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

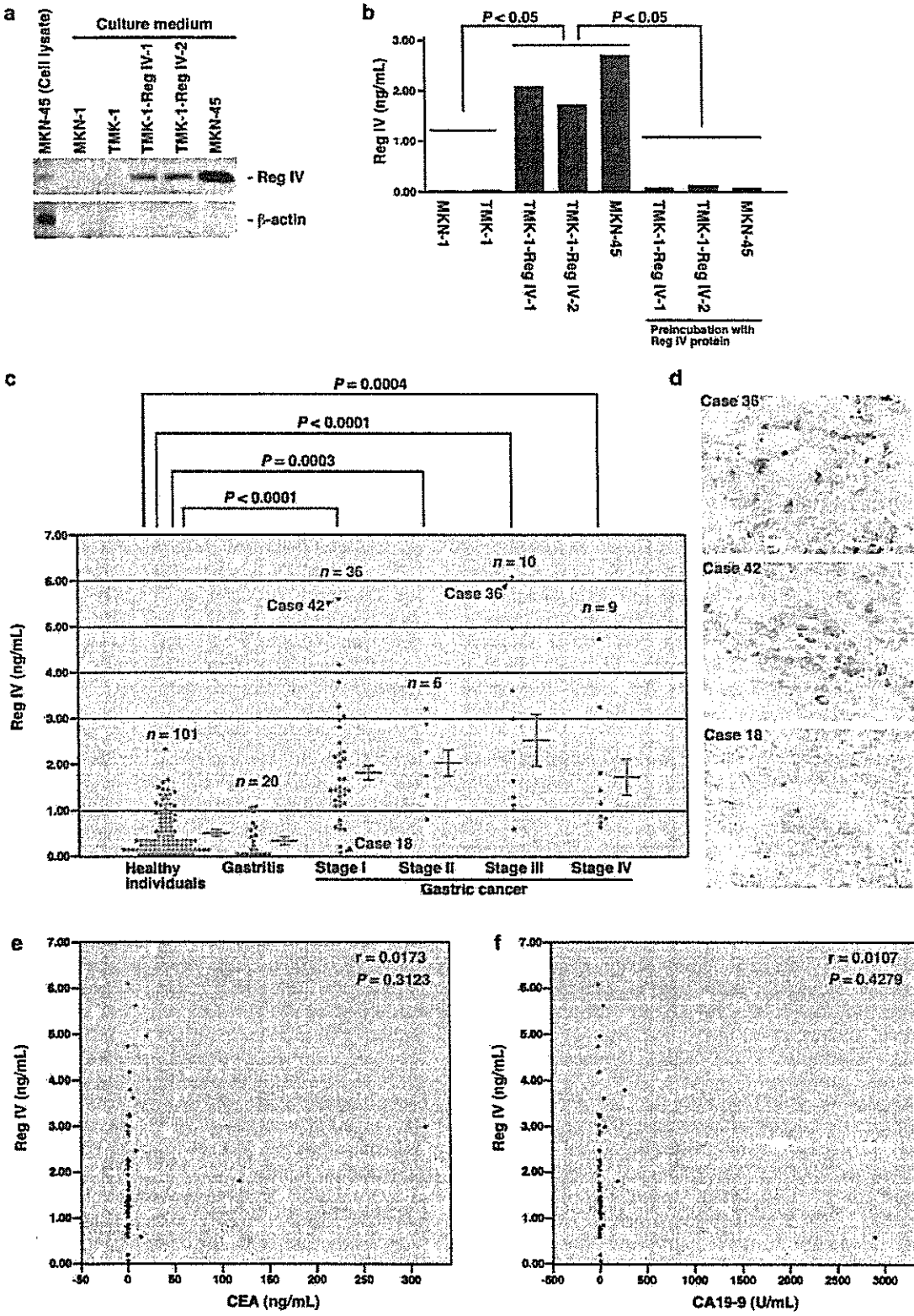

Table 3 Diagnostic sensitivity of serum Reg IV, CEA and CA19-9 with respect to tumor stage

	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.

