

reports indicated that the expression of CDH17 was correlated with lymph node metastasis [8, 9]. Therefore, CDH17 could be a novel prognostic factor of gastric carcinoma. However, no study has demonstrated the prognostic impact of CDH17 in gastric carcinoma. In the present study, we examined the expression of CDH17 protein in primary gastric carcinoma tissues and corresponding non-neoplastic mucosa by immunohistochemistry, and analyzed the correlation of CDH17 expression with clinicopathological characteristics and patient's prognosis. We also compared CDH17 expression with Cdx2 in gastric carcinoma tissue.

Materials and methods

Tissue samples

We used a total of 94 gastric adenocarcinoma cases. Tumor tissues and corresponding non-neoplastic gastric mucosa were obtained by surgery at the Hiroshima University Hospital and its affiliated hospitals. For immunohistochemistry, tissues were fixed in 10% buffered formalin and embedded in paraffin.

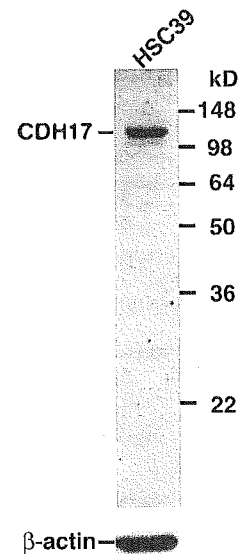
Histological classification of gastric cancer was done according to the Lauren classification system [11]. Staging of gastric carcinoma was done according to the Tumor-Node-Metastasis Stage Grouping [16]. Classification of the quantity of stroma was made according to the criteria of the Japanese Classification of Gastric Cancer [7] as follows: scirrhous type (sci), stroma is abundant and fibrous; medullary type (med), stroma is scanty; intermediate type (int), the quantity of stroma is intermediate between sci and med.

Western blotting and immunohistochemistry

To confirm the specificity of the anti-CDH17 antibody, Western blotting was carried out as described [14]. Human gastric carcinoma cell line, HSC39, was used. The cells were lysed in lysis buffer [50 mM Tris-HCl pH 7.4, 125 mM NaCl, 0.1% (v/v) NP-40, 5 mM ethylene diamine tetraacetic acid (EDTA), 50 mM NaF, 50 µg/ml phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor, and 1 µg/ml aprotinin]. Protein (50 µg) was subjected to Western blot analysis. Anti-CDH17 polyclonal antibody (C-17, sc-6978; Santa Cruz Biotechnology, USA) at a 1:100 dilution was used in primary reaction. As expected, a single band of 120 kDa was detected (Fig. 1).

Consecutive 4-µm tissue sections were prepared from paraffin blocks and immunostained for CDH17 and Cdx2. Each section was mounted on a MAS-coated glass slide, deparaffinized, and soaked for 15 min at room temperature in 3% H₂O₂/methanol to block endogenous peroxidase. Anti-CDH17 polyclonal antibody at a 1:200 dilution and anti-Cdx2 monoclonal antibody (CDX2-88, BioGenex,

Fig. 1 The specificity of CDH17 antibody. Western blot analysis was performed as described in Materials and methods. A single band of 120 kDa was detected in HSC-39 gastric carcinoma cell lines

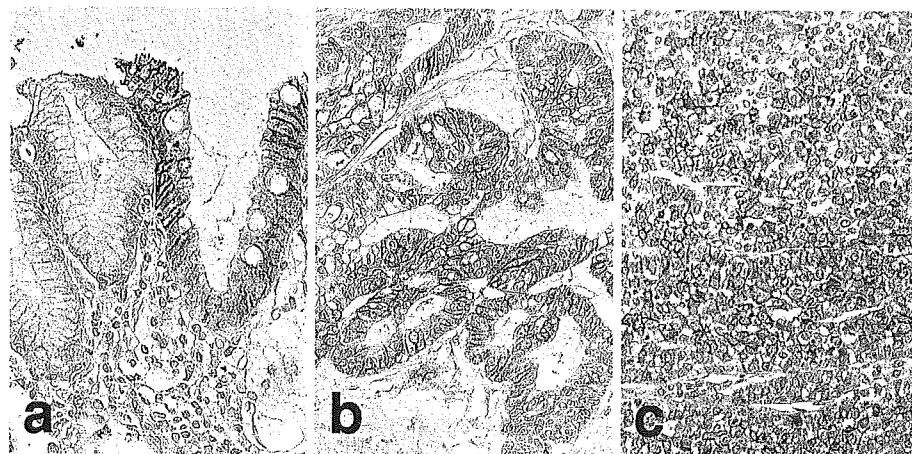


USA) at a 1:100 dilution were used as primary antibody and was applied for overnight at 4°C. The primary antibody was visualized using the Histofine Simple Stain MAX-PO (MULTI) kit (Nichirei, Tokyo, Japan) according to the instruction manual. The slide was counterstained with hematoxylin. Negative control staining was done using non-specific IgG in the primary reaction. The stained slides were observed without clinicopathological information. Reproducibility of staining was confirmed by re-immunostaining via the same method in multiple, randomly selected specimens. To determine the definition of CDH17 expression, we counted the number of positive cells that showed immunoreactivity on the cell membrane among tumor cells in the representative ten microscopic fields, and calculated the percentage of positive cells. The definition of staining was as follows: positive, more than 40% of tumor cells showed immunoreactivity; weak, 5–40% of tumor cells showed immunoreactivity; negative, from 0% to less than 5% of tumor cells showed immunoreactivity. To define Cdx2 expression, we counted the number of nuclear stained cells. Definition of staining was as follows: positive, over 40% of tumor cells showed immunoreactivity; negative, from 0% to less than 40% of tumor cells showed immunoreactivity.

Statistical analysis

Statistical analyses were performed with non-parametric test and *P* values were shown. If the *P* value is less than 0.05, it means that each parameter has a significant correlation with the level of CDH17 expression. Survival rates were calculated via the Kaplan–Meier method. The difference between the survival curves was analyzed by means of log-rank test.

Fig. 2 Expression of CDH17 protein in gastric carcinoma tissues and non-neoplastic mucosa. CDH17 immunohistochemical staining: **a** non-neoplastic mucosa with intestinal metaplasia, **b** intestinal-type carcinoma, **c** diffuse-type carcinoma. CDH17 expression was found in cell membrane and cytoplasm at various levels in gastric carcinoma tissues. In non-neoplastic mucosa, the expression of CDH17 was not recognized in normal foveolar epithelia, while intestinal metaplasia revealed expression of CDH17



Results

Immunohistochemical detection of CDH17 in gastric carcinoma tissues and non-neoplastic mucosa

We immunohistochemically examined the expression and localization of CDH17 protein in 94 cases of gastric carcinoma and non-neoplastic mucosa. Representative stainings of CDH17 are shown in Fig. 2, and the results are summarized in Table 1. CDH17 was expressed in 67% (63/94) of gastric carcinomas at various levels (Fig. 2b, c and Table 1). CDH17 expression was mainly localized in cell membrane and cytoplasm. In non-neoplastic gastric mucosa, CDH17

was also detected in the cell membrane of epithelial cells with intestinal metaplasia (Fig. 2a).

