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
hMLH1		
	F: 5′-TAGTAGTYGTTTTAGGGAGGGA -3′ R: 5′-TCTAAATACTCAACRAAAATACCTT-3′	55°C
MGMT (unmethylated)		
	F: 5′-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3′ R: 5′-AACTCCACACTCTTCCAAAAACAAAACA-3′	59°C
MGMT (methylated)		
	F: 5′-TTTCGACGTTCGTAGGTTTTCGC-3′ R: 5′-GCACTCTTCCGAAAACGAAACG-3′	59°C
$p16^{INK4a}$ (unmethylated)		
	F: 5′-TTATTAGAGGGTGGGGTGGATTGT-3′ R: 5′-CCACCTAAATCAACCTCCAACCA-3′	60°C
$p16^{INK4a}$ (methylated)		
	F: 5′-TTATTAGAGGGTGGGGCGGATCGC-3′ R: 5′-CCACCTAAATCGACCTCCGACCG-3′	65°C
RAR-beta (unmethylated)		
	F: 5′-TTAGTAGTTTGGGTAGGGTTTATT -3′ R: 5′-CCAAATCCTACCCCAACA-3′	55°C
RAR-beta (methylated)		
	F: 5′-GGTTAGTAGTTCGGGTAGGGTTTATC-3′ R: 5′-CCGAATCCTACCCCGACG-3′	64°C
CDH1 (unmethylated)		
	F: 5′-TAATTTTAGGTTAGAGGGTTATTGT-3′ R: 5′-CACAACCAATCAACAACACA-3′	53°C
CDH1 (methylated)		
	F: 5′-TTAGGTTAGAGGGTTATCGCGT-3′ R: 5′-TAACTAAAAATTCACCTACCGAC-3′	57°C

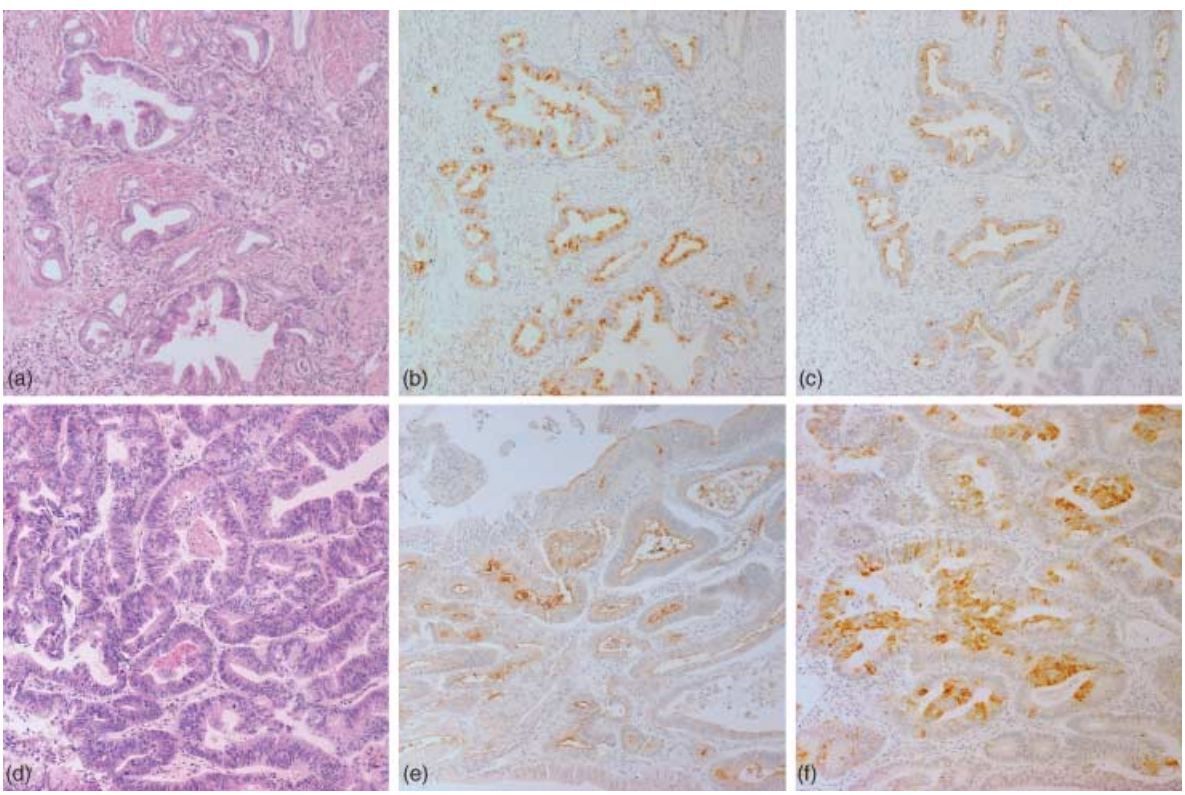

Fig. 1. G type (case 3: a, b, c) and I type (case 10: d, e, f) gastric carcinomas. (a,d) Hematoxylin and eosin staining. (b) MUC5AC and (c) M-GGMC-1 were detected in the cytoplasm of cancer cells. (e) CD10 was expressed on the luminal surfaces of cancer cells. (f) MUC2 is positive in the cytoplasm of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

