CpG Island Promoter Methylation (CIHM) Status of Tumor Suppressor Genes Correlates with Morphological Appearances of Gastric Cancer

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Abstract. *CpG island hypermethylation (CIHM) of tumor suppressor genes is one of the major events in the gastric carcinogenesis. We aimed to investigate the association between CIHM status of tumor suppressor genes and clinicopathological and morphological characteristics of gastric cancer. Patients and Methods: CIHM of p14, p16, Death-associated protein kinase (DAPK) and E-cadherin (CDH1) genes were determined by methylation-specific-polymerase chain Reaction in 146 gastric cancer tissues. CIHM-high was defined as three or more methylated CpG islands. Results: CIHM pf p14 was found in 70 (47.9%) cases, in 26 (17.8%) for p16, in 104 (71.2%) for CDH1 and in 127 (87.0%) for DAPK. CIHM-high was also found in 63 cases (43.2%). No association was found between between CIHM status and different staging, Lauren's subtypes, anatomic location, venous and lymphatic invasion, lymph node metastasis, distant metastasis, or peritoneal dissemination. However, among early gastric cancer cases, the depressed type with ulceration presented a significantly lower prevalence of CIHM of DAPK. In addition, Borrmann type IV cases presented significantly lower prevalence of CIHM-high among advanced gastric cancer. The Borrmann type IV cases also presented lower mean methylation number. Conclusion: The present results suggest that CIHM of DAPK and CIHM-high were associated with the morphological appearance of depressed type with ulceration in early gastric cancer, and Borrmann type IV advanced gastric cancer, respectively.*

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CpG island hypermethylation (CIHM) has been shown to be an important mechanism in gene silencing. In many kinds of cancer, several genes acquire CIHM. p16(INK4a) and p14(ARF) are involved cell cycle regulation *via* the pRb and p53 pathways, respectively. These two proteins have an independent first exon (exon 1a and 1β, respectively) but share exon 2 and 3 (1, 2). Methylation of *p16* and *p14* has been shown to be present in gastric cancer as well as premalignant lesions (3, 4). E-cadherin (*CDH1*) is an adhesion molecule involved in tumour invasion/metastasis. Silencing of *CDH1* by promoter CpG methylation has also been shown in gastric cancer (5). Death-associated protein kinase (*DAPK*) is a calcium/calmodulin-dependent serine/threonine kinase, and participates in various apoptosis systems. Methylation of *DAPK* has been reported in many types of cancer (6) including gastric cancer (7, 8).

CIHM of these four genes frequently occur in gastric cancer tissue as well as in premalignant lesions (3-8). Because methylated genes rarely occurs singly but in groups (9), the concept of a CpG island methylator phenotype (CIMP) through the study of aberrant methylation in gastric and colorectal cancer was introduced (3, 10), in which five to seven methylation sensitive genes were included for evaluating the methylation status in cancer and for correlating the CIMP with tumor risk and prevention (11, 12).

Gastric cancer is still one of the most common malignancies worldwide and remains a leading cause of cancer death in Asia and some European countries (13, 14). Therefore, it is necessary to clarify whether clinical characteristics of gastric cancer are associated with the extent of CIHM.

In this study, we investigated the prevalence of CIHM of 4 *p14, p16, CDH1* and *DAPK*, which are thought to be most susceptible for methylation in the stomach (3-8) among gastric cancer tissues and its relation to various clinicopathological characteristics. We also investigated the