Correlation between CDH17 expression and clinicopathological characteristics of gastric carcinoma

We next analyzed the relationship between the expression of CDH17 and the clinicopathological parameters of gastric carcinoma. As shown in Table 1, the expression of CDH17 tended to be associated with intestinal histology. The incidence of cases with positive expression of CDH17 was significantly higher in medullary type (37/47, 79%),

Table 1 Expression of CDH17 in gastric carcinomas and its correlation with clinicopathological parameters

	Case no.	CDH17 expression ^a		<i>P</i> value
		Positive	Weak and negative	
Gastric carcinomas	94	63 (67%)	31 (33%)	
Histology ^b				
Intestinal type	53	39 (74%)	14 (26%)	
Diffuse type	41	24 (59%)	17 (41%)	0.0621
Quantity of stroma ^c				
med	47	37 (79%)	10 (21%)	
sci and int	47	26 (55%)	21 (45%)	0.01581
Depth of invasion ^d				
t1	47	26 (55%)	21 (45%)	
t2-4	47	37 (79%)	10 (21%)	0.01581
Lymph node metastasis				
n0	47	31 (66%)	16 (34%)	
n1-4	47	32 (68%)	15 (32%)	0.08263
Stage ^e				
I	45	24 (53%)	21 (47%)	
II-IV	49	39 (80%)	10 (20%)	0.0683

^aGrades of CDH17 expression were classified as "positive" and "weak and negative", as described in Materials and methods

^bHistological classification of gastric carcinoma was done according to the Laure classification system [11]

^cAccording to the criteria of the Japanese Classification of Gastric Cancer [7]

^dTumor staging of gastric carcinoma was done according to be Tumor-Node-Metastasis Stage Grouping [16]

^eCorrelation was analyzed by non-parametric test and *P* values are shown. *P* values less than 0.05 were regarded as statistically significant

than in scirrhous and intermediate type (26/47, 55%). Positive expression of CDH17 was significantly associated with deep invasion of tumor cells in gastric wall ($P=0.01581$). The incidence of positive expression of CDH17 tended to be higher in carcinomas with lymph node metastasis. Further more, cases with positive CDH17 expression were significantly more frequent in carcinomas of stages II–IV than stage I ($P=0.00683$).

Prognosis of gastric carcinoma cases with or without CDH17 expression

We studied the survival rate of gastric carcinoma patients after surgery. If all patients with carcinoma of different stages were included, the cases with positive expression of CDH17 showed worse prognosis than the cases with negative expression of CDH17 ($P=0.0314$) (Fig. 3). However, we did not find any significant correlation between CDH17 expression and the prognosis of the patients with “stage I and II” carcinoma ($P>0.5$) or “stage III and IV” carcinoma ($P=0.1072$). With multivariate survival analysis, the factor that gave the strongest influence was not CDH17 expression, but tumor stage (data not shown). Thus CDH17 expression must be one of the effective factors for gastric cancer prognosis.

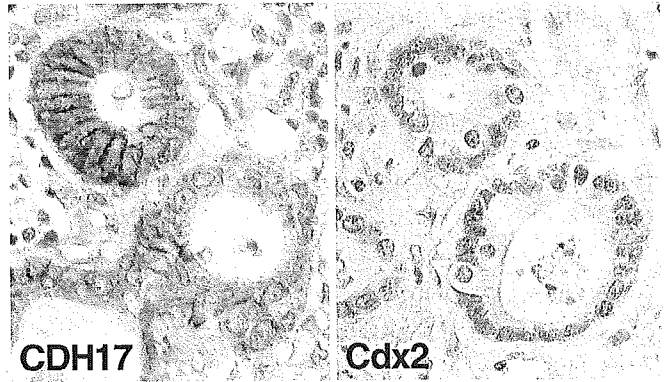
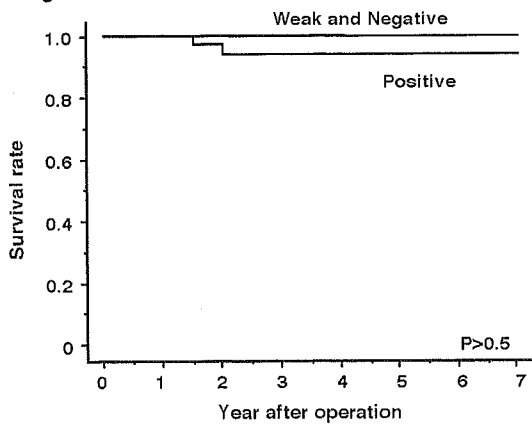


Fig. 4 Relationship of the expression of CDH17 and Cdx2. Synchronous expression of CDH17 and Cdx2 was detected in intestinal type gastric carcinoma

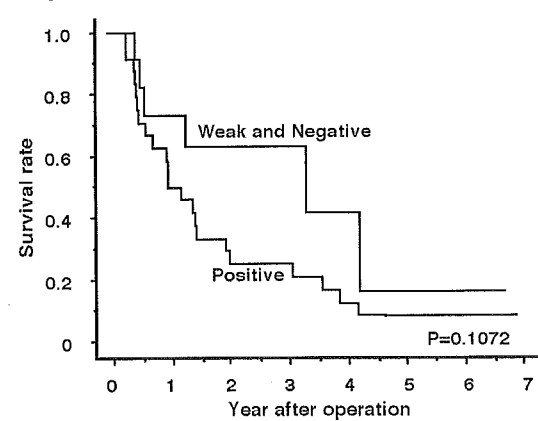
Comparison of the Cdx2 expression with CDH17 expression

We finally compared CDH17 expression with Cdx2 protein expression. Cdx2 immunohistochemical staining was performed in 52 gastric cancer cases extracted from cases that had been investigated with CDH17 immunohistochemical staining. Representative stainings are shown in Fig. 4. Seven

Stage I and II



Stage III and IV



All Stages

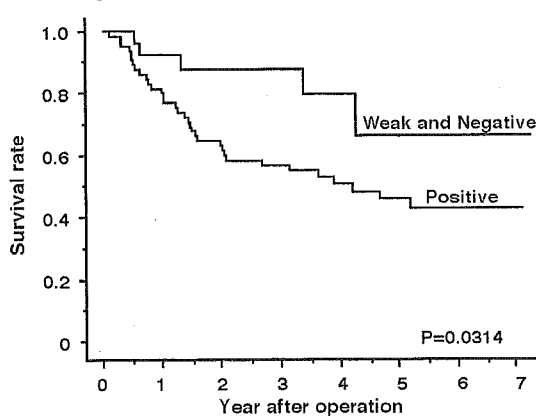


Fig. 3 Comparison of survival rates in patients between CDH17 positive cases, and weak and negative cases. The prognosis of patients with CDH17 positive cases was significantly poorer than that

of the patients weak and negative for CDH17, whereas there is no significance between survival rate and CDH17 expression when divided the cases into “stage I and II” and “stage III and IV”

Cdx2 positive cases (7/52, 13%) were found in 52 gastric cancer cases, and all of them were intestinal-type gastric carcinoma. Six of the seven cases (86%) expressed CDH17 simultaneously, and five of these six cases were stage I (data not shown).