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 $p21^{WAF1/CIP15}$ 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*-

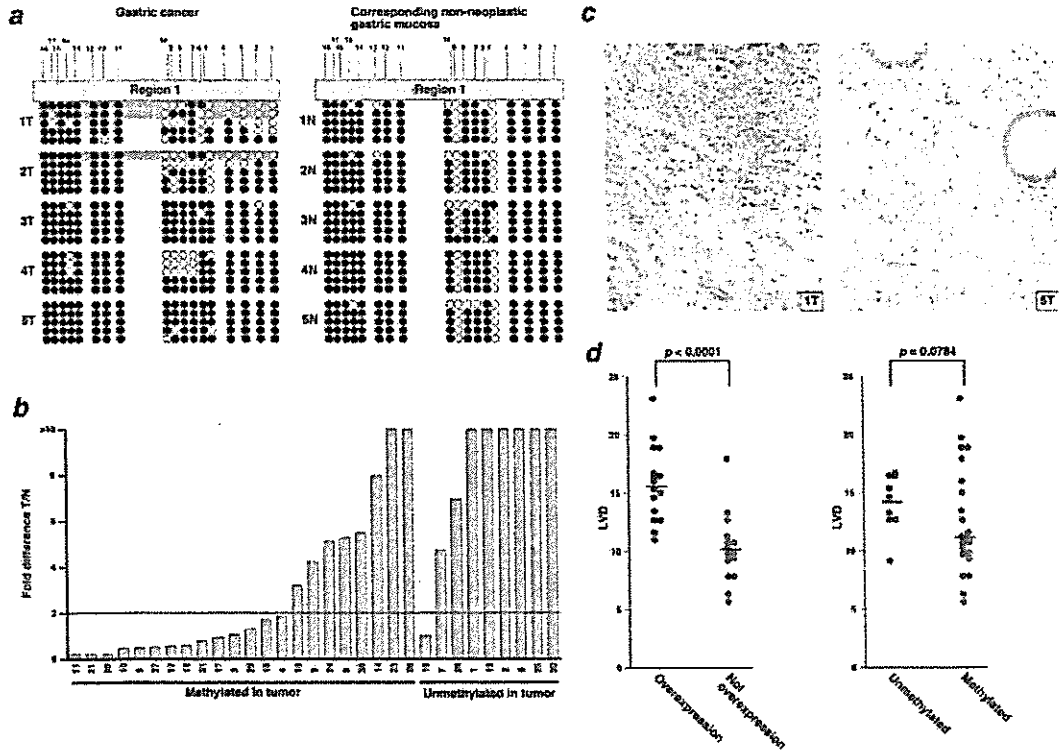

FIGURE 2 – DNA methylation and expression of *VEGF-C* and LVD analysis in GC tissue. (*a*) Representative results of bisulfite genomic DNA sequencing for GC tissues. In cases 1T and 2T, the unmethylated allele (allele contains unmethylated CpG sites 1, 2, 3 and 4, red box) was detected. In corresponding 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.

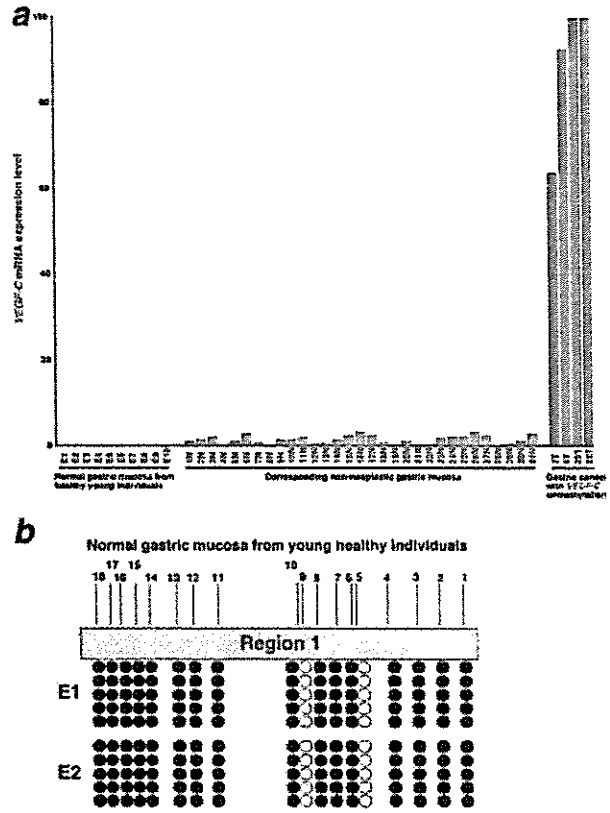

FIGURE 3 – mRNA expression and DNA methylation of *VEGF-C* in non-neoplastic tissue. (*a*) Quantitative RT-PCR analysis of *VEGF-C* in normal gastric mucosa, corresponding non-neoplastic mucosa, and GC. Units are arbitrary, and we calculated *VEGF-C* mRNA levels by standardization against 1 μg total 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.

