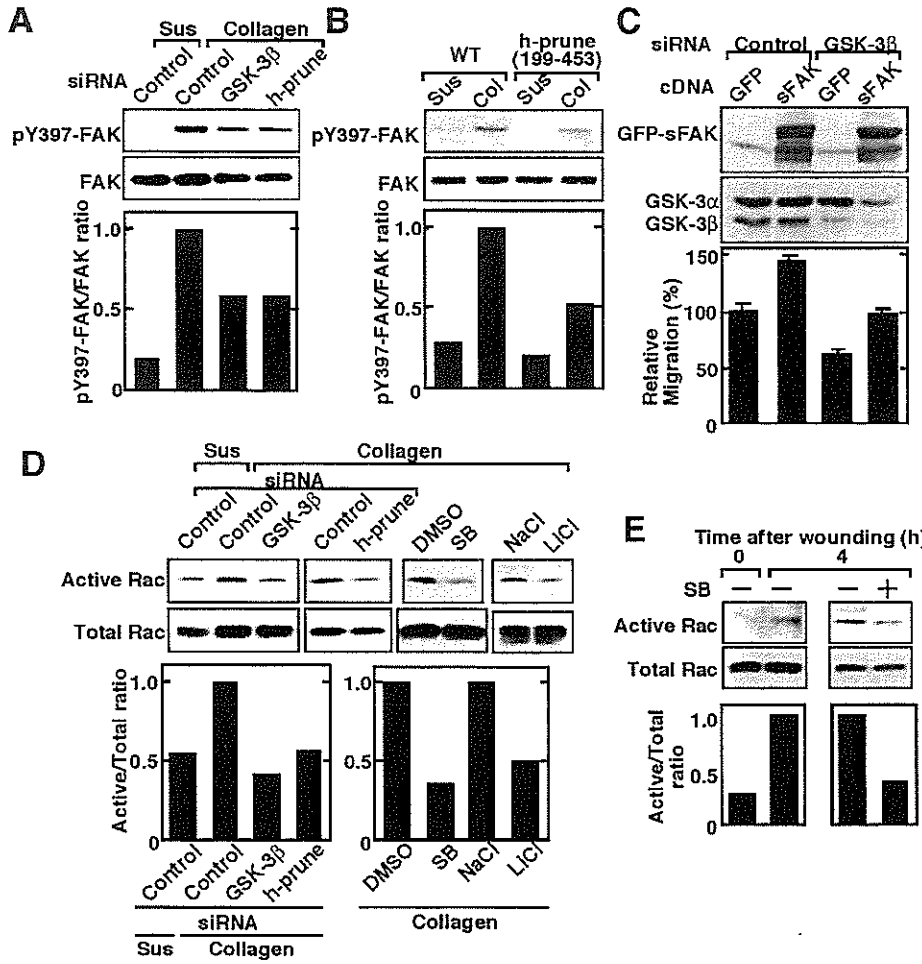

FIG. 6. Involvement of GSK-3 and h-prune in the activation of FAK and Rac. (A and B) FAK activation. HeLa S3 cells transfected with the indicated siRNAs (A) or expressing Myc-h-prune(199–453) (B) were suspended in serum-free medium and were kept in suspension (Sus) or replated onto collagen-coated dishes (Col). Top panel, the cells were lysed at 1 h after plating, and the lysates were probed with the phospho-specific antibody to FAK pTyr397 (pY397 FAK) or anti-FAK antibody. Bottom panel, the FAK activity was measured as the ratio of phosphorylated FAK (active FAK) to total FAK. (C) GFP or GFP-FAK$^{K578E/K581E}$ (sFAK) was expressed in NIH 3T3 cells transfected with siRNA for GSK-3β, and the cells were subjected to the Transwell migration assay. Migrated GFP-labeled cells were normalized with transfection efficiency. Top panel, the protein levels of GFP-FAK$^{K578E/K581E}$ and GSK-3β were shown by anti-FAK and GSK-3 antibodies. Bottom panel, migration ability of the cells used in this assay. (D) Rac activation. HeLa S3 cells were transfected with the indicated siRNAs or treated with the indicated GSK-3 inhibitors. The cells were replated onto collagen-coated dishes, and the lysates were incubated with GST-CRIB immobilized on glutathione-Sepharose. Top panel, the total lysates and precipitates were probed with anti-Rac-1 antibody. Bottom panel, the Rac activity was measured as the ratio of the amount of CRIB-bound Rac (active Rac) to that of Rac in total cell lysates (total Rac). (E) Multiple wounds were made several times in HeLa S3 cells treated with 10 μM SB216763 (SB). The Rac activity was measured at 4 h after wounding. DMSO, dimethyl sulfoxide.

context of the engagement of integrins at the cell surface (34). Activation of FAK results in the recruitment of a number of SH2-domain- and SH3-domain-containing proteins. Among them, p130Cas and Crk are involved in cell migration (34). Dominant negative Rac blocks the increased migration in response to the expression of p130Cas and Crk, probably through DOCK180, which suggests that Rac is an important downstream effector of the FAK-Cas-Crk complex.

We showed that the phosphorylation of Tyr397 in FAK and the activation of Rac induced by collagen are reduced in the GSK-3 or h-prune knocked-down cells. Furthermore, treatment with GSK-3 inhibitors or overexpression of the C-terminal region of h-prune also showed the same results as those obtained in the GSK-3 or h-prune knocked-down cells. It has been demonstrated that FAK plays a prominent role in integrin signaling and that Rac is required for adhesion turnover (34, 39). Therefore, FAK and Rac could act downstream of GSK-3 and h-prune. Consistent with these observations, expression of a constitutively active form of FAK rescued the inhibition of cell migration in GSK-3 knocked-down cells although it was partial. The phosphorylation of proteins by GSK-3 at focal adhesions may be required to mediate the integrin signal. The substrates of GSK-3 that regulate FAK activity remain to be identified.

Several reports have shown that GSK-3 negatively regulates cell migration. For example, integrin inhibits GSK-3 through the activation of integrin-linked kinase and PKB/Akt, and activation of PKB/Akt promotes cell migration (26). Further-

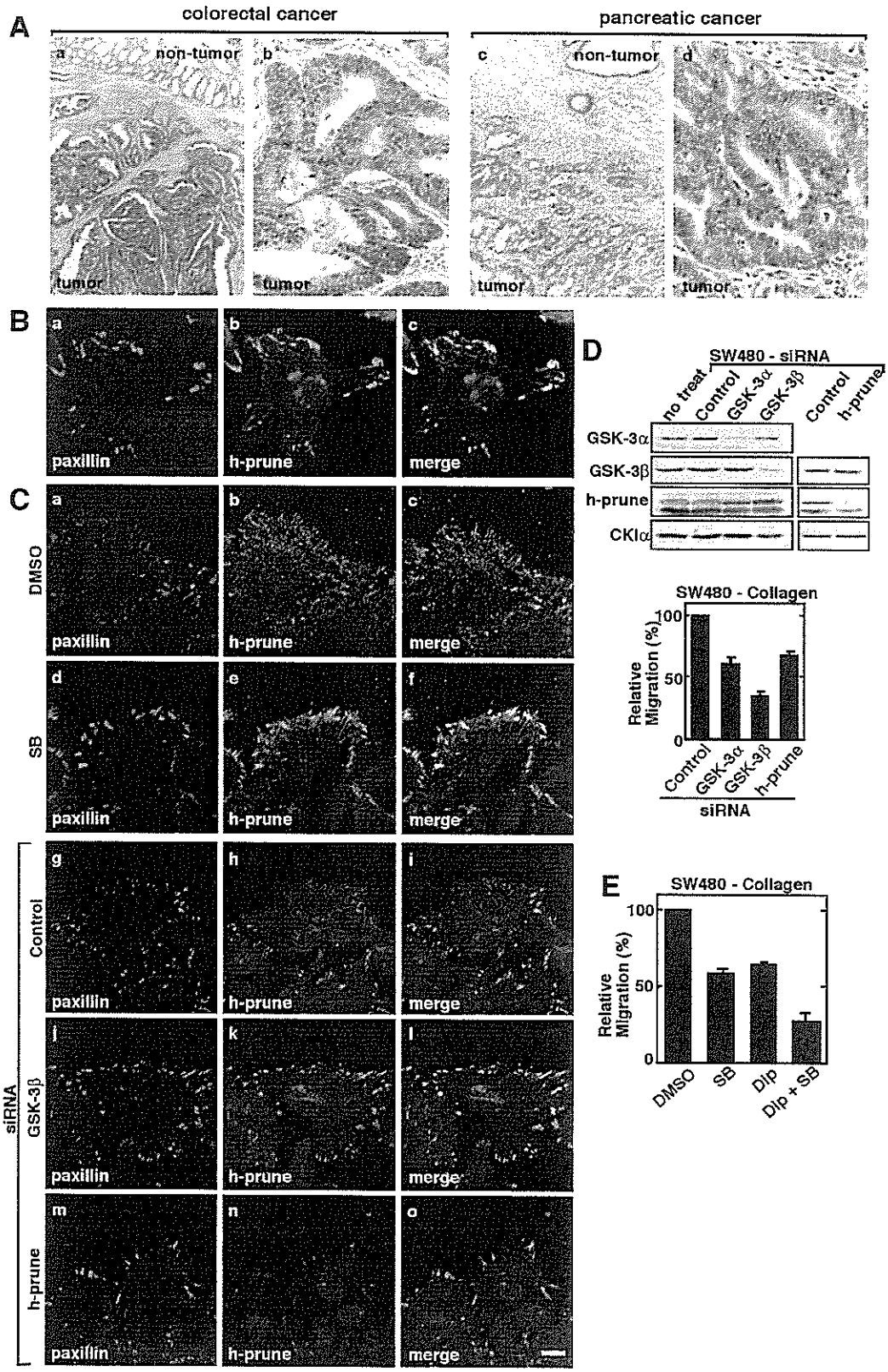

FIG. 7. Correlation of h-prune expression with tumor aggressiveness. (A) Immunohistochemical analyses of h-prune in human colorectal cancer (a and b) and human pancreatic cancer (c and d). (a) Magnification, ×13; (c) magnification, ×33. Expression levels of h-prune in the nontumor and tumor regions were compared. (b and d) Magnification, ×135. The tumor regions were enlarged. (B) SW480 cells were stained with antipaxillin (a) or anti-h-prune (b) antibody. The merged image is shown in panel c. (C) SW480 cells treated with 10 μM SB216763 (d to f) or transfected with the siRNA for GSK-3β (j to l) or h-prune (m to o) were wounded. Twelve hours after wounding, the cells were stained with antipaxillin