Discussion

In the present study, we examined the expression of CDH17 in human gastric carcinoma and compared it with clinicopathological parameters, prognosis and Cdx2 expression. The expression of CDH17 protein was detected in 67% of gastric carcinomas that were correlated with depth of tumor invasion, tumor stage and amount of stromal tissue. It was recently reported that CDH17 mRNA expression is an independent factor associated with lymph node metastasis [8, 9]. Furthermore, our study demonstrated an association between CDH17 expression and poor prognosis. Although CDH17 was not an independent prognostic factor by multivariate analysis, CDH17 expression may be associated with aggressiveness of gastric carcinoma and can be a good marker for high-grade malignancy. On the other hand, in pancreas carcinoma, the survival prognosis was significantly poorer in CDH17 negative tumors compared with CDH17 positive tumors, and CDH17 might participate in the early stage through interaction with galectin-3 [17]. CDH17 may have a distinct organ-specific distribution and the role of CDH17 may be different through distinct intracellular interacting molecules.

Dysregulation of the classical cadherin-catenin adhesion network has been implicated in the progression of gastrointestinal tract cancer [3]. CDH17 is structurally distinct from its E-cadherin counterpart, and there is no direct association with the β -catenin network [10]. According to a previous report, tumor aggressiveness is associated with changes in cadherin expression. For example, P-cadherin, one of the classical cadherins, is highly expressed in well-differentiated adenocarcinoma, and its expression decreased in the advanced stage [19]. E-cadherin expression is also down-regulated in advanced gastric carcinoma cases [12]. LI cadherin is capable of mediating Ca^{2+} -dependent homophilic cell-cell adhesion independent of interactions with the cytoskeleton, indicating that the adhesive function of CDH17 may be complementary to that of E-cadherin. At present, while the role of CDH17 in tumor progression remains unknown, overexpression of CDH17 might be complementary to the co-expressed classical cadherins that are down-regulated in advanced gastric carcinoma.

Cdx2 is one of the transcription factors regulating CDH17 gene expression in normal, metaplastic and neoplastic tissues of the gastrointestinal tract via binding to elements in the 5'-flanking region of the gene [6]. In gastric carcinoma, Cdx2 gene is known to be methylated [21], and therefore epigenetic alteration of CDH17 may be one of the important steps in gastric carcinogenesis. In our study, most of the gastric carcinomas with simultaneous expression of Cdx2 and CDH17 were in the early stage (stage I).

All the seven Cdx2 positive cases showed intestinal phenotype and CDH17 positive expression. Moreover, six of seven cases were early stage. Mizoshita et al. [13] examined Cdx2 protein expression with T1 (intramucosal and submucosal invasion) gastric carcinoma cases and the data suggest that Cdx2 is expressed in the very early stage of gastric carcinogenesis in association with the shift from gastric to intestinal phenotypic expression. Because our samples were constructed from all stages of gastric carcinomas, the number of Cdx2 positive cases is lower in our study. Alternatively, CDH17 may regulate not only Cdx2 but also other transcriptional factors.

Our results suggest that CDH17 may participate not only in stomach carcinogenesis, but also in tumor progression which is associated with poor prognosis. CDH17 and Cdx2 may serve as molecular targets for gastric carcinoma treatment.

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DNA methylation profiles of differentiated-type gastric carcinomas with distinct mucin phenotypes

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Gastric carcinomas (GC) are classified into four phenotypes according to mucin expression. Previous studies revealed the association of distinct genetic profiles in GC with mucin phenotypic expression; however, the roles of epigenetic changes, such as DNA methylation, are poorly understood. We examined whether the phenotypic expression of GC was associated with DNA methylation of *hMLH1*, *MGMT*, *p16^{INK4a}*, *RAR-beta* or *CDH1*. Expression of HGM, M-GGMC-1, MUC2, and CD10 was analyzed immunohistochemically in 33 advanced GC with differentiated histology. HGM was expressed in 14 (42.4%) cases, M-GGMC-1 in five (15.2%) cases, MUC2 in 15 (45.5%) cases and CD10 in 18 (54.5%) cases. DNA methylation was detected in five (15.2%) cases for *hMLH1*, 11 (33.3%) cases for *MGMT*, 13 (39.4%) cases for *p16^{INK4a}*, 17 (51.5%) cases for *RAR-beta* and 14 (42.4%) cases for *CDH1* by bisulfite-polymerase chain reaction and methylation-specific polymerase chain reaction. DNA methylation of *hMLH1* occurred more frequently in MUC2-negative GC than in MUC2-positive GC ($P = 0.0488$, Fisher's exact test). In contrast, *MGMT* was more frequently methylated in MUC2-positive GC than in MUC2-negative GC ($P = 0.0078$, Fisher's exact test). There was no correlation between gastric or intestinal-markers and methylation of the *p16^{INK4a}*, *RAR-beta* and *CDH1* genes. These results indicate that DNA methylation of specific genes, such as *hMLH1* and *MGMT*, may be involved partly in the distinct phenotypic expression of GC. (*Cancer Sci* 2005; 96: 474–479)

Gastric carcinoma (GC) is one of the most common malignancies worldwide. GC are often classified histologically into two major types: the differentiated and undifferentiated types described by Nakamura *et al.*⁽¹⁾ or the Lauren intestinal and diffuse types⁽²⁾ based on glandular structure. Various genetic and epigenetic alterations are associated with GC; some are found in both the intestinal and diffuse types, whereas others are type specific.^(3,4) It was previously reported that GC can be subdivided according to mucin expression into four phenotypes:^(5–7) (i) gastric or foveolar phenotype (G type); (ii) intestinal phenotype (I type); (iii) intestinal and gastric mixed phenotype (GI type); and (iv) neither gastric nor intestinal phenotype (N type). Despite the usefulness of the Lauren classification, there are several variations of the intestinal-type GC described by Lauren. To better understand the development of GC at the molecular level, it is important to analyze molecular alterations in

intestinal-type GC according to the mucin phenotype. Distinct genetic changes appear to be associated with I type and G type GC. *p53* mutations and allelic deletions of the adenomatous polyposis coli (*APC*) gene are detected more frequently in I type GC than in G type GC,^(8–11) whereas microsatellite instability (MSI) is detected more frequently in G type GC than in I type GC.^(10,12) We reported previously that alterations of *p73*, including loss of heterozygosity and abnormal expression, play important roles in the genesis of G type GC.⁽¹³⁾

Several lines of evidence suggest that changes in DNA methylation patterns, such as hypermethylation of CpG islands, are common changes in human cancers.⁽¹⁴⁾ Hypermethylation of CpG islands in promoters is associated with silencing of some tumor-related genes.^(15–17) We previously reported DNA methylation of the *hMLH1*,⁽¹⁸⁾ *MGMT*,⁽¹⁹⁾ *p16^{INK4a}*, *RAR-beta* and *CDH1*⁽²⁰⁾ genes. In contrast to the many studies of genetic alterations in G type and I type GC, epigenetic alterations in G type and I type GC are poorly understood. Associations between genetic and epigenetic alterations have been reported. DNA methylation of *hMLH1* is associated with MSI,^(21,22) and DNA methylation of *MGMT* is associated with G to A mutations in the *K-ras*⁽²³⁾ and *p53*⁽²⁴⁾ genes. Because MSI occurs frequently in G type GC, it is possible that DNA methylation of *hMLH1* may occur frequently in G type GC. In fact, it has been reported that DNA hypermethylation of *hMLH1* occurs frequently in G type GC.⁽²⁵⁾ Because *p53* mutations are detected frequently in I type GC, it is possible that DNA methylation of *MGMT* occurs in I type GC. However, the association between DNA methylation and the mucin phenotypic expression of GC has been investigated only for *hMLH1*.

