1*. However, of the remaining 43 cancers without nuclear accumulation of p53, DNA methylation of *RIZ1* was observed in only 16 (37.2%) specimens ($P = 0.0272$). Of the three PCa cell lines, only the PC3 cell line showed loss of *RIZ1* mRNA due to DNA methylation, and this loss was rectified by treatment with a demethylating agent, 5-Aza-2′-deoxycytidine. These results suggest that transcriptional inactivation of *RIZ1* by aberrant DNA methylation may contribute to prostate carcinogenesis. Genetic alterations are likely associated with epigenetic alterations in PCa. (*Cancer Sci* 2007; 98: 32–36)

Prostate cancer (PCa) is one of the most common cancers and the second leading cause of cancer death in men in the USA.[1] An understanding of the genetic and epigenetic pathways involved in the pathogenesis of PCa is essential for development of improved diagnostic and treatment modalities. A variety of genetic and epigenetic alterations are associated with PCa.[2,3] Epigenetic changes, such as DNA methylation of CpG islands, are detected commonly in human cancers. Hypermethylation of CpG islands is associated with silencing of many genes, especially defective tumor-related genes, and has been proposed as an alternative way to inactivate tumor-related genes in human cancers.[4,5] Identification of methylated genes may be useful in the diagnosis and treatment of PCa and may provide insight into prostate carcinogenesis. Prior studies have shown that DNA hypermethylation is a crucial mechanism in transcriptional silencing of tumor-related genes in PCa.[6,7]

The retinoblastoma protein-interacting zinc finger gene, *RIZ1*, was isolated with a functional screen for retinoblastoma (Rb)-binding proteins.[8] Domain analysis suggested that RIZ1 is a histone methyltransferase (HMT) specific for the lysine 9 residue of histone H3, an activity known to be linked with transcriptional repression.[9] *RIZ1* is considered to be a tumor suppressor gene because it can induce G_2–M arrest and apoptosis of several types of cancer cells.[10,11] *RIZ1* plays an important role in human cancers, as evidenced by genetic mutations.[12–14] The *RIZ1* gene is located on human chromosome 1p36, a region deleted in many human cancers,[15] and chromosome 1p36 is a potential hereditary PCa susceptibility locus.[16] In addition to genetic alterations, DNA methylation of *RIZ1* has been shown to be a common mechanism for inactivation of *RIZ1* expression in human cancers.[17,18] In PCa, DNA methylation of *RIZ1* is present in 31% of tumor tissues.[19]

A knockout study showed that *RIZ1* is a tumor susceptibility gene in mice.[14] *RIZ1* and *p53* deficiencies likely cooperate in tumor formation in mice and are expected to occur in human cancers as well.[14] In fact, many sporadic human cancers carry both *p53* mutations and a silenced *RIZ1* gene.[10,14] The *p53* gene is involved in the tumorigenesis of many human cancers,[20] including PCa.[21] p53 functions as a transcriptional regulator involved in G_1 phase growth arrest of cells in response to DNA damage. p53 also has roles in regulation of the spindle checkpoint, centrosome homeostasis and G_2–M phase transition.[22] Several lines of evidence suggest associations between genetic and epigenetic alterations. *p53* mutations have been found frequently in colorectal and gastric cancers without DNA methylation.[23,24] However, the association between genetic and epigenetic alterations has not been investigated in PCa.

In the present study, we investigated the relationship between *RIZ1* methylation status and *p53* mutation status in 47 PCa tissues. To determine whether transcriptional silencing of the *RIZ1* gene is caused by DNA hypermethylation, we compared the methylation status with expression of *RIZ1* mRNA in PCa cell lines.

Materials and Methods

Tissue samples. Subjects were 47 patients with PCa who were referred to the Department of Urology, Hiroshima University Hospital (Hiroshima, Japan). Forty-seven PCa tissues from these 47 patients were analyzed for DNA methylation of *RIZ1* and localization of p53. PCa samples were obtained by radical prostatectomy, and all PCa cases were confirmed to be node negative by pathological examination. None of the 47 patients with PCa received preoperative treatment. All 47 specimens were archival, formalin-fixed, paraffin-embedded tissues. It was confirmed microscopically that the tumor specimens consisted mainly (>50%) of cancer cells. Tumor staging was according to the TNM classification system.[25] In the present study, PCa were graded by the reporting pathologists on the radical surgery specimen, according to the system of the Gleason score.[26] After prostatectomy, the serum prostate-specific antigen (PSA) level was measured by *E*-test Tosoh II Assay (Tosoh, Tokyo, Japan). Patients were followed up by PSA measurement monthly during the first 6 months after prostatectomy and then every 3 months

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thereafter. Biochemical relapse was defined as a PSA level of 0.2 ng/mL or greater. Because written informed consent was not obtained, for strict privacy protection, identifying information for all samples was removed before analysis. This procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research of the Japanese Government.