TABLE 1. Relation between h-prune expression and clinicopathologic characteristics in colorectal cancer

Characteristic[a]	No. of cases (%) with indicated h-prune expression		*P* value
	Positive	Negative	
T grade			
T1/2	3 (11.1)	24	0.0132
T3/4	24 (36.9)	41	
N grade			
N0	10 (17.9)	46	0.0044
N1/2	17 (47.2)	19	
M grade			
M0	22 (25.9)	63	0.0215
M1	5 (71.4)	2	
Stage			
I/II	10 (17.9)	46	0.0044
III/IV	17 (47.2)	19	

[a] T1, tumor invades submucosa; T2, tumor invades muscularis propria; T3, tumor invades through muscularis propria into subserosa or into nonperitonealized pericolic or perirectal tissue; T4, tumor directly invades other organs or structures and/or perforates visceral peritoneum. N0, no regional lymph node metastasis; N1, metastasis in one to three regional lymph nodes; N2, metastasis in four or more regional lymph nodes. M0, no distant metastasis; M1, distant metastasis.

TABLE 2. Relation between h-prune protein expression and clinicopathologic characteristics in pancreatic cancer

Characteristic[a]	No. of cases (%) with indicated h-prune expression		*P* value
	Positive	Negative	
T grade			
T1/2	2 (15.4)	11	0.0208
T3/4	16 (55.2)	13	
N grade			
N0	5 (25.0)	15	0.0334
N1	13 (59.1)	9	
M grade			
M0	10 (35.7)	18	0.2079
M1	8 (57.1)	6	
Stage			
I/II	10 (35.7)	18	0.2079
III/IV	8 (57.1)	6	

[a] T1, tumor limited to pancreas (2 cm or less in greatest dimension); T2, tumor limited to pancreas (more than 2 cm in greatest dimension); T3, tumor extends beyond pancreas but without involvement of celiac axis or superior mesenteric artery; T4, tumor invades celiac or superior mesenteric artery. N0, no regional lymph node metastasis; N1, regional lymph node metastasis. M0, no distant metastasis; M1, distant metastasis.

more, PKB/Akt promotes integrin recycling by inactivating GSK-3 (37), and hypoxia-induced tumor cell invasion is mediated by inhibiting GSK-3 (53). However, inhibition of GSK-3 has been demonstrated to prevent the accumulation of Rac at lamellipodia and to inhibit epidermal growth factor-dependent wound closure (29), consistent with our results showing that GSK-3 positively regulates cell migration. Although the exact reasons for the differences between our results and those of others are not known, it has been demonstrated that GSK-3 is rapidly and transiently activated, followed by its inhibition by extracellular stimuli, including insulin and epidermal growth factor, or cell adhesion (6, 31). Therefore, cell migration may involve cyclic transient activation and inactivation of GSK-3 as well as modulation of the cellular localization of GSK-3. Since our results suggest that GSK-3 forms a complex with focal adhesions through h-prune, the GSK-3 activity may be necessary to trigger the integrin signal. Another possibility is that GSK-3 binds to h-prune at a site other than focal adhesion. In this model, when GSK-3 is inactivated by integrin, h-prune dissociates from GSK-3 and locates to focal adhesions. Then, h-prune may promote cell migration with GSK-3 after the kinase activity is recovered.

We showed that h-prune overexpression in colorectal and pancreatic cancers is correlated with the depth of invasion and the degree of lymph node metastasis. Taken together with the observations that h-prune is highly expressed in invasive breast cancer (55), this suggests that h-prune might be used as a marker for the identification of subsets of the cancer patients with higher tumor aggressiveness. h-prune has cyclic nucleotide PDE activity, and inhibition of the PDE activity by dipyridamole suppresses cell motility (8). Although a correlation between an h-prune PDE activity and cellular motility has been shown, GSK-3 did not affect the PDE activity of h-prune. Inhibition of GSK-3 and h-prune additively suppressed the cell migration of colon cancer cells, suggesting that h-prune regulates cell motility by two different actions through the PDE activity and the GSK-3 binding activity. Therefore, the identification of highly specific inhibitors of GSK-3 and h-prune might be useful for developing medicines to prevent or treat cancer metastasis.

It has been reported that *Drosophila* prune genetically interacts with $awd^{k\text{-}pn}$, which encodes a nucleotide diphosphate kinase as well as mammalian nm23-H1 (4), and that h-prune and nm23-H1 protein levels are unbalanced in sarcoma and breast cancers (12), suggesting that h-prune may negatively regulate nm23-H1 antimetastatic activity. These results are consistent with the previous observations that nm23-H1 is downregulated in certain cancer cells with high metastasis (33). However, the expression levels of nm23-H1 show no relationship with metastasis of other cancer cells, such as colorectal cancer (17). Since we could not detect the presence of nm23-H1 in the GSK-3 immune complexes (data not shown), whether the complex of GSK-3, h-prune, and nm23-H1 is present and whether this ternary complex is involved in cell migration are not known.

Protein complexes containing GSK-3 regulate the functions of GSK-3 in different subcellular locations. Frat-1, which is known to be involved in the regulation of β-catenin stability, binds to GSK-3 and facilitates its nuclear export (13). p53 interacts with GSK-3 in the nucleus. This association activates

(a, d, g, j, and m) or anti-h-prune (b, e, h, k, and n) antibody. Merged images are shown in panels c, f, i, l, and o. Scale bar, 10 μm. (D) Upper panel, the lysates of SW480 cells transfected with the indicated siRNAs were probed with the indicated antibodies. Lower panel, SW480 cells transfected with the indicated siRNAs were subjected to the Transwell migration assay. (E) SW480 cells treated with 10 μM SB216763 and/or 10 μM dipyridamole were subjected to the Transwell migration assay on collagen. SB, SB216763; Dip, dipyridamole; DMSO, dimethyl sulfoxide. The results shown are means ± standard errors of the means from three independent experiments.

GSK-3, and GSK-3 promotes the transcriptional and apoptotic actions of p53 (45). Further studies to identify additional GSK-3-binding proteins will be necessary to clarify how regulatory mechanisms are integrated to achieve substrate-specific regulation of GSK-3 activity.

ACKNOWLEDGMENTS

We are grateful to K. Matsumoto, S. Takada, J. R. Woodgett, Y. Matsuura, H. Sabe, K. Kaibuchi, and M. D. Schaller for donating cells, plasmids, and baculoviruses.

This work was supported by Grants-in-Aid for Scientific Research and for Scientific Research on Priority Areas from the Ministry of Education, Science, and Culture, Japan (2002, 2003, 2004, 2005); by grants from the Yamanouchi Foundation for Research on Metabolic Disorders (2003) and the Sankyo Foundation of Life Science (2004, 2005); and by grants from EU FP6-BRECOSM-LSH-CT-503234 (M.Z.).

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Accumulation of DNA Methylation Is Associated with Tumor Stage in Gastric Cancer

Naohide Oue, M.D., Ph.D.[1]
Yoshitsugu Mitani, D.D.S.[1]
Junichi Motoshita, M.D.[1]
Shunji Matsumura, M.D., Ph.D.[1]
Kazuhiro Yoshida, M.D., Ph.D.[2]
Hiroki Kuniyasu, M.D., Ph.D.[3]
Hirofumi Nakayama, M.D., Ph.D.[1]
Wataru Yasui, M.D., Ph.D.[1]

[1] Department of Molecular Pathology, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima, Japan.

[2] Department of Surgical Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan.

[3] Department of Molecular Pathology, Nara Medical University, Kashihara, Japan.

Supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Culture, Science, Sports, and Technology of Japan and from the Ministry of Health, Labor, and Welfare of Japan.

The authors thank Masayoshi Takatani for excellent technical assistance and advice. This work was carried out with the kind cooperation of the Research Center for Molecular Medicine, Faculty of Medicine, Hiroshima University.

Address for reprints: Wataru Yasui, M.D., Ph.D., Department of Molecular Pathology, Hiroshima University Graduate School of Biomedical Sciences, 1-2-3 Kasumi, Minami-ku, Hiroshima, 734-8551, Japan; Fax: (011) 81-82-257-5149; E-mail: wyasui@hiroshima-u.ac.jp

Received April 26, 2005; revision received September 20,2005; accepted October, 19, 2005.

BACKGROUND. The authors purpose in this study was to clarify the difference in terms of clinicopathologic features between gastric cancer (GC) with high numbers of DNA methylated genes and CpG island methylator phenotype (CIMP)-positive GC as originally defined.

METHODS. We analyzed DNA methylation of 12 tumor-related genes (*hMLH1, MGMT, p16^{INK4a}, CDH1, RAR-beta, HLTF, RIZ1, TM, FLNc, LOX, HRASLS, HAND1*) in 75 samples of GC from 75 patients, 25 samples of corresponding nonneoplastic mucosa from 25 patients, and 10 samples of normal gastric mucosa from 10 healthy young individuals by methylation-specific polymerase chain reaction (PCR) and bisulfite PCR. We also investigated CIMP status by examining the methylation of five *MINT* loci and *p53* mutation status by PCR single-strand conformation polymorphism. We measured levels of expression of mRNAs for these 12 genes by quantitative reverse transcription PCR in 50 GC specimens.

RESULTS. The average number of methylated genes per tumor was 4.83. DNA methylation of each gene was correlated with low expression of the respective mRNA. High methylation (GC with 5 or more methylated genes) was detected in 39 (52.0%) of 75 GCs. Twenty-nine (37.8%) of 75 GCs were CIMP-positive. DNA methylation of each of the 12 genes was observed more frequently in the high-methylation group than in the low-methylation group. Methylation of 6 specific genes occurred more frequently in CIMP-positive GC than in CIMP-negative GC. Methylation of the remaining 6 genes was not correlated with CIMP-status. High methylation was found more frequently in Stage III/IV GC (26 of 40 cases, 65.0%) than in Stage I/II GC (13 of 35 cases, 37.1%, $P = 0.029$).