In the present study, we investigated the association between expression of gastric-type and intestinal-type markers and DNA methylation status of *hMLH1*, *MGMT*, *p16^{INK4a}*, *RAR-beta* and *CDH1* in differentiated-type GC.

Materials and Methods

Tissue samples

Thirty-three samples of differentiated-type GC from 33 patients were examined. All GC samples were not early GC

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but advanced GC, that had invaded beyond the muscularis propria.⁽²⁶⁾ Samples were obtained at time of surgery at Hiroshima University Hospital (Hiroshima, Japan) and affiliated hospitals. Tissue samples for molecular analyses were frozen immediately in liquid nitrogen and stored at -80°C until use. We confirmed microscopically that the tumor specimens consisted mainly of carcinoma tissue ($> 50\%$). For immunohistochemical staining, tissues were fixed in 10% buffered-formalin and embedded in paraffin. Tumor staging was carried out according to the tumor-node-metastasis stage grouping.⁽²⁷⁾ Because written informed consent was not obtained, for strict privacy protection, all samples were dis-identified before analyzing DNA methylation status. This procedure is in accordance with the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government.

Phenotypic analysis of gastric carcinomas

Tissue sections (4 μm thick) were prepared from paraffin blocks, and representative sections were immunostained for human gastric mucin (HGM), M-GGMC-1, MUC2 and CD10. Immunostaining was by the immunoperoxidase technique with a Histofine Simple Stain Kit (Nichirei Biosciences, Tokyo, Japan). Deparaffinized tissue sections were immersed in methanol containing 3% hydrogen peroxide for 15 min to block endogenous peroxidase activity. Microwave pretreatment in citrate buffer was carried out for 15–30 min to retrieve the antigenicity. The sections were then incubated with antibodies against gastric-type markers HGM (NCL-HGM-45M1; Novocastra, Newcastle, UK; dilution 1:50) and M-GGMC-1 (HIK1083; Kanto Kagaku, Tokyo, Japan; dilution 1:50), and intestinal-type markers MUC2 (Ccp58; Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:200) and CD10 (NCL-CD10-270; Novocastra; dilution 1:50), for 1.5 h at 37°C followed by incubation with the secondary antibody for 30 min. The immunocomplexes were visualized with 3,3'-diaminobenzidine. Sections were then counterstained with hematoxylin. GC were classified as G type, I type, GI type or N type. G type comprised those samples in which $> 30\%$ of the tumor cells were positive for gastric-type markers and showed little staining with intestinal-type markers. I type comprised those specimens in which $> 30\%$ of the tumor cells were positive for MUC2 or in which $> 5\%$ of the tumor cells were positive for CD10 and showed little staining with gastric-type markers. GC that showed positive staining for both gastric-type and intestinal-type markers were classified as GI type, and those that showed no staining with those markers were classified as N type.

Genomic DNA extraction and methylation analysis

To examine DNA methylation patterns in the 5' CpG islands of the *hMLH1*, *MGMT*, *p16^{INK4a}*, *RAR-beta* and *CDH1* genes, we extracted genomic DNA with a genomic DNA purification kit (Promega, Madison, WI, USA) and treated the genomic DNA with sodium bisulfite, as described previously.⁽²⁸⁾ In brief, 2 μg of genomic DNA was denatured by treatment with NaOH and modified with 3 M sodium bisulfite for 16 h. DNA samples were purified with Wizard DNA purification resin (Promega), treated with NaOH, precipitated with ethanol and resuspended in 25 μL water.

Aliquots (2 μL) were used as templates for methylation-specific polymerase chain reaction (MSP) amplification of the *MGMT*, *p16^{INK4a}*, *RAR-beta* and *CDH1* genes. MSP primers for *MGMT*, *p16^{INK4a}*, *RAR-beta* and *CDH1* were described previously.^(28–30) For analysis of DNA methylation of *hMLH1*, we carried out bisulfite-polymerase chain reaction (PCR) followed by restriction digestion as described previously.⁽³¹⁾ Primers and PCR conditions used for amplifying specific DNA fragments of various target genes are listed in Table 1. PCR products (15 μg) were loaded onto 8% non-denaturing polyacrylamide gels, stained with ethidium bromide and visualized under UV light. According to the corresponding literature, CpG island hypermethylation in the regions examined revealed good correlation with epigenetic silencing of the respective target genes.^(31–35)

Statistical methods

Fisher's exact test was used for statistical analysis. *P*-values less than 0.05 were regarded as statistically significant.

Results

Association between gastric-type and intestinal-type markers and DNA methylation

We carried out immunohistochemical analysis of 33 advanced differentiated-type GC (Fig. 1). Of the 33 GC, expression of gastric and intestinal markers was detected in 14 (42.4%) cases for HGM, five (15.2%) cases for M-GGMC-1, 15 (45.5%) cases for MUC2 and 18 (54.5%) cases for CD10. Next, DNA methylation status was investigated. Representative data for bisulfite-PCR followed by restriction digestion of the *hMLH1* gene and MSP of the *MGMT*, *p16^{INK4a}*, *RAR-beta* and *CDH1* genes are shown in Fig. 2. Of the 33 GC, DNA hypermethylation was detected in five (15.2%) cases for *hMLH1*, 11 (33.3%) cases for *MGMT*, 13 (39.4%) cases for *p16^{INK4a}*, 17 (51.5%) cases for *RAR-beta* and 14 (42.4%) cases for *CDH1*. Although recent evidence suggests that methylation of certain genes such as *hMLH1* and *CDH1* is associated with aging,^(36,37) there was no correlation between age and DNA methylation of a specific gene (Table 2). We compared DNA methylation status with each marker (Tables 3–6). DNA methylation of *hMLH1* was detected more frequently in MUC2-negative GC (5/18, 27.8%) than in MUC2-positive GC (0/15, 0.0%, *P* = 0.0488, Fisher's exact test). In contrast, DNA methylation of *MGMT* was detected more frequently in MUC2-positive GC (9/15, 60.0%) than in MUC2-negative GC (2/18, 11.1%, *P* = 0.0078, Fisher's exact test) (Table 5). There was no correlation between gastric and intestinal markers and methylation of the *p16^{INK4a}*, *RAR-beta* and *CDH1* genes.

Phenotypic expression of gastric carcinomas

On the basis of the combinations of expression of these four mucin markers, the 33 GC were classified phenotypically as five (15.2%) G type, 14 (42.4%) I type, 9 (27.2%) GI type and five (15.2%) N type. There was no apparent correlation between mucin phenotypic expression and clinicopathological findings (data not shown). No apparent association was observed between DNA methylation of a specific gene and phenotypic expression of GC (data not shown).