Cell lines and drug treatment. LNCaP, PC3 and DU145 PCa cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). All cell lines were maintained in RPMI-1640 (Nissui Pharmaceutical, 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. Cells were treated with a final concentration of 1 µM 5-aza-2′-deoxycytidine (Aza-dC; Sigma Chemical, St Louis, MO, USA) for 5 days before they were harvested for DNA or RNA extraction.

Genomic DNA extraction and methylation-specific polymerase chain reaction. For extraction of DNA from the archival, formalin-fixed, paraffin-embedded tissue samples, PCa samples were dissected manually from different sets of 10 serial, 10 µm-thick, formalin-fixed, paraffin-embedded tissue sections with a fine needle. The dissected samples were lysed by incubation in 200 mg/mL proteinase K at 55°C for 3 days. Genomic DNA was purified by three rounds of phenol–chloroform extraction followed by ethanol precipitation. For DNA extraction from cell lines, genomic DNA was extracted with a Genomic DNA Purification Kit (Promega, Madison, WI, USA). To examine the DNA methylation pattern, genomic DNA was treated with 3 M sodium bisulfite, as described previously.[27] For analysis of DNA methylation of the *RIZ1* gene, methylation-specific polymerase chain reaction (MSP) was carried out as described previously.[17] Polymerase chain reaction (PCR) products (15 µL) were loaded onto 8% non-denaturing polyacrylamide gels, stained with ethidium bromide, and visualized under ultraviolet light.

Immunohistochemistry. Formalin-fixed, paraffin-embedded samples were sectioned, deparaffinized, and stained with hematoxylin–eosin to ensure that the sectioned block contained tumor cells. Adjacent sections were then stained immunohistoschemically. For immunostaining of p53, a Dako LSAB Kit (Dako, Carpinteria, CA, USA) was used in accordance with the manufacturer's recommendations. In brief, sections were pretreated by microwaving in citrate buffer for 30 min to retrieve antigenicity. After peroxidase activity was blocked with 3% H_2O_2–methanol for 10 min, sections were incubated with normal goat serum (Dako) for 20 min to block non-specific antibody binding sites. Anti-p53 antibody (DO7, 1 : 100; Novocastra, Newcastle, UK) was incubated with tissue samples for 60 min at room temperature followed by incubations with biotinylated antimouse IgG and peroxidase-labeled streptavidin for 10 min each. Staining was completed with a 10-min incubation with the substrate–chromogen solution. The sections were counterstained with 0.1% hematoxylin. p53 staining was classified according to the percentage of stained cancer cells. When more than 10% of cancer cells were stained, the immunostaining was considered positive.

PCR–single-strand conformation polymorphism analysis. Exons 5–8 of the *p53* gene were examined for mutations by PCR–single-strand conformation polymorphism (SSCP) analysis with 10 sets of primers, as described previously.[28] Each target sequence was amplified in a 20-µL reaction volume containing 10–20 ng genomic DNA, 0.2 µM dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.3 µM of each primer and 0.2 µL of Ampli Taq Gold (Applied Biosystems, Foster City, CA, USA). PCR amplification consisted of 35 cycles of 94°C for 30 s, 60°C or 55°C for 30 s, and 72°C for 30 s after the initial activation step of 94°C for 10 min. PCR products were diluted 10-fold with formamide dye solution, denatured at 85°C for 10 min, and separated by electrophoresis on 6% polyacrylamide gels. Gels were stained, and bands were visualized with a Silver Staining II kit (WAKO, Osaka, Japan).

Reverse transcription–polymerase chain reaction. Expression of *RIZ1* mRNA was analyzed by reverse transcription (RT)-PCR. 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). Primer sequences and amplification conditions were as described previously.[18] RT-PCR products were subjected to 1.5% agarose gel electrophoresis, stained with ethidium bromide, and examined under ultraviolet light. *ACTB*-specific PCR products were amplified from the same RNA samples and served as internal controls.

Statistical methods. Associations between clinicopathological parameters and DNA methylation of *RIZ1* were analyzed by Fisher's exact test. A *P*-value of less than 0.05 was considered statistically significant.