CONLUSIONS. These findings indicate that GCs with higher numbers of methylated genes have more distinct DNA methylation profiles than the originally defined CIMP-positive GCs. DNA methylation of tumor-related genes accumulates in conjunction with tumor progression. ***Cancer* 2006;106:1250–9.**

KEYWORDS: CIMP, p53, DNA methylation, progression, gastric cancer.

A variety of genetic and epigenetic alterations are associated with gastric cancer (GC).[1,2] Epigenetic changes, such as DNA methylation of CpG islands, are detected commonly in human cancers.[3,4] 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. Previous studies have indicated that DNA hypermethylation is a crucial mechanism in transcriptional silencing of tumor-related genes in GC. DNA methylation of tumor-related genes has been shown to occur in early stages of stomach carcinogenesis,[5] and it increases in parallel with stomach carcinogenesis.[6] Although several genes have been implicated in tumor progression and in

DOI 10.1002/cncr.21754
Published online 13 February 2006 in Wiley InterScience (www.interscience.wiley.com).

prognosis,[7,8] recent studies have indicated that methylation of a single gene has little or no prognostic significance.[9]

In contrast, methylation of multiple genes has been shown to be associated with a poor prognosis. This phenomenon has been observed in esophageal adenocarcinoma,[9] bladder cancer,[10] and acute lymphocytic leukemia. Several studies have investigated the methylation status of multiple tumor-related genes in GC;[6,12–14] however, little is known about the correlation between DNA methylation of multiple genes and clinicopathologic features of GC.

GCs show the CpG island methylator phenotype (CIMP).[13] Originally CIMP-positive GC was defined as a tumor with methylation at more than 3 loci *methylated in tumors* (*MINT*: *MINT1*, *MINT2*, *MINT12*, *MINT25*, and *MINT31*).[13] CIMP-positive GCs also tend to show DNA methylation of the $p16^{INK4a}$,[13] *hMLH1*,[15] and *RIZ1* genes,[16] suggesting that CIMP is an important pathway involved in stomach carcinogenesis. However, limited numbers of genes have been shown to be associated with CIMP-positive GC, and the role of CIMP has not been clarified in detail. Recent studies have found no evidence to support the CIMP model in esophageal cancer[17] or in colorectal cancer.[18] Furthermore, it is impossible to draw a precise line between CIMP-positive and CIMP -negative tumors because of the gradual distribution pattern of CpG island hypermethylation,[19] which is far from the bimodal distribution originally reported.[20] The discrepancy may be due, at least in part, to the finding that the current definition of simultaneous methylation of CpG islands is not very precise. Furthermore, because DNA methylation of multiple genes often contributes to a poor outcome, lack of association between CIMP and clinical features raises the question of whether DNA methylation of multiple loci could yield high malignant potential.

In the present study, we investigated DNA methylation status of 12 genes (*mutL homolog 1* [*hMLH1*], *O-6-methylguanine-DNA methyltransferase* [*MGMT*], $p16^{INK4a}$, *cadherin-1* [*CDH1*, encoding E-cadherin], *retinoic acid receptor-beta* [*RAR-beta*], *helicase-like transcription factor* [*HLTF*], *retinoblastoma protein-binding zinc finger protein* [*RIZ1*], *thrombomodulin* [*TM*], *gamma-filamin* [*FLNc*], *Lysyl oxidase* [*LOX*], *HRAS-like suppressor* [*HRASLS*], and *heart-and neural crest derivatives-expressed 1* [*HAND1*]) in 75 GC tissues because CpG island hypermethylation in the examined genes has been investigated in GC and has revealed good correlation with epigenetic silencing of respective target genes.[16,21–27] Inactivation of the *hMLH1* gene by hypermethylation is associated with microsatellite instability in GC.[21] *MGMT* is a DNA repair gene that removes mutagenic and cytotoxic adducts from the O^6 position of guanine induced by alkylating agents such as MNNG and MNU.[28] Therefore, inactivation of the *MGMT* gene can lead to G to A mutation. $p16^{INK4a}$, which is a CDK inhibitor, negatively regulates the G1-S transition.[29] *RAR-beta*, which acts as a retinoic acid-dependent transcriptional activator in heterodimeric association with the retinoid X receptors (RXR-alpha, -beta, and -gamma) and in conjunction with multiple corepressors.[30] *CDH1*, which plays a role in invasion suppression, has been found methylated in diffuse-type GC.[31] *CDH1* germ line inactivating mutations have been shown to underlie about 30% of hereditary diffuse GC families of various ethnic backgrounds.[32] *HLTF* contains a DNA-binding domain, a RING finger domain, and 7 helicase domains and is a homologue to SWI/SNF proteins. SWI/SNF proteins are ATP-dependent chromatin remodeling enzymes that have been implicated in regulation of gene expression in yeast and higher eukaryotes.[33] *RIZ* was isolated with a functional screen for Rb-binding protein.[34] The partial response/SET domain is involved in chromatin-mediated gene expression and plays an important role in human cancers as evidenced by genetic mutations of several family members.[35] *TM* encodes an endothelial cell receptor that binds thrombin to activate protein C and works as a member of anticoagulant pathway.[36] *FLNc* is a member of the filamin family, which is known to organize actin polymerization in response to various signals.[37] It has been reported that selective inactivation of *LOX* causes transformation of rat fibroblasts.[38] *HRASLS* is a human homologue of mouse A-C1, which has been reported to inhibit growth of *ras*-transformed NIH3T3 cells.[39] *HAND1* encodes a basic helix-loop-helix transcriptional factor, which is essential for placental development and cardiac morphogenesis.[40]

We examined whether CIMP and the number of methylated genes correlates with clinical features such as age, sex, histology, and tumor stage to clarify the difference between the GC with a high number of methylated genes and CIMP-positive GC. In addition, we investigated *p53* mutation status because distinct combinations of epigenetic and genetic alterations have been reported in CIMP-positive and -negative tumors.[41]

MATERIALS AND METHODS

Tissue Samples

In a retrospective study design, frozen tissue samples were collected from 75 patients 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. Seventy-five GC tissue specimens from 75 patients (age range, 34–87 yrs; mean, 68.6 yrs) and 25 corresponding nonneoplastic mucosae from 25 patients (age range, 46–87 yrs; mean, 72.5 yrs) were analyzed for methylation of 12 genes (*hMLH1*, *MGMT*, *p16^{INK4a}*, *CDH1*, *RAR-beta*, *HLTF*, *RIZ1*, *TM*, *FLNc*, *LOX*, *HRASLS*, *HAND1*), CIMP status, and *p53* mutation status. Among 75 GC samples, total RNA was available for 50 pairs of tumor and corresponding nonneoplastic mucosae. Tumors and corresponding nonneoplastic mucosae were removed surgically, frozen immediately in liquid nitrogen, and stored at −80 °C until use. We confirmed microscopically that the tumor of specimens consisted mainly of cancer tissue (> 50%, on a nuclear basis) and that specimens of nonneoplastic mucosa did not contain GC cells and high-grade dysplasia. Several samples of corresponding nonneoplastic mucosa contained intestinal metaplasia and *H. pylori* associated inflammation. *H. pylori* status was confirmed by histology and/or ^{13}C-urea breath test. Tumor staging was performed according to the TNM stage grouping.[42] Histologic classification of GC was performed according to the Lauren classification system.[43] In addition, because recent evidence suggests that methylation of certain genes is associated with aging,[44] we examined methylation status of the 12 genes in 10 samples of normal gastric mucosae obtained endoscopically with informed consent from 10 healthy young individuals (age range, 22–35 yrs; mean, 26.4 yrs) with no clinical symptoms and no microscopic changes.

Because written informed consent was not obtained from 75 patients with GC, for strict privacy protection, all samples were disidentified before analyzing DNA methylation status. This procedure is in accord with Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government.

Bisulfite Polymerase Chain Reaction (PCR) and Methylation-Specific PCR (MSP)

Genomic DNAs were extracted with a genomic DNA purification kit (Promega, Madison, WI). To examine DNA methylation patterns, genomic DNA was treated with sodium bisulfite as described previously.[45] For analysis of DNA methylation of the *MGMT*, *p16^{INK4a}*, *CDH1*, *RAR-beta*, *HLTF*, *RIZ1*, *TM*, *FLNc*, *LOX*, *HRASLS*, and *HAND1* genes, we performed MSP. MSP was carried out with primers for these genes as described previously.[27,45–49] For analysis of DNA methylation of *hMLH1*, *MINT1*, *MINT2*, *MINT12*, *MINT25*, and *MINT31*, we performed bisulfite-PCR followed by restriction digestion as previously described.[13,50] PCR products (15 μL) were loaded onto 8% nondenaturing polyacrylamide gels, stained with ethidium bromide, and observed under ultraviolet (UV) light. Because of contamination with stromal and inflammatory cells, unmethylated alleles were also simultaneously detected in all tumor samples. We regarded the methylation status of a case as "methylated" if methylated PCR products were detected in that case. We considered cases with methylation at more than 3 of 5 *MINT* loci to be positive for CIMP.[13] The presence or absence of CIMP and DNA methylation of 5 genes (*hMLH1*, *MGMT*, *p16^{INK4a}*, *CDH1*, and *RAR-beta*) was determined previously.[15]