Table I. *Primer sequences for MSP.*

Primer name	Forward sequence	Reverse sequence	Product size (bp)	Reference
p14 methylated	5'-gtgttaaagggcggcgtagc-3'	5'-aaaaccctcactcgcgacga-3'	122	(17)
p14 unmethylated	5'-tttttggtgttaaagggtggtgtagt-3'	5'-cacaaaaaccctcactcacaacaa-3'	132	(17)
p16 methylated	5'-ttattagagggtggggcggatcgc-3'	5'-gaccccgaaccgcgaccgtaa-3'	150	(18)
p16 unmethylated	5'-ttattagagggtggggtggattgt-3'	5'-caaccccaaaccacaaccataa-3'	151	(18)
CDH1 methylated	5'-ttaggttagagggttatcgcgt-3'	5'-taactaaaaattcacctaccgac-3'	115	(18)
CDH1 unmethylated	5'-taattttaggttagagggttattgt-3'	5'-cacaaccaatcaacaacaca-3'	97	(18)
DARK methylated	5'-ggatagtcggatcgagttaacgtc-3'	5'-ccctcccaaacgccga-3'	98	(19)
DARK unmethylated	5'-ggaggatagttggattgagttaatgtt-3	5'-caaatccctcccaaacaccaa-3'	106	(19)

association between the CIHM status of genes and the morphological appearance of gastric cancer.

Patients and Methods

Patients, tissue samples, DNA extraction, and Helicobacter pylori infection status. The studied population comprised 146 patients with gastric cancer being seen by the Endoscopy Center of Fujita Health University Hospital. All cases of gastric cancers were diagnosed histologically and were classified according to Lauren (15). Detailed information were obtained regarding anatomic location, distant metastasis and peritoneal dissemination. Information about venous and lymphatic invasion, and lymph node metastasis were also obtained in resected cases. Based on this information, early gastric cancer was defined as gastric cancer localized within the mucosa or submucosa, irrespective of lymph node metastasis (16), and all other cases were defined as advanced gastric cancer.

Patients with severe systemic diseases were excluded. All patients underwent an upper endoscopy with biopsy of the cancer lesions and the biopsy specimens were immediately frozen and stored at –80˚C. Genomic DNA was isolated from frozen specimens using proteinase K. *H. pylori* infection status was assessed by serological or histological analysis, or urea breath test. Patients were diagnosed as infected when at least one of the diagnostic tests was positive. The Ethics Committee of the Fujita Health University School of Medicine approved the protocol, and written informed consent was obtained from all participants.

Classification of gastric cancer according to morphological appearance. According to the morphological appearance by endoscophic imaging or of the surgically resected specimen, early gastric cancer was divided into three groups: polypoid or elevated type, depressed type, and depressed type with ulceration. Advanced gastric cancer was also classified according to Borrmann's classification.

Bisulfite modification and methylation-mpecifc polymerase chain reaction (MSP). For the examination of DNA methylation, genomic DNA from the cancer lesion was treated with sodium bisulfite using BislFast DNA Modification Kit for Methylated DNA Detection (TOYOBO, Co., Ltd., Osaka, Japan). MSP was carried out with primers as shown in Table I.

Annealing temperatures and times were determined using DNA from peripheral blood of a young individual without *H. pylori* infection and DNA methylated with *SssI* methylase (New England BioLabs Inc., Beverly, MA, USA). The MSP was carried out in a volume of 20 μl containing 0.1 μg of bislufite-modified DNA. The DNA was denatured at 95˚C for 5 minutes, followed by 33-35 cycles at 95˚C for 30 seconds and 57-69˚C for 1 minute with the primers, and 72˚C for 1 minute with a final extension at 72˚C for 5minutes. MSP reactions were carried out using EX Taq HS (TAKARA BIO INC., Shiga, Japan). PCR products (10 μl) were separated by electrophoresis in 2.5% agarose gels, and visualized by UV illumination using ethdium bromide staining.