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 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 immunohistochemically. 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

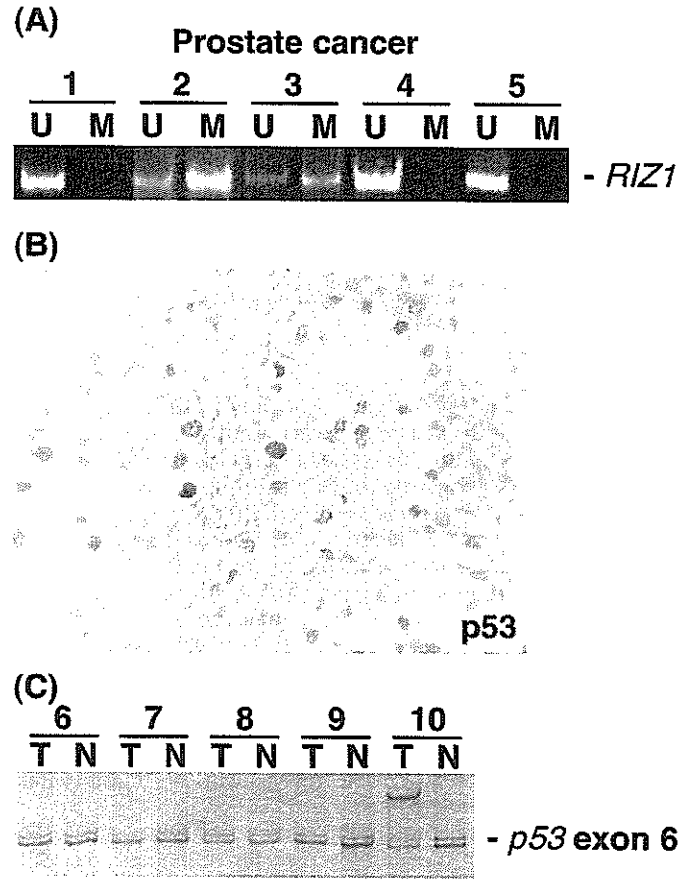

Fig. 1. (A) Methylation-specific polymerase chain reaction (PCR) of *RIZ1* in prostate cancer (PCa). Methylated *RIZ1* was detected in two cases (cases 2 and 3) of PCa. U, unmethylated PCR product; M, methylated PCR product. (B) Immunostaining of p53 in PCa. Nuclear accumulation of p53 was observed in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

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

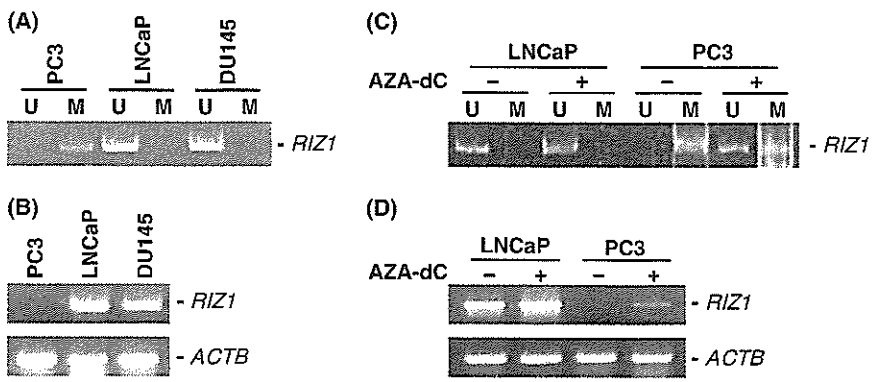

Fig. 2. DNA methylation status and expression of *RIZ1* in prostate cancer (PCa) cell lines. (A) Methylation-specific polymerase chain reaction (MSP) of *RIZ1*. The methylated allele was detected only in the PC3 cell line. U, unmethylated polymerase chain reaction (PCR) product; M, methylated PCR product. (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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important role in inactivation of the *RIZ1* gene. Previous studies have demonstrated that CpG island hypermethylation in the region examined was well correlated with epigenetic silencing of the *RIZ1* gene.[17,18] In the present study, DNA methylation of the *RIZ1* gene was not associated with clinicopathological features. Because DNA methylation of *RIZ1* is a rare event in non-malignant prostate tissues,[19] these results suggest that DNA methylation of the *RIZ1* gene may be associated with prostate carcinogenesis.