TABLE 1
Primer Sequences for Quantitative RT-PCR

Gene	Primer sequence	Annealing temperature
MGMT	F: 5′-GGATGGATGTTTGAGCGACA-3′ R: 5′-CGGTGCCTCCACGCC-3′	55 °C
CDH1	F: 5′-GCCAAAGACAGAGCGGAACT-3′ R: 5′-GCCAGGCTCAATGACAAGCT-3′	55 °C
RAR-beta	F: 5′-ACCACTGGACCATGTAACTCTAGTGT-3′ R: 5′-GGCATCAAGAAGGGCTGGA-3′	55 °C
HLTF	F: 5′-TTTTCTGAGAAGGACCGACCAG-3′ R: 5′-TGCAATGGCGTAAGAGTTTT-3′	55 °C
RIZ1	F: 5′-ATTGATGCCACTGATCCAGAGA-3′ R: 5′-GCTCTGTTGATTTCCAGTGGGA-3′	55 °C
TM	F: 5′-ATTTCAGAGAGGCCTTTTGGAA-3′ R: 5′-TTCTAACCAGCTCCCATGGG-3′	55 °C
FLNc	F: 5′-GGAAGCACAATCAGAGAAGAAAACA-3′ R: 5′-GCCGGTCCATGTGCCA-3′	55 °C
LOX	F: 5′-TGACCTGCTTGATGCCAACA-3′ R: 5′-GTGTCTTCAAGACAGAAACTTGCTTT-3′	55 °C
HRASLS	F: 5′-GCATTCCTGCGTCCTTTACAA-3′ R: 5′-TCAAGAGCTGCATTTTCACCA-3′	55 °C
HAND1	F: 5′-ATCCCGAGGCCTTCAAGG-3′ R: 5′-TCCGCTTGCTCTCACGG-3′	55 °C

Sequencing Analysis of Methylated PCR Products

The PCR products were purified and cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA). Plasmid DNA was extracted from individual clones by alkaline lysis plasmid minipreparation. The inserted PCR fragments obtained from each sample were sequenced with M13 forward primer using the PRISM Ampli*Taq* DNA polymerase FS Ready Reaction Dye Terminator Sequencing kit (Applied Biosystems, Foster City, CA). Reamplified DNA fragments were purified with CENTRI-SEP COLUMNS (Applied Biosystems) and were sequenced with an ABI PRISM 310 genetic analyzer (Applied Biosystems).

p53 Mutation Analysis

To examine mutations in exons 5–8 of the *p53* gene, we performed PCR-single strand conformation poly-

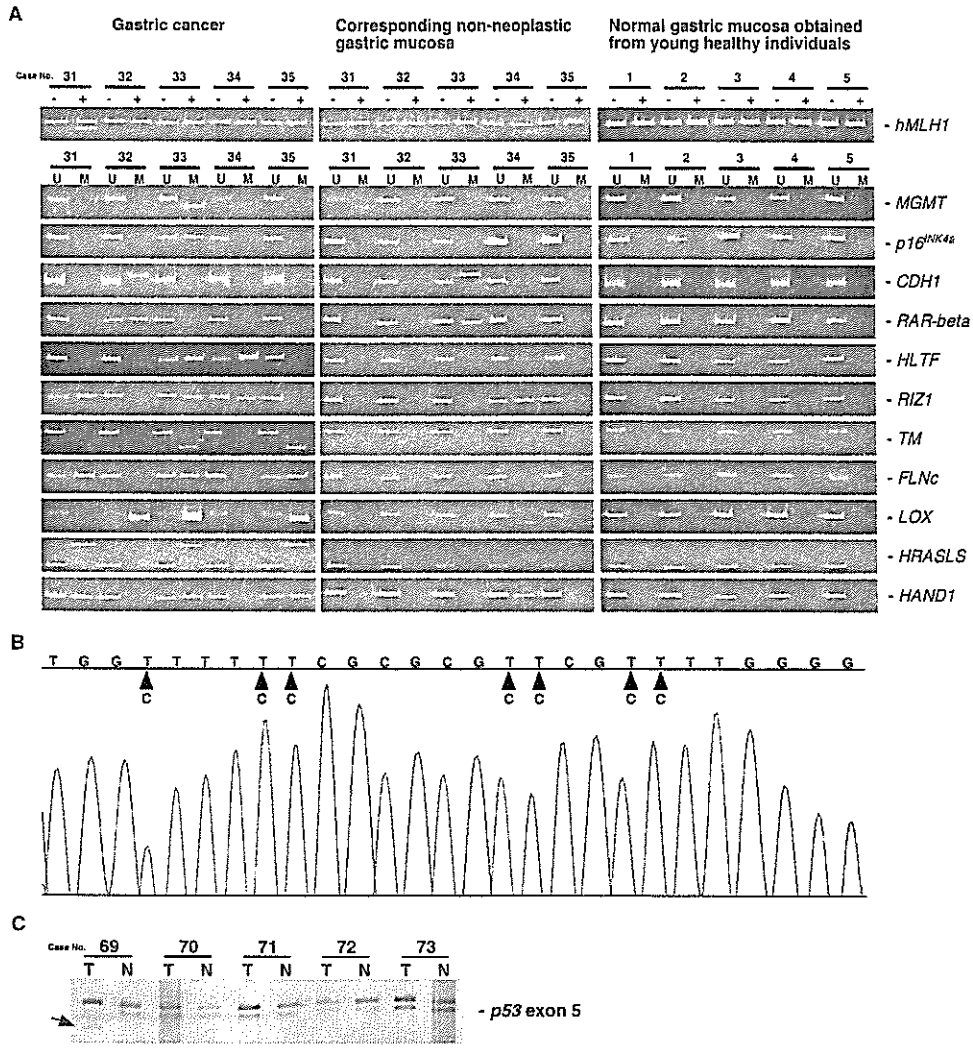

FIGURE 1. (A) Methylation analysis of 12 genes in GC, corresponding nonneoplastic mucosa, and normal gastric mucosae by bisulfite-PCR followed by restriction digestion (*hMLH1*) and MSP (*MGMT*, *p16^{INK4a}*, *CDH1*, *RAR-beta*, *HLTF*, *RIZ1*, *TM*, *FLNc*, *LOX*, *HRASLS*, *HAND1*). The gene studied is indicated at the right of each panel. (B) Sequencing analysis of methylated PCR products of *HLTF* (GC Case 34). All CpG sites were methylated and C to T transition was observed by bisulfite modification (arrowhead). (C) PCR-SSCP analysis of *p53*. A *p53* mutation was detected in 1 case (Case 69). +: after digestion of restriction enzyme (RsaI); −: before digestion of restriction enzyme; M: methylated; units: unmethylated; T: tumor; N: normal.

morphism (SSCP) analysis with 10 sets of primers as described previously.[22] In brief, each target sequence was amplified in a 20 μL reaction volume containing 10–20 ng of genomic DNA, 0.2 μM 2′deoxynucleoside-S′triphosphate, 10 mM Tris-HCl (pH 8.3), 50 mM potassium chloride, 2 mM MgCl2, 0.3 μM of each primer, and 0.75 units (U) of Ampli Taq Gold (Perkin-Elmer, Norwalk, CT). PCR amplification consisted of 35 cycles of 94 °C for 30 seconds, 60 °C or 55 °C for 30 seconds, and 72 °C for 30 seconds, after the initial activation step of 94 °C for 10 minutes. PCR products were diluted 10-fold with formamide dye solution, denatured at 85 °C for 10 minutes, and separated by electrophoresis on 6% polyacrylamide gels. Gels were stained, and bands were observed with a Silver Staining II kit (WAKO, Osaka, Japan). The presence or absence of *p53* mutations was determined previously in 45 of 75 GC samples.[31]

RNA Extraction and Quantitative Reverse Transcription (radiotherapy [RT])-PCR Analysis

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 Corp., Piscataway, NJ). To analyze expression of the 12 genes, we performed real-time RT-PCR as described previously.[7] We used TaqMan Pre-Developed Assay Reagents Human *hMLH1* and *p16^{INK4a}*, and TaqMan beta-actin Control Reagents (Applied Biosystems) in *hMLH1* and *p16^{INK4a}* expression analysis. Primer sequences of the remaining 10 genes and annealing temperatures are shown in Table 1. PCRs were performed with the SYBR Green PCR Core Reagents Kit (Applied Biosystems). Reference samples were included on each assay plate to verify plate-to-plate consistency. At the end of 40 PCR cycles, reaction products were separated electro-

TABLE 2
Frequency of DNA Methylation in Gastric Cancer, Corresponding Nonneoplastic Mucosa, and Normal Gastric Mucosa

Gene	Gastric cancer (n = 75) No. cases (%)	Corresponding nonneoplastic mucosa (n = 25) No. cases (%)	Normal gastric mucosa (n = 10) No. cases (%)
hMLH1	8 (10.7)	0 (0.0)	0 (0)
MGMT	20 (26.7)	2 (8.0)	0 (0)
p16^{INK4a}	16 (21.3)	3 (12.0)	0 (0)
CDH1	41 (54.7)	4 (16.0)	0 (0)
RAR-beta	38 (50.7)	4 (16.0)	0 (0)
HLTF	40 (53.3)	3 (12.0)	0 (0)
RIZ1	50 (66.7)	5 (20.0)	0 (0)
TM	29 (38.7)	3 (12.0)	0 (0)
FLNc	31 (41.3)	2 (8.0)	0 (0)
LOX	31 (41.3)	3 (12.0)	0 (0)
HRASLS	30 (40.0)	0 (0.0)	0 (0)
HAND1	28 (37.3)	0 (0.0)	0 (0)

phoretically on 8% nondenaturing polyacrylamide gels, stained with ethidium bromide, and observed under UV light for visual confirmation of PCR products.

Statistical Methods

Differences were analyzed statistically by chi-square tests and Mann–Whitney U tests. P values less than 0.05 were considered statistically significant.