Table 1. Primer sequences for DNA methylation analysis

Primer sequence	Primer sequence	Annealing temperature
<i>hMLH1</i>	F: 5'-TAGTAGTYGTTTTAGGGAGGGA -3' R: 5'-TCTAAATACTCAACRAAAATACCTT-3'	55°C
<i>MGMT</i> (unmethylated)	F: 5'-TTTGTGTTTTGATGTTTGTAGGTTTTGT-3' R: 5'-AACTCCACACTCTTCCAAAAACAAAACA-3'	59°C
<i>MGMT</i> (methylated)	F: 5'-TTTCGACGTTTCGTAGGTTTTCGC-3' R: 5'-GCACTCTTCCGAAAACGAAACG-3'	59°C
<i>p16^{INK4a}</i> (unmethylated)	F: 5'-TTATTAGAGGGTGGGGTGGATTGT-3' R: 5'-CCACCTAAATCAACTCCAACCA-3'	60°C
<i>p16^{INK4a}</i> (methylated)	F: 5'-TTATTAGAGGGTGGGGCGGATCGC-3' R: 5'-CCACCTAAATCGACTCCGACCG-3'	65°C
<i>RAR-beta</i> (unmethylated)	F: 5'-TTAGTAGTTTGGGTAGGGTTTATT -3' R: 5'-CCAAATCCTACCCCAACA-3'	55°C
<i>RAR-beta</i> (methylated)	F: 5'-GGTTAGTAGTTCGGGTAGGGTTTATC-3' R: 5'-CCGAATCCTACCCGACG-3'	64°C
<i>CDH1</i> (unmethylated)	F: 5'-TAATTTAGGTTAGAGGGTTATTGT-3' R: 5'-CACAAACCAATCAACAACACA-3'	53°C
<i>CDH1</i> (methylated)	F: 5'-TTAGGTTAGAGGGTTATCGCGT-3' R: 5'-TAACTAAAAATTCACCTACCGAC-3'	57°C

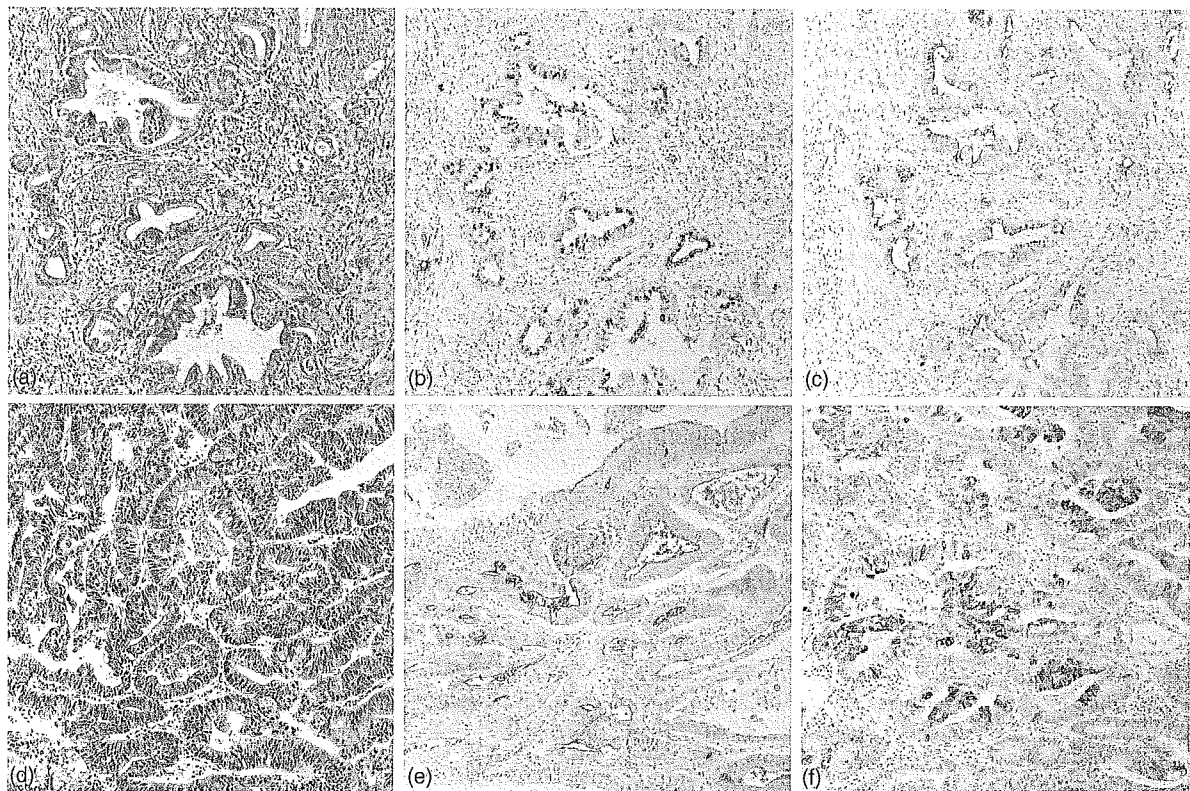


Fig. 1. G type (case 3: a, b, c) and I type (case 10: d, e, f) gastric carcinomas. (a,d) Hematoxylin and eosin staining. (b) MUC5AC and (c) M-GGMC-1 were detected in the cytoplasm of cancer cells. (e) CD10 was expressed on the luminal surfaces of cancer cells. (f) MUC2 is positive in the cytoplasm of cancer cells. (Original magnification, $\times 100$).

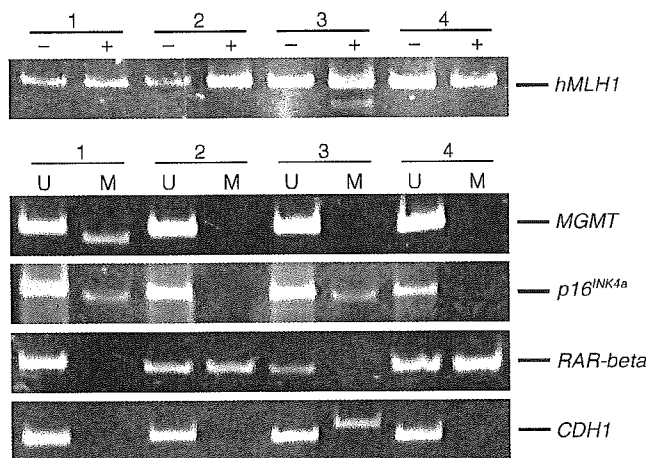


Fig. 2. Bisulfite-polymerase chain reaction followed by restriction digestion of the *hMLH1* gene and methylation-specific polymerase chain reaction of the *MGMT*, *p16^{INK4a}*, *CDH1* and *RAR-beta* genes. Methylated allele was detected in case 3 (*hMLH1*), case 1 (*MGMT*), cases 1 and 3 (*p16^{INK4a}*), cases 2 and 4 (*RAR-beta*) and case 3 (*CDH1*). M, methylated; U, unmethylated; +, after restriction enzyme digestion; -, before restriction enzyme digestion.

Table 2. Association between age and DNA methylation

Gene	Methylation status	Age		P-value [†]
		> 61	≤ 60	
<i>hMLH1</i>	Methylated	5 (100.0%)	0	0.5663
	Unmethylated	22 (78.6%)	6	
<i>MGMT</i>	Methylated	7 (63.6%)	4	0.1458
	Unmethylated	20 (90.9%)	2	
<i>p16^{INK4a}</i>	Methylated	11 (84.6%)	2	1.0000
	Unmethylated	16 (80.0%)	4	
<i>RAR-beta</i>	Methylated	13 (76.5%)	4	0.6562
	Unmethylated	14 (87.5%)	2	
<i>CDH1</i>	Methylated	11 (78.6%)	3	1.0000
	Unmethylated	16 (84.2%)	3	

[†]Fisher's exact test.