It has been reported that *p53* is mutated in late-stage PCa.[21] In the present study, nuclear accumulation of p53 was observed only in stage III PCa, and all PCa tissues with nuclear accumulation of p53 showed DNA methylation of *RIZ1*. In the present study, of the four PCa specimens with nuclear accumulation of p53, only two exhibited a *p53* mutation. These inconsistent results between immunohistochemistry and PCR-SSCP may be due to methodological differences. Because only exons 5–8 were examined by PCR-SSCP in the present study, the mutation of *p53* may be in the remaining exons. It is also possible that the sensitivity of PCR-SSCP may not be sufficient to detect a small number of mutant alleles in a background of wild-type alleles from stromal cells and normal epithelial cells. It has been reported that the correlation between nuclear accumulation of p53 and the presence of *p53* gene mutations can vary.[30]

RIZ1 has HMT activity.[9] HMT activity is thought to be important to the tumor suppression function of RIZ1, because this activity is reduced by *RIZ1* mutations found in human cancers.[9] Because histone modification is thought to affect chromatin structure directly,[31] aberrant chromatin structure may induce mutation of the *p53* gene. In fact, Suv39h (an HMT) -deficient mice display severely impaired viability and chromosomal instabilities.[32] A subset of PCa cells with mutations in *p53* may arise from PCa cells with DNA methylation of *RIZ1*. Therefore, DNA methylation of *RIZ1* may predict development of *p53* mutant PCa cells. In the PCa cell lines, *p53* mutation status has been described previously (PC3, mutant-type *p53*; LNCaP, wild-type *p53*; DU145, mutant-type *p53*).[33] Because DNA hypermethylation of *RIZ1* was detected in PC3 cells, mutation of the *p53* gene may be induced by inactivation of *RIZ1*. In contrast, DNA hypermethylation of *RIZ1* was not detected in DU145 cells despite the presence of *p53* mutation. Whether silencing of *RIZ1* induces *p53* mutation should be verified experimentally in the near future.

Treatment options for the early stages of PCa have been limited to local treatment. Treatments for more advanced disease rely on suppression of testosterone production, primarily with hormonal therapy. Until the introduction of luteinizing hormone-releasing hormone agonist therapy, estrogen therapy was often used for hormonal manipulation of PCa.[34] Because estradiol treatment produces a selective decrease in *RIZ1* expression,[35] it is interesting to investigate the *RIZ1* expression in PCa tissues from patients who received preoperative hormone treatment.

In conclusion, our results suggest that genetic alterations are associated with epigenetic alterations and that these alterations are not random events in PCa. To better understand the development of PCa at the molecular level, molecular classification of PCa based on genetic and epigenetic alterations may be useful.

Acknowledgments

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MOLECULAR PATHOBIOLOGY OF GASTRIC CANCER

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ABSTRACT

Gastric carcinogenesis is a multistep process, during which numerous genetic and epigenetic alterations accumulate: there are abnormalities of growth factors/receptors, angiogenic factors, cell cycle regulators, DNA mismatch repair genes etc. These abnormalities define, at the same time, the biological character of the cancer cell and may thus serve as therapeutic targets. Genetic instability may cause accumulation of genetic abnormalities. The most important epigenetic alterations are DNA methylation, histone modification and chromatin remodeling. Some of these changes are common in gastric cancer, regardless of subtype, and some differ by histological type or (gastric or intestinal) mucin phenotype. Genetic polymorphism is a crucial endogenous cause and fundamental aspect of cancer risk. Importantly, genetic polymorphisms are also associated with the therapeutic efficacy and toxicity of anti-cancer drugs. Genomic science and technology such as Serial Analysis of Gene Expression (SAGE) allows the identification of novel genes and molecules specifically up-regulated or down-regulated in gastric cancer, e.g., RegIV and claudin-18 can be identified. Advances in our understanding of the genetic and molecular bases lead to improved diagnosis, personalised medicine and prevention of gastric cancer.