RESULTS

Frequency of DNA Methylation

Representative results of bisulfite PCR followed by restriction digestion of *hMLH1* and MSP of *MGMT, p16^{INK4a}, CDH1, RAR-beta, HLTF, RIZ1, TM, FLNc, LOX, HRASLS,* and *HAND1* are shown in Figure 1A, and overall results are summarized in Table 2. The majority (74 of 75, 98.7%) of GCs showed methylation of at least 1 gene, and 10 (13.3%) showed methylation of 9 to 11 genes. The average number of methylated genes per tumor was 4.83. We confirmed that DNA methylation of each gene was correlated with low expression of the respective mRNA (Table 3). Bisulfite genomic DNA sequencing (representative result is shown in Fig. 1B) of representative methylated PCR products of each of the 12 genes showed that all cytosines at non-CpG sites were converted to thymine. This excluded the possibility that successful amplification could be attributable to incomplete bisulfite conversion. All methylated PCR products showed extensive methylation of CpG sites that are located inside the amplified genomic fragments. The results of bisulfite sequencing analyses were, thus, consistent with those of MSP, indicating that it is appropriate to

TABLE 3
Relation between DNA Methylation and mRNA Expression in Gastric Cancer

Gene	Methylation status	No. cases	mRNA expression level in gastric cancer[a]	P^b
hMLH1	M	4	0.07 ± 0.06[c]	0.004
	U	46	0.38 ± 0.03	
MGMT	M	13	0.23 ± 0.04	0.005
	U	37	0.85 ± 0.14	
p16^{INK4a}	M	9	0.06 ± 0.02	0.005
	U	41	0.89 ± 0.19	
CDH1	M	30	0.17 ± 0.02	0.001
	U	20	0.36 ± 0.05	
RAR-beta	M	26	0.42 ± 0.08	0.024
	U	24	1.02 ± 0.20	
HLTF	M	23	0.04 ± 0.01	0.027
	U	27	0.09 ± 0.02	
RIZ1	M	17	0.13 ± 0.04	0.029
	U	33	0.27 ± 0.10	
TM	M	16	7.70 ± 4.30	0.029
	U	34	20.00 ± 2.35	
FLNc	M	19	3.50 ± 0.74	0.001
	U	31	15.90 ± 5.45	
LOX	M	22	5.30 ± 0.95	0.001
	U	28	18.30 ± 3.66	
HRASLS	M	21	0.04 ± 0.01	0.001
	U	29	0.54 ± 0.18	
HAND1	M	17	3.49 ± 0.84	0.004
	U	33	22.56 ± 4.27	

M: methylated; U: unmethylated.
[a] Mean values and standard errors for 50 GC samples including those that are methylated and unmethylated.
[b] Statistical significance determined using the Mann-Whitney U test.
[c] The units are arbitrary, and we calculated the respective mRNA expression level by standardization with 1 μg total RNA of the HSC-39 GC cells, taken as 1.0.

conclude the methylation status of each of the 12 genes from results of MSP assay. In corresponding nonneoplastic mucosa from GC patients, DNA methylation was observed in 0–20.0% of 25 cases. Nineteen (76.0%) of 25 samples of corresponding nonneoplastic mucosa showed methylation of at least 1 gene. The average number of methylated genes per sample was 1.16. In normal gastric mucosa, DNA methylation was not detected. When we focused on the number of methylated genes, GCs could be subdivided into 2 groups (Fig. 2A). To classify GCs by their methylation status, we arbitrarily divided the specimens into high-methylation (GCs with 5 or more methylated genes) and low-methylation (GCs with 4 or fewer methylated genes) groups because the average number of methylated genes per tumor was 4.83. We previously investigated CIMP status in these 75 GC cases, and as expected, CIMP-positive GC was found more frequently in the high-methylation group than in the low-methylation group (P = 0.010, chi-square test, Fig.

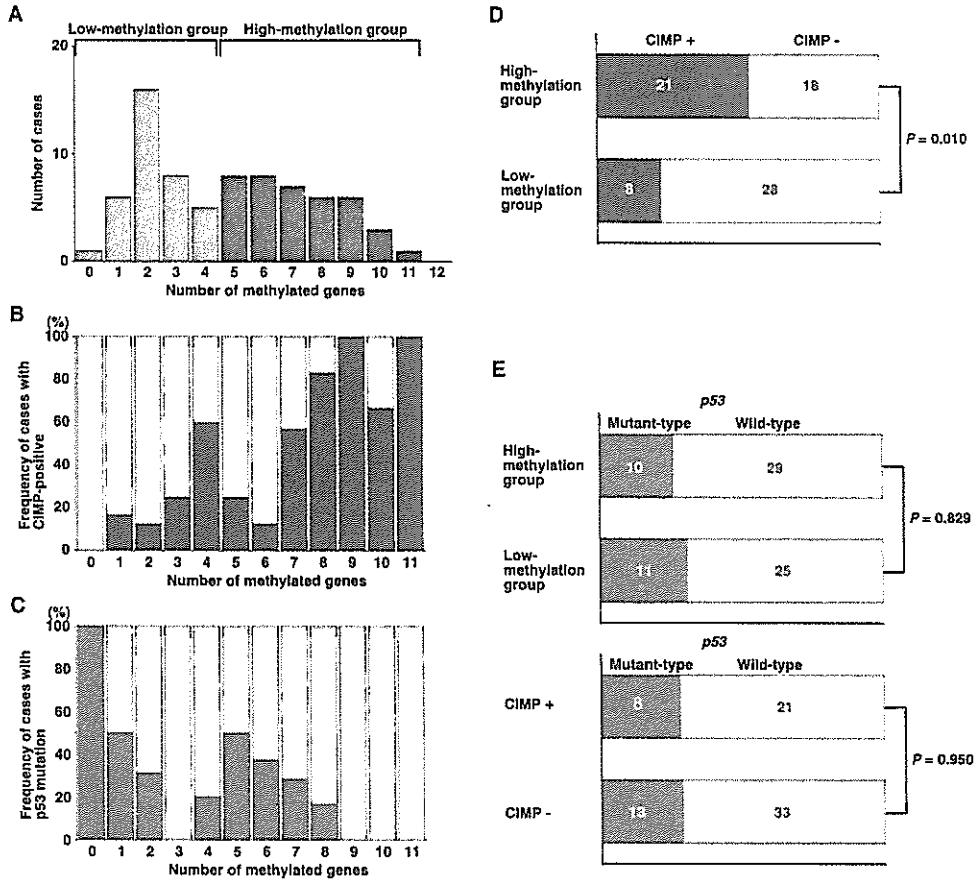

FIGURE 2. Distribution of DNA methylation, CIMP, and *p53* mutation in GC. (A) Distribution of the number of methylated genes per tumor. GC cases appeared to either show high methylation (GC with 5 or more methylated genes, red color) or low methylation (GC with 4 or fewer methylated genes). (B) Distribution of CIMP. The frequency of the CIMP-positive GC (blue) increases in parallel with the number of methylated genes. (C) The frequency of *p53* mutations (green) decreases as the number of methylated genes increases. (D) Relation between number of methylated genes and CIMP status. CIMP is strongly associated with high methylation. The numbers in panels represent numbers of cases in these subgroups. *P* values were calculated by chi-square test. (E) *p53* mutation is not associated with DNA methylation level or CIMP status. The numbers in panels represent numbers of cases in these subgroups. *P* values were calculated by chi-square test.

2B, D). Mutations in *p53* were detected in 28.0% (21 of 75) of the GCs (Fig. 1C), and the frequency of *p53* mutations decreased in parallel with the increase in the number of methylated genes in both the high- and low-methylation groups (Fig. 2C). The number of methylated genes was significantly higher in GC cases without *p53* mutation (mean ± standard error; 5.35 ± 0.39) than in those with *p53* mutation (3.47 ± 0.49; $P = 0.009$, Mann–Whitney *U* test). However, there was no significant correlation between *p53* mutation status and the methylation group ($P = 0.829$, chi-square test, Fig. 2E) or CIMP status ($P = 0.950$, chi-square test, Fig. 2E).

Concordant Methylation

DNA methylation of 6 genes (*hMLH1*, *p16^{INK4a}*, *HLTF*, *RIZ1*, *TM*, and *FLNc*) was observed frequently in CIMP-positive GCs, whereas DNA methylation of the remaining genes (*MGMT*, *CDH1*, *RAR-beta*, *LOX*, *HRASLS*, and *HAND1*) was not (Table 4). In contrast, DNA methylation of each of the 12 genes occurred more frequently in the high-methylation group than in the low-methylation group (Table 4).

Association between Clinicopathologic Features and DNA Methylation

We analyzed the relation of DNA methylation of individual genes in GC samples to clinical data (age, sex, histology, T grade [depth of tumor invasion], N grade [degree of lymph node metastasis], and tumor stage). Hypermethylation of both *CDH1* and *RAR-beta* occurred more frequently in GC cases showing T classifications 3 and 4 than in those showing T classifications 1 and 2 ($P = 0.038$ and $P = 0.020$, chi-square test, respectively). However, DNA methylation of each of the 12 genes was not correlated with tumor stage (data not shown). We analyzed the relation of CIMP status and the number of methylated genes to the clinical data (age, sex, histology, T grade, N grade, and tumor stage) (Table 5). There was no correlation between CIMP status and any clinicopathologic characteristic (Table 5). No significant correlation was found between DNA methylation and age or sex. However, the high-methylation group contained a greater number of advanced N-grade tumors ($P = 0.025$, chi-square test, Table 5) than the low-methylation group. High methylation was detected more frequently in Stage III/IV. GC (26 of 40, 65.0%)

TABLE 4
Distribution of DNA Methylation of 12 Genes with Respect to CIMP and High-Methylation Group in Gastric Cancer

		CIMP (%)			Methylation group (%)		
		Positive	Negative	P^a	High	Low	P^a
hMLH1	M	7 (87.5)	1	0.009	8 (100.0)	0	0.012
	U	22 (32.8)	45		31 (46.3)	36	
MGMT	M	10 (50.0)	10	0.344	15 (75.0)	5	0.032
	U	19 (34.5)	36		24 (43.6)	31	
p16^{INK4a}	M	12 (75.0)	4	0.002	15 (93.8)	1	0.001
	U	17 (28.8)	42		24 (40.7)	35	
CDH1	M	19 (46.3)	22	0.207	30 (73.2)	11	0.001
	U	10 (29.4)	24		9 (26.5)	25	
RAR-beta	M	17 (44.7)	21	0.392	30 (78.9)	8	0.001
	U	12 (32.4)	25		9 (24.3)	28	
HLTF	M	22 (55.0)	18	0.004	26 (65.0)	14	0.029
	U	7 (20.0)	28		13 (37.1)	22	
RIZ1	M	25 (50.0)	25	0.009	31 (62.0)	19	0.027
	U	4 (16.0)	21		8 (32.0)	17	
TM	M	19 (65.5)	10	0.001	25 (86.2)	4	0.001
	U	10 (21.7)	36		14 (30.4)	32	
FLNc	M	19 (61.2)	12	0.002	27 (87.1)	4	0.001
	U	10 (22.7)	34		12 (27.3)	32	
LOX	M	14 (45.2)	17	0.466	27 (87.1)	4	0.001
	U	15 (34.1)	29		12 (27.3)	32	
HRASLS	M	15 (50.0)	15	0.160	24 (80.0)	6	0.001
	U	14 (31.1)	31		15 (33.3)	30	
HAND1	M	14 (50.0)	14	0.190	23 (82.1)	5	0.001
	U	15 (31.9)	32		16 (34.0)	31	

CIMP: CpG island methylator phenotype; M: methylated; U: unmethylated.
[a] Statistical significance determined using the chi-square test.

than in Stage I/II GC (13 of 35, 37.1%, $P = 0.029$, xhi-square test, Table 5).