Table 3. Association between human gastric mucin (HGM) expression and DNA methylation status

Gene	Methylation status	HGM expression		P-value [†]
		Positive	Negative	
<i>hMLH1</i>	Methylated	2 (40.0%)	3	1.0000
	Unmethylated	12 (42.9%)	16	
<i>MGMT</i>	Methylated	5 (45.5%)	6	1.0000
	Unmethylated	9 (40.9%)	13	
<i>p16^{INK4a}</i>	Methylated	7 (53.8%)	6	0.4720
	Unmethylated	7 (35.0%)	13	
<i>RAR-beta</i>	Methylated	8 (47.1%)	9	0.7283
	Unmethylated	6 (37.5%)	10	
<i>CDH1</i>	Methylated	7 (50.0%)	7	0.4969
	Unmethylated	7 (36.8%)	12	

[†]Fisher's exact test.

Table 4. Association between M-GGMC-1 expression and DNA methylation status

Gene	Methylation status	M-GGMC-1 expression		P-value [†]
		Positive	Negative	
<i>hMLH1</i>	Methylated	1 (20.0%)	4	1.0000
	Unmethylated	4 (14.3%)	24	
<i>MGMT</i>	Methylated	1 (9.1%)	10	0.6431
	Unmethylated	4 (18.2%)	18	
<i>p16^{INK4a}</i>	Methylated	1 (7.7%)	12	0.6253
	Unmethylated	4 (20.0%)	16	
<i>RAR-beta</i>	Methylated	2 (11.8%)	15	0.6562
	Unmethylated	3 (18.8%)	13	
<i>CDH1</i>	Methylated	3 (21.4%)	11	0.6285
	Unmethylated	2 (10.5%)	17	

[†]Fisher's exact test.

Table 5. Association between MUC2 expression and DNA methylation status

Gene	Methylation status	MUC2 expression		P-value [†]
		Positive	Negative	
<i>hMLH1</i>	Methylated	0 (0.0%)	5	0.0488
	Unmethylated	15 (53.6%)	13	
<i>MGMT</i>	Methylated	9 (81.8%)	2	0.0078
	Unmethylated	6 (27.3%)	16	
<i>p16^{INK4a}</i>	Methylated	7 (53.8%)	6	0.4928
	Unmethylated	8 (40.0%)	12	
<i>RAR-beta</i>	Methylated	8 (47.1%)	9	1.0000
	Unmethylated	7 (43.8%)	9	
<i>CDH1</i>	Methylated	5 (35.7%)	9	0.4824
	Unmethylated	10 (52.6%)	9	

[†]Fisher's exact test.

Table 6. Association between CD10 expression and DNA methylation status

Gene	Methylation status	CD10 expression		P-value [†]
		Positive	Negative	
<i>hMLH1</i>	Methylated	1 (20.0%)	4	0.1523
	Unmethylated	17 (60.7%)	11	
<i>MGMT</i>	Methylated	8 (72.7%)	3	0.2659
	Unmethylated	10 (45.5%)	12	
<i>p16^{INK4a}</i>	Methylated	7 (53.8%)	6	0.7332
	Unmethylated	11 (5.0%)	9	
<i>RAR-beta</i>	Methylated	7 (41.2%)	10	0.1663
	Unmethylated	11 (68.8%)	5	
<i>CDH1</i>	Methylated	6 (42.9%)	8	0.3041
	Unmethylated	12 (63.2%)	7	

[†]Fisher's exact test.

Discussion

Gastric carcinomas are classified into the G, I, GI, and N phenotypes according to gastric-type and intestinal-type markers. In this study, expression of HGM, M-GGMC-1, MUC2 and CD10 was investigated. We observed that *hMLH1* was rarely methylated, whereas *MGMT* was frequently methylated in MUC2-positive GC. Therefore, DNA methylation, especially of the *hMLH1* and *MGMT* genes, may participate partly in the distinct phenotypic expression of GC. In fact, recent studies showed that the *MUC2* gene is also a target of DNA methylation.⁽³⁸⁾ Changes in genome-wide DNA methylation may also affect DNA methylation of these genes. To our knowledge, there is no report regarding DNA methylation of HGM, M-GGMC-1 and CD10.

DNA hypermethylation of *MGMT* occurred frequently in MUC2-positive GC. Previously reported data indicate that DNA hypermethylation of *MGMT* is associated with a G to A mutation in the *K-ras* and *p53* genes.^(23,24) Although we found no association between DNA methylation of *MGMT* and I type GC, MUC2 is a marker of intestinal epithelial cells. Thus, frequent *p53* mutations in I type GC⁽⁸⁻¹¹⁾ may be due to DNA methylation of *MGMT*.

The *hMLH1* gene was rarely methylated in MUC2-positive GC in this study. Endoh *et al.* reported that DNA hypermethylation of *hMLH1* occurs frequently in G type GC,⁽²⁵⁾ which does not express MUC2. Our findings support the notion that DNA methylation of *hMLH1* occurs frequently in G type GC. On the other hand, MUC2-positive GC were reported to show MSI more frequently than MUC2-negative GC.⁽³⁹⁾ DNA methylation of *hMLH1* is associated with MSI, indicating that MUC2-positive GC may have frequent DNA methylation of *hMLH1*. The reason for the discrepancy between our results and those of Lee *et al.* is unclear; however, the discrepancy may be due to differences in the samples analyzed.⁽³⁹⁾ Lee *et al.* studied the MUC2 expression and MSI in both differentiated-type and undifferentiated-type GC, whereas we analyzed the phenotypic expression and DNA methylation in differentiated-type GC only. Taken together, MUC2-positive undifferentiated-type GC

may show frequent MSI and DNA methylation of *hMLH1*. In addition, because of a phenotypic shift from G type to I type expression in conjunction with tumor progression,⁽⁷⁾ G type early GC showing *hMLH1* methylation may lose G type expression along with tumor progression.

There was no correlation between mucin marker expression and DNA methylation of *p16^{INK4a}*, *CDH1* and *RAR-beta*. Hypermethylation of the *p16^{INK4a}* gene is more common in differentiated-type GC than in undifferentiated-type GC, whereas *CDH1* and *RAR-beta* hypermethylation is observed more frequently in undifferentiated-scattered-type GC than in other types.⁽²⁰⁾ Thus, DNA methylation of these three genes may be involved in histogenesis, but not in phenotypic expression of GC.

Although MUC2 expression was correlated with DNA methylation of *hMLH1* and *MGMT* in this study, the number of cases we studied was too small to clarify correlation between phenotypic expression of GC and DNA methylation status. Additional studies are needed to obtain the definite association between DNA methylation and G and I phenotypes of GC.

In conclusion, our data show that DNA methylation of specific genes, such as *hMLH1* and *MGMT*, may be associated with the distinct phenotypic expression of GC. Because DNA methylation of tumor-related genes has been shown to occur in the early stages of stomach carcinogenesis⁽⁴⁰⁾ and to increase in parallel with stomach carcinogenesis,⁽⁴¹⁾ the association between DNA methylation and GC phenotypes in early GC should be investigated.