Key words: Gastric cancer; molecular pathology; cell cycle regulator; angiogenic factor; genetic instability; epigenetics; serial analysis of gene expression

INTRODUCTION

Gastric cancer is one of the most common human cancers worldwide. Although recent diagnostic and therapeutic advances have provided excellent survival for patients with early gastric cancer, the prognosis of patients with advanced cancer is still poor. The pathogenesis of gastric cancer involves *Helicobacter pylori* (*H. pylori*) infection, chronic active or atrophic gastritis and intestinal metaplasia. Integrated research in molecular pathology has shown that gastric cancer is a chronic proliferative disease characterised by multiple genetic and epigenetic alterations, i.e., a disease of altered gene expression (1-5). Genetic polymorphism is an important endogenous cause and a modulator of the risk of a person of developing gastric cancer. Advances of genomic science, including global analysis of gene expression, have uncovered detailed molecular mechanisms of gastric carcinogenesis (5, 6). A better understanding of the molecular bases of gastric cancer may lead to new diagnostic, therapeutic and preventive approaches to the disease. Recently identified molecules that participate in carcinogenesis appear to be useful targets for cancer therapy.

This review describes an outline of the molecular pathway of stomach carcinogenesis, of the importance of epigenetic alterations, of the significance of genetic polymorphism, and of the biological and clinical roles of recently identified genes involved in gastric cancer.

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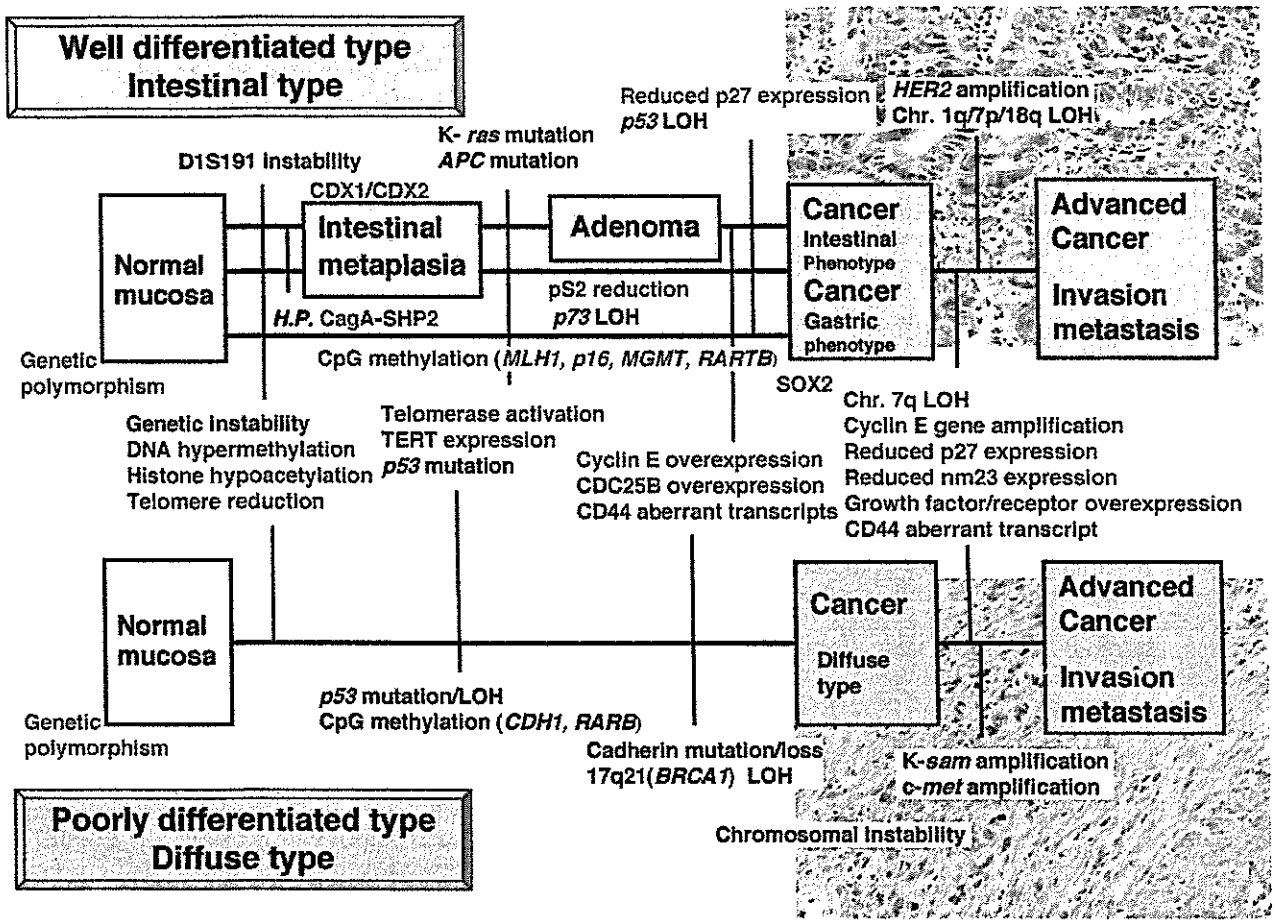