We next analyzed the relation of DNA methylation of individual genes in corresponding nonneoplastic mucosa and normal gastric mucosa obtained from 10 healthy young individuals to the *H. pylori* infection. *H. pylori* infection was found in 17 (68.0%) of 25 corresponding nonneoplastic mucosa samples. *H. pylori* infection was not detected in 10 normal gastric mucosa samples obtained from 10 healthy young individuals. No correlation was found between methylation of any of 12 genes and presence of *H. pylori* infection (data not shown).

DISCUSSION

The hypermethylator phenotype is thought to be related to patient-specific factors, such as exposure to carcinogens or genetic predisposition.[51] In GC, a relation between Epstein–Barr virus and DNA methylation has been reported.[52,53] DNA methylation occurs early in the multistep process of stomach carcinogenesis,[5,54] and CIMP is detected in some normal gastric mucosa.[13] Thus, DNA methylation may contribute to carcinogenesis but not progression. As expected, we found no association between tumor stage and DNA methylation of individual genes or initially defined CIMP. However, methylation of the larger number of genes was significantly associated with advanced tumor stage, suggesting that methylation of tumor-related genes accumulates with the progression of GC. Consistent with our present results, methylation of multiple tumor-related genes is associated with a poor prognosis in various types of tumors.[9–11] In addition, the cumulative loss of expression of tumor-related genes has been reported to be associated with tumor stage in GC.[55] Therefore, consecutive inactivation of multiple tumor-related genes by DNA methylation appears to be important in GC progression.

Although methylation of several genes was associated with tumor stage, initially defined CIMP was not. Although the number of methylated genes was higher in CIMP-positive GCs than in CIMP-negative GCs, statistically significantly concordant methylation was observed for only 6 of the 12 genes, suggesting that initially defined CIMP may not reflect overall DNA methylation status. This may be why we found

TABLE 5
Associations of CIMP and High-Methylation Group with Clinicopathologic Features in Gastric Cancer

	CIMP (%)			Methylation group (%)		
	Positive	Negative	P^a	High	Low	P^a
Age						
> 65 yrs	20 (39.2)	31	0.887	30 (58.8)	21	0.140
< 65 yrs	9 (37.5)	15		9 (37.5)	15	
Gender						
Male	21 (40.4)	31	0.840	26 (50.0)	26	0.787
Female	8 (34.8)	15		13 (56.5)	10	
T classification[b]						
T1/T2	11 (32.4)	23	0.433	13 (38.2)	21	0.0523
T3/T4	18 (43.9)	23		26 (63.4)	15	
N status[b]						
N0	7 (30.4)	16	0.474	7 (30.4)	16	0.025
N1/N2/N3	22 (42.3)	30		32 (61.5)	20	
Stage[b]						
Stage I/II	12 (44.7)	23	0.623	13 (37.1)	22	0.029
Stage III/IV	17 (32.4)	23		26 (65.0)	14	
Histologic type[c]						
Intestinal	18 (55.0)	21	0.251	20 (51.3)	19	0.897
Diffuse	11 (20.0)	25		19 (52.8)	17	

CIMP: CpG island methylator phenotype; M: methylated; U: unmethylated.
[a] Statistical significance was determined using the chi-square test.
[b] Stage was classified according to the criteria of the International Union Against Cancer TNM classification of malignant tumors, 6th edition, 2002.
[c] Histology was classified according to the criteria of Lauren.

no association of initially defined CIMP with tumor stage. In the present study, we divided GC into 2 groups, and concordant methylation of each of the 12 genes was observed in the high-methylation group. We found significant association between CIMP and hypermethylation of the *hMLH1*, *p16^{INK4a}*, *HLTF*, *RIZ1*, *TM*, and *FLNc* genes, suggesting that methylation of these genes is not a random event in stomach carcinogenesis.

The molecular mechanisms of the association between high-methylation group and tumor stage are unclear. Because DNA methylation of each gene was associated with low expression of the respective mRNA, it seems reasonable that the associated biologic functions were also simultaneously lost. Thus, selection for the loss of specific biologic functions might have played a role in the accumulation of DNA methylation. Moreover, the finding that the frequency of *p53* mutation tended to decrease with the simultaneous increase of the number of methylated genes suggests that gene methylation may affect cancer-associated functions with some overlap with *p53* regulated functions. In this study, we investigated DNA methylation of 12 tumor-related genes, of which only 2, *CDH1* and *RAR-beta*, have been shown to be involved in tumor invasion or metastasis.[56,57] In the present study, DNA methylation of *CDH1* and *RAR-beta* was correlated with advanced T grade. However, DNA methylation of each of the 12 genes was not associated with tumor stage but occurred frequently in the high-methylation group, and therefore, the association between high-methylation group and tumor stage is not a secondary effect of the loss of specific biologic functions. Conversely, several lines of evidence suggest that DNA methylation of some genes may be caused by inflammatory proliferative stimuli. For example, it has been reported that cirrhosis is associated with multiple DNA methylation.[58] Similar results were also found in ulcerative colitis, where chronic inflammation was associated with increased methylation of multiple genes.[50] Thus, accumulation of DNA methylation may be caused by proliferative changes during tumor progression.

H. pylori infection is an important etiologic risk factor in GC, and it has been classified as a Group I, or definite carcinogen, by the World Health Organization International Agency for Research on Cancer.[59] In GC, DNA methylation of *CDH1* has been reported to be correlated with *H. pylori* infection.[56] Conversely, it has been reported that in nonneoplastic gastric mucosa, *H. pylori* infection is not correlated with the number of methylated genes.[60] In the present study, no correlation was found between methylation of any of 12 genes and presence of *H. pylori* infection in corresponding nonneoplastic gastric mucosa and normal gastric mucosa. It also has been reported that vitamins, such as folic acid and B12, are crucial for DNA methylation.[61,62] In the present study, we did not investigate the relation between DNA methylation and intake of vitamins because of the lack of clinical information.

In summary, we show that GCs with a higher number of methylated genes have a distinct DNA methylation profile that differs from that of CIMP-positive GC as originally described. DNA methylation of tumor-related genes, some of which are associated with CIMP, may contribute to development of a majority of GCs and appears to accumulate in parallel with tumor progression.

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Virchows Arch (2006) 448: 428–434
DOI 10.1007/s00428-005-0120-2

ORIGINAL ARTICLE

Phyu Phyu Aung · Yoshitsugu Mitani ·
Yuichi Sanada · Hirofumi Nakayama ·
Keisuke Matsusaki · Wataru Yasui

Differential expression of claudin-2 in normal human tissues and gastrointestinal carcinomas

Received: 3 October 2005 / Accepted: 10 October 2005 / Published online: 17 November 2005

Abstract Claudins are involved in the formation of tight junctions in epithelial and endothelial cells. Claudins form a family of 24 members displaying organ- and tissue-specific patterns of expression. In the present study, we evaluated the specificity of the claudin-2 expression in various normal human tissues and gastrointestinal cancers by quantitative reverse transcriptase–polymerase chain reaction and immunohistochemistry. In 14 various normal tissues, *claudin-2* mRNA was expressed in the kidney, liver, pancreas, stomach, and small intestine; the highest level of which was detected in the kidney. Colorectal cancers (CRCs) expressed *claudin-2* mRNA at high levels. Immunohistochemical analysis of claudin-2 in 146 gastric cancers (GCs) and 99 CRCs demonstrated claudin-2 expression in 2.1% of GCs and 25.3% of CRCs, respectively. There was no obvious correlation between claudin-2 expression and clinicopathological parameters of CRCs. These results suggest that the expression of claudin-2 may involve organ specificity, and increased expression of claudin-2 may participate in colorectal carcinogenesis.

Keywords Claudin-2 · Gastric · Colorectal cancers

P. P. Aung · Y. Mitani ·
Y. Sanada · H. Nakayama · W. Yasui (✉)
Department of Molecular Pathology,
Graduate School of Biomedical Sciences,
Hiroshima University,
1-2-3 Kasumi, Minami-ku,
Hiroshima 734-8551, Japan
e-mail: wyasui@hiroshima-u.ac.jp
Tel.: +81-82-2575145
Fax: +81-82-2575149

K. Matsusaki
Department of Surgery,
Hofu Institute of Gastroenterology,
14-33 Ekiminami, Hofu,
Yamaguchi 747-0801, Japan

Introduction

Claudin-1 and claudin-2 were the first members of the transmembrane tetraspan family of proteins identified as being involved in tight junction formation with the reruitment of occludin [4] and binding to other tight junction constituents [8]. Claudins form a family of at least 24 members displaying organ- and tissue-specific patterns of expression [9, 17]. Among the claudin family members, expression of claudin-2 is found in the liver, pancreas, and gut in normal rat tissues [17]. Claudin-2 expression is ubiquitous in the epithelial cells at the crypts of the small intestine but restricted to the undifferentiated cell compartment of the colon in rats [17]. Claudin-2 is also known to be expressed in mouse nephron [3, 9]. However, the expression pattern of claudin-2 remains to be elucidated in normal human tissues.