CIHM was defined as the presence of positive methylation band, showing signals approximately equivalent to or greater than that of the size marker (10 ng/μl: 100 bp DNA Ladder, TAKARA BIO INC.,), irrespective of the presence of un-methylated bands. Samples giving faint positive signals were analyzed a further two

Table II. *Clinicopathologic characteristics of 146 gastric cancer.*

Variable (n)	
Mean age±SD (years)	65.2±12.4
Gender (m:f)	
Lauren's histological subtype	
Intestinal type	81
Diffuse type	65
H. pylori infection status	
+	115
–	31
Stage	
Early cancer	68
Advanced cancer	78
Anatomical location	
Cardiac	5
Non cardiac	141
Morphology	
Early cancer	
Polypoid or elavated type	20
Depressed type	38
Depressed type with ulceration	10
Advanced cancer	
Borrmann type I	5
Borrmann type II	30
Borrmann type III	30
Borrmann type IV	13

Table III. *Methylatio status of CpG islands in GC, in relation to age, gender, H. pylori infection status, Lauren's classification, and stage.*

Variable (n)	Age (yer)[a]	Gender[b]		*H. pylori* infection status[c]		Lauren's classification		Stage	
	Mean±SD	Male	Female	–	+	Intestinal	Diffuse	Early	Advanced
p14									
Unmethylated (76)	64.3±12.1	61	15	14	62	40	36	31	45
Hypermethylated (70)	66.1±12.8	44	26	17	53	41	29	37	33
p16									
Unmethylated (120)	65.7±12.7	86	34	27	93	69	51	57	63
Hypermethylated (26)	62.9±11.2	19	7	4	22	12	14	11	15
CDH1									
Unmethylated (43)	67.4±12.1	36	7	13	30	23	20	21	22
Hypermethylated (104)	64.2±12.5	69	34	18	85	58	45	47	56
DAP-kinase									
Unmethylated (19)	69.8±14.9	11	8	4	15	10	9	7	12
Hypermethylated (127)	64.5±11.9	94	33	27	100	71	56	61	66
CIMH-high									
– (83)	64.8±12.6	62	21	18	65	48	35	35	48
+ (63)	65.5±12.4	43	20	13	50	33	30	33	30

[a]p=0.08; [b]*p14*, p=0.02, *CDH1*, p=0.045; [c]*CDH1*, p=0.09. [a]Student's t-test; [b,c]χ^2 test.

times and only those samples with consistent positive methylation band were considered as CIHM. In addition, the fluorescence intensities of methylated bands were measured for 50 randomly selected CHIM samples using digital densitometer (Lane Analyzer, ATTO, Tokyo, Japan), and the fluorescence intensities of all 50 methylated bands were confirmed as being approximately equivalent to or greater than that of the size marker (data not shown). The number of CIHM among each subjects were calculated, and CIHM-high was defined as three or more CpG islands being methylated.

Statistical analysis. Statistical analysis was done with χ^2 test for the comparison of CIHM in two different groups. Student's t-test was used for the association between CIHM and age, and for the association between mean methylation number, based on number of CIHM among each subjects between two different groups. A probability value of less than 0.05 was considered statistically significant.

Results

Characteristics of patients, association between CIHM and age, gender, H. pylori infection status Lauren's classification, and stage. The characteristics of 146 gastric cancer patients are shown in Table II. All 146 gastric cancer samples were available for MSP analysis. CIHM was found in 70 cases (47.9%) for *p14*, 26 (17.8%) for *p16*, 104 (71.2%) for *CDH1* and 127 (87.0%) for *DAPK*. CIHM-high was also found in 63 cases (43.2%). CIHM of *DAPK* was weakly correlated with lower age. CIHM of *p14* and *CDH1* was significantly more frequent among females. Weak correlation was also found between CIHM of *CDH1* and *H. pylori*-positive gastric cancer. No association was found between between CIHM status and different staging and Lauren's subtypes (Table III). We also investigated the association between CIHM status and other clinicopathological factors, such as anatomic location, venous and lymphatic invasion, lymph node metastasis distant metastasis, and peritoneal dissemination, but no association was found (data not shown).