Fig. 1. Multiple genetic and epigenetic alterations during gastric carcinogenesis. Texts written in dark-blue stand for genetic alterations and those in green stand for epigenetic alterations.

OUTLINE OF MOLECULAR CARCINOGENESIS

An accumulation of genetic and molecular abnormalities occurs during gastric carcinogenesis, which is a multistep process (Fig. 1) (2, 4). The steps include activation of oncogenes; overexpression of growth factors/receptors and matrix metalloproteinases; inactivation of tumour suppressor genes, DNA repair genes and cell adhesion molecules; and abnormalities of cell cycle regulators that define biological characteristics of cancer cells (and could thus serve as therapeutic targets). Common genetic alterations in gastric cancer include loss of heterozygosity and point mutations of tumour suppressor genes, while common epigenetic changes are gene silencing of tumour suppressors by CpG island methylation and overexpression of tumour promoting genes at the transcriptional level. Genetic polymorphisms predispose to cancer and alter the person's susceptibility to cancer. Genetic instability, DNA methylation, telomerase activation and *p53* mutations participate in the early stage of gastric carcinogenesis. Increased hTERT expression and strong telomerase activity confer cellular immortality in a majority of gastric cancers. Amplification and overexpression of the c-*met*, the EGF receptor (EGFR) and the cyclin E genes are associated with the late stage. Overexpression of growth factors and angiogenic factors lead to cancer progression and metastasis. Some of these changes occur often in well differentiated and in poorly differentiated types of cancers and some differ by histological type or (gastric or intestinal) mucin phenotype. Intestinal metaplasia and adenomas share abnormalities similar to those in the well-differentiated type of gastric cancer, such as the *APC* and K-*ras* mutation albeit at low frequency.

GROWTH FACTORS, CYTOKINES, ANGIOGENIC FACTORS

Gastric cancer cells produce various growth factors and express their receptors to bring about biological events, including cancer-stromal interactions (2, 4). These factors induce not only cell growth but also extracellular matrix degradation and angiogenesis which facilitate tumour invasion and metastasis. Simultaneous expression of EGF/TGF-alpha and EGFR

is associated with cancer progression and correlates with poor patient prognosis. IL-alpha acts as an autocrine growth factor of gastric cancer and it interacts with the EGFR system. Gene amplification of the c-*met* (hepatocyte growth factor [HGF]) receptor frequently occurs in the scirrhous type of gastric cancer. HGF produced by stromal cells stimulates cancer cell motility. Gene amplification and overexpression of *HER-2/c-erbB2* and K-*sam* are preferentially found in well differentiated and poorly differentiated gastric cancer, respectively.

Angiogenesis is regulated by several angiogenic factors produced by cancer cells and stromal cells (4). Neovascularisation stimulates the growth of the primary tumour and provides an avenue for haematogenous metastasis. In gastric cancer, there is a positive association between microvessel count, metastatic potential and poor prognosis. Among the best known angiogenic factors of gastric cancer cells are vascular endothelial growth factor (VEGF), interleukin (IL-8), basic fibroblast growth factor (bFGF) and platelet-derived endothelial cell growth factor (PD-ECGF). Most gastric cancers express IL-8/receptor systems, and IL-8 levels correlate closely with neovascularity. Well differentiated gastric cancer expresses VEGF at high levels, while poorly differentiated scirrhous cancer expresses strongly bFGF.