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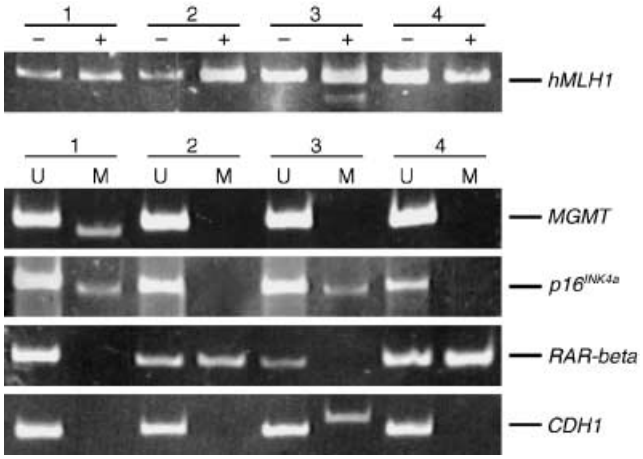

Fig. 2. Bisulfite-polymerase chain reaction followed by restriction digestion of the *hMLH1* gene and methylation-specific polymerase chain reaction of the *MGMT*, *p16*^{INK4a}, *CDH1* and *RAR-beta* genes. Methylated allele was detected in case 3 (*hMLH1*), case 1 (*MGMT*), cases 1 and 3 (*p16*^{INK4a}), 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	
hMLH1	Methylated	5 (100.0%)	0	0.5663
	Unmethylated	22 (78.6%)	6	
MGMT	Methylated	7 (63.6%)	4	0.1458
	Unmethylated	20 (90.9%)	2	
p16^{INK4a}	Methylated	11 (84.6%)	2	1.0000
	Unmethylated	16 (80.0%)	4	
RAR-beta	Methylated	13 (76.5%)	4	0.6562
	Unmethylated	14 (87.5%)	2	
CDH1	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	
hMLH1	Methylated	2 (40.0%)	3	1.0000
	Unmethylated	12 (42.9%)	16	
MGMT	Methylated	5 (45.5%)	6	1.0000
	Unmethylated	9 (40.9%)	13	
p16^{INK4a}	Methylated	7 (53.8%)	6	0.4720
	Unmethylated	7 (35.0%)	13	
RAR-beta	Methylated	8 (47.1%)	9	0.7283
	Unmethylated	6 (37.5%)	10	
CDH1	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	
hMLH1	Methylated	1 (20.0%)	4	1.0000
	Unmethylated	4 (14.3%)	24	
MGMT	Methylated	1 (9.1%)	10	0.6431
	Unmethylated	4 (18.2%)	18	
p16^{INK4a}	Methylated	1 (7.7%)	12	0.6253
	Unmethylated	4 (20.0%)	16	
RAR-beta	Methylated	2 (11.8%)	15	0.6562
	Unmethylated	3 (18.8%)	13	
CDH1	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	
hMLH1	Methylated	0 (0.0%)	5	0.0488
	Unmethylated	15 (53.6%)	13	
MGMT	Methylated	9 (81.8%)	2	0.0078
	Unmethylated	6 (27.3%)	16	
p16^{INK4a}	Methylated	7 (53.8%)	6	0.4928
	Unmethylated	8 (40.0%)	12	
RAR-beta	Methylated	8 (47.1%)	9	1.0000
	Unmethylated	7 (43.8%)	9	
CDH1	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	
hMLH1	Methylated	1 (20.0%)	4	0.1523
	Unmethylated	17 (60.7%)	11	
MGMT	Methylated	8 (72.7%)	3	0.2659
	Unmethylated	10 (45.5%)	12	
p16^{INK4a}	Methylated	7 (53.8%)	6	0.7332
	Unmethylated	11 (5.0%)	9	
RAR-beta	Methylated	7 (41.2%)	10	0.1663
	Unmethylated	11 (68.8%)	5	
CDH1	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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