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

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

Esophageal squamous cell carcinoma (ESCC) is one of the most common cancers worldwide, but the prognosis for patients with this condition is extremely poor because of difficulties with early diagnosis and a lack of effective treatments.⁽¹⁾ The development of ESCC is a multi-step and progressive process, an early indicator of which is the increased proliferation of epithelial cells, including basal cell hyperplasia and dysplasia, which are regarded as precancerous

lesions. Multiple genetic alterations are involved, including amplification/overexpression of the *epidermal growth factor (EGF)/EGF receptor (EGFR)*^(2,3) and *cyclin D1/hst-1/int-2* genes,⁽⁴⁾ abnormal retention of intron 9 in the *CD44* gene,⁽⁵⁾ loss of heterozygosity (LOH) at multiple chromosomal loci,^(6,7) microsatellite instability,⁽⁸⁾ and mutation of the *TP53* gene.⁽⁹⁾

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

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

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

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

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

Materials and Methods

Tissue samples

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

Genomic DNA extraction and methylation analysis

To examine the DNA methylation patterns of the *RAR-beta*, *CRBP1*, and *TIG1* genes, we extracted genomic DNA with a Genomic DNA Purification Kit (Promega, Madison, WI, USA) and performed methylation-specific PCR (MSP).⁽³⁹⁾ In brief, 2 μg of genomic DNA was denatured by treatment with 2 M NaOH and modified with 3 M sodium bisulfite for 16 h. DNA samples were purified with Wizard DNA Purification Resin (Promega), treated with 3 M NaOH, precipitated with ethanol, and resuspended in 25 μL water. Two-microliter aliquots were used as templates for PCR reactions. The sequences of primers and the annealing temperature for *RAR-beta*, *CRBP1*, and *TIG1* MSP were as described previously

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

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

F, forward; R, reverse.

(Table 1).^(33,36,40) We determined the number of PCR cycles according to the correlation between the mRNA expression and DNA methylation of each gene in gastric cancer cell lines.⁽⁴¹⁾ Hot-start PCR with a total cycle number of 30 was used in all MSP DNA amplifications.

Quantitative reverse transcription-PCR analysis

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

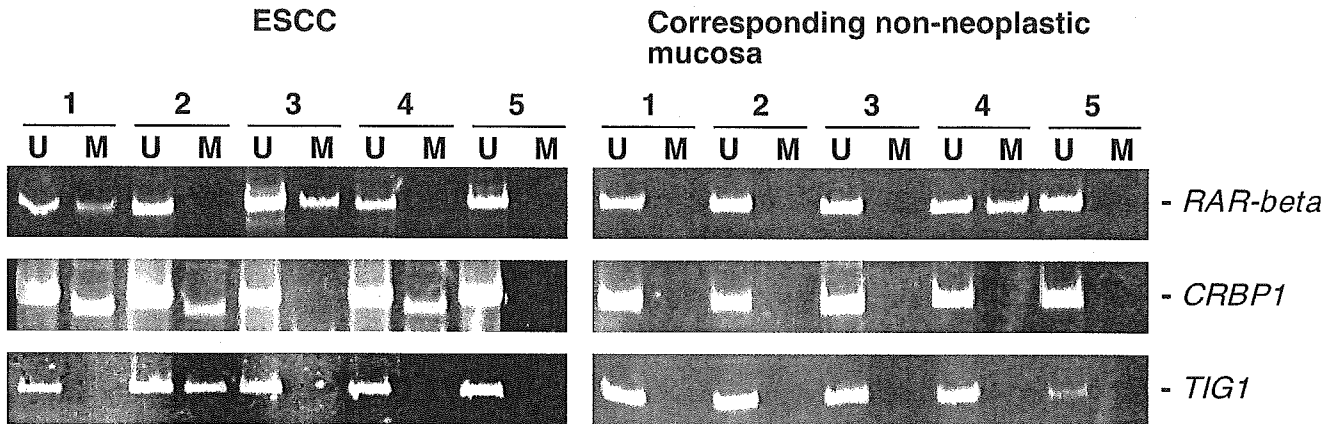


Fig. 1. Methylation-specific polymerase chain reaction (PCR) analysis of *RAR-beta*, *CRBP1*, and *TIG1* genes in esophageal squamous cell carcinoma and corresponding non-neoplastic mucosa. U, unmethylated PCR product; M, methylated PCR product. A methylated allele of the *RAR-beta* gene was detected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

Statistical methods

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

Results

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

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

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

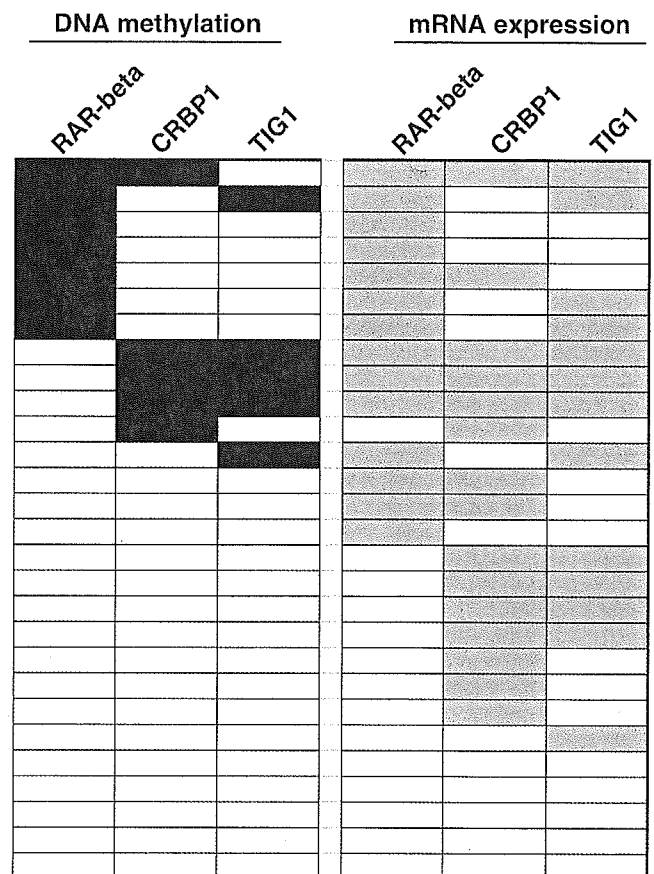


Fig. 2. Summary of DNA methylation and mRNA expression of *RAR-beta*, *CRBP1*, and *TIG1* in esophageal squamous cell carcinoma (ESCC) tissues. DNA methylation of each gene was associated with low expression of the respective mRNA. Concordant hypermethylation of *CRBP1* and *TIG1* was noted. Concordant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

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

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

*Fisher's exact test.

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

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

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

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

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

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

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

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

We used quantitative reverse transcription (RT)-PCR analysis to determine whether DNA methylation of the *RAR-beta*, *CRBP1*,

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

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

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

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

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

Discussion

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

Reduced expression of *RAR-beta* was detected in 50.0% of ESCC, and half of these cases showed DNA methylation of *RAR-beta*. Although DNA methylation of *RAR-beta* was detected in the corresponding non-neoplastic samples (10.0%), the frequency of methylation in ESCC (25.0%) was higher, suggesting that methylation of the *RAR-beta* gene may contribute to esophageal carcinogenesis. DNA methylation

occurs in premalignant and histologically normal squamous epithelium of the esophagus.^(19,44) The frequency of *RAR-beta* methylation did not differ significantly between early-stage and late-stage ESCC in this study. However, among the 14 ESCC with reduced expression of *RAR-beta*, 12 (85.7%) were positive for lymph nodes metastasis. A previous study indicated that retinoic acid induces the expression of *nm23-H1*,⁽⁴⁵⁾ which is known to reduce cell motility.^(46,47) Reduced expression of *RAR-beta* followed by reduced expression of *nm23-H1* may occur frequently in ESCC with lymph node metastasis.