Gastrointestinal cancers including gastric cancer (GC) and colorectal cancer (CRC) are the most common malignancies worldwide. A better knowledge of changes in gene expression during gastrointestinal carcinogenesis may lead to new paradigms and possible improvements in diagnosis, treatment, and prevention. On the other hand, relatively little is known about the expression of claudins in human tumors, and only little information is available on the influence of claudin expression on tumor behavior. It was reported that the expression of claudin-7 was decreased in high-grade breast cancer [10]. Overexpression of claudin-4 has been found in pancreatic adenocarcinoma and its precursor lesions [15, 22], while overexpression of claudin-3 and claudin-4 has been found in prostate and ovarian carcinomas [7, 13]. Concerning the expression of claudin-2 in tumor tissue, it has been reported that claudin-2 expression was detected in 98 (52%) of 188 breast carcinomas [20]. There is one report showing claudin-2 expression in gastrointestinal tumors, but the sample number was small [21].

In the present study, the expression of claudin-2 was investigated in various normal tissues, GCs, and CRCs by

quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) and immunohistochemistry. The aim of this study is to clarify whether claudin-2 expression is specific for cancer by comparing the expression level of claudin-2 in various normal tissues with that in cancer tissues.

Materials and methods

Tissue samples

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

For immunohistochemical analysis, we used archival formalin-fixed, paraffin-embedded tissues from 245 patients who had undergone surgical excision or removal of the tumor by polypectomy for GC (n=146) and CRC (n=99). The 146 GCs were either histologically classified (n=85) or poorly (n=61) differentiated. Ninety-nine CRCs were either histologically classified (n=47) or moderately (n=45) or poorly (n=7) differentiated. Tumor staging was carried out according to the tumor–node–metastasis (TNM) staging system [12]. 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.

Cell lines

Eight cell lines derived from human GC were used. The TMK-1 cell line was established in our laboratory [16]. Five GC cell lines of the MKN series were kindly provided by Dr. T. Suzuki. KATO-III and HSC-39 cell lines were kindly provided by Dr. M. Sekiguchi and Dr. K. Yanagihara [25], respectively. All cell lines were maintained in RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% fetal bovine serum (Whittaker, Walkersville, MA, USA) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Quantitative RT-PCR analysis

Total RNA was extracted with an RNeasy Mini kit (Qiagen, Valencia, CA, USA), and 1 μg of total RNA was converted to cDNA with a First-Strand cDNA Synthesis kit (Amersham Pharmacia, Little Chalfont, UK). PCR was performed with an SYBR Green PCR Core Reagents kit (Applied Biosystems, Foster City, CA, USA). Real-time detection of the emission intensity of SYBR green bound to double-stranded DNA was performed with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) as described previously [11]. *Claudin-2* cDNA and internal control cDNAs [β-actin gene (*ACTB*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)] were PCR-amplified separately. Relative gene expression was determined by the threshold cycles for the *claudin-2* and *ACTB* or *GAPDH* genes. Reference samples (GC cell line, KATO-III) were included on each assay plate to verify plate-to-plate consistency. Plates were normalized to each other by these reference samples. PCR amplification was performed according to the manufacturer's instructions in 96-well optical trays with caps with a 25-μl final reaction mixture. Quantitative RT-PCRs were performed in triplicate for each sample primer set, and the mean of the three experiments was used as the relative quantification value. *Claudin-2* primer sequences are forward primer 5′-TCCCCAAACCC ACTAATCACA-3′ and reverse primer 5′-CCAACCTCAG CCAGAGAGAGG-3′. *ACTB* primer sequences were 5′-T CACCGAGCGCGGCT-3′ and 5′-TAATGTCACGCAC GATTTCCC-3′ [11]. *GAPDH* primer sequences were 5′-GGTGAAGGTCGGAGTCAACG-3′ and 5′-AGAGTTAA AAGCAGCCCTGGTG-3′. The units are arbitrary, and we calculated *claudin-2* mRNA expression by standardization to 1.0 μg total RNA from KATO-III as 1.0. We found a similar result in both quantitative RT-PCR analyses of *claudin-2* and *ACTB* or *GAPDH* of 8 GC cell lines, 14 various normal tissues, 5 GC tissues, and 9 CRC tissues. Therefore, throughout this article, we will describe and discuss the results obtained using *ACTB* as an internal control in quantitative RT-PCR analysis.

Western blot analysis

Preparation of whole cell lysates from GC cell lines was made and Western blotting was performed as described previously [26]. Protein concentrations were determined by Bradford protein assay (Bio-Rad, Hercules, CA, USA) with bovine serum albumin used as the standard. Lysates (20 μg) were solubilized in Laemmli's sample buffer by boiling and then subjected to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by electrotransfer onto a nitrocellulose filter. Anti-claudin-2 polyclonal antibody was purchased from Zymed (South San Francisco, CA, USA), and anti-β-actin mouse monoclonal antibody was purchased from Sigma (USA). Peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG was used in the secondary reaction, respectively. The immunocomplex was visualized with an ECL Western Blot Detection System (Amersham Pharmacia Biotech).

Immunohistochemistry

A Dako LSAB kit (Dako, Carpinteria, CA, USA), which is based on the LSAB method, was used for the immunohistochemical analysis. In brief, microwave pretreatment in citrate buffer was performed for 15 min to retrieve antigenicity. After blocking the peroxidase with 3% H_2O_2–methanol for 10 min, sections were incubated with antibody/rabbit polyclonal anti-claudin-2, 1:100 (Zymed).

Sections were treated consecutively at room temperature with primary antibody for 2 h, followed by sequential 10 min incubation with biotinylated anti-rabbit IgG and peroxidase-labeled streptavidin. Staining was completed after 10 min incubation with the substrate–chromogen solution. The sections were counterstained with 0.1% hematoxylin. The results of staining with each antibody were evaluated with reference to the percentage of stained cancer cells. The results of immunohistochemistry were graded as follows: "–," 0% to 25% of tumor cells showed immunoreactivity; "+," 25–50% of tumor cells showed immunoreactivity; "++," more than 50% of tumor cells showed immunoreactivity. We regarded "++" as positive throughout this report.

Statistical methods

Associations between clinicopathologic parameters and claudin-2 expression were analyzed by Fisher's exact test. *P* values less than 0.05 were considered statistically significant.

Results

Measurement of mRNA expression of *claudin-2* in various normal tissues, GCs, and CRCs by quantitative RT-PCR

To measure the expression levels of *claudin-2* mRNA, we performed quantitative RT-PCR on 5 GCs, 5 CRCs, and 14 normal tissue samples (heart, lung, stomach, small intestine, colon, liver, pancreas, kidney, bone marrow, peripheral leukocytes, spleen, skeletal muscle, brain, and spinal cord). The results are shown in Fig. 1. In general, the expression levels in CRCs were higher than those in GCs and normal tissues. Among the 14 normal tissues mentioned, the highest level of expression was detected in the kidney. Although obvious expression of *claudin-2* was also detected in the liver, pancreas, stomach, and small intestine, it was not as high as compared with the kidney. In the lung and skeletal muscle, a faint expression of *claudin-2* was found. There was no expression in the remaining normal tissues. Expression levels of *claudin-2* were not so different between normal stomach and GCs. While no expression of *claudin-2* was found in normal colon, two of five CRCs expressed *claudin-2* at significantly higher levels (more than 5 arbitrary units).

Expression and localization of claudin-2 in cancer cell lines

To confirm the mRNA expression of *claudin-2* in cancer cells, we performed quantitative RT-PCR in eight GC cell

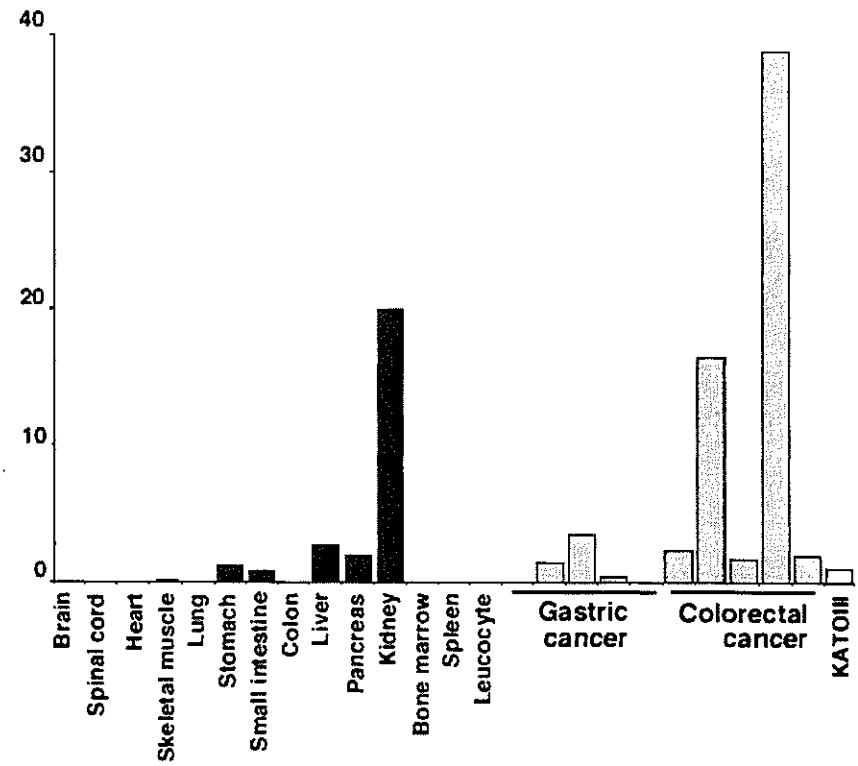

Fig. 1 Quantitative RT-PCR analysis of *claudin-2* in 14 various normal tissues as well as in 5 GC and 5 CRC samples. Among the various normal tissues, the highest level of *claudin-2* expression was found in the kidney; low expression was detected in the stomach, small intestine, liver, and pancreas; and faint expression was seen in the lung and skeletal muscle. In GCs, the expression levels were not so different from that in normal stomach. In CRCs, the expression levels were higher than those in normal colon

lines. As shown in Fig. 2a, an obvious expression of *claudin-2* was detected in MKN-45, MKN-74, and KATO-III, whereas only a low level of expression was seen in HSC-39; there was no expression detected in the remaining four GC cell lines. The anti-claudin-2 antibody detected an approximately 22-kDa band on Western blot of cell extracts from MKN-45, MKN-74, and KATO-III (Fig. 2b). We also confirmed these results by immunohistochemical staining in MKN-28 and MKN-45 cell lines. Claudin-2 staining was detected in cell membranes in MKN-45 cells but not in MKN-28 cells (Fig. 2c). Thus, this antibody was considered to be useful in the detection of claudin-2 protein in situ.