Association between CHIM status and morphological appearance of gastric cancer. We then investigated whether CIHM status would be associated with morphological appearance of gastric cancer. Among early gastric cancer cases, it was revealed that the depressed type with ulceration presented a significantly lower prevalence of CIHM of *DAPK* when compared to other morphological appearances (depressed type *vs*. depressed type with ulceration: p<0.0001, polypoid or elevated type *vs*. depressed type with ulceration: p<0.0001; polypoid or elevated type + depressed type *vs*. depressed type with ulceration: p<0.0001: Table IV). In addition, it was also revealed that the Borrmann type IV presented significantly lower prevalence of CIHM-high among advanced gastric cancer (Borrmann type III *vs*. type IV: p=0.009, Borrmann type I + type II + type III *vs*. type IV: p=0.016).

Association between mean methylation number and morphological appearance of gastric cancer. We also investigated the association between mean methylation number and morphological appearance of gastric cancer. No association was found in early-stage gastric cancer, however, it was revealed that the Borrmann type IV cases presented significantly lower mean methylation number (Borrmann type

Table IV. *Methylation status of CpG islands and morphological subtypes of GC.*

Variables (n)	Early stage GC			Advanced stage GC			
	Polyroid or elevated type	Depressed type	Depressed type with ulceration	Borrmann classification Type I	Type II	Type III	Type IV
p14							
Unmethylated (76)	8	18	5	4	15	17	9
Hypermethylated (70)	12	20	5	1	15	13	4
p16							
Unmethylated (120)	19	30	8	4	22	24	13
Hypermethylated (26)	1	8	2	1	8	6	0
CDH1							
Unmethylated (43)	9	11	1	1	8	8	5
Hypermethylated (104)	11	27	9	4	22	22	8
DAP-kinase[a]							
Unmethylated (19)	2	5	10	1	1	6	4
Hypermethylated (127)	18	33	0	4	29	24	9
CIMH-high[b]							
– (83)	14	18	4	3	16	12	11
+ (63)	7	20	6	2	14	18	2

[a]Depressed type *vs*. depressed type with ulceration: $p<0.0001$; polyroid or elevated type *vs*. depressed type with ulceration: $p<0.0001$. [b]Borrmann type II *vs*. type IV: $p=0.09$; Borrmann type III *vs*. type IV: $p=0.009$; Borrmann type I + type II + type III *vs*. type IV: $p=0.016$. Statistical analysis was performed using the χ^2 test.

II *vs*. type IV: $p=0.008$; Borrmann type I + type II + type III *vs*. type IV: 2.28±1.03 *vs*. 1.62±0.97, $p=0.008$; Table V).

Discussion

CIHM of *p14, p16, CDH1* and *DAPK* assessed in this study is frequent in gastric cancer tissue as well as premalignant lesions (3-8). It is suggested that this epigenetic change may also be relevant in the biological behavior of gastric cancer. In this study, we investigated the prevalence of CIHM of these four genes among gastric cancer tissues and its relation to various clinicopatological characteristics and morphological appearance of gastric cancer. Our results showed that CIHM of *DAPK* was weakly correlated with lower age. On the other hand, CIHM of *p14* and *CDH1* were significantly more frequent among females. Weak correlation was also found between CIHM of *CDH1* and *H. pylori*-positive gastric cancer.

It has been reported that CIHM in non-neoplastic gastrointestinal tissues correlates with higher age, male gender (20), and *H. pylori* infection (21, 22). The discrepancies of our result of correlation between CIHM of *DAPK* and lower age, CIHM of *p14*, *CDH1* and female gender may be partly due to the difference of CIHM status between cancerous and non-cancerous tissues.

One of the most important factors causing CIHM in the stomach is *H. pylori* infection (21, 22), which induces chronic inflammation, causing oxidative stress to the gastric epithelium (23, 24). Since CIHM of *CDH1* in non-neoplastic gastric mucosa correlates with *H. pylori* infection (5, 22), our result of correlation between CIHM of *CDH1* and *H. pylori*-positive gastric cancer seems to be reasonable.