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

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

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

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GASTROENTEROLOGY

Somatic mutations of mitochondrial DNA in digestive tract cancers

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Abstract

Background: Somatic mutations of mitochondrial DNA (mtDNA) have been reported to play an important role in the carcinogenesis of several human cancers. However, there are few reports on mtDNA mutations in digestive tract cancers, including esophageal, gastric and colorectal cancers. The present study examined somatic mtDNA mutations in these cancers.

Methods: Samples of 82 esophageal cancers, 96 gastric cancers and 138 colorectal cancers were collected. Mutations in the D310 mononucleotide repeat of mtDNA were examined by microsatellite assay.

Results: Frequencies of mtDNA mutations were similar in each digestive tract cancer: 14% (7/51) in esophageal cancers, 15% (14/94) in gastric cancers and 8% (11/133) in colorectal cancers. There were no significant relationships between mtDNA mutations and clinicopathological features, such as patient age or sex, tumor location, depth of tumor invasion and lymph node metastasis in each digestive tract cancer.

Conclusions: The results suggest that mtDNA mutations play a role in the development but not progression in each digestive tract cancer, and that the role of mtDNA mutations might be similar among the digestive tract cancers.

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Key words: D310, mutation, digestive tract cancer, mitochondrial DNA.

INTRODUCTION

Human cells contain two types of DNA; nuclear DNA, comprising approximately six billion base pairs, and mitochondrial DNA (mtDNA), comprising 100–1000 copies of 16 569 base pairs per cell.^{1,2} Mitochondrial DNA is susceptible to mutations and has at least a 10-fold higher mutation rate than nuclear DNA. This is because of the high level of reactive oxygen species in mitochondria products of oxidative phosphorylation in the generation of cellular adenosine triphosphate (ATP) and the low level of DNA repair systems.

Carcinogenesis and the progression of human malignancies are multistep processes involving the accumulation of genetic alterations. Much attention has been directed to the study of genetic events such as the

activation of oncogenes, the inactivation of tumor suppressor genes and defects in mismatch repair genes. Recently, somatic mutations of mtDNA have been reported in several types of human cancers.^{3–6} Fliss *et al.* reported mtDNA mutations in 9/14 (64%) bladder cancers, 6/13 (46%) head and neck cancers and 6/14 (43%) lung cancers.⁷ In addition, frequent mutation of the D-loop region of mtDNA has been reported. The D-loop region is critical for replication and expression of the mitochondrial genome because it contains the leading-strand origin of replication and the major promoters for transcription. There are few reports on mtDNA mutations in digestive tract cancers including esophageal, gastric and colorectal cancers. Therefore, the present study examined mtDNA mutations in these cancers.

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METHODS

Tissue samples

Eighty-two esophageal cancers, 96 gastric cancers and 138 colorectal cancers were collected at the Hiroshima University Hospital between 1988 and 2001. In each case, both cancerous and non-cancerous tissues were obtained. The present study was approved by the local ethical committee (No. I-RIN-HI-45), and informed consent was obtained from each patient.

Histological examination

Four micron-thick sections were prepared from formalin-fixed, paraffin-embedded specimens. The sections were stained with hematoxylin and eosin (HE) for histological examination.

Clinicopathological features

All esophageal cancers were squamous cell carcinomas. Esophageal cancers were classified into two groups according to depth of invasion; superficial type (mucosa and submucosa) and advanced type (muscularis propria or deeper). To analyze the relationship between tumor location and genetic alterations, the esophagus was divided into three parts; the upper, the middle and the lower.

Gastric cancers were classified histologically as either intestinal type or diffuse type as defined by Lauren.^{8,9} Depth of invasion was defined as early stage (mucosa and submucosa) or advanced stage (muscularis propria or deeper). The stomach was divided into two parts: the upper and the lower.

Colorectal cancers were classified into two groups according to depth of invasion: to the muscularis propria or subserosa, and the serosa or deeper. The colorectal cancers were divided into two parts; the right (from the cecum to the transverse colon) and the left (from the descending colon to the rectum).

DNA extraction

Ten micron-thick tissue sections were placed onto glass slides and stained with HE. The sections were then dehydrated in graded ethanol and dried without a cover glass. Cancerous and normal tissues on the slides were scraped up separately with sterile needles. DNA was extracted from the tissues with 20 μ L extraction buffer (100 mmol Tris-HCl; 2 mmol ethylene diamine tetraacetic acid [EDTA], pH 8.0; 400 μ L/mL proteinase K) at 50°C overnight. The tubes were boiled for 7 min to inactivate the proteinase K, and 2 μ L of the extracts were used for each polymerase chain reaction (PCR) amplification.

Mutations of the mtDNA

A 109-bp fragment containing the D310 repeat of mtDNA (D-loop region) was amplified. The primer sequences were as follows: 5'-ACAATT^oGAATGTCTGCACAGCCACTT-3' for the sense primer and 5'-GGCAGAGATGTGTTTAAGTGCTG-3' for the antisense primer.⁶ Microsatellite assays were carried out as described previously.¹⁰ Briefly, each 15 μ L reaction mixture containing 10–20 ng of genomic DNA, 6.7 mmol Tris-HCl (pH 8.8), 6.7 mmol EDTA, 6.7 mmol MgCl₂, 0.33 μ mol primer labeled with (gamma-³²P) dATP, 0.175 μ mol unlabeled primer, 1.5 mmol of each deoxynucleotide triphosphate and 0.75 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Branchburg, NJ, USA) was amplified for 40 cycles as follows: denaturation at 94°C for 30 s, annealing at 55°C for 30 s and strand elongation at 72°C for 30 s. The PCR products were electrophoresed on 6% polyacrylamide, 8 mol urea, 32% formamide gels and subjected to autoradiograph overnight at -80°C on Fuji RX film (Fuji Photo Film, Minamiashigara, Japan).

Statistical analysis

Fisher's exact test was used. A value of $P < 0.05$ was regarded as significant.

RESULTS

Samples

The male-to-female ratio of the patients with esophageal cancer was 50/9. The mean age of the patients was 66.0 years (range, 52–80). Twenty-three cases were of the superficial type, and 36 were of the advanced type. Four were located in the upper esophagus, 39 were in the middle, and 16 were in the lower.

The male-to-female ratio of patients with gastric cancer was 68/28. The mean age of the patients was 63.8 years (range, 33–90). Sixty cases were of early stage cancer and 36 were of advanced stage cancer. Thirty were located in the upper stomach, and 66 were in the lower. Seventy cases were negative for lymph node metastasis, and 26 were positive.

The male-to-female ratio of patients with colorectal cancer was 85/53. The mean age of the patients was 63.8 years (range, 37–88). In 43 cases, the depth of invasion was the muscularis propria or subserosa. In the remaining 95 cases, the depth was the serosa or deeper. Thirty-three cancers were located in the right side and 105 were in the left. Sixty-nine cases were negative for lymph node metastasis and 69 were positive.

Mutations of the mtDNA

Somatic mutations in the D310 mononucleotide repeat of mtDNA were detected in 32/278 (12%) of all digestive tract cancers studied. The frequencies of mtDNA