CELL CYCLE REGULATORS

Cell cycle checkpoints are regulatory pathway that control cell cycle transitions, and ensure DNA replication and chromosome segregation with high fidelity. Any dysbalance in the actions of cell cycle regulators results in genomic instability and unbridled cell proliferation and will contribute to tumour development (2, 4). Several abnormalities of positive and negative regulators of the cell cycle are associated with gastric cancer. Gene amplification of cyclin E is present in 15-20% of gastric cancer samples and an increased expression of cyclin E correlates with tumour progression. p53 is multifunctional and participates in cell cycle regulation in part through p21 induction. Inactivation of p53 by loss of heterozygosity (LOH) and through mutations occurs in over 60% of all gastric cancers. Expression of p27 is reduced in gastric adenoma, and further reduction is associated with metastasis, indicating participation in development as well as in progression of gastric cancer. Aberrant (reduced or increased) expression of p16 is frequently found in gastric cancer, but the biological role is unknown. An important downsteam target for cyclins/cyclin-dependent kinases (CDKs) at the G1/S checkpoint is the retinoblastoma (RB) protein. The expression of RB is weaker in nodal metastases of primary gastric cancers. Checkpoint kinase 1 (Chk1) and Chk2 are DNA damage-activated kinases involved at the G2/M checkpoint. Both Chk1 and Chk2 are overexpressed in more than 70% of gastric cancers associated with the *p53* mutation. Chk1 and Chk2 may play a role in the checkpoint function of gastric cancers with the *p53* mutation.

GENETIC INSTABILITY

Genomic instability is classified into microsatellite instability (MSI) and chromosome instability. Dysfunction of DNA mismatch repair causes MSI. The target genes for MSI include *TGFBRII, IGFIIR, BAX, hMSH3, hMSH6* and *MBD4* (2, 4). MSI or genetic instability leads to accumulation of genetic alterations and contributes to the pathogenesis not only of hereditary non-polyposis colorectal cancer but also of sporadic gastric cancer. CpG island hypermethylation of *hMLH1* and loss of expression are the main mechanisms of MSI in sporadic gastric cancer. The frequency of MSI is especially high in well differentiated gastric cancer of the foveolar phenotype with a papillary structure. There is a tight association between MSI and tumour multiplicity; the frequency of MSI is apparently high in patients with multiple primary cancers. Therefore, detection of MSI serves as a good predictor of another cancer in a patient with gastric cancer. Many reports have, on the other hand, indicated that MSI is associated with less aggressive behaviour of the tumour and a more favourable prognosis.

EPIGENETIC ALTERATIONS

Among the epigenetic alterations that occur in cancer, abnormalities of the transcription step are important; post-translational modifications (such as ubiquitin-dependent degradation) are also involved. For regulation of gene expression on a transcriptional level, DNA methylation, histone modification and chromatin remodeling function as an on-off switch, while the transcription factors act as a volume switch. The abnormal methylation of CpG islands – usually associated with tumour suppressor genes – leads to transcriptional silencing, inactivation of gene function and, ultimately, to carcinogenesis (3, 4). In gastric cancer, aberrant methylation occurs in several tumour suppressor genes, such as *p16, CDH1* (E-cadherin), *hMLH1, RAR-beta, RUNX3, MGMT* (O^6-methylguanine methyltransferase), *TSP1* (thrombospondin-1), *HLTF* (helicase-like transcription factor), and *RIZ1* (retinoblastoma protein-interacting zinc finger gene-1), with the incidence of methylation raging from 10% to 70%. When DNA methylation associated with 12 tumour-related genes (*hMLH1, MGMT, p16, CDH1, RAR-beta, HLTF, RIZ1, TM, FLNs, LOX, HRASLS, HAND1*) was analysed, a high methylation rate (5 or more methylated genes) was observed more frequently in stage III/IV cancers than in stage I/II cancers. Thus it indicates that DNA methylation of tumour-related genes accumulates as the tumour progresses (7). On the other hand, some gene promoters become hypermethylated in non-neoplastic conditions, like aging. The incidence of promoter hypermethylation of *hMLH1* and *p16* is higher in non-neoplastic gastric mucosa of gastric cancer patients than in the gastric mucosa of healthy individuals.

The amino terminals of histones are subjected to acetylation and methylation, which recruit downstream regulatory factors, influence chromatin struc-