Results

DNA methylation of *RIZ1* and p53 mutation status in PCa tissues. DNA methylation status of the *RIZ1* gene was examined in a total of 47 PCa tissue specimens from 47 patients. DNA methylation of *RIZ1* was detected in 20 (42.6%) of 47 PCa tissues. Representative results of MSP for *RIZ1* are shown in Fig. 1A. No association was detected between the methylation status of

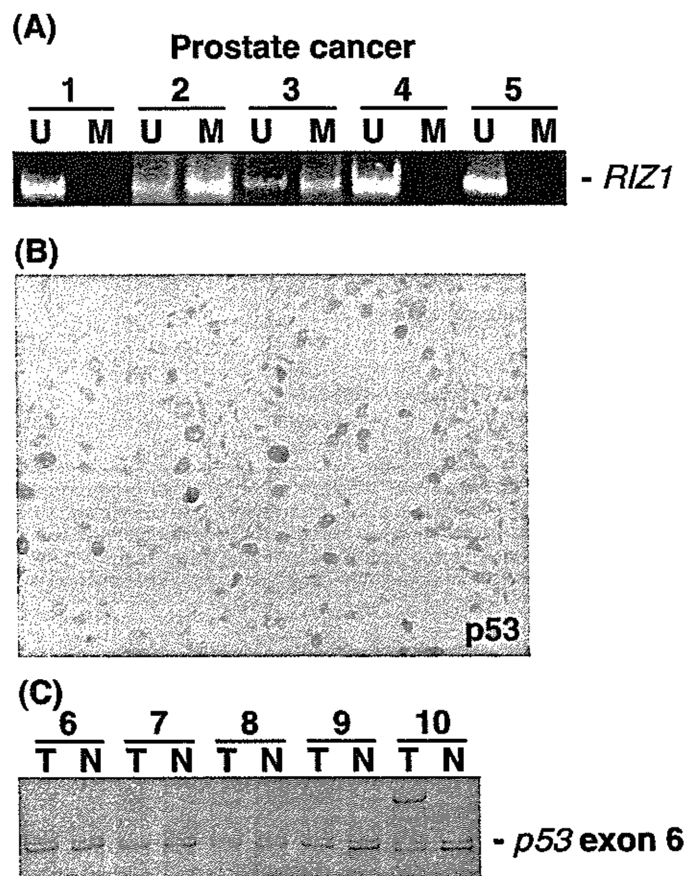

Fig. 1. (A) Methylation-specific polymerase chain reaction (PCR) of *RIZ1* in prostate cancer (PCa). Methylated *RIZ1* was detected in two cases (cases 2 and 3) of PCa. U, unmethylated PCR product; M, methylated PCR product. (B) Immunostaining of p53 in PCa. Nuclear accumulation of p53 was observed in PCa cells. Original magnification, ×400. (C) PCR–single-strand conformation polymorphism analysis of *p53*. A *p53* mutation was detected in one case (case 10).

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RIZ1 and age (P = 1.000), T grade (P = 0.1425), stage (P = 0.1425), preoperative PSA concentration (P = 0.7674), or relapse (P = 0.3917) (Table 1). DNA methylation of *RIZ1* tended to occur more frequently in PCa cases with a high Gleason score (16/30, 53.3%) than in those with a low Gleason score (4/17, 23.5%); however, the difference was not statistically significant (P = 0.0675; Table 1). We next investigated the nuclear accumulation of p53 in the 47 PCa tissues by immunostaining. Nuclear accumulation of p53 typically indicates the presence of *p53* gene mutations.[29] Immunostaining revealed nuclear accumulation of p53 in four (8.5%) of 47 PCa tissues (Fig. 1B).

Table 1. Association between *RIZ1* methylation status and clinicopathological features and nuclear accumulation of p53 in prostate cancer

Feature	*RIZ1* methylation status			*P*-value
	Methylated	n%	Unmethylated	
Age (years)				
>70	10	43.5	13	1.0000
≤70	10	41.7	14	
Tumor grade				
T1/2	7	30.4	16	0.1425
T3	13	54.2	11	
Stage†				
I/II	7	30.4	16	0.1425
III	13	54.2	11	
Gleason score‡				
2–6	4	23.5	13	0.0675
7–10	16	53.3	14	
Preoperative PSA (ng/mL)§				
<10	13	44.8	16	0.7674
>10	7	38.9	11	
Relapse¶				
Positive	7	35.0	13	0.3917
Negative	13	48.1	14	
Nuclear accumulation of p53				
Positive	4	100.0	0	0.0272
Negative	16	37.2	27	
p53 mutation determined by PCR-SSCP				
Mutant-type	2	100.0	0	0.1758
Wild-type	18	40.0	27	

†Tumor stage according to TNM classification. ‡Tumor grade according to Gleason criteria. §Prostate-specific antigen (PSA) concentration was determined as described in the Materials and Methods. ¶Relapse was defined as serum PSA concentration of 0.2 ng/mL or higher. PCR-SSCP, polymerase chain reaction–single-strand conformation polymorphism.