Table V. *Association between mean methylation number of CpG islands and morphological subtypes of GC.*

Variable (n)	All GC (146) Mean methylation number
Early stage GC	
Polyroid or elevated type (20)	2.10±0.97
Depressed type (38)	2.32±1.04
Depressed type + ulceration (10)	2.60±0.84
Advanced stage GC	
Borrmann type I (5)	2.00±1.00
Borrmann type II (30)	2.47±0.97
Borrmann type III (30)	2.14±1.09
Borrmann type IV (13)	1.62±0.97

All data were expressed as mean±SD. Borrmann type II *vs*. type IV, $p=0.008$; Borrmann type I + type II +type III *vs*. type IV, 2.28±1.03 *vs*. 1.62±0.97; $p=0.008$. Statistical analysis was performed using Student's *t*-test.

We have also shown that CIHM status is associated with the morphological appearance of gastric cancer. Among early gastric cancer cases, the depressed type with ulceration presented a significantly lower prevalence of CIHM of *DAPK* when compared to other morphological appearances. In addition, Borrmann type IV cases also presented significantly lower prevalence of CIHM-high. Furthermore,

Borrmann type IV cases showed significantly lower mean methylation number. It is well known that the morphological appearance of gastric cancer is closely associated with its biological behavior and prognosis. Gotoda *et al*. showed that ulcer findings are significantly associated with lymph node metastasis in early gastric cancer (16).

Borrmann type IV gastric cancer has been reported to have more aggressive characteristic clinicopathological features including a young female prominence, detection at an advanced stage, high frequency of peritoneal or distant metastasis at diagnosis, a low rate of curative surgery, and frequent peritoneal recurrence, even after a curative resection (25-29). Our data indicate that different gastric cancer subgroups may have different epigenetic backgrounds. It may be suggested that CIHM is one of the major events in gastric carcinogenesis, however, it may not be frequent in patients with aggressive disease behavior. Our result of association between CIHM status and certain clinical phenotypes of gastric cancer also indicates the potential usefulness of CIHM status as a molecular marker to conduct more appropriate clinical implementation reflecting an individual's pathophysiology. However, we did not find significant association between CIHM status and other clinicopathological factors, such as anatomic location, venous and lymphatic invasion, lymph node metastasis distant metastasis, and peritoneal dissemination. Why only morphological appearance was correlated with CIHM status needs to be explained.

Concerning the association between CIHM status and clinicopatological characteristics of gastric cancer, it should be noted that some investigators produced different results. Zhang *et al*. showed that CIHM of *CDH1* correlated with lymph node metastasis (30). Similarly, Kim *et al*. showed that CIHM of *CDH1* more frequently occurred in early gastric cancer with lymph node metastasis (31). Furthermore, Luo *et al*. shows a much higher proportion of methylated *p16* in gastric cancer with metastasis (local or distant) than without metastasis (32). These data indicate that CIHM, at least for CDH1 and *p16* may be closely associated with more aggressive phenotypes of gastric cancer. However, in this study, we did not find any association between CIHM of *CDH1* or *p16* and clinicopathological characteristics of gastric cancer, and CIHM of *DAPK* and CIHM-high were associated with morphological appearances of depressed type with ulceration in early gastric cancer, and Borrmann type IV advanced gastric cancer, respectively. It is possible that the association seen in other studies may be due to population stratification and different patient groups, or to the different CpG islands and genes assessed in other studies. Our observation indicates that the CIHM of *DAPK* and CIHM-high were closely associated with the morphological appearances of gastric cancer.

In conclusion, we have shown that CIHM of *DAPK* and CIHM-high were associated with the depressed type with ulceration in early gastric cancer, and Borrmann type IV advanced gastric cancer, respectively.

However, our data did not provide the detailed mechanisms of CIHM in morphological appearances of gastric cancer. Only a more extensive understanding of the regulation of methylation in relation to gene expression and carcinogenesis will allow us to fully interpret our findings.

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