Expression and localization of claudin-2 in CRC tissues

To predict the sensitivity of anti-claudin-2 rabbit polyclonal antibody in immunohistochemistry, we examined the mRNA expression and protein expression of claudin-2 in an additional four CRC tissues. Firstly, we performed quantitative RT-PCR to detect *claudin-2* mRNA expression level and found an obvious expression of *claudin-2* (more than 5 arbitrary units) in three of four examined CRC tissue samples (Fig. 3a). The anti-claudin-2 antibody detected an approximately 22-kDa band on Western blot of protein extracts from the three CRC tissues, which showed obvious high expression in quantitative RT-PCR analysis (Fig. 3b). We also confirmed these results by immunohistochemical staining. Claudin-2 staining was detected in cell membranes of the same three CRC tissue samples, which expressed claudin-2 at a significantly higher level in both quantitative RT-PCR and Western blotting analyses (Fig. 4c). Therefore, in these CRC tissue samples, we found a good correlation between mRNA and protein levels. This result also suggests that in immunohistochemical analysis, our antibody may detect claudin-2 protein expression at levels higher than 5 arbitrary units measured by quantitative RT-PCR analysis.

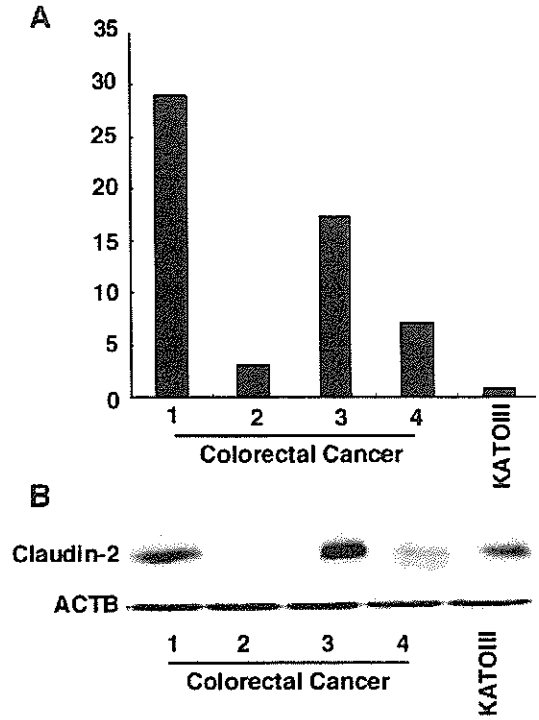

Fig. 3 Expression of claudin-2 in CRC tissue samples. Claudin-2 expression was analyzed at the mRNA level by quantitative RT-PCR (**a**) and at the protein level by Western analysis (**b**)

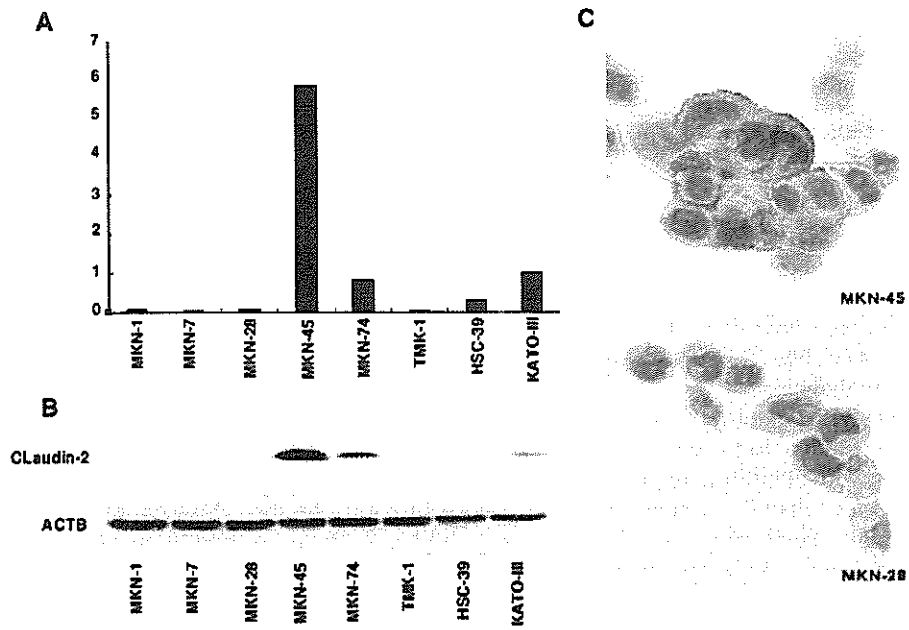

Fig. 2 Expression and localization of claudin-2 in GC cell lines. Claudin-2 expression was analyzed at the mRNA level by quantitative RT-PCR (**a**) and at the protein level by Western analysis (**b**) and immunohistochemistry (**c**); claudin-2 immunoreactivity was evident in the cell membranes of MKN-45 cells but not MKN-28 cells

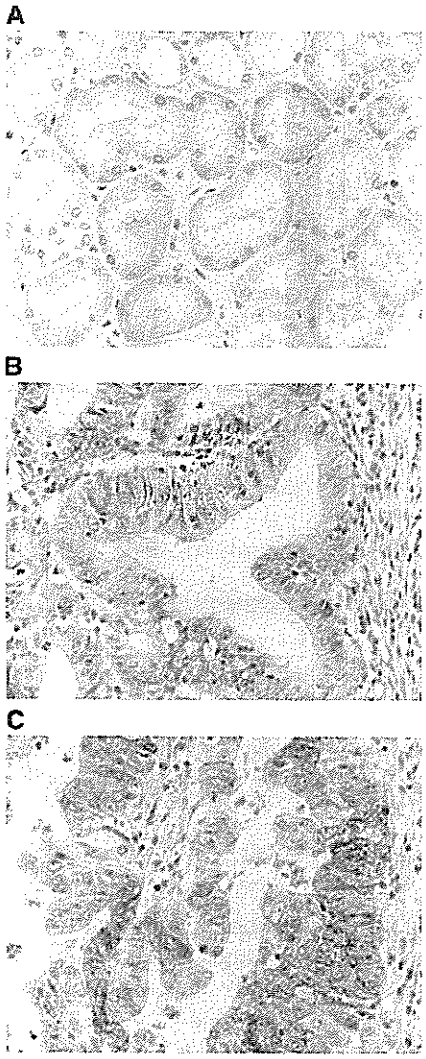

Fig. 4 Immunohistochemical analysis of claudin-2 in normal kidney (as a control) as well as in GC and CRC tissues. Staining for claudin-2 was observed in the basal membranes of the proximal tubule of the kidney (**a**), the cellular membrane of GC cells forming a tubular structure (**b**), and the cellular membrane of CRC cells forming a papillotubular structure (**c**). **c** is case number 1, which expressed *claudin-2* at a high level (more than 5 arbitrary units measured by quantitative RT-PCR), as shown in Fig. 3a,b. Original magnification was ×400

Expression of claudin-2 protein in GCs and CRCs by immunohistochemistry

We then examined the expression of claudin-2 protein in 146 GC and 99 CRC samples by immunohistochemistry. Immunostaining was also performed in normal kidney to serve as a positive control because our real-time RT-PCR revealed a high expression level and a previous report showed claudin-2 expression in mouse nephron. In the kidney, claudin-2 positivity was found strongest in the basal membranes of the proximal tubule, which is consistent with the result of previous reports [3, 9] (Fig. 3a). No obvious staining of claudin-2 was found in normal liver, stomach, and small and large intestines (data not shown). Of the 146 cases of GC, only 3 (2.1%) were positive for claudin-2. Immunoreactivity of claudin-2 was mainly observed in the cell membranes of GC cells forming a tubular structure (Fig. 3b). In CRCs, 25 (25.3%) of 99 cases were positive for claudin-2. Claudin-2 was mainly localized in the cell membranes of tumor cells forming a papillotubular structure (Fig. 3c).

We analyzed the relation between the expression of claudin-2 and clinicopathologic characteristics of CRC. There was no clear correlation between claudin-2 staining and clinicopathological parameters such as location, T grade, N grade, M grade, stage, and histologic differentiation (Table 1).

Table 1 Relation between claudin-2 protein expression and clinicopathologic characteristics in CRCs

	Claudin-2 expression	*P* value[a]
Location		
Right	8/32 (25.0%)	1.0000
Left	17/67 (25.4%)	
T grade		
T1/2	7/29 (24.1%)	1.0000
T3/4	18/70 (25.7%)	
N grade		
N0	17/58 (29.3%)	0.3494
N1/2	8/41 (19.5%)	
M grade		
M0	25/95 (26.3%)	0.5695
M1	0/4 (0.0%)	
Stage		
I	6/25 (24.0%)	0.1453
II	11/33 (33.3%)	
III	8/37 (21.6%)	
IV	0/4 (0.0%)	
Histology		
Well	14/47 (29.8%)	0.4774
Moderate	9/45 (20.0%)	
Poor	2/7 (28.6%)	

[a]Fisher's exact test

Discussion

Cellular tight junctions are structures that help preserve the integrity of cellular layers and regulate their permeability [23, 24]. It may be hypothesized that changes in expression of tight junctional proteins can lead to cellular disorientation and detachment, which are commonly seen in neoplasia. In this study, we demonstrated for the first time the expression of *claudin-2* mRNA in normal human kidney, liver, pancreas, and so on. Among these, the highest level of expression was detected in the kidney. In addition, an obvious expression of *claudin-2* was also detected in the stomach and small intestine. These results are consistent with a previous report in rat and mice; *claudin-2* is expressed in nephron, liver, pancreas, and gut