We also carried out PCR-SSCP analysis of *p53*. Representative results are shown in Fig. 1C. Of the four PCa specimens with nuclear accumulation of p53, two (50.0%) exhibited a *p53* mutation. No mutation was found in the 43 PCa specimens without nuclear accumulation of p53. All PCa specimens with nuclear accumulation of p53 showed DNA methylation of *RIZ1* whereas only 16 (37.2%) of 43 PCa specimens without nuclear accumulation of p53 showed DNA methylation of *RIZ1* (P = 0.0272, Fisher's exact test; Table 1). We found no association between the methylation status of the *RIZ1* and *p53* mutations determined by PCR-SSCP analysis (P = 0.1758, Fisher's exact test; Table 1).

DNA methylation status and expression of *RIZ1* in PCa cell lines. To determine whether DNA hypermethylation of *RIZ1* inactivates transcription of the gene, DNA methylation and expression of *RIZ1* were investigated in three PCa cell lines (Fig. 2A). MSP revealed DNA hypermethylation of *RIZ1* in PC3 cells, whereas hypermethylation of *RIZ1* was not detected in LNCaP or DU145 cells. To study the relationship between DNA methylation status and *RIZ1* expression levels, we carried out RT-PCR of mRNA from PC3 cells. Transcriptional inactivation of *RIZ1* was observed in PC3 cells with DNA hypermethylation (Fig. 2B). LNCaP and DU145 cells expressed *RIZ1*. To investigate whether transcriptional inactivation of *RIZ1* was caused by DNA methylation in PC3 cells, we treated PC3 cells and LNCaP cells (unmethylated control) with Aza-dC and carried out MSP (Fig. 2C) and RT-PCR (Fig. 2D) analyses. Unmethylated *RIZ1* was detected in PC3 cells after Aza-dC treatment. Expression of *RIZ1* was restored in PC3 cells after treatment with Aza-dC. *RIZ1* expression in LNCaP cells was not changed significantly by Aza-dC treatment.

Discussion

A variety of genetic and epigenetic alterations are associated with human cancers. Although there have been several reports regarding genetic and epigenetic changes in various genes in PCa, in most of these studies, the alteration was investigated for just a single gene. In the present study, the relationship between DNA methylation of *RIZ1* and mutation of *p53*, as measured by nuclear accumulation of p53, was investigated, and we found that PCa tissues with nuclear accumulation of p53 also showed DNA methylation of the *RIZ1* gene.

In the present study, DNA methylation of *RIZ1* was found in 42.6% of PCa cases analyzed, a frequency slightly higher than that reported previously.[19] In the PCa cell lines, DNA hypermethylation of *RIZ1* was detected in PC3 cells, which expressed undetectable levels of the *RIZ1* mRNA. After 5 days of Aza-dC treatment, unmethylated *RIZ1* was observed, and expression of *RIZ1* mRNA followed. Thus, hypermethylation of *RIZ1* plays an

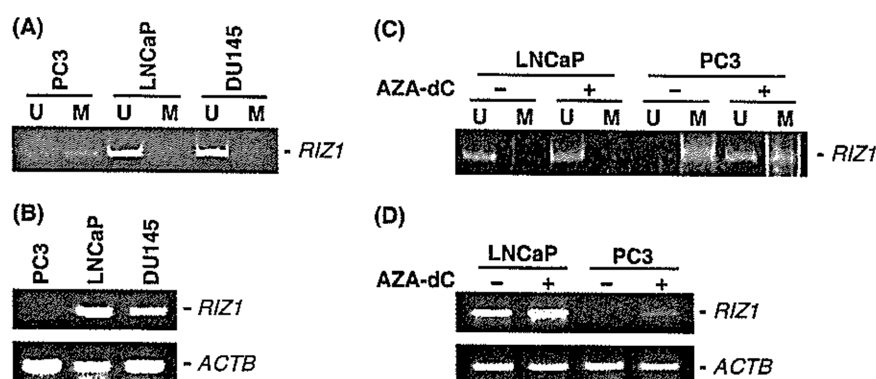

Fig. 2. DNA methylation status and expression of *RIZ1* in prostate cancer (PCa) cell lines. (A) Methylation-specific polymerase chain reaction (MSP) of *RIZ1*. The methylated allele was detected only in the PC3 cell line. U, unmethylated polymerase chain reaction (PCR) product; M, methylated PCR product. (B) Reverse transcription–PCR of mRNA from PCa cell lines. *RIZ1* is not expressed in the PC3 cell line. (C) Effect of 5-Aza-2′-deoxycytidine (Aza-dC) treatment. Aza-dC-treated LNCaP and PC3 cells and untreated LNCaP and PC3 cells were analyzed by MSP. The unmethylated allele was detected in Aza-dC-treated PC3 cells but not in untreated PC3 cells (D) Expression of *RIZ1* was analyzed in Aza-dC-treated LNCaP and PC3 cells and untreated LNCaP and PC3 cells. RIZ1 mRNA is expressed in Aza-dC-treated PC3 cells but not in untreated PC3 